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OXIDATION-REDUCTION POTENTIALS AND COFACTOR BINDING IN THE Clostridium beijerinckii FLAVODOXIN: EFFECTS OF METHIONINE 56

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

Lawrence James Druhan, M.S.

* * * * *

The Ohio State University
1998

Dissertation Committee:
Dr. Richard P. Swenson, Advisor
Dr. Edward J. Behrman
Dr. Gary E. Means
Dr. Smita Patel

Approved by

[Signature]
Advisor
The Department of Biochemistry
ABSTRACT

Flavodoxins separate the two oxidation-reduction potentials of the flavin mononucleotide (FMN) cofactor through the stabilization of the one-electron reduced semiquinone form and the destabilization of the fully reduced hydroquinone. This thesis is a comprehensive study of how the residue interacting with the re face of the FMN alters the function of the Clostridium beijerinckii flavodoxin. Oligonucleotide-directed mutagenesis facilitated the generation of thirteen mutant flavodoxins, replacing the wild-type Met56 with leucine, isoleucine, valine, alanine, glycine, cysteine, serine, tryptophan, phenylalanine, tyrosine, histidine, aspartic acid, and asparagine. These mutations altered both one-electron oxidation-reduction potentials, and produced changes in the dissociation constant between the oxidized FMN and the apoflavodoxin. As such, the residue interacting with the re-face of the FMN influences the stability of the holoflavodoxin in all three oxidation states. The changes in the oxidized (OX) to semiquinone (SQ) couple were due to alterations in the protein structural energy, and to the removal of an electrostatic sulfur-FMN interaction that preferentially stabilized the oxidized wild-type holoflavodoxin. Thiols, hydroxyls, and aromatic rings, had electrostatic side chain-flavin interactions as well, but the oxidation state specificity was unique to methionine. The
reduction potential of the semiquinone to hydroquinone (HQ) couple shifted to less negative values in all of the mutant flavodoxins. This change was due, at least partially, to the removal of an unfavorable interaction between the methionine thioether and the anionic hydroquinone. Additionally, electrostatic interactions between the fully reduced FMN and the formally charged side chains, at position 56, affected the stability of the hydroquinone complex. This interaction modified both the SQ/HQ midpoint potential and the pKa values of the interacting side chain. As such, the SQ/HQ couple was dependent upon the proton concentration. However, it was not a single residue producing this pH dependence, but rather a cooperative interaction between multiple charged residues and the anionic hydroquinone.

Of the fourteen amino acids sampled at position 56, methionine produced the greatest SQ/HQ stability difference. Therefore, Met56 is the uniquely suited for the production of the very negative SQ/HQ reduction potential necessary for the physiological function of these proteins.
Dedicated to my family and friends
ACKNOWLEDGMENTS

I would like to thank my advisor, Richard Swenson, without whom this thesis would have been impossible. His insight, encouragement, and support was deeply appreciated, and his love for science was inspirational.

I thank Dr. Mesut Eren, who synthesized the C. beijerinckii flavodoxin structural gene, making my studies possible. Thanks to Drs. David Myzka and Larry Helms for teaching me many of the techniques used during the course of my studies.

Thanks also go to my other labmates, Dr. Zhimin Zhou, Dr. Dawei Chen, Yucheng Feng, Fu-chung Chang, Luke Bradley, and Mumtaz Kasim, for many hours of helpful discussion.
VITA

November 27, 1965 ............... Born - Camp Zama, Japan

1988 ........................................ B.S. Biology, Spring Hill College

1993 ................................. M.S. Biochemistry, The Ohio State Biochemistry Program

1993 - present ....................... Graduate teaching and Research Assistant, The Ohio State University

FIELDS OF STUDY

Major Field: Biochemistry
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CHAPTER 1

INTRODUCTION

Flavodoxins are members of a large family of proteins collectively known as flavoproteins. These proteins demonstrate the common property of utilizing derivatives of vitamin B₂, or riboflavin, as essential cofactors for activity. Riboflavin contains the redox-active isoalloxazine ring and a D-ribityl side chain at the N10 position (see Figure 1 for the isoalloxazine numbering). The isoalloxazine ring consists of three ring systems: a dimethylbenzene ring, a central pyrazine ring, and a pyrimidine ring. The flavin mononucleotide (FMN) derivative is riboflavin with a phosphate group esterified to the 5'-hydroxyl group of the D-ribityl side chain. It can be argued that although FMN possesses all the components of a nucleotide, it does not contain a glycosidic bond between the D-ribityl and the isoalloxazine ring. Thus, the more precise nomenclature for FMN is riboflavin-5'-phosphate (Beinert, 1960). Another member of the flavin family is FAD, or flavin adenine dinucleotide. This chromophore has adenosine diphosphate esterified to the 5' position of the D-ribityl side chain of riboflavin. For the comparison of the chemical structures of these molecules see Figure 1.
Figure 1. The Chemical Structures of Riboflavin, Flavin Mononucleotide, and Flavin Adenine Dinucleotide. The standard numbering of the isoalloxazine ring is illustrated on the FMN ring system.
All three chromophores have similar visible absorption spectra and exhibit a yellow color when fully oxidized, with absorbance maxima in the 450 nm and 375 nm regions (Figure 2). This yellow color persists in the holoprotein, giving many flavoproteins characteristic visible absorption spectra. As expected, the fluorescence of FMN and riboflavin are also very much alike with a single emission maximum at ~ 520 nm (Mayhew & Ludwig, 1975; Beinert, 1960). In solution, FAD exhibits an equilibrium between an extended form and an intramolecular complex between the adenine and the isoalloxazine ring systems. The extended form exhibits typical flavin fluorescence which is quenched upon the formation of the intramolecular complex (Weber, 1950).

One of the principal chemical properties of flavin is its capacity to undergo oxidation-reduction reactions. Flavins can be reduced in a single step by two electrons, often by the transfer of a hydride ion from substrate or pyridine nucleotides. Also, it is possible for the isoalloxazine ring system to undergo two independent one-electron reductive steps, that may or may not be associated with proton transfer. The addition of the first electron produces the flavin semiquinone radical, and the subsequent one-electron reduction generates the fully reduced hydroquinone. The two one-electron reductions and the two physiologically relevant ionizations of the isoalloxazine ring are depicted in Figure 3. The addition of an electron and/or proton alters the electronic structure of the conjugated ring system, resulting in significant spectral changes in the visible absorbance region. As a consequence, the oxidation-reduction and the protonation states of the
Figure 2. The Near UV/Visible Absorbance Spectra of the Three Flavin Cofactors in the Oxidized State. The y-axis is in extinction (M⁻¹cm⁻¹). Spectrum 1 is riboflavin, spectrum 2 is FMN, and Spectrum 3 is FAD.
Figure 3. The Three Oxidation States of the Isoalloxazine Ring. From left to right, the oxidation states are oxidized, semiquinone, and hydroquinone. Also shown are the physiologically relevant ionizations for the semiquinone and the hydroquinone.
molecule can be conveniently determined by monitoring these spectral changes in an ordinary spectrophotometer (Figure 4).

The semiquinone radical undergoes a physiologically relevant ionization with a $pK_a$ of 8.4 (Figure 3). The protonated form, which predominates at pH 7, is blue in color and is thus termed the blue neutral semiquinone, while the deprotonated form has a reddish color and is called the red anion semiquinone (Land & Swallow, 1969). Upon addition of the second electron, the chromophore becomes almost colorless, displaying a nondescript absorption spectrum (Figure 4), and thus was designated the leuco form (Beinert, 1960). The fully-reduced isoalloxazine ring has a $pK_a$ of 6.5, forming either the neutral or anionic hydroquinone. The visible absorbance spectra of both hydroquinone protonation states are very similar but are distinguishable (Draper & Ingraham, 1968; Ghisla et al., 1974). Even though it is possible for these redox-active molecules to undergo two one-electron steps, in aqueous solution at pH 7.0 the fully reduced hydroquinone is the energetically favored reduced form. Therefore, the semiquinone forms of the cofactors do not accumulate in free solution at equilibrium.

The first flavoprotein, Old Yellow enzyme, was identified approximately 65 years ago (Warburg & Christian, 1933). In the intervening 65 years, numerous flavin-containing proteins have been purified, the sources of which range from single-celled bacteria to humans. As the metabolic pathways evolved, there was a need to transfer electrons from one substrate to another. The enzymes that catalyzed these oxidation-reduction reactions emerged to accomplish this important task. Because proteins contain a very limited
Figure 4. The Near UV/Visible Spectra of FMN in All three Oxidation States. Spectrum 1 is oxidized FMN, spectrum 2a is the red anionic semiquinone FMN, spectrum 2b is the blue neutral semiquinone, spectrum 3a is the neutral hydroquinone, and spectrum 3b is the anionic hydroquinone.
ability to accept and donate electrons, the addition of cofactors was necessary. Flavins, along with pyrimidine nucleotides, such as nicotinamide adenine dinucleotide (NAD$^+$), and bound metals, filled the need for redox-active centers. One might ask, why is more than one type of redox-active cofactor necessary? The answer may lie in a need for more than one electron transfer mechanism, *i.e.*, two-electron transfers *versus* one-electron transfers. While NADH and NADPH are limited to the transfer of two electrons at a time (through a hydride ion) and metals can generally transfer one at a time, flavins are able to uniquely bridge this gap. Flavins can accept a hydride ion (or equivalent) from an organic substrate and then donate the two electrons, in two one-electron transfer steps, to obligatory one-electron acceptors such as cytochromes, or *visa versa*. Consequently, flavoproteins are found in nearly every metabolic pathway.

Flavoproteins nearly always utilize FMN or FAD as cofactors rather than riboflavin directly. In most cases, the flavin chromophores are bound non-covalently, but there are examples where there is a covalent bond between the protein and the flavin. Covalently bound flavins are usually attached to the protein through the 8-methyl position of the isoalloxazine ring, but there are examples where the attachment is at the 6 position. Three amino acid residues have been found to be involved in these types of interactions, histidine, cysteine and tyrosine (Edmondson & De Francesco, 1987; Singer & McIntire, 1984).

While all flavoproteins generally serve the same basic function (oxidation-reduction), they catalyze a myriad of different types of reactions. The types of reactions
and the substrates acted upon have been used to categorize the many proteins into distinctive groups (Hemmerich et al., 1977). Transhydrogenases catalyze a two-electron, two-proton transfer from one substrate to another. Dehydrogenase-oxidases catalyze the two-electron oxidation of substrate with the concomitant reduction of molecular oxygen to hydrogen peroxide. Dehydrogenase-oxygenases insert one atom of molecular oxygen into a substrate, with the other oxygen atom reduced to water. Dehydrogenase-electron transferases receive two electrons from one substrate and transfer them in two one-electron steps to a second substrate. Pure electron transferases, which include the flavodoxins, carry out one-electron transfers between other oxidation-reduction proteins. These classes have been labeled class 1 through class 5, respectively (Hemmerich et al., 1977).

The fact that the enzymes employ two different cofactors, FMN and FAD, is not sufficient to explain how such a diverse number of reactions are catalyzed by a single cofactor type. The explanation must clearly lie in the proteins' capability to tune the oxidation-reduction properties and chemical reactivity of the cofactor. Since the redox-active portion of both cofactors is the isoalloxazine ring system, it is not surprising that it is the interactions between the protein and this portion of the molecules that determine the specific reactivity of each individual enzyme (Massey et al., 1983).

It would seem that each enzyme provides a different milieu for the flavin and thereby alters the cofactor's ability and mechanism by which it accepts and donates electrons, and other chemical reactivities which may favor or disfavor the formation of
covalent intermediates involving the flavin. The specific interactions that cause the alteration of the flavin properties include, but are not limited to: varying the electrostatic environment, hydrogen bonding interactions to N1, O2, N3H, O4, and N5H, steric interactions that modify the protonation of the N5 or N1 atoms or limit the accessibility of the cofactor, aromatic-aromatic and other non-bonding interactions. The possible hydrogen bonding and electrostatic interactions are illustrated in Figure 5. All of these interactions not only change the reactivities of the flavins but may also alter their spectrophotometric properties as well. Absorbance maxima, extinction coefficients and overall spectra shape all reflect changes in the flavin environment. One of the most important characteristics of the cofactor that can be altered by the flavoprotein is the midpoint potentials of the two oxidation-reduction couples. The potentials of the two couples have been found to be perturbed by as much as ± 400 mV, equivalent to a change in free energy of almost 7 kcal/mol. This astounding shift in potential has been the focus of a vast amount of investigation and is of great interest to our laboratory.

Of the flavoproteins, the flavodoxin family has been among the most vigorously studied. The name flavodoxin evolved from the fact that this FMN-binding protein is physiologically analogous to another small acidic electron transferase, the ferredoxin. The flavodoxin name was first applied to a protein purified from the bacteria Clostridium pasteurianum (Knight et al., 1966). This was not the first flavodoxin to be purified, however. An FMN-containing pure electron transferase protein was isolated from Anacystis nidulans, a blue-green alga (Smillie, 1963). These authors called the protein
Figure 5. Cartoon Demonstrating the Possible Electrostatic and Hydrogen Bonding Interactions for the FMN Isoalloxazine Ring. The panel outline represents the FMN environment provided by the protein. The circled negative sign in the panel outline indicates the surrounding monopoles. The top panel is oxidized FMN, and the bottom panel is anionic hydroquinone FMN. Dotted lines indicate possible H-bonds. The solid arrow indicates the possibility for non-bonded multipole interactions, these interactions are not limited to the o-xylene subnucleus. The open arrow indicates a specific interaction between the surrounding monopoles and the anionic hydroquinone.
phytoflavin, but this term gave way to the currently used nomenclature, and this protein is now also designated as a flavodoxin. From that time, numerous flavodoxins from a diverse number of sources have been purified. A common feature of all the flavodoxins is the stabilization of the oxidized/semiquinone (OX/SQ) couple and the destabilization of the semiquinone/hydroquinone (SQ/HQ) couple relative to the free FMN values. Table 1 lists a number of these proteins, along with the midpoint potentials for each one-electron oxidation-reduction couple. The three-dimensional structures for several flavodoxins have been determined either by x-ray crystallography or more recently by NMR spectroscopy [see Table 1 for references].

The physiological roles of many flavodoxins have not been definitively established, in most cases. They have been implicated in nitrogen fixation, sulfate reduction, photosynthesis, and the oxidation of pyruvate, by both in vivo and in vitro experiments (Bennett et al., 1988; Drummond et al., 1983; Smillie. 1965; Knight et al., 1966; Hatchian et al., 1972). There is also evidence that flavodoxins can substitute for ferredoxins when organisms encounter an iron-poor environment, i.e., the growth of some organisms in an iron poor medium causes the over expression of flavodoxin and a decline in ferredoxin expression (Knight et al., 1966; Mayhew, 1971; Mayhew & Ludwig, 1975). The flavodoxin from Azotobacter vinelandii donates electrons to purified assimilatory nitrate reductase to catalyze the reduction of nitrate to nitrite (Gangeswaran & Eady, 1996). A precise role for flavodoxin has been determined in Klebsiella pneumoniae. In this bacterium, flavodoxin accepts electrons from the oxidation of pyruvate by pyruvate...
<table>
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<td>Anabaena 7120$^a$</td>
<td>-196</td>
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<td></td>
<td>-314</td>
<td>-124</td>
<td>Anderson, 1983</td>
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</table>


Table 1. Various Flavodoxins and Their Oxidation-Reduction Potentials. The three-dimensional structure of the flavodoxin from each species highlighted in bold has been solved and the superscript indicates the structural reference. The E$_{OX/\Sigma}$ values have been adjusted to pH 7 using 59 mV/pH.
oxidoreductase and subsequently reduces nitrogenase (Nieva-Momez et al., 1980; Shah et al., 1983). In *Escherichia coli*, flavodoxin takes part in at least four metabolic reactions. Flavodoxin supplies an electron to the *E. coli* cobalamin-dependent methionine synthase during the *de novo* biosynthesis of methionine (Fujii & Huennekens, 1974; Osborn et al., 1991; Drummond et al., 1993). The *E. coli* flavodoxin is involved in the activation of pyruvate formate lyase in the dissimulation of pyruvate to acetyl-CoA and formate (Blaschkowski et al., 1982; Knappe et al., 1993). Through a similar mechanism of activation (production of a glycyl radical), ribonucleotide reductase, which catalyses the reduction of ribonucleoside triphosphate to deoxyribonucleoside triphosphate, is also activated by the *E. coli* flavodoxin (Biachi et al., 1993; Reichard, 1993; Mullies et al., 1995). Lastly, the *E. coli* flavodoxin has been implicated in the conversion of dethiobiotin to biotin (Ifuku et al., 1994). It has been shown that the SQ/HQ couple is usually the physiologically active couple (Mayhew & Tollin, 1992; Mühlenhoff et al., 1996).

Flavodoxins can be separated into two distinct groups based on their molecular weight. The so called "long chain flavodoxins" have a molecular weight of 20 to 23 kilodaltons, while the "short chain flavodoxins" have a molecular weight of 15 to 12 kilodaltons. In general, flavodoxins from photosynthetic organisms are the long chain type, while short chain flavodoxins are isolated from non-photosynthetic organisms (Mayhew & Ludwig, 1975). Another method to divide the flavodoxins relies upon differences in fluorescence excitation spectra, circular dichroism, and FMN binding constants. These properties divide the flavodoxins into the *rubrum*-type and the
pasteurianum-type. These two rules of classification are not mutually exclusive, in that there are both long chain and short chain, rubrum-types and both rubrum and pasteurianum, long chain types, etc.

Flavodoxins provide an excellent choice for the study of flavin/protein interactions for a number of important reasons. Flavodoxins drastically alter the redox properties of the bound FMN, separating the two redox couples by \(-300\) mV. This separation is produced by an increase in the OX/SQ couple (\(\sim +100\) mV) and a decrease in the SQ/HQ couple (\(\sim -300\) mV), relative to the free flavin. These large and differential changes in reduction potential are indicative of very strong protein-flavin interactions. The amino acid composition of flavodoxins is very high in acidic residues, giving them very negative isoelectric points. This property allows ease of purification through the use of anionic exchange chromatography. Flavodoxins are small and stable and can be prepared at high solution concentrations, lending themselves to many experimental techniques including nuclear magnetic resonance spectroscopy. Apoflavodoxin is easy to produce, allowing the reconstitution of holoprotein with chemically modified or isotopically enriched FMN. Flavodoxins from many different sources have been purified, each with slightly different properties. Therefore, the comparison of the primary sequences can identify residues important in the regulation of the flavin. The three-dimensional structure of many of these proteins is known, in some cases in all three oxidation states, making structure/function studies possible. Also, the structural genes coding for several flavodoxins have been cloned or synthesized. Thus, not only can the
proteins be overexpressed, the primary structure of the proteins can be manipulated at the genetic level using, now classical, protein engineering techniques.

The flavodoxin from Clostridium beijerinckii (previously classified as Clostridium MP) is a short chain, pasteurianum-type flavodoxin. It has a molecular weight of 15,300 daltons, and contains 138 amino acid residues. The primary structure of this flavodoxin has been determined (Tanaka, et al., 1974), and its three-dimensional structure has been solved in all three oxidation states for both the wild type and a number of single amino acid mutants (Burnett, et al., 1974; Smith et al., 1977; Ludwig & Luschinsky, 1992; Ludwig et al., 1997). Figure 6 illustrates the primary sequence of the C. beijerinckii flavodoxin. While the natural gene coding for this flavodoxin has not been cloned, a synthetic gene was constructed and the product from this gene was shown to be identical to the natural flavodoxin (Eren & Swenson, 1989).

The general features of the three-dimensional structure of the C. beijerinckii (MP) flavodoxin are conserved in all the flavodoxins (Figure 7). It has a central parallel beta-sheet, which is composed of five strands, and four alpha-helices that flank this central sheet. The structure of the FMN bound to the clostridial flavodoxin has also been determined. The ribityl moiety is in a trans-extended conformation, with all three ribose hydroxyl groups involved in hydrogen bonds, two to the apoprotein and one to solvent. The isoaalloxazine ring system is bent along the N5-N10 axis by just 2°, with only a portion of the dimethyl benzene ring being exposed to the solvent.
Figure 6. The Amino Acid Sequence of the *Clostridium beijerinckii* (MP) Flavodoxin. The underlined residues delineate the FMN binding site.
Figure 7. The Three-Dimensional Structure of the *Clostridium beijerinckii* Flavodoxin. This is a protein cartoon with the FMN represented in ball-and-stick configuration at the bottom of the structure.
The FMN binding site, found near the surface at one end of the protein, is composed of three loops: the teen's loop, the 60's loop and the 90's loop (named for the residue numbers that comprise these structures). The teen's loop binds the 5'-phosphate moiety, while the 60's and 90's loops provide the residues which interact with the peptide (re) and solvent (si) side of the isoalloxazine ring system, respectively. There are also numerous hydrogen bonds formed between the isoalloxazine ring and the 60's and 90's loops. As in many flavodoxins, the acidic residues outnumber the basic residues surrounding the cofactor binding site.

The teen's loop is highly conserved in the flavodoxins, being largely composed of hydroxyl-containing residues that, along with five backbone amine groups, provide the majority of the interactions with the phosphate of the cofactor. A distinctive feature of this phosphate-binding site is the absence of compensating charges to neutralize any charge on the phosphate. Preliminary calculations indicated that the uncompensated phosphate charge, initially thought to be dianionic, was responsible for a large portion of the destabilization of the SQ/HQ couple (Moonen et al., 1984). However, recent work has demonstrated that any charge present on the phosphate has only a small effect in the flavodoxin from Desulfovibrio vulgaris. The magnitude of this effect was similar to the electrostatic destabilization produced by each of the surrounding acidic residues [see below], suggesting that the phosphate is not dianionic (Zhou & Swenson, 1996a). The 5'-phosphate moiety is, however, crucial in cofactor binding for flavodoxins as riboflavin
binding is much weaker than FMN binding (Mayhew & Tollin, 1992; Curley et al., 1991; Zhou & Swenson, 1996a).

The structures of the 60's and 90's loops, while conserved throughout the flavodoxin family, vary considerably. Table 2 illustrates the primary sequence of the 60's and 90's loops from many of the flavodoxins, demonstrating the differences in the residues that interact with the re- and the si-face of the cofactor. It can be seen that the residues which interact with the planar faces of the FMN are all hydrophobic in character.

The C. beijerinckii (MP) flavodoxin has a methionine (M56) contacting the re face of the isoalloxazine ring, and a tryptophan (W90) flanking the si face. This is different from several flavodoxins that have aromatic residues on both sides of the cofactor. There is also hydrogen bonding between these loops and the flavin. In the C. beijerinckii flavodoxin, N1 and O2 are hydrogen bonded to the peptide amides of glycine 89 and glycine 91, N3 to the side chain carboxylate of glutamate 59, and O4 to the peptide amide of glutamate 59. As the C. beijerinckii protein is reduced to the semiquinone form, an additional hydrogen bond is thought to form between a peptide carbonyl group (glycine 57 in the C. beijerinckii) and the N5H of the semiquinone, through a conformational change. This new interaction is the consequence of a conformational change that has been demonstrated to occur in at least two other flavodoxins (Laudenbach et al., 1988; Luschinsky et al., 1991; Watenpaugh et al., 1976; Watt et al., 1991). The structure of the hydroquinone form of the flavodoxin is essentially identical to the semiquinone form in the short chain flavodoxins. A summary of all the hydrogen bonding contacts in the C.
### Table 2. Amino Acid Sequences of the 60’s and 90’s Loops from Several Flavodoxins

The residues which interact with the planar faces of the isoalloxazine ring in each loop are in bold. The numbering is from the *A. nidulans* flavodoxin.

<table>
<thead>
<tr>
<th>Source</th>
<th>Loop Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anacystis nidulans</td>
<td>G C P T W N V G E L</td>
</tr>
<tr>
<td>Anabaena 7120</td>
<td>G C P T W N I G E L</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>G T P T L G D G Q L</td>
</tr>
<tr>
<td>Azotobacter chroococcum</td>
<td>G T P T L G E G E L</td>
</tr>
<tr>
<td>Azotobacter vinelandii</td>
<td>G T P T L G E G E L</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>G I P T W Y Y G E A</td>
</tr>
<tr>
<td>Chondrus crispus</td>
<td>G A P T W N T G A D</td>
</tr>
<tr>
<td>Megasphaera elsdenii</td>
<td>G C P A M G S E E L</td>
</tr>
<tr>
<td>Clostridium beijerinckii</td>
<td>G C S A M G D E V L</td>
</tr>
<tr>
<td>Desulfovibrio vulgaris</td>
<td>G C S T W G E L</td>
</tr>
<tr>
<td></td>
<td>D D S I</td>
</tr>
</tbody>
</table>

Anacystis nidulans       | G A G D Q V G Y S D N F Q D A |
Anabaena 7120            | G T G D Q I G Y A D N F Q D A |
Klebsiella pneumoniae    | G L G D Q R G Y P D N F V S G |
Azotobacter chroococcum  | G L G D Q V G Y P E N F L D A |
Azotobacter vinelandii   | G L G D Q V G Y P E N Y L D A |
Escherichia coli         | G C G D Q E D Y A E Y F C D A |
Chondrus crispus         | G L G D A E G Y P D N F C D A |
Megasphaera elsdenii     | G S Y G W G S G E W |
Clostridium beijerinckii | G S Y G W G D G K W |
Desulfovibrio vulgaris   | G C G D S S Y E Y F C G A |

beijerinckii flavodoxin is given in Table 3, with a drawing of the hydrogen bonds in the reduced state shown in Figure 8 (Ludwig et al., 1997).

All of these protein/cofactor interactions are likely to have the effect of altering the midpoint potentials of one or both oxidation-reduction couples of the FMN. In the wild-type C. beijerinckii flavodoxin, the midpoint potential of the OX/SQ couple, $E_{\text{ox/sq}}$ (some literature refers to this couple as the E2 couple) becomes less negative, shifting from -238 mV to -92 mV. In contrast, the midpoint potential of the SQ/HQ couple, $E_{\text{sq/hq}}$ (some literature refers to this couple as the E1 couple) becomes more negative, moving from -172 mV to -399 mV (Draper & Ingraham, 1968; Mayhew, 1971). Thus, the two couples are very effectively separated, facilitating the two independent one-electron reduction electron-transfer pathway in this protein. Figure 9 shows the spectral changes associated with the reduction of the C. beijerinckii (MP) flavodoxin. The appearance of the long wavelength absorbance around 580 nm indicates the accumulation of the blue neutral semiquinone, demonstrating the separation of the E1 and E2 couples. The holoprotein hydroquinone spectrum is indicative of a planar anionic hydroquinone cofactor (Ghisla et al., 1974)

The protonation of each oxidation state has been investigated. The OX/SQ couple demonstrates a dependence on the hydrogen ion concentration of 60 mV/pH unit between pH 5-9. This is consistent with a single protonation linked to the one-electron reduction of the flavin at 25 °C (Mayhew, 1971; Ludwig et al., 1990; Clark, 1972). Since there is no change in the slope of such a plot, and no major change in the visible absorbance spectra
<table>
<thead>
<tr>
<th>FMN atom</th>
<th>Contact Atom</th>
<th>Oxidized</th>
<th>Semiquinone</th>
<th>Reduced</th>
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<tbody>
<tr>
<td>N1</td>
<td>G89 N</td>
<td>3.08</td>
<td>3.09</td>
<td>3.02</td>
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<td></td>
<td>W90 N</td>
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</tr>
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<td>2.90</td>
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<tr>
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<td></td>
<td>Wat253 O</td>
<td>3.28</td>
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</tr>
<tr>
<td>OP1</td>
<td>G8 N</td>
<td>2.62</td>
<td>2.80</td>
<td>2.80</td>
</tr>
<tr>
<td></td>
<td>S54 Oγ</td>
<td>2.80</td>
<td>2.82</td>
<td>2.88</td>
</tr>
<tr>
<td></td>
<td>Wat245 O</td>
<td>2.87</td>
<td>2.82</td>
<td>2.89</td>
</tr>
<tr>
<td>OP2</td>
<td>S7 Oγ</td>
<td>2.82</td>
<td>2.80</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
<td>T12 N</td>
<td>2.80</td>
<td>2.72</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td>T12 Oγ</td>
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<td>2.83</td>
<td>2.92</td>
</tr>
<tr>
<td>OP3</td>
<td>T9 N</td>
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<td>2.70</td>
<td>2.76</td>
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<td></td>
<td>T9 Oγ</td>
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<td>2.57</td>
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<tr>
<td></td>
<td>N11 N</td>
<td>3.02</td>
<td>3.05</td>
<td>3.08</td>
</tr>
</tbody>
</table>

Table 3. Summary of the Protein-FMN Hydrogen Bonding Contacts in the *Clostridium beijerinckii* Flavodoxin. (Ludwig & Luschinsky, 1992).
Figure 8. The Hydrogen Bonding in the Reduced *Clostridium beijerinckii* Flavodoxin. The protein structure is from 5ull.pdb (Ludwig et al., 1997). Small solid circles represent nitrogen atoms and open circles represent oxygen atoms. Dotted lines indicate a hydrogen bond. Water and some protein atoms not involved in hydrogen bonding have been omitted for clarity. The FMN nitrogen numbering is shown ant the phosphate oxygen numbering is, starting at “12 O’clock” and moving counter-clockwise, I, II, III.
Figure 9. Spectral Changes Associated with the Reduction of the *Clostridium beijerinckii* Flavodoxin. The titration proceeds from the oxidized state (spectrum 1) through the semiquinone form (spectrum 4) to approximately 95% fully reduced (spectrum 8).
between these pH values, the $pK_a$ of the semiquinone FMN must be substantially shifted to a more basic value in the holoflavodoxin (the $pK_a$ of the one-electron reduced FMN in free solution is 8.3). Using stopped flow spectrophotometry this $pK_a$ has been estimated to be greater than 13 in the *Megasphaera elsdenii* flavodoxin (Ludwig *et al.*, 1990). The SQ/HQ couple of the flavodoxin is pH independent from pH 10 to ~ 7, but becomes progressively less negative between pH 7 to 5 (Mayhew, 1971; Ludwig *et al.*, 1990).

This data was initially interpreted as the protonation of the FMN around the normal free solution $pK_a$ of 6.5. However, NMR spectroscopic analysis of the FMN has indicated that the hydroquinone cofactor remains unprotonated in the flavodoxins to pH values below 5, indicating a shift of the $pK_a$ to below 4 (Franken *et al.*, 1984; Vervoort *et al.*, 1985; Vervoort *et al.*, 1986). Thus, protonation of the flavin cannot be responsible for the observed pH dependence of the SQ/HQ couple. As such, this dependency has been assigned to the redox-linked ionization of a residue, or residues, in proximity to the FMN (Ludwig *et al.*, 1992; Swenson & Zhou, 1996)

Many of the specific protein-cofactor interactions that are responsible for these observed changes in FMN chemistry have been studied in the flavodoxins. Among the interactions that have been investigated is the conformational change that occurs upon reduction to the semiquinone form. As indicated above, this conformational change is limited to the 60's loop in the *C. beijerinckii* flavodoxin, wherein the carbonyl of a conserved glycine (G57 in *C. beijerinckii*) points away from the N5 position of the flavin in a type II beta turn. In the semiquinone form, this loop converts to a structure similar
to a type II' beta turn, with the carbonyl pointing toward the N5H of the semiquinone. forming a new hydrogen bond (Mayhew & Ludwig, 1975; Smith et al., 1977). Those flavodoxins that do not have this conserved glycine (usually replaced with asparagine) demonstrate a more negative OX/SQ midpoint potential.

Oligonucleotide-directed mutagenesis was used to create a number of mutants at this conserved position in both _C. beijerinckii_ and _D. vulgaris_ flavodoxins (Swenson et al., 1991; Ludwig et al., 1997; Mayhew et al., 1996). In the _C. beijerinckii_ flavodoxin, G56A, G56N, and G56T produce $E_{ox/sq}$ values of -143 mV, -162 mV, and -270 mV, respectively. Relative to wild type ($E_{ox/sq} = -92$ mV), these values represent increases in free energy of 0.9, 1.2, and 3.0 kcal/mol. In the _D. vulgaris_ flavodoxin, G61N produced an OX/SQ couple of -248 mV, 100 mV more negative than wild type, in agreement with the sequence comparisons and _C. beijerinckii_ mutant data. Additionally, G61A, G61V and G61L produced $E_{ox/sq}$ values of -311 mV, -333 mV, and -338 mV. These studies show that as the side chain becomes larger, the midpoint potential of the OX/SQ couple becomes more negative. The geometry of the hydrogen bond formed between the carbonyl group and the N5H atom of the isoalloxazine ring is dependent upon the nature of the residues involved in the conformational change (Ludwig et al., 1991; Ludwig et al., 1997). These results lead to the formulation of a hypothesis that defined the stabilization of the OX/SQ couple as a function of two energy terms, an energy of protein-cofactor interaction (which includes the strength of the newly formed hydrogen bond) and a difference in protein conformational energy between the oxidized and the semiquinone.
forms of the holoprotein (which is independent of any interaction with the flavin) (Ludwig et al., 1997).

Recent work has demonstrated that the G57-D58 peptide bond in the C. beijerinckii flavodoxin is composed of a mixture of the unusual cis as well as the more typical trans conformations in the oxidized state, this reverts to all trans in the semiquinone and hydroquinone states (Ludwig et al., 1997). The energetic significance of this change in peptide bond conformation is unclear. However, it has been demonstrated that the cis/ trans population distribution is influenced by the residues that are involved in the peptide bond, indicating a possible structural energy dependence (Ludwig et al., 1997). Yet, the rate of oxidation from semiquinone to oxidized is unaffected by changes in these amino acids (Swenson, unpublished results). The formation of a single hydrogen bond would not appear to be enough to account for the 146 mV change in the $E_{\text{ox/sq}}$ couple, or the observed shift in the pKₐ of the semiquinone FMN. However, NMR spectroscopic analysis of the relative N5H - CO57 H-bond strength in a number of the G57X mutant flavodoxins does indeed indicate that as the strength of this hydrogen bond becomes weaker the OX/SQ couple becomes more negative (Chang & Swenson, unpublished results).

Conformational changes can not justify the large destabilization of the hydroquinone form, since there are no significant structural differences between the one-electron reduced protein and the two-electron reduced state. As indicated above, there is evidence that the hydroquinone is an anion at physiological pH. Experiments involving
the reconstitution of the holoflavodoxin with 1-deaza-FMN demonstrated that there
appears to be steric hindrance to the protonation of the hydroquinone (at N1) in the
flavodoxin from C. beijerinckii, thereby lowering the pKₐ (Ludwig et al., 1990). Because
there are no compensating positive charges in the vicinity of the flavin N1 atom, it was
hypothesized that the observed destabilization of the hydroquinone was due to repulsive
electrostatic interactions. Through the creation of a large number of mutants, in which
many of the acidic residues surrounding the FMN were permanently neutralized by
substitution with their amide equivalents, the midpoint potential of the SQ/HQ couple of
the D. vulgaris flavodoxin was shown to depend upon the number of charges in the
vicinity of the cofactor. Each negative charge produces a shift in the SQ/HQ couple of
approximately -15 mV, while the OX/SQ couple displayed no such trend (Zhou &
Swenson, 1995). Additionally, the pH dependence of the SQ/HQ couple has been shown
to be contingent upon the number of acidic residues surrounding the FMN. This
dependence decreases as the total number of surrounding charges is reduced,
demonstrating almost pH independent behavior when six negative charges are neutralized
(Swenson & Zhou, 1996).

Site-directed mutagenesis has been used to study several specific residues within
the flavin binding site. From sequence comparisons, it was found that there was a
conserved tyrosine residue found on the solvent side of the isaloalloxazine ring. This fact
lead to the substitution of this tyrosine (Y98) in the cloned structural gene of
Desulfovibrio vulgaris with amino acid residues of aliphatic, charged or different aromatic
character. These studies gave some preliminary evidence that the presence of an aromatic residue at this position may alter the midpoint potentials of the redox couples through selective stabilization of specific oxidation states (Swenson et al., 1990; Swenson & Krey, 1994). These interactions have been hypothesized to be charge transfer, electrostatic, or hydrophobic in nature and have been shown to be additive with the electrostatic destabilization caused by the surrounding acidic residues (Zhou & Swenson, 1996b; Chang & Swenson, 1997). These substitutions also indicated that the hydroquinone anion was greatly stabilized by the presence of a positively charge residue and by the removal of unfavorable aromatic-aromatic interactions between the isoalloxazine ring and any of the three aromatic side chains.

While there has been considerable progress, there are still many uncertainties concerning the protein-FMN interactions and/or differences in protein structural stability that separates the two one-electron reduction potentials in flavodoxins. This dissertation is part of the continuing effort toward the elucidation of the means by which flavoproteins alter the chemistry of the bound flavin cofactors. Methionine 56 is located in a critical position, interacting with the re face of the flavin cofactor and participating in a conformational change. As such, this residue can affect the stability of the holoflavodoxin complex by two different mechanisms, altering the protein-cofactor interaction energy and influencing the structural energy of the flavodoxin. Through a comprehensive examination of the effects residue 56 elicits on the oxidation-reduction potentials and the cofactor binding energy, the functionality of this residue was
thoroughly dissected. These examinations included not only those effects specific to
methionine, but also to nearly every possible type of amino acid.

This work strives to answer the following specific questions: 1) Are there
specific interactions with the re face of the isoalloxazine ring, such as sulfur-π
interactions, hydrophobic interactions, and π-π interactions, that serve to stabilize a
particular FMN oxidation state? 2) How do changes in the amino acid sequence of the
60’s loop affect protein stability and thus alter the OX/SQ oxidation-reduction potential?
3) Is cofactor solvent accessibility a factor in the stability of the holoprotein complex? 4)
How does the introduction of a formally charged residue, both positive and negative, at
the re face affect the redox behavior of the flavodoxin. In addition to these specific aims,
this work also extended our knowledge of mechanisms thought to be common to all
flavodoxins.

In order to address these and other questions, methionine 56 was replaced with
thirteen of the remaining naturally-occurring amino acids using oligonucleotide-directed
mutagenesis. The resultant mutant flavodoxins, along with the wild type, were studied
using a variety of experimental and mathematical techniques. The presentation and
analysis of the data have been divided into three sections, each group including those
mutants that have similar chemical characteristics. The first set, M56L, M56I, M56V,
M56A, and M56G, create a series of aliphatic mutants that alters the size and flexibility
of the side chain. The second group, M56C, M56S, M56W, M56Y, and M56F, include
those amino acids that introduce possible electrostatic interactions without the insertion
of a monopole. The last group consists of M56H and M56D, residues that introduce a
formal charge, and M56N as a steric control.
CHAPTER 2

THE ALIPHATIC SERIES

Introduction

Those flavodoxins which have a methionine residue flanking the re (inner) face of the flavin ring generally have less negative midpoint potentials, particularly when compared to those proteins which have a leucine at this position (Table 4). The sulfur atom of methionine 56 in the C. beijerinckii flavodoxin is situated directly over the edge of the o-xylene ring of the FMN (Figure 10). Additionally, this amino acid is the first residue in the β-turn in the 60’s loop that is involved in the conformational change associated with the first one-electron reduction of the FMN. Furthermore, there apparently is a small oxidation state-specific conformational change in the methionine side chain in which the ε-methyl group moves from a position close to the C7 of the isoalloxazine ring in the oxidized form to a position close to the 7-methyl group of the FMN (Figure 10) (Ludwig et al., 1997). Therefore, the observed differences in oxidation-reduction potentials could stem from specific interactions between the methionine side chain and the cofactor, or from differences in the energy associated with each protein structure.
Table 4. Primary Structure and Midpoint Potential\textsuperscript{a,b} Comparisons within the Flavodoxin Family\textsuperscript{c}. \textsuperscript{a} Values reported in mV versus the SHE. \textsuperscript{b} \(E_{\text{OX/SQ}}\) values were adjusted, using 59 mV/pH unit, such that all reported values are at pH 7.0. \textsuperscript{c} See Ludwig and Luschinsky, 1992 and references therein. \textsuperscript{d} The residue which interacts with the \textit{re} face of the FMN is in bold. \textsuperscript{e} \textit{C. pasteurianum} primary sequence is from Fox et al., 1972.

<table>
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<tr>
<th>Source</th>
<th>Aligned 60s Loop Sequences\textsuperscript{d}</th>
<th>(E_{\text{OX/SQ}})</th>
<th>(E_{\text{SQ/HQ}})</th>
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<td>Clostridium beijerinckii</td>
<td>G C S A M G D E V L</td>
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<tr>
<td>Clostridium pasteurianum\textsuperscript{e}</td>
<td>G S P S M G S E V</td>
<td>-132</td>
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</tr>
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</table>
Figure 10. Representations of the Crystal Structure of the *Clostridium beijerinckii* Flavodoxin Highlighting the Conformations of the Peptide Backbone Involving Residues 55-60 and the Side Chain of Met56. Panel A is the oxidized structure and panel B is the semiquinone structure, viewed from the *re* face of the FMN. Note the change in orientation of the carbonyl of Gly57 from the O-down in the oxidized state to the O-up in the semiquinone state with the potential for the formation of a new hydrogen bond at N(5)H of the cofactor. Also note the slight change in the structure of the Met56 side chain. Coordinates and terminology are from Ludwig et al., 1977.
It has been demonstrated that the interaction between the residue flanking the si (outer) face of the FMN in the *D. vulgaris* flavodoxin affects the midpoint potentials of both the OX/SQ and the SQ/HQ couples (Swenson & Krey, 1994; Zhou & Swenson, 1996b). In those studies, tyrosine 98 was replaced by a number of different amino acids, including alanine and methionine. Both of these aliphatic substitutions caused the midpoint potential of the SQ/HQ couple to increase by approximately +140 mV. Conversely, these mutants decreased the potential of the OX/SQ couple by approximately -40 mV and -60 mV for Y98A and Y98M, respectfully. There has also been a limited study of the re-face residue in the *D. vulgaris* flavodoxin, wherein tryptophan 60 was replaced by alanine and methionine (Mayhew et al., 1996). These mutations indicated that W60M lowered the midpoint potential of the OX/SQ couple \(E_{\text{OX/SQ}} = -136 \text{ mV}\) more than W60A \(E_{\text{OX/SQ}} = -157 \text{ mV}\). Both of these mutants shifted the SQ/HQ potential to less negative values relative to the wild-type *D. vulgaris* flavodoxin, *i.e.* -357 mV for W60A and -418 mV for W60M. These changes in redox potentials are likely to be dependent upon the geometry of interaction between the isoalloxazine ring and the interacting amino acid side chain, which is known to differ between the *C. beijerinckii* and the *D. vulgaris* flavodoxins.

The 60's loop, which includes a four-residue β-turn (residues 56-59) in the clostridial flavodoxin, undergoes a conformational change at the peptide bond between position \(i+1\) (glycine 57) and \(i+2\) (aspartate 58) during the one-electron reduction of the oxidized cofactor in which the carbonyl oxygen "flips" from being oriented away from the
flavin to pointing toward the N5H of the blue neutral semiquinone, forming a new hydrogen bond (Figure 10) (Smith et al., 1977, Ludwig & Luschinsky, 1992; Ludwig et al., 1997). The central peptide bond of this four residue turn is found to be in a mixture of the cis and trans conformers. The ratio of cis:trans varies with oxidation state and is affected by mutations at residues 57 and 58 (Ludwig et al., 1997). The structural properties of the residues in the $i+1$ and $i+2$ positions of this turn are critical in the stabilization of the semiquinone form of the flavodoxin (Ludwig et al., 1991; Ludwig et al., 1997). The “flip” of the carbonyl oxygen of the conserved glycine has been observed in other flavodoxins (Laudenbach et al., 1988; Luschinsky et al., 1991; Watenpaugh et al., 1976; Watt et al., 1991) and this glycine has also been shown to be influential in the stabilization of the semiquinone FMN in the flavodoxin from D. vulgaris (Mayhew et al., 1996). The phi and psi angles of methionine 56 (position $i$ in the $\beta$-turn) are also affected by this conformational change and changes in these angles will affect the newly formed cofactor-protein interactions (Ludwig et al., 1997). Significant conformational changes associated with the reduction of the cofactor to the fully reduced hydroquinone are not apparent in any of the flavodoxins (Ludwig & Luschinsky, 1992).

This set of mutants was designed to investigate how specific methionine-FMN interactions in the C. beijerinckii flavodoxin affect the stability of the holoprotein in each oxidation state and to explore how the size of the side chain at this particular position in the protein structure alters these stabilities. Methionine 56 could alter the redox potentials by at least two mechanisms. First, specific side chain-FMN interactions may
selectively stabilize or destabilize a particular oxidation state of the cofactor. These
interactions could include specific sulfur-aromatic interactions, dipole interactions, and
alterations in the polarity and/or solvation of the isoalloxazine ring binding pocket.
Second, because of its location in the critical β-turn, this residue could influence the
conformational change and/or the relative energetics of the preferred protein structures
associated with the oxidized or reduced states, indirectly causing a shift in the midpoint
potentials. Norleucine would be an ideal methionine substitution for the determination of
any effects the thioether moiety elicits. However, the production of this mutant would
require complicated in vitro translation methods which do not produce large amounts of
protein (Noren et al., 1989). Therefore, using oligonucleotide-directed mutagenesis
technology, both structural and sulfur-specific roles of methionine 56 were investigated
by the systematic substitution with the aliphatic amino acid series leucine, isoleucine,
valine, alanine, and glycine. This series of flavodoxin mutants not only gradually
decreases the size of the side chain at position 56 but also provides a means of probing
the role of specific sulfur-flavin interactions in the regulation of the redox properties of
the FMN without the addition of other types of interactions.

Materials and Methods

Materials. Indigo di- and trisulfonate were purchased from Fluka Chemicals.
Benzyl viologen was obtained from Serva Chemicals. Safranine T was purchased from
Fluka chemical company and recrystallized from 95% hot ethanol before use. Flavin
mononucleotide was extracted from a preparation of *C. beijerinckii* flavodoxin and purified via anion exchange chromatography. All other chemicals were of analytical reagent grade.

**Bacterial Strains and Plasmids.** The chemically synthesized artificial gene encoding the flavodoxin from *Clostridium beijerinckii* (MP) has been previously constructed, and cloned into both the pKK223-3 expression vector and the phagemid pBluescript (Eren & Swenson, 1989). The *Escherichia coli* strains XL-1 and AG-1 were used for expression. The CJ236 strain of *E. coli* was used in the production of uracil-enriched, single-stranded DNA for mutagenesis.

**Oligonucleotide Directed Mutagenesis.** The Kunkel method was used to accomplish the oligonucleotide-directed mutagenesis (Kunkel et al., 1987). Single-stranded DNA was generated from the phagemid pBSFlasy (pBluescript with the synthetic flavodoxin gene cloned into the *EcoRI* and *HindIII* sites) in the *E. coli* strain CJ236. Two degenerate oligonucleotides were used:

5′-ATCCTGATCCTGGGTTGCTCTGC(G)XTCGGCGATGAAGTTC-3′ for M56L, M56I, and M56V, and 5′-GGTTGCTCTGCCYZGGGCGATGAAG-3′ for M56A and M56G. The nucleotide in parenthesis is a silent mutation introduced for screening purposes. The underlined nucleotides represent the mutations that introduce the desired amino acid substitutions; X indicates C, G and A, Y indicates G and T, and Z indicates G and C. Each mutation was identified by restriction mapping. All of the mutants remove an *Ncol* site, M56L creates an *Hhal* site, M56I creates an *PvuI* site, M56A creates an
SstII site, and M56G creates an NciI site. All of the mutants were confirmed by dideoxy termination DNA sequencing using the Sequenase protocol (Sanger et al., 1977). The second oligonucleotide also has the possibility to generate two additional mutants, M56W and M56S.

Expression and Purification of Mutant Flavodoxin Proteins. The flavodoxin structural genes were subcloned into the pKK223-3 expression vector using the HindIII and EcoRI restriction sites. The entire reading frame of each mutant subclone was confirmed by dideoxy termination DNA sequencing using the Sequenase protocol (Sanger et al., 1977). The subclones were then transformed into either the XL-1 Blue or AG-1 strain of E. coli for expression. The transformed cells were cultured for between 36 to 48 hours at 37 °C in NZY medium containing 100 μg/mL ampicillin. The cells were then harvested by centrifugation, lysed in a French cell press, and the cellular debris removed by high speed centrifugation. Nucleic acids and flavodoxin were precipitated by slowly adding a stock solution of 1% (w/v) polyethylene imine, with stirring on ice, until the polyethylene imine concentration of the supernatant was 0.15% (w/v). The solution was incubated, with stirring, for 15 minutes. The precipitate was collected by centrifugation and the flavodoxin resolubilized in 50 mM Tris, pH 7.0, 300 mM NaCl. Insoluble material was removed by centrifugation and the supernatant was diluted 3-fold with 50 mM Tris, pH 7.0 and loaded onto a DEAE column equilibrated to pH 7.0. The flavodoxin holoprotein was eluted from the DEAE column, chromatographed on a Sephadex G-50 column and concentrated by ultrafiltration, as previously described (Krey
et al., 1988; Zhou & Swenson, 1995). The Sephadex G-50 column was not used in the purification of the M56I mutant. Instead, a second round of anion-exchange chromatography was applied using a Bio-Rad 5 mL High Q anion exchange cartridge. The column was eluted with a 0-250 mM NaCl gradient at 1.5 mL/min over 60 minutes. Fractions with an $A_{272}/A_{448}$ ratio of 4.9 or less were then desalted and concentrated by ultrafiltration. All mutant preparations were judged to be greater than 90-95% pure by SDS-PAGE (Laemmli, 1970).

**UV/Visible and Fluorescence Spectroscopy.** All UV/visible spectra were recorded using a Hewlett-Packard HP 8452A diode-array spectrophotometer. Fluorescence measurements were made at an excitation wavelength of 445 nm by monitoring the emission at 520 nm on a Perkin Elmer LS50B luminescence spectrophotometer.

**Determination of the Extinction Coefficients of Mutant Flavodoxin Proteins.** Extinction coefficients of the fully oxidized flavodoxin holoproteins were determined using a modified protocol previously described by Mayhew and Massey (1969). The flavodoxin was diluted in 0.1 M potassium phosphate, 0.3 mM EDTA, pH 7.0 such that the $A_{448} = 1.0$. A 300 μL aliquot of this solution was then added to 300 μL 10% (w/v) TCA and incubated, on ice, in the dark. The mixture was centrifuged in a microcentrifuge at 4 °C for 10 min. The supernatant was removed and added to a 2.0 mL volumetric flask containing 600 μL 2 M K$_2$HPO$_4$, 0.6 mM EDTA. The pellet was washed twice with 300 μL 5% (w/v) TCA, 0.3 mM EDTA, each resultant supernatant being added to the volumetric flask. Finally, the flask was filled to 2.0 mL with the 2 M phosphate buffer.
The spectrum of the resultant solution was assumed to contain a quantity of FMN equal to the initial quantity of mutant holoprotein. An extinction coefficient of 12,500 M$^{-1}$cm$^{-1}$ at 445 nm was used for the determination of the FMN concentration (Whitby, 1953).

_Determination of the One-Electron Reduction Potentials._ Oxidation-reduction potentials were determined at 25 °C in 50 mM sodium phosphate, pH 7.0 as previously described (Zhou and Swenson, 1995). The indicator dyes (with their midpoint potentials at pH 7 and 25 °C vs. the standard hydrogen electrode) used were indigo disulfonate (-116 mV), indigo trisulfonate (-75 mV), safranine T (-280 mV), and benzyl viologen (-359 mV) (Michaelis & Hill, 1933; Clark, 1972). The experimental error in the experimental midpoint potential values for the various flavodoxins is estimated to be less than ± 5 mV.

_Determination of the Binding Constants for FMN._ The dissociation constants for the FMN$_{ox}$ complex of the wild-type and the flavodoxin mutants were determined using either ultraviolet-visible absorption spectroscopy or fluorescence spectroscopy. In each case, apoflavodoxin [prepared as previously described (Wassink & Mayhew, 1975; Zhou & Swenson, 1996b)] was titrated into a solution of known FMN concentration until there were no further changes in the absorption spectra or fluorescence. Values for the change in the ultraviolet-visible spectra were determined using the peak-to-valley values from difference spectra generated at each point in the titration. Fluorescence intensity was monitored as emission at 520 nm, with excitation at 445 nm. Corrections for dilution were applied as necessary and the spectral changes were plotted as a function of
apoflavodoxin added. The $K_d$ was then determined by fitting the resultant data to a quadratic solution of the equation describing a normal binding isotherm as follows:

$$I = I_{\text{max}} \cdot \left(1 - \frac{([\text{FMN}]_r + [\text{Apo}]_r + K_d) - \sqrt{([\text{FMN}]_r + [\text{Apo}]_r + K_d)^2 - 4 [\text{FMN}]_r [\text{Apo}]_r}}{2 [\text{FMN}]_r}ight) + I_{\text{res}}$$

or

$$\Delta A = \Delta A_{\text{max}} \cdot \frac{([\text{FMN}]_r + [\text{Apo}]_r + K_d) - \sqrt{([\text{FMN}]_r + [\text{Apo}]_r + K_d)^2 - 4 [\text{FMN}]_r [\text{Apo}]_r}}{2 [\text{FMN}]_r}$$

$I$ or $\Delta A$ are the fluorescence intensity or peak to valley absorbance difference. $[\text{FMN}]_r$ and $[\text{Apo}]_r$ are the total flavin concentration and the total apoflavodoxin concentration at each titration point. $I_{\text{max}}$ and $\Delta A_{\text{max}}$ are the maximal fluorescence intensity and the maximal peak to valley value. $I_{\text{res}}$ is the residual fluorescence. Note that these experiments were carried out under conditions identical to those used for the determination of the midpoint potentials. The $K_d$ values for the semiquinone and hydroquinone forms of the cofactor were determined using a thermodynamic box, using the binding constant of the oxidized cofactor, the determined holoprotein midpoint potentials, and the published one-electron reduction potentials for free FMN (Draper & Ingraham, 1968).
Molecular Modeling. Each mutant protein structure was generated by replacing methionine 56 with the appropriate amino acid using the HyperChem molecular modeling software (Autodesk, Inc.). The coordinates for the oxidized (3fxn) and semiquinone (4fxn) forms of the flavodoxin were obtained from the Brookhaven Protein Data bank. Mutant structures for each major side chain rotomer conformation were generated by altering the appropriate torsion angle. Each of these structures was subjected to the following energy minimization strategy using the AMBER force field (Weiner et al., 1984) implemented by the HyperChem modeling package (Autodesk, Inc.). All the residues within a 7 Å radius of the Cα of residue 56 were selected and extended to the next sp3 atom. Next, 100 cycles of energy minimization using the steepest descent algorithm were applied, using a distance dependent dielectric with a scale factor of 1, 1-4 scale factors of 0.5, and a switched cutoff with outer and inner radii of 14 Å and 10 Å, respectively. The minimization was completed by the application of the Polak-Ribiere conjugate gradient, using the same parameters, converging to a RMS gradient endpoint of 0.01 kcal Å⁻¹ mole⁻¹. The rotomer structure of each mutant with the lowest energy was used for further analysis. Although the FMN atoms were included in the calculation, the cofactor itself was not allowed to be geometry optimized during the energy minimization. This was done because of the uncertainty for several parameters for the flavin molecule. Single point energy calculations for residues of interest (extended to sp3) were performed similarly. The solvent exposed surface of the cofactor in each energy minimized mutant
structure was determined with the Quanta software package (Molecular Simulations Inc.). using a rolling ball algorithm with a probe radius of 1.4 Å (Connolly, 1983).

Results

Generation, Expression and Characterization of the Mutants. A series of five flavodoxin mutants in which methionine 56 was substituted with other aliphatic amino acids and glycine was generated by the Kunkel method of oligonucleotide-directed mutagenesis using two sets of degenerate oligonucleotide primers. The flavodoxin holoproteins were over-expressed in *E. coli* using the pKK223-3 expression vector. The color of the cellular pellet ranged from green to dark blue, indicative of the *in vivo* accumulation to various extents of the blue neutral semiquinone form of the flavodoxin, just as for wild type (Eren & Swenson, 1989). Each flavodoxin mutant was purified as the holoprotein in the fully oxidized state. A modification to our standard purification procedure was necessary, however, to purify the M56I mutant as holoprotein, due to the relatively low FMN binding affinity. The flavodoxin preparations were judged to be greater than 90-95% pure based on SDS-PAGE. The $A_{280} : A_{450}$ ratio of all the mutants was $4.5 \pm 0.2$, similar to wild type and indicative of the stoichiometric binding of the FMN cofactor.

The visible absorbance spectra of each mutant were determined in all three redox states during reductive titrations with sodium dithionite under anaerobic conditions (Figure 11). The overall spectral characteristics of each mutant remain very similar to
Figure 11. The Near UV/Visible Absorbance Spectra of the Recombinant Wild-type *C. beijerinckii* and the Aliphatic M56X Mutant Flavodoxins. Wild type, M56A, M56V, M56I, M56L, and M56G flavodoxin mutants are shown in panel A-F, respectively. Spectra were determined in 50 mM phosphate buffer, pH 7.0. In each panel, the solid line represents the spectrum of the oxidized state, the dot-dashed line represents the blue neutral semiquinone spectrum, and the dashed line the fully reduced hydroquinone spectrum. *Insets* are plots of the changes in absorbance in the 580 nm region (representing the $\lambda_{\text{max}}$ of the semiquinone species) vs. those in the 450 nm region (largely representing the oxidized species) during the course of the reductive titration.
that of wild type in all three redox states; however, some subtle differences were noted. The \( \lambda_{\text{max}} \) for the oxidized flavin was shifted slightly to 448 nm in the M56L mutant, to 446 nm for M56V, M56I, and wild type, and to 444 nm for M56A (based on the 2 nm resolution of the spectrophotometer). A shift to lower wavelengths as side chain size decreases may be consistent with a slight increase in the polarity of the flavin environment. However, a concomitant shift in the \( \lambda_{\text{max}} \) of the second transition was not apparent, so this conclusion is somewhat tentative (Müller, 1991). The characteristics of the hydroquinone spectrum for each mutant are similar to wild type. Shoulders at 450 and 310 nm and a distinct peak around 370 nm were noted. The extinction coefficient of the 370 nm peak generally decreases as the side chain size increases. These spectral characteristics have been interpreted as indicating a planar, anionic hydroquinone (Ghisla et al., 1974). The flavin fluorescence spectra for all the mutants were similar with the fluorescence intensity quenched to values less than 1% of that of the free FMN as in wild type.

**Determination of the Oxidation-Reduction Potentials.** The one-electron reduction potentials for both couples of the FMN cofactor for each mutant were determined at pH 7.0 and 25 °C by equilibration with redox indicator dyes of known \( E_m \) values (Clark, 1972). The midpoint potentials of the OX/SQ couple for M56L, M56I and M56V were all more negative than wild type, poised around -125 ± 8 mV (Table 5). The midpoint potentials for the M56A (-72 mV) and M56G (-84 mV) mutants are less negative than wild type. All of these data conformed well to the linearized form of the Nernst equation.
Table 5. Oxidation-Reduction Midpoint Potentials\(^a\) and FMN Dissociation Constants\(^b\) of the *C. beijerinckii* Wild Type and the Aliphatic M56X Mutant Flavodoxins. \(^a\) Values are reported in millivolts versus SHE, pH 7.0, 25 °C; the error in the midpoint potentials is estimated to be ± 5 mV. \(^b\) The dissociation constants for FMNO\(_{OX}\) are measured at pH 7.0, 25 °C, the FMNSQ and FMNHQ values are calculated using published FMN midpoint potential values (Draper and Ingraham, 1968). \(^c\) Determined via fluorescence spectroscopy. \(^d\) Determined via visible spectroscopy. \(^e\) From Ludwig *et al.* (1997). \(^f\) Potentials determined using indigo disulfonate, indigo trisulfonate, benzyl viologen, and safranine T as the redox indicator, respectively.

<table>
<thead>
<tr>
<th>Flavodoxin</th>
<th>(K_d^{OX}) (μM)</th>
<th>(K_d^{SQ}) (nM)</th>
<th>(K_d^{HQ}) (μM)</th>
<th>(E_{OX-SQ})</th>
<th>(E_{SQ-HQ})</th>
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<td>WT</td>
<td>0.018 ± 0.002(^d)</td>
<td>0.06 ± 0.01</td>
<td>0.42 ± 0.15</td>
<td>-92(^g)</td>
<td>-399(^g)</td>
</tr>
<tr>
<td>M56L</td>
<td>0.180 ± 0.02(^d)</td>
<td>2.46 ± 0.8</td>
<td>2.1 ± 0.8</td>
<td>-128(^f)</td>
<td>-345(^h)</td>
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<tr>
<td>M56I</td>
<td>1.500 ± 0.2(^d)</td>
<td>16.2 ± 3.5</td>
<td>3.3 ± 1.1</td>
<td>-122(^f)</td>
<td>-308(^h)</td>
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<tr>
<td>M56V</td>
<td>0.480 ± 0.02(^d)</td>
<td>6.31 ± 1.6</td>
<td>2.8 ± 1.0</td>
<td>-127(^f)</td>
<td>-328(^h)</td>
</tr>
<tr>
<td>M56A</td>
<td>0.044 ± 0.008(^c,d)</td>
<td>0.07 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>-72(^i)</td>
<td>-331(^h)</td>
</tr>
<tr>
<td>M56G</td>
<td>0.270 ± 0.03(^d)</td>
<td>0.66 ± 0.2</td>
<td>0.3 ± 0.11</td>
<td>-84(^i)</td>
<td>-326(^h)</td>
</tr>
</tbody>
</table>
generating slopes of $59 \pm 6$ mV, indicative of a one-electron equilibrium at 25°C (Figure 12).

The midpoint potentials for the SQ/HQ couple of all the flavodoxin mutants, as determined by equilibration with benzyl viologen ($E_{m,7} = -359$ mV), were less negative than wild type (Table 5). The M56L mutant, likely to be the most structurally conservative mutation, displayed the smallest shift from the wild-type value, increasing by 50 mV. Three mutants, M56V, M56A and M56G, had intermediate midpoint increases of $65 \pm 4$ mV. Substitution with isoleucine resulted in the largest effect, increasing the midpoint by more than 90 mV to a value of -308 mV. This value, which is near the limit of the range for benzyl viologen, was confirmed using safranine T ($E_{m,7} = -280$) as the redox indicator dye.

These results demonstrate that the one-electron reduction potential of both couples are highly dependent upon the nature of the side chain at position 56. These dependencies could result from two factors, 1) the chemistry of the interaction between the side chain and each oxidation state of the cofactor, and 2) the relative stability of the different protein structure found in each redox state. The contribution of each factor will be discussed in detail below. It is also important to note that in no case was the midpoint potential for the SQ/HQ couple more negative than for wild type.

**Determination of the FMN Dissociation Constants.** The dissociation constants for $\text{FMN}_\text{ox}$ have been determined for each mutant by visible absorption spectroscopy or alternatively by spectrofluorimetry in the cases where the very low flavin concentrations
Figure 12. Determination of the Midpoint Potentials for the Aliphatic Met56 Mutants. Panel A is the OX/SQ couple. Panel B is the SQ/HQ couple. In each case, the one-electron reduction potential was determined in 50 mM phosphate, pH 7.0, at 25 °C. Symbols in each panel represent the data obtained for each flavodoxin as follows: M56L, •; M56I, ■; M56V, ▲; M56A, ▼; M56G, ◆.
necessary produced absorbance changes too low to obtain reliable data (i.e. fluorescence is much more sensitive). Examples of each type of binding data are shown in Figure 13. The titration data could be fit accurately to a quadratic form of the binding isotherm as shown by the binding curves associated with each plot, generating reliable values for the dissociation constant for each mutant holoprotein. The $K_d$ for the FMN$_{ox}$ was increased in all of the aliphatic mutations (Table 5). The two mutations that introduce a $\beta$-methyl group, M56I and M56V, cause the largest decrease in FMN$_{ox}$ affinity relative to wild type, increasing the $K_d$ to $1.5 \pm 0.4 \mu M$ and $0.48 \pm 0.02 \mu M$, respectively, with the smaller valine side chain allowing tighter binding than the more bulky isoleucine. The M56L and M56A mutants increase the $K_d$ to $0.18 \pm 0.02 \mu M$ and $0.044 \pm 0.008 \mu M$, respectively, following the trend that a smaller side chain at position 56 in the C. beijerinckii flavodoxin favors binding of the FMN$_{ox}$. If the side chain is completely removed, as in the M56G mutant, the binding is weakened by approximately six-fold relative to the M56A mutant and 15-fold relative to wild type.

Because free energy changes associated with binding are pathway independent, a thermodynamic cycle can be employed for the calculation of the dissociation constants of both the semiquinone and hydroquinone forms of the cofactor (Figure 14). Such calculations require knowledge of the binding constant for the oxidized cofactor, the midpoint potentials for each redox couple for each mutant, and the established one-electron midpoint potentials for unbound FMN (DuBourdieu et al., 1975). The calculated values for the dissociation constants for the reduced forms of the FMN are
Figure 13. Determination of the Dissociation Constants for the M56L mutant (A) and Wild-type C. beijerinckii (B) Flavodoxins. In each case data was collected in 50 mM phosphate buffer, pH 7.0, at 25 °C. For the M56L flavodoxin, difference spectra were generated for each titration point (inset shows representative difference spectra) and the peak-to-valley values were plotted as a function of apoprotein concentration. For wild-type flavodoxin, the formation of holoprotein was monitored by the quenching of the flavin fluorescence. Each data set of spectral changes were fit to a quadratic solution to the equation describing a single-binding site binding isotherm.
Figure 14. Thermodynamic Box Describing the Relationship Between Oxidation-Reduction Potentials and Dissociation Constants in Flavodoxins. Apo represents the free apoprotein. The midpoint potentials for free flavin are from Draper & Ingraham, 1968. If the dissociation constant for the oxidized cofactor is known, the values for the semiquinone and hydroquinone FMN can be calculated.
included in Table 5. The M56A mutant binds the blue neutral FMN semiquinone very tightly, comparable to wild type, while all the rest of the mutants form significantly weaker complexes with the flavin semiquinone. In the case of M56I, the $K_d$ for the semiquinone has increased by over 270-fold. In all cases, however, the semiquinone form of the FMN is the most tightly bound oxidation state, just as in wild type. The FMN hydroquinone complex is typically significantly destabilized relative to the oxidized and semiquinone states in the flavodoxin, representing an important means of establishing the low reduction potentials that typify this class of flavoprotein electron transferase (Ludwig & Luschinsky, 1992). This trend is generally true for this series of flavodoxin mutants. However, it is interesting to note that for the M56A and M56G mutants the hydroquinone is bound with approximately equal affinity to the oxidized state. In the case of M56A, this is accomplished by the significant improvement (by about 14-fold) in the binding of the hydroquinone relative to wild type. Wild type and M56G bind the hydroquinone FMN to an equal extent. The M56L, M56I, and M56V mutants all displayed approximately 6-fold weaker binding affinities for the FMN hydroquinone than wild type (Table 5).

**Molecular Modeling.** Molecular modeling and molecular mechanical calculations were initiated in an attempt to gain further insight into the structural consequences of these amino acid substitutions. In any energy minimizing calculation, it is important to ensure that the final structure is the structure at the global energy minimum. These types of calculations take a given structure and alter the coordinates of the atoms, comparing the
initial energy to the energy of the newly generated structure. The calculation continues, searching for lower energy conformations, until the energy difference is less than a given constraint. Therefore, it is important to sample the entire energy surface of a given structure by using a number of initial structures in an energy calculation. If there is only one energy minimum, all of the starting structures will minimize to the same conformation. However, if there are local energy minima the starting structures will minimize to different final configurations. Figure 15 graphically illustrates how the choice of the starting structure can affect the final energy-minimized structure.

There are two methods for generating a sample of the protein energy surface for use as starting structures in energy minimizations. The first procedure involves the use of molecular dynamics, wherein an increase in the energy of the system is simulated at constant temperature. This simulation will cause the positions of the atoms to fluctuate, producing differing conformations as a function of time. The structure of the system is saved at designated time intervals and successively used as starting structures in energy minimizations. The second method, and the one employed in this study, is to manually generate starting structures which represent the range of possible protein conformations. For this series of mutants, the starting structures were generated by altering the torsion angles of the mutant side chains, producing nine starting structures for both M56L and M56I and three structures for M56V. Since there are no side-chain torsion angles for either alanine or glycine, M56A and M56G had only one side-chain specific starting structure. In each case, the backbone atom coordinates were identical to the wild-type
Figure 15. A Two-Dimensional Representation of a Protein Structural Energy Surface. The open star represents the global minimum. The open circles represent local energy minima. The filled circles represent possible starting points in an energy minimization.
structure. This exploration was successful in identifying the most probable global energy minimum for all of the mutants (Figure 16). The structures found at these global energy minima were used for all subsequent calculations and discussion.

Given the rather conservative nature of the substitutions introduced, such calculations are likely to yield reasonably reliable indications of the highly localized structural effects. All of the mutant structures converged to similar energy values and none of the substitutions introduced large changes in either the oxidized or the semiquinone structure. The small changes that were observed were localized within the immediate vicinity of the substitution, with no atom moving more than 1.0 angstroms relative to the equivalent position in the wild-type structure if applicable. These predictions are consistent with the actual three-dimensional structure data which indicated that mutations in this region produce very minor structural changes (Ludwig et al., 1997) and with the minor changes in the spectral properties of the bound FMN. The relative energy of each mutant in both the oxidized and semiquinone states was calculated by including only residues 55, 56 and 57 (extended to the next sp3 atom) in a single point energy calculation using the AMBER force field with the same parameters used in the minimizations. The difference in conformational energies between the oxidized and semiquinone states can be used as a measure of the relative stability of the semiquinone structure for each mutant in this localized region only. The differences in the single point energies for this region, between the semiquinone and the oxidized state, varied from 0.2 kcal/mol for M56G to 2.2 kcal/mol for the M56L mutant, suggesting that in the
Figure 16. Representations of the Energy-Minimized Structures of the Aliphatic M56X Mutants. The structure of the backbone (residues 55-60) and residue 56 side chain atoms from the energy-minimized structure of M56M (wild type), M56L, M56I, M56V, M56A, and M56G (A-F respectively).
absence of other non-bonding interactions a smaller side chain at position 56 favors the formation of the semiquinone structure (Table 6). While one must exercise some caution in the interpretation of geometry-optimized structures, the substitutions also seemed to introduce small changes in the hydrogen bond stabilizing the β-turn involving residues 56 to 59. For example, the M56A and M56G mutants seem to have slightly more favorable intra-loop hydrogen bonding geometry (shorter N to O distance and/or a CO to N angle closer to 180°), in the semiquinone state while the M56L, M56I, and M56V mutants have slightly better hydrogen-bond geometry in the oxidized state (Table 6).

The modeled structures also suggested that changing the side chain size at position 56 could significantly affect the solvent exposure of the bound FMN cofactor. As expected, based on the rolling ball algorithm (Connolly, 1983), the exposed surface of the flavin systematically increased from 141 to 168 Å² (wild type vs. M56G) as the size of the side chain at position 56 decreases, with the change in exposed area being localized mainly to the o-xylene subnucleus of the isoalloxazine ring (Table 6).

Discussion

Flavodoxins have served as an exceptionally good system in which to investigate the roles of specific flavin-protein interactions in the control of the physio-chemical properties of the bound cofactor. In particular, the means by which the one-electron reduction potentials are modulated is especially significant in flavoprotein electron
Table 6. Results from the Analysis of the Energy-minimized Aliphatic M56X Mutant Structures.  

<table>
<thead>
<tr>
<th>Mutant</th>
<th>ΔΔG_{SQ/OX} $^a$</th>
<th>Exposed FFMN $^b$</th>
<th>CO57 - N5$^{FMN}_M$ $^c$</th>
<th>CO56 - N59 $^d$</th>
<th>CO56 - N60 $^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>M56L</td>
<td>+2.2</td>
<td>145</td>
<td>3.1</td>
<td>131</td>
<td>2.8 / 2.9</td>
</tr>
<tr>
<td>M56I</td>
<td>+1.4</td>
<td>149</td>
<td>3.0</td>
<td>130</td>
<td>2.8 / 3.1</td>
</tr>
<tr>
<td>M56V</td>
<td>+0.7</td>
<td>157</td>
<td>2.9</td>
<td>138</td>
<td>2.8 / 3.3</td>
</tr>
<tr>
<td>M56A</td>
<td>+0.4</td>
<td>166</td>
<td>3.0</td>
<td>129</td>
<td>2.7 / 2.9</td>
</tr>
<tr>
<td>M56G</td>
<td>+0.2</td>
<td>168</td>
<td>3.0</td>
<td>129</td>
<td>2.7 / 2.9</td>
</tr>
</tbody>
</table>

$^a$ Calculated as the difference in the single point energies of residues 55-57 in the SQ and the OX states (see Materials and Methods).

$^b$ The solvent accessible surface area of the FMN in each mutant semiquinone structure, expressed in Å².

$^c$ This H-bond is only present upon reduction of the flavodoxin to the blue neutral semiquinone.

$^d$ These are the H-bonds present within the turn which undergoes the conformational change between the OX and the SQ states.
transferred. In the flavodoxin, the FMN binding site provides a limited number of different types of molecular contacts with either face of the isoalloxazine ring system. The interactions are generally apolar and either aliphatic or aromatic in nature. Aromatic interactions predominate, particularly at the $si$ face of the flavin; however, the details of these interactions are significantly different. The nature of the residue flanking the $re$ face varies to a greater extent. Several flavodoxins have a methionine residue at this position and, in general, these proteins display more positive oxidation-reduction potentials, particularly for the OX/SQ couple (Table 4). Given the rather conservative structural differences between this methionine and the leucine residue found in other flavodoxins such as from *A. vinelandii*, the reduction potential differences are intriguing and raise the question as to whether the unique properties of the methionine play a significant role in establishing these differences. Therefore, in this classification of mutants the role of methionine 56 flanking the $re$ face of the FMN cofactor in the *C. beijerinckii* flavodoxin was investigated. The substitution of other aliphatic amino acids at this position was designed to determine if interactions between the methionine side chain and the flavin differentially stabilize or destabilize each oxidation state of the cofactor either through specific non-bonding effects such as sulfur-aromatic interactions, through differential conformational stabilities, and to probe the effect of side chain size and/or solvent exposure, ultimately evaluating how these interactions might influence the oxidation-reduction potentials in this flavodoxin.
The results of this study demonstrate quite clearly that methionine 56 plays an important role in the control of the one-electron reduction potentials in this flavodoxin. The midpoint potentials for each redox couple were seen to vary by > 50 mV for the OX/SQ couple to nearly 100 mV for the SQ/HQ couple depending on the amino acid at position 56 (Table 5). This was particularly surprising given the rather conservative nature of these substitutions. Of course, the observed changes in midpoint potentials must result from changes in the relative binding energies for each oxidation state of the FMN cofactor (Table 7). However, it is evident from the analyses that the substitutions affect the stability of each oxidation state through different mechanisms. The comparison of these binding energies is facilitated by the free energy diagram shown in Figure 17, which depicts the free energy of cofactor binding in each of the oxidation states. One deficiency in this type of comparison is the uncertainty of the "ground state" for each flavodoxin species which, as will be subsequently discussed, is not necessarily the same in each case. In this presentation, all of the free energy levels are relative to the free energy for the oxidized wild-type holoflavodoxin complex which has been set to zero. In the discussion of this diagram, we have considered a model very similar to one previously formulated to assist in the interpretation of results obtained from amino acid substitutions at positions 57 and 58 in this flavodoxin (Ludwig et al., 1997). In this model, the stability of the holoflavodoxin in each oxidation state is determined by three equilibria: the formation of the apoprotein from a random state which is incapable of cofactor binding (ΔGr), the conversion of the apoprotein structure into the protein structure found in the
Table 7. Gibbs Free Energy of FMN Binding\textsuperscript{a,b,c} for \textit{C. beijerinckii} Wild Type and the M56X Mutant Flavodoxins. \textsuperscript{a} Values are reported in kcal/mol. at 25 °C. \textsuperscript{b} The error in these values is estimated to be 0.1-0.2 kcal/mol. \textsuperscript{c} Values determined using: \( \Delta G = RT\ln K_d \).
Figure 17. Free Energy Diagram Comparing the Relative Changes in the Free Energy of Binding for the Various Oxidation States of the FMN Cofactor by Wild-type and the Aliphatic Met56 Mutant Flavodoxins. The horizontal bars represent the differences in the free energies from the oxidized wild-type holoprotein complex. The numbers represent the differences in the free energy of binding between the oxidized (ox) and semiquinone (sq) as well as semiquinone and hydroquinone (hq) states as indicated by the dashed lines. The free energy set depicted by the dotted lines has been adjusted to correct for entropic or steric factors expected to contribute equally to the destabilization of all three holoprotein oxidation states.
holoflavodoxin ($\Delta G_a$), and the interactions formed between the protein and the FMN ($\Delta G_i$) (**Figure 18**). The total free energy change associated with FMN binding as represented in **Figure 17** will be $\Delta G_a + \Delta G_i + RT\ln (1 + K_f)$, where $K_f$ is the equilibrium constant defined by $\Delta G_f$. The following discussion will analyze the possible contributions of each of these energies and how they differ with redox state and discuss the affects these differing stabilities elicit on the midpoint potentials.

**Possible Factors Affecting $\Delta G_i$.** The specific molecular interactions which contribute to $\Delta G_i$ include protein-FMN hydrogen bonding, hydrophobic interactions, steric effects, possible sulfur-aromatic and electrostatic interactions. It is expected that several of these types of interactions could be altered by the amino acid substitutions introduced in the methionine 56 mutants. It is important, however, to distinguish changes that occur similarly in all three oxidation states from those changes that differentially effect one or more. It is only the latter type that results in shifts in reduction potentials. Several hydrogen-bonding interactions between the *C. beijerinckii* flavodoxin protein and the FMN cofactor are evident in the X-ray crystal structure including interactions with the 5'-phosphate, the ribityl hydroxyls, and the isoalloxazine ring (Smith *et al.*, 1977; Ludwig *et al.*, 1997). Changes in the hydrogen bonding to the 5'-phosphate and ribityl hydroxyls are likely to be independent of the oxidation state (van Mierlo *et al.*, 1990); however, because the partial charges on the atoms of the isoalloxazine ring change upon reduction, any alteration in the strength of a hydrogen bond involving an isoalloxazine ring atom is apt to change with redox state, (Zeng & Ornstein, 1996). Examination of the three-
Figure 18. Energy Diagram Demonstrating the Energies Affecting the FMN Binding Energy. Random, Apoprotein, Protein, and Holoprotein are protein structural forms identical to those described in Figure 17. All free energies also correspond to those described in Figure 17, except $\Delta G_{kd}$. This latter free energy is associated with the measured FMN dissociation constant ($i.e. \Delta G_{kd} = RT\ln K_d$). Note, the random to apoprotein equilibrium is in gray to emphasize that it has only a small effect on the binding energy.
dimensional structure of other flavodoxin mutants and the energy-minimized structures of the methionine 56 mutants in the oxidized and semiquinone states, indicated that the structural changes caused by mutations in this region of the protein are small. Hydrogen-bonding geometries including bond length and angle, if affected, are not altered by more than 6%, including the hydrogen bond between N5H of the FMN semiquinone and the carbonyl of glycine 57 (Ludwig et al., 1997). Therefore, it is reasonable to conclude that the methionine 56 substitutions do not significantly alter the strength of the hydrogen bonding between the FMN and the flavodoxin in the any of the oxidation states. It has, however, been postulated that the CO$_{\text{57}}$-N5H$_{\text{FMN}}$ hydrogen bond is extremely sensitive to mutations in this region (Ludwig et al., 1997). Additionally, small changes which are undetectable by this analysis, summed over a number of hydrogen-bonds, could produce changes in free energy of the magnitude observed in these mutations. Therefore, while it seems unlikely that these mutations substantially alter any flavin-protein hydrogen bonds, additional data (perhaps using NMR spectroscopy) are necessary in order to confirm this conclusion.

Since the size of the aliphatic side chain which interacts with a planar face of the cofactor is being altered, the strength of the hydrophobic interaction energy might be expected to be modified by these mutations. As anticipated, the solvent accessible surface area of the FMN, determined by the application of a rolling ball algorithm to the energy minimized structures, increases as the size of the re-face side chain decreases. Thus, if the hydrophobic effect is a major factor influencing FMN binding, as proposed
by Janin & Chothia (1978), M56L and M56I should bind tighter than M56A and M56V, respectively. This is not the case (Tables 5 & 7 and Figure 17). Our results agree with the calorimetric work done on the *Azotobacter vinelandii* flavodoxin, which indicate that hydrophobic interactions are not the thermodynamic driving force in cofactor binding (Carlson & Langerman, 1984). Additionally, the majority of the FMN binding energy is contributed by the 5'-phosphate moiety in the wild type *C. beijerinckii* flavodoxin (Mayhew & Ludwig, 1975). Therefore, if the mutants did alter the hydrophobic interaction energy of the isoalloxazine ring, the overall changes in binding energy should be relatively small.

The methionine side chain is very flexible and thus can pack against the isoalloxazine ring so as to minimize any steric repulsion. The introduction of branched methyl groups reduces side chain flexibility, a β-methyl more so than a γ-methyl, and could cause steric interference toward flavin binding. The introduction of a γ-methyl branched side chain (M56L) significantly reduces the stability of the holoprotein complex by 1.4 kcal/mol (Figure 17). Comparison of the binding energies of M56L and M56I indicates that moving the methyl group to the β-position increases the destabilization of the holoprotein by an additional 1.2 kcal/mol. These results are consistent with the introduction of steric repulsive forces by the substitutions. If this is the case, the removal of side chain mass should produce tighter binding. Comparison of binding energy of M56L to that of M56A and of M56I to M56V demonstrates that indeed a smaller side chain produces greater holoprotein stability. Thus, it seems that repulsive steric forces
are at least partially responsible for the decrease in binding affinity for M56L, M56I and M56V. This steric reduction of binding affinity is likely to be oxidation-state independent, contributing equally to the destabilization of each oxidation state of these three mutants. But for the M56A mutant, which can not introduce any steric repulsion, the oxidized holoprotein complex is also less stable than wild type. Why?

Because hydrophobic interactions do not seem to be major contributors to cofactor binding, the major difference between wild type and M56A is the removal of the thioether moiety. It has been hypothesized that there is an attractive electrostatic interaction between sulfur atoms and aromatic rings in proteins (Morgan et al., 1978; Reid et al., 1985). The possibility of an electrostatic interaction between sulfur and an aromatic ring system stems from the quadrupole moment generated by the π-electron cloud, generating a negative potential above and below and positive potential along the plane of the ring (Dougherty, 1996; Hunter & Sanders, 1990). The nature of the interaction is dependent upon the polarization of the aromatic ring produced by the heteroatoms and the geometry of the sulfur-aromatic interactions (Dougherty, 1996; Reid et al., 1985). These types of interactions have been measured to be between -0.2 and -0.75 kcal/mol in model peptides (Viguera & Serrano, 1995; Stapley et al., 1995). The sulfur-aromatic interaction geometry found in the C. beijerinckii flavodoxin (Figure 10) could lead to a favorable interaction if the isoalloxazine ring is polarized such that the dimethyl benzene subnucleus has a positive surface potential, very probable considering the location of the heteroatoms. Therefore, the loss of ~0.5 kcal/mol in binding energy
associated with M56A, relative to wild type, could be ascribed to the removal of the sulfur atom. Any such sulfur-aromatic interactions is likely to be dependent upon the oxidation state of the holoprotein. Reduction to the semiquinone increases the electron density of the FMN, and is accompanied by protein conformational changes which seems to alter the sulfur-aromatic interaction geometry (Ludwig et al., 1997). The addition of the second electron generates the anionic FMN hydroquinone which should significantly decrease any attractive sulfur-aromatic interactions, possibly even becoming repulsive.

The striking destabilization of the hydroquinone that is characteristic of the flavodoxins is largely due to electrostatic interactions and, perhaps to a lesser extent, the polarity of the isooalloxazine ring binding site (Zhou & Swenson, 1995, 1996a, 1996b). The polarity of the binding site will affect the solvation free energy, or self-energy, of the hydroquinone anion, the self-energy being the energy associated with moving a charged species between two media of differing dielectric constants. Moving an ion from a polar environment to an apolar environment is endothermic (Born, 1920). Therefore, as the polarity of the binding site increases, the self-energy decreases, and the hydroquinone anion-apoflavodoxin complex becomes more stable. So, based on the FMN solvent exposure determined for each mutant and the hydrophobicity of the residue 56 side chain, it was expected that the hydroquinone stability of this series would follow as, WT < M56L < M56I < M56V < M56A < M56G. The data does not fit with this trend. But when the binding energies for each mutant are adjusted by subtracting out increases expected to contribute equally to the destabilization of all three holoprotein oxidation
states (see discussion below), the free energy levels for the hydroquinone complex do seem to follow the expected trend. (This "adjustment" is represented by the corresponding sets of free energy levels shown in dashed lines in Figure 17. The free energy level for the oxidized complex was set to 0.5 kcal/mol to reflect the loss of the sulfur-aromatic interaction and, therefore, to correspond to that of the M56A mutant.) It is very difficult to determine the self-energy of the anionic hydroquinone FMN from these data. However, there are electrostatic models capable of such calculations and the application of these models to this system could greatly enhance this analysis (Sharp & Honig, 1990; Warshel & Aquist, 1991).

Possible Factors Affecting $\Delta G_f$ and $\Delta G_a$. Both of these energies are related to protein structural stability. $\Delta G_f$ is the energy difference between an unfolded or denatured state and the folded apoprotein (Figure 18). This equilibrium is known to be affected by single amino acid substitutions, and can be altered by changes in any of a number of non-covalent forces that are dependent upon side chain size (Shortle & Meeker, 1986; Shortle et al., 1990; Zhu et al., 1993; Cornish et al., 1994). It is difficult to predict how this series of mutants might affect this equilibrium without more detailed structural and thermodynamic data. It is tempting to assign the ~1 kcal/mol destabilization demonstrated by M56G compared to M56A (Table 7) to an increase in the entropy of the unfolded state (Shortle et al., 1990; D'Aquino et al., 1996). However, it is important to consider that the amount by which changes in the unfolded-folded equilibrium affect the measured dissociation constant are directly related to the magnitude
of $K_f$. If this equilibrium constant is very small, effectively isolating this equilibrium, changes in this equilibrium will have only minor effects on the measured $K_d$. While there is no hard data concerning this equilibrium for the *C. beijerinckii* flavodoxin, anecdotal evidence (gained through handling of the apoprotein) indicates that the apoprotein is very stable. Additionally, the Gibbs free energy for the thermal unfolding of the *Anabaena* 7119 apoflavodoxin is $\sim 15$ kJ/mol (Genzor *et al.*, 1996a). Thus, changes in $\Delta G_f$ are unlikely to affect the measured FMN dissociation constant.

$\Delta G_a$ is the energy difference between two apoprotein structures; the structure found in the FMN free apoprotein and a virtual apoprotein structure equivalent to the structure of the holoprotein but lacking the cofactor (Figure 18) (see also Ludwig *et al.*, 1997). There is evidence for a conformational change between the apoprotein and holoprotein structures in flavodoxins (Barman & Tollin, 1972; DuBourdieu *et al.*, 1974; Leenders *et al.*, 1993). In the flavodoxin from *Anabaena* 7119, this structural change has been identified as being small and localized around tryptophan 57, the residue analogous in location to methionine 56 in the *C. beijerinckii* flavodoxin (Genzor *et al.*, 1996b). Additionally, it has been demonstrated that methionine 56 is involved in a conformational change which occurs in the holoprotein upon reduction to the semiquinone state (Smith *et al.*, 1977; Ludwig *et al.*, 1997). Therefore, these mutants might alter this equilibrium in all three oxidation states.

A mutation which affects the stability of the folded apoprotein (Apoprotein in Figure 18) without altering the stability of any of the three virtual apoprotein
conformations, will alter the cofactor affinity equally in all three oxidation states. An increase in this relative stability will increase the measured dissociation constant. Since glycine is known to increase the entropy of proteins (see above), the observed changes in cofactor affinity in M56G could indicate an increase in the stability of the folded apoprotein (and thus can be subtracted equally from each oxidation state for comparison, as in Figure 17). In fact, if the −1 kcal/mol difference between M56A and M56G is uniformly subtracted from the free energy levels of all three oxidation states of the M56G mutant, a free energy diagram nearly superimposable with that for the M56A mutant is generated suggesting that factors responsible for the differences from wild type in both are nearly identical. This seems reasonable given the absence or near absence of side chain interactions with the FMN in both mutants. The observed changes in M56L, M56I, and M56V, could also contain some differences in this apoprotein conformation stability.

Because there is no major conformational change between the SQ and HQ states, any change in $\Delta G_a^{sq}$ will likely be accompanied by an equal change in $\Delta G_a^{ha}$ ($\Delta G_c^{sq/ha} = 0$ in Figure 18). The determination of any alteration in these equilibria will require more data, however changes in the differences in these energies ($\Delta G_a^{ox} - \Delta G_a^{sq} = \Delta G_c^{ox/sq}$) can be predicted using statistical data and molecular modeling as follows.

Through the examination of the protein database, the statistical preference for each amino acid at all four positions of many common β-turns has been determined (Hutchinson & Thornton, 1994). This preference, termed the turn potential, can be used to predict the probability a particular sequence has of forming a particular type of turn.
and is related to the energy each residue contributes to turn formation (Chou & Fasman, 1974; Wilmot & Thornton, 1988, 1990). The four-residue \( \beta \)-turn found in the 60's loop with methionine 56 at position 1 in the \( C. \) \( \text{beijerinckii} \) flavodoxin, has \( \phi \) and \( \psi \) angles which correspond to a type II turn in the oxidized state and type II' in the semiquinone state (Ludwig \textit{et al.}, 1997). Therefore, the relative free energy change associated with the conformational change occurring upon reduction (\( \Delta G_e^{\text{ox/sq}} \)) might be predicted using the turn potentials for position 1 in type II and type II' turns. Unfortunately, the predictive values determined using the turn potentials for each mutant do not correspond well to the observed changes in holoprotein stability, possibly due to specific local structural requirements present in the flavodoxin. This hypothesis works well if applied to mutants which create changes in the \( i+1 \) and \( i+2 \) positions. However, there are very few statistically significant values for position 1 in these turn types, thus this type of analysis may improve as more statistically significant turn potentials are established.

Previous molecular modeling calculations have indicated that there are energetic differences between type II and type II' turns which are sequence dependent (Yang \textit{et al.}, 1996). Single point energy calculations were used to compare the structural energy of the oxidized state to that of the semiquinone state of each mutant. These calculations predicted that a large side chain at position 56 is less favorable than a smaller side chain (Table 6), so it is possible that these mutants produce changes in \( \Delta G_e^{\text{ox/sq}} \).

\textit{Changes in the Oxidation-Reduction Potentials.} Oxidation-reduction potentials are directly proportional to the free energy difference between the two oxidation states. In
the *C. beijerinckii* flavodoxin, this change in energy has been divided into two contributing factors; \( \Delta \Delta G_a \) \((\Delta G_a)\), the energy difference between the two redox dependent protein structural states, and \( \Delta \Delta G_i \), the difference in interaction energy between the two oxidation states (Figure 18). A change in a midpoint potential must arise from changes in the stability of either oxidation state. For example, an amino acid substitution that results in a decrease in \( E_{ox/sq} \), such as observed for the M56I, M56L, and M56V mutants, would either stabilize the oxidized form \((\Delta G_a^{ox} + \Delta G_i^{ox} < 0)\), or destabilize the semiquinone form \((\Delta G_a^{sq} + \Delta G_i^{sq} > 0)\). Using the data and analysis of the FMN dissociation constants for each oxidation state, the following discussion will endeavor to elucidate how the wild type and these mutants poise the determined redox potentials. The M56L, M56I and M56V mutants all have the about same \( E_{ox/sq} \) value. This indicates that \( \Delta G_c^{ox/sq} + \Delta \Delta G_i^{ox/sq} \) is equivalent in each mutant, thus the observed changes in stability must imply changes in some energy \((\Delta G_i, \Delta G_a\), or portions of \( \Delta G_i \)) which contribute equally to the stability of FLV\(_{ox}\), FLV\(_{sq}\) and FLV\(_{hq}\). Therefore, a direct comparison of these stabilities as they relate to changes in midpoint potential can be made if the free energies which are independent of oxidation state and specific to each mutant are subtracted out (cf. the binding energy sets shown in dashed lines Figure 17). Some conclusions concerning the mechanisms by which the midpoint potentials are poised can be made from the examination of these "adjusted" stabilities.

This study demonstrates that the less negative \( E_{ox/sq} \) values of M56A and M56G, compared to wild type are due to a destabilization of the oxidized holoflavodoxin (Figure...
It is reasonable to assign this loss in stability to the removal of an attractive sulfur-aromatic interaction. While this destabilization is also produced by M56L, M56I and M56V, these mutants also greatly destabilize the holoprotein in the semiquinone state, producing a more negative OX/SQ couple. The source of this destabilization is still unclear, but two likely causes can be implicated. First, it is known that this residue affects the interaction geometry found between the FMN and the peptide bond involved in the N5H hydrogen bond (Figure 10). A change in this geometry can affect both the hydrogen-bond strength and the strength of a repulsive oxygen-oxygen interaction between the carbonyl group of glycine 57 and C4O of the FMN in the O-up configuration of the semiquinone state, thus altering $\Delta G_{i}^{\text{ox/sq}}$ (Ludwig et al., 1997). Second, the single point energy calculations in this study did indicate that there are possible structural energy differences between the oxidized and semiquinone holoprotein which are dependent upon the size of the side chain at position 56, specifying a possible change in $\Delta G_{c}^{\text{ox/sq}}$. The less negative $E_{\text{sq/hq}}$ exhibited by all of the mutants could be produced by either the destabilization of the semiquinone state, the stabilization of the hydroquinone state, or a combination of both. The series of methionine 56 mutants studied here demonstrate all of these possibilities (Figure 17).

Conclusion

This series of mutants indicates that methionine 56 alters the stability of the holoprotein complex each of the three oxidation states by different mechanisms. The
stability of the oxidized state is increased by a putative sulfur-aromatic interaction, which may be electrostatic in nature. The semiquinone state is stabilized through alterations in the protein structural energy, either by the passive lowering of the energy through this side chains inherent flexibility, or possibly by a unique structural change produced only when methionine is at position 56. The relative destabilization of the hydroquinone demonstrated by methionine 56 is most likely produced by shielding the FMN from solvent, increasing the self energy associated with the anionic hydroquinone.

Therefore, the observed stabilization of the OX/SQ potential in those flavodoxins with a re-face methionine, compared to those proteins with a re-face leucine, can be ascribed to this singular amino acid difference. The observed increase (less negative values) in the SQ/HQ couple in the re-face methionine flavodoxins must be due to other amino acid differences destabilizing the HQ for two reasons: 1) M56L demonstrates no destabilization of the hydroquinone relative to wild type. 2) The observed stabilization of the SQ (comparing wild type to M56L) would produce more negative values for the SQ/HQ couple in the methionine flavodoxins, compared to the leucine flavodoxins. This result is not totally surprising, considering that this couple is highly dependent upon other FMN-protein interactions which differ greatly between the re-face methionine and the re-face leucine flavodoxins, including number of acidic residues surrounding the cofactor binding site and the interactions at the si face of the isoalloxazine ring.

This study also demonstrates that the comparison of the changes produced in any mutant flavodoxin to another requires the establishment of a suitable "ground state".
This is particularly important when the mutation is introduced at a position which either interacts directly with the cofactor or alters the protein structural energy (in any conformation). The determination of the FMN dissociation constants begins to define this ground state, but the comparisons still rely upon some assumptions. A definitive comparison may never be possible, but would be aided by a detailed calorimetric study of the apoprotein stability, the determination of the kinetic mechanism and rate constants which govern FMN binding, the determination of the apoprotein structure, and the application of detailed microscopic electrostatic models.
CHAPTER 3

THE NON-MONOPOLE ELECTROSTATIC SERIES

Introduction

In the last chapter, the series of aliphatic amino acid substitutions at methionine 56 indicated the possibility of a specific, attractive sulfur-FMN interaction in the C. beijerinckii flavodoxin. This prediction was compromised by the differences in side-chain size and flexibility for each of the amino acid substitutions introduced. These differences made it difficult to unambiguously assign the observed differences in cofactor affinity to a specific sulfur-FMN interaction. Indeed, it was found that both the size and the structure of the side chain at position 56 in the C. beijerinckii flavodoxin influenced FMN binding affinity. Thus, a second set of mutations was designed to determine if there is a specific interaction between sulfur and the re face of the FMN cofactor in the C. beijerinckii flavodoxin, and to determine the nature of such an interaction. In addition to providing corroborating evidence as to the nature of the sulfur-FMN interactions, the aromatic mutants are aimed toward the investigation of quadrupole and \( \pi-\pi \) interactions at the re face of the FMN. This group of mutants is comprised of M56C, M56S, M56W, M56Y and M56F. Through the comparison of M56C to the previously generated M56A, the
differences in side chain size are brought to a minimum, allowing a more definitive identification of any specific sulfur-FMN interaction. The aromatic mutants provide additional evidence for an electrostatic attraction between the isoalloxazine ring and the side chain of the residue at position 56. The aromatic mutants are also of interest as all flavodoxin proteins thus far characterized have at least one aromatic residue flanking the flavin isoalloxazine ring, one of which shields the re face of the cofactor.

Specific interactions between sulfur and aromatic systems in proteins were first identified by Morgan and coworkers (Morgan et al., 1978). These types of interactions have been hypothesized to be electrostatic in nature, and have been shown to be geometry dependent (Morgan et al., 1978; Morgan & McAdon, 1980; Reid et al, 1985). An electrostatic interaction between sulfur and an aromatic ring system is possible due to the quadrupole moment generated by the π electrons of the ring system. This quadrupole moment generates negative potential on either side, and positive potential along the plane of the ring system (Figure 19) (Dougherty, 1996; Hunter & Sanders, 1990). Thus, the electrostatic field produced by an aromatic ring system is symmetric around the plane of the ring system, and any electrostatic interactions involving such systems will occupy a specific interaction geometry (Hunter & Sanders 1990). The surface potential, and thus the geometric requirements for interaction of aromatic ring systems are influenced by any heteroatoms within the ring system. These atoms polarize the π-electron cloud, altering both the shape and the magnitude of the ring system surface potential (Dougherty, 1996; Kearney et al., 1993; Hunter & Sanders, 1990). Thus, the prediction of any electrostatic
Figure 19. Cartoon of the Quadrupole Moment of Aromatic Rings. The orientation of the ring is indicated in panel A. Panel B demonstrates that the quadrupole moment created by the ring $\pi$ electrons generates positive potential along the plane, and negative potential above and below the ring.
interaction with an aromatic ring system requires knowledge of the surface potential of the system and the geometry of interaction.

A detailed examination of a large three-dimensional structural data set allowed for the determination of the preferred geometry of interaction between sulfur atoms and aromatic ring systems in proteins (Reid et al., 1985). The majority of such interactions occurred at a distance of approximately 5.5 angstroms. There are two angles (together with this distance) which define the interaction geometry of a point (sulfur atom) and a plane (aromatic ring). These angles have been termed $\phi_1$, for the out-of-plane angle, and $\phi_2$, for the in-plane angle (Figure 20). There does not seem to be any statistical preference for the in-plane angle, while an out-of-plane angle greater than $60^\circ$ is highly disfavored. Since aromatic ring systems have quadrupolar electrostatic fields, with the positive potential located along the plane of the ring, this geometric preference indicates that these ring systems are interacting electrostatically with the $\delta$-negative sulfur.

In this work, the geometries of interaction have been determined either from the published x-ray crystal structures or from the structures predicted using molecular mechanical energy-minimization calculations. The surface potential of the FMN has been determined using \textit{ab initio} molecular orbital calculations. In addition to M56C and M56S, three other mutants were created to further test any possible quadrupole interactions with the electrostatic surface potential of the flavin. These mutants are M56F, M56Y and M56W. It has been demonstrated that the three aromatic amino acids have differing electrostatic surface potentials, increasing in the order, phenylalanine $<$ tyrosine $<$
Figure 20. Parameters Defining the Sulfur-Ring Interaction Geometry. The distance between the sulfur and the ring centroid is defined by $D$. $\phi_1$ is the angle of elevation between vector $D$ and the plane of the ring. $\phi_2$ is the equatorial angle defined by the projection of $D$ into the ring plane and the vector through one of the ring atoms (adapted from Reid et al., 1985).
tryptophan (Dougherty, 1996). Therefore, if the proper geometry is present and the interaction has electrostatic character, the strength of interaction between the aromatic M56X mutants and FMN should increase with the same trend.

**Materials and Methods**

Unless otherwise noted all materials and methods used in this study are identical to those described in Chapter 1.

*Oligonucleotide-Directed Mutagenesis.* The Kunkel method was used to accomplish the oligonucleotide-directed mutagenesis (Kunkel et al., 1987). Single-stranded DNA was generated from the phagemid pBSFlasy in the *E. coli* strain CJ236. The pBSFlasy construction is the synthetic gene for the *C. beijerinckii* flavodoxin previously prepared (Eren & Swenson, 1989) cloned into the *EcoRI* and *HindIII* sites of the vector pBluescript SK(-). Two degenerate oligonucleotides were designed:

5'-'GGTTGCTCTGCCTXCGGCGATGAAG-3' for the generation of the mutants M56F, M56Y, and M56C, and 5'-GGTTGCTCTGCCYZGGGCGATGAAG-3' for the generation of M56S and M56W. The underlined nucleotides represent the mutations that introduce the desired amino acid substitutions, where X indicates G, A and T; Y indicates G and T; Z indicates G and C. A successful mutation event was determined by restriction mapping in that all of the mutants remove an *NcoI* site. The M56F, M56Y and M56C mutants were identified by dideoxy termination DNA sequencing. The M56S and M56W mutants were identified by restriction mapping such that M56S creates a new
\textit{AvaI} site while M56W creates an \textit{EcoRII} site. The first degenerate oligonucleotide has the capability to produce the M56F mutant, however, even after exhaustive screening of the transformant mixture, the M56F mutation was not identified. Therefore, a third oligonucleotide was used to create this mutant:

\begin{equation}
5'\text{-GGTTGCTCTGC( A)TTCGGCG ATGAAG-3'}.
\end{equation}

The nucleotide in parenthesis is a silent mutation introduced for screening purposes and the underlined nucleotides create the mutation. The M56F mutant was identified by restriction mapping through the creation of a new \textit{BsmI} site. All of the mutations as well as the sequence of the entire open reading frames were confirmed by dideoxy termination DNA sequencing using the Sequenase protocol (Sanger \textit{et al.}, 1977; USB). It should be noted that the second oligonucleotide also has the possibility to generate two additional mutants, M56A and M56G, which were, in fact, prepared for other studies described elsewhere in this document.

\textit{Molecular Modeling and Molecular Mechanical Calculations.} Molecular models for each mutant protein structure were generated by replacing methionine 56 with the appropriate amino acid using the HyperChem molecular modeling software (Autodesk, Inc.) as described in Chapter 2. The x-ray crystal structural coordinates for the oxidized (5nll), semiquinone (2fox) and hydroquinone (5ull) forms of the flavodoxin were obtained from the Brookhaven Protein Data bank (Ludwig \textit{et al.}, 1997). Multiple starting structures for each mutant were generated by altering the C-Cα-Cβ-Xγ torsion angle (where X = C, S or O, as applicable). The FMN cofactor was altered to represent the appropriate oxidation
state and charges. The oxidation state was changed by editing the bonds, protonation state, and atom types of the cofactor using HyperChem script files. Charges on each atom of the FMN isoalloxazine ring, as determined by ab initio calculations (see below), were assigned using HyperChem script files. The solvent-exposed surface area of the cofactor in each energy-minimized mutant structure was determined with the Quanta software package (Molecular Simulations Inc.) using a rolling ball algorithm with a probe radius of 1.4 Å (Connolly, 1983).

Ab Initio Calculations. In order to minimize the calculation time, lumiflavin was used as a model for the isoalloxazine ring moiety of FMN for use in ab initio molecular orbital calculations (ab initio calculations using the entire FMN molecule are time prohibitive and the side chain ribityl group should contribute very little to the overall electronic structure of the flavin ring.) All of the following calculations were carried out using the Spartan chemical simulation package (Wavefunctions Inc.). Structures representing the oxidized, blue neutral semiquinone (protonated at N5), and anionic hydroquinone (N1 unprotonated) lumiflavin were constructed by model building and geometry optimized using the AM1 semi-empirical method (Dewar et al., 1985). The ab initio molecular orbital calculations were carried out employing the 6-31G* basis set using the restricted Hartree-Fock (RHF) wavefunction for the singlet oxidized and singlet hydroquinone states and unrestricted Hartree-Fock (UHF) for the doublet semiquinone state (Szabo & Ostlund, 1989). Atomic charges were obtained from fits to the electrostatic potentials (Chirlian & Francl, 1987; Breneman & Wiberg, 1990). The
electrostatic potential was mapped onto the total electron density surface calculated for each oxidation state.

**Results**

*Generation, Expression and Characterization of the Mutants.* Degenerate oligonucleotide primers have been used with the Kunkel method of oligonucleotide-directed mutagenesis to create five mutants of the flavodoxin from *C. beijerinckii*, replacing methionine 56 with cysteine, serine, and the three naturally-occurring aromatic amino acids: phenylalanine, tyrosine, and tryptophan. Although the initial plan called for the use of only two primers for the creation of all five mutants, a third primer was necessary to generate M56F because this mutation was not identified, even after extensive screening of the transformant population (over 80 putative mutants from 120 transformants). This antipathy could stem from many different factors including, chemical preferences involved in the synthesis of the oligonucleotide, handling errors in the primer synthesis, differential oligonucleotide primer annealing, or, perhaps due to some selective pressure against this particular mutant. Thus, a third non-degenerate oligonucleotide was used for the production of the M56F mutant.

The mutant flavodoxins were overexpressed in *E. coli* using the hybrid *tac* promoter within the *pKK223-3* expression vector. Just as for wild type, all of the mutant proteins accumulated *in vivo* as the holoflavodoxin in the semiquinone form, as determined by the dark blue color of the cellular pellet (Swenson & Eren, 1989). During
the purification procedure, all of the flavodoxins reoxidized to the fully oxidized state.
The $A_{280}:A_{450}$ ratio of M56S, M56C and M56F was 4.3 ± 0.2, similar to wild type and indicative of the stoichiometric binding of FMN. This ratio was elevated in both M56Y (4.7) and M56W (5.1) mutants, most likely due to the additional absorbance at 280 nm contributed by the extra aromatic residue. Each mutant was judged to be greater than 95% pure based on SDS-PAGE analysis.

The visible absorbance spectra representing the three oxidation-reduction states of each mutant flavodoxin were determined during anaerobic titrations with sodium dithionite. These mutants demonstrated nearly identical FMN absorbance transitions in all three redox states compared to wild type (Figure 21). A long wavelength band at wavelengths >530 nm indicative of a charge transfer complex between the introduced tryptophan and the oxidized FMN was not observed for the M56W mutant. This absorbance band has been observed for several aromatic-flavin complexes in solution and was also observed in the Y98W mutant of the \textit{D. vulgaris} flavodoxin (Pereira & Tollin, 1967; Swenson & Krey, 1994). Charge-transfer complexes are though to require extensive $\pi$-orbital overlap between an electron-rich donor molecule and the electron-deficient acceptor. The absence of such an absorbance band in the M56W mutant suggests that the interaction geometry between Trp56 and the flavin is non-parallel or that there is not extensive overlap between these two residues (more on this later). The quenching of the flavin fluorescence which normally occurs upon binding of the FMN to the wild-type apoprotein was determined during the measurement of the dissociation constant of the
Figure 21. The Near UV/Visible Absorbance Spectra of the M56X Mutant Flavodoxins. M56C, M56S, M56W, M56Y, and M56I flavodoxin mutants are shown in panel A-E, respectively. Spectra were determined in 50 mM phosphate buffer, pH 7.0. In each panel, the solid line represents the spectrum of the oxidized state, the dot-dashed line represents the blue neutral semiquinone spectrum, and the dashed line the fully reduced hydroquinone spectrum. Insets are plots of the changes in absorbance in the 580 nm region (representing the λ_{max} of the semiquinone species) vs. those in the 450 nm region (largely representing the oxidized species) during the course of the reductive titration.
oxidized cofactor to these mutants. Just as in the wild-type *C. beijerinckii* flavodoxin, all mutants quench the flavin fluorescence emission at 520 nm to less than 1% of that of free oxidized flavin in solution under similar conditions.

*Determination of the Oxidation-Reduction Potentials.* The one-electron reduction potentials for both couples of the FMN cofactor were determined at pH 7.0 and 25 °C by equilibration with redox indicator dyes of known $E_{m7}$ values (Clark, 1972). All five mutants demonstrated a less negative value for the midpoint potential of the OX/SQ couple compared to wild type which has a value of -92 mV (Table 8). The observed midpoint potentials of M56C and M56S were similar, poised around -69 ± 2 mV. The midpoint potentials for the OX/SQ couple of the aromatic series increased with the order M56F < M56Y < M56W, with M56W demonstrating the largest increase at -61 mV. All of redox titration data conformed well to the linearized form of the Nernst equation, generating slopes of 54 ± 5 mV, consistent with a one-electron equilibrium at 25 °C (Figure 22). It was noted that any residual oxygen present in the titration cuvette caused the partial destruction of both indigo di- and trisulfonate dyes (*i.e.* the oxidized form of the dye could not be quantitatively recovered after reoxidation of the system by introduction of air or potassium ferricyanide). This apparent anomalous degradation appeared to be independent of reduction method. The chemical reactions(s) involved in this phenomenon are not known, and to our knowledge, have not been previously described. Therefore, special care was taken to ensure that exceptionally stringent anaerobic conditions were maintained throughout the titrations while using these dyes.
<table>
<thead>
<tr>
<th>Flavodoxin</th>
<th>$K_d^\text{OX}$ (µM)</th>
<th>$K_d^\text{SO}$ (µM)</th>
<th>$K_d^\text{HQ}$ (µM)</th>
<th>$E_{\text{OX-SO}}$</th>
<th>$E_{\text{SO-HQ}}$</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.018 ± 0.002</td>
<td>0.06 ± 0.01</td>
<td>0.42 ± 0.15</td>
<td>-92$^a$</td>
<td>-399$^d$</td>
</tr>
<tr>
<td>M56L$^c$</td>
<td>0.180 ± 0.02</td>
<td>2.46 ± 0.8</td>
<td>2.1 ± 0.8</td>
<td>-128$^e$</td>
<td>-345$^g$</td>
</tr>
<tr>
<td>M56A$^c$</td>
<td>0.044 ± 0.008</td>
<td>0.07 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>-72$^{ef}$</td>
<td>-331$^g$</td>
</tr>
<tr>
<td>M56C</td>
<td>0.018 ± 0.003</td>
<td>0.026 ± 0.008</td>
<td>0.009 ± 0.004</td>
<td>-70$^{ef}$</td>
<td>-323$^g$</td>
</tr>
<tr>
<td>M56S</td>
<td>0.025 ± 0.005</td>
<td>0.030 ± 0.01</td>
<td>0.011 ± 0.005</td>
<td>-67$^f$</td>
<td>-322$^g$</td>
</tr>
<tr>
<td>M56F</td>
<td>0.11 ± 0.02</td>
<td>0.190 ± 0.06</td>
<td>0.057 ± 0.025</td>
<td>-75$^f$</td>
<td>-318$^g$</td>
</tr>
<tr>
<td>M56Y</td>
<td>0.072 ± 0.010</td>
<td>0.099 ± 0.03</td>
<td>0.046 ± 0.02</td>
<td>-70$^g$</td>
<td>-329$^g$</td>
</tr>
<tr>
<td>M56W</td>
<td>0.020 ± 0.004</td>
<td>0.020 ± 0.007</td>
<td>0.008 ± 0.004</td>
<td>-61$^f$</td>
<td>-327$^g$</td>
</tr>
</tbody>
</table>

Table 8. Oxidation-Reduction Midpoint Potentials$^a$ and FMN Dissociation Constants$^b$ of the *C. beijerinckii* Wild Type, M56C, M56S and the Aromatic M56X Mutant Flavodoxins. $^a$ Values are reported in millivolts versus the SHE, pH 7.0, 25°C; the error in the midpoint potentials is estimated to be ± 5 mV. $^c$ The dissociation constants for FMN$_{\text{OX}}$ are measured at pH 7.0, 25°C, the FMN$_{\text{SO}}$ and FMN$_{\text{HQ}}$ values are calculated using published FMN midpoint potential values (Draper and Ingraham, 1968). $^d$ From Chapter 2. $^e$ From Ludwig *et al.*, 1997. $^{ef-g}$ Potentials determined using indigo disulfonate, indigo trisulfonate, and benzyl viologen, as the redox indicator, respectively.
Figure 22. Determination of the Midpoint Potentials for the M56C, M56S, M56W, M56Y and M56F Mutants. Panel A is the SQ/HQ couple. Panel B is the OX/SQ couple. In each case, the one-electron reduction potential was determined in 50 mM phosphate, pH 7.0, at 25 °C. Symbols in each panel represent the data obtained for each flavodoxin as follows: M56C, ●; M56S, ■; M56F, ○; M56Y, □; M56W, Δ.
This was accomplished by including the commonly used PCA/PCD oxygen scrubbing system in the titration mixture. Protochatechuate dioxygenase (PCD), having a very low $K_m$ value for molecular oxygen, efficiently consumes residual oxygen in the titration mixture by reaction with its substrate protochatechuic acid (PCA). The reversibility of the reductive reaction, the absence of dye degradation, and the proper equilibrium between the flavodoxin and the indicator dye was ensured by reoxidation of the system using potassium ferricyanide. When this $O_2$-scrubbing system was employed, 95-100% of the indicator dye was recoverable after reoxidation.

The oxidation-reduction potentials for the SQ/HQ couple of these flavodoxin mutants as determined using the indicator dye benzyl viologen were poised around $-324 \pm 6 \text{ mV}$ (Table 8). These values were all significantly less negative than wild type ($-399 \text{ mV}$). Again, the titration data conformed well to the linear equation describing the equilibration between the flavodoxin and benzyl viologen (Figure 22) (Zhou & Swenson, 1995). The M56S and M56C mutants have nearly identical midpoint. The midpoint potentials for the M56Y and M56W mutants were very similar, while M56F exhibited the least negative potential at $-318 \text{ mV}$.

**Determination of the FMN Dissociation Constants.** The dissociation constant for the oxidized FMN for each mutant was determined using spectrofluorimetry. Titration data were collected in triplicate and fit to the quadratic equation describing a single binding site isotherm (Figure 23). While none of the mutants displayed cofactor binding that was tighter than that of wild type, the M56C mutant exhibited a $K_d$ value equal to that of
Figure 23. Determination of the Dissociation Constants for the M56C, M56S, M56W, M56Y, and M56F Flavodoxin Mutants. Panel A shows the M56C (▲) and M56S (■) data in comparison to the M56A mutant (●). Panel B shows the M56W (●), M56Y (▲) and M56F (■) data. In each case data was collected in 50 mM phosphate buffer, pH 7.0, at 25 °C.
wild type in these conditions (1.8 x 10^{-8} M) (Table 8). M56S bound FMN with only slightly less affinity (K_d = 2.5 x 10^{-8} M). The aromatic series demonstrated increasing binding affinity, with the K_d values following the trend: M56F > M56Y > M56W, with M56W having the greatest FMN affinity with a dissociation constant of 2.0 x 10^{-8}M. As stated previously, all of these mutants quenched the flavin fluorescence to less than 1% of the free flavin value, similar to the wild type and other mutants with substitutions at methionine 56.

**Molecular Modeling.** In order to aid in the interpretation of the effects these amino acid substitutions have on the function of the flavodoxin, molecular modeling has been used to generate energy-minimized structures of each mutant protein. To explore the entire range of possible conformations in each mutant structure, the torsion angles governing the position of the side chain were altered in 30° increments, generating at least 12 initial structures. This type of study provides a method that predicts the mutant structure without any prejudice, sampling the entire energy surface of the protein structure to find the global, rather than a local, energy minimum. Through this analysis, unique minimal energy conformations have been determined for the M56C, M56S, M56Y and M56W mutants. The phenylalanine mutant demonstrated a range of minimal energy structures which have small differences in both the axial and planar angles between the isoalloxazine and side-chain ring systems. There were no major differences between the wild type and any of the mutant structures, outside of the mutated side chains, demonstrating that all of these alterations can be accommodated in the protein without
significant disruption of the tertiary structure of the flavodoxin. This information is consistent with experimental data including the tight binding of the FMN cofactor.

Examination of the energy-minimized protein structures of M56S and M56C show that the heteroatoms of each side chain are located in essentially identical positions, approximately 4 angstroms above the C6 atom of the o-xylene subnucleus of the isalloxazine ring, with an out-of-plane angle ($\phi_1$ in Figure 20) of approximately 75°. These structures place the side chain heteroatoms approximately the same distance from the cofactor, but offset by ~ 2 Å towards the C6 and C7 atoms of the FMN, relative to the S-sulfur of the wild type methionine (Figure 24). In both of these mutants, the side chains shift by 1 angstrom in the hydroquinone form, placing the heteroatoms 4 angstroms above the FMN C7 atom. The energy-minimized structure of M56Y and the average structure of M56F share very similar conformations in both the oxidized and reduced states. The side-chain aromatic rings are centered over the FMN 7-methyl group in a non-parallel geometry relative to the plane of the isalloxazine ring, with an interplanar angle of ~ 30° (Figure 25).

Molecular mechanical calculations of the M56W mutant generates two structures with minimal energies, designated W-in and W-out (Figure 26). The Amber field energy associated with W-in is lower than W-out. The majority of the energy difference is contributed by the formation of a hydrogen bond between the Trp Nε and an Oε of a nearby glutamate (Glu63) which is not possible in the W-out conformation. However, molecular mechanic calculations performed using a distant dependent dielectric constant,
Figure 24. Representations of the Energy-minimized Structures of the *C. beijerincki* Wild-type, M56C, and M56S Mutant Flavodoxins. The three structures have been superimposed upon each other for comparison. For clarity, only the FMN and residue 56 have been shown. The dotted surface indicates the van der Walls radius of the FMN and side-chain heteroatoms. The side-chains and FMN bonds are shown in black, and the solid black circles represent side-chain heteroatoms. The heteroatom of M56S is furthest away from the FMN.
Figure 25. Representations of the Energy-Minimized Structures of the Oxidized M56F (A) and M56Y (B) Mutant Flavodoxins. In each case only the FMN and residue 56 is shown. For each mutant, fifteen different side-chain conformations (differing in the C-Cα-Cβ-Cγ torsion) were used as starting structures for energy minimization. The structures labeled with a star are lowest in energy. Note that some high energy structures have been omitted for clarity.
Figure 26. Representations of the Energy-Minimized Structures of the Oxidized M56W Mutant Flavodoxin in the Trp-in (A) and Trp-out (B) Conformations. Only the FMN and the side-chain of Trp56 are shown, for clarity.
with no periodic box, will tend to overestimate the strengths of hydrogen bonds at the protein surface. Indeed, these geometry optimization analyses also indicate the formation of a hydrogen bond between residues Asp58 and Trp90, which is not predicted in the crystal structure (Ludwig et al., 1997). Therefore, there is some uncertainty as to which structure is the most reflective of the actual protein structure. Of course, it is possible that in the dynamic protein structure the tryptophan side chain may move rapidly between both positions.

Single-point energy calculations were used to evaluate the flexibility and conformation of the amino acid side chains in the binding site. The torsion angles governing the position of the side chains relative to the FMN in each energy-minimized structure were altered and the potential energy of the resultant structures was determined. A plot of this energy as a function of torsion indicates that all of the aromatic mutants are able to accommodate a range of interplanar angles between the side chain and isoalloxazine rings without a large increase in energy (Figure 27). The distances between the isoalloxazine ring and the heteroatoms in the M56C and M56S mutants are also energetically adaptable. In these mutants, the distance between the isoalloxazine ring and either the sulfur or the oxygen atom is able to change without any appreciable increase in energy. Conversely, the flexibility of the methionine side chain in wild type is much more limited as it is packed more tightly against the cofactor and, as such, the δ-sulfur atom is more constrained (Figure 28).
Figure 27. Amber Field Energy of the M56W (○) and M56F (▲) Mutant Flavodoxins at Various Side-Chain Torsion Angles. The ordinate is the Amber field energy, i.e. the single point energy calculated in each flavodoxin with the specified side-chain torsion angle. The specific side-chain torsion which was altered is illustrated for Trp56. The identical torsion was altered for Phe56.
Figure 28. The Amber Field Energy for Various Side-chain Conformations for the Wild-type (■) and M56C (●) Flavodoxins. The ordinate is the Amber field energy, i.e. the single point energy calculated in each flavodoxin with the specified side-chain torsion angle. The specific side-chain torsion which was altered is illustrated for each residue. The black atoms indicate the sulfur atoms in each side chain.
The solvent exposure of the FMN group in each of the energy-minimized structures was determined using the rolling ball algorithm in the Quanta protein analysis software package. The FMN cofactor in the M56S and M56C mutants have similar solvent exposure and are much like that for M56A determined previously in Chapter 2. As stated above, M56F did not have a unique minimal energy structure. However, the FMN solvent exposure did not change by more than 5 Å² for the range of possible M56F structures. The average of these values, 140 ± 5 Å², is comparable to the cofactor solvent accessibility determined for M56Y. The FMN accessibility for M56W is smaller for W-in, ~ 130 Å², than for W-out, ~145 Å².

Ab Initio and Semi-empirical Calculations. The accurate prediction of the electronic properties of a molecule requires *ab initio* molecular orbital calculations. Lumiflavin has been used successfully as a model system for the isoalloxazine ring of flavin cofactors (Zheng & Ornstein, 1996; Hall et al., 1987; Pullman & Pullman, 1958). Therefore, lumiflavin was chosen as the model system for this study. Geometry optimization using the AM1 semi-empirical method predicted that both the oxidized and semiquinone structures were planar, and that the hydroquinone structure was bent along the N10-N5 axis. The predicted planarity of the oxidized lumiflavin is consistent with previous molecular orbital calculations and crystallographic studies of several 10-alkylisoalloxazines (Zheng & Ornstein, 1996; Kuo et al., 1974; Trus, et al., 1971; Fritchie & Johnstone, 1975). The bent structure of the hydroquinone predicted by this work is in agreement with previous *ab initio* calculations and with recent electrochemical
studies of lumiflavin analogs (Zheng & Ornstein, 1996, Hasford et al., 1997). The precise conformation of the semiquinone form has not been determined by any physical method; however, the calculations of Zheng & Ornstein are in agreement with the planarity predicted by this work.

Using the AM1 geometry-optimized structures, *ab initio* molecular orbital calculations were used to determine the theoretical charges on each atom of the isoalloxazine ring, the total electron surface density, and the electrostatic potential for each oxidation-reduction state of the lumiflavin molecule (*i.e.* the oxidized, the blue neutral semiquinone radical and the anionic hydroquinone). The charges of each atom predicted for oxidized lumiflavin by these calculations are in good agreement with previously reported values, differing by an average of only 0.006 charge units per atom (Zheng & Ornstein, 1996). There have been no previously reported values for the atomic charges for the anionic hydroquinone or the neutral flavin semiquinone atoms. The partial atomic charges determined by these calculations, summarized in Table 9, were used to assign charges to the FMN atoms in the molecular mechanical calculations described above. The electrostatic field potential determined by these calculations, was mapped onto the total electron density surface (analogous to the van der Walls surface). This field is calculated by moving a point charge over the surface of the sample and calculating the electrostatic interaction energy between the point charge and the molecule at each position. Mapping the field onto a surface is convenient for visualization. Figure 29 illustrates the electrostatic surfaces of the oxidized, blue neutral semiquinone and anionic hydroquinone
<table>
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<th>Isoalloxazine Atom</th>
<th>Oxidized</th>
<th>Neutral Semiquinone</th>
<th>Anionic Hydroquinone</th>
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Table 9. Partial Charges Determined for the Isoalloxazine Ring Atoms by Ab Initio Calculations. These charges were calculated using the 6-31 G* basis set. Atom nomenclature is identical to that used for FMN. The C1* atom is the lumiflavin N10 methyl carbon.
Figure 29. The Electrostatic Surface Potential of Lumiflavin in the Three Oxidation States. The electrostatic potential of lumiflavin calculated using the 6-31G* basis set; mapped to the electron surface density of oxidized lumiflavin (A), the neutral one-electron reduced lumiflavin (B), and the fully reduced anionic lumiflavin (C). Red indicates negative potential, and blue indicates positive.
species of the lumiflavin molecule. Red indicates negative potential and blue indicates positive potential. As expected, the hydroquinone with a formal negative charge displays the most negative field. The oxidized and semiquinone fields are generally very similar, with some minor variations. In each, the system is polarized such that the o-xylene ring is positive and the pyrimidine subnucleus is more negative.

**Discussion**

There has been a great deal of work done toward the elucidation of the means by which proteins regulate the redox chemistry of cofactors. It seems quite obvious that the specific molecular interactions between the protein and the cofactor are important in this regard. Numerous different types of interactions are possible, including electrostatics, solvent shielding, and hydrogen bonding. Indeed, any interaction between the protein and the redox active cofactor which preferentially stabilizes or destabilizes one particular oxidation state will serve to alter the midpoint potential. One such interaction, between a sulfur atom and an aromatic ring, has not been subjected to a great deal of investigation. The series of mutants in which methionine 56 in the *C. beijerinckii* flavodoxin has been substituted with a number of aliphatic amino acids (Chapter 2) suggested that there is an important interaction between the sulfur atom of methionine 56 and the FMN in the binding of the cofactor to this flavodoxin. However, the conclusions drawn from these results must be considered to be somewhat tentative, owing to the differences in side chain size and/or flexibility of the amino acid replacements introduced in these mutants.
Therefore, the set of five mutants generated in this study were designed to more fully explore the role of specific sulfur-cofactor interactions, to determine if such an interaction is electrostatic in nature, as well as to compare such interactions to aromatic-aromatic or π-π quadrupole interactions. In order to predict the necessary interaction parameters, extensive molecular mechanical modeling and ab initio molecular orbital calculations have been employed. The results of this study provide evidence that there is an attractive quadrupolar electrostatic interaction between sulfur atoms and other polarized aromatic moieties and the flavin isoalloxazine ring system of the FMN. The following discourse will examine the accumulated evidence for the sulfur-FMN interaction, demonstrate that said interaction is most probably electrostatic in character, and finally will analyze the effects these mutations have on the oxidation-reduction potentials.

*The Sulfur-FMN Interaction.* Statistical analysis of the protein tertiary structural database suggested early on that there may be energetically favorable interactions between the sulfur atom and the aromatic ring systems of amino acids in proteins (Morgan et al., 1978). There appears to be a statistical preference for the sulfur atoms of cysteine and methionine to be located proximal to an aromatic ring system, forming chains of alternating S-π- S-π moieties. The *C. beijerinckii* flavodoxin was one of the proteins in which this geometric propensity was identified, in this case with one terminus of the described S-π chain being the isoalloxazine ring of the bound FMN cofactor itself. Comparison of the oxidation-reduction potentials of the flavodoxin family indicates that those flavodoxins which have a methionine (*versus* Leu or Trp) in contact with the re
face of the FMN have less negative midpoint potentials for both couples. This observation raised the question as to whether the methionine sulfur atom was responsible for the elevated midpoint potentials. However, it has been demonstrated that there are many interactions between the apoflavodoxins and FMN that alter the midpoint potentials (Swenson & Krey, 1994, Swenson & Zhou, 1995, Ludwig et al., 1997).

Therefore, in order to implicate any specific interaction, it is important to observe the effects produced by removing or adding said interaction without altering any other protein-FMN contacts. Towards this end, protein engineering using oligonucleotide-directed mutagenesis has proved to be a powerful approach.

Two flavodoxin systems have been used in the study of the affects produced when methionine is in position to interact with the planar faces of the FMN. In the \textit{C. beijerinckii} flavodoxin, the \textit{re}-face methionine has been replaced with all of the naturally-occurring aliphatic amino acids (this work). For the flavodoxin from \textit{D. vulgaris}, the two residues which interact with both planar faces of the flavin isoalloxazine ring (a tryptophan and a tyrosine) have been replaced with alanine and methionine. In studies directed toward the investigation of the effects produced by the tyrosine 98 residue flanking the \textit{si}-face of the FMN in the flavodoxin from \textit{D. vulgaris}, this residue has been replaced with both alanine and methionine (Swenson & Krey, 1994). These substitutions have large effects on the oxidation-reduction potentials of both couples (Table 10). The Y98A mutant has a less negative $E_{\text{ox/sq}}$ (-186 mV) than for the Y98M mutant (-211 mV), with both being considerably more negative than for wild type (-150 mV). A similar
Flavodoxin & $E_{\text{OX/SQ}}$ & $E_{\text{SQ/HQ}}$ & $K_d^{\text{OX}}$ (nM) & $K_d^{\text{SQ}}$ (nM) & $K_d^{\text{HQ}}$ (nM) \\
WT C. beijerinckii $^a$ & -92 & -399 & 18.0 & 0.06 & 420 \\
M56A C. beijerinckii & -72 & -331 & 44.0 & 0.07 & 30 \\
WT D. vulgaris $^b,c$ & -148 & -443 & 0.24 & 0.006 & 206 \\
Y98M D. vulgaris $^b$ & -211 & -302 & ND & ND & ND \\
Y98A D. vulgaris $^b$ & -186 & -304 & 3.2$^d$ & 0.42$^d$ & 73$^d$ \\
W60M D. vulgaris $^c$ & -136 & -418 & 0.31 & 0.006 & 86 \\
W60A D. vulgaris $^c$ & -157 & -357 & 18.3 & 0.49 & 660 \\

Table 10. Oxidation-Reduction Midpoint Potentials and FMN Dissociation Constants of the Methionine-Alanine Flavodoxin Pairs. Midpoint potentials are reported in mV, at pH 7.0, 25 °C; the error in the midpoint potentials is estimated to be ± 5 mV. ND indicates values which have not been determined. $^a$ Ludwig et al., 1997. $^b$ Swenson & Krey, 1994. $^c$ Mayhew et al., 1996. $^d$ Zhou & Swenson, 1996b.
trend was observed in the comparison of M56A to the wild-type *C. beijerinckii* flavodoxin. This shift in potential could be interpreted as an increase in stability of the oxidized form relative to semiquinone, which is likely to be the consequence of the specific sulfur-FMN interaction in this flavodoxin. However, the analysis for the *D. vulgaris* flavodoxin is hindered at this point by the lack of information concerning the FMN_{OX} dissociation constant for the Y98M mutant. Both Y98A and Y98M produced a less negative oxidation-reduction potential for the SQ/HQ couple compared to wild type, both approximately -300 mV. The fact that the potentials for these two mutants are approximately equal is not consistent with the *C. beijerinckii* data. In the *C. beijerinckii* flavodoxin, replacement of methionine with a smaller side chain, as in the M56A mutant, caused a stabilization of the hydroquinone holoflavodoxin (producing a less negative E_{SQ/HQ}). However, a re-face side chain is sterically limited and must pack against the isoalloxazine ring, while the methionine of Y98M is less hindered and could allow a more solvent exposed cofactor or differing sulfur-FMN geometry in the reduced flavodoxin.

Thus, as the negative charge develops on the hydroquinone cofactor, any attractive electrostatic interaction that may exist between the side-chain sulfur atom and the more oxidized state of the flavin is eliminated. In the absence of this interaction, the side chain is free to move aside, altering the sulfur-flavin interaction geometry and increasing the solvent exposed surface of the flavin.

The mutants constructed at the re face of the isoalloxazine ring in the *D. vulgaris* flavodoxin, W60A and W60M (Mayhew et al., 1996) also altered the redox properties of
the FMN, but to a different extent (Table 10). Both mutants demonstrated a less negative $E_{SQ/HQ}$ relative to wild type, -357 and -418 mV for W60A and W60M, respectively. These results are in agreement with the aliphatic series of M56X mutants, wherein M56A has a less negative $E_{SQ/HQ}$ value than for the wild type C. beijerinckii flavodoxin. The $E_{O/S}$ of the re-face mutants of the D. vulgaris flavodoxin was also modified, with the W60A mutant having a more negative (-157 mV) and W60M a less negative midpoint potential (-136 mV) compared to the wild-type flavodoxin. These results conflict with those of the aliphatic M56X mutants, which indicated that re-face methionine produced a more negative potential for the OX/SQ couple than alanine. However, as discussed previously, there are other factors (such as oxidation state dependent structural energy differences) that govern the reduction potential of this couple. Such factors may be specific to a particular flavodoxin species.

The data produced by all the alanine / methionine mutant pairs are summarized in Table 10. This table demonstrates that, for the reasons described above, the effects elicited by the substitution of alanine for methionine are dependent upon both the location of the residue (i.e. at the re- or si- face) and the species of flavodoxin. It is interesting that while neither two sets match exactly, there are similar shifts among all three. These data suggest that there are specific interactions between methionine and both faces of the FMN, but that this interaction is site-specific, most likely being governed by both the geometry of interaction and local structural constraints. In addition to modifications in redox properties of the FMN, it has been established in Chapter 2 that.
compared to alanine (in the M56A mutant) or indeed any aliphatic residue, methionine produces a lower dissociation constant for the oxidized FMN in the flavodoxin holoprotein from C. beijerinckii (Table 8). This phenomenon has also been observed in the re-face mutants of the D. vulgaris flavodoxin, wherein the formation of the holoflavodoxin is more favorable in the W60M mutant than in W60A (Table 10). Thus, it is clear that some protein-cofactor interaction specific to methionine favors cofactor binding.

While the above analyses are complicated by differences in the size of the side chain which interacts with the FMN, the comparison of the M56C and M56S mutants to M56A brings the differences in side chain to a minimum within the limits of the naturally-occurring amino acids. As such, if the specific interaction between the oxidized flavin and the apoflavodoxin determined from the above data persists, the sulfur atom is more fully implicated as the causal moiety. The binding energy of FMNOX to the M56C and M56S mutants is approximately 0.5 kcal/mol more favorable than M56A (Table 11). The magnitude of this energy difference is within the range determined for the S-π interaction in model peptides (Viguera & Serrano, 1995; Stapley et al., 1995). Additionally, the interaction energy of the M56C apoprotein for FMNOX is equivalent to that of wild type. Because there is a large variance in FMN solvent exposure between these two structures, these observations suggest that hydrophobic interaction between the protein and the FMN is not a major diving force in cofactor binding for this flavodoxin. This comparison provides more compelling evidence that there is an attractive interaction between a thiol
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Table 11. Gibbs Free Energy of FMN Binding\textsuperscript{a,b,c} for the *C. beijerinckii* Wild-type, M56C, M56S, and the Aromatic M56X Flavodoxins. \textsuperscript{a} Values are reported in kcal/mol at 25 °C. \textsuperscript{b} The error in these values is estimated to be 0.1-0.2 kcal/mol. \textsuperscript{c} Values determined using: \( \Delta G = RT\ln K_d \).
(or hydroxyl) moiety and the FMN\textsubscript{OX} in the binding site milieu provided by the C. *beijerinckii* flavodoxin. By inference then, it is indeed the sulfur atom of the methionine side chain which is responsible for the increase in cofactor affinity in the methionine containing holoflavodoxins.

To this point, the M56C and M56S mutants have been treated as interchangeable; however, there are chemical differences in the side chains. A sulfur atom has a larger and more polarizable electron cloud than does an oxygen atom, and a cysteine residue is ionizable. Indeed there is a small (0.2 kcal/mol), but reproducible, difference in the interaction energy between FMN and the apoflavodoxin of the M56C and M56S mutants that is oxidation-state independent. This small variance in binding energy might be indicative of the differences in the side chain involved in the interaction with the FMN (more on this below).

The increase in holoflavodoxin stability observed in M56C and M56S (relative to M56A) is not limited to the oxidized form, but is also observed in both the semiquinone and the hydroquinone forms (Table 8). This differs from the trend observed in the comparison of M56A or M56L to the wild-type *C. beijerinckii* flavodoxin, which indicated that the presence of the electron-rich sulfur atom (of Met56) destabilized the hydroquinone complex. The reasons for this variance will be discussed below.

*The Electrostatic Nature of the Sulfur-FMN Interaction.* The accumulated data strongly suggest that the presence of an electron-rich atom such as sulfur or oxygen in close association with the FMN, particularly at the *re* face of the isoalloxazine ring,
affects the stability of the holoprotein complex. Nonetheless, it is important to determine the nature of the interactions which are responsible for the observed stability differences. The geometry between the sulfur (oxygen) atoms in the wild type C. beijerinckii flavodoxin, M56C and M56S, and the isoalloxazine would predict a repulsive interaction. However, the electrostatic field of the o-xylene subnucleus is equivalent to similar benzyl derivatives. However, the \textit{ab initio} calculations demonstrate that the presence of the heteroatoms in the fused ring system of lumiflavin, and by analogy FMN, polarize the electrostatic surface potential of the isoalloxazine ring (\textbf{Figure 29}). Furthermore, the presence of hydrogen bonds between the pyrimidine subnucleus and the protein will lead to additional polarization of the flavin ring. This polarization alters the surface potential of the isoalloxazine ring such that the surface of the o-xylene ring carries a positive potential. As such, the $\phi_1$ and $\phi_2$ angles (angles which define the heteroatom-ring geometry) in the wild-type C. beijerinckii flavodoxin, M56C, and M56S, are consistent with an attractive electrostatic force.

A geometry-dependent electrostatic force similar in nature to the S-$\pi$ or O-$\pi$ interaction exists between two aromatic ring systems (Dougherty, 1996; Hunter & Sanders, 1990). This $\pi$-$\pi$ interaction is repulsive in a face-to-face interactions, and attractive in the edge-to-face configuration. However, this energy-dependent conformation is altered by the presence of heteroatoms in the ring systems (Kearney et al., 1993). The energy-minimized structures of the aromatic M56X mutants, which places the negative surface of the side-chain aromatic ring in contact with the calculated
positive surface potential of the o-xylene subnucleus of the flavin (based on lumiflavin). predict that the side chain-FMN interaction should be attractive in nature. The fact that the energy of interaction increases with the expected trend, \textit{i.e.} M56W > M56Y > M56F (\textbf{Table 11}) (Dougherty, 1996), gives strong supporting evidence for electrostatic interactions between the electrostatic field generated by the \pi electrons at the o-xylene subnucleus of the isoalloxazine ring and other polarized atoms or ring systems which are positioned in the proper geometry. The FMN isoalloxazine ring appears to be more fully shielded from solvent in the M56W mutant, raising the possibility of more enhanced hydrophobic interaction energies in this protein. The lower $K_d$ value for FMN$_{OX}$ in this mutant is consistent with this idea. However, previous results indicated that differences in FMN solvent exposure did not appreciably affect the binding of oxidized FMN (see previous chapter). Additionally, based on the structural predictions, the M56Y and M56F mutants appear to have nearly identical FMN solvent exposure, while differing in FMN$_{OX}$ affinity by almost 0.4 kcal/mol. These observations quite reasonably suggest that the observed increase in FMN$_{OX}$ affinity (M56W > M56Y > M56F) is not caused by differences in the solvent accessibility of the FMN, but rather by the differences in the surface potential of the aromatic ring involved in the side chain-FMN interaction. These data are consistent with the hypothesis that the observed increase in affinity for oxidized FMN in those flavodoxins with a sulfur (or oxygen in the M56S mutant) atom positioned to interact with the \textit{re} face of the FMN is caused by an electrostatic interaction between the heteroatom and the FMN.
Effects on the Oxidation-reduction Potentials. As stated in Chapter 2, the oxidation-reduction potentials are directly proportional to the sum of two contributing energy factors, $\Delta G_c$ and $\Delta G_i$, the free energy change associated with protein conformation and the differences in the free energy change associated with the interactions between the flavin cofactor and the protein in each redox state, respectively (Figure 18).

In the aliphatic M56X mutants, $\Delta G_i$ was held to a minimum, because these mutations did not introduce any functional groups which could engage in specific interactions with the FMN (other than those caused by differences in side chain size). In contrast, the side chains introduced in the series of mutants discussed in this chapter have characteristics that could lead to a preferential interaction with a specific FMN oxidation state. Therefore, the interpretation of the observed oxidation-reduction potentials in this series of mutants is more complicated. This series of mutants is subdivided into two groups for the following analyses of the data. The first group includes M56C and M56S, and these mutants will be compared to M56A, M56L, and wild type. The second group includes the aromatic mutants, M56F, M56Y, and M56W.

As expected, the M56C and M56S mutants have very similar $E_{\text{OX/SQ}}$ and $E_{\text{SQ/HQ}}$ values (Table 8), owing to the similarity in the side-chain functional groups. Comparison of these mutants to the M56A mutant indicates that the addition of either a thiol or a hydroxyl to the side chain of residue 56 has practically no influence on the $E_{\text{OX/SQ}}$ and only a very small affect on the $E_{\text{SQ/HQ}}$. The most straightforward conclusion that might be drawn is that these functional groups do not affect any of the relative energy differences
that poise these potentials; however, this deduction is almost certainly too simplistic. To illustrate, it is possible that these mutants produce changes of similar magnitudes in $\Delta G^\text{Ox/SQ}_c$ and $\Delta A\Delta G^\text{Ox/SQ}_i$, but opposite in sign, producing no net change in the sum (i.e. no change in the $E^\text{Ox/SQ}$).

As stated previously, it is difficult to predict the changes in $\Delta G^\text{Ox/SQ}_c$ produced by mutations at the first position in a $\beta$-turn without additional experimental data. However, it is theoretically possible to get a crude estimate of this energy difference using the positional turn potentials as defined by Hutchinson and Thornton (Hutchinson & Thornton, 1994). Both cysteine and serine have larger type II' potentials than the corresponding type II potentials. The opposite is true for alanine. Consequently the structural energy stability gained (i.e. a negative $\Delta G^\text{Ox/SQ}_c$) upon formation of the FMN semiquinone should be greater for M56C and M56S relative to M56A. Therefore, in order for the $E^\text{Ox/SQ}$ values to remain constant among the three mutants, as observed, the difference in the interaction energy between the apoflavodoxin and the FMN must decrease upon reduction of the cofactor to the semiquinone state in the M56C and M56S mutants (leading to a positive $\Delta A\Delta G^\text{Ox/SQ}_i$). This suggests that the attractive electrostatic force between the electronegative side-chain atoms, demonstrated in the dissociation constant data above, decreases in the semiquinone state. Such an observation would not be unexpected, since the surface potential of the o-xylene ring of lumiflavin becomes less positive and the electron density of the cofactor becomes greater as the oxidized cofactor is reduced to the neutral semiquinone (Figure 29).
Since the predicted surface potential of the \( o \)-xylene ring of lumiflavin becomes decidedly negative upon the addition of the second electron, it is be reasonable to predict that any electrostatic interaction between the electronegative atoms of the side-chain of residue 56 and the isoalloxazine ring would become repulsive upon the formation of the anionic hydroquinone. As such, the M56C and M56S mutants should have more negative \( E_{SQ\text{HQ}} \) values when compared to M56A. However, the experimental results clearly show the opposite to be true. There are at least two possible explanations as to why the presence of the electronegative atoms does not cause a decrease in affinity for the anionic hydroquinone. First, the \textit{ab initio} calculations performed for the determination of the lumiflavin surface potentials were performed on a free lumiflavin molecule. Interactions between the flavodoxin and the cofactor could alter the electronic distribution of the cofactor and change the isoalloxazine ring surface potential. The flavodoxin structures established by X-ray crystallography and NMR spectroscopy clearly indicate that there are hydrogen bonds between the protein and all of the heteroatoms of the isoalloxazine ring. These hydrogen bonds are expected to polarize the isoalloxazine ring and delocalize the developing negative charge, perhaps to such an extent that the \( o \)-xylene ring remains positive, even in the fully anionic hydroquinone state.

Second, molecular modeling suggests that the side chains of the M56C and M56S mutants have a greater conformational freedom compared to the wild type (see Figure 28). As such, the electronegative atom of these side chains could move from a location
which is in close contact with the isoalloxazine ring (in position for the attractive interaction) to a position perhaps at least seven angstroms away. The interaction energy of these types of electrostatic interactions fall off sharply with distance (Buckingham, 1967). Consequently, it is possible that as the FMN is reduced to the anionic hydroquinone the electronegative atom simply moves away in order to minimize the now unfavorable repulsive interaction with the isoalloxazine ring. It is interesting to note that because of its larger size the methionine side chain of the wild-type flavodoxin is more sterically hindered. In this case the sulfur atom is not able to move significantly away from the cofactor upon addition of the second electron. This conclusion is supported both by the molecular modeling analyses done here and by the x-ray crystal structures which indicate no significant structural differences between the semiquinone and hydroquinone species (Ludwig et al., 1997). Thus, the sulfur atom of the methionine side chain in the wild-type flavodoxin may be partially responsible for some of the destabilization of the anionic hydroquinone. Similar arguments can be made in the explaining why the mutants with aromatic residues at position 56 also have less negative $E_{SQ/HQ}$ values compared to the M56A mutant. In these mutants the freedom of the side-chain aromatic ring systems observed in the molecular modeling (Figures 25, 26 & 27) could allow for aromatic ring-FMN geometries that give rise to a favorable interaction (perhaps allowing for more favorable edge-to-face interactions). It would be interesting to determine if such possible conformational changes actually occur in these proteins, either by x-ray crystallography or NMR spectroscopy.
Examination of the $E_{\text{OX}/\text{SQ}}$ values for the aromatic M56X mutants reveals a small trend, with the potentials becoming progressively less negative: $F < Y < W$ (Table 8). As with the M56C and M56S mutants, the aromatic mutants will alter both $\Delta G_c^{\text{OX}/\text{SQ}}$ and $\Delta \Delta G_i^{\text{OX}/\text{SQ}}$. As state above, it is difficult to assign values to these energies without additional experimental evidence. Nonetheless, if the statistical turn potentials are used to predict $\Delta G_c^{\text{OX}/\text{SQ}}$ for the aromatic mutants, the M56W mutant should produce a more stable semiquinone structure than the M56Y mutant, and both Trp56 and Tyr56 should be more stable than the M56F mutant. Therefore, either this estimation of $\Delta G_c^{\text{OX}/\text{SQ}}$ is not representative of the actual structural energy differences or there are other factors involved in the $\Delta \Delta G_i^{\text{OX}/\text{SQ}}$ which combine with the $\Delta G_c^{\text{OX}/\text{SQ}}$ to produce the observed results.

It has been demonstrated that aromatic amino acids have different affinities for each oxidation state of free FMN and free riboflavin (Draper & Ingraham, 1970). Since the 5'-phosphate moiety of the bound FMN is sequestered from interaction with an aromatic residue at position 56, the minimal equilibrium constants between the aromatic amino acids and free riboflavin best represent the specific side chain-cofactor interaction in flavodoxin. Thus, a comparison of the association constants for each aromatic amino acid and the semiquinone and oxidized states of free riboflavin should be indicative of which oxidation state a particular amino acid stabilizes in the aromatic flavodoxin mutants (i.e. indicative of the $\Delta \Delta G_i^{\text{OX}/\text{SQ}}$). An amino acid which demonstrates greater affinity for the semiquinone than for the oxidized state of free riboflavin has a negative $\Delta \Delta G_i^{\text{OX}/\text{SQ}}$ and...
should produce a less negative \( E_{\text{OX/SQ}} \) than for the unbound cofactor. In solution, all of the aromatic amino acids preferentially stabilize the semiquinone state of free riboflavin, with the relative association constants \( (K_{\text{SQ}}/K_{\text{OX}}) \) as follows: \( F(2.4) > Y(+1.8) > W(+1.4) \). Since this trend does not replicate that observed for the \( E_{\text{OX/SQ}} \) of the aromatic mutants, it is evident that there are other factors (as discussed previously) which govern the one-electron reduction potential of the \( \text{OX/SQ} \) couple. It is interesting to note that the equilibrium constants determined between the free amino acids and oxidized riboflavin mirror the trend of the determined \( K_{d}^{\text{OX}} \) for the aromatic mutants, \textit{i.e.} tryptophan binds riboflavin tighter than tyrosine which binds tighter than phenylalanine.

Taken individually, neither the statistical turn potentials nor the riboflavin association constants sufficiently replicate the \( E_{\text{OX/SQ}} \) values of the aromatic mutants, emphasizing the cooperatively between structural and interaction energies in the poising of this midpoint potential. Since there is no observed conformational change between the semiquinone and hydroquinone states of the wild type flavodoxin (Ludwig \textit{et al.}, 1997), \( \Delta G_{c}^{\text{SQ/HQ}} \) can be assumed to be approximately zero. As such, the interpretation of the \( E_{\text{SQ/HQ}} \) couple is possibly more easily explained. If the differences in the riboflavin associations constants reported by Draper & Ingraham are used to estimate the \( \Delta G_{i}^{\text{SQ/HQ}} \), tyrosine and tryptophan should produce a more negative \( E_{\text{SQ/HQ}} \) compared to phenylalanine. This prediction is born out by the experimental data obtained from the aromatic M56X mutants (Table 8). What's more, this same trend has been noted in a series of mutants at the \textit{si}-face of the isoalloxazine ring in the \textit{D. vulgaris} flavodoxin.
(Swenson & Krey, 1994). The association constants of aromatic amino acids with flavins are known to be pH dependent (Draper & Ingraham, 1970), indicating that electrostatic interactions are involved in these equilibria. This observation, together with the known difference in aromatic amino acid ring system surface potential, provides further support for the premise that aromatic-aromatic (π-π) electrostatic interactions influence the reduction potentials of the SQ/HQ couple in flavodoxins.

**Conclusion**

The data obtained for the set of mutants described in this chapter demonstrate quite convincingly that there is a specific interaction between a sulfur atom and the isoalloxazine ring of the FMN cofactor in the flavodoxin from *C. beijerinckii*. Additionally, it has been shown that the nature of such an interaction is likely to be mainly an electrostatic force between the partially charged sulfur and the electrostatic surface potential of the isoalloxazine ring. Such interactions with the electrostatic surface potential of the flavin isoalloxazine ring are apparently not limited to partially charged point atoms, but are also evident with the electrostatic surfaces provided by aromatic amino acids. Such interactions affect both the redox potentials of the FMN and the stability of the holoflavodoxin complex. The holoprotein complex is stabilized by the interaction between an electronegative moiety (a sulfur atom, an oxygen atom, or the electrostatic surface of the flanking aromatic side chain) with the positive surface of the o-xylene subnucleus of the oxidized FMN cofactor.
While it is evident that the oxidation-reduction potentials of the flavin cofactor are also affected by these mutations, the results are not easily interpreted. The data suggests that it is not simply the nature of the cofactor environment that contributes to the stabilization of the semiquinone. Rather, it seems more likely that the observed changes in midpoint potentials are the results of the combination of the specific interactions between the flavin and the amino acid side chain at position 56 and the structural stability produced by a particular amino acid at this specific location in the protein structure. If the simplest case is assumed in comparing M56A to M56C (i.e. no structural energy differences between the two mutants) then, by virtue of the similarity in the $E_{OX/SQ}$ values, the presence of the sulfur atom does not produce any additional stabilization of the semiquinone form of the cofactor. This assumption is almost certainly too simplistic, as outlined above, and the elucidation of this problem will require the application of more sensitive methods, including NMR spectroscopy and mathematical electrostatic modeling.

The midpoint potential values for the SQ/HQ couple also indicate some interesting possibilities. Inasmuch as it is known that the anionic hydroquinone is destabilized by the presence of negatively charged residues in proximity to the cofactor (Swenson & Krey, 1994; Zhou & Swenson, 1995), the presence of the additional negative electrostatic potential, either from the electron-rich sulfur or oxygen atoms or the planar face of the aromatic surface, should cause a destabilization of the fully reduced cofactor. Such a destabilization would result in a shift in the $E_{SQ/HQ}$ toward more negative values. However, the observations do not fit with this simple static model. Therefore, it was
necessary to predict that small conformational changes, which alter the interaction
gometry between the cofactor and the re-face side chain, minimize and possibly reverse
the magnitude and polarity of these interactions upon reduction of the flavin. It would be
exciting to solve the three-dimensional structure of these mutant flavodoxins to determine
if these predictions hold true.

Inspection of the primary sequences of the flavodoxin family demonstrated that
those flavodoxins with a methionine residue in contact with the re face of the
isoalloxazine ring had elevated oxidation-reduction potentials (particularly for the $E_{OX/SQ}$).
This generated the hypothesis that the presence of a methionine at the re face of the
cofactor should result in the thermodynamic stabilization of the semiquinone form.
presumably through a specific interaction between the thioether moiety and the flavin.
However, these mutants together with the aliphatic mutant series discussed in Chapter 2.
demonstrate that the sulfur atom of the methionine does not produce the less negative
midpoint potentials for the OX/SQ couple through a direct stabilization of the
semiquinone. Rather, the less negative potentials observed in the methionine-containing
flavodoxins more likely stem from a combination of at least two factors. First, there may
be a structural energy preference for a methionine residue at this specific location in the
protein tertiary structure. Second, the oxidized form of the FMN may be stabilized
through an attractive interaction between the sulfur atom and the electrostatic surface of
the FMN isoalloxazine ring. Such an interaction is not specific to sulfur, but rather to any
electron-rich moiety which is in the proper geometry to favorably interact with the
positive surface of the oxidize cofactor, or, upon reduction, generate an unfavorable 
electrostatic interaction with the anionic FMN hydroquinone.
CHAPTER 4

THE MONOPOLE ELECTROSTATIC SERIES

Introduction

The oxidation-reduction potentials of redox active proteins are affected by the
location and nature of the charged amino acids which surround the redox active sites
(Rees, 1985; Gunner & Honig, 1991). Flavodoxins provide a unique model system for the
study of the oxidation-reduction altering effects of the charged amino acids. Flavodoxins
undergo two oxidation reduction steps, one which involves the formation of a charged
oxidation state (the SQ/HQ couple) and another in which both oxidation states are neutral
at physiologic pH (the OX/SQ couple). This provides an ideal internal control which
allows the experimenter to demonstrate that alterations in the charged amino acids are
eliciting their changes in the oxidation-reduction potentials via electrostatic interactions.
Additionally, the large number of flavodoxin primary and tertiary structures solved to
date and the cloning of several flavodoxin structural genes make the flavodoxin family well
suited toward site directed mutagenesis and structural analysis (see Table 1 and
references there in).
The phenomenon of electrostatic control of oxidation-reduction potentials has been examined in the flavodoxin from *D. vulgaris*. In this flavodoxin, six acidic residues surrounding the FMN have been replaced by the corresponding amides (Zhou & Swenson, 1995). This experiment demonstrated that the formation of the hydroquinone was destabilized by an average of -15 mV per acidic residue. While these mutants did affect the absolute value of the OX/SQ couple, there was no apparent trend in the changes, lending convincing evidence that electrostatic interactions did not affect the formation of the semiquinone flavodoxin. These mutations altered rather long ranged interactions, thus producing small individual affects.

Short-ranged interactions have also been studied in this flavodoxin. Tyrosine 98, which is known to interact with the *si* face of the FMN (Watenpaugh *et al.*, 1976; Watt *et al.*, 1991), was replaced with a number of amino acids, including histidine and arginine (Swenson & Krey, 1994). In both of the Y98H and Y98R mutants, just as in the wild type, the pH dependency of the OX/SQ couple was approximately 59 mV/pH, indicative of the uptake of a proton during the one-electron reduction of the flavin at 25 °C (protonation of the FMN N5, see Figure 3). Moreover, the $E_{OX/SQ}$ values at pH 7 for both of these mutants are very similar to the value of the Y98A mutant. The spectrally distinct blue neutral form of the flavin semiquinone was observed throughout the entire pH range for all of the tyrosine 98 mutants. Thus, alterations in the electrostatic environment of the FMN does not affect either the formation of the blue neutral semiquinone or the OX/SQ midpoint potential, even at such short distances.
Conversely, these mutants did significantly alter the SQ/HQ couple. The $E_{SQ/HQ}$ value for the Y98R mutant was independent of pH (between pH 5.5-8.0), with a value of around -250 mV, increasing by over +90 mV from the wild-type value of -443 mV (Swenson & Krey, 1994). The Y98H mutant demonstrated a marked pH dependence. A plot of pH vs. $E_{SQ/HQ}$ produced a curve which indicated two $pK_a$ values for the histidine (one in each oxidation state) and an $E_0$ of ~ -250 mV, where $E_0$ is the reduction potential at pH = 0. Thus, both residues contribute approximately the same stabilization of the anionic hydroquinone at acidic pH values, owing to the fact that the protonated forms of both amino acids carry a +1 charge. The pH independence of the SQ/HQ couple seen in the Y98R mutant is not surprising in that the $pK_a$ of the arginine side chain is naturally very high, as such any shift in said $pK_a$ will not be observed within the pH range of the study. The pH dependent curve generated with the Y98H data is indicative of a shift in the $pK_a$ of the histidine side chain as a result of the developing negative charge on the FMN. This spectrophotometric data, together with direct NMR spectroscopic analysis, indicated that the $pK_a$ of the histidine shifted from 7.0 in the semiquinone form to 8.5 in the anionic hydroquinone form (Swenson & Krey, 1994; Chang & Swenson, 1997). Thus, it has been dramatically illustrated that electrostatic interactions, particularly in such proximity to the FMN, produce large shifts in the midpoint potential of the SQ/HQ couple, with a concomitant shift in the $pK_a$ of the interacting ionizable group(s).

The pH dependency of the wild type *C. beijerinckii* flavodoxin reduction potentials has been studied previously (Mayhew, 1971). A plot of the pH vs. OX/SQ
midpoint potential produced a 59 mV/pH unit slope, as expected for an equilibrium involving the transfer of one proton (protonation of the FMN N5). The SQ/HQ couple was found to be independent of proton concentration above pH 7, but below neutrality the potential became increasingly less negative as the system was shifted to more acidic values. Two hypothesis emerged which endeavored to explain this observation. The first theorized that this data was indicative of the protonation of the anionic FMN hydroquinone. However, subsequent NMR and UV/visible spectroscopic data indicated that the hydroquinone was unprotonated throughout the pH range under study (van Schagen & Müller, 1981; Franken et al., 1984; Vervoort et al., 1985, 1986; Ludwig et al., 1990). This spawned the second hypothesis, that the ionization of a residue in close proximity to the FMN is altered by the formation of the anionic hydroquinone, which in turn changes the oxidation-reduction potential of the SQ/HQ couple (i.e. a redox-linked protonation equilibrium). Analysis of the x-ray crystal structure indicated that the most likely candidate was glutamate 59, which is found to be hydrogen bonded to the N3H of the pyrimidine subnucleus (Ludwig et al., 1990). However, recent work in our laboratory in has indicated that although Glu59 is responsible for a portion of the destabilization of the hydroquinone anion, it is not solely responsible for the observed pH dependence (Bradley & Swenson, in press).

The SQ/HQ couple of the flavodoxin from *D. vulgaris*, in which there is no acidic group hydrogen bonded to the FMN, demonstrates a very similar response to changes in pH. Since there is no single acidic group which can be identified as the sole residue
involved in the redox-linked ionization, it was hypothesized that the pH dependent behavior of the SQ/HQ couple was caused by a combination of all the ionizable residues surrounding the FMN. This hypothesis was investigated using the acid to amide mutants in the *D. vulgaris* flavodoxin (Swenson & Zhou, 1996). The apparent pKₐ shifted to a more acidic value with the successive neutralization of increasing acidic residues, finally falling below the observable pH range in the mutant which neutralized six acidic residues. Thus it is quite clear that the observed pH dependency is caused not by one single charge residue, but rather by a combination of all the charged residues surrounding the FMN. Therefore, using the E59Q data as a foundation, it may be inferred that a similar cooperative destabilization of the anionic hydroquinone by the surrounding charged residues should also exist in the *C. beijerinckii* flavodoxin.

In order to extend our knowledge and insight into how electrostatic interactions control the oxidation-reduction potentials of the redox active FMN, the re-face methionine of the *C. beijerinckii* flavodoxin has been replaced by both histidine and aspartic acid, sterically similar but oppositely charged residues. Both one-electron midpoint potentials for these mutants and the wild-type protein have been explored as a function of pH, the resultant data fit to the equations of Clark which describe a redox-linked protonation equilibrium (Clark, 1972). The M56N mutant was generated to serve as an uncharged control.

By fitting the data produced in the present study to different theoretical equations, both the number of ionizable groups involved in the equilibrium and apparent
pKₐ values for these groups in each oxidation state can be determined. These analyses indicated both the M56H and the M56D mutant data were more accurately fit by an equation describing an equilibrium involving more than one ionizable group. This is significant, and is in support of the hypothesis that multiple acidic residues are involved in determining the pH dependence of the SQ/HQ couple in the wild-type flavodoxin. The pKₐ of the M56H histidine shifts to a more basic value upon the formation of the anionic hydroquinone, in accordance with hypothetical models and previous data. This increase in pKₐ was confirmed using ¹H NMR spectroscopy.

This set of mutants demonstrates quite clearly that the formation of the hydroquinone form of the *C. beijerinckii* flavodoxin is affected by the electrostatic influences of charged residues, leading to alterations in the midpoint potential of the SQ/HQ couple. Conversely, the OX/SQ couple remains relatively unaffected by the electrostatic character of the residue which interacts with the *re* face of the flavin. Additionally, this work provides data to support the hypothesis that the pH dependence of the SQ/HQ couple of the wild-type *C. beijerinckii* flavodoxin is governed not by a single residue, but rather by a combination of all the ionizable amino acids which surround the flavin binding site.

**Materials and Methods**

Unless otherwise stated the materials and methods used in this section were identical to those described in the preceding chapters.
Determination of the oxidation-reduction potentials. In this study the reduction potentials have been determined at various pH values ranging from 5 - 9. In order to effectively buffer the reaction solution at each specific pH it was necessary to employ buffers with the appropriate pK_a values. Sodium acetate was used for pH values between 5 and 6, sodium phosphate between 6 and 8, and sodium pyrophosphate for experiments above pH 8. In order to keep the ionic strength of the reaction mixture constant, the buffer concentration was altered so as to produce an ionic strength of 120 ± 5 mM at each pH value. In order to insure the accuracy of the proton concentration, the pH was measured before and after each experiment.

Oligonucleotide-Directed Mutagenesis. The Kunkel method was used to accomplish the oligonucleotide-directed mutagenesis (Kunkel et al., 1987). Single-stranded DNA was generated from the phagemid pBSFlasy (pBluescript with the synthetic flavodoxin gene cloned into the EcoRI and HindIII sites) in the E. coli strain CJ236. A single degenerate oligonucleotide was used in the production of M56H, M56D and M56N:

5'-GGTTGCTCTGC(G)VACGGCGATGAAG-3'

where V = G, A or C. The nucleotide in parentheses is a silent mutation introduced for screening purposes. All of the mutations destroy an Styl site and M56H creates an FspI site; therefore, restriction mapping was used in screening transformants. M56D and M56N were positively identified by limited dideoxy chain-termination sequencing. As
always, the sequence integrity of the entire coding region was confirmed by nucleotide sequence analysis.

$^1$H NMR Spectroscopy. All NMR measurements were carried out as previously described, with minor modifications (Chang & Swenson, 1997). Purified flavodoxins were exhaustively dialyzed against 5 mM sodium phosphate, pH 6.5 (oxidized sample) or 50 mM sodium pyrophosphate, pH 7.5 (samples to be reduced). Samples were lyophilized, resuspended in D$_2$O and incubated at 42 °C. This procedure was repeated three times in order to replace the exchangeable protons with deuterons. The final samples were dissolved in D$_2$O to a final protein concentration of approximately 1 mM. When necessary, the pH was adjusted using 2.5% (w/w) DCl or 2.5% (w/w) NaOD. For the reduced flavodoxin samples, each was made anaerobic by using several cycles of partial vacuum and argon gas in NMR tubes with screw caps, and reduced by the anaerobic addition of sodium dithionite.

$^1$H NMR measurements were performed using a Bruker DMX-600 spectrometer at 600.13 Mhz, 300 K. Proton chemical shifts were compared to an internal standard of sodium 2,2-dimethyl-2-silapentane (DSS). Spectra were ported to a Silicon Graphics Indigo workstation and processed using Felix 95.0 software (Biosym Technologies).

$pK_a$ Curve Fitting Analysis and Modeling. The pH dependencies of the SQ/HQ couple were fit to equations describing an ionization-linked oxidation-reduction reaction (Clark, 1972). The general form of the equation is:

135
\[ E_m = E_0 + 59 \log \left( \frac{[H^+]^n + K_1^n[H^+]^{n-1} + K_1^nK_2^n[H^+]^{n-2} + \cdots + K_1^nK_2^nK_3^n \cdots K_n^n}{[H^+]^n + K_0^n[H^+]^{n-1} + K_0^nK_0^n[H^+]^{n-2} + \cdots + K_0^nK_0^nK_0^n \cdots K_0^n} \right) \]

where \( E_m \) is the midpoint potential at a specific pH value, \( E_0 \) is equal to the midpoint potential at pH 0, \( n \) is a constant equal to the number of ionizable residues in each oxidation state, and \( K_r^n \) and \( K_o^n \) are the ionization constants of each residue in the reduced and oxidized states, respectively. When fitting data to a model that included a \( pK_a \) value below the observable pH range (below pH 5) this value was treated as a constant (3.9).

### Results

*Generation, Expression and Characterization of the Mutants.* A single degenerate oligonucleotide primer has been used with the Kunkel method of oligonucleotide-directed mutagenesis to create three mutants of the flavodoxin from *C. beijerinckii*, replacing methionine 56 with histidine, aspartic acid, or asparagine. The mutant flavodoxins were over-expressed in *E. coli* using the hybrid tac promoter of the pKK223-3 expression vector, and purified as described. All mutant flavodoxins were judged to be ~95% pure by SDS-PAGE. All three mutant flavodoxins show very similar UV/visible absorption spectra in all three oxidation states (Figure 30). Of particular interest is the semiquinone spectra of M56H. It was thought that the introduction of a positively charged residue in such close proximity to the FMN (and partially removed from solvent) might lead to the stabilization of the red anionic semiquinone form. However, it is clear that the one-
Figure 30. The Near UV/Visible Absorbance Spectra of the M56H (A), M56N (B), and M56D (C) Mutant Flavodoxins. Spectra were determined in 50 mM phosphate buffer, pH 7.0. In each panel, the solid line represents the spectrum of the oxidized state, the dot-dashed line represents the blue neutral semiquinone spectrum, and the dashed line the fully reduced hydroquinone spectrum. Insets are plots of the changes in absorbance in the 580 nm region (representing the $\lambda_{\text{max}}$ of the semiquinone species) vs. those in the 450 nm region (largely representing the oxidized species) during the course of the reductive titration.
electron reduced form of this flavodoxin is virtually 100% blue neutral semiquinone, regardless of pH.

**Oxidation-reduction Potentials.** The oxidation-reduction potentials of each mutant were determined using UV/visible spectroscopy in 50 mM phosphate at pH 7, 25 °C, through equilibration with the redox indicator dye indigo trisulfonate ($E_{m,7} = -75$ mV). The OX/SQ couple of M56H, M56D, and M56N were all less negative than wild type, with values at pH 7 of -73 mV, -83 mV, and -77 mV respectively (Table 12). Each of these titrations produced slopes of around 50 mV/log unit, which are slightly lower than, but within experimental limits of the 59 mV slope expected for a single electron equilibrium (Figure 31). Additionally, the reversibility of each titration was demonstrated by reoxidation with potassium ferricyanide. The pH dependence of the OX/SQ couple of M56H demonstrates a slope of 55 mV/pH unit, consistent with a single proton transfer linked to the reduction of the flavodoxin to the semiquinone form (inset in Figure 31). The midpoint potential of indigo trisulfonate at each pH value was determined using the equations of Clark (1972). During the course of this investigation it was noted that the UV/visible spectrum of the indigo trisulfonate was pH dependent. These alteration in the absorbance spectrum were limited to transitions below 400 nm, with no change in the shape, or $\lambda_{\text{max}}$, for the prominent transition centered at 600 nm. As such, the multicomponent analysis of data involving this indicator were performed between 400 - 800 nm, effectively eliminating any differences in absorption.
Table 12. Oxidation-Reduction Midpoint Potentials\(^d\) of the *C. beijerinckii* Wild Type and the M56H, M56N, and M56D Mutant Flavodoxins. \(^a\) Values are reported in millivolts *versus* SHE. pH 7. 25 °C; the error in the midpoint potentials is estimated to be ± 5 mV. \(^b\) Values from Ludwig *et al.*, 1997. \(^c\) Potentials determined using indigo trisulfonate, indigo trisulfonate, safranine T. or benzyl viologen, as the redox indicator, respectively.

<table>
<thead>
<tr>
<th>Flavodoxin</th>
<th>(E_{OX-SQ})</th>
<th>(E_{SQ-HQ})</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>-92(^b)</td>
<td>-399(^b)</td>
</tr>
<tr>
<td>M56H</td>
<td>-73(^c)</td>
<td>-303(^d)</td>
</tr>
<tr>
<td>M56N</td>
<td>-77(^c)</td>
<td>-329(^e)</td>
</tr>
<tr>
<td>M56D</td>
<td>-83(^c)</td>
<td>-349(^e)</td>
</tr>
</tbody>
</table>
Figure 31. The Determination of the $E_{\text{OX/SQ}}$ Values for the M56H (■), M56N (▲), and M56D (●) Mutant Flavodoxins. The data shown is for pH 7, 25 °C, using indigo trisulfonate as the indicator dye. The inset shows the pH dependency of the $E_{\text{OX/SQ}}$ values for the M56H and M56D mutant flavodoxins.
The SQ/HQ couple of all the mutants were determined spectrophotometrically at pH 7, 25 °C, in 50 mM phosphate by equilibration with benzyl viologen \((E_{m,7} = -359\) mV), hydroxyethyl viologen \((E_{m,7} = -408\) mV) or safranine T \((E_{m,7} = -280\) mV). The midpoint potentials of the viologens are pH independent, while that of safranine T at each pH value was determined using the equations of Clark (1972). Nernst plots of the titrations using safranine T had slopes of \(58 \pm 5\) mV/log unit, consistent with a single electron reduction of the flavodoxin from the semiquinone to the hydroquinone (Figure 32). All of the mutants produced SQ/HQ values that were less negative than wild type (Table 12). The SQ/HQ couple was also studied as a function of pH for the wild-type, M56H, and M56D C. beijerinckii flavodoxins.

In order to properly buffer the titration solutions over the pH range of interest, it was necessary to use a number of buffering agents. Also, oxidation-reduction reactions can be affected by changes in the ionic strength of the reaction (Clark, 1972). In these studies it was noted that the ionic strength of the reaction altered the \(E_{SQHQ}\) of the M56H mutant by +1 mV for each 10 mM increase, at pH 7.5. Therefore, in order to produce comparable values for the pH dependency of this couple, it was necessary to maintain a constant ionic strength throughout the pH range under study. Two methods can be employed to maintain a constant ionic strength over a range of pH values, either through the addition of a salt or by altering the concentration of the buffering agent. The pH dependency of the wild-type flavodoxin (Mayhew, 1971a) was repeated in this study to confirm published results and to insure a good comparison to the mutant data. The data
Figure 32. The Determination of the $E_{SQ/HQ}$ Values for the M56H, M56D, and M56N Mutant Flavodoxins. Panel A shows titrations of the M56H mutant flavodoxin using safranine T as the indicator dye at various pH values. From top to bottom the pH values are, 5, 6, 7, 8, and 8.8. Panel B shows titrations of the M56D (●) and M56N ( ○ ) mutant flavodoxins in equilibrium with benzyl viologen.
generated for the wild type agrees with the previous results, indicating that above pH 7 there is very little change in potential (Figure 33). It should be noted, however, that there is still a slight reduction in potential between pH 7 - 8 (approximately -10 mV). thus the potential does not become truly independent of proton concentration until pH > 8. During the application of curve fitting analysis to the wild-type data, it became evident that the data could be fit equally well to equations including single or multiple ionizing residues (pKₐ values) in each oxidation state. These data are limited to the pH range tolerated by the protein, and with the limited data set it is only possible to assign value to the highest pKₐ value (or average value) present within the system. Thusly, curve fitting predicts that the highest pKₐ value in the wild-type C. beijerinckii flavodoxin is ~6.4.

The pH dependence of M56D was found to be very similar, but not identical, to that of wild type (Figure 33). Somewhat surprisingly, the pH independent portion of the M56D curve was 60 mV less negative than wild type, -349 mV vs. -409 mV. This result was unexpected because previous work demonstrated that the introduction of an additional acidic residue in proximity to the FMN produced a more negative SQ/HQ couple (Zhou & Swenson, 1995). However, as will be discussed below, the results of the other M56 mutants indicate that this position of the C. beijerinckii flavodoxin affects the thermodynamic stability of all three oxidation states of the flavoprotein, and substitution of the methionine residue is more complex than an acid-to-amide mutation. Therefore, a direct comparison of the hydroquinone stabilities cannot be made between the wild type
Figure 33. The pH dependence of the $E_{SQ/HQ}$ values for the M56D (○) and the Wild-type (○) C. beijerinckii Flavodoxins. The solid lines are the results of curve fitting analysis. The wild type fit was of a model with one ionizing residue, $pK_a^{HQ} = 6.4$. The M56D fit was produced using a model with two ionizing residues, $pK_a^{SQ} = 3.9$ and 5.7, $pK_a^{HQ} = 5$ and 6.4. The dashed line represents a theoretical single ionizing residue for the M56D data. $pK_a^{HQ} = 6.4$. 
and the M56D using only the SQ/HQ potential values. As such, it is more valid to
compare the SQ/HQ midpoint potential value of the M56D mutant (-349 mV) and to that
of the M56N mutant (-329 mV). This comparison, along with a similar comparison to
the M56H mutant [see the Discussion section], demonstrates that the introduction of a
negatively charged residue at this position does indeed produce a destabilization of the
anionic hydroquinone (Table 12). While the overall shape of the two pH dependencies
(wild type and M56D) were found to be similar, there is a subtle difference. The M56D
curve has a more gradual transition to pH independence, with a shift in the apparent pKa
to a more acidic value, <6.4. The M56D pH vs. E_sq/hq curve was fit best to an equation
representing two ionizations (two pKa values) in each oxidation state (Figure 33). The
pKa values which describe the curve in Figure 33 are 3.9 and 5.7 for the semiquinone, 5
and 6.4 for the hydroquinone form. The 3.9 value was not predicted by curve fitting,
rather this value was entered as a constant. It should be noted that if this constant is
assigned any value ≤ 3.9, an identical curve will be produced for the observable pH range.
Thus 3.9 is a maximal, rather than an absolute value for this pKa.

The pH dependence of M56H showed a marked difference from both wild type
and M56D (Figure 34). While wild-type and the M56D data included only one
apparent inflection, M56H had three observable apparent pKa values. The M56H data
was fit to an equation which describes the redox-linked ionization of two residues, the
histidine and a "virtual" acidic residue. This "virtual" acidic residue could represent at
least three different ionizing moieties, a single acidic residue, the average of a number of

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Figure 34. The pH dependence of the $E_{50/HQ}$ Values for the M56H Mutant Flavodoxin. The solid line represents the curve fitting analysis using a model with two ionizing residues. $pK_a^{SO} = 3.9$ and $6.4$, $pK_a^{HQ} = 5.3$ and 7.5.
acidic residues, or perhaps the anionic hydroquinone [see the Discussion section]. Using this model, the observable pKₐ values were determined to be 5.3, 6.4, and 7.5. The 6.4 and 7.5 values were assigned to the histidine residue in the oxidized and semiquinone form, respectively, and the 5.3 value represents the pKₐ of the "virtual" acidic residue in the hydroquinone form of the flavodoxin. It should be noted that while this model included only one "virtual" acidic residue, equations describing models that included multiple acidic acids (i.e. 3 or more ionizations in each oxidation state) fit the data equally well. The SQ/HQ couple of the M56H mutant was independent of pH above pH ~8.5, displaying a value of -332 mV.

¹H NMR Spectroscopy. The ¹H NMR spectra of oxidized M56H at various pH values are shown in Figure 35. Histidine 56 is the only histidine residue in this C. beijerinckii flavodoxin. Three resonance peaks have been identified within the aromatic region of the spectrum (a, b, and c). Peak a can be assigned to the C2H of histidine 56 by both the absolute chemical shift values and the direction of change in chemical shift as a function of pH (Markley, 1975). While peak b has the proper chemical shift and response to pH change which would lead to the assignment of this resonance to the histidine C4H, its assignment is still in question (see below). Peak c is unusual, compared to a and b, in that it shifts downfield. It is difficult to assign this peak without additional data; however the FMN C6H in the D. vulgaris and M. elsdenii flavodoxins (the latter is homologous to the C. beijerinckii flavodoxin) resonates at a similar chemical shift (Knauf et al., 1993; van Mierlo et al., 1990). So, while three peaks that respond to changes in pH
Figure 35. The $^1$H NMR Spectra of the Oxidized M56H Mutant Flavodoxin at Various pH Values. The peak labeled $a$ has been assigned to the C2H of His56. The spectra are ordered from lowest pH (5.91) to highest pH (8.51), top to bottom.
have been identified, only peak \( a \) can be reasonably assigned (to the histidine C2H). The assignment of the other two peaks will require additional data. Histidine C2H and C4H assignments can be verified using homonuclear 2D correlation spectroscopy (COSY) analysis (King & Wright, 1982; Wüthrich, 1986). Confirmation of the speculation that peak \( c \) is due to the FMN C6H will require more detailed analysis, and can be facilitated using isotopically enriched FMN (van Mierlo et al., 1990).

The \( pK_a \) of histidine 56 was determined by fitting the NMR titration data of the C2H resonance (peak \( a \)) to the Hill equation (Markley, 1973). The data were fit by curve-fitting analysis, generating a \( pK_a \) value of 6.5 (Figure 36). For reasons discussed below, it is reasonable to assume that the \( pK_a \) of oxidized FMN is equivalent to the \( pK_a \) of the semiquinone FMN. As such, the value of 6.5 corresponds very well with the value determined by fitting the pH dependency of the \( E_{SQ/HQ} \) to the equation describing a redox-linked ionization of the histidine. The titration data of peak \( c \) produced a similar value (\( pK_a = 6.3 \)). While this peak cannot be assigned to H56, it is intriguing that this resonance demonstrates a pH dependence that matches the histidine protonation. If, through the collection of additional data, peak \( c \) is assigned to the FMN C6H, this response to pH would be indicative of the histidine protonation altering the chemical shift of this proton. The peak \( b \) data also fit well to the Hill equation, generating a \( pK_a \) value of 5.8. This value is quite different from that determined for the His56 C2H (peak \( a \)). Therefore, it is possible that this resonance is not due to the His56 C4H, or, because the determination of the chemical shift values in this region of the spectrum is made difficult
Figure 36. Curve Fitting Analysis of the $^1$H NMR Peaks $a$, $b$, and $c$ from the pH Titration of the Oxidized M56H Mutant Flavodoxin. Panel A shows the fits of peaks $a$ (●) and $c$ (■) to the Hill equation. Panel B shows the fit of peak $b$. In each case the open symbols represent back titration data points. Note that peak $a$ has been assigned to the C2H of His56.
due to the large number of resonances, the resultant value is in error. As such, any conclusions concerning peak \( b \) will require additional data.

The \(^1\)H NMR spectra of fully reduced M56H at various pH values are shown in Figure 37. A single peak, labeled \( a \), was assigned to the C2H of H56. This assignment was based on the position of the chemical shifts and response of the resonance to change in pH. This data was fit to the Hill equation, giving a \( pK_a \) of 7.6 for histidine 56 in the fully reduced flavodoxin (Figure 38), which corresponds well to the value obtained from curve fitting of the pH dependency of \( E_{SQ/HQ} \). Thus, the \( pK_a \) of the histidine increases by \( \sim 1 \) pH unit upon reduction of the flavodoxin. The significance of this large redox-linked shift will be discussed below.

**Discussion**

Electrostatic effects have been implicated in the control of the oxidation-reduction potentials of redox active proteins. These types of interactions have been examined in detail in the flavodoxin from *D. vulgaris* (Swenson & Krey, 1994; Zhou & Swenson, 1995, 1996a, 1996b). It is important to extend our knowledge of how charged amino acids alter the redox chemistry of the flavin in other flavodoxins, in order to produce a standard model for electrostatic control of oxidation-reduction potential. When such a model is developed, it can be extended to include other flavoproteins and redox-active proteins in general. The data produced with the M56H, M56D and M56N mutants begin to elucidate how such interactions alter the potentials of the FMN in the *C. beijerinckii* flavodoxin. The following section will first describe how these mutants affect each of the
Figure 37. The $^1$H NMR Spectra of the Fully Reduced M56H Mutant Flavodoxin at Various pH Values. The peak labeled $a$ has been assigned to the C2H of His56. The spectra are ordered from lowest pH (6) to highest pH (9), top to bottom.
Figure 38. pH Dependence of the $^1$H NMR Chemical Shifts for the C2H of His56 in the Fully Reduced M56H Mutant Flavodoxin. This is the pH dependence of the peak labeled $a$ in the $^1$H NMR spectra generated in the pH titration of the fully reduced M56H flavodoxin mutant. The data are fit to the Hill equation.
two one-electron oxidation-reduction steps. Next, the pH dependency of these couples will be analyzed in detail for the wild-type, the M56H and the M56D *C. beijerinckii* flavodoxins. Finally, the $^1$H NMR data will be examined.

*Effects on the Oxidized/Semiquinone Couple.* The oxidation-reduction potentials of flavodoxins are directly related to the relative stability of the holoprotein complex in each of the two oxidation states. The preceding chapters demonstrated that residue 56 can change the stability of the holoprotein complex by altering either the protein structural energy or the strength of the interactions between the apoflavodoxin and the FMN cofactor. The mutants in this series have the possibility of altering both of these factors as well. All three mutants increase the potential of the OX/SQ couple at pH 7. by 10-20 mV. An increase of similar magnitude observed in the M56A mutant has been attributed to the removal of a stabilizing electrostatic interaction between the sulfur atom of methionine 56 and the oxidized FMN. While the removal of the methionine sulfur atom is certainly one factor contributing to the observed alterations in the $E_{\text{OX/SQ}}$ of these mutants, these mutants introduce additional functional groups which may interact with a particular oxidation state of the FMN.

Histidine residues are known to interact specifically with the aromatic ring systems in proteins (Loewenthal *et al.*, 1992; Bromme *et al.*, 1996). A specific aromatic-histidine interaction in barnase stabilizes the protein by about 1 kcal/mol when the histidine is protonated (Loewenthal *et al.*, 1992). Thus, it is possible for a specific interaction to exist between the flavin ring system and His56. If this interaction is
preferential for either oxidized or FMN semiquinone, then the presence of a histidine residue in proximity to the flavin will alter the $E_{\text{OX/SQ}}$. This, however, is not the case. The OX/SQ midpoint potentials for the M56H mutant, the M56A, and the more sterically similar M56N mutant are all very similar. Thus, the imidazole side chain does not appear to introduce any interaction that is preferential for either the oxidized or the FMN semiquinone. Moreover, the pH dependency of the OX/SQ couple in the M56H mutant is linear, indicating that there is no redox-linked ionization for this couple (i.e. neither the protonated nor the unprotonated histidine interacts specifically with the FMN in these two oxidation states). These data agree with solution studies, which indicated that both protonated and unprotonated imidazole rings interact very weakly with flavins (Johnson & McCormick, 1973; Draper & Ingraham, 1970), and with mutants created at the $si$ face of the FMN in the $D.\ vulgaris$ flavodoxin in which both the Y98A and the Y98H mutant had nearly identical $E_{\text{OX/SQ}}$ values (Swenson & Krey, 1994).

Similar arguments also suggest that an aspartic acid at position 56 has no preferential interaction with the oxidized or semiquinone flavin. The $E_{\text{OX/SQ}}$ of the M56D mutant has a linear pH dependency and is very similar to that for both M56A and M56N. This data seems to be contrary to the electrostatic nature of the sulfur-FMN interaction. If there is an attractive force between a partially charged electronegative sulfur atom and the FMN, it seems logical that a residue which carries a formal negative charge should have a similar, if not greater, interaction energy. However, in order to affect the midpoint potential, the energy of any such interaction must differ between
oxidation states. Thus, the data generated in the present study does not preclude such an interaction, it simply indicates that the strength of any specific side chain-flavin interaction is unchanged upon the formation of the FMN semiquinone (similar to the M56C and M56S mutants).

*Effects on the Semiquinone/Hydroquinone Couple.* This couple is known to be affected by the electrostatic character of the residues surrounding the FMN binding site. The neutralization of a negatively-charged residue, or the introduction of a positively-charged residue, leads to an increase in the SQ/HQ values in the *D. vulgaris* flavodoxin through an electrostatic interaction with the anionic hydroquinone (Swenson & Krey, 1994; Zhou & Swenson, 1995). Thus, the introduction of a negatively-charged residue (i.e. M56D) should produce a more negative $E_{SQ/HQ}$ in the flavodoxin from *C. beijerinckii*. However, all three mutants in this series shifted the SQ/HQ midpoint potential to much less negative values in comparison to wild type. Does this observation indicate that the SQ/HQ couple in the *C. beijerinckii* flavodoxin is not affected by electrostatic interactions? The answer is a definite no.

If the SQ/HQ couples of the three mutants in this series are compared to each other, it becomes obvious that the formation of the fully-reduced hydroquinone is facilitated by decreasing the negative electrostatic field surrounding the FMN binding site. This electrostatic field becomes progressively less negative on going from M56D to M56N to M56H, and the $E_{m,7}$ values of the SQ/HQ couple follow the expected increasingly less negative trend, -342 mV, -329 mV, and -303 mV, respectively.
Additionally, the pH dependencies of this couple in the wild type, the M56H mutant, and the M56D mutant, demonstrate quite clearly that the values of the SQ/HQ potential in these flavodoxins are affected by the ionization state of the residues surrounding the FMN (see below). The question that remains is, why do all three mutants demonstrate an SQ/HQ value which is more positive than wild type?

Midpoint potentials are determined by the relative stability of each oxidation state involved in the oxidation-reduction. In order to produce a very negative $E_{SQ/HQ}$, flavodoxins thermodynamically stabilize the FMN semiquinone and destabilize the hydroquinone. Thus, mutations which produce less negative $E_{SQ/HQ}$ values have either lessened the stabilization of the semiquinone or reduced the destabilization of the hydroquinone (or a combination of both effects). A change in the stabilization of the semiquinone can be observed in the $E_{OX/SQ}$ data. If a mutation decreases the stabilization of the FMN semiquinone, relative to the oxidized, a more negative $E_{OX/SQ}$ value will be produced (e.g. the M56L mutant). Since all of the mutants in this series produce a less negative midpoint potential for the OX/SQ couple, it is reasonable to conclude that these mutations do not significantly decrease the stabilization of the FMN semiquinone in these flavodoxins. Thus, the shift in $E_{SQ/HQ}$ to less negative values observed in the M56D, M56N, and M56H mutants must be attributed to the removal of some interaction which serves to destabilize the FMN hydroquinone in the wild-type flavodoxin. The data generated with the series of aliphatic M56X mutants agree with this conclusion, and implicate the methionine sulfur atom as a causal moiety leading to the destabilization of
the hydroquinone. It should be noted that these conclusion must be considered preliminary without information concerning the FMN dissociation constants.

*The pH Dependencies of the Wild-type, M56D and M56H flavodoxins.* Any oxidation-reduction reaction that also involves the transfer of a proton will necessarily be affected by the proton concentration by mass action. These affects are easily visualized by a plot of pH versus midpoint potential. **Figure 39** depicts three theoretical pH dependencies, illustrating how the protonation of each ionization state affects the shape of the pH vs. $E_m$ function. The shape of this graph is affected by both the number of protons being transferred per electron and the oxidation state that is undergoing ionization. Dividing the first derivative of the function defined by such a plot by 59 mV/pH unit gives the number of protons being transferred per electron at any particular pH (e.g. a slope of 59 indicates one proton, 118 two protons etc.). The sign of the second derivative at any pH in which there is a change in the slope of the graph denotes which ionization state is being protonated, positive and negative indicating the protonation of the oxidized and the reduced forms, respectively. Therefore, inflections that alter the slope toward a smaller value are due to an ionization of the reduced form and *vice versa*. This information, along with knowledge of the system under study, enables the generation of a model for any specific oxidation-reduction reaction.

The wild-type, M56D, and M56H flavodoxins all produced linear pH dependencies for the OX/SQ couple, with slopes of 59 ± 5 mV (**Figure 31, inset**). As stated above, this is indicative of a single proton transfer linked to the reduction of these
Figure 39. Theoretical pH versus $E_m$ Plots. The solid line represents a reaction involving one ionization for the oxidized species ($pK_a = 5$) and one for the reduced species ($pK_a = 8$). The dashed line represents a reaction in which only the reduced species is protonated ($pK_a = 8$). The dot-dash line represents a reaction in which only the oxidized species is protonated ($pK_a = 4$).
flavodoxins, proceeding from the oxidized to the blue neutral semiquinone. Thus, it can be concluded that the neutral semiquinone is the only one-electron reduced form in these flavodoxins within this pH range (i.e. no formation of the red anionic semiquinone). Since the formation of the blue neutral semiquinone requires the addition of a proton, it is obvious that the proton acceptor is the FMN (at N5). The linearity of the M56H and M56D data also indicates that the OX/SQ couple is not significantly affected by the ionization state of the surrounding residues, as expected for an equilibrium in which both oxidation states are neutral. This observation agrees with data collected from the *D. vulgaris* flavodoxin mutants (Swenson & Krey, 1994; Zhou & Swenson, 1995; Chang & Swenson, 1997). These pH dependencies are expected to be linear because the pKₘ of the N5H of the FMN semiquinone is estimated to be > 13, well above the observable pH range (Ludwig *et al.*, 1990). If it were possible to extend the pH range of these data to very basic values, the pKₘ of the N5H could be determined from the inflection of the curve. The formation of the red anionic SQ does not require a proton transfer and the ionizing species is the reduced member of the redox couple. Therefore, if it was observable, this inflection would change the slope of the function from 59 mV/pH unit to zero.

It was hypothesized that the M56H mutant might stabilize the formation of the red anionic semiquinone. Any such stabilization would be evidenced by a shift in the pKₘ of the N5H of the FMN semiquinone (pKₘSQ) to a more acidic value. Additionally, at pH values near the pKₘSQ, the UV/visible spectrum of the one-electron reduced flavodoxins
would contain a mixture of the absorption transitions characteristic of both protonated and unprotonated FMN semiquinone (see Figure 4). The linear pH dependence, along with the UV/visible absorption data, indicate that the one-electron reduced M56H mutant is entirely blue neutral semiquinone up to pH 8.4, within the limits of detection. Therefore, this mutant does not produce enough stabilization of the red anionic semiquinone to shift the $pK_a^{SQ}$ into the pH range of this study.

The data collected for the pH dependency of the SQ/HQ couple of the wild-type *C. beijerinckii* flavodoxin agrees very well with previous values (Mayhew, 1971a). These data can be fit equally well to a number of different equations, each describing equilibria containing from one to several ionizing residues in each oxidation state (Figures 33 & 40). The simplest model is one in which the FMN N1 is being protonated, forming the neutral hydroquinone (*i.e.* one $pK_a$, assigned to the reduced member of the redox couple). A second reasonable model involves the redox-linked protonation of a nearby acidic residue, as illustrated in Figure 41 (*e.g.* Glu59 as has been proposed by Ludwig and coworkers) (Ludwig *et al.*, 1992). In this model, the destabilizing electrostatic interaction between the glutamate and the anionic hydroquinone increases linearly as the residue becomes unprotonated (a single proton transfer). Once the acid is fully deprotonated, the equilibrium becomes pH independent. The equation describing this model has one $pK_a$ for the residue in the oxidized protein and one $pK_a$ in the reduced form. There is no data establishing the $pK_a$ in the oxidized state, as such, either the $pK_a^{OX}$ or the $E_0$ value (the midpoint potential at pH = 0) must be treated as a constant (*i.e.* supplied to the curve
Figure 40. Theoretical Curves Modeling the SQ/HQ pH Dependence of the Wild-type *C. beijerinckii* Flavodoxin. The solid line depicts the model in which the FMN hydroquinone is protonated at N1 (pK$_a^{HQ}$ = 6.4). The dotted line is generated using a redox-linked model with a single acidic residue (pK$_a$ in the semiquinone = 3.9; pK$_a$ in the hydroquinone = 6.4). The dash-dot line represents a redox-linked model with multiple ionizing residues (semiquinone pK$_a$ values = 4.9, 5.2, 5.5, 5.8, 6.1; hydroquinone pK$_a$ values = 5.2, 5.5, 5.8, 6.1, 6.4). Note that the curves are superimposable but have been separated for clarity.
Figure 41. Equilibria Demonstrating a Redox-Linked Ionization. FLD represents the flavodoxin in each ionization state (denoted by the subscript). XH and X' represent an ionizable residue that is in proximity to the FMN binding site in the protonated and unprotonated forms, respectively. $E_p$ and $E_u$ represent the midpoint potential in the protonated and unprotonated forms, respectively. $pK_a^{SQ}$ and $pK_a^{HQ}$ are the ionization constants for the linked residue in the semiquinone and hydroquinone states.
fitting algorithm). If the $pK_a$ of the residue in the oxidized protein is assigned a value $\geq 4.5$, a curve identical to the single $pK_a$ model is generated between the pH values of this study. Curve fitting analysis of both models indicated a $pK_a = -6.4$ for the reduced moiety (Figure 40). While both of these models successfully fit the wild-type C. beijerinckii flavodoxin data, there is evidence that neither is correct. NMR and UV/visible spectroscopic data indicates that the FMN remains unprotonated to pH values below 5 (van Schagen & Müller, 1981; Franken et al., 1984; Vervoort et al., 1985, 1986; Ludwig et al., 1990), and the E59Q mutant has a pH dependency very similar to wild type (Bradley & Swenson, in preparation). Thus, a model which includes multiple ionizing residues is most likely the appropriate representation.

Two pieces of evidence argue that the multiple-ionization model correctly portrays the system in flavodoxins. First, the charged residues surrounding the flavin binding site alter the stability of the anionic hydroquinone. Second, this model fits reasonably well to the pH dependencies of the $E_{SQ/HQ}$ for both wild type and the charge neutralization mutants in the D. vulgaris flavodoxin (Swenson & Zhou, 1996). Therefore, a multiple-ionization model has been applied to the wild-type C. beijerinckii flavodoxin. This model included five $pK_a$ values in each of the semiquinone and hydroquinone forms of the flavodoxin. Five values were used because there are five acidic residues within 13 Å of the FMN N1 atom, and acidics within this range have been shown to affect the formation of the anionic hydroquinone (Zhou & Swenson, 1995). Each of these five residues were predicted to alter the $E_{SQ/HQ}$ by $-15$ mV, using the D. vulgaris
data and comparisons between the M56D, M56N and M56H reduction potentials (see below). Thus, the shift in the $pK_a$ values of these acidics, produced by the formation of the hydroquinone, can be calculated \[
\Delta E_m = (2.303RT/nF)(pK_a^{\text{HQ}} - pK_a^{\text{SO}})\]. Using these limitations, a curve which fit very well to the data was produced (Figure 40). Although all three of these models fit the wild-type data with equal accuracy, the collective evidence argues that a multiple-ionization model, including all of the surrounding ionizable groups, best represents the effects causing the pH dependence of the SQ/HQ oxidation-reduction potential in flavodoxins.

The $E_{\text{SQ/HQ}}$ data from the M56H mutant was fit to an equation which included ionization constants for the histidine and a "virtual" acidic residue, in each oxidation state. This "virtual" residue represents the moiety (or moieties) which causes the pH dependence observed in the wild-type flavodoxin data. As indicated above, the pH dependence of $E_{\text{SQ/HQ}}$ for the wild-type flavodoxin is most likely not caused by a single unique residue (Bradley & Swenson, in preparation). Thus, this residue was termed "virtual" in this model to emphasize that the determined $pK_a$ is not assignable to any one specific moiety. Application of curve fitting analysis using this model produced three $pK_a$ values, 5.3, 6.4, and 7.5. From the direction of the inflections, both the 5.3 and the 7.5 values were assigned to ionizations in the reduced flavodoxin, and the 6.4 value to an ionization of the semiquinone M56H flavodoxin. The 5.3 value has been assigned to the "virtual" acidic residue, and the 6.4 and 7.5 values to His56. The shift in $pK_a$ of the "virtual" acidic residue from 6.4 in the wild type to 5.3 in the M56H mutant is logical.
The introduction of the positively charged histidine will likely stabilize the unprotonated form of the "virtual" acidic residue, through electrostatic interactions, causing a shift in the $pK_a$ to more acidic values.

The histidine interacts with the anionic hydroquinone in a similar fashion, hence the difference between the $pK_a$ values of His56 in each oxidation state. This change in $pK_a$ predicts that the protonation of the histidine is responsible for an ~ 65 mV increase in the $E_{SQ/HQ}$ value. This increase is smaller than that noted for the Y98H *D. vulgaris* mutant, not surprising considering that the Y98H histidine is located closer to the developing negative charge (Swenson & Krey, 1994). What is surprising is that this 65 mV change is not replicated by the introduction of a negatively charged residue in exactly the same location. At very basic pH values, the histidine of the M56H mutant carries no charge, while the aspartic acid of M56D carries a full -1 charge. The difference between the $E_{SQ/HQ}$ values, at pH > 8.5, of these two mutants is ~ -20 mV. Thus, the introduction of a +1 charge causes 65 mV of stabilization, while a -1 charge at the same location causes only -20 mV of destabilization. Clearly, there must be some side-chain specific factors affecting the interaction energy which differ between the histidine and the aspartic acid. Two possibilities are, 1) a variance in the solvation state of the interacting residue, or 2) perhaps a difference in the side-chain flexibility. If the positively charged histidine is packed closer to the anionic flavin than the aspartic acid, the discrepancy in interaction energy could be explained by a shorter interaction distance and/or a smaller dielectric constant between the side chain and the hydroquinone. It is interesting to note that the
value determined for the $E_{S Q/H Q}$ of the M56H mutant when the histidine is unprotonated (-332 mV), is approximately equal to that of the M56N mutant at pH 7 (-329 mV).

The overall shape of the pH vs. $E_{S Q/H Q}$ plot for the M56D mutant is similar to the wild-type data. There is, however, an important difference. At no point does the slope approach 59 mV/pH unit. This observation indicates three things. First, there is more than one ionizable residue responsible for the observed pH dependency. Second, some moiety in the semiquinone state of the M56D mutant has an ionization constant within the observable pH range. Third, the $pK_a^{SQ}$ of this residue must be larger than the next highest $pK_a^{HQ}$. Using these limitations, the data was fit using a two-residue, redox-linked ionization model, very similar to the model used for the M56H mutant. The results of this fit indicated that one residue had $pK_a$ values of ≤ 3.9 in the semiquinone state and 5.0 in the fully-reduced flavodoxin. The values predicted for the second residue were 5.7 and 6.4, in the semiquinone and hydroquinone states, respectively. A multiple-residue redox-linked models fit these data equally well, producing similar shifts in the overall $pK_a$ values. As such, both residues in the two-residue model discussed here must be considered "virtual" residues (see above).

The comparisons to the M56H and the M56N mutants indicated that Asp56 destabilizes the formation of the hydroquinone by ~ 20 mV, an amount similar to the average destabilization associated with each acidic residue in the *D. vulgaris* flavodoxin (Zhou & Swenson, 1995). This corresponds to an increase in the $pK_a$ of Asp56 by ~ 0.3 units upon the formation of the fully-reduced flavodoxin. As such, neither of these
virtual residues modeled for the M56D mutant, both with $pK_a$ shifts larger that 0.3. can be defined as being only due to Asp56. Rather, these observed $pK_a$ values must include one, or more, of the acidic residues native to the flavodoxin. If it is assumed that the FMN is not responsible for any of the observed pH dependency, there must be a minimum of three acidic residues (including Asp56) which have ionization constants within this pH range (i.e. at least three acidics responsible for the observed pH dependence). By extrapolation, then, there must be at least two residues which affect the wild-type pH vs. $E_{SQ/HQ}$ plot.

While it is impossible to assign these $pK_a$ values to any specific amino acid(s) with these data, a number of conclusions can be drawn that do not require any such assignment. First, it is quite reasonable to conclude that the introduction of Asp56 differentially destabilizes the unprotonated form of one or more of the surrounding acidic groups. Such a destabilization will shift the corresponding ionization constants to more basic values, the magnitude of the shift dependent upon the amount of destabilization. Second, these data indicate that there is more than one acid residue affecting the stability of the hydroquinone in both M56D and wild type. This observation adds to the mounting evidence supporting the multiple-residue redox-linked ionization model for the pH dependence of the SQ/HQ couple in flavodoxins.

$^1H$ NMR of the M56H C. beijerinckii Flavodoxin. The $pK_a$ values of His56 determined from the oxidation-reduction titration data included a number of assumptions. In order to directly measure these values and to reinforce the conclusions drawn from

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them, $^1$H NMR spectroscopy was used to directly measure the ionization of histidine in both the oxidized and the fully-reduced holoflavodoxin. The results of these experiments indicate that His56 has $pK_a$ values of 6.5 and 7.6 in the oxidized and hydroquinone states, respectively. Determination of the $pK_a$ in the semiquinone form was not attempted because previous results indicated that the paramagnetic shielding effect of the unpaired electron on the flavin caused severe line broadening (Chang & Swenson, 1997). However, the linear pH dependence of $E_{OXSQ}$ of the M56H mutant indicates that this couple is not affected by a change in the protonation state of His56, and by inference the protonation state His56 is not affected by the formation of the semiquinone. Thus, it is reasonable to assume that the $pK_a$ of the histidine is very similar in both the oxidized and one-electron reduced states. Additionally, in a mutant flavodoxin from *D. vulgaris* (Y98H), the $pK_a^{OX}$ value measured by $^1$H NMR spectroscopy corresponded very well to the $pK_a^{SQ}$ value determined from the redox titration data (Chang & Swenson, 1997). Therefore the $pK_a$ value of His56 in the one-electron reduced flavodoxin is assumed to be equal to the measured $pK_a$ value in the oxidized form, *i.e.* 6.5.

The $pK_a$ values of His56 as determined by NMR match those calculated by fitting the pH dependent $E_{SQHQ}$ data to the redox-linked ionization model. This model included three ionization constants, the third representing the ionization of some “virtual” acidic residue. Since, two out of the three $pK_a$ values predicted by curve fitting have been proven correct by direct spectroscopy, the third $pK_a$ (5.3) is also likely to be correct. Thus, the “virtual” acidic residue shifts from a $pK_a$ of 6.4 in the fully-reduced wild type
to 5.3 in the fully-reduced M56H mutant. This is quite reasonable, and is indicative of
the histidine stabilizing the unprotonated form of the acidic residues which in turn
produces the observed decrease in $pK_a$. In the Y98H mutant there is no $pK_a$ observed for
this “virtual” acidic residue (Swenson & Krey, 1994). This indicates that the introduction
of a positively charged histidine at this position in the flavodoxin structure shifts $pK_a$
values of all the acidic residues which destabilize the hydroquinone below the observable
pH range. It would be intriguing to measure the $pK_a$ values of the acidic residues
surrounding the flavin binding site directly, possibly using heteronuclear NMR techniques
(Forman-Kay et al., 1992; Oda et al., 1994).

**Conclusion**

This work has demonstrated that the midpoint potential for the $E_{OX/SQ}$ couple of
the *C. beijerinckii* flavodoxin is unaffected by charged residues located in the flavin
binding site. While all of these mutations increased the reduction potential of the OX/SQ
couple, this increase was independent of the charge on the residue introduced. An
increase of similar magnitude in the M56A mutant has been ascribed to the removal of a
favorable electrostatic interaction between the methionine sulfur and the surface of the
flavin. It is logical that the removal of this interaction by these mutations also causes a
destabilization of the oxidized holoflavodoxin complex producing the observed potential
changes. This speculation could be proven through the determination of the $FMN_{OX}$
dissociation constants for these mutant flavodoxins.
The M56D, M56H and M56N mutants demonstrate, convincingly, that electrostatic interactions are involved in poising the reduction potential of the SQ/HQ couple in the *C. beijerinckii* flavodoxin. While all of the mutants produced a less negative $E_{SQ/HQ}$, this increase is almost certainly caused by the removal of some other destabilizing interaction common to all three mutants (and to all the previous M56X mutants). The relative comparisons of these three mutants indicated that the hydroquinone is stabilized by the introduction of a positively charged residue and destabilized by a negatively charge residue. Additionally, the pH dependence of this couple is altered drastically in both the M56H and the M56D mutants. These changes are due to the electrostatic effects the introduced residue exerts upon both the anionic hydroquinone and the surrounding ionizable residues. Examination of the accumulated data demonstrated that the pH dependence of the $E_{SQ/HQ}$ in the wild-type flavodoxin is caused by the collective destabilization produced by the acidic residues surrounding the FMN binding site. The differential interaction energy between the side chain at position 56 and the anionic hydroquinone, noted for the M56D and M56H mutants, seems to indicate a redox-dependent conformational change (similar to the mutants in Chapter 3). It would be interesting to determine if such structural changes actually occur, through x-ray crystallography or perhaps NMR spectroscopy.
CHAPTER 5

SUMMARY AND FUTURE DIRECTION

Flavoproteins are involved in nearly every metabolic pathway. In order to take part in such a myriad of different reactions, the chemistry of the cofactor is modulated through flavin-protein interactions. Flavodoxins have proven an excellent model system for the study of a number of these interactions. These pure electron transferases separate the two one-electron reduction potentials of the sole redox active FMN through the selective stabilization of the blue neutral semiquinone and destabilization of the anionic hydroquinone. This work adds to the body of knowledge being produced leading toward the elucidation of the mechanisms by which such selective stability of each oxidation state of the cofactor is produced. Comparison of the oxidation-reduction potentials and the primary sequences of many flavodoxins indicated that the methionine at position 56 in the C. beijerinckii flavodoxin was important in the function of this protein.

Summary of Conclusions. Methionine 56 has a number of unique properties, based both on the characteristics of its position in the tertiary structure and the properties of its side chain, that allow this residue to alter the oxidation-reduction potentials of both FMN couples. In a comprehensive study to investigate the nature
these mechanisms, oligonucleotide-directed mutagenesis was used to replace Met56 with thirteen of the other naturally-occurring amino acids. These thirteen residues included all of the aliphatics and all of the aromatics, as well as glycine, cysteine, serine, histidine, aspartic acid, and asparagine. These substitutions span nearly all of the possible chemical characteristics at this position. Through the examination of all of the mutant flavodoxins and data generated for the wild type, a number of interesting conclusions were made.

The data presented here both confirm and add to our basic understanding of the mechanisms controlling the reduction potentials of both redox couples of the bound FMN cofactor. The $E_{\text{OX/SQ}}$ is a function of two energy differences, both of which are affected by Met56. The potential of the OX/SQ couple is affected slightly by both the size and the flexibility of residue 56. These affects are likely produced by an energetic preference for a particular side chain in the protein structures of each oxidation state ($\Delta G_{\text{c,ox/sq}}$). The sulfur moiety of Met56 also has a preferential interaction with the oxidized FMN not found in the semiquinone form, effectively lowering the $\Delta G_{\text{i,ox/sq}}$. Thus, while Met56 is involved in the poising of the $E_{\text{OX/SQ}}$, its contribution to the stabilization of the semiquinone form is relatively small. On the other hand, Met56 plays a rather large role in the destabilization hydroquinone (on the order of -1.0 to -1.5 kcal/mol). In all the mutants the $E_{\text{SQ/HQ}}$ is less negative than the wild-type value. Hence, there is some factor specific to methionine that destabilizes the anionic hydroquinone which is not reproduced in any of the mutant flavodoxins. It is logical to conclude that the thioether moiety of Met56 is important in the destabilization of the hydroquinone through electrostatic
interactions. The function of Met56 is in stark contrast to Gly57, which is directly involved in the conformational change associated with the reduction of the oxidized FMN to the semiquinone (Ludwig et al., 1997). This difference in functionality is dramatically illustrated through a comparison of the wild-type, M56A, and G57A flavodoxins (Figure 42). While the Gly57 to Ala substitution significantly decreases the stability of the semiquinone, it has almost no effect on the stability of the hydroquinone complex. In contrast, substitution of an alanine for Met56 drastically alters the stability of the hydroquinone complex while having only a small effect on the semiquinone. The overall conclusion is that the re-face residue contributes most of its functionality toward poising the Esq/HQ through the destabilization of the hydroquinone.

There appears to be an attractive interaction between a sulfur atom and the o-xylene subnucleus of the oxidized FMN, on the order of -0.5 kcal/mol. This interaction is not limited to sulfur, but is also present for oxygen and the planar faces of side-chain aromatic rings. As such, the nature of the interaction is almost certainly electrostatic. It is unusual that this electrostatic attraction for the oxidized FMN is translated into a putative repulsive interaction for the anionic hydroquinone only in the methionine-containing wild-type C. beijerinckii flavodoxin. This lead to the speculation that small, oxidation-state dependent, structural changes in the side chains of residue 56 in the mutant flavodoxins served to minimize, or remove, any such repulsive interactions. If these predicted alterations in side-chain structure are proven to be correct, then electrostatic forces are implicated as the casual factor for the difference in conformation
Figure 40. Comparison of the Free Energy of Binding for the Various Oxidation States of the FMN Cofactor by the Wild-type, M56A, and G57A Mutant Flavodoxins. The horizontal bars represent the differences in the free energies from the oxidized wild-type holoflavodoxin complex. The numbers represent the differences in the free energy of binding between the oxidized (ox) and semiquinone (sq) as well as semiquinone and hydroquinone (hq), as indicated by the dashed line. The stars indicate the holoflavodoxin complex that is affected the most by each particular mutant.
between the oxidized and semiquinone forms observed within the methionine side chain of the wild-type *C. beijerinckii* flavodoxin (see Figure 10) (Burnett, *et al.*, 1974; Smith *et al.*, 1977; Ludwig & Luschinsky, 1992).

It is clear that the change in stability of the anionic hydroquinone produced by the introduction of a charged residue (both positive and negative) is similar in both the *C. beijerinckii* and the *D. vulgaris* flavodoxins each with distinctively different FMN-binding sites. Therefore, it is likely that all the other flavodoxins will respond in a similar fashion to alterations in the charged residues surrounding the flavin binding site of these proteins. The pH dependency of the $E_{SQHQ}$ couple in flavodoxins has been controversial. This work provides evidence supporting the multiple-residue, redox-linked ionization model (Swenson & Zhou, 1996). In this model, the observed pH dependency is produced through the cooperative interaction between the ionizing residues surrounding the flavin binding site. This interaction includes both the destabilization of the anionic hydroquinone and the mutual destabilization between the surrounding residues. In this way, the $pK_a$ values of the surrounding residues are shifted into the pH range observable for flavodoxins.

*Why Has "Nature" Chosen Methionine for Position 56?* The evidence, taken collectively, appear to be consistent with an important role for sulfur-flavin interactions in the modulation of the one-electron reduction potentials in this flavodoxin. This conclusion is necessarily somewhat qualified because each substitution could have more than one structural consequence. However, the differing properties of each group of
mutants do seem to largely exclude the effects of steric hindrance, changes in hydrophobicity, and in solvent exposure and/or polarity of the cofactor binding site. Despite these qualifications, the unique functional role of Met56 in this flavodoxin is unequivocal. Flavodoxins function as low potential electron transfer proteins, using the SQ/HQ couple of the FMN cofactor \textit{in vivo}. Therefore, one of the primary functions of these proteins is to thermodynamically separate these two oxidation states, either through the stabilization of FMN$_{\text{SQ}}$, destabilization of FMN$_{\text{HQ}}$, or both, generating the low one-electron reduction potential. \textbf{Figure 17} graphically demonstrates that while some mutants destabilize FMN$_{\text{HQ}}$ complex better than methionine (\textit{i.e.} M56L, M56I and M56V), these mutants less readily stabilize FMN$_{\text{SQ}}$ complex, and thus they produce a midpoint potential that is not as negative as wild type. Conversely, the semiquinone complex of M56A is just as stable as wild type, but this mutant does not destabilize the FMN$_{\text{HQ}}$ relative to the oxidized state. So, it is quite intriguing that methionine is the only residue, out of all the thirteen amino acids introduced in these mutants, that was able to maximally stabilize the semiquinone while at the same time significantly destabilizing the hydroquinone, an optimal situation for the generation of low reduction potentials for the SQ/HQ couple, the couple thought to be physiologically relevant in this flavodoxin. Additionally, a methionine at the \textit{re} face of the cofactor produces a more stable holoprotein complex in the oxidized and semiquinone states, insuring saturation of the flavodoxin apoprotein at a low intracellular FMN concentration. It is not surprising, then, that a methionine residue has been retained at this position of the \textit{C. beijerinckii}
flavodoxin and perhaps others, as it produces the lowest $E_{sq/hq}$ while maintaining reasonable cofactor binding levels.

**Future Directions.** This work suggests a number of very interesting avenues of continued exploration into the function of flavodoxins. The aliphatic series demonstrated two fascinating structural properties. Firstly, the putative difference in structural energy for residues at position 56 correlated, albeit weakly, to the positional dependence of the statistical turn potentials. This correlation should be much stronger at positions 57 and 58, as these residues are more intimately involved in the conformational change and the statistical turn potentials are much stronger for positions $i+1$ and $i+2$ in $\beta$-turns. The investigation of this possible effect is currently underway in our laboratory (Kasim & Swenson, unpublished results). Secondly, the M56G mutant demonstrated an equal destabilization of the holoprotein in all three oxidation states, relative to the M56A mutant. This was ascribed to an increase in entropy of the protein structure. It would be fascinating to determine the structural energies of both of these mutants in the apo- and holoprotein forms.

The identified sulfur-flavin interaction, present in both wild type and M56C, is most likely electrostatic in nature. However, a detailed calorimetric study of the FMN binding in the wild-type, M56C, and M56A flavodoxins would allow the precise identification of the forces involved. Another interesting possibility involving the M56C flavodoxin, would be the mutation of the three native cysteine residues (Cys53, Cys108, and Cys128) in the M56C mutant to serine. The resultant quadruple mutant could then
be used to introduce a number of different moieties at the re face of the FMN via thio-specific reagents (Schindler & Viola, 1996).

As stated above, the multiple-residue redox-linked ionization model best fits the collective data. However, this model does not rule out the ionization of the FMN. While the majority of the data indicate that the FMN remains unprotonated to very acidic values, there are some discrepancies (Yalloway et al., 1996). Therefore, a detailed study of the ionization state of the FMN is necessary. Such a study is currently being done for the *D. vulgaris* flavodoxin, using $^{15}$N enrich FMN. This work predicts that the pKa of the HQ is shifted to a more basic value in the M56D mutant, and more acidic in the M56H mutant. Therefore, it would be very informative to also determine the ionization state of the FMN in the fully reduced wild-type, and the mutant, *C. beijerinckii* flavodoxins.
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