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NUCLEAR MAGNETIC RESONANCE SPECTROSCOPIC STUDIES OF HYDROGEN BONDING AND ELECTROSTATIC INTERACTIONS IN THE FLAVODOXINS FROM Desulfovibrio vulgaris AND Clostridium beijerinckii

DISSERTATION

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

By

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* * * * *

The Ohio State University
1999

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ABSTRACT

Regulation of redox potentials through hydrogen-bonding interactions and redox-linked ionization in \textit{Clostridium beijerinckii} and \textit{Desulfovibrio vulgaris} flavodoxins has been studied by using NMR spectroscopy. In the \textit{C. beijerinckii} flavodoxin, the structural differences between the oxidized and one-electron reduced states are the formation of the N(5)H•••O57 hydrogen bond and the Gly57-Asp58 peptide bond flipping from cis O-down to trans O-up. Gly57 has been suggested that it is the only residue at position 57 to stabilize the type II’ turn and to favor the N(5)H•••O57 interaction in the SQ state. Study the hydrogen-bonding interaction is based on the measurement of the temperature coefficients of the N(5)H of the bound $^{15}$N-labeled FMN in the wild-type and G57 mutant proteins in the reduced state. Weakening of the hydrogen bond at N(5)H in G57 mutants has been shown by the increasing of the temperature dependency of N(5)H. The temperature coefficients of the N(5)H in the wild-type, G57A, G57N, and G57T were found to correlate with their $E_{ox/sq}$ as well as the binding free energy of FMN SQ. These results support the hypothesis that the hydrogen bonding interaction at N(5)H is an important factor of regulating the $E_{ox/sq}$ couple in which the strength of this hydrogen bond can be optimized by having the Gly at position 57.
Control of the $E_{sq/hq}$ in the *D. vulgaris* flavodoxin is mainly due to the electrostatic and $\pi-\pi$ stacking interactions in the HQ state. Stabilization of the FMN HQ anion can be achieved by neutralization of the acidic residues clustering the isoalloxazine ring or changing the coplanar Tyr98 to a positive charge residue such as Arg or His. In the Y98H mutant, the $E_{sq/hq}$ increases 180 mV relative to that of wild-type and displays a pH dependency. This phenomenon has been described by a redox-linked ionization model. Based on this model, the pKa of His98 would increase 1.5 units upon the reduction of flavin from SQ to HQ form. The pKa of His98 measured by NMR spectroscopy shows that it has the value of 7.02 and 8.43 in the OX and HQ state, respectively. These results confirm the role of His98 in the redox-linked ionization model and demonstrate that this short-range electrostatic interaction represents an important mechanism in the modulation of redox potentials in flavoproteins and other redox systems.

The effects of the 5'-phosphate of FMN on the binding affinity and redox potentials were studied by using the *D. vulgaris* flavodoxin reconstituted with $^{15}$N-labeled riboflavin. The $^{15}$N NMR and temperature coefficient data indicated that the N(5) is more solvent accessible, the N(5)H hydrogen bonding becomes weaker, and the N(3)H···O=C_{100} interaction becomes stronger in the riboflavin complex. Weakening of the N(5)H hydrogen bonding interaction is responsible for the decrease of the $E_{ox/sq}$ in this complex. Without the dianionic phosphate group, the flavin-protein interactions are changed in which they could regulate the $E_{sq/hq}$ couple.
Dedicated to my parents, my sister, and brother
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I would like to express my sincere gratitude to my adviser, Professor Richard P. Swenson, for his support, encouragement, and enthusiasm. The invaluable experience I have gained in his laboratory will always be with me.

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correlate with changes in the hydrogen-bonding interaction with the proton on N(5) of
the reduced flavin mononucleotide cofactor as measured by NMR chemical shift

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[Hildenborough] flavodoxin: Direct \(^{1}\)H NMR spectroscopy evidence for the redox-

3. Shen, H.-D., Choo, K.-B., Yu, K.-W., Ling, W.-L., Chang, F.-C., & Han, S.-H.
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Major Field: Biophysics
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapters:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction ................................................................................. 1</td>
</tr>
<tr>
<td>2. The Midpoint Potentials for the Oxidized-Semiquinone</td>
</tr>
<tr>
<td>Couple for Gly57 Mutants of the <em>Clostridium beijerinckii</em></td>
</tr>
<tr>
<td>Flavodoxin Correlate with Changes in the Hydrogen-Bonding</td>
</tr>
<tr>
<td>Interaction with the Proton on N(5) of the Reduced Flavin</td>
</tr>
<tr>
<td>Mononucleotide Cofactor As Measured by NMR Chemical Shift Temperature</td>
</tr>
<tr>
<td>Dependencies ...................................................................................... 36</td>
</tr>
<tr>
<td>Introduction ...................................................................................... 36</td>
</tr>
<tr>
<td>Experimental Procedures ..................................................................... 44</td>
</tr>
<tr>
<td>Results and Discussion ..................................................................... 47</td>
</tr>
<tr>
<td>Conclusion ......................................................................................... 70</td>
</tr>
<tr>
<td>3. Regulation of Oxidation-Reduction Potentials through Redox-Linked</td>
</tr>
<tr>
<td>Ionization in the Y98H Mutant of the <em>Desulfovibrio vulgaris</em> [Hildenborough] Flavodoxin: Direct Proton Nuclear Magnetic Resonance Spectroscopic Evidence for the Redox-Dependent Shift in the pKa of Histidine-98 .......... 72</td>
</tr>
</tbody>
</table>

Abstract ...................................................................................................................... ii
Dedication ........................................................................................................................ iv
Acknowledgments ............................................................................................................... v
Vita ................................................................................................................................... vi
List of Schemes ................................................................................................................ ix
List of Tables .................................................................................................................... x
List of Figures .................................................................................................................. xi
Introduction ...............................................................................................................72
Experimental Procedures .........................................................................................79
Results ........................................................................................................................82
Discussion ................................................................................................................101

   Introduction ..............................................................................................................111
   Experimental Procedures .......................................................................................113
   Results and Discussion ..........................................................................................117

5. General Conclusions and Future Directions ..........................................................132

List of References ........................................................................................................147
### LIST OF SCHEMES

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The structure of riboflavin, FMN, FAD, and their derivatives lumiflavin and lumichrome</td>
<td>2</td>
</tr>
<tr>
<td>2. The biosynthesis of FMN and FAD from riboflavin</td>
<td>3</td>
</tr>
<tr>
<td>3. The structures of the neutral, anionic, and cationic flavin species in the oxidized, one-electron reduced, and two-electron reduced states</td>
<td>5</td>
</tr>
<tr>
<td>4. Depiction of the linked equilibria relating the midpoint potentials, dissociation constants, and conformational changes for <em>C. beijerinckii</em> flavodoxin</td>
<td>38</td>
</tr>
<tr>
<td>5. Equilibria linking the ionization of His98 to shifts in the midpoint potential for the sq/hq couple in the Y98H flavodoxin mutant</td>
<td>104</td>
</tr>
<tr>
<td>6. Thermodynamic cycles that describe the midpoint potential in relate to the free energies from flavin-protein interactions (<em>ΔG_i</em>) and conformation change between the oxidized and semiquinone states (<em>ΔG_c^ox/sq</em>)</td>
<td>134</td>
</tr>
<tr>
<td>7. Equilibria linking the ionization of Asp266 or His266 to shifts in the midpoint potential for the ox/sq couple in the ETF mutant</td>
<td>146</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. $^{15}$N Chemical Shifts of Free FMN and Flavodoxin-Bound Oxidized FMN in the Wild-type and Gly57 Mutants</td>
<td>48</td>
</tr>
<tr>
<td>2. Comparison of the Temperature Coefficients for the Wild-type and Mutant <em>C. beijerinckii</em> Flavodoxins</td>
<td>59</td>
</tr>
<tr>
<td>3. Comparison of One-Electron Reduction Potentials, Dissociation Constants, and Binding Free Energy Changes for Each Oxidation State of the FMN Cofactor in the Wild-type and Mutant Flavodoxins from <em>C. beijerinckii</em></td>
<td>66</td>
</tr>
<tr>
<td>4. $^{15}$N Chemical Shifts of Free FMN and Flavodoxin-Bound Reduced FMN in the Fld$^{16}$ and Y98A/Fld$^{16}$ Mutants</td>
<td>84</td>
</tr>
<tr>
<td>5. $^{15}$N Chemical Shifts of Free and Flavodoxin-Bound Oxidized and Reduced FMN and Riboflavin</td>
<td>119</td>
</tr>
<tr>
<td>6. Comparison of One-Electron Reduction Potentials, Temperature Coefficients, Dissociation Constants, and Binding Free Energy Changes for Each Oxidation State of the FMN and Riboflavin Cofactor in the Wild-type Flavodoxins from <em>Desulfovibrio vulgaris</em></td>
<td>124</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Visible spectra of riboflavin, FMN, and FAD</td>
<td>6</td>
</tr>
<tr>
<td>2. Absorption spectra of FMN (dashed line) and FAD in the oxidized and two-electron reduced states</td>
<td>7</td>
</tr>
<tr>
<td>3. Absorption and fluorescence spectra of different methyl-substituted isoalloxazine in acetonitrile</td>
<td>8</td>
</tr>
<tr>
<td>4. Changes in the computed partial atomic charges of flavin in going from the oxidized to reduced states and computed dipole moment vectors for oxidized lumiflavin and dihydrolumiflavin</td>
<td>10</td>
</tr>
<tr>
<td>5. Three dimensional structure of the flavodoxin from <em>C. beijerinckii</em></td>
<td>15</td>
</tr>
<tr>
<td>6. Representation of the hydrogen bonding scheme in the phosphate binding site of <em>D. vulgaris</em> flavodoxin</td>
<td>17</td>
</tr>
<tr>
<td>7. Orientation of the aromatic amino acids flanking the isoalloxazine ring of the FMN in the flavodoxin from <em>D. vulgaris</em></td>
<td>18</td>
</tr>
<tr>
<td>8. Comparison of the sequences of FMN binding sites</td>
<td>19</td>
</tr>
<tr>
<td>9. Thermodynamic cycles relating midpoint potentials and dissociation constants for free and bound FMN</td>
<td>20</td>
</tr>
<tr>
<td>10. Diagrams of type II and II' turns</td>
<td>22</td>
</tr>
<tr>
<td>11. Conformation inversion of the reduced flavin</td>
<td>24</td>
</tr>
<tr>
<td>12. Representation of the six acidic amino acid residues clustered around the FMN in the <em>D. vulgaris</em> flavodoxin</td>
<td>28</td>
</tr>
<tr>
<td>13. $^{15}$N NMR spectrum of recombinant wild-type <em>C. beijerinckii</em> apoflavodoxin reconstituted with $^{15}$N-enriched FMN in the oxidized state</td>
<td>49</td>
</tr>
</tbody>
</table>
14. Correlation diagram of $^{15}$N chemical shifts of free FMN and FMN bound to wild-type and mutant apoproteins from *C. beijerinckii* flavodoxin in the oxidized state.............. 51

15. Contour plot of the $^1$H-$^{15}$N HSQC NMR spectrum of the G57A mutant flavodoxin reconstituted with $^{15}$N-labeled FMN in the fully reduced state at 296K.......................... 55

16. Temperature dependence of the chemical shift for the N(5)H of the bound $^{15}$N-labeled FMN for the wild-type and the G57A, G57N, and G57T mutant flavodoxins ............. 58

17. Correlation between the changes in midpoint potential (relative to wild type) for the ox/sq couple and the sq/hq couple and the free energy of binding of the FMN semiquinone and FMN hydroquinone with the temperature coefficient of the chemical shift for the N(5)H of the bound $^{15}$N-labeled FMN in the fully reduced state for the wild-type, G57A, G57N, and G57T flavodoxins..................................................... 62

18. Representative determinations of the dissociation constant for the G57A, G57N, D58P, and G57T mutant *C. beijerinckii* flavodoxins ............................................. 65

19. Partial view of the structure of the flavin mononucleotide binding site in the flavodoxin from *D. vulgaris* highlighting the aromatic residues flanking the flavin isoalloxazine ring ............................................................................................................. 75

20. $^1$H NMR spectra (600MHz) in the aromatic region for the oxidized Y98H mutant flavodoxin as a function of pH.............................................................................. 85

21. $^1$H NMR spectra (600MHz) in the low-field region for the oxidized wild-type flavodoxin as a function of pH.......................................................................................... 87

22. Histidine region of a 600 MHz COSY spectrum of oxidized Y98H mutant flavodoxin at pH 7.35, 300K................................................................................................. 89

23. pH dependence of the $^1$H NMR chemical shifts for the histidine C2H and C4H assigned to His98 and His142 in the Y98H mutant flavodoxin in the oxidized and fully reduced states................................................................. 91

24. Downfield region of the 600 MHz $^1$H NMR spectra for the two-electron reduced Y98H mutant flavodoxin as a function of pH................................................................. 93

25. Aromatic region of the 600 MHz $^1$H NMR spectra for the Y98H mutant flavodoxin in each of the three redox states of the FMN cofactor .............................................. 95

26. $^1$H NMR spectra (600MHz) in the region containing the histidine C2H and C4H resonances for the fully reduced wild-type flavodoxin at different pH ......................... 97

27. Experimental values for the midpoint potential for the sq/hq couple of the Y98H mutant as a function of pH and the fit to the redox-linked ionization model............. 100

xii
28. HPLC chromatogram of $^{15}$N-riboflavin................................................................. 115

29. Representation of the FMN binding site in the oxidized *D. vulgaris* flavodoxin..... 121

30. $^{15}$N NMR spectra of *D. vulgaris* apoflavodoxin reconstituted with $^{15}$N-labeled riboflavin in the oxidized state and two-electron reduced state........................................ 118

31. Temperature dependence of the chemical shift for the protons on the N(3) and N(5) atoms of the cofactor in the *D. vulgaris* flavodoxin reconstituted with $^{15}$N-labeled FMN or riboflavin .......................................................... 122

32. Binding energy diagram for the riboflavin and FMN bound to *D. vulgaris* wild-type apoflavodoxin in three different oxidation states................................................... 128

33. Relationship between the $E_{ox/sq}$ and the temperature coefficient of the chemical shift for the N(5)H of the bound $^{15}$N-labeled FMN in the fully reduced state in the *C. beijerinckii* wild-type, G57 mutants, E59Q mutant, and *D. vulgaris* wild-type ...... 136
CHAPTER 1

INTRODUCTION

Flavin is a yellow compound discovered a long time ago. Several different names were given to this class of compounds such as avoflavin, lactoflavin, and verdoflavin, depending on the source of isolation. It turns out that all these compounds are riboflavin. It was found that riboflavin (vitamin B_2) is the most abundant material of flavin in nature. Chemical synthesis and structural studies of riboflavin were completed by Karrer et al. (1935) and Kuhn et al. (1934). The structure of riboflavin shows that it contains two components, the 7,8-dimethylisoalloxazine and the reduced form of D-ribose (Scheme 1). The term riboflavin was deduced from these components. The numbering system in the isoalloxazine ring used here is based on the official International Union of Pure and Applied Chemistry (IUPAC) numbering. Riboflavin is an important source for the flavin cofactor found in many flavoenzymes and flavoproteins. As shown in Scheme 1, the riboflavin is a precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Humans and other higher animals obtain riboflavin from their diets because they can not synthesize the isoalloxazine ring. However, they are able to synthesize the FMN and FAD from riboflavin by utilizing flavokinase and FAD pyrophosphorylase (Scheme 2) (Sabu et al., 1990; Yukiko et al., 1990).
Scheme 1: The structure of riboflavin, FMN, FAD, and their derivatives lumiflavin and lumichrome. (from Müller, 1992)
Scheme 2: The biosynthesis of FMN and FAD from riboflavin.
Flavin Chemistry/Properties

The most outstanding feature of the flavin molecule is its ability to undergo oxidation-reduction reactions. The oxidized flavin can accept one electron to form the flavin semiquinone radical. The reduction of flavin semiquinone by an addition of a second electron produces fully reduced hydroquinone. The chemical structures for the redox reactions of flavin are summarized in Scheme 3.

Oxidized flavin has the visible absorption spectrum with maximum absorption bands around 375 nm and 445 nm (Figure 1). A value of 12,500 M\(^{-1}\)cm\(^{-1}\) was reported for the molar extinction coefficient at 445 nm for the riboflavin and FMN (Whitby, L. G., 1953). Theoretical calculation data indicated that both these two maximum bands are predominantly \(\pi \rightarrow \pi^*\) transitions while that at 375 nm may have some \(n \rightarrow \pi^*\) character (Eweg et al., 1980; Song, P. S., 1969). The color of the flavin hydroquinone is pale yellow due to its significantly lower absorption from 300 to 500 nm (Figure 2).

The oxidized flavins exhibit a strong fluorescence with a maximum emission around 520 nm upon excitation at 445 nm (Figure 3). The quantum yields of riboflavin fluorescence are 0.26 in water (pH 7.0), 0.52 in 90% dioxane-water, and 0.71 in dioxane (Koziol & Knobloch, 1965; Weber & Teale, 1957). While riboflavin and FMN have about the same quantum efficiencies, the fluorescence of FAD is about 1/10 that of riboflavin. This difference is the result of the quenching effects of the static interaction between the isoalloxazine ring and adenine moiety of FAD (Wahl et al., 1974). The complete quenching of flavin fluorescence is observed upon binding to some...
Scheme 3: The structures of the neutral, anionic, and cationic flavin species in the oxidized, one-electron reduced, and two-electron reduced states. (from Müller, 1992)
Figure 1: Visible spectra of flavins. 1, Riboflavin; 2, FMN (dashed line); 3, FAD (from Whitby, L. G., 1953)
Figure 2: Absorption spectra of FMN (dashed line) and FAD in the oxidized and two-electron reduced states. (from Walsh, C., 1977)
Figure 3: Absorption (—) and fluorescence (—) spectra of different methyl-substituted isoalloxazines in acetonitrile (excitation wavelength, 440nm) (Visser & Müller, 1979).
apoflavoproteins. This phenomenon may be due to the hydrogen bonding and aromatic stacking interactions between the flavin and apoprotein.

The isoalloxazine ring in all three different oxidation states can undergo ionizations upon a change in pH. Deprotonation at N(3)H in the oxidized states occurs above pH 9.0 (Dudley et al., 1964; Kavanagh & Goodwin, 1949; Kunio et al., 1976). The flavin semiquinone is neutral at pH 7.0. The color of the solution of the neutral flavin semiquinone is blue in apolar solvents (Müller et al., 1972). With a pKa of 8.3 at N(5)H, the red anionic flavin semiquinone can be observed when the pH is above this value (Ehrenberg et al., 1967). The N(1) of flavin has a different pKa in each redox state. The pKa of N(1) in the oxidized and one-electron reduced states is very low. A pKa value of 6.7 was determined for the N(1)H in the two-electron reduced state (Dudley et al., 1964; Van Schagen & Müller, 1981).

Theoretical calculations of the molecular orbital structure of flavins provide valuable information for understanding the chemical and physical properties of the flavin moiety. Molecular orbital calculations show that upon reduction, the charge density at N(1) decreases significantly but increases only slightly at N(5). Deprotonation of the reduced flavin at N(1) results in redistribution of electron density from N(1) to C(10a). The charge density on the N(3) does not change upon reduction (Figure 4A) (Hall et al., 1987a). Calculation of the dipole moment of the isoalloxazine ring in the oxidized and reduced states show that it is largely determined by the O=C-NH-C=O moiety in addition to N(1) and C(10a) (Figure 4B & 4C) (Hall et al., 1987a). Because the N(1) and N(5) are very important for the redox properties of the flavins, changes in the interactions to
Figure 4: (A) Changes (greater than 0.05e) in the computed partial atomic charges in going from the oxidized to reduced states. + indicates gain of electron density; - indicates loss of electron density. (Dashed lines to N(1)-H and N(5)-H indicate that these atoms are only present in the reduced state). Computed dipole moment vectors for (B) oxidized lumiflavin and (C) dihydrolumiflavin (Hall et al., 1987a).
these two nitrogen atoms such as binding of the flavin to apoflavodoxin will result in a redox potential shift as well as affect the stability of the flavin.

Reduction of flavins can be achieved by photoreduction in the presence of electron donors such as EDTA or deazaflavins, by adding a reducing agent such as dithionite or borohydride, or by potentiometric methods (Massey & Palmer, 1966; Massey & Hemmerich, 1978; Mayhew, S. G., 1978; Stankovich & Massey, 1976). Therefore, any one of these methods can be used to determine the midpoint potentials for the flavin. The midpoint potentials of the ox/sq couple \( E_{ox/sq} \) for riboflavin and FMN at pH 7.0 are -231 mV and -238 mV, respectively (Draper & Ingraham, 1968). The midpoint potentials of the sq/hq couple \( E_{sq/hq} \) are -167 mV for riboflavin and -172 mV for FMN (Draper & Ingraham, 1968). However, these properties are modulated when bound to flavoproteins.

A goal of our laboratory is to elucidate the mechanisms by which the protein regulates the redox properties of the bound flavin cofactor. Flavodoxin is being exploited as a model system for other FMN-binding proteins. For example, cytochrome P450 reductase (CPR), a flavoprotein with multidomains, contains two flavin cofactors, FAD and FMN. The folding pattern of the FMN-binding domain is very much like flavodoxin (Wang et al., 1997; Sevrioukova et al., 1999). However, the bacterial CPR modulates the \( E_{ox/sq} \) and \( E_{sq/hq} \) of the bound FMN in an opposite way compared with those of flavodoxins (Sevrioukova et al., 1996). The conclusions drawn from the study of electrostatic interactions in our lab have been used to point out a possible mechanism for the switching of these two couples in bacterial CPR (Sevrioukova et al., 1999). Another example is nitric oxide synthase (NOS). This enzyme catalyzes the conversion of L-arginine to nitric
oxide and L-citrulline. Its carboxyl-terminal reductase domain displays significant
sequence homology to CPR and contains one equivalent each of FAD and FMN. Two
aromatic residues, Phe810 and Tyr890, were expected to stack with the FMN ring
(Brunner et al., 1998). These aromatic stacking interactions have also been found in
flavodoxins and have been demonstrated to be an important factor to regulate the redox

General Properties of the Flavodoxin

Flavodoxins are small electron transfer proteins (MW~15kd) that contain a single
noncovalently bound FMN cofactor as their only redox center and function as low-
potential electron carriers. They have acidic residues asymmetrically distributed over the
surface of the protein. Typically, the redox potentials of flavodoxins are well separated
such that the $E_{\text{ox/eq}}$ being more positive while the $E_{\text{eq/eq}}$ is substantially more negative.
Flavodoxins are good model systems to study for the following reasons. 1) They are
stable and soluble in aqueous solution. 2) They are easy to purify and produce large
quantities of proteins in a short period of time. 3) Various spectroscopic methods can be
applied to study these proteins including UV/Vis spectroscopy, X-ray crystallography,
NMR, fluorescence, Raman, and stopped-flow spectroscopy. 4) Besides site-directed
mutagenesis, different flavin analogues or isotopically-labeled flavin such as $^{15}$N- or $^{13}$C-
labeled FMN can be synthesized and incorporated into apoflavodoxins for structure-
function studies.

Flavodoxins have been isolated from a variety of microorganisms but none have been
found in higher animals and plants. Very little is known about the physiological roles of
most flavodoxins. However, flavodoxins have been implicated as the electron donors for methionine synthetase and trithionate reductase (Fujii & Huennekens, 1974; Kim & Akagi, 1985). In cyanobacteria and eukaryotic algae, flavodoxin is the electron donor for the ferredoxin-NADP⁺ reductase associated with photosynthesis. The physiological electron donor for nitrogenase in *Klebsiella pneumoniae* has been identified to be flavodoxin (Nieva-Gomez *et al.*, 1980; Shah *et al.*, 1983). It was also found that flavodoxins can substitute for ferredoxins when microorganisms are grown in iron-deficient medium (Mayhew & Ludwig, 1975). Flavodoxins can be classified as either short chain or long chain flavodoxins based on their size. The molecular weights for the short chain and long chain flavodoxins are in the range from 14,000 to 17,000 and 20,000 to 23,000 Da, respectively. Sequence comparisons show that the long chain flavodoxins have 20 extra residues in the β₃ strand. The redox and electron-transferring properties of flavodoxins have been extensively studied because of the highly perturbed redox potentials for both the ox/sq and sq/hq couples for the FMN cofactor (Mayhew & Tollin, 1992). A distinguishing feature of the flavodoxin family is their exceptionally low reduction potential for the sq/hq couple of the bound FMN. Thus, structure-function studies have become critical in flavodoxin research.

structures from NMR spectroscopy have been solved for the flavodoxins from \textit{A. nidulans} (Clubb \textit{et al.}, 1991), \textit{Anabaena 7120} (Stockman \textit{et al.}, 1990), \textit{D. vulgaris} (Knauf \textit{et al.}, 1993; Peelen & Vervoort, 1994; Stockman \textit{et al.}, 1993), \textit{D. desulfuricans} (Pollock \textit{et al.}, 1996), \textit{Azotobacter chroococcum} (Peelen \textit{et al.}, 1996), and \textit{Megasphaera elsdenii} (Mierlo \textit{et al.}, 1990). The overall folding patterns for all of the flavodoxins are similar. They all consist of a central five-stranded parallel $\beta$-sheet surrounded by four $\alpha$-helices. The FMN is located at the periphery of the molecule with the ribityl side chain extending towards the center of the molecule (Figure 5). The binding affinity of FMN to the apoflavodoxins in the oxidized state is high, with a dissociation constant (Kd) in the nM range (Curley \textit{et al.}, 1991; Druhan & Swenson, 1998). It has been suggested that hydrophobicity is the major source of stabilization free energy for the association of FMN. Based on accessible surface area calculations, the total areas buried in \textit{C. beijerinckii} flavodoxin for FMN and the protein are 736 $\text{Å}^2$ (Janin & Chothia, 1978). Assuming a hydrophobic energy of 25 cal/mol per $\text{Å}^2$, hydrophobic interactions could contribute about 18 kcal/mol favorable binding free energy. The free energy for the association of FMN with this protein is 10.6 kcal/mol. Therefore, it seems that hydrophobicity makes the major contribution to the binding of FMN. However, when they calculated the protein surface area shielded from the solvent due to the presence of the FMN, the cofactor was simply removed from the flavodoxin and did not take into account the apoflavodoxin structure. In addition, the unfavorable energy for transferring the negative charge phosphate group of FMN from polar to nonpolar environment was not considered in their calculations. Favorable binding energies such as aromatic stacking have to be considered for the association of FMN and
Figure 5: Three dimensional structure of the flavodoxin from *Clostridium beijerinckii*. The structure is adapted from the coordinates published by Smith *et al.* (1977).
apoflavodoxin because they represent two major interactions commonly found in flavodoxins.

Three peptide segments of the apoflavodoxins are involved in FMN binding. The first segment, sometimes referred to as the “teens loop”, contributes to the binding of the phosphate group through an extensive hydrogen bonding network (Figure 6). This binding site in the flavodoxin is quite unusual because it does not have any positively charged residues to interact with the negatively charged phosphate group. The isoalloxazine ring of FMN interacts with the second and third segments of the protein, the so-called 60’s and 90’s loops. At least one aromatic residue from either one of these two loops interacts with either face of the FMN ring. In *D. vulgaris* flavodoxin, the isoalloxazine ring is sandwiched by two aromatic residues, with Trp 60 on the *re* or inner face and Tyr 98 on the *si* or outer face (Figure 7) (Watenpaugh *et al*., 1973; Watt *et al*., 1991). Both the phosphate group and isoalloxazine ring are shielded from solvent. Most of the residues involved in the FMN binding site are conserved (Figure 8).

**Mechanism by Which Flavodoxins Modulate the Redox Properties of the FMN.**

The thermodynamic stability of the bound FMN in each oxidation state is determined by the interactions between the FMN and apoflavodoxin. These differential stabilities establish the redox potentials for the ox/sq and sq/hq couple (Figure 9). In the *C. beijerinckii* flavodoxin, the redox potential for the ox/sq couple is 146 mV more positive than that for free FMN in solution, representing a stabilization of 3.4 kcal/mol. A conformation change associated with the reduction of the oxidized FMN to the semiquinone has been shown based on the comparison of the X-ray structures of oxidized
Figure 6: Representation of the hydrogen bonding scheme in the phosphate binding site of *D. vulgaris* flavodoxin. Hydrogen bonds are indicated by dashed lines. The representation is taken from X-ray crystal structural data (Watt *et al.*, 1991).
Figure 7: Orientation of the aromatic amino acids flanking the isoalloxazine ring of the FMN in the flavodoxin from *D. vulgaris*. Representation is taken from the coordinates published by Watt *et al.* (1991).
Figure 8: Comparison of the sequences of FMN binding sites. Flavodoxins from *Ch. Crispus* (Wakabayashi *et al.*, 1989), *A. nidulans* (Laudenbach *et al.*, 1988), and *Anabaena 7120* (Leonhardt & Straus, 1989) belong to the long chain type, and those of *D. vulgaris* (Krey *et al.*, 1988), *D. salexigens* (Helms *et al.*, 1990), *D. desulfuricans* (Helms & Swenson, 1992), *C. beijerinckii* (Tanaka *et al.*, 1974), and *M. elsdenii* (Tanaka *et al.*, 1973) to the short chain type.
Figure 9: Thermodynamic cycles relating midpoint potentials and dissociation constants for free and bound FMN.
and semiquinone forms of *C. beijerinckii* flavodoxin (Smith et al., 1977). In the oxidized state, the backbone carbonyl group of Gly57 points away from the flavin but reorients to form a hydrogen bond with the N(5)H of the FMN semiquinone in the one-electron reduced state. A similar structural rearrangement has also been observed in *D. vulgaris* and *A. nidulans* flavodoxins (Luschinsky, 1991; Watt et al., 1991). Stabilization of FMN semiquinone by this newly formed hydrogen bond increases the binding affinity of FMN semiquinone by 300-fold relative to that in the oxidized state in the *C. beijerinckii* flavodoxin (Durban & Swenson, 1998; Ludwig & Luschinsky, 1992). Recent refinements of structure from *C. beijerinckii* flavodoxin have shown that the peptide of the 50's loop in the oxidized form resembles the type II turn conformation but it adopts the type II' turn conformation in the one-electron reduced state (Ludwig et al., 1997). Type II’ turn is the mirror image of type II turn with the inverse $\phi$, $\varphi$ values (Figure 10). Due to the unfavorable non-bonding interactions between the C$\beta$ of the second residue ($i$+1) and the NH of the third residue ($i$+2), type II’ turn strongly prefers glycine in the second position (Richardson, 1981; Rose et al., 1985).

Flavodoxin from *A. nidulans* has an Asn residue at the position equivalent to the Gly57 in *C. beijerinckii* (Laudenbach et al., 1987). In *C. crispus* flavodoxin, a hydrogen bond between the O'\(H\) of Thr58 and the N(5) atom of FMN is already observed in the oxidized state (Fukuyama et al., 1992). The redox potentials for the ox/sq couple for both the *A. nidulans* and *C. crispus* flavodoxins are significantly more negative than *C. beijerinckii* (Fukuyama et al., 1992; Laudenbach et al., 1987). It was proposed that the ox/sq potential may be a function of the conformation stability of the type II’ turn and the
Figure 10: Diagrams of type II and II' turns.
newly formed hydrogen bonding interaction between the N(5)H and C=O57 (Swenson et al., 1991). This hypothesis has been confirmed by studying the redox properties, the structures, and the NMR chemical shift temperature dependencies for the Gly57 mutants of the *C. beijerinckii* flavodoxin (Chang & Swenson, 1999; Ludwig et al., 1997).

Another prominent feature of the redox properties of the flavodoxins is the very negative reduction potential for the sq/hq couple of the bound FMN cofactor. For the *D. vulgaris* flavodoxin, the one-electron reduction potential for this couple of the flavin is shifted from -172 mV (Draper & Ingraham, 1968) to approximately -445 mV when associated with the apoflavodoxin, representing a Gibbs free energy change of over 6 kcal/mol (Curley et al., 1991; Swenson & Krey, 1994). One of the early hypotheses proposed that the very low redox potential for the sq/hq couple could be due to the energy cost to overcome the activation barrier for the transition from the bent to the planar configuration for the FMN (Simonsen & Tollin, 1980; Tauscher et al., 1973; Van Schagen & Muller, 1981). The isoalloxazine ring of FMN in the oxidized state is planar, but it is bent along the N(5)-N(10) axis in the fully reduced state, with an angle that varies from 9° to 36° depending on the number and size of the N(5) substituents (Tauscher et al., 1973). A ¹H NMR study of the dynamics of the reduced flavin (Figure 11) in aqueous solution indicated an energy barrier of about 13 kcal/mol for the inversion of the N(5) atom (Tauscher et al., 1973). However, theoretical calculations indicated that the barrier for reversal of this bend is 4 kcal/mol (Dixon et al., 1979). The low intrinsic barrier for the ring inversion was supported by the results from the ¹³C NMR study on the dynamics of the conformation of reduced flavin (Moonen et al., 1984a). Moreover, a
Figure 11: Conformation inversion of the reduced flavin. A and B represent the xylene and the pyrimidine side of the isoalloxazine ring, respectively.
recent study of the flavin model systems showed that the energy driven by the 
conformational effects is about 1.7 kcal/mol (Hasford et al., 1997). It is unlikely, 
therefore, that the conformation inversion between the planar and nonplanar of the FMN 
or so called “butterfly motion” contributes significantly to the energetics that establish the 
redox potential for the sq/hq couple. But the type of interactions between apoprotein and 
flavin that force the bound FMN hydroquinone to be planar are still unclear.

Electrostatic forces may play an important role in the regulation of the redox potential 
for the sq/hq couple due to the charge that develops on the FMN hydroquinone at N(1) 
atom during reduction. The pKa of N(1) for the FMN hydroquinone free in solution is 6.7 
(Scheme 3) (Dudley et al., 1964; Müller, 1992; Van Schagen & Müller, 1981). A very 
similar ionization for the bound FMN in the D. vulgaris flavodoxin has been suggested 
based on the observation of the pH-dependent spectroscopic changes (Mayhew et al., 
1969; Mayhew & Ludwig, 1975; Mayhew & Tollin, 1992; Yalloway et al., 1999). 
However, 15N NMR studies of the reduced flavodoxins from A. vinelandii (Vervoort et 
al., 1986), Anabaena 7120 (Stockman et al., 1988), C. beijerinckii (Vervoort et al., 
1986), D. vulgaris (Vervoort et al., 1985), M. elsdenii (Franken et al., 1984; Vervoort et 
al., 1986), and old yellow enzyme (Beinert et al., 1985a & 1985b) indicate that the FMN 
hydroquinone is anionic at pH 8.0. In the D. vulgaris and M. elsdenii flavodoxins, the 
FMN hydroquinone still remains in its anionic form even at pH 6.0 and 5.5, respectively 
(Franken et al., 1984; Vervoort et al., 1985). Estimation of N(1) pKa in the M. elsdenii 
flavodoxin from reduced flavodoxin spectra indicated that its value is lower than 4 
(Ludwig et al., 1990). While the pKa of the FMN hydroquinone in the flavodoxin is still a
matter of debate, it nevertheless is clear that the bound FMN hydroquinone possesses a negative charge at pH 7.0. According to Coulomb’s law, the electrostatic interaction energy among charge groups can be calculated based on the following equation:

\[ E = \frac{q_1 q_2}{4 \pi \varepsilon_0 \varepsilon_{\text{eff}}} \]

where \( \varepsilon_0 \) is the permittivity of vacuum, \( \varepsilon_{\text{eff}} \) is the effective dielectric constant, \( r \) is the distance between two charges, \( q_1 \) and \( q_2 \) are the charges of the two groups, and \( E \) is the electrostatic interaction energy. Since the FMN has a dianionic phosphate group and the flavodoxins have many acidic residues surrounding the cofactor, charge-charge interactions could arise between the negative charge on N(1) of FMN hydroquinone and the phosphate dianion as well as the acidic amino acid residues. The phosphate group of FMN is buried in an environment in which no positively charged residues from the apoprotein are near the binding site to compensate the negative charges of phosphate (Figure 6). Moonen et al. calculated the unfavorable electrostatic interaction energy between the phosphate dianion and the FMN hydroquinone anion and found it to closely match the redox potential difference for the sq/hq couple between the free and bound FMN for several flavodoxins (Moonen et al., 1984b). They therefore proposed that this type of electrostatic interaction could be considered as a major determinant of the sq/hq couple. Also, their hypothesis could reasonably explain the 180 mV positive shift for the sq/hq couple for the complex of the \textit{D. vulgaris} apoflavodoxin with riboflavin, which lacks the terminal phosphate (Draper & Ingraham, 1968). To test these hypotheses, Zhou and
Swenson made two mutant *D. vulgaris* flavodoxins, T12H and N14H, in an attempt of offset the negative charge of the 5'-phosphate through ion pairing. They found that the redox potentials for the sq/hq couple only increased 28 and 15 mV, respectively, compared to that of the wild-type protein (Zhou & Swenson, 1996a). From this study, they concluded that the negative charge on the phosphate group of FMN does not play a disproportionate role in decreasing the redox potential for the sq/hq couple.

Another unfavorable electrostatic interaction between the FMN hydroquinone anion and negatively charged residues has been investigated by Swenson’s group. In their studies, six acidic residues from *D. vulgaris* flavodoxin clustered within 13 Å of the N(1) of the FMN were irreversibly neutralized in various combinations through the substitution of Asn for Asp and Gln for Glu (Figure 12). An average of about 15 mV per substitution was established for the contribution of the sq/hq couple from these charge residues (Zhou & Swenson, 1995). For the long-chain flavodoxin from *A. chroococcum*, the greater number of negatively charged residues surrounding the FMN may be one reason for the more negative redox potential of the sq/hq couple for this flavodoxin (Peelen et al., 1996). According to Zhou and Swenson’s studies, the contribution from each acidic residue and the phosphate group of FMN to the sq/hq couple shift are very similar.

One of the most important forces involving in the control of the conformations and ligand binding properties of nucleic acids and proteins is aromatic stacking interactions (Burley & Petsko, 1988; Hangauer *et al.*, 1984; Saenger, 1984). A face-to-face
Figure 12: Representation of the six acidic amino acid residues clustered around the FMN in the *D. vulgaris* flavodoxin. The acidic residues are depicted as the van der Waals surfaces. The structure is adapted from the coordinates published by Watt *et al.* (1991).
orientation between two aromatic residues will result in unfavorable quadrupolar electrostatic interactions because the \( \pi-\pi \) repulsions overcome the \( \pi-\sigma \) attractions (Hunter & Sanders, 1990; Hunter et al., 1991). For the flavodoxin from \textit{D. vulgaris}, the FMN hydroquinone in the fully reduced state is an electron-rich molecule and carries a formal negative charge. Therefore, the coplanar orientation of the \( \pi \)-electron rich Tyr98 and the FMN isoalloxazine ring should significantly increase the unfavorable electrostatic repulsions. The redox properties of this flavodoxin have been shown to be sensitive to mutations at Tyr98 that change the \( \pi \)-electron density at this position (Swenson & Krey, 1994). A very similar result was also observed for the flavodoxin from \textit{Anabaena 7119} (Lostao et al., 1997). In addition, Breinlinger and Rotello have shown in their model system studies that \( \pi \)-stacking interactions can modulate the redox properties of flavin (Breinlinger & Rotello, 1997). The magnitude of the effect of the elimination of the unfavorable \( \pi-\pi \) contact accounts for 50\% of the total shift in the sq/hq couple (Swenson & Krey, 1994). Together with the contribution from acidic residues, they represent the major determinant for the reduction potential for the sq/hq couple (Zhou & Swenson, 1996b). Investigation of the backbone dynamics of \textit{D. vulgaris} flavodoxin by NMR spectroscopy indicated that the fully reduced protein is more flexible, especially for the FMN binding site, than the oxidized protein (Hrovat et al., 1997). This result is consistent with the more repulsive environment of the FMN binding site in the fully reduced state.

RESEARCH GOALS AND CHALLENGES
Based on the study of X-ray structures and redox potentials of flavodoxins from *D. vulgaris* and *C. beijerinckii*, the molecular basis of hydrogen bonding and the electrostatic interactions in modulating the $E_{\text{ox/sq}}$ and $E_{\text{sq/sq}}$ appears to be very significant. The goal of this thesis is to further investigate and evaluate the relative importance of these interactions primarily by utilizing nuclear magnetic resonance spectroscopy as a powerful investigative tool. The following three different parts of studies are the focus of this thesis.

1. Investigation of the role of hydrogen-bonding at N(5)H.

   In the *C. beijerinckii* flavodoxin, the introduction of the first electron of the FMN cofactor is associated with a local structural change: the Gly57-Asp58 peptide bond rotates from primarily the cis O-down configuration in the oxidized state to the trans O-up configuration in the semiquinone state such that a new hydrogen bond can be formed between the carbonyl group of Gly57 and the proton on N(5) of the neutral FMN semiquinone radical (Smith *et al.*, 1977). This new interaction between the protein and the cofactor is thought to contribute to the increase of the FMN semiquinone association constant and the ox/sq redox potential. X-ray structure shows that the Gly57 and Asp58 are located in a $\beta$ turn in which the Gly57 is the second residue in that turn. However, the residue that participates in the formation of this hydrogen bond in *A. nidulans* flavodoxin is Asn58 and its $E_{\text{ox/sq}}$ is 129 mV more negative than that of *C. beijerinckii* flavodoxin. Several single-site mutations were made to test the importance of Gly57 (Eren & Swenson, 1989). The ox/sq potentials for all the G57 mutants are lower than that of wild-type protein which agree with predictions that a side chain at position 57 should make
addition of the first electron more difficult. The relative change of $E_{\text{ox/sq}}$ comes from two kinds of free energies, the energy difference from the conformation change in the apoprotein ($\Delta G_c$) (e.g. cis O-down vs. trans O-up), and the FMN-protein interactions ($\Delta G_i$) (e.g. N(5)H•••O57 hydrogen bonding interaction). Weakening of the N(5)H•••O57 hydrogen bonds in G57 mutants was thought to be an important contribution in lowering the $E_{\text{ox/sq}}$. However, it has not been directly established experimentally that altering the strength of this hydrogen-bonding interaction is responsible for the changes in $E_{\text{ox/sq}}$. The role of N(5)H•••O57 hydrogen bond in modulating the $E_{\text{ox/sq}}$ in C. beijerinckii flavodoxin is investigated in the first part of thesis.

(2) Investigation of the role of redox-linked ionization mechanism.

Regulation of the $E_{\text{sq/hq}}$ in D. vulgaris flavodoxin is primarily controlled by electrostatic interactions. Formation of the anionic FMN hydroquinone at pH 7.0 has been demonstrated by $^{15}$N NMR data. The charge-charge interactions between the acidic amino acid residues and the anionic FMN hydroquinone are unfavorable. Besides the unfavorable electrostatic environment provided by the negatively-charged residues, the $\pi$-$\pi$ interaction between the Tyr98 and anionic FMN hydroquinone has been shown to be unfavorable (Swenson & Krey, 1994; Zhou & Swenson, 1996b). Substitution of an alanine residue for this residue to eliminate this unfavorable aromatic stacking interaction significantly increases (by 139 mV) the $E_{\text{sq/hq}}$ (Swenson & Krey, 1994). However, an even larger increase in the $E_{\text{sq/hq}}$ (by 180 mV) has been shown when a histidine is introduced at this position (Swenson & Krey, 1994). In that study, the $E_{\text{sq/hq}}$ couple Y98H mutant displays a pH dependency which is consistent with a redox-linked ionization model. A pH
dependency for \( E_{sq/q} \) was also observed in *C. beijerincki* flavodoxin. The carboxyl group of Glu59 is hydrogen-bonded to N(3)H FMN and is the nearest ionizable group to the flavin ring. Change of the pKa of Glu59 upon the reduction of the flavin from semiquinone to hydroquinone was proposed for the redox-linked ionization in *C. beijerincki* flavodoxin (Ludwig *et al*., 1990). In the Y98H mutant, the redox-linked ionization model suggests that the favorable electrostatic coupling between the imidazolium cation and the flavin hydroquinone anion is responsible for the higher \( E_{sq/q} \) (Swenson & Krey, 1994). Such a model predicts an increase in pKa of 1.5 units for His98 on going from the semiquinone to hydroquinone state (Swenson & Krey, 1994). However, direct measurements of the intrinsic pKa of His98 are needed to support and confirm the redox-linked ionization model. The shift of the pKa of His98 was determined in this thesis to establish the role of redox-linked ionization mechanism.

One question has been raised in the charge neutralization mutants (Fld\(^{+1}\)−Fld\(^{+6}\)) is the ionization state of FMN hydroquinone. Both the \( E_{ox/q} \) and \( E_{sq/q} \) display pH dependence. The pH dependency of the \( E_{ox/q} \) has been known due to the protonation at N(5) FMN semiquinone. The \( E_{sq/q} \) displays a pH dependence in several flavodoxins below pH 7.0 (Caldeira *et al*., 1994; Curley *et al*., 1991; Ludwig *et al*., 1990). It was thought early on that this pH dependency was due to protonation at N(1). However, \(^{15}\)N NMR data show that the FMN hydroquinone remains in its anionic form at low pH (Franken *et al*., 1984; Vervoort *et al*., 1985). From a study of redox-linked pH dependence of the charge neutralization mutants (Fld\(^{+1}\)−Fld\(^{+6}\)) in *D. vulgaris* flavodoxin, it has been shown that the pH dependence of \( E_{sq/q} \) can be described by the redox-linked ionization of collectively
charged groups (Swenson & Zhou, 1996). The more positive $E_{sqhq}$ in these mutants is due to a decrease in the unfavorable electrostatic interactions by neutralization of acidic residues instead of protonation of FMN hydroquinone anion. Direct measurements of the ionization state of FMN hydroquinone in the Fld$^{+6}$ and Y98A/Fld$^{+6}$ mutants by $^{15}$N NMR were conducted in this part of thesis to establish the role of electrostatic interactions.

(3) Investigation of the role of the FMN 5'-phosphate and the riboflavin

An early hypothesis suggested that the unfavorable electrostatic interactions between the phosphate dianion and the FMN hydroquinone anion could be responsible for the $E_{sqhq}$ difference between the free and bound FMN (Moonen et al., 1984b). The observation that the significant increases of the $E_{sqhq}$ (by 186 mV) in the riboflavin complex relative to that of wild-type *D. vulgaris* protein seems to support this suggestion. However, the average contribution of one nearby acidic residue to the $E_{sqhq}$ in *D. vulgaris* flavodoxin is about 15 mV (Zhou & Swenson, 1995). To clarify the uncertainty in the contribution of the phosphate group in establishing the $E_{sqhq}$, the electrostatic effect of the FMN terminal phosphate group of the *D. vulgaris* flavodoxin has been investigated. The introduction of histidine residues adjacent to the 5'-phosphate was found to only modestly increase the $E_{sqhq}$ by 15-28 mV (Zhou & Swenson, 1996a). In that study, substantial destabilization of the riboflavin semiquinone was found. However, detail information about the riboflavin-apoflavodoxin interactions was not available at the time. Both FMN-protein and riboflavin-protein interactions in *D. vulgaris* flavodoxin were investigated in this part of thesis.
The overall goal of this thesis project is to use NMR spectroscopy to thoroughly study the roles of hydrogen bonding interactions and redox-linked ionization in regulation of the reduction-oxidation potentials for flavodoxins. NMR spectroscopy was useful for this project for the following reasons:

1. The size of flavodoxin from *D. vulgaris* and *C. beijerinckii* is suitable for NMR study.

2. Both the flavodoxin genes from *D. vulgaris* and *C. beijerinckii* have been cloned and successfully expressed (Eren & Swenson, 1989; Krey *et al.*, 1988). Sufficient amounts of proteins can be easily purified for NMR study.

3. Several mutants of these flavodoxins have been prepared and characterized previously.

4. NMR spectroscopy can provide useful information such as the ionization state of flavin, the hydrogen bonding interactions, the pKa of ionizable amino acid residues, the energy barrier of the structure transition, solvent accessibility, and structural dynamics.

5. NMR spectroscopy has been applied to study the flavodoxins from *A. nidulans* (Clubb *et al.*, 1991), *A. vinelandii* (Vervoort *et al.*, 1986a), *Anabaena 7120* (Stockman *et al.*, 1990), and *M. elsdenii* (Mierlo *et al.*, 1990). It would be valuable to compare the results of NMR spectroscopy from *D. vulgaris* and *C. beijerinckii* flavodoxins to other flavodoxins.

Strategies used in this project are as follows:
(1) Study the hydrogen bonding interaction at N(5)H of the reduced bound FMN by measurement of NMR chemical shift temperature dependencies.

(2) Determination of the pKa of His98 in Y98H mutant of the *D. vulgaris* flavodoxin.

(3) Investigation of the effect of the hydrogen bonding interactions on the reduction potentials for the riboflavin bound to *D. vulgaris* apoflavodoxin.

More detailed information on each NMR technique and its application to the flavodoxin problem will be given in the introduction to each appropriate chapter. The midpoint potentials for the ox/sq couple for G57 mutants of the *C. beijerinckii* flavodoxin in regulating the changes in the hydrogen-bonding interaction with the N(5)H of the reduced flavin are described in Chapter 2. Chapter 3 describes the regulation of midpoint potentials through redox-linked ionization in the Y98H mutant of the *D. vulgaris* flavodoxin. The effect of hydrogen bonding interactions for the FMN and riboflavin bound to *D. vulgaris* apoflavodoxin on the oxidation-reduction potentials are discussed in Chapter 4.
CHAPTER 2

THE MIDPOINT POTENTIALS FOR THE OXIDIZED-SEMIQUINONE COUPLE FOR GLY57 MUTANTS OF THE Clostridium beijerinckii FLAVODOXIN CORRELATE WITH CHANGES IN THE HYDROGEN-BONDING INTERACTION WITH THE PROTON ON N(5) OF THE REDUCED FLAVIN MONONUCLEOTIDE COFACTOR AS MEASURED BY NMR CHEMICAL SHIFT TEMPERATURE DEPENDENCIES

INTRODUCTION

As described in Chapter 1, flavodoxins are typified by the thermodynamic stabilization of the blue neutral form of the flavin semiquinone radical while the anionic hydroquinone state is substantially destabilized (Ludwig & Luschinsky, 1992; Mayhew & Tollin, 1992). For the Clostridium beijerinckii flavodoxin, the midpoint potentials for the oxidized-semiquinone (ox/sq) couple and sq/hq couple are shifted from -238 and -172 mV for FMN in aqueous solution at pH 7 to -92 and -399 mV when bound, respectively (Draper & Ingraham, 1968; Mayhew, 1971). The early X-ray crystal structures of flavodoxins from Desulfovibrio vulgaris and C. beijerinckii revealed a conformational difference between the oxidized and reduced states of these proteins
Ludwig & Luschinsky, 1992; Ludwig et al., 1997; Smith et al., 1977; Watenpaugh et al., 1973; Watenpaugh et al., 1976; Watt et al., 1991). In the flavodoxin from C. beijerinckii, the carbonyl group of the peptide bond between Gly57 and Asp58 primarily points away from the flavin in the oxidized state ("O-down" configuration). This group rotates or "flips" to form an apparent hydrogen bond with N(5)H of the reduced FMN ("O-up" configuration) (Scheme 4) (Ludwig & Luschinsky, 1992; Smith et al., 1977).

The unique structural properties of the frequently conserved glycine residue involved in this conformational change are critical for the unique structure of this loop (Ludwig et al., 1997). In the C. beijerinckii flavodoxin, the Gly57-Asp58 peptide bond adopts a mixture of conformations, including primarily the unusual cis O-down configuration in crystals of the oxidized protein. Furthermore, structural analyses suggest that glycine is the only residue that can optimally accommodate the trans O-up conformation adopted by this loop in the semiquinone state. To more thoroughly test these ideas, several amino acid substitutions have been made at positions 57 and 58 in the C. beijerinckii flavodoxin (Ludwig et al., 1991; Ludwig et al., 1997; Swenson et al., 1991). These substitutions introduce side chains having different structural constraints or introduce the more cis-to-trans restricted X-Pro bond within this turn. The midpoint potentials for the ox/sq couple in the G57A, G57N, G57D, and D58P mutants were found to be 50-70 mV lower than for the wild-type protein. A more pronounced decrease of 180 mV was noted for the G57T mutant, most likely the consequence of the more restricted structural characteristics of the β-branched side chain at this position (Ludwig et al., 1997). A somewhat similar series of substitutions in the D. vulgaris flavodoxins involving what appears to be a functionally equivalent glycine residue, Gly61, also results in a more negative midpoint potential for
Scheme 4: Depiction of the linked equilibria relating the midpoint potentials, dissociation constants, and conformational changes for *C. beijerinckii* flavodoxin as adapted from Ludwig and Luschinsky (1992). The value of the midpoint potential for the ox/sq couple for free FMN used here was taken from Draper and Ingraham (1968). Druhan and Swenson (1998) determined the Kd value for the oxidized state of the recombinant wild-type flavodoxin. The Kd value for the semiquinone state was calculated based on this thermodynamic box. The conformational change is illustrated by the “flipping” of the carbonyl group and the establishment of a new hydrogen bond between the N(5)H and O57.
Scheme 4
the ox/sq couple than for the wild type (Mayhew et al., 1996). In contrast, the *A. nidulans* flavodoxin contains an asparagine at the equivalent position as this glycine and has a midpoint potential for the ox/sq couple of $-221 \text{ mV}$, a value significantly lower than for the above-mentioned flavodoxins (Laudenbach et al., 1988). Interestingly, the N58G mutation in this flavodoxin results in an increase in the reduction potential (Ludwig & Luschinsky, 1992). These results clearly emphasize the importance of structural adaptability of the glycine residue at this critical position in establishing the higher potential for the ox/sq couple.

An extensive structural characterization of the *C. beijerinckii* mutants has revealed some interesting aspects (Ludwig et al., 1991; Ludwig et al., 1997). In all cases, the amino acid substitutions introduce only minor structural changes. Just as in wild type, the cis O-down conformation is primarily found in the oxidized state of the mutant flavodoxins, except for G57T, which adopts the trans O-down configuration. In all cases, the carbonyl group was able to rotate to the trans O-up conformation in the semiquinone state, forming a hydrogen bond with N(5)H as in wild type. Rather surprisingly, the substitution of a proline residue at position 58, creating an X-Pro peptide bond thought to have a high free energy barrier for cis/trans transitions, still demonstrated the characteristic “flip” of the Gly57 carbonyl group. Thus, the decrease in the midpoint potential for the ox/sq couple in these mutants could not be ascribed to the prevention of the hydrogen-bonding interaction itself. Instead, this decrease in midpoint potential was considered to be the result of changes in the strength of the interaction between the protein and the N(5)H of the FMN and/or in the conformation equilibrium of this loop (Ludwig et al., 1997). Evaluation of the geometries of flavin-protein interactions and the
pKa of N(5)H in flavodoxin semiquinone for G57T mutant protein did suggest that the
N(5)H•••O57 interaction might be weakened. However, more direct experimental
evidence for these assertions was not available in that study. Such evidence is crucial for
the unequivocal assignment of the functional importance of this interaction in
establishing the reduction potentials of this and other flavoproteins.

The use of NMR spectroscopy for the investigation of hydrogen bond began several
decades ago. In simple molecules and in the absence of other effects, formation of
hydrogen bond causes downfield shifts of the proton resonances due to the decrease in
electron density surrounding the particular hydrogen nucleus (Emsley et al., 1965).
Because $^1$H chemical shifts in peptides and proteins are sensitive not only to the local
electronic structure but also to shielding effects that result from the conformational
effects such as formation of secondary and tertiary structure, hydrogen bonds in peptides
and proteins may not be easier to identify unambiguously in this way. Nevertheless, the
relationships between the $^1$H chemical shifts and protein secondary structures as well as
hydrogen bond length have been investigated in order to provide useful information for
structure determination (Asakura et al., 1995; Oldfield, 1995; Wagner et al., 1983;
Wishart et al., 1991; Zhou et al., 1992). For the Gly57 mutants in C. beijerinckii
flavodoxin, the change of $^1$H chemical shift of N(5)H FMN could be due to a multitude
environmental effects rather than just solely to the change of the hydrogen bonding
interaction. Therefore, it is difficult to extract the contribution of the hydrogen bond by
comparison of the $^1$H chemical shifts among these mutants.

Besides $^1$H chemical shifts, NMR measurement of the exchange rate of amide
protons has been widely used to estimate solvent accessibility. The rate of amide proton
exchange can be estimated by dissolving the sample in D$_2$O and following the rate of the deuterium exchange of the NH to ND as manifested by a decrease in area of the NH peak. Hydrogen bonds are then ascribed to the slower exchanging protons. To determine the deuterium exchange rate of the N(5)H FMN in G57 mutants would greatly assist in the comparison of the strengths of the N(5)H···O57 interactions among these proteins. However, the oxidized FMN picks up a proton from solvent upon reduction. Thus, it is not possible to measure the exchange rate of N(5)H for the flavodoxin reduced in D$_2$O. Another possibility is to reduce flavodoxin in H$_2$O and then to add anaerobic D$_2$O to the sample. But this approach is technically very challenging due to the instability of the fully reduced flavodoxin and its marked tendency to rapidly reoxidize even in the presence of small amount of air or oxygen. Thus, it would be difficult to know whether the decrease of the N(5)H signal is due to the exchange with D$_2$O or reoxidation of the reduced protein. Therefore, the result may ambiguous and inaccurate.

Temperature dependence of proton resonances study is another common approach to evaluate hydrogen bonds. It has been observed that the amide proton signal of N-methylacetamide shifts upfield upon raising the temperature. This phenomenon was explained by decreasing in the fraction of the amide protons, which are weakly hydrogen bonded to the solvent (Jardetzky & Roberts, 1981). Amide protons involved in a stable intramolecular hydrogen bond, which will be much less likely to break when the temperature is increased, have been shown to have their chemical shifts change to a lesser extent with temperature than solvent exposed amide protons (Kopple et al., 1969; Ohnishi & Urry, 1969). The temperature coefficient can be determined by calculating the slope of proton chemical shift vs. temperature. In many cases, the temperature
coefficients correlate with the deuterium exchange rate. For example, protons that exchange slowly have smaller temperature coefficients. Therefore, it seems that the temperature dependence of $^1$H chemical shifts shows a great potential for being applied to study the N(5)H hydrogen bond interactions in the G57 mutants. This approach has the following advantages. 1) It can provide valuable information on the N(5)H hydrogen bond interactions. 2) The results can be compared to the X-ray structure data. 3) To prepare $^{15}$N-labeled FMN-reconstituted flavodoxin is inexpensive. 4) It can be used to study other flavodoxin species such as *M. elsdenii*, or *A. nidulans* to obtain more information about the N(5)H hydrogen bond. 5) There is no size limitation for this kind of approach because the only molecule that is isotopically-labeled is flavin. So this approach can be used to study bigger flavoproteins such as cytochrome P450 reductase. However, a disadvantage of this method is that the N(5)H resonance in the semiquinone state will be broadened due to the paramagnetic effect of the flavin radical.

The temperature dependency of the chemical shift for the amide proton in the peptide bond has been used to provide information on the solvent exposure, hydrogen bonding, and structural transitions in peptides (Andersen *et al*., 1997; Baxter & Williamson, 1997; Dyson *et al*., 1988; Merutka *et al*., 1995; Rothemund *et al*., 1996). In the study reported here, a similar approach was used. The $^1$H-$^{15}$N HSQC nuclear magnetic resonance (NMR) technique was applied to the wild-type and mutant flavodoxin proteins reconstituted with $^{15}$N-labeled FMN to determine the temperature coefficient of the proton on N(5) of reduced FMN. In this way, a more quantitative measure of the effects of the amino acid replacements for Gly57 on the strength of the N(5)H•••O57 interaction in the reduced *C. beijerinckii* flavodoxin can be established. The results show that the
absolute value for the temperature coefficients for these mutants correlate quite well with the changes in the midpoint potentials of the FMN in this group of mutants. These results provide more direct quantitative evidence for the critical role of the N(5)H•••O57 interaction in establishing the reduction potentials of the FMN cofactor in this flavodoxin as hypothesized previously based on the X-ray structure of this flavodoxin in all three oxidation states (Ludwig & Luschinsky, 1992; Ludwig et al., 1997; Smith et al., 1977).

EXPERIMENTAL PROCEDURES

Materials. Deuterium oxide (D$_2$O) was obtained from Fluka Chemicals. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate and $^{15}$NH$_4$Cl (99%) were purchased from Cambridge Isotope Laboratories. Sodium dithionite was from Aldrich Chemical Co. Isopropylthio-β-D-galactoside (IPTG) was acquired from Gibco BRL. All other chemicals were of reagent grade.

Preparation of $^{15}$N-Enriched FMN. $^{15}$N-enriched FMN was purified from recombinant *Clostridium beijerinckii* flavodoxin obtained from transformed *E. coli* AG-1 cells grown on minimal medium for up to 40 h at 37 °C with $^{15}$NH$_4$Cl as the sole nitrogen source. Each liter of minimal medium contained 6 g of Na$_2$HPO$_4$, 3 g of KH$_2$PO$_4$, 1 g of $^{15}$NH$_4$Cl, 0.5 g of NaCl, 1 mM MgSO$_4$, 6 g of dextrose, 0.5 mg of thiamin, and 100 mg of ampicillin. Isopropylthio-β-D-galactoside (IPTG) was added after approximately 28 h to a final concentration of 0.5 mM to induce the expression of flavodoxin. The flavodoxin was partially purified by poly-(ethylenimine) precipitation (Druhan & Swenson, 1998) and dialyzed against 10 mM NH$_4$HCO$_3$. The $^{15}$N-enriched FMN was dissociated from
the holoprotein by incubation in 6 M urea at 40 °C for 30 min in the dark and the flavin separated from the apoprotein by ultrafiltration. The $^{15}$N-enriched FMN was purified by chromatography on an Econo-Pac Hi-Q anion exchange cartridge (Bio Rad) equilibrated with 10 mM NH$_4$HCO$_3$ using a step gradient from 50 to 250 mM NH$_4$HCO$_3$, repeatedly lyophilized to remove the NH$_4$HCO$_3$, and redissolved in H$_2$O.

Reconstitution of the Apoflavodoxin with $^{15}$N-Labeled FMN. Recombinant flavodoxins were purified by established procedures (Eren & Swenson, 1989; Ludwig et al., 1997; Swenson & Krey, 1994). Apoflavodoxin was prepared by the procedure of Wassink and Mayhew (Wassink & Mayhew, 1975), dissolved in a minimum volume of 10 mM potassium phosphate buffer (pH 7.0), and dialyzed against the same buffer at 4 °C. The apoflavodoxin solution was lyophilized and dissolved in a solution containing an equal molar ratio of $^{15}$N-enriched FMN to prepare the $^{15}$N-enriched (>95%) FMN-reconstituted flavodoxin.

$^{15}$N and $^1$H-$^{15}$N HSQC NMR Spectroscopy. Samples for $^{15}$N NMR contained approximately 2.0 mM oxidized flavodoxin in 50 mM potassium phosphate buffer (pH 7.0) prepared in 10% D$_2$O. The $^{15}$N NMR spectra of the samples contained in Wilmad 5 mm thin wall 7740 Pyrex NMR sample tubes were recorded at 300 K on a Bruker MSL-300 spectrometer operating at 30.4 MHz. For $^{15}$N NMR measurements, $^{15}$N inverse gated decoupling was acquired using the 100 μs $^1$H 90° pulse for the WALTZ-16 decoupling sequence (Shaka et al., 1983) with a recycle time of 2.00 s. $^{15}$N chemical shifts are referenced to an external standard of 1.5 M $^{15}$NH$_4$NO$_3$ in 1 M HNO$_3$ (21.6 ppm relative to liquid ammonia set by convention to 0.0 ppm) (Srinvasan & Lichter, 1977). Samples for $^1$H-$^{15}$N HSQC experiments contained approximately 1.0 mM fully reduced flavodoxin.
in 50 mM potassium phosphate buffer (pH 7.0) in 10% D₂O. Reduction was achieved by adding an appropriate amount of a freshly prepared sodium dithionite solution to the anaerobic solution of flavodoxin. Anaerobic flavodoxin solutions were prepared by gaseous exchange with several cycles of a partial vacuum and prepurified argon. The ¹H-¹⁵N HSQC NMR spectra (Bodenhausen & Ruben, 1980) were acquired on a Bruker DMX-600 spectrometer using the water flip-back versions (Grzesiek & Bax, 1993) with ¹H and ¹⁵N sweep widths of 8802 and 7298 Hz, respectively. Sixteen scans were recorded for each of 128 t₁ values. Quadrature in t₁ was accomplished by using the TPPI-States method (Marion et al., 1989). GARP decoupling (Shaka et al., 1985) was used during acquisition to decouple the ¹⁵N. Temperature calibration was performed using methanol and ethylene glycol (VanGeet, 1969). Proton chemical shifts were referenced to an internal standard of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) set at 0.0 ppm.

**Determination of the Dissociation Constant for the Oxidized Form of Mutant Flavodoxins.** The dissociation constants (Kₐ) for the oxidized form of the FMN cofactor were determined by spectrofluorometric titrations in which quenching of the fluorescence of an FMN solution was monitored during the addition of apoflavodoxin (Mayhew, 1971). In a typical titration, a 0.2 μM solution of purified FMN (extracted from flavodoxin) in 50 mM phosphate buffer (pH 7.0) was progressively titrated with substoichiometric quantities of 35-75 μM apoflavodoxin stock solutions at 25 °C. The intensity of fluorescence emission at 522 nm excited at 445 nm was recorded on a Perkin-Elmer LS50B luminescence spectrometer after equilibration. The average Kₐ values from two separate titrations are reported.
RESULTS AND DISCUSSION

\(^{15}\text{N} \text{NMR of FMN, both Free and Bound to Wild-Type and Mutant } C. \text{ beijerinckii} \)

Apo flavodoxin in the Oxidized and Fully Reduced States. X-ray crystallographic and \(^{15}\text{N} \) NMR analyses of \( C. \text{ beijerinckii} \) flavodoxin indicate that N(1) and N(3) atoms, but not N(5), have hydrogen-bonding contacts with apoflavodoxin in the oxidized state (Burnett \textit{et al.}, 1974; Ludwig & Luschinsky, 1992; Vervoort \textit{et al.}, 1986). In this study, changes in the environment of the FMN cofactor and hydrogen-bonding interactions at N(1), N(3), and N(5) were evaluated in response to the amino acid replacements at positions 57 and 58. These residues are part of a four-residue loop that undergoes a conformational change upon reduction of the flavin cofactor in this flavodoxin (Ludwig & Luschinsky, 1992). These changes were evaluated by both one-dimensional \(^{15}\text{N} \) and two-dimensional \(^{1}\text{H}-^{15}\text{N} \) HSQC nuclear magnetic resonance spectroscopic analyses of the recombinant wild-type flavodoxin and four mutants in which the cofactor had been replaced with uniformly \(^{15}\text{N}\)-enriched (\(>95\%\)) FMN. Such enrichment facilitated the determination of the chemical shifts of each nitrogen atom of the FMN as well as the temperature dependencies of the slowly exchanging protons on N(5) (in the reduced state) and N(3).

The \(^{15}\text{N} \) chemical shifts of free FMN and recombinant wild-type \( C. \text{ beijerinckii} \) flavodoxin in the oxidized state determined in this work (Table 1 and Figure 13) agree well with those reported by Vervoort \textit{et al.} (Vervoort \textit{et al.}, 1986) except we were unable to make an assignment for N(3) of the unbound FMN. As this is a uniformly labeled \(^{15}\text{N} \) derivative and this signal clearly appears in our protein samples, it may be that the N(3)
<table>
<thead>
<tr>
<th>atom</th>
<th>FMN(^a)</th>
<th>FMN(^b)</th>
<th>TARF(^b)</th>
<th>rC. b.(^a)</th>
<th>C. MP(^b)</th>
<th>G57A(^a)</th>
<th>G57N(^a)</th>
<th>G57T(^a)</th>
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<td>190.8</td>
<td>199.9</td>
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<td>185.3</td>
<td>185.5</td>
<td>187.2</td>
<td>185.7</td>
</tr>
<tr>
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<td>160.3</td>
<td>161.1</td>
<td>-(^c)</td>
<td>160.4</td>
<td>161.9</td>
</tr>
<tr>
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<td>334.7</td>
<td>344.3</td>
<td>350.9</td>
<td>351.5</td>
<td>343.1</td>
<td>343.6</td>
<td>344.0</td>
<td>342.8</td>
</tr>
<tr>
<td>N(10)</td>
<td>163.1</td>
<td>164.6</td>
<td>150.2</td>
<td>163.9</td>
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<td>163.4</td>
<td>163.3</td>
<td>164.3</td>
<td>163.8</td>
</tr>
</tbody>
</table>

\(^a\)This work. \(^b\)From Vervoort et al. (1986). \(^c\)Chemical shift values could not be assigned from this work. Abbreviations: rC. b., recombinant *Clostridium beijerinckii* flavodoxin; C. MP, *Clostridium MP (beijerinckii)* flavodoxin; TARF, tetraacetylriboflavin in CHCl\(_3\).

Table 1: \(^{15}\)N Chemical Shifts of Free and Flavodoxin-Bound Oxidized FMN at pH 7, 300 K.
Figure 13: $^{15}\text{N}$ NMR spectrum of recombinant wild-type *Clostridium beijerinckii* apoflavodoxin reconstituted with $^{15}\text{N}$-enriched FMN in the oxidized state at pH 7, 300 K.
resonance overlaps with that of N(10), although this was not documented. The N(1) and N(5) atoms in the oxidized FMN represent pyridine-type nitrogen atoms in heteroaromatic ring systems. Hydrogen bonding and protonation of the pyridine-type nitrogen atom result in an increase in the nitrogen shielding and an upfield shift in its resonance (Witanowski et al., 1981; Witanowski et al., 1986; Witanowski et al., 1993). The $^{15}$N chemical shift of the N(5) atom of wild-type flavodoxin is shifted downfield compared to that of tetraacetylriboflavin in apolar solutions (Table 1). This suggests that the N(5) atom of protein-bound FMN does not form a hydrogen bond and is in a relatively nonpolar environment (Vervoort et al., 1986). Significant upfield shifts for the N(5) atom compared to that of wild-type flavodoxin were noted in all of the mutant flavodoxins, bringing the value slightly upfield of that of tetraacetylriboflavin in CHCl$_3$, but still well below that of FMN in an aqueous environment. These upfield shifts are unlikely to be the result of the formation of a hydrogen bond to the apoflavodoxin. Instead, they may be due to slight changes in the local environmental leading to small increases in polarity near N(5) and/or to differences in the mixture of peptide conformations in the 50's loop region among the mutants in the oxidized state (Ludwig et al., 1997). The $^{15}$N chemical shifts of the N(1) atom of wild-type and mutant flavodoxins are all shifted upfield relative to that of free FMN in an aqueous solvent, consistent with the formation of a strong hydrogen bond between the N(1) atom of FMN and apoflavodoxin (Vervoort et al., 1986). The N(1) chemical shifts of the mutants appear further downfield than for the wild-type protein (Figure 14 and Table 1), which might imply that the hydrogen-bonding interactions of N(1) are weaker in these proteins.
Figure 14: Correlation diagram of $^{15}$N chemical shifts of free FMN and FMN bound to wild-type and mutant apoproteins from *C. beijerinckii* flavodoxin in the oxidized state. The value for N(3) of FMN was not assigned in this work, but was taken from Vervoort *et al.*, 1986. A resonance peak for the FMN N(3) in the G57A mutant was not observed. As indicated in the diagram, it is tentatively suggested to overlap with the N(10) resonance in this protein.
The N(3) and N(10) atoms in the oxidized FMN represent pyrrole-type nitrogen atoms in heteroaromatic ring systems. Considering the similarity of the N(3) chemical shifts to wild-type and free FMN for all the mutants except G57A, it seems likely that a hydrogen bond is maintained between the FMN N(3) and the apoprotein in these mutants as well. The N(3) signal for G57A could not be unambiguously assigned, perhaps because it overlaps with that of N(10). If this is the case, then for this mutant the N(3) signal has shifted slightly downfield. Thus, except for the N(5) atom, the $^{15}$N NMR data in this study suggest that the amino acid substitutions at positions 57 and 58 do not cause significant structural changes to the FMN binding site in the oxidized state. It is perhaps not surprising that the greatest differences in the $^{15}$N NMR spectra are associated with the N(5) atom that is adjacent to the amino acid replacements.

The chemical shift values for the nitrogen atoms of the fully reduced FMN bound to each of the mutant flavodoxins are, for the most part, nearly identical to those of wild type. It was noted that the chemical shift for N(5) in the G57T mutant at 300 K is shifted upfield by about 1.6 ppm compared to that of the rest of the flavodoxin mutants, suggesting that the environment of N(5) in this mutant is significantly different. It is possible that this shift is reflecting a weaker hydrogen-bonding interaction with the G57T apoprotein, a conclusion that is consistent with the temperature dependency data that will follow. However, it should be noted that the chemical shifts of pyrrole-type nitrogen atoms (as with all nitrogens of the fully reduced FMN) are relatively insensitive to hydrogen-bonding interactions (Vervoort et al., 1986), making it difficult to accurately evaluate hydrogen bond strengths based on the chemical shift data alone.
Temperature-Dependent $^1\text{H}-^{15}\text{N}$ HSQC Spectroscopy Studies of the Fully Reduced FMN Cofactor Bound to the Wild-Type and Various Mutants of the C. beijerinckii Apoflavodoxin. Along with D/H fractionation factors and hydrogen exchange rates, the temperature coefficient of proton chemical shifts from NMR data has been used as an indicator of hydrogen-bonding strength (Dyson et al., 1988; Loh & Markley, 1994; Markley & Westler, 1996). For the protons involved in hydrogen bonding, an upfield shift in resonance with increasing temperature has been rationalized as due to the weakening of hydrogen bonds, leading to an alteration in the distribution between the hydrogen-bonded and nonhydrogen-bonded species (Liddel & Ramsey, 1951; Muller & Reiter, 1965). The most studied temperature coefficients in peptides and proteins are the backbone amide protons (Andersen et al., 1997; Baxter & Williamson, 1997; Dyson et al., 1988; Merutka et al., 1995; Rothemund et al., 1996). The temperature coefficients for the backbone amide protons in random-coil peptides have values greater than -6.5 ppb/K and may indicate hydrogen-bonding interactions between the amide protons and solvent (Merutka et al., 1995). Backbone amide protons having temperature coefficient magnitudes less than from -3 to -5 ppb/K are considered to be involved in intramolecular hydrogen bonding (Baxter & Williamson, 1997). By comparison of the average value for D/H fractionation factors for backbone amides, the strength of intramolecular hydrogen bonding within the staphylococcal nuclease is thought to be stronger than protein-solvent and solvent-solvent interactions (Markley & Westler, 1996). Therefore, for the protons involved in hydrogen bonding at the same site in proteins, the value of the temperature coefficient has been used as a good indicator of the relative strength of such hydrogen-bonding interactions.
This type of analysis was applied in this study to the wild-type and mutant flavodoxins in the fully reduced state to evaluate the strength of the hydrogen bond between the N(5)H and the carbonyl oxygen of residue 57. Our principal interest is in evaluating the hydrogen-bonding strength with the FMN semiquinone because of the importance that has been placed on its stabilization (Ludwig & Luschinsky, 1992; Ludwig et al., 1997). Unfortunately, it is not possible to determine the temperature coefficient of N(5)H of the FMN semiquinone because the paramagnetic effect of the unpaired electron in the flavin radical significantly broadens the proton resonances close to the isoalloxazine ring (Chang & Swenson, 1997; Peelen & Vervoort, 1994). However, the X-ray crystal structures of this flavodoxin indicate that the semiquinone and hydroquinone states are nearly identical in structure (Ludwig & Luschinsky, 1992; Ludwig et al., 1997; Smith et al., 1977). Furthermore, the hydrogen-bonding interaction between O57 and N(5)H appears to be retained in both reduced states. It would seem quite reasonable, then, that the hydrogen-bonding interaction with the FMN hydroquinone is representative of that in the semiquinone state, although this interaction could be weaker in the fully reduced state due to the decrease in charge at the N(5)H (Hall et al., 1987b; Ludwig et al., 1997).

The two-dimensional $^1$H-$^{15}$N HSQC spectrum of fully reduced G57A mutant flavodoxin at pH 7.0, 296 K shown in Figure 15 is representative of the other flavodoxins. Since the $^1$H-$^{15}$N HSQC spectrum only reveals the connectivity between nitrogen and hydrogen atoms, only two intense and well-resolved cross-correlation peaks are noted. Each could unambiguously be assigned to the hydrogen atoms associated with N(3) and N(5) of the FMN. A signal associated with N(1) was not observed because it
Figure 15: Contour plot of the $^1$H-$^{15}$N HSQC nuclear magnetic resonance spectrum of the G57A mutant flavodoxin reconstituted with $^{15}$N-labeled FMN in the fully reduced state at 296 K. The FMN $^{15}$N$_3$-$^1$H$^{N3}$ and $^{15}$N$_5$-$^1$H$^{N5}$ cross-correlation peaks are labeled 'FMN N3' and 'FMN N5'. No other cross-correlation peaks, such as for N(1), were observed.
Figure 15

$^{1}H$ Chemical Shift (ppm)

$^{15}N$ Chemical Shift (ppm)

FMN N5

FMN N3

\( \theta \)
remains unprotonated at this pH (Vervoort et al., 1986). The persistent HSQC signal for the protons on N(3) and N(5) implies that these protons are exchanging slowly with solvent, due to their relative inaccessibility to solvent and strong hydrogen-bonding interactions with the apoprotein as are evident in the X-ray crystal structure (Ludwig & Luschinsky, 1992; Ludwig et al., 1997; Smith et al., 1977).

The changes in chemical shift of the hydrogen atom on N(5) of the bound FMN cofactor plotted as a function of temperature are shown in Figure 16. The resonances for the N(5)H atom in all of the proteins studied shift upfield as the temperature increases, displaying a linear dependence with temperature in the range from 273 to 314 K. The values of the temperature coefficient (Δδ/ΔT) are listed in Table 2. The wild-type flavodoxin has the smallest temperature coefficient (-0.8237 ppb/K) among this group of flavodoxins. This value is substantially smaller than that of amide protons involved in intramolecular hydrogen bonding found in reverse turns (Dyson et al., 1988), suggesting that a rather strong hydrogen bond is formed with the N(5)H atom in this protein as has been proposed previously (Burnett et al., 1974; Ludwig & Luschinsky, 1992; Vervoort et al., 1986).

Compared with the wild-type protein, the magnitudes of the temperature coefficients for N(5)H in the fully reduced G57A, G57N, and G57T mutants have all increased, by approximately 3-, 5-, and 11-fold, respectively (Table 2). The G57T mutant exhibits the largest temperature coefficient of -9.264 ppb/K, a value similar to that of amide protons not involved in intramolecular hydrogen bonding (Merutka et al., 1995). By this criterion, the hydrogen bonding is very weak or nonexistent in this mutant. Also, it was noted that the HSQC signal for N(5)H for the G57T mutant disappeared upon raising the
Figure 16: Temperature dependence of the chemical shift for the N(5)H of the bound $^{15}$N-labeled FMN for the wild-type (circles) and the G57A (squares), G57N (inverted triangles), and G57T (triangles) mutant flavodoxins. A value at 314 K was not obtained for the G57A mutant because of reoxidation of the sample at this temperature. The N(5)H cross-correlation peak for the G57T mutant reproducibly and reversibly disappeared at 314 K, implicating the loss of hydrogen bonding and/or rapid exchange at this temperature.
<table>
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<th>flavodoxin</th>
<th>temperature coefficient(^d) ((\Delta \delta/\Delta T)) (ppb/K)</th>
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<tr>
<td></td>
<td>N(5)H</td>
<td>N(3)H</td>
<td></td>
</tr>
<tr>
<td>wild type</td>
<td>-0.8237 (1)</td>
<td>-0.07184 (0.087)</td>
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<td>G57A</td>
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<td>-0.827 (1.00)</td>
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<tr>
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<td>-4.129 (5.01)</td>
<td>-0.512 (0.62)</td>
<td></td>
</tr>
<tr>
<td>G57T</td>
<td>-9.264 (11.25)</td>
<td>-0.681 (0.83)</td>
<td></td>
</tr>
</tbody>
</table>

\(^d\)Numbers in parentheses are the temperature coefficients relative to that of the N(5)H of the wild-type flavodoxin.

**Table 2:** Comparison of the Temperature Coefficients for the Wild-type and Mutant *C. beijerinckii* Flavodoxins.
temperature above 310 K and reappeared upon cooling the sample to one of the lower
temperatures. This reversible phenomenon again suggests that the hydrogen bond in this
mutant may have been weakened to such an extent that the proton on N(5) more rapidly
exchanges with solvent at the elevated temperatures.

Despite repeated efforts, a HSQC signal was not observed for N(5)H in the D58P
mutant in the reduced state, and, therefore, the temperature coefficient data cannot be
compared with other mutants. We are not certain of the reason(s) for the absence of the
HSQC signal in this mutant. Strong $^15$N resonance signals were obtained for each FMN
nitrogen atom in the oxidized (Table 1) and reduced states of the mutant. It is possible
that the solvent exchange rate for both N(5)H and N(3)H may again be too rapid in this
mutant. This is particularly unfortunate in that this mutant was constructed previously to
specifically evaluate the free energy differences for the cis-trans isomerization of this
critical X-Pro peptide bond (Ludwig et al., 1997). Changes in hydrogen-bonding
interactions in this mutant might have been particularly revealing.

In contrast to N(5)H, very small temperature dependencies were noted for the proton
on N(3) of the FMN in the reduced state (Table 2). The temperature coefficient for wild
type was about one-tenth that for N(5)H, suggesting that by this criteria hydrogen-
bonding interactions at this location are quite strong. The temperature coefficients for the
N(3)H atom of the G57A, G57N, and G57T mutants were all somewhat higher than for
wild type, but well below those observed for the N(5)H in each mutant. Also, the trend
that was noted among this group of flavodoxins for N(5)H temperature coefficients was
not apparent for this hydrogen atom. Thus, these amino acid substitutions may only
slightly weaken the hydrogen-bonding interactions at N(3)H. This is not surprising
because hydrogen bonding at this location involves the side chain of Glu59 that is part of this unusual binding loop (Ludwig & Luschinsky, 1992; Smith et al., 1977). However, it is quite clear that these substitutions primarily affect the interactions at N(5)H as predicted by the structural studies of these proteins (Ludwig et al., 1991; Ludwig et al., 1997).

The Temperature Coefficients Correlate with Changes in the Midpoint Potential and with the Binding Affinity of the FMN Semiquinone and Hydroquinone in Response to the Amino Acid Substitutions at Position 57. The substitution of amino acids of various types for Gly57 affects the reduction potentials of each couple of the FMN cofactor in opposite ways (Ludwig et al., 1991; Ludwig et al., 1997; Swenson et al., 1991). The midpoint potential of the ox/sq couple decreases substantially while that for the sq/hq couple increases to a lesser extent. These changes correlate very well with the temperature coefficients for the N(5)H of the FMN in the fully reduced state as shown in Figure 17A. Several conclusions can be made. First, because the temperature coefficients vary inversely with the strength of hydrogen bonding, the data strongly suggest that the amino acid substitutions for Gly57 alter the midpoint potentials of both couples of the FMN primarily by weakening the hydrogen-bonding strength at N(5)H. Second, the alteration of the hydrogen bonding at N(5)H (as reflected in the temperature coefficients) has a much larger effect on the midpoint potential for the ox/sq couple than for the sq/hq couple. This observation suggests that the N(5)H•••O57 interaction primarily favors the stabilization of the FMN semiquinone. Because the semiquinone is a common intermediate for both flavin couples, the preferential destabilization of the semiquinone by the amino acid replacements should disfavor the first one-electron reduction step.
Figure 17: Correlation between the changes in midpoint potential (relative to wild type) for the ox/sq couple (closed symbols) and the sq/hq couple (open symbols) (panel A) and the free energy of binding of the FMN semiquinone (closed symbols) and FMN hydroquinone (open symbols) (panel B) with the temperature coefficient of the chemical shift for the N(5)H of the bound $^{15}$N-labeled FMN in the fully reduced state for the wild-type (circles), G57A (squares), G57N (inverted triangles), and G57T (triangles) flavodoxins. The midpoint potentials used in this analysis are from Ludwig et al., 1997.
Figure 17
forming the semiquinone while facilitating the second one-electron reduction forming the hydroquinone. This should affect the midpoint potentials of each of the couples in an opposite manner, just as is observed. The fact that the midpoint potential for the sq/hq couple is affected by the substitutions is also consistent with differences between the semiquinone and hydroquinone states in terms of the hydrogen-bonding strength at N(5)H.

That the N(5)H•••O57 interaction primarily favors the stabilization of the FMN semiquinone is perhaps more clearly illustrated by examining the dissociation constant (Kd) for each redox state of the FMN for each mutant. The Kd for the oxidized FMN complex of the G57A/N/T and D58P mutant flavodoxins was determined by titration of FMN solutions with freshly prepared apoprotein while monitoring the quenching of flavin fluorescence associated with flavin binding (Figure 18). The Kd values for the FMN semiquinone and hydroquinone for each apoprotein were calculated from the linked equilibria described by Dubourdieu et al. (Dubourdieu et al., 1975) (Chapter 1, Figure 9). The Kd values for the G57A/N/T and D58P complexes in the oxidized state have increased by only about 2-3-fold compared to that of wild-type protein, suggesting that these substitutions have a rather small effect on the binding of the oxidized cofactor (Table 3). In contrast, the FMN semiquinone binds to G57A, D58P, G57N, and G57T apoproteins with Kd values about 20-, 40-, 50-, and 1800-fold greater than for wild type, respectively. The Kd value for the FMN hydroquinone in each of the mutant proteins is also higher than that of wild type, but these increases, while in the same relative order as for the semiquinone, are significantly smaller. The free energy of binding of each reduced state of the FMN for each mutant derived from these Kd values also correlates to
Figure 18: Representative determinations of the dissociation constant (Kd) for the G57A, G57N, D58P, and G57T mutant *C. beijerinckii* flavodoxins. For each mutant flavodoxin, the formation of holoprotein was monitored by the quenching of the flavin fluorescence. Each data set of spectral changes was fit to a quadratic solution to the equation describing a single binding site.
Table 3: Comparison of One-Electron Reduction Potentials, Dissociation Constants, and Binding Free Energy Changes for Each Oxidation State of the FMN Cofactor in the Wild-type and Mutant Flavodoxins from *Clostridium beijerinckii*.

<table>
<thead>
<tr>
<th>flavodoxin</th>
<th>$E_{ox/sq}$ (mV)</th>
<th>$E_{sq/hq}$ (mV)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G(ox)^f$</th>
<th>$\Delta G(sq)^f$</th>
<th>$\Delta G(hq)^f$</th>
<th>$\Delta \Delta G_{(sq-hq)}^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>-92$^a$</td>
<td>-399$^a$</td>
<td>18$^c$</td>
<td>0.06</td>
<td>425</td>
<td>-10.6</td>
<td>-13.9</td>
</tr>
<tr>
<td>G57A</td>
<td>-143$^a$</td>
<td>-373$^a$</td>
<td>52$^e$</td>
<td>1.27</td>
<td>3243</td>
<td>-9.9</td>
<td>-12.1</td>
</tr>
<tr>
<td>D58P</td>
<td>-155$^a$</td>
<td>-360$^a$</td>
<td>59$^e$</td>
<td>2.32</td>
<td>3556</td>
<td>-9.9</td>
<td>-11.8</td>
</tr>
<tr>
<td>G57N</td>
<td>-162$^a$</td>
<td>-372$^a$</td>
<td>59$^e$</td>
<td>3.02</td>
<td>7418</td>
<td>-9.9</td>
<td>-11.6</td>
</tr>
<tr>
<td>G57T</td>
<td>-270$^a$</td>
<td>-320$^a$</td>
<td>32$^e$</td>
<td>110.69</td>
<td>35694</td>
<td>-10.2</td>
<td>-9.5</td>
</tr>
<tr>
<td>FMN</td>
<td>-238$^b$</td>
<td>-172$^b$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$From Ludwig *et al.* (1997).  $^b$From Draper and Ingraham (1968).  $^c$From Druhan & Swenson (1998).  $^d$Determined directly by spectrofluorometric titration of FMN with apoflavodoxin.  $^e$The $K_d$ values for the semiquinone and hydroquinone states of the FMN were calculated from the observed shifts in the midpoint potentials of the cofactor upon binding to flavodoxin [see Dubourdieu *et al.* (1975) for details].  $^f$Free energy change of binding, $\Delta G$ (kcal/mol) = -RT ln($1/K_d$).

For Table 3, the values are presented in a tabular format, showing the flavodoxins, their midpoint potentials, dissociation constants, and the corresponding changes in free energy for each oxidation state of the FMN cofactor. The data includes references for the determination of the dissociation constants and the free energy changes.
the temperature coefficient for N(5)H of the reduced FMN (Figure 17B), with the free energy values increasing along with the increase in the temperature coefficient. These plots clearly reveal a strong correlation between a decrease in the hydrogen-bonding interaction at N(5)H of the reduced flavin (as measured by the temperature coefficients) and a decrease in the free energy of binding of the reduced states of the cofactor. A loss of approximately 4 kcal/mol of binding energy was noted in comparing the semiquinone complex of wild type to the G57T mutant, which has the weakest interaction at N(5)H based on the elevated temperature coefficient. The increase in binding free energy for the hydroquinone as a function of the temperature coefficient is about half that for the semiquinone. Because the hydrogen bond interaction between N(5)H of FMN and O57 also exists in the fully reduced state, it is not surprising that the weakening of this interaction by the amino acid substitutions also reduces the binding of flavin hydroquinone. This observation also supports our previous assumption that the temperature dependency for the N(5)H of the hydroquinone is reflective of that in the semiquinone state. It must be emphasized once again that the temperature coefficients for the N(5)H of the FMN semiquinone are derived from the fully reduced state. It is quite likely that if they could be determined, the temperature coefficients for the semiquinone would be affected to an even larger extent by the amino acid replacements for Gly57. Thus, the correlation between the temperature coefficients and the free energy of binding with the FMN semiquinone shown in Figure 17B may be understated relative to that of the hydroquinone. It is also important to noted that factors other than the interaction at N(5)H, such as unfavorable aromatic stacking and longer range electrostatic interactions, also contribute to the destabilization of the hydroquinone and the low
midpoint potential of the sq/hq couple in the flavodoxin (Zhou & Swenson, 1995; Zhou & Swenson, 1996b).

The Temperature Dependency Data Are Consistent with the Previously Proposed Role of Gly57 and Associated Conformational Changes. An important functional role of Gly57 and the “flipping” of its carbonyl group in the thermodynamic stabilization of the blue neutral form of FMN semiquinone through hydrogen bonding was an early postulate of the pioneering studies by Ludwig and co-workers on the flavodoxin from C. beijerinckii (Ludwig & Luschinsky, 1992; Smith et al., 1977). More recent highly refined, high-resolution crystal structures now further reveal a mixture of conformations, including an unusual cis configuration, for the peptide bond between Gly57 and Asp58 in this flavodoxin and, based on several mutants of Gly57, emphasizing the importance of this glycine residue in this interaction (Ludwig et al., 1997). The reverse turn involving residues 56-59 adopts a type II' turn conformation (trans O-up) in the semiquinone state in the wild-type and all mutant flavodoxins. Due to the unfavorable contact between Cβ of position i+1 and the backbone amide proton of position i+2, glycine is strongly preferred in position i+1 in such turns (Richardson, 1981). Therefore, the substitution of amino acid residues with side chains, even as small as a methyl group but certainly as bulky as a β-branched side chain, is expected to destabilize the type II' turn. According to the thermodynamic cycles developed by Ludwig et al. (Ludwig et al., 1997), the relative changes in the ox/sq potential in response to the addition of this side chain can be rationalized by the contribution of the free energy changes associated with the conformational change in the protein upon reduction of the FMN to the semiquinone state ($\Delta G_e^{ox/sq}$) and of the molecular interactions, including hydrogen bonding, between the
FMN cofactor in each oxidation state and the protein (ΔGi). These two aspects are obviously interrelated, but have been formally separated to assist in the functional interpretation of this phenomenon (Ludwig et al., 1997). Based on the X-ray crystallographic analyses of primarily the G57D mutant, but also for G57A, the decreased ox/sq potential in these proteins has been rationalized as being primarily due to changes in ΔGc^ox/sq induced by the presence of the side chain rather than in ΔGi (Ludwig et al., 1997). If this is the case, the different temperature coefficients observed for the Gly57 mutants may result from a shift of the equilibrium between the hydrogen-bonded and non-hydrogen-bonded species due to alterations to the conformational energy of the turn introduced by the side chain, causing an overall net change in the strength of the N(5)H•••O57 interaction, a conclusion now supported more directly by this study.

The substantially more negative potential for the ox/sq couple and the weaker hydrogen-bonding interaction for the G57T flavodoxin have been attributed to significant differences in the relative energies of the cis O-down, trans O-down, and trans O-up conformations, with this mutant exhibiting only the trans O-down conformation in the oxidized state (Ludwig et al., 1997). It was therefore suggested that the ΔGc term is considerably more positive for this mutant, perhaps as a consequence of the more conformationally restrictive nature of the β-branched side chain of the threonine residue. It is reasonable to conclude that this effect could also be primarily responsible for the larger temperature coefficient for G57T observed in this study. However, changes in ΔGi could also contribute significantly to the larger temperature coefficient for this mutant. A weakening of the hydrogen-bonding interaction at N(5)H is suggested by the alteration of
the geometry of the N(5)H•••O57 interaction evident in the X-ray crystal structure of the G57T mutant (Ludwig et al., 1997). Also, the apparent pKa value for N(5)H appears to be lowered by approximately 2 pH units in this mutant (Ludwig et al., 1997). However, it was noted that the pKa is still higher than for free FMN. It is important to note that this study cannot truly distinguish between a conformational effect (changing ΔG_e) and a direct effect on hydrogen bonding strength itself (altering ΔG_i). Nevertheless, it is quite apparent from this study that the strength of the N(5)H•••O57 interaction is functionally linked to the structural features of the amino acid residue at position 57 and directly to the modulation of the midpoint potentials of primarily the ox/sq couple of the FMN cofactor. It is of significance that the presence of a glycine residue, as found in the wild-type flavodoxin, provides the strongest interaction and the most negative potential for the sq/hq couple.

CONCLUSION

The results of this study provide direct experimental evidence that strongly supports several important long-standing hypotheses of Ludwig and co-workers (Ludwig & Luschinsky, 1992; Smith et al., 1977). The formation of the critical hydrogen-bonding interaction with the N(5)H of the reduced FMN cofactor in conjunction with a conformational change within the 50’s loop in the C. beijerinckii flavodoxin represents an important means of modulating the midpoint potential of the bound cofactor, primarily through the thermodynamic stabilization of the neutral flavin semiquinone (Ludwig & Luschinsky, 1992; Ludwig et al., 1997; Smith et al., 1977). This study, in conjunction
with our previous characterization of this group of flavodoxin mutants (Ludwig et al., 1991; Ludwig et al., 1997; Swenson et al., 1991), more completely discloses the critical role of the conserved Gly57 and the unique structure of this loop in establishing this interaction. Because similar conformational changes are observed in other flavodoxins, this phenomenon may represent a general means of regulation of the redox properties of flavodoxins. The modulation of the strength of hydrogen bonds with the various donor/acceptor atoms of the flavin undoubtedly plays an important role in establishing the reduction potentials in other flavoproteins as well.
CHAPTER 3


INTRODUCTION

As described in Chapter 1, shuttling between the one-electron (semiquinone) and fully reduced (hydroquinone) states, flavodoxins play an important role in mediating low-potential electron transfer between other redox proteins in such notable physiological processes as photosynthesis, nitrogen fixation, and microbial respiration, often substituting for the low-potential ferredoxin in these metabolic pathways (Mayhew & Tollin, 1992). These proteins exhibit the lowest reduction potentials among the flavoprotein family, with values recorded as low as -520 mV (Mayhew & Tollin, 1992). Thus, a characteristic and essential role of the flavodoxin is to dramatically lower the midpoint potential of the semiquinone/hydroquinone (sq/hq) couple. In the case of the Desulfovibrio vulgaris flavodoxin, the one-electron reduction potential for this couple of
the flavin shifts from $-124$ mV observed for FMN in aqueous solution (Anderson, 1983) to approximately $-450$ mV (at pH 7, 25 °C vs SHE) (Curley et al., 1991; Swenson & Krey, 1994). X-ray crystallographic analyses of flavodoxin from *D. vulgaris* in its three redox states indicate that the semiquinone and fully reduced states are nearly identical in structure (Watenpaugh et al., 1973, 1976; Watt et al., 1991). Therefore, conformational differences are unlikely to account for the large shift in the reduction potential of the sq/hq couple. More likely to affect the redox potentials are the differential stabilization/destabilization of each redox state by common features of the cofactor binding site such as long-range electrostatic, π-π aromatic stacking, and hydrogen-bonding interactions as has been directly demonstrated recently (Ludwig et al., 1990, 1997; Swenson & Krey, 1994; Zhou & Swenson, 1995, 1996).

An important physical property that seems to be of critical importance in the regulation of the one-electron reduction potentials of flavodoxins is the formation of the anionic form of the FMN hydroquinone when the flavodoxin is fully reduced. Optical and $^{15}$N NMR spectroscopic data suggest that the pKa of the hydroquinone anion has been shifted substantially from 6.7 found in solution to $<4$ when bound by the protein (Vervoort et al., 1985, 1986; Ludwig et al., 1990). Structural studies with a flavodoxin reconstituted with 1-deaza-FMN suggest structural reasons for the lower pKa value (Ludwig et al., 1990). This situation seems somewhat paradoxical in that the anionic form of the hydroquinone selectively accumulates in an environment provided by the protein in which its formation is energetically unfavorable. The importance of unfavorable electrostatic interactions has been demonstrated through the systematic substitution of the six acidic residues surrounding the cofactor binding site in the
flavodoxin from *D. vulgaris* and shown that the unfavorable electrostatic environment destabilizes the flavin hydroquinone anion, contributing about one-third of the large redox potential shift for the sq/hq couple (Zhou & Swenson, 1995). Significant increase of the $E_{sq/hq}$ couple was also found in the *D. vulgaris* Y98A mutant. Elimination of unfavorable $\pi-\pi$ interaction between the $\pi$-rich Tyr98 and FMN hydroquinone anion was attributed to the change of $E_{sq/hq}$ in this mutant (Swenson & Krey, 1994; Zhou & Swenson, 1996b). It is clear that the FMN hydroquinone remains anionic at pH 7.0 in the wild-type protein. However, the N(1) FMN hydroquinone was assumed to be unprotonated in the Y98A and charge neutralization mutants (Fld$^{+1}$-Fld$^{+6}$) (Swenson & Krey, 1994; Zhou & Swenson, 1995, 1996b). $^{15}$N NMR was used to investigate the ionization state of FMN hydroquinone in the Fld$^{+6}$ and Y98A/Fld$^{+6}$ mutants. Protonation of N(1) FMN hydroquinone will shift the N(1) chemical shift upfield by about 60 ppm (Vervoort et al., 1986). The N(1) chemical shift in these two mutants shows that the FMN hydroquinone anion did not change its ionization state. These data further confirm the role of electrostatic interactions in regulating the $E_{sq/hq}$ couple.

However, flavoproteins may not only utilize unfavorable electrostatic interactions to modulate redox potentials. Recent studies have demonstrated that the introduction of a basic residue such as a histidine or an arginine near the flavin isoalloxazine ring can significantly increase the redox potential for the sq/hq couple (Helms, 1990; Swenson & Krey, 1994). For example, Tyr98 is one of two aromatic residues that flanks the flavin in the flavodoxin from *D. vulgaris*, the other being Trp90 (Watenpaugh et al., 1973, 1976). The phenolic side chain of Tyr98 is nearly coplanar with the outer or $si$ face of the isoalloxazine FMN ring, making extensive van der Waals contacts with it (Figure 19A).

74
Figure 19: Partial view of the structure of the flavin mononucleotide binding site in the flavodoxin from *D. vulgaris* highlighting the aromatic residues flanking the flavin isoalloxazine ring. (Panel A) Wild-type structure is adapted from X-ray crystal structural data (Watt *et al.*, 1991). (Panel B) The Y98H mutant structure is a geometry-optimized model based on the NMR solution structure of this protein (Stockman *et al.*, 1994). Dots represent the van der Waals surfaces of each residue. Hydrogen atoms have been omitted for clarity. The side chain of the amino acid at position 98 flanks in a coplanar manner the *si* face of the flavin ring.
Figure 19

A

W60
FMN
Y98

B

W60
FMN
H98
Substitution of a histidine or an arginine for this residue significantly increases (by 180 mV) the midpoint potential of the sq/hq couple (Swenson & Krey, 1994). Such large increases do not seem to be the result of significant structural changes in these mutants (Stockman et al., 1993, 1994). However, the general pH dependency of the one-electron reduction potential for the sq/hq couple in the Y98H mutant suggests that the reduction of the semiquinone to the hydroquinone is closely coupled to the ionization of His98 and is consistent with the electrostatic stabilization of the flavin hydroquinone anion by the flanking cationic imidazolium side chain of His98 (Figure 19B). As a necessary consequence of the redox-linked ionization model, the pKa of His98 was predicted to shifted from 7.0 in the semiquinone state to approximately 8.5 in the fully reduced state as a consequence of the electrostatic coupling (Swenson & Krey, 1994). However, direct experimental evidence of this pKa shift was not available at the time and the analysis was compromised somewhat by the inability to extend the redox titrations beyond pH 8.5.

Thus, an important aspect of the redox-linked ionization scheme is that the ionization of the coupled amino acid residue is directly influenced by the redox state of the flavin cofactor and vice versa. NMR spectroscopy was chosen in this study because of the following reasons. 1) It can provide intrinsic pKa of ionizable groups such as histidine or aspartic acid (Jeng & Dyson, 1996; Markley, 1975). 2) The pKa of histidine has been successfully determined in many proteins by this method (Bos et al., 1989; Griffin et al., 1973; Kubal et al., 1994; Markley, 1973). 3) The size of the D. vulgaris flavodoxin is within NMR limit. 4) There are two histidine residues in the Y98H mutant and one in the wild-type protein. By using the high field NMR to increase resolution, it would not be
difficult to resolve the C2H and C4H resonances of imidazole ring. 5) There is no need to prepare $^{13}$C- or $^{15}$N-enriched histidine sample to study the pKa of histidine.

There are three different approaches to study the pKa of histidine by NMR spectroscopy. The first approach is to monitor the resonance shift of C2H and C4H upon changing the pH. This approach is suitable for the low molecular weight proteins. To make the assignment of C2H and C4H easily in using this method, the sample is usually dissolved in D$_2$O to minimize the influence of the solvent peak and slowly exchanging amide proton peaks. The second approach is to follow the change of histidine NH resonance as a function of pH if the imidazole NH exchange slowly with water (Griffin et al., 1973). The third approach is to use proteins incorporated with $^{13}$C- or $^{15}$N-labeled histidine (Bachovchin & Roberts, 1978; Hunkapiller et al., 1973). In this study, the first approach was used to determine the intrinsic pKa of histidine in the Y98H mutant. On protonation of the imidazole ring of histidine in simple peptides, the C2H resonance shifts downfield from 7.70 to 8.68 ppm and the C4H shifts from 7.0 to 7.4 ppm. $^1$H NMR titration curves for the histidine can be obtained by plotting the chemical shift of the C2H and C4H as a function of pH. The titration data were used to fit the following equation (Hill equation) to determine the pKa of histidine.

$$(\delta_{H^+} - \delta_{\text{obsd}}) / (\delta_{H^+} - \delta_{H^o}) = K_a^n / (K_a^n + [H^+]^n)$$

where $\delta_{H^+}$ and $\delta_{H^o}$ are the chemical shifts of protonated and unprotonated form of histidine, respectively. $n$ is the Hill coefficient. $K_a$ is the dissociation constant of the histidine. $[H^+]$ is the hydronium ion concentration from glass electrode measurements.
In this study, NMR spectroscopy was used to directly determine the pKa of His98 in oxidized and fully reduced states in order to provide direct experimental proof for this linkage and to support and confirm the redox-linked ionization model. The close agreement between the theoretical and experimental results obtained in this and the previous study for the linked equilibria involved provides convincing evidence for this type of control of the oxidation-reduction potentials of the flavin cofactor in flavoproteins. Redox-linked ionization may provide an important means through which the change in the ionization state of an amino acid residue in the active site during catalysis and/or the alteration of its pKa by environmental effects may indirectly alter the redox state of the flavin during catalytic turnover, perhaps facilitating electron transfer. Such mechanisms will be discussed.

EXPERIMENTAL PROCEDURES

Materials. Deuterium oxide (D₂O) was obtained from Fluka Chemicals. Sodium deuter oxide (NaOD), deuterium chloride (DCl), sodium 3-(trimethylsilyl)propionate-2,2,3,3-d₄ (TMSP), and sodium 2,2-dimethyl-2-silapentane-5-sulfonate and ^15NH₄Cl (99%) were obtained from Cambridge Isotope Laboratories. Sodium dithionite was from Aldrich Chemical Co. Isopropylthio-β-D-galactoside was acquired from Gibco BRL. All other chemicals were of analytical reagent grade.

Expression and Purification of the Flavodoxin Proteins. The expression plasmids containing the coding region for the pseudo-wild-type (P2A) and the mutant flavodoxins used in this study have been described previously (Swenson and Krey, 1994).
Escherichia coli AG-1 cells transformed with these plasmids were cultured for 36-48 h at 37 °C in NZY medium containing 100 μg/mL ampicillin. Under these conditions, induction of the tac promoter with isopropyl-β-D-thiogalactopyranoside was not necessary. Flavodoxin proteins were purified by established procedures (Krey et al., 1988). Column fractions containing flavodoxin having $A_{274}/A_{454}$ ratios ≤4.4 were pooled and concentrated by ultrafiltration. The purity of each flavodoxin preparation was confirmed by SDS-polyacrylamide gel electrophoresis.

*Preparation of $^{15}$N-enriched FMN.* $^{15}$N-enriched FMN was purified from recombinant Clostridium beijerinckii flavodoxin obtained from transformed E. coli AG-1 cells grown on minimal medium for up to 40 hours at 37 °C with $^{15}$NH$_4$Cl as the sole nitrogen source. Each liter of minimal medium contained 6 g Na$_2$HPO$_4$, 3 g KH$_2$PO$_4$, 1 g $^{15}$NH$_4$Cl, 0.5 g NaCl, 1 mM MgSO$_4$, 6 g dextrose, 0.5 mg thiamine, and 100 mg ampicillin. Isopropylthio-β-D-galactoside (IPTG) was added after ~28 hrs to a final concentration of 0.5 mM to induce the expression of flavodoxin. The flavodoxin was partially purified by poly (ethylene imine) precipitation (Druhan & Swenson, 1998) and dialyzed against 10 mM NH$_4$HCO$_3$. The $^{15}$N-enriched FMN was dissociated from the holoprotein by incubation in 6 M urea at 40 °C for 30 min in the dark and the flavin separated from the apoprotein by ultrafiltration. The $^{15}$N-enriched FMN was purified by chromatography on an Econo-Pac ion exchange cartridge (Bio Rad) equilibrated with 10 mM NH$_4$HCO$_3$ using a step gradient from 50-250 mM of NH$_4$HCO$_3$, repeatedly lyophilized to remove the NH$_4$HCO$_3$, and re-dissolved in H$_2$O.
Reconstitution of the Apoflavodoxin with \(^{15}\text{N}\)-labeled FMN. Recombinant flavodoxin from \textit{D. vulgaris} was purified by established procedures (Krey \textit{et al}., 1988; Eren & Swenson, 1989). Apoflavodoxin was prepared by the procedure of Wassink and Mayhew (1975), dissolved in a minimum volume of 10 mM sodium phosphate buffer (pH 7.0), and dialyzed against the same buffer at 4 °C. The apoflavodoxin solution was lyophilized and dissolved in a solution containing an appropriate molar ratio of \(^{15}\text{N}\)-enriched FMN to prepare the \(^{15}\text{N}\)-enriched (>95%) FMN-reconstituted flavodoxin.

\textit{NMR Sample Preparation of Y98H Mutant.} All NMR samples contained approximately 1.0 mM flavodoxin. Exchangeable protons were replaced with deuterons by incubation of the flavodoxin in D\(_2\)O at 42 °C for 3.5 h followed by lyophilization. This procedure was repeated at least three times. NMR samples of flavodoxin in the oxidized state were prepared in 20 mM sodium phosphate buffer and the pH was adjusted by the addition of appropriate amount of 5\% (w/w) NaOD or DCl in D\(_2\)O during the course of the pH titration. Individual NMR samples of flavodoxin in the reduced state were prepared in 50 mM sodium phosphate buffer for pH values below 8.0 and in 50 mM sodium pyrophosphate buffer for pH values above 8.0. The flavodoxin solutions were made anaerobic by purging with several cycles of a partial vacuum and prepurified argon in NMR sample tubes equipped with septum seals. The flavodoxin was reduced with the addition of an appropriate volume of a freshly prepared sodium dithionite solution. The pH measurements were performed immediately after the NMR measurements. By convention, the reported pH values (designated pH\(^*\)) are not corrected for the deuterium isotope effect. In this way, the isotope effect at the glass electrode (~0.4 unit)
approximately offsets the isotope effect on the acid dissociation equilibrium of the histidine, generating pKa values comparable to those in H₂O (Markley, 1975).

$^{15}$N NMR Spectroscopy. Samples for $^{15}$N NMR contained approximately 2.0 mM flavodoxin in 150 mM sodium phosphate buffer (pH 7.0) for the reduced state prepared in 10% D₂O. The $^{15}$N NMR spectra of the samples contained in Wilmad 5mm thin wall 7740 Pyrex NMR sample tubes were recorded at 300 K on a Bruker AM-500 spectrometer operating at 50.6 MHz. For $^{15}$N NMR measurements, $^{15}$N inverse gate decoupling was acquired using the 100us $^1$H 90° pulse for the WALTZ-16 decoupling sequence (Shaka et al., 1983) with a recycle time of 2.00 s. $^{15}$N chemical shifts are referenced to an external standard of 100mM $^{15}$N-urea in DMSO (76 ppm relative to liquid ammonia). Reduction was achieved by adding an appropriate amount of a freshly prepared sodium dithionite solution to the anaerobic solution of flavodoxin. Anaerobic flavodoxin solutions were prepared by gaseous exchange with several cycles of a partial vacuum and prepurified argon.

$^1$H NMR Spectroscopy. All $^1$H NMR spectra were recorded at 300 K on a Bruker DMX-600 spectrometer operating at 600.13 MHz. Proton chemical shifts in D₂O were referenced to internal standard of TMSP set at 0.0 ppm. Spectra were processed on a Silicon Graphics Indigo workstation using Felix 95.0 software (Biosym Technologies).

RESULTS

$^{15}$N NMR of FMN, Bound to Mutant D. vulgaris Apoflavodoxin in the Reduced State.

All nitrogen atoms in the reduced FMN represent pyrrole-type nitrogen atoms in
heteroaromatic ring systems. Deprotonation of the pyrrole-type nitrogen atom results in a downfield shift in its resonance (Vervoort et al., 1985). It was shown that the FMN hydroquinone in the flavodoxins from *Megasphaera elsdenii*, *Clostridium beijerinckii*, *Azotobacter vinelandi*, and *Desulfovibrio vulgaris* are ionized (Vervoort et al., 1986). The $^{15}$N chemical shifts ofFld$^{+6}$ and Y98A/Fld$^{+6}$ mutant flavodoxins are shown in Table 4. It is clearly that the FMN hydroquinone in these two mutants are also ionized because the chemical shifts of the FMN N(1) in these mutants are very close to that of FMNH (Table 4).

**Determination of the pKa of His98 of the Y98H Mutant in the Oxidized State.** The $^1$H NMR spectra of oxidized Y98H mutant in D$_2$O at different pH values are shown in Figure 20. This flavodoxin contains two histidine residues: His142, which is also present in wild type, and His98, which has been introduced by site-directed mutagenesis (Swenson & Krey, 1994). Four resonance peaks (designated $a$, $a'$, $b$, and $b'$) were observed in the aromatic region of the $^1$H NMR spectrum that could be assigned to the C2H ($a$ and $b$) and C4H ($a'$ and $b'$) of histidine on the basis of their general chemical shift values and titration shifts (Markley, 1975). Peaks $b$ and $b'$ were present in the $^1$H NMR spectra of oxidized wild-type protein at different pH values (Figure 21) and were assigned to the C2H and C4H, respectively, of His142. Peaks $a$ and $a'$ in the spectra of the Y98H mutant (Figure 20) and absent in the wild-type spectra were assigned to the C2H and C4H of His98, respectively. These assignments were further confirmed by homonuclear 2D correlation spectroscopy (COSY) analysis in which a correlation or cross-peak between C2H and C4H of histidine can be observed in the $^1$H COSY spectrum (King & Wright, 1982; Wüthrich, 1986). Figure 22 shows the COSY spectrum of Y98H.
Reduced $^{15}$N chemical shifts (ppm) in atom

<table>
<thead>
<tr>
<th>atom</th>
<th>$D. v.$</th>
<th>Fld$^{16b}$</th>
<th>Y98A/Fld$^{16b}$</th>
<th>FMNH$^a$</th>
<th>FMNH$_2^c$</th>
<th>TARFH$_2^e$</th>
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<tr>
<td>N(1)</td>
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<td>184.7</td>
<td>185.5</td>
<td>182.6</td>
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<td>146.9</td>
<td>148.1</td>
<td>149.3</td>
<td>149.7</td>
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<td>59.7</td>
<td>60.0</td>
<td>57.7</td>
<td>58.0</td>
<td>60.4</td>
</tr>
<tr>
<td>N(10)</td>
<td>98.4</td>
<td>96.6</td>
<td>96.2</td>
<td>97.2</td>
<td>87.3</td>
<td>72.2</td>
</tr>
</tbody>
</table>

$^a$From Vervoort et al. (1985). $^b$This Work. $^c$From Moonen et al. (1984).

**Table 4:** $^{15}$N Chemical Shifts of Free and Flavodoxin-Bound Reduced FMN.
Figure 20: $^1$H NMR spectra (600MHz) in the aromatic region for the oxidized Y98H mutant flavodoxin as a function of pH. Peaks $a$ and $a'$ were assigned to the C2 and C4 protons, respectively, of the imidazole side chain of His98. Similarly, peaks $b$ and $b'$ were assigned to the C2 and C4 protons of His142.
Figure 20
Figure 21: $^1$H NMR spectra (600 MHz) in the low-field region for the oxidized wild-type flavodoxin as a function of pH. Peaks $b$ and $b'$ were assigned to the C2 and C4 protons, respectively, of His142.
Figure 21
Figure 22: Histidine region of a 600 MHz COSY spectrum of oxidized Y98H mutant flavodoxin at pH 7.35, 300 K. The cross-correlations between the C2H and C4H of His98 and of His142 are labeled.
mutant flavodoxin. The C2H-C4H four-bond connectivities, as manifested by the cross-peaks at 7.86 and 6.98 ppm for His98 and 8.01 and 7.00 for His142 between C2H and C4H, respectively, confirm the previous resonance assignments for the C2H and C4H of these residues.

Both C2H and C4H of His98 and His142 shift upfield as the pH is increased (Figure 23, panels A and B, respectively). The pKa values of His98 and His142 in the oxidized Y98H mutant were determined by fitting the titration data to the Hill equation (Markley, 1973). The pKa values of His142 determined from this plot were 7.23 from the C2H signal and 7.22 from the C4H. These values are similar to those obtained from the titration curve for His142 of the wild-type flavodoxin (7.19 from C2H and 7.15 from C4H). The pKa values of His98 were 6.94 from the C2H and 7.11 from the C4H (Figure 23, panels A and B, respectively), giving an average value of 7.02 ± 0.08.

Determination of the pKa of His98 of the Y98H Mutant in the Two-Electron Reduced State. Figure 24 shows the aromatic proton resonance region of the NMR spectrum of the two-electron reduced form of Y98H mutant at different pH values. Two narrow resonance peaks (labeled a and b) corresponding to imidazole C2 hydrogens were observed. Peak a was assigned to the C2H of His98 and peak b was assigned to the C2H of His142 according to the following two approaches. Resonance assignments were initially based on the comparison of NMR spectrum for the two-electron reduced, one-electron reduced, and oxidized states of this flavodoxin (Figure 25). Due to the paramagnetic effect of the flavin radical generated in the one-electron reduced state, proton resonances close to the isoalloxazine ring will be strongly broadened (Peelen & Vervoort, 1994). Peak a is present as a narrow band in the two-electron reduced
Figure 23: pH dependence of the $^1$H NMR chemical shifts for the histidine C2H (Panel A) and C4H (Panel B) assigned to His98 and His142 in the Y98H mutant flavodoxin in the oxidized state. Panel C represents the pH dependency of the chemical shift for the C2H in the fully reduced Y98H flavodoxin. The chemical shifts assigned to His98 and His142 are represented by the ■ and ● respectively, in each panel.
Figure 23
Figure 24: Downfield region of the 600 MHz $^1$H NMR spectra for the two-electron reduced Y98H mutant flavodoxin as a function of pH. Peaks $a$ and $b$ were assigned to the C2H hydrogens of His98 and His142, respectively.
Figure 24
Figure 25: Aromatic region of the 600 MHz $^1$H NMR spectra for the Y98H mutant flavodoxin in each of the three redox states of the FMN cofactor at pH 6.3. Peaks $a$ and $b$ were assigned to the C2H of His98 and His142, respectively.
spectrum and the oxidized spectrum but appears broadened in the one-electron reduced (semiquinone) spectrum. Also, this peak appears to shift upfield upon full reduction the flavin. Both observations are consistent with the behavior of the C2H of His98 close to the FMN ring. However, peak b was observed in the spectra of all three redox states, and its chemical shift did not change significantly, consistent with its assignment to the C2H of His142, which is located on nearly the opposite side of the protein, over 25 Å from the cofactor. It is interesting to note that the doublet at approximately 8.21 ppm appearing in both the oxidized and fully reduced spectra is also broadened in the semiquinone spectrum and may represent other protons near the FMN in this flavodoxin.

A second approach for the assignment of resonance peaks was based on the comparison of the pH dependency of the spectra to the two-electron reduced wild-type flavodoxin (representative spectra are shown in Figure 26). The C2H and C4H (peaks b and b') of His142 shift upfield as the pH is increased and the pH shift of C2H correlated very well to that of peak b in the fully reduced spectra of the Y98H mutant and to peak b in the oxidized spectra (assigned to His142, Figure 21). The pH titration of the C2H resonance of His142 generates a pKa value of 7.32 (see Figure 23C), which is also very similar to that for the oxidized wild-type protein.

It is quite clear from the pH dependency of the resonance assigned to the C2H of His98 (peak a) that the pKa of this residue has increased substantially in the fully reduced flavodoxin. Unlike resonance peak b (C2H of His142), peak a does not shift upfield to any significant extent below pH 7.5 (Figure 24). This behavior is also in marked contrast to the response of this resonance in the oxidized flavodoxin, in which peak a has almost completed its upfield shift by the time pH 7.5 was reached (Figure 20). Above
Figure 26: $^1$H NMR spectra (600 MHz) in the region containing the histidine C2H and C4H resonances for the fully reduced wild-type flavodoxin at several different pH values. Peaks $b$ and $b'$ were assigned to the C2 and C4 protons of His142, respectively.
this pH, this peak began to shift upfield, started to broaden noticeably above pH 7.9, and became quite broad at the highest pH tested, pH 8.7 (Figure 24). The reason for the broadening of this resonance peak is not completely understood. This phenomenon was very reproducible, consistently appearing with different samples and on different times of analysis. It may result from a decrease in rotational freedom of the neutral form of the imidazole ring of His98, perhaps as the result of the hydrogen bonding between the neutral form of the imidazole ring and the carboxyl group of either Asp62 or Asp95, which are located nearby. Alternatively, the neutral imidazole may exist in two or more slowly exchanging conformations or environments. The pKa value of His98 in the two-electron reduced state of the Y98H flavodoxin was obtained from the fit of the titration curve of peak a to the Hill equation. The pKa was determined to be 8.43 ± 0.11 (Figure 23C).

*pH Dependency of the Midpoint Potential for the sq/hq Couple Is Described by a Redox-Linked Ionization Model Using the Experimentally Determined pKa Values for His98.* The midpoint potential for the Y98H mutant is dependent on pH in a manner that appears to be consistent with the favorable electrostatic coupling of the ionization of the imidazole side chain with the hydroquinone anion as the flavodoxin is reduced (Swenson & Krey, 1994). The relationship of the midpoint potential to the ionization of a single ionizable group can be described by the standard equation as follows (Clark, 1972):

\[
E_m = E_0 + \frac{RT}{nF} \ln \left\{ \frac{[H^+] + K_a^{HQ}}{[H^+] + K_a^{SQ}} \right\}
\]

98
Therefore, in order to calculate the pH dependency of the midpoint potential of the sq/hq couple, it is necessary to know the acid dissociation constant for the redox-linked ionizable group, in this case His98, in both the semiquinone and hydroquinone reduced states. However, the determination of the pKa of His98 by NMR when the FMN in the semiquinone state was not possible because the C2H and C4H resonances could not be identified, undoubtedly due to extensive line broadening through the paramagnetic shielding effect of the nearby unpaired electron in the flavin radical (for example, see Figure 25). It was necessary, therefore, to assume that the pKa of His98 in the semiquinone state is very similar to that in the oxidized state. This assumption seems reasonable because the redox-linked ionization effect described here is most likely propagated through electrostatic interactions between the flavin and His98. Because the FMN in the blue neutral semiquinone form, like the oxidized state, does not carry a net charge, we suggest that the pKa values will not differ appreciably between these two redox states. Also, it was established spectrophotometrically that the semiquinone is maintained in its blue neutral form under the conditions and throughout the pH range used in this study. Given this assumption, the experimental data conform very well to the theoretical curve generated by the redox-linked ionization model using the experimental values for the pKa\textsuperscript{SQ} (7.02) and for the pKa\textsuperscript{HQ} (8.43) for His98 generated in this study and an $E_0$ value of $-242$ mV, where $E_0$ represents the midpoint potential when the ionizable group is in the fully protonated state (Figure 27). In our previous report, similar pKa values were assigned to His98 through the redox-linked model; however, direct experimental proof of this assignment was not available (Swenson & Krey, 1994).
Figure 27: Experimental values for the midpoint potential for the sq/hq couple of the Y98H mutant (●) as a function of pH [data from Swenson & Krey (1994)] and the fit (solid line) to the redox-linked ionization model described under Results using the experimental values for the pK_a of His98 of 7.02 ± 0.08 and 8.43 ± 0.11 in the semiquinone (pK_a^{SQ}) and hydroquinone states (pK_a^{HQ}), respectively, from this study and a value of -242 mV for E_0.
The midpoint potentials for the sq/hq couple of Flad
 and Y98A/Flad are 93 mV and 220 mV less negative than that of the wild type. It has been demonstrated that the cumulative effect of the unfavorable electrostatic interactions introduced by π-π stacking interactions and the negative electrostatic environment of the FMN binding site contribute 81% of the total redox potential shift for the sq/hq couple of the *D. vulgaris* flavodoxin (Zhou & Swenson, 1995, 1996b). However, there is an another possibility that the substantial increase in $E_{sq/hq}$ for these two mutants may be due to the protonation of the N(1) of FMN hydroquinone, which will also eliminate the unfavorable aromatic stacking and/or electrostatic interactions. The $^{15}$N NMR data in this study show that the N(1) of the reduced FMN in these two mutants are still ionized at pH 7.0, supporting the concept that the less negative values of $E_{sq/hq}$ are due to the neutralization of the acidic residues and/or elimination of π-π interactions (Zhou & Swenson, 1995, 1996b).

Besides the $^{15}$N NMR data in this study, there is another piece of evidence that can also support this concept. The midpoint potential for the sq/hq couple was found to be pH dependent in several flavodoxins such as *D. vulgaris, D. desulfuricans*, and *D. salexigens* (Calderia et al., 1994; Curley et al., 1991; Swenson & Zhou, 1997). $^{15}$N NMR studies show that the FMN hydroquinone still remains in its anionic form at pH 6.0 and 5.5 in the *D. vulgaris* and *M. elsdenii* flavodoxin, respectively (Vervoort et al., 1985; Franken et al., 1984). The redox-linked pH dependence of the sq/hq couple for the *D. vulgaris* flavodoxin can be reasonable fitted based on the ionization of the charged residues clustered around the FMN N(1) atom (Swenson & Zhou, 1997). As the number
of neutralized charges increases, the \( E_{aq/hq} \) couple becomes much less pH dependent. No redox-linked pH-dependency is observed between pH 6.0 and 7.0 for the Fld\(^{+6} \) mutant (Swenson & Zhou, 1997). If the less negative value of the \( E_{aq/hq} \) couple in Fld\(^{+6} \) mutant results from the protonation of reduced FMN N(1), then the \( E_{aq/hq} \) couple should display a pH dependence with a slope of 59 mV/pH unit below pH 7.0 due to an uptake a proton at N(1). The observed results of no pH-dependency above pH 6.0 for the Fld\(^{+6} \) mutant indicate that the pKa of N(1) FMN hydroquinone is lower than 6.0 and the electrostatic interactions between the FMN hydroquinone and the negative charge residues are responsible for the redox-linked pH dependence of the \( E_{aq/hq} \) couple in the \textit{D. vulgaris} flavodoxin. Taken the \(^{15}\text{N} \) NMR and pH-dependency data together, these results support the previous concept and eliminate the possibility of the protonation of reduced FMN N(1) at pH 7.0 in Fld\(^{+6} \) and Y98A/Fld\(^{+6} \) mutant flavodoxins (Zhou & Swenson, 1995, 1996b; Zhou, 1996).

The differential stabilization/destabilization of the various redox states of the flavin cofactor through both short- and long-range electrostatic interactions with ionizable amino acid residues within and around the cofactor binding site represents an important mechanism in the modulation of the one-electron reduction potentials of the cofactor. Both favorable and unfavorable electrostatic interactions have been demonstrated to have significant effects on the midpoint potentials of the FMN cofactor in the flavodoxin system. The placement of a basic residue immediately adjacent to the flavin by the substitution of Tyr98 with either a histidine or an arginine was found to substantially increase the one-electron reduction potential for this couple (Swenson & Krey, 1994).
In this study, we have experimentally established the pKa of His98 in the oxidized and reduced states of the Y98H flavodoxin mutant in an effort to more directly support a general redox-linked ionization model for the regulation of the oxidation-reduction potentials of the flavin cofactor in flavoproteins. In such a model, the ionization properties of His98, if coupled to the reduction of the neutral FMN semiquinone to the hydroquinone anion, should be described by the linked equilibria shown in Scheme 5. This linkage predicts that the pKa of His98 should be directly dependent on the redox state of the flavin and, concurrently, the pH-dependent shift in the midpoint potential of the sq/hq couple ($\Delta E_m$) is determined by the difference in the pKa values for this residue in each redox state according to

$$\Delta E_m = E_p - E_n = (2.303RT/nF) (pK_a^{HQ} - pK_a^{SQ})$$

where $E_p$ and $E_n$ represent the midpoint potentials for this couple when His98 is protonated or neutral, respectively.

The pKa of His98 established by NMR spectroscopy in this study was quite clearly dependent on the redox state of the FMN cofactor, providing direct experimental proof of the redox-linked ionization phenomenon. The pKa shifted from a value of $7.02 \pm 0.08$ in the oxidized state to $8.43 \pm 0.11$ in the fully reduced state. Because the pKa of His98 in the semiquinone state could not be determined directly by NMR due to paramagnetic line broadening, we were forced to assume that the pKa is similar to that in the oxidized state, in our opinion, a reasonable assumption (see Results for justification). Given this assumption, the experimental pKa values determined in this work correlate very well...
Scheme 5: Equilibria linking the ionization of His98 to shifts in the midpoint potential for the sq/hq couple in the Y98H flavodoxin mutant. $E_n$ and $E_p$ refer to the midpoint potentials for the sq/hq couple when His98 is either neutral or protonated, respectively. $K_a^{\text{SQ}}$ and $K_a^{\text{HQ}}$ represent the ionization constants for His98 in the one-electron (semiquinone) and fully reduced (hydroquinone) states, respectively.
with the previous theoretical values and accurately predict the observed pH dependency of the midpoint potential of the sq/hq couple in this mutant. These results establish with greater certainty that it is the redox-linked ionization of His98 in this flavodoxin mutant that is responsible for the pH-dependent changes in the midpoint potential.

The pKa shift from 7.0 to 8.43 for the His98 upon reduction demonstrate the model that the flavin hydroquinone anion was stabilized by the favorable electrostatic interaction from the positive charge on the imidazole ring of histidine (Swenson & Krey, 1994). The contribution of Gibbs free energy by the electrostatic interaction upon the ionization of His98 can be determined by

\[ \Delta G = 2.303RT(pK_a^{\text{HQ}} - pK_a^{\text{SQ}}) \]

The Gibbs free energy change calculated by this equation is 2.0 kcal/mol equivalent to a shift in reduction potential of about 85 mV, consistent with the shift of the midpoint potential of the sq/hq couple from -240 mV to approximately -325 mV upon deprotonation of His98. In agreement with our original prediction based on curve-fitting to the experimental data to the redox-linked ionization model (Swenson & Krey, 1994), the NMR results confirm that the midpoint potential of the sq/hq couple for the Y98H mutant at high pH when His98 is fully deprotonated is still about 100 mV less negative than that for the wild-type flavodoxin. This represents a stabilization of the FMN hydroquinone anion of about 2 kcal/mol by the neutral imidazole side chain of His98 relative to the tyrosine in wild type. This phenomenon is not completely understood but
may in part be the result of the destabilization of the of the semiquinone state in this mutant (Swenson & Krey, 1994).

Electrostatic interaction energies of this magnitude resulting in large pKa shifts are not uncommon in proteins. For example, the ion pairing between His31 and Asp70 in T4 lysozyme results in the increase in the pKa of the histidine to a rather high value of 9.1 as compared to a more typical value of 6.8 in the unfolded state, contributing between 3 and 5 kcal/mol of stabilization to the native structure of this protein (Anderson et al., 1990). The catalytically relevant imidazolium-cystine thiolate ion pair in papain and various chemically mutagenized subtilisins results in the shift of the histidine pKa to rather high values (>8.6) (Lewis et al., 1981; Plou et al., 1996). For papain, the methylthiolation of the active-site cysteine, Cys25, results in the decrease in the pKa of the adjacent histidine by more than 4 pKa units, demonstrating the effect of destroying the ion-pairing interaction between these two residues. In the flavoprotein family, the histidine in the analogous ion pair in glutathione reductase and lipoamide dehydrogenase displays apparent pKa values as high as 9.3 (Sahlman & Williams, 1989).

It is of importance to note that the underlying mechanism of the redox-linked ionization model in which His98 modulates the oxidation-reduction potential of the FMN in the Y98H flavodoxin (Scheme 5) is conceptually different from that reported for the covalent flavin analog 8-α-(N-imidazolyl)riboflavin. This derivative is of physiological importance as it represents a model for an important class of flavoproteins with covalently bound flavin cofactors attached through either 8-α-(N^δ1-histidinyl) or 8-α-(N^ε2-histidinyl)flavin linkages. The pKa values for the imidazole group of 8-α-(N-imidazolyl)riboflavin in all three redox states have been established through the analysis
of the pH dependency of its oxidation-reduction potential and more directly by \(^1\)H NMR spectroscopy of the oxidized and fully reduced states (6.0 ± 0.1 and 7.0 ± 0.1, respectively) (Williamson & Edmondson, 1985a,b). The increase in the pKa is attributed to the change in the more direct through-bond inductive effect in going from the electron-withdrawing characteristics of the oxidized flavin to that of an electron-donating properties of the fully reduced flavin in this covalent analog. The pKa of the imidazole in the semiquinone form is estimated to be intermediate between that of the oxidized and fully reduced states (Williamson & Edmondson, 1985a,b). Thus, the ionization of the imidazolyl group in such flavin adducts may represent an important means by which redox potentials are regulated in this class of covalently linked flavoproteins (Williamson & Edmondson, 1985a). For example, in the *E. coli* fumarate reductase, which contains an 8-α-(N\(^{\alpha2}\)-histidinyl)FAD with an elevated midpoint potential, substitution of His44 (to which the FAD is attached) with other amino acids results in an enzyme that still retains the ability to bind FAD tightly but noncovalently yet is unable to oxidize succinate, presumably because the redox potential of the flavin has now become too negative, although no direct evidence was given (Blaut et al., 1989).

The situation presented in this study is fundamentally different from that of the 8-α-(N-histidinyl)linked flavoproteins. In our case, the effect of the ionization of His98 are necessarily indirect, involving through-space electrostatic interactions rather than more direct through-bond inductive effects. Thus, the Y98H flavodoxin mutant provides a good example of perhaps the more general redox-linked ionization phenomenon in which ionizable groups adjacent to the flavin cofactor in flavoproteins indirectly modulate the reduction potential of the flavin. This is likely to be a relatively common situation in
flavoproteins. Such groups could represent catalytically essential amino acid residues in the active site of the flavoenzyme that as a consequence of their participation in acid-base catalysis change ionization state during the catalytic cycle. A good example is found in flavocytochrome $b_2$ (yeast L-lactate dehydrogenase) and the structurally (and perhaps mechanistically) related enzymes glycolate oxidase and L-lactate oxidase (Ghisla & Massey, 1991). The active-site histidine [His373, His254, and His290 in flavocytochrome $b_2$, glycolate oxidase, and L-lactate oxidase (by homology), respectively] in these flavoenzymes is stacked in a coplanar fashion over the pyrimidine ring of the $si$ face of the FMN in a manner not unlike that in the Y98H flavodoxin (Lindqvist & Brändén, 1989; Xia & Mathews, 1990; Ghisla & Massey, 1991; Stockman et al., 1994). The replacement of His373 by a glutamine residue in flavocytochrome $b_2$ by site-directed mutagenesis provides evidence that this residue is essential for activity, serving as a general base and perhaps influencing electron transfer between substrate and the flavin (Gaume et al., 1995). Various experiments suggest that the pKa of this histidine is dramatically increased upon reduction of the FMN such that proton exchange from the imidazolium cation is very slow, leading to estimates of the pKa as high as 10 or greater. Such a large shift is very unusual and needs to be confirmed; however, it apparently has not been possible to measure its pKa directly by NMR spectroscopy because of the size of this protein (Lederer, 1992; Balme & Lederer, 1994). These observations have led to the conclusion that His373, after assisting proton abstraction from substrate, facilitates the reduction of the FMN through favorable ion-pairing interaction with the flavin hydroquinone.
The studies reported for the Y98H flavodoxin mutant seem to provide a good model for certain features of this type of mechanism, providing direct experimental evidence for the redox-linked ionization aspect and a measure of the magnitude of such effects (Swenson & Krey, 1994; this work). In this protein, it has been possible to directly determine the extent of the substantial shift in the pKa of His98 upon reduction of the flavin and to attribute the substantial pH-dependent increase in the midpoint potential of the bound FMN of nearly 100 mV to the favorable ion-pairing interactions between the protonated His98 and the flavin hydroquinone anion as required by a redox-linked ionization mechanism. Such an increase should significantly facilitate the entry of electrons into the flavin. One important structural difference in the Y98H flavodoxin mutant must be considered, however. Located on the surface of the Y98H flavodoxin, His98 is much more exposed to solvent than is likely to be the situation in the active center of a flavoenzyme (Stockman et al., 1994). Therefore, the magnitude of the pKa and midpoint potential shifts observed in the Y98H flavodoxin, while substantial, may actually be more modest than could occur within an active-site cavity. The more restricted solvent exposure of His373 in reduced flavocytochrome $b_2$ could explain its substantially higher pKa; however, hydrogen bonding to Asp282 and perhaps its closer proximity to the N1/C2O region of the FMN, which carries the formal charge of the hydroquinone anion, may also contribute (Balme & Lederer, 1994). Whether these and other interactions are significant enough to raise the pKa of His373 to values greater than 10 when the flavin is reduced remains to be established.

Ionizable groups affecting the oxidation-reduction potentials of the flavin cofactor are not necessarily limited to amino acid residues within the active site but could also
represent the substrates and/or products involved in catalysis. A good example of the mechanistic importance of such modulation may be provided by the medium-chain acyl CoA dehydrogenase, in which the binding of substrate/product has been shown to increase the midpoint potential of the FAD cofactor in this enzyme, perhaps through favorable electrostatic interactions between the partially charged transition state and the flavin (Johnson et al., 1995). This induced shift in the reduction potential of the flavin cofactor of approximately 100 mV is critical for catalysis as the potential of the uncomplexed enzyme is significantly lower than that of its substrates, a thermodynamically unfavorable situation for electron transfer (Lenn et al., 1990).

Thus, both short- and long-range electrostatic interactions must be considered as part of an important general mechanism for the modulation of reduction potentials in flavoproteins and other redox systems. The deviation of the pH dependency of the midpoint potentials from that expected for the direct protonation of the flavin upon reduction in various flavoproteins has been reported and generally explained by the influence of ionizable groups in the flavin binding site. It is likely, therefore, that the redox-linked ionization mechanism is of general significance in flavoenzymes, and the thorough characterization of this phenomenon is of importance.
EVALUATION OF THE HYDROGEN BONDING INTERACTIONS FOR THE RIBOFLAVIN COMPLEX OF THE Desulfovibrio vulgaris FLAVODOXIN AND THEIR EFFECTS ON THE REDOX POTENTIALS

INTRODUCTION

As described in Chapter 1, the unfavorable π-π stacking interactions between the Tyr 98 and anionic FMN hydroquinone and the negative electrostatic environment of the FMN binding site in flavodoxin from Desulfovibrio vulgaris have been shown to have significant contribution to the low midpoint potential for the sq/hq couple (Figure 7 and 12) (Zhou & Swenson, 1995, 1996b; Swenson & Krey, 1994). The D. vulgaris apoflavodoxin can also form a complex with riboflavin. However, the binding of the oxidized riboflavin to the wild-type apoprotein is 100-fold weaker than the binding of the FMN to the same apoprotein and the midpoint potential for the sq/hq couple for the riboflavin-wt complex is about 180 mV less negative than the wild-type protein. The phosphate group of the FMN has been indicated to be very important for the association of flavin and apoflavodoxin (Pueyo et al., 1996), but the mechanism of binding is still unclear. The unfavorable electrostatic interactions between the dianionic phosphate and
the negative charge on the FMN hydroquinone N1 atom was proposed to have significant effect on the regulation of the midpoint potential for the sq/hq couple based on the results of the calculated values for this couple from several flavodoxins (Moonen et al., 1984). Because the unfavorable electrostatic environment provided by the clustered acidic residues contributes about one-third of the midpoint potential shift for the sq/hq couple (Zhou & Swenson, 1995), the effect of the interactions between the phosphate group and FMN N1 atom was investigated. Introducing a basic charge residue in the T12H and N14H mutant flavodoxins from D. vulgaris to neutralize the negative charge of the 5'-phosphate was found to increase the sq/hq couple by 28 and 15 mV compared to that of the wild-type protein (Zhou & Swenson, 1996a). Apparently, the midpoint potential shift for the sq/hq couple contributed by the negative charge on the phosphate group is similar to the acidic residues flanking around the flavin ring. Therefore, the large redox potential shift for the sq/hq couple in the riboflavin-wt complex is not primarily due to the stabilization of hydroquinone by elimination of the unfavorable electrostatic interactions between the hydroquinone anion and the dianionic phosphate. However, it was found that the riboflavin-wt complex in the semiquinone state is substantially destabilized compared to the wild-type protein (Zhou & Swenson, 1996a). The formation of the hydrogen bonding interaction between the N(5)H of FMN and the C=O group of Gly57 in the C. beijerinckii flavodoxin has been demonstrated to be an important factor to regulate the thermodynamic stabilization of the blue neutral flavin semiquinone and the midpoint potentials for the ox/sq and sq/hq couple (Chang & Swenson, 1999; Ludwig et al., 1997). This has led to speculation that the modulation of the redox potentials in the
riboflavin-wt complex could be partially due to the weakening of the hydrogen bonding interaction between the N(5)H of riboflavin and the C=O group of Gly61.

In this study, the $^1$H-$^{15}$N HSQC NMR was applied to the wild-type and riboflavin-wt flavodoxin proteins reconstituted with $^{15}$N-labeled FMN or riboflavin to investigate the temperature dependencies of the N(5)H. The N(5)H hydrogen bond interaction and its role in the regulation of the redox potentials in both proteins are evaluated and discussed. The results of this study are compared to the crystal structure of the riboflavin-wt complex (Walsh et al., 1998). Also, the ionization state of FMN hydroquinone N(1) in Fld$^{+6}$ and Y98A/Fld$^{+6}$ mutant flavodoxins were investigated by $^{15}$N NMR.

EXPERIMENTAL PROCEDURES

**Materials.** Deuterium oxide ($\text{D}_2\text{O}$) was purchased from Fluka chemicals. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate and $^{15}\text{NH}_4\text{Cl}$ (99%) were obtained from Cambridge Isotope Laboratories. Sodium dithionite was from Aldrich Chemical Company. Isopropylthio-β-D-galactoside and Calf Intestinal Alkaline Phosphatase were acquired from Gibco BRL. All other chemicals were of reagent grade.

**Preparation of $^{15}$N-enriched FMN.** $^{15}$N-enriched FMN was purified from recombinant *Clostridium beijerinckii* flavodoxin obtained from transformed *E. coli* AG-1 cells grown on minimal medium for up to 40 hours at 37 °C with $^{15}\text{NH}_4\text{Cl}$ as the sole nitrogen source. Each liter of minimal medium contained 6 g Na$_2$HPO$_4$, 3 g KH$_2$PO$_4$, 1 g $^{15}\text{NH}_4\text{Cl}$, 0.5 g NaCl, 1 mM MgSO$_4$, 6 g dextrose, 0.5 mg thiamine, and 100 mg
ampicillin. Isopropylthio-β-D-galactoside (IPTG) was added after ~28 hrs to a final concentration of 0.5 mM to induce the expression of flavodoxin. The flavodoxin was partially purified by poly (ethylene imine) precipitation (Druhan & Swenson, 1998) and dialyzed against 10 mM NH₄HCO₃. The ¹⁵N-enriched FMN was dissociated from the holoprotein by incubation in 6 M urea at 40 °C for 30 min in the dark and the flavin separated from the apoprotein by ultrafiltration. The ¹⁵N-enriched FMN was purified by chromatography on an Econo-Pac ion exchange cartridge (Bio Rad) equilibrated with 10 mM NH₄HCO₃ using a step gradient from 50-250 mM of NH₄HCO₃, repeatedly lyophilized to remove the NH₄HCO₃, and re-dissolved in H₂O.

Preparation of ¹⁵N-enriched Riboflavin. ¹⁵N-labeled FMN (1.5 umole) was incubated with calf intestinal alkaline phosphatase (300 units) at 37 °C for 4 hrs. 60% CH₃CN was added to a final concentration of 30% CH₃CN. The ¹⁵N-labeled riboflavin was purified by HPLC (Phenomenex) on a Bondcldne C₁₈ column. The buffer system utilized 5 mM sodium phosphate buffer, pH 7.0 as solvent A and 60% CH₃CN as solvent B. A linear gradient of 0 to 30% solvent B in 25 min was used for separation. The effluent was monitored by visible absorbance at 445 nm (Figure 28). The ¹⁵N-riboflavin was lyophilized repeatedly to remove the CH₃CN.

Reconstitution of the Apoflavodoxin with ¹⁵N-labeled FMN and Riboflavin. Recombinant flavodoxin from D. vulgaris was purified by established procedures (Krey et al., 1988; Bren & Swenson, 1989). Apoflavodoxin was prepared by the procedure of Wassink and Mayhew (1975), dissolved in a minimum volume of 10 mM sodium phosphate buffer (pH 7.0), and dialyzed against the same buffer at 4 °C. The
Figure 28: HPLC chromatogram of $^{15}$N-riboflavin.
apoflavodoxin solution was lyophilized and dissolved in a solution containing an appropriate molar ratio of $^{15}$N-enriched FMN or riboflavin to prepare the $^{15}$N-enriched (>95%) FMN- and riboflavin-reconstituted flavodoxin.

$^{15}$N and $^1$H-$^{15}$N HSQC NMR Spectroscopy. Samples for $^{15}$N NMR contained approximately 2.0 mM flavodoxin in 50 mM and 150 mM sodium phosphate buffer (pH 7.0) for the oxidized and reduced state, respectively, prepared in 10% D$_2$O. The $^{15}$N NMR spectra of the samples contained in Wilmad 5mm thin wall 7740 Pyrex NMR sample tubes were recorded at 300 K on a Bruker Avance-600 spectrometer operating at 60.8 MHz, respectively. For $^{15}$N NMR measurements, $^{15}$N inverse gate decoupling was acquired using the 100us $^1$H 90° pulse for the WALTZ-16 decoupling sequence (Shaka et al., 1983) with a recycle time of 2.00 s. $^{15}$N chemical shifts are referenced to an external standard of 1.5 M $^{15}$NH$_4$NO$_3$ in 1 M HNO$_3$ (21.6 ppm relative to liquid ammonia set by convention to 0.0 ppm) (Srinvasan & Lichter, 1997) for $^1$H-$^{15}$N HSQC NMR and 100mM $^{15}$N-urea in DMSO (76 ppm relative to liquid ammonia) for $^{15}$N NMR. Samples for HSQC experiments contained approximately 1.0 mM fully reduced flavodoxin in 50 mM sodium phosphate buffer (pH 7.0) in 10% D$_2$O. Reduction was achieved by adding an appropriate amount of a freshly prepared sodium dithionite solution to the anaerobic solution of flavodoxin. Anaerobic flavodoxin solutions were prepared by gaseous exchange with several cycles of a partial vacuum and prepurified argon. The $^1$H-$^{15}$N HSQC NMR spectra (Bodenhausen & Ruben, 1980) were acquired on a Bruker DMX-600 spectrometer using the water flip-back versions (Grzesiek & Bax, 1993) with $^1$H and $^{15}$N sweep widths of 8802 and 7298 Hz, respectively. 16 scans were recorded for each 128 $t_1$ value. Quadrature in $t_1$ was accomplished by using the TPPI-states (Marion et al.,
GARP decoupling (Shaka et al., 1985) was used during acquisition to decouple the $^{15}\text{N}$. Temperature calibration was performed using methanol and ethylene glycol (VanGeet, 1969). Proton chemical shifts were referenced to internal standard of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) set at 0.0 ppm.

RESULTS AND DISCUSSION

$^{15}\text{N} \text{NMR of the Riboflavin Complex in the Oxidized and Reduced States.}$ The $^{15}\text{N}$ chemical shift of the N(1) atom of the riboflavin complex in the oxidized state is similar to FMN, both unbound and in the native flavodoxin complex (Table 5 and Figure 30A). However, the N(5) resonance for the riboflavin complex is shifted upfield compared to that of native $D$. vulgaris flavodoxin and is similar to that of unbound FMN in aqueous solutions (Table 5 and Figure 30A). Because both are pyridine-type nitrogen atoms, such shifts suggest (Witanowski et al., 1993) that the N(1) of the riboflavin, like for FMN in the native protein, is strongly hydrogen bonded to the protein backbone (Watt et al., 1991). In contrast, the N(5) atom of riboflavin complex is likely to be in a more polar environment than for the FMN, perhaps forming a hydrogen bond with solvent. Both observations conform very well to the crystal structure of the riboflavin complex (Walsh et al., 1998).

The chemical shift of N(5) in the riboflavin complex in the fully reduced state shifts upfield about 4.4 ppm relative to the native flavodoxin (Table 5). Just as for the oxidized state, this shift implies that the hydrogen bond between the N(5)H in the reduced riboflavin and the apoprotein is weaker in the riboflavin complex. Further evidence is provided by the temperature dependency data described below. The chemical shift of N(1)
Figure 30: $^{15}$N NMR spectra of *D. vulgaris* apoflavodoxin reconstituted with $^{15}$N-labeled riboflavin in the oxidized state (Panel A) and two-electron reduced state (Panel B).
<table>
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<th>atom</th>
<th>D. v.&lt;sup&gt;a&lt;/sup&gt; riboflavin-wt complex&lt;sup&gt;c&lt;/sup&gt;</th>
<th>FMN&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TARF&lt;sup&gt;b&lt;/sup&gt;</th>
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<th>FMNH&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>87.3</td>
<td>72.2</td>
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<sup>a</sup>From Vervoort <i>et al.</i> (1985).  <sup>b</sup>From Moonen <i>et al.</i> (1984).  <sup>c</sup>This work. Abbreviations: TARF, tetraacetylriboflavin in CHCl<sub>3</sub>.

Table 5: $^{15}$N Chemical Shifts of Free and Flavodoxin-Bound Oxidized and Reduced FMN and Riboflavin.
of riboflavin bound to the apoflavodoxin is close to that of anionic FMNH\textsuperscript{aq} (FMNH\textsuperscript{-} in Table 5, Figure 30B), indicating that like the FMNH\textsuperscript{aq} in the native flavodoxin, the Ribo\textsubscript{HQ} is fully ionized at pH 7.0. Thus, its protonation can not be responsible for the significant shift in E\textsubscript{sq/aq} for the riboflavin complex, further arguing against the role of diminished electrostatic interactions in dramatic increase in E\textsubscript{sq/aq} for the riboflavin complex.

Temperature Dependent \textsuperscript{1}H-\textsuperscript{15}N HSQC Spectroscopy Studies of FMN and Riboflavin Complexes. The temperature coefficients of the backbone amide protons in peptides and proteins have been well characterized and have been used as a measure of the strength of intra-peptide hydrogen bonds involving these atoms (Andersen \textit{et al.}, 1997; Baxter & Williamson, 1997; Dyson \textit{et al.}, 1988; Merutka \textit{et al.}, 1995; Rothemund \textit{et al.}, 1996). Furthermore, the temperature coefficient of the N(5)H of reduced FMN, representing the strength of the N(5)H\textsuperscript{aq}O57 interaction, have been shown to correlate very well with E\textsubscript{ox/aq} for Gly57 mutants of the \textit{C. beijerinckii} flavodoxin (Chang & Swenson, 1999). As in that study, the temperature dependencies of the chemical shifts of the hydrogen atoms on N(3) and N(5) of the bound FMN and riboflavin in the oxidized and fully reduced states were determined (Figure 31). The temperature coefficient (\(\Delta \delta/\Delta T\)), derived from the slopes from that plot, reveal significant differences between the two complexes (Table 6). In the \textit{D. vulgaris} flavodoxin, the N(3)H forms a hydrogen bond with the backbone carbonyl oxygen of Tyr100 in all three oxidation states (Figure 29) (Watt \textit{et al.}, 1991). Relative to the native FMN complex, the smaller temperature coefficients for the N(3)H in the riboflavin complex in the oxidized and particularly in the
Figure 29: Representation of the FMN binding site in oxidized *D. vulgaris* flavodoxin based on the X-ray crystal coordinates (3fx2) of Watt *et al.* (Watt *et al.*, 1991). For clarity, only the residues that form the neutral 5-phosphate binding site and those that interact with the N(3) and N(5) regions of the isoalloxazine ring are shown. Upon reduction, the carbonyl oxygen of Gly61 rotates to form a hydrogen bond with the N(5)H of the FMN (Watt *et al.*, 1991).
Figure 31: Temperature dependence of the chemical shift for the protons on the N(3) and N(5) atoms of the cofactor in the *D. vulgaris* flavodoxin reconstituted with $^{15}$N-labeled FMN (*filled circles*) or riboflavin (*open circles*). Panel A: N(3)H chemical shifts in the oxidized flavodoxin. Panel B: N(3)H chemical shifts in the fully reduced flavodoxin. Panel C: N(5)H chemical shifts in the fully reduced flavodoxin. Note: A signal was not observed the N(5)H in the riboflavin complex (see text).
Figure 31
<table>
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<th>flavodoxin</th>
<th>$E_{ox/n}(mV)$</th>
<th>$E_{sq/h}(mV)$</th>
<th>temperature coefficient $(\Delta \delta/\Delta T)$ (ppb/K)</th>
<th>$K_d$ (nM)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G_{ox}^{\dagger}$</th>
<th>$\Delta G_{sq}^{\dagger}$</th>
<th>$\Delta G_{hq}^{\dagger}$</th>
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<td>N(3)H</td>
<td>N(5)H</td>
<td>ox</td>
<td>sq</td>
</tr>
<tr>
<td>riboflavin complex</td>
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<td>-257$^b$</td>
<td>fully reduced</td>
<td>N(3)H</td>
<td>N(3)H</td>
<td>N(5)H</td>
<td>ox</td>
<td>sq</td>
</tr>
<tr>
<td>FMN</td>
<td>-238$^c$</td>
<td>-172$^c$</td>
<td>$\Delta G_{ox}^{\dagger}$</td>
<td>$\Delta G_{sq}^{\dagger}$</td>
<td>$\Delta G_{hq}^{\dagger}$</td>
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<td></td>
</tr>
<tr>
<td>riboflavin</td>
<td>-231$^c$</td>
<td>-167$^c$</td>
<td>$\Delta G_{ox}^{\dagger}$</td>
<td>$\Delta G_{sq}^{\dagger}$</td>
<td>$\Delta G_{hq}^{\dagger}$</td>
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Table 6: Comparison of One-Electron Reduction Potentials, Temperature Coefficients, Dissociation Constants, and Binding Free Energy Changes for Each Oxidation State of the FMN and Riboflavin Cofactor in the Wild-type Flavodoxins from *Desulfovibrio vulgaris*. 
fully reduced state suggest that the N(3)H•••O=C100 interaction is stronger in riboflavin complex. Because the charge density of the respective nitrogen atoms on the riboflavin and FMN are identical, this stronger hydrogen bonding interaction must be primarily due to the change of the geometry of this hydrogen bond. In fact, Walsh et al. (Walsh et al., 1998) report a shorter distance and an altered geometry between N(3)H and the carbonyl oxygen of Tyr100 in the oxidized riboflavin complex. Unfortunately, it is not possible to evaluate the contribution of the strength of the N(3)H•••O=C100 interaction in the stabilization of the SQ state via the temperature coefficients of N(3)H for the FMN and riboflavin because their determination is precluded by the paramagnetic effect of the unpaired electron of the flavin radical (Chang & Swenson, 1997; Chang & Swenson, 1999; Peelen & Vervoort, 1994). It would be of interest to learn more about its contribution to the stabilization of this oxidation state of the flavin cofactor.

In the native protein, the temperature coefficient for N(5)H in the reduced FMN is -4.907 ppb/K (Table 6). This value is significantly higher than that observed for the C. beijerinckii flavodoxin (Chang & Swenson, 1999). It was also noted that as for the G57T mutant of that flavodoxin the 1H-15N cross-correlation peak for the N(5)H disappears above ~300 K (Figure 31C) (Chang & Swenson, 1999). Both observations are consistent with a weaker hydrogen bonding interaction at N(5)H in the reduced native D. vulgaris flavodoxin. Based on the relationships between the midpoint potentials and the temperature coefficient of N(5)H of the reduced FMN for the clostridial flavodoxin (Chang & Swenson, 1999), these results also seem to correlate with the lower E_{ox/sq} value
for this flavodoxin. An HSQC signal was not observed for N(5)H in the fully reduced riboflavin complex throughout the temperature range of 273 to 314 K. It is very unlikely that the absence of an HSQC signal is due to the dissociation of the proton from N(5) of the riboflavin HQ because of its high pKₐ (Ludwig et al., 1992). It is more likely that the loss of ¹H-¹⁵N HSQC signal is the consequence of the rapid exchange of the proton between the N(5) and solvent or perhaps to a moderately rapid conformational equilibrium involving the flipping of the carbonyl group of Gly61 which is associated with the formation of the N(5)H•••O=C₆₁ interaction in the reduced states (Watt et al., 1991). Either situation is consistent with a substantially weaker or even the absence of the N(5)H•••O=C₆₁ interaction in the reduced riboflavin complex than for FMN in the native protein, which itself appears to be weaker than in the C. beijerinckii flavodoxin. This conclusion is also supported by the substantial upfield shift in the signal for ¹⁵N(5) discussed above (Table 5). These results are consistent with the movement of the 60's-loop away from the flavin ring and its greater solvent exposure that is noted in the oxidized riboflavin complex, although it is not yet known if such changes persist in the reduced state (Walsh et al., 1998).

Changes in MidpointPotentials Correlate with Changes in Binding Interactions.

In the flavodoxin, the neutral FMNₛₒ is nearly always thermodynamically stabilized while the anionic FMNₜₒ is significantly destabilized relative to the unbound cofactor, generating the characteristic low reduction potential for these proteins (Ludwig et al., 1992; Mayhew & Ludwig, 1975). The midpoint potentials for each couple are established by the relative binding free energy for each oxidation state of the cofactor (Dubourdieu et
al., 1975). Figure 32 clearly illustrates that both the OX and SQ states of riboflavin are substantially less stable than for FMN in the native protein; however, the increase for the SQ state is more pronounced (4.7 vs. 2.7 kcal/mol). Much of the loss of binding affinity is most likely due to the elimination of the hydrogen bonding interactions between the protein and the 5'-phosphate (Pueyo et al., 1996; Watt et al., 1991); however, this should be similar for all three redox states. Variations specific to each redox state are more likely attributable to changes in the orientation of the riboflavin ring in the binding site and to other observed structural changes (Walsh et al., 1998). Based on the NMR data for the reduced riboflavin complex, the preferential destabilization of the SQ can be at least partially attributed the substantial weakening of the hydrogen bonding interaction at N(5)H (assuming the observations for the fully reduced state can be extrapolated to the SQ) (Chang & Swenson, 1999) and greater solvent exposure, leading to an $E_{\text{ox/sq}}$ value that is very similar to unbound riboflavin and substantially lower than that for native protein (Table 6). These results are consistent with those obtained for the Gly57 mutants in the C. beijerinckii flavodoxin in which the increased temperature coefficients for the N(5)H of the reduced FMN in response to the amino acid replacements correlate with the increases in $E_{\text{ox/sq}}$ (Chang & Swenson, 1999).

Much of the increase of 180 mV in $E_{\text{sq/hq}}$ for the riboflavin complex (a decrease in free energy of 4.1 kcal/mol relative to the native protein) can be attributed to the much less stable SQ state. However, from the binding free energy changes (Figure 32), it also appears that the HQ state is destabilized to a much less extent in the riboflavin complex than the OX and SQ states. Why is this? Unfavorable electrostatic interactions between the Ribo_HQ and apoprotein should continue to play their important role in regulating $E_{\text{sq/hq}}$
Figure 32: Binding energy diagram for the riboflavin (square) and FMN (circle) bound to *D. vulgaris* wild-type apoflavodoxin in three different oxidation states.
(Zhou & Swenson, 1996a, 1996b) because the $^{15}$N NMR data (Table 5 and Figure 30B) clearly indicate that the RiboHQ remains anionic at pH 7.0. However, these interactions may be reduced in a number of ways. 1) The absence of the electrostatic effects of the anionic 5'-phosphate group could contribute up to 40 mV of the increase in $E_{sq/hq}$ (based on the introduction of basic residues in the phosphate-binding loop in this flavodoxin) (Zhou & Swenson, 1996a). 2) The $^{15}$N NMR and temperature coefficient data for the riboflavin complex in the oxidized state both indicate that the N(5) atom is more solvent accessible, just as was noted in the crystal structure (Walsh et al., 1998). Greater solvent exposure and an increase in the polarity of the flavin-binding site should reduce the unfavorable electrostatic interactions (Swenson & Krey, 1994; Zhou & Swenson, 1996b). 3) The reorientation of the isoalloxazine ring in the riboflavin complex may change the π-π interactions between the isoalloxazine ring and Tyr98. Aromatic stacking interactions are an important force in ligand binding and protein stability (Hunter & Sanders, 1990; Hunter et al., 1991; Serrano et al., 1991) and in the modulation of flavin redox properties (Breinlinger & Rotello, 1997; Lostao et al., 1997; Swenson & Krey, 1994; Zhou & Swenson, 1996b). It is possible that the unfavorable π-π interactions in the reduced riboflavin complex are reduced because of an altered geometry between the isoalloxazine ring and Tyr98. This interaction is crucial. The total elimination of the aromatic interaction in the Y98A mutant of this flavodoxin increases $E_{sq/hq}$ by 139 mV (Swenson & Krey, 1994). Even the introduction of the less electron-rich benzene ring in the Y98F mutant significantly increases the stability of the FMNHQ (Swenson & Krey, 1994; Zhou & Swenson, 1996b). In addition, favorable aromatic stacking interactions can occur when
both aromatic residues reorient in such a geometry that the π-σ attractions dominate the π-
π repulsion and can contribute from -0.6 ~ -1.3 kcal/mol to the stability of proteins
(Burley & Petsko, 1985; Hunter & Sanders, 1990; Hunter et al., 1991; Serrano et al.,
1991). However, it was noted that in the oxidized state the phenol ring actually seems to
overlap more strongly with the riboflavin ring (Walsh et al., 1998). It is not yet known if
this interaction is significantly altered when the riboflavin is reduced. 4) As discussed
above, the N(3)H•••O=C_{100} hydrogen bonding interaction appears to be stronger in the
riboflavin complex and its strength (relative to FMN) actually seems to increase upon
reduction to the HQ state (Tables 5 and 6, Figure 31B). The stronger N(3)H•••O=C_{100}
interaction for the riboflavin complex in the HQ state could contribute to its greater
relative stability, possibly partially offsetting the loss or substantial weakening of the
N(5)H•••O=C_{e1} interaction that normally develops upon reduction of the native protein
(Watt et al., 1991).

In conclusion, the 5'-phosphate group of the FMN appears to assist in establishing
an optimal binding configuration of the cofactor through the hydrogen bonding network
(Walsh et al., 1998; Watt et al., 1991). Without this anchoring group, the binding of
riboflavin to D. vulgaris apoflavodoxin becomes weaker and the position of the
isoalloxazine ring in the binding site and the structure of the 60's-loop are altered (Walsh
et al., 1998). This change leads to a greater solvent exposure of the N(5) and a weakening
of the N(5)H•••O=C_{e1} in the reduced riboflavin complex. These effects contribute to the
destabilization of the OX and, to a greater extent, the SQ states, leading to a more negative
E_{ox/sq} and an E_{sq/hq} that is increased by 180 mV for the riboflavin complex. This increase is
clearly not the result of the elimination of unfavorable electrostatic interactions between the 5'-phosphate and the negative charge on N(1), but is the result of the substantial destabilization of the SQ relative to the HQ. The fact that the HQ is destabilized to the least extent in the riboflavin complex is more difficult to explain with certainty, but could be the result of observed changes in hydrogen bonding interactions at the N(3)H and N(5)H, greater solvent exposure, and more favorable π-π interactions between the flavin ring and Tyr98 due to changes in the orientation of the isoalloxazine ring in the binding site in response to the elimination of the 5'-phosphate. Because these interactions are commonly found in flavin-binding sites, one can conclude that the "tuning" of these interactions represent an important means of the regulation of the binding and redox properties of flavin cofactor in flavoproteins.
CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Summary of Conclusions. Flavodoxins are one of the important proteins that mediate low-potential electron transfer between other redox proteins. They separate the two oxidation-reduction couples of the FMN cofactor through the thermodynamic stabilization of the one-electron reduced semiquinone form and the destabilization of the anionic hydroquinone. Structure-function studies of these proteins have revealed that they use different and delicate mechanisms to alter the midpoint potentials of both FMN couple to accomplish their goals (Bradley & Swenson, 1999; Druhan & Swenson, 1998; Feng & Swenson, 1997; Ludwig et al., 1997; Swenson & Krey, 1994; Zhou & Swenson, 1995, 1996a, 1996b). In this thesis, NMR spectroscopy was used to primarily study the hydrogen bonding and electrostatic interactions to more thoroughly explore their roles in regulating the redox properties of FMN cofactor.

The first part of the thesis studied the N(5)H•••O57 hydrogen bonding interaction in regulating the $E_{ox/sq}$ for the wild-type and mutant flavodoxins from C. beijerinckii. For the ox/sq couple, the free flavin accepts one electron and one proton at N(5) to become the blue neutral semiquinone. To increase the midpoint potential for this couple in the protein-bound flavin, nature has to find a way to stabilize this flavin radical. Because of
the opposite donor/acceptor characteristics at N(1) in the oxidized versus the reduced states, hydrogen bonding could effectively be used to accomplish this. It has been shown that flavodoxins use a local conformation change by flipping the backbone C=O group to form a new hydrogen bond with N(5)H FMN to stabilize the blue neutral semiquinone (Ludwig et al., 1997; Watt et al., 1991). Flipping of this carbonyl group involves the glycine residue in the cofactor-binding site. In the *C. beijerinckii* flavodoxin, this glycine residue (Gly57) is located at the second position in a well-defined β turn in which it can best accommodate the type II' turn in the one-electron reduced state. To test the sequence preference at position 57 in the *C. beijerinckii* flavodoxin, several amino acid substitutions have been made and their structures and redox properties have also been studied (Ludwig et al., 1991, 1997; Swenson et al., 1991). All the mutants have the $E_{\text{ox/sq}}$ more negative than the wild-type protein. Weakening of the N(5)H⋯O57 hydrogen bonding interactions was thought to be responsible for the lowering $E_{\text{ox/sq}}$ in these mutants. In this study, the temperature dependencies of the chemical shift for the N(5)H FMN in the wild-type and mutant proteins were determined to provide information on the strength of the N(5)H⋯O57 interaction. The results show that the temperature coefficients correlate well with the changes in $E_{\text{ox/sq}}$ as well as the binding free energy for the semiquinone state in this group of mutants. According to the thermodynamic cycles developed by Ludwig *et al.* (Ludwig *et al.*, 1997), redox potential shifts reflect the altered free energies from flavin-protein interactions and the conformation change of the β turn. For example, $\Delta E_{\text{ox/sq}} = \Delta G_c^{\text{ox/sq}} + \Delta G_i^{\text{sq}} - \Delta G_i^{\text{ox}}$ (Scheme 6). Changing the Gly57 residue to a bulky side chain amino acid will increase the conformation energy of the type II' turn.
Scheme 6: Thermodynamic cycles that describe the midpoint potential in relate to the free energies from the flavin-protein interactions ($\Delta G_i$) and conformation change between the oxidized and semiquinone states ($\Delta G_{c_{\text{ox/sq}}}$).
in the semiquinone state. As a result, the C=O57 group would not be in a perfect position to optimize the N(5)H•••O57 hydrogen bond. The binding affinity of FMN in the oxidized state in the wild type and mutants are almost the same, indicating no change for the $\Delta G^\text{ox}_i$. So the shifts of $E_{\text{ox/sq}}$ are from the $\Delta G_{\text{c}$ox/sq$}$ and $\Delta G_{\text{i}$sq$}$. But the temperature dependence study cannot distinguish between the contribution from both terms because affecting either one of these two terms or both will reflect on the alteration of the strength of the N(5)H•••O57 interaction. Weakening of the strength of the N(5)H hydrogen bond has not only been observed in the G57 mutants.

In the *C. beijerinckii* flavodoxin, the E59Q mutant was primarily designed to eliminate the redox-linked ionization without altering the $E_{\text{ox/sq}}$ and $\Delta G_{\text{c}$ox/sq$}$. However, the more negative $E_{\text{ox/sq}}$ relative to wild-type was found and was attributed to the substantial decrease binding affinity of FMN in the semiquinone state (Bradley & Swenson, 1999). Study the N(5)H hydrogen bonding interaction in this mutant shows that the temperature coefficient is larger than that of wild-type and its value is correlated with the $E_{\text{ox/sq}}$ (Figure 33) (Bradley & Swenson). These results suggest that the $\Delta G_{\text{i}}$ makes the major contribution to the weakening of the N(5)H hydrogen bond. For the *D. vulgaris* flavodoxin, it seems that the temperature coefficient for this protein also correlates with the $E_{\text{ox/sq}}$ (Figure 33). Could this mean that the strength of the hydrogen bond at N(5) is the only factor to regulate the $E_{\text{ox/sq}}$ in flavodoxins? In the G57T mutant, the $E_{\text{ox/sq}}$ is 32 mV lower than that of free FMN (Ludwig *et al.*, 1997). If the N(5)H hydrogen bond were the only factor to control the $E_{\text{ox/sq}}$, then this hydrogen bond interaction in G57T mutant would be weaker than that of free FMN. However, the pKa
Figure 33: Relationship between the $E_{\text{ox/sq}}$ and the temperature coefficient of the chemical shift for the N(5)H of the bound $^{15}$N-labeled FMN in the fully reduced state in the *C. beijerinckii* wild-type and G57 mutants (filled circles), *C. beijerinckii* E59Q mutant (open circle) (Bradley & Swenson, 1999), *D. vulgaris* wild-type (open triangle, $E_{\text{ox/sq}}$ = -185 mV; open square, $E_{\text{ox/sq}}$ = -148 mV), and riboflavin complex (open inverted triangle, the temperature coefficient (-7.169) was calculated based on the linear regression obtained from G57 mutants (solid line)). The value of $E_{\text{ox/sq}}$ = -185 mV is from Feng (1998) and -148 mV from Swenson & Krey (1994).
of N(5)H of this mutant is still higher than that of free FMN (Ludwig et al., 1997). Apparently some other factors can contribute to the regulation of the $E_{\text{ox}/\text{sq}}$. More temperature coefficient data from different flavodoxin species such as $D. \text{desulfuricans}$, $D. \text{gigas}$, and $M. \text{elsdenii}$ are needed to confirm the role of this hydrogen-bonding interaction. For the riboflavin complex, the temperature coefficient could not be obtained possibly due to the rapid hydrogen exchange of N(5)H with solvent. The $^{15}$N NMR data indicated that the N(5)H hydrogen bond is weakened in this complex. A value of $-7.169$ of the temperature coefficient was predicted for the riboflavin complex based on the correlation obtained from the G57 mutants (Figure 33). If this value could be determined, then it would further demonstrate that the N(5)H hydrogen bond is the most important interaction in regulating the $E_{\text{ox}/\text{sq}}$.

The $E_{\text{ox}/\text{sq}}$ of riboflavin complex is 42 mV more positive than that of G57T mutant. From the correlation between the temperature coefficient and $E_{\text{ox}/\text{sq}}$ (Figure 33), it suggests that the N(5)H hydrogen bond in the riboflavin complex is stronger than that of G57T mutant. But why is the N(5)H exchange with water faster in the riboflavin complex than the G57T mutant? A more solvent accessible environment at N(5) in riboflavin complex could be one of the possibilities. Hydrogen exchange rate can be influenced by many factors such as change in the amino acid sequence or temperature. The 60’s loop in the $D. \text{vulgaris}$ flavodoxin has two extra amino acid residues than the $C. \text{beijerinckii}$ flavodoxin, which has been suggested to have more flexibility. It is then possible that the increase of hydrogen exchange rate in riboflavin complex could be due to the increase fluctuation of the local structure in the ring-binding site.
While the other methods have not been successfully utilized in direct measurement of the N(5)H hydrogen bond, the temperature dependence study clearly demonstrate that the temperature coefficient of N(5)H is a good approach to investigate the hydrogen-bonding interactions in flavodoxins. The *M. elsdenii* flavodoxin has 46% sequence homology compared to that of *C. beijerinckii* flavodoxin. The polypeptide backbone folding patterns between these two proteins are almost identical (Ludwig & Luschinsky, 1992). But the $E_{\text{ox/sq}}$ for the *M. elsdenii* flavodoxin is 23 mV more negative than that of *C. beijerinckii* species (Mayhew et al., 1969). The peptide bond flipping associated with reduction in *M. elsdenii* involves Gly58-Ser59. No structural information in regard to the difference of N(5)H hydrogen bond interaction between these two proteins has been suggested yet. Therefore, determination of the N(5)H temperature coefficient for the *M. elsdenii* would be a perfect choice to study the N(5)H hydrogen bond. Study the hydrogen-bonding interaction by this approach is not limited to the N(5)H but can be also applied to the N(3)H and N(1)H (if N(1) is protonated). Hydrogen bonds between these atoms and proteins are constantly found in flavoproteins. Temperature dependence study of these hydrogen bonds would certainly help to understand their roles in flavin-protein interactions and flavin redox properties.

However, evaluation of these hydrogen bonds in the semiquinone state by this method is not possible. The hydrogen bond interactions in the hydroquinone state still can represent that in the semiquinone state because there are no significant structural differences between these two states. The correlation between the temperature coefficient and $E_{\text{ox/sq}}$ (Figure 33) suggests that this assumption is valid. The N(5)H---O57 hydrogen bonding interaction has been proposed to play an important role
in modulating the \( E_{\text{ox}/\text{sq}} \) in \( C. \text{beijerinckii} \) flavodoxin (Ludwig & Luschinsky, 1992; Smith et al., 1977). This hypothesis now has been supported by the temperature dependence study. Application of this method to study other flavoproteins would greatly assist in understanding the flavin-protein interactions.

The second part of the thesis involved the study of the regulation of the \( E_{\text{sq}/\text{hq}} \) couple through redox-linked ionization in the \( D. \text{vulgaris} \) flavodoxin and the ionization state of bound FMN hydroquinone. Regulation of the \( E_{\text{sq}/\text{hq}} \) is different in mechanism from that of the \( E_{\text{ox}/\text{sq}} \). Both the O-up configuration of Gly C=O group and the N(5)H hydrogen bond in the semiquinone state are also preserved in the hydroquinone state. However, the Kd for the FMN in the hydroquinone state in the \( D. \text{vulgaris} \) and \( C. \text{beijerinckii} \) flavodoxins have increased by about 39000- and 7000-fold, respectively, compared to that in the semiquinone state. Thus, conformation changes and N(5)H hydrogen bond are unlikely to account for the change in the affinity for FMN. Formation of the anionic FMN hydroquinone in several flavodoxins at pH 7.0 has been demonstrated by \( ^{15}\text{N} \) NMR (Franken et al., 1984; Vervoort et al., 1985). Therefore, the anionic FMN hydroquinone is situated in an unfavorable environment provided by the protein. Unfavorable electrostatic interactions between the anionic FMN hydroquinone and acidic residues as well as the aromatic residue (Y98) have been proved to play a significant role in lowering the \( E_{\text{sq}/\text{hq}} \) in \( D. \text{vulgaris} \) flavodoxin (Swenson & Krey, 1994; Zhou & Swenson, 1995, 1996b). Based on these conclusions, the absence of negative charge residues and the presence of positive-charged amino acids near the flavin ring have been suggested to contribute to the stabilization of the anionic hydroquinone and to cause the increase of the \( E_{\text{sq}/\text{hq}} \) in bacterial cytochrome P450 reductase (Sevrioukova et al., 1999). Electrostatics
affect the redox potential has been also found in other flavoproteins. Neutralization of a positively charge residue (Lys297) 8 Å away from N(1) FAD in para-hydroxybenzoate hydroxylase was found to decrease the redox potential by 13 mV, in good agreement with the change of redox potential caused by one negative charge alteration in D. vulgaris flavodoxin (Moran et al., 1997; Zhou & Swenson, 1995). Regulation of the electrostatic interactions between the cofactor and charged residues can be also achieved by change the ionization state of the ionizable residues. In the flavocytochrome b2 (L-lactate dehydrogenase), it has been suggested that the active-site histidine (His373) has its pKa significantly increased in the reduced enzyme leading to the favorable interaction between the positive-charged histidine and anionic FMN hydroquinone (Gondry & Lederer, 1996).

Favorable electrostatic interaction in D. vulgaris flavodoxin can be created by substitution of a histidine for Tyr98. The $E_{sq/hq}$ of Y98H mutant is substantially increased compared to that of wild-type protein (Swenson & Krey, 1994). A pH dependency of $E_{sq/hq}$ was also observed in this mutant and a redox-linked ionization model was proposed (Swenson & Krey, 1994). In that model, the His98 is the redox-linked ionizable group in which protonation of His98 is coupled to the reduction of the FMN from semiquinone to hydroquinone state. As a consequence of the stabilization of anionic FMN hydroquinone by this electrostatic coupling, the model predicted that the pKa of His98 would shift from 7.0 in the semiquinone state to 8.5 in the fully reduced state. The intrinsic pKa of His98 measured by $^1$H NMR in this thesis have the values of 7.02 and 8.43 in the oxidized and fully reduced flavodoxin, respectively, which conforms with the previous prediction. These results provide direct experimental proof of the redox-linked ionization of this
residue and provide further evidence of the crucial role of electrostatic interactions in the stabilization of the FMN hydroquinone anion.

The Y98H mutant study demonstrates an important mechanism in which change the ionization state of charged residues can regulate the redox potential. But why is it important for the enzymes to regulate their redox potential by this way? This question can be answered by the study of human medium-chain acyl-CoA dehydrogenase, a flavoenzyme containing a FAD cofactor. This enzyme catalyzes the dehydrogenation of fatty acids in mitochondria via the abstraction of both a proton and a hydride ion from the α- and β-carbons of the thioester-CoA substrate, respectively (Ghisla et al., 1984). The redox potential of the uncomplexed enzyme is more negative than that of its substrates. It was found that binding of the substrate significantly increases the redox potential of this enzyme making the electron transfer possible (Lenn et al., 1990). The Glu376, which serves as the proton abstracting base, has its pKa increase by at least 2 units upon binding of ligands (Vock et al., 1998). Study of the redox properties of this enzyme shows that change of the ionization state of Glu376 is important in the increase of the redox potential in this enzyme, consistent with the regulation of the redox potential through redox-linked ionization (Chang & Swenson, 1997; Mancini-Samuelson et al., 1998).

In the Y98H mutant, the change of the $E_{sq/hq}$ is coupled with the ionization of His98 residue (Swenson & Krey, 1994; Chang & Swenson, 1997). For the wild-type protein, the pH dependence of $E_{sq/hq}$ can be reasonably well explained by a model in which the change of the $E_{sq/hq}$ at low pH is linked to the ionization of the acidic residues (Swenson & Zhou, 1996). The permanent neutralization of acidic residues by replacement by their respective amide analogs in D. vulgaris flavodoxin not only increases the $E_{sq/hq}$ but also
makes this couple less pH dependent than the wild-type protein. The $^{15}$N NMR data show that the N(1) FMN hydroquinone in the Fld$^{+6}$ and Y98A/Fld$^{+6}$ mutants is still ionized at pH 7.0. These results support the redox-linked ionization of collective acidic residues model and the role of electrostatic interactions in regulating the $E_{sq/hq}$.

The third part of the thesis investigates the riboflavin-apoflavodoxin interactions. One of the structural characteristics in flavodoxins is the unusual neutral binding site for the 5'-phosphate group of the FMN. How this group affects the binding and redox properties of FMN is an important issue in study the flavodoxins. The 5'-phosphate group has been suggested to regulate the $E_{sq/hq}$ by up to 250 mV (Moonen et al., 1984). This value is about 17-fold higher than that of the average contribution from one negative charge residue in *D. vulgaris* flavodoxin (Zhou & Swenson, 1995). Study the riboflavin-apoflavodoxin complex would provide good comparison to reveal the role of 5'-phosphate. *D. vulgaris* flavodoxin is the only short-chain protein that has been found to bind riboflavin. Moreover, the role of electrostatic interactions in modulating the $E_{sq/hq}$ in this protein has been well established (Chang & Swenson, 1997; Feng & Swenson, 1997; Swenson & Krey, 1994; Zhou & Swenson, 1995, 1996a, 1996b).

For the riboflavin complex, the $^{15}$N NMR data suggest a weaker hydrogen bonding and more solvent accessible environment at N(5). Weakening of the N(5)H hydrogen bond is consistent with the decrease of $E_{ox/sq}$ in this complex and the binding affinity of riboflavin semiquinone. However, the $^1$H-$^{15}$N HSQC data suggest that the N(3)H⋯O=C$_{100}$ interaction in the riboflavin complex is stronger than that of wild-type in both the oxidized and fully reduced state. Both the $^{15}$N NMR and $^1$H-$^{15}$N HSQC indicate that the riboflavin ring slides outward in the complex relative to that of FMN in
the wild-type protein. The change of the position of riboflavin ring in the complex is consistent with the X-ray crystal structure (Walsh et al., 1998). Regulation of the stability of riboflavin hydroquinone could be due to the changes in hydrogen bonding interactions at N(3)H and N(5)H, greater solvent exposure, and more favorable π-π interactions between the FMN ring and Tyr98. The NMR study of riboflavin complex provides important information with respect to change of environment and hydrogen-bonding interactions in the riboflavin ring-binding site. These results are consistent with the X-ray crystal structure of the riboflavin complex (Walsh et al., 1998) and more importantly, support the role of hydrogen bond and electrostatic interactions in regulating the redox potentials (Chang & Swenson, 1999; Swenson & Krey, 1994; Zhou & Swenson, 1995, 1996a, 1996b).

**Future Directions.** The temperature dependence study of the *C. beijerinckii* G57 mutants confirms the role of Gly57 in the type II' turn. X-ray crystal structure shows that the N(3)H FMN hydrogen bonds to the carboxyl side chain of Glu59, the fourth residue in the type II' turn. The role of the N(3)H hydrogen bond in regulating the binding and redox properties of FMN in this protein is being investigated by Luke Bradley in our lab. Temperature dependencies have been applied in that study (Bradley & Swenson, 1999). Besides the residue in the fourth position in this turn, both the Gly57 and Asp58 have been systematic mutated to study the effects of conformation change between type II and II' turn on the $E_{ox/sq}$ (Kasim, unpublished results). Temperature dependencies will be also used to investigate the hydrogen-bonding interactions in these mutants.
Determination of the temperature coefficient of N(5)H FMN in the *C. beijerinckii* flavodoxin were conducted in the fully reduced state. Evaluation of the N(5)H•••O57 hydrogen bonding interaction in the one-electron reduced state by NMR spectroscopy is not possible. Resonance Raman spectroscopy is a valuable technique to study the hydrogen bonding interactions between the flavin and apoflavodoxin in the one-electron reduced state. Similar studies have been carried to study the hydrogen bonding environment of flavin ring for various flavoproteins (Copeland & Spiro, 1986; Dutta & Spiro, 1980; Hazekawa *et al.*, 1997; Kim & Carey, 1993; Schmidt *et al.*, 1983; Tegoni *et al.*, 1997; Visser *et al.*, 1983). Estimation of the strength of N(5)H hydrogen bonding interaction from Resonance Raman study would allow for comparison to the temperature coefficient data. The significance of the hydrogen-bonding and electrostatic interactions in contribution to the binding affinity of the FMN indicates that they could play an important role in dynamics. Besides computer simulation, NMR relaxation experiment is the most important tool to study the dynamics of flavodoxins from *D. vulgaris* and *A. nidulans* (Hrovat *et al.*, 1997; Zhang *et al.*, 1997). NMR relaxation study the wild-type and mutant flavodoxins from *C. beijerinckii* and *D. vulgaris* to get dynamics information would greatly help in understanding the role of the hydrogen-bonding and electrostatic interactions from a different aspect of view.

Besides flavodoxins, other flavoproteins also use similar hydrogen bonding interactions to alter the $E_{ox/sq}$. Characterization of electron transfer flavoproteins (ETF) has shown that their $E_{ox/sq}$ is very positive and the one-electron reduced form of flavin is anionic semiquinone FAD (Byron *et al.*, 1989; Husain *et al.*, 1984; Watmough *et al.*, 1992). X-ray crystal structure and NMR spectroscopy data reveal that the N(5) FAD in
the oxidized state hydrogen bonds to the hydroxyl group side chain of Thr266, and the C(4)=O, C(2)=O, N(3)H, and N(1) are all hydrogen bond to the main chain or side chain atoms from the protein (Griffin et al., 1998; Roberts et al., 1996). It appears that ETF use similar mechanism to stabilize the red anion semiquinone. It would be an interesting subject to study the change of $E_{ox/sq}$ in response to the mutation of Thr266 to ionizable residues such as His or Asp. For the T266D mutant, if the protein still wants to stabilize the red anion semiquinone by forming a hydrogen bond between the N(5) and a hydroxyl group, then the pKa of Asp266 should increase in the one-electron reduced state due to the redox-linked ionization on going from the oxidized to semiquinone state (Scheme 7). For the T266H mutant, the pH dependency of $E_{ox/sq}$ and the pKa of His in the oxidized and semiquinone states will also be studied to compare to that of T266D mutant. The differences of the $E_{ox/sq}$ between the wild-type and mutant proteins (T266D and T266H) and the shifts of the pKa of ionizable groups (Asp266 and His266) between the oxidized and semiquinone states can be used to estimate the free energy changes stabilized by the hydrogen bonding interaction (N(5)⋯HOC$_{Asp}$ or N(5)⋯His$^{+}$) or charge-charge interaction (FMNSq⋯His$^{+}$). The results can be also compared to that of *C. beijerinckii* flavodoxin.
Scheme 7: Equilibria linking the ionization of Asp266 or His266 to shifts in the midpoint potential for the ox/sq couple in the ETF mutant.
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