Articles

Role of Hydrogen Bonding Interactions to N(3)H of the Flavin Mononucleotide Cofactor in the Modulation of the Redox Potentials of the *Clostridium beijerinckii* Flavodoxin†

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ABSTRACT: The role of the hydrogen bonding interaction with the N(3)H of the flavin cofactor in the modulation of the redox properties of flavoproteins has not been extensively investigated. In the flavodoxin from *Clostridium beijerinckii*, the γ-carboxylate group of glutamate-59 serves as a dual hydrogen bond acceptor with the N(3)H of flavin mononucleotide (FMN) cofactor and the amide hydrogen of the adjacent polypeptide backbone in all three oxidation states. This "bridging" interaction serves to anchor the FMN in the binding site, which, based on the E59Q mutant, indirectly affects the stability of the neutral flavin semiquinone by facilitating a strong and critical interaction at the FMN N(5)H [Bradley, L. H., and Swenson, R. P. (1999) *Biochemistry 38*, 12377−12386]. In this study, the specific role of the N(3)H interaction itself was investigated through the systematic replacement of Glu59 by aspartate, asparagine, and alanine in an effort to weaken, disrupt, and/or eliminate this interaction, respectively. Just as for the E59Q mutant, each replacement significantly weakened the binding of the cofactor, particularly for the semiquinone state, affecting the midpoint potentials of each one-electron couple in opposite directions. ^{1}H-^{15}N HSQC nuclear magnetic resonance (NMR) spectroscopic studies revealed that not only was the N(3)H interaction weakened as anticipated, but so also was the hydrogen bonding interaction with the N(5)H. Using the temperature coefficients of the N(5)H to quantify and correct for changes in this interaction, the contribution of the N(3)H hydrogen bond to the binding of each redox state of the FMN was isolated and estimated. Based on this analysis, the N(3)H hydrogen bonding interaction appears to contribute primarily to the stability of the oxidized state (by as much as 2 kcal/mol) and to a lesser extent the reduced states. It is concluded that this interaction contributes only modestly ($<$45 mV) to the modulation of the midpoint potential for each redox couple in the flavodoxin. These conclusions are generally consistent with ab initio calculations and model studies on the non-protein-bound cofactor.

The oxidation−reduction properties of the flavin cofactor in flavoproteins are controlled by a variety of noncovalent flavin−protein interactions (*1*, *2*). A very common, but not well understood, interaction is the hydrogen bond that is generally found between the protein and the N(3)H of the cofactor. Molecular orbital calculations for free flavin suggest that N(3) plays a limited role in flavin redox chemistry and the primary function of this interaction is to contribute to cofactor binding, with a preference for the oxidized state (*3*). Recent experimental evidence obtained for flavin derivatives in apolar solvents appears to support this preference (*4*). Based on studies using the N(3)-methyl derivative, which precludes hydrogen bonding interactions at N(3), and small organic molecular hosts, that study suggests that such interactions preferentially stabilize the oxidized rather than the anionic semiquinone state, contributing to a lower midpoint potential for this couple (by as much as 80 mV) (*4*). However, experimental evidence for the role of this interaction in flavoproteins as well as for other redox forms of the flavin is lacking.

The flavodoxin has served as an excellent model system in which to study how flavin−protein interactions control the oxidation−reduction properties of the cofactor. These small acidic flavoproteins function as one-electron carriers in low-potential oxidation−reduction reactions (*1*). The flavodoxin protein significantly perturbs the oxidation−reduction potentials of the noncovalently bound flavin mononucleotide (FMN)[1] cofactor, resulting in a substantial separation of the two one-electron couples. This separation is in part the result of the thermodynamic stabilization of the neutral form of the flavin semiquinone radical and the

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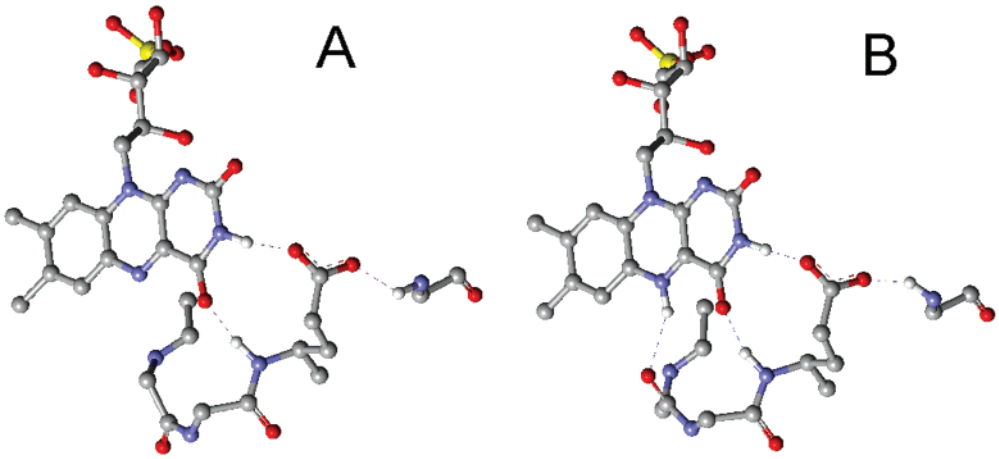

FIGURE 1: Structures of the FMN binding site in the *Clostridium beijerinckii* flavodoxin in the oxidized (panel A) and hydroquinone (panel B) states. For clarity, only the backbone atoms of residues 56−59 and Trp95 (to the right in each panel) are shown as well as the side chain of Glu59. Only hydrogen atoms involved in hydrogen bonding are shown. The Glu59 side chain carboxylate serves as a dual hydrogen bond acceptor for both N(3)H of FMN and the amide hydrogen of Trp95. The Glu59 amide hydrogen serves as a hydrogen bond donor with the C(4)O of FMN. In the reduced flavodoxin, the Gly57 backbone carbonyl group forms a hydrogen bonding contact with N(5)H of FMN (panel B). Carbon atoms are shown in gray, nitrogen atoms in blue, oxygen atoms in red, the phosphate in yellow, and hydrogen atoms in white.

substantial destabilization of the anionic hydroquinone (*2*, *5*). The midpoint potential for the sq/hq couple is among the lowest for all flavoproteins. High-resolution X-ray crystal structures have been determined for several short- and long-chain flavodoxins, often in all three redox states (*6*−*9*).

In most flavodoxins, the N(3)H of the FMN is hydrogen bonded directly to polypeptide backbone carbonyl groups, making the study of this specific interaction rather difficult. However, in the homologous flavodoxins from *Clostridium beijerinckii* (MP) and *Megasphera elsdenii*, the carboxylate group of the side chain of Glu59 or Glu60, respectively, appears to serve as a dual hydrogen bonding acceptor with the N(3)H of the flavin and the amide hydrogen of the adjacent polypeptide backbone (of Trp95 in the clostridial protein) in all three oxidation states (Figure 1) (*10*−*12*). These acidic residues have also been implicated as being responsible for the pH dependency of the sq/hq couple, possibly through a redox-linked ionization mechanism (*13*). The disruption of this interaction through the conservative replacement of Glu59 with a glutamine residue in the *C. beijerinckii* flavodoxin has led to a greater understanding of this residue's functional role (*14*). While the midpoint potentials for both couples in the E59Q mutant of the *C. beijerinckii* flavodoxin mutant shifted in opposite directions by about 80 mV due to the preferential destabilization of the semiquinone, there was very little apparent affect on the pH dependency for the midpoint potential for sq/hq couple (*14*). The analogous Glu60 to glutamine substitution in the *M. elsdenii* flavodoxin also resulted in similar shifts of the midpoint potentials for both redox couples; however, in this case, the pH dependency of $E_{sq/hq}$ was altered (*15*). These apparently conflicting results do not fully resolve the redox-linked ionization role of this acidic residue in these proteins. It is readily apparent, however, that the hydrogen bonding bridge provided by the carboxylate group of Glu59 serves to "anchor" the FMN in the binding pocket and to stabilize the structure of the 50's loop in such a way so as to promote critical hydrogen bonding interactions, primarily at the N(5)H of the FMN, which contributes substantially to the stabilization of the semiquinone state (*14*). These effects for the most part masked the direct evaluation of the involvement of the N(3)H interaction in the redox properties of this flavodoxin.

To understand the role of this interaction more completely, a series of mutants of the *C. beijerinckii* flavodoxin in which Glu59 was replaced by aspartate, asparagine, and alanine were prepared and characterized in this study. This series together with the previously characterized E59Q mutant were designed to either alter or remove the dual hydrogen bonding interactions at N(3)H of the cofactor. While it was not possible to introduce these changes without also affecting the anchoring effects of Glu59, the systematic analyses of the entire group enabled us to approximate the direct effects on the N(3)H interaction itself in each oxidation state.

MATERIALS AND METHODS

Materials. Anthraquinone-2,6-disulfonate, safranine T, indigo disulfonate, and deuterium oxide were obtained from Fluka Chemicals. Safranine T was recrystallized from ethanol before use. Benzyl viologen was obtained from Serva Chemicals. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate and $^{15}NH_4Cl$ (99%) were purchased from Cambridge Isotope Laboratories. Sodium dithionite was purchased from Aldrich Chemical Co. Isopropylthio-β-D-galactoside and all restriction enzymes were obtained from Gibco BRL. All other chemicals were reagent grade.

Oligonucleotide-Directed Mutagenesis. The Glu59 to aspartate, asparagine, and alanine substitutions were introduced by oligonucleotide-directed mutagenesis using the Kunkel method (*16*). Single-stranded DNA was generated from phagemid p*BSFlasy* (pBluescript with synthetic flavodoxin gene cloned into *Eco*RI and *Hin*dIII sites) in *E. coli*

[1] Abbreviations: FMN, flavin mononucleotide; ox or FMN_{OX}, oxidized state of the flavin cofactor; sq or FMN_{SQ}, one-electron-reduced or blue-neutral semiquinone state; hq or FMN_{HQ}, two-electron-reduced or anionic hydroquinone state; $E_{ox/sq}$, midpoint potential of the ox/sq couple; $E_{sq/hq}$, midpoint potential of the sq/hq couple; TARF, tetra-acetylriboflavin.

strain CJ236. Three separate oligonucleotides were used to generate each of the mutants:

E59D:
5′-GGGCGATGATGT(A)CTCGAGGAAAGCG-3′
E59N:
5′-GCCATGGGCGATAATGT(A)CTCGAGGAAAGCG-3′
E59A:
5′-GCCATGGGCGATGCAGT(A)CTCGAGG-3′

Underscored nucleotides represent base changes necessary to introduce the amino acid replacements, while those in parentheses are silent mutations that were incorporated for screening by restriction mapping, with the Glu59 to Asp and Asn substitutions introducing a new *Rsa*I site, while replacement by Ala introduces a *Sca*I site. All mutations were confirmed by dideoxy DNA termination sequencing (*17*).

Expression and Purification of Mutant Flavodoxin Proteins. The flavodoxin structural genes for each mutant were subcloned into the pKK223-3 expression vector using the *Hin*dIII and *Eco*RI restriction sites, and this construction was used to transform *E. coli* XL-1 Blue cells for expression (*18*). The flavodoxin holoprotein was purified as described previously (*14*, *19*). Each mutant flavodoxin protein migrated similar to the recombinant wild-type protein during SDS−PAGE electrophoresis and was estimated to be >95% pure.

Midpoint Potential Determinations. The midpoint potentials for the ox/sq and sq/hq couples were determined in 50 mM sodium phosphate buffer, pH 7.0, at 25 °C as described previously (*14*, *20*). The indicator dyes indigo disulfonate ($E_{m,7} = -130$ mV) and anthraquinone-2,6-disulfonate ($E_{m,7} = -185$ mV) (*21*) were used for establishing $E_{ox/sq}$. Safranine T ($E_{m,7} = -280$ mV) and benzyl viologen ($E_{m,7} = -360$ mV) (*21*) were used to determine $E_{sq/hq}$. Midpoint potentials for both couples were calculated by fitting the plot of the FMN_{SQ} concentration versus the system potentials that were determined at each point in the titration (*14*).

Determination of the Dissociation Constant for FMN_{OX}, FMN_{SQ}, and FMN_{HQ}. The dissociation constant (K_d) for the binding of the FMN cofactor to the apoflavodoxin was determined as described previously using either fluorescence (for wild type and E59D) or UV−visible spectroscopy (for E59N, E59Q, and E59A) to monitor the spectral changes that occur upon binding of the cofactor (*22*). Due to the instability of the reduced states of the flavin in solution, the K_d values for FMN_{SQ} and FMN_{HQ} were calculated from the differences in midpoint potentials for each couple of the FMN bound to flavodoxin versus free FMN according to the relevant thermodynamic cycles involved (*23*). The midpoint potentials for FMN in solution were those determined by Anderson (*24*), which have recently (*25*) been shown to be more reliable than those of Draper and Ingraham (*26*).

^{15}N and ^{1}H-^{15}N HSQC NMR Spectroscopy. Flavodoxin apoproteins reconstituted with >99% enriched ^{15}N-FMN and ^{15}N-FMN were prepared as previously described (*27*, *28*). Samples for ^{15}N NMR and ^{1}H-^{15}N HSQC analyses contained approximately 1.5 mM oxidized flavodoxin in 50 mM sodium phosphate, pH 7.0, containing 10% D_2O. The ^{15}N NMR spectra were recorded at 298 K on a Bruker DMX 600 MHz spectrophotometer. The ^{15}N chemical shifts were referenced to an external standard of 1.5 mM $^{15}NH_4NO_3$ in 1 M HNO_3. The ^{1}H-^{15}N HSQC NMR spectra (*29*) were acquired on either a Bruker DMX 600 MHz spectrophotometer or a Bruker DRX 800 MHz spectrophotometer using the water flip-back and WATERGATE for water suppression (*30*, *31*). ^{15}N NMR and ^{1}H-^{15}N HSQC experiments were performed under the same conditions as described previously (*14*, *28*). When appropriate, full reduction of the holoprotein was achieved by adding an appropriate amount of a freshly prepared sodium dithionite solution to the anaerobic solution of the flavodoxin.

RESULTS

This study was designed to investigate more fully the role within a protein framework of hydrogen bonding interactions at the N(3)H position of FMN in establishing the oxidation−reduction properties of the flavin cofactor. In many flavoproteins, this interaction is often made directly with the protein backbone itself, making its study more difficult. However, in the *C. beijerinckii* flavodoxin, the side chain carboxylate group of Glu59 forms an intervening hydrogen bonding "bridge" between the N(3)H of the cofactor and the adjacent polypeptide backbone. This interaction assists in "anchoring" the flavin ring in the binding site, which primarily contributes to the stabilization of the semiquinone (*14*). In that study, it was not possible to establish whether the N(3)H interaction itself directly contributes to the regulation of the oxidations−reductions. Here we describe the characterization of three additional site-directed mutants in which Glu59 was replaced by aspartate, asparagine, and alanine, which, along with the E59Q mutant previously characterized, provides a systematic alteration of the hydrogen bonding contacts with N(3)H of the FMN.

Physical and Spectral Properties of the E59X Mutants. The ultraviolet/visible absorbance spectra of each mutant were determined in all three redox states during reductive titration with sodium dithionite under anaerobic conditions. Overall, the spectral characteristics of each mutant remain similar to those of wild type in all three redox states; however, some slight differences were noted (data not shown). The λ_{max} for the oxidized flavodoxin was shifted from 446 nm in wild type to 452 nm for the E59D mutant and to 454 nm for the E59N, E59Q, and E59A mutants. These spectral changes are similar to those observed for the E60Q mutant of the homologous *M. elsdenii* flavodoxin (*15*). The blue-neutral form of the FMN_{SQ} having spectral features similar to the wild type was observed for each mutant. The spectra for the hydroquinone state were also similar to wild type with a noticeable shoulder near 370 nm, which has been interpreted as evidence for the anionic form of the hydroquinone (*32*). However, small spectral differences in the 300−350 nm region were noted. These changes have been interpreted as reflecting changes in the ionization state, polarization, conformation, and/or FMN hydrogen bonding with protein (*33*).

Midpoint Potentials and Binding Affinities for Each Redox State. The one-electron reduction potentials for both couples of the FMN cofactor for each mutant were determined at pH 7.0 and 25 °C by equilibrium with redox indicator dyes with known $E_{m,7}$ values (*21*). The $E_{ox/sq}$ values for E59D, E59N, E59Q, and E59A mutants were all more negative than wild type (Table 1) with values of −130, −146, −159, and −186 mV, respectively. The $E_{sq/hq}$ values for each mutant were all significantly less negative than the wild-type

Table 1: One-Electron Reduction Potentials, Dissociation Constants, and Gibb's Binding Free Energies for Each Oxidation State of the FMN Cofactor for Wild-Type and Glu59 Mutant Flavodoxins from *Clostridium beijerinckii* at pH 7.0, 25 °C

	E_m (mV)		K_d (nM)[a]					
flavodoxin	$E_{ox/sq}$	$E_{sq/hq}$	OX	SQ	HQ	ΔG_{ox}[b]	ΔG_{sq}[b]	ΔG_{hq}[b]
FMN	−314[c]	−124[c]	–	–	–	–	–	–
WT	−92[d]	−399[d]	18[e]	0.0031	140	−10.6	−15.7	−9.3
E59D	−130	−352	65	0.049	360	−9.8	−14.1	−8.8
E59N	−146	−318	420	0.60	1160	−8.7	−12.6	−8.1
E59Q[f]	−159	−313	770	1.8	2900	−8.3	−11.9	−7.5
E59A	−186	−298	580	3.9	3500	−8.5	−11.5	−7.4

[a] K_d^{OX} values were determined experimentally while the K_d^{SQ} and K_d^{HQ} values were calculated as described under Materials and Methods. [b] Units of kcal/mol. [c] From Anderson, 1983 (*24*). [d] From Mayhew, 1971 (*45*). [e] From Druhan and Swenson, 1998 (*22*). [f] All values for E59Q are from Bradley and Swenson, 1999 (*14*).

flavodoxin. The $E_{sq/hq}$ values of −352 mV for E59D, −318 mV for E59N, −313 mV for E59Q, and −298 mV for E59A were obtained. It was apparent that the retention of the properties associated with a carboxylate group (i.e., E59D and wild type) was more important than a more sterically conservative replacement (i.e., E59Q vs wild type or E59N vs E59D). Also, the midpoint potentials for both couples were observed to shift in opposite directions, suggesting once again that the semiquinone state has been preferentially destabilized by these substitutions (*14*).

The midpoint potentials for each couple of the cofactor are established by the relative binding affinities of each redox state. The dissociation constants for FMN_{OX}, as determined by visible absorption or fluorescence spectroscopy, were higher than wild type for the neutral mutants E59N, E59Q, and E59A (0.42 ± 0.05, 0.77 ± 0.08, and 0.58 ± 0.07 μM, respectively) (Table 1**)**. These values are about 23-, 43-, and 32-fold larger than the 0.018 μM value obtained under identical conditions for recombinant wild-type flavodoxin (*22*). The E59D mutant displayed a more moderate 3.6-fold increase (K_d^{OX} of 0.065 ± 0.005 μM). The dissociation constants for the two reduced states of the FMN cofactor (Table 1) were determined using these K_d values and the thermodynamic cycle describing the shift in midpoint potentials of the two couples relative to those of the unbound cofactor (*23*). Each mutant binds the neutral FMN semiquinone and the (anionic) hydroquinone significantly weaker than wild type. The E59N, E59Q, and E59A mutants show the largest increase in K_d^{SQ} and K_d^{HQ} with values of 0.6 and 1160 nM for the E59N, 1.8 and 2900 nM for E59Q, and 3.9 and 3500 nM for the E59A mutants, respectively. Once again, the Glu59 to aspartate substitution leads to a more modest increase in both K_d^{SQ} and K_d^{HQ} with values of 0.049 and 360 nM, respectively. Thus, the K_d for FMN_{SQ} was observed to increase by 16-, 190-, 580-, and 1260-fold over wild type for the E59D, E59N, E59Q, and E59A flavodoxins in turn, confirming that the stability of semiquinone was the most sensitive to alterations at this position.

^{15}N NMR Spectroscopy. The effects of the Glu59 substitutions on the hydrogen bonding interactions and the FMN environment were investigated by ^{15}N NMR spectroscopy. For these studies, the mutant flavodoxins were replaced with uniformly enriched ^{15}N-FMN so that the chemical shifts of each FMN nitrogen atom could be established (*28*). For each mutant, four well-resolved resonance peaks were observed for the reconstituted holoprotein in the oxidized state (data not shown). The ^{15}N chemical shifts for N(1), N(5), and N(10) were assigned by comparison to the wild-type flavodoxin (Table 2) (*34*). Because the N(3) atom is the only protonated nitrogen in oxidized FMN, its resonance was confirmed using ^{15}N NMR proton coupled data and/or by the ^{15}N chemical shift of the N(3) ^{1}H-^{15}N HSQC signal in the oxidized states. The proton coupling experiments yielded a doublet peak replacing the furthest upfield peak in the one-dimensional ^{15}N NMR decoupled spectra (data not shown). Unfortunately, neither N(3)H coupling nor a ^{1}H-^{15}N HSQC cross-correlation peak was observed for the E59A mutant, so definitive assignments for the N(3) and N(10) nuclei were not possible. The N(3) nucleus for the E59A mutant was tentatively assigned to the most upfield chemical shift by analogy to the others. This potential ambiguity does not compromise our interpretations, however.

Table 2: ^{15}N NMR Chemical Shifts of FMN, TARF[a], Recombinant *C. beijerinckii* Wild-Type and Glutamate-59 Mutant Flavodoxins at pH 7.0, 25 °C

nuclei	FMN[b]	TARF[a,b]	rWT[c]	E59D	E59N	E59Q[d]	E59A
N(1)	190.8	199.9	183.7	184.7	185.5	185.7	185.9
N(3)	160.5	159.8	160.3	157.4	157.0	156.9	157.9[e]
N(5)	334.7	344.3	350.9	340.8	338.7	336.7	335.5
N(10)	164.6	150.2	163.9	159.1	158.4	159.3	159.0[e]

[a] TARF, tetraacetylriboflavin in $CHCl_3$. [b] From Vervoort et al., 1986 (*34*). [c] Recombinant wild-type flavodoxin values are from Chang and Swenson, 1999 (*28*). [d] From Bradley and Swenson, 1999 (*14*). [e] Tentative assignments (see text).

The ^{15}N chemical shifts for the pyrrole-like N(3) and N(10) atoms of FMN_{OX}, although not typically highly sensitive to hydrogen bonding interactions, are shifted slightly upfield in response to hydrogen bonding interactions with polar solvent (*35*) (Table 2). The ^{15}N(3) chemical shifts for all of the mutants are shifted upfield relative to FMN, to tetraacetylriboflavin (TARF)[1] in chloroform, and to wild-type flavodoxin, consistent with the weakening or loss of the hydrogen bonding interaction at the N(3)H. The ^{15}N chemical shifts of the N(10) atom in the oxidized state for this group of mutants are shifted upfield relative to FMN in aqueous medium, but are shifted downfield compared to TARF in chloroform (Table 2). The downfield shift for the chemical shift of ^{15}N(10) in the transfer of FMN from apolar to polar solvents (and wild-type flavodoxin) has been attributed to the increase in sp^2 hybridization at this atom due to the stabilization of the isoalloxazine ring polarization by hydrogen bonding interactions at C(2)O and C(4)O (*34*). Previous ^{13}C NMR and Raman spectroscopy data suggested that C(2)O and C(4)O are the main π-electron acceptors of the polarized oxidized flavin, with C(4)O receiving its π-electron density primarily from N(10) (*36*, *37*). In this flavodoxin, the hydrogen bond between the backbone amide group of Glu59 and C(4)O may contribute to the downfield shift of the N(10) resonance. Small local perturbations in the 50's loop made by these Glu59 mutations could alter this C(4)O interaction, which may be responsible for the observed N(10) resonance upfield shift (*14*).

The crystal structure of this flavodoxin reveals a potentially strong hydrogen bonding interaction between N(1) and the backbone amide group of Gly89 (*10*−*12*). ^{15}N chemical shifts for the pyrimidine-like atoms N(1) and N(5) in oxidized

FMN shift upfield in response to hydrogen bond formation (*35*). Indeed, the chemical shift value for the ^{15}N(1) atom in each mutant was shifted upfield relative to that of FMN in aqueous solvent, but remained somewhat downfield compared to wild type, however (Table 2). Thus, a strong hydrogen bonding interaction was retained at N(1), but may be slightly weaker than for wild type, particularly for the E59A mutant. The ^{15}N-chemical shifts for the N(5) atom were observed to be the most affected by the amino acid replacements, being shifted substantially upfield relative to both TARF in chloroform and the wild-type flavodoxin (Table 2). The chemical shift values for N(5) are sensitive to many factors including the polarity of its environment, hydrogen bonding, and the π-electron density localized on this atom (*34*), so it is difficult to interpret with certainty what these shifts are reflecting. It is possible that the polarity near N(5) has increased through new hydrogen bonding interactions with solvent. However, similar upfield shifts were noted in the Gly57 mutants for this flavodoxin, and X-ray crystal structures indicate no apparent increase in solvent exposure or significant alterations in the structure of the cofactor-binding site (*12*). It is perhaps more reasonable to conclude that the ^{15}N(5) chemical shifts are reflecting perturbations of the resonance structure of the flavin ring that result in small changes in the π-electron density localized on N(5) and/or altered ring current effects (*34*).

^{1}H-^{15}N HSQC Temperature Dependencies for the FMN in the Oxidized and Reduced States. The temperature dependencies of the chemical shifts for protons involved in hydrogen bonding interactions at the same site in proteins have been used as an indicator of relative hydrogen bonding strength (*38*). The weakening of hydrogen bonding interactions at elevated temperatures alters the distribution between the hydrogen bonded and nonbonded species, resulting in a temperature-dependent upfield shift of the resonance of the proton involved (*39*, *40*). Temperature coefficients range from ~-3 ppb/K for intramolecular hydrogen bonding for backbone amide protons to greater than -6.5 ppb/K for random coil amide protons involved with hydrogen bonding with solvent (*41*, *42*).

This technique has been previously used in our laboratory to evaluate the effects of various amino acid replacements in the *C. beijerinckii* flavodoxin on the hydrogen bonding interactions with N(5)H and N(3)H of the reduced FMN (*14*, *28*). As in those studies, the cofactor in the E59D, E59N, and E59A mutant flavodoxins was replaced with uniformly enriched (>95%) ^{15}N-FMN and subjected to ^{1}H-^{15}N HSQC NMR spectroscopy. Using a 600 MHz spectrometer, a ^{1}H-^{15}N HSQC signal for the N(3)H was observed only for the oxidized wild-type and E59Q flavodoxins. In an 800 MHz instrument, a ^{1}H-^{15}N HSQC cross-correlation peak was observed for the N(3)H of the E59D and E59N flavodoxins. A signal was not observed for the E59A mutant on either instrument. These observations suggest that the N(3)H has an intermediate exchange rate in these mutants. A minor ^{1}H-^{15}N HSQC peak (<30% of the intensity of the major peak) was also present adjacent to the N(3)H cross-correlation peak for the wild-type and E59D flavodoxins in the fully oxidized state. The origin of this minor peak is not known. For both the E59D and E59N mutants, the chemical shift for the N(3)H moved downfield as the temperature was increased. This unusual positive temperature coefficient might imply that the hydrogen bonding interaction at N(3)H actually strengthens at higher temperatures. However, the actual chemical shift at each temperature for each mutant was found significantly upfield relative to wild type (Figure 2A), consistent with the N(3)H becoming more shielded in the mutant structure, which was not expected for a strong hydrogen bonding interaction. Nonetheless, the data indicate that this interaction has been altered in this group of mutants, and is likely to be weaker in the oxidized state.

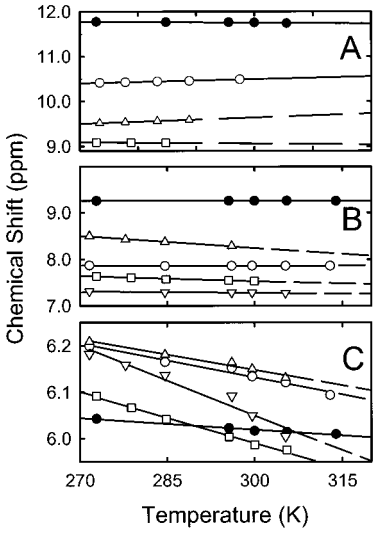

FIGURE 2: Temperature dependencies of the proton chemical shift for the N(3)H oxidized (panel A), N(3)H fully reduced (panel B), and N(5)H (panel C) of the bound ^{15}N-labeled FMN for the wild-type (closed circles), E59Q (open squares), E59D (open circles), E59N (open triangles), and E59A (open-inverted triangles) flavodoxins. The E59D (open circles) and E59N (open triangles) mutant oxidized flavodoxin data (panel A) were acquired on an 800 MHz NMR instrument. All other data were acquired on a 600 MHz spectrometer.

Both the N(3)H and N(5)H cross-correlation signals were observed in all of the fully reduced mutants in the 600 MHz NMR spectrometer. The signals for both protons shift upfield to varying extents with increased temperature (Figure 2B,C). The absolute values for the N(3)H temperature coefficient derived from these plots were all larger than for wild type (Table 3), increasing by 2-, 14-, 52-, and 120-fold for the aspartate, asparagine, glutamine, and alanine substitutions, respectively. These results are consistent with the increasingly weaker N(3)H hydrogen bonding interactions across this series in the reduced state. The temperature coefficient for E59A of -8.6 ppb/K falls within the range that represents a weak transient hydrogen bonding interaction of a backbone amide group of a random-coiled peptide forming a hydrogen bond with solvent (*42*). Thus, it is possible that a water molecule occupies the void created by the alanine substitution. Similarly, the absolute values for the temperature coefficient for the N(5)H are also larger than for wild type, increasing 3-fold for E59D and E59N, and about 4−6-fold for the E59Q and E59A mutants (Table 3). These values are similar to the G57N and G57A mutants which were designed to directly alter the interaction between the carbonyl group of Gly57 and the N(5)H in the reduced FMN (*28*).

DISCUSSION

Although the roles of a variety of flavin−protein interactions in establishing the properties of the cofactor in

Table 3: ^{1}H-^{15}N HSQC Temperature Coefficients for the *C. beijerinckii* Wild-Type and Glutamate-59 Mutant Flavodoxins at pH 7.0[a]

	temperature coefficient ($\Delta\delta/\Delta T$) (ppb/K)							
proton	wild type	E59Q[b]	E59D	E59N	E59A	G57A	G57N	G57T
N(3)H ox	−0.889[b]	−1.00	+3.11[d]	+4.60[d]	−	−0.248	−0.307	−0.093
N(3)H red	−0.0718[c]	−3.76	−0.138	−0.985	−8.56	−0.827[c]	−0.512[c]	−0.681[c]
N(5)H red	−0.824[c]	−3.61	−2.42	−2.19	−5.01	−2.24[c]	−4.13[c]	−9.26[c]

[a] All experiments performed on a Bruker 600 MHz DMZ spectrometer unless otherwise denoted. [b] From Bradley and Swenson, 1999 (*14*). [c] From Chang and Swenson, 1999 (*28*). [d] Experiment performed on a Bruker 800 MHz DMX spectrometer.

flavoproteins have been investigated, one that is not well understood or explored is the hydrogen bonding interaction at the N(3)H of the flavin cofactor. Geometry-optimized molecular orbital calculations of isolated molecules of lumiflavin in the gas phase suggest that N(3) plays a limited role in the flavin redox chemistry and the function of this interaction is to serve as an anchor for flavin binding, with a preference for the oxidized state (*3*). In a more recent study using a novel host−flavin model system, the formation of a hydrogen bond at N(3)H in apolar solvents was found to stabilize the oxidized state relative to the red anionic semiquinone, decreasing the one-electron reduction potential by 80 mV (*4*). The study of this interaction within a protein structure has been limited due in large part to the involvement of main chain heteroatoms that are difficult to alter. However, in the flavodoxin from *C. beijerinckii*, the side chain carboxylate group of Glu59 interacts directly with the N(3)H. This residue has been conservatively substituted with glutamine to evaluate the effect of the glutamate's ionization characteristics on the reduction potentials of the FMN cofactor (*14*). Although the pH dependencies of both couples were not appreciably affected by this change, the midpoint potentials were substantially affected in opposite directions, demonstrating the functional importance of this residue as an "anchor". Although one might logically attribute these changes to alterations in hydrogen bonding with the N(3)H, ^{15}N NMR and ^{1}H-^{15}N HSQC data together establish that interactions at C(4)O and N(5)H (in the reduced state) are also affected (*14*). Thus, it was not possible in that study to isolate the individual contribution by the N(3)H hydrogen bond in the modulation of the reduction potentials.

Isolation of the Effects of the Amino Acid Replacements on the FMN N(3)H Interaction Only—The Model. In an effort to more completely understand the specific effect of the N(3)H hydrogen bond on the modulation of the midpoint potentials in flavoproteins, one must separate the indirect and direct effects associated with alterations at Glu59 in the flavodoxin. The binding of FMN to the flavodoxin can be attributed to many factors (for review, see ref *2*). The 50's and 90's loops of the *C. beijerinckii* flavodoxin contribute most of the contacts with the isoalloxazine ring of the FMN, including direct hydrogen bonding contacts with the pyrimidine ring as well as aromatic and donor atom interactions (*10*−*12*, *22*). The 50's loop provides several important interactions with the flavin ring, including hydrogen bonding contacts with the N(3)H and C(4)O in all three oxidation states. A critical hydrogen bond is formed between the carbonyl group of Gly57 and the N(5)H in the reduced states (*10*−*12*). Other more indirect interactions may also play a role such as long-range electrostatic effects (*20*).

The effects of substitutions at position 59 seem to be localized to the 50's loop and its interactions with the FMN, without altering the overall protein structure. The movements upfield of the chemical shift values for ^{15}N(5) in the oxidized FMN observed for this set of mutants are similar to those observed for the Gly57 mutants, although somewhat larger (Table 2 and ref *28*). One might conclude that these changes are signaling a change in the structure of the FMN binding site. However, high-resolution X-ray crystal structures of the Gly57 mutants show no obvious changes in the overall structure of the oxidized flavodoxin as compared to wild type (*12*). The only apparent changes are localized to the peptide bond configuration in this region (*12*). Therefore, the ^{15}N(5) chemical shifts are more likely reflecting changes in the π-electron density localized on N(5), altered ring current effects, changes in protein dynamics near N(5), and/or the polarity of its environment (*14*, *28*, *34*). Also, other protein−flavin interactions not associated with the 50's loop did not appear to be affected by substitutions at position 59. The chemical shifts for the ^{15}N(1) are not substantially altered in any of the Glu59 mutants, indicating that a strong hydrogen bond is maintained with the main chain of the 90's loop (Table 2). In addition, although less sensitive to localized structural changes, circular dichroism data suggest that substitutions at this position do not substantially alter the protein structure (*14*). Collectively, these data suggest that the effects of substitutions at position 59 are localized to the 50's loop and that the overall protein structure was not affected by these substitutions.

The ^{15}N NMR and ^{1}H-^{15}N HSQC temperature dependency data for the reduced state indicated that the Glu59 substitutions not only disrupt the N(3)H interaction as expected, but also disrupt interactions at N(5)H and possibly C(4)O of the cofactor. Therefore, the observed free energy of binding of FMN in a given oxidation state can be simplified as the sum of the free energy contributions of interactions at N(5)H (in the reduced states), C(4)O, and N(3)H, or

$$\Delta G_{\mathrm{obs}}^{(\mathrm{ox,\ sq,\ or\ hq})} = \Delta G_{\mathrm{N(5)H}}^{(\mathrm{sq\ or\ hq})} + \Delta G_{\mathrm{C(4)O}}^{(\mathrm{ox,\ sq,\ or\ hq})} + \Delta G_{\mathrm{N(3)H}}^{(\mathrm{ox,\ sq,\ or\ hq})} + \Delta G_{\mathrm{other}}^{(\mathrm{ox,\ sq,\ or\ hq})} \quad (1)$$

The amino acid replacements characterized in this study were designed to primarily affect this N(3)H interaction. The C(4)O and the N(3)H interactions, which are both made with the Glu59 residue, cannot be adequately separated as will be discussed below. So for our discussions, it is necessary to combine these terms. The differences in the free energy of binding between the mutant and wild type can then be expressed as

$$\Delta\Delta G_{\mathrm{obs}} = \Delta\Delta G_{\mathrm{N(5)H}} + \Delta\Delta G_{\mathrm{N(3)H/[C(4)O]}} + \Delta\Delta G_{\mathrm{other}} \quad (2)$$

Although it is difficult to completely isolate each interaction, in the discussion that follows, we attempt to deduce at least

an upper limit for the contribution of the N(3)H hydrogen bond to the modulation of the midpoint potentials by correcting for changes in the N(5)H interaction through the use of the NMR data.

(A) The Oxidized State. The binding free energies, the chemical shift for the ^{15}N(3), and the temperature dependency of the N(3)H chemical shift of the FMN in the oxidized state are all significantly affected by the amino acid replacements for Glu59 (Table 2). It will be argued below that these changes can be primarily attributed to changes in the interactions at N(3)H. The Glu59 to alanine substitution should eliminate the hydrogen bonding with the protein. (A water molecule could substitute as a hydrogen bond donor but would likely not contribute significantly to the binding energy.) This substitution resulted in a loss of about 2.1 kcal/mol to the binding free energy for FMN_{OX} (Table 1). A smaller loss of 0.8 kcal/mol was noted for the more conservative replacement with an aspartyl residue. This seems reasonable. Molecular modeling suggested that like Glu59 the side chain carboxylate group of the aspartate can still participate in the double hydrogen bond "bridging" interaction, but with longer and less optimal bonding distances due to the shorter side chain. The loss of binding energy for the amide mutants (E59N and E59Q) is similar to that observed for E59A, consistent with a complete loss of hydrogen bonding with N(3)H. In principle, the carbonyl of the amide group could continue to contribute to binding by serving as a hydrogen bond acceptor with N(3)H. However, the "bridging" or "anchoring" function of the side chain would be destroyed due to the absence of one of the donor groups. Also, molecular modeling and dynamics calculations suggest that the amide group can form alternative interactions through the hydrogen bond donor amide $-NH_2$ group [such as to the C(4)O] that disrupts the interaction of the carbonyl with N(3)H.

Do the amino acid replacements affect other interactions that also contribute to this loss in binding? NMR data provide some insights here. First, some differences in the ^{15}N(5) chemical shifts were noted (Table 2), yet there are no apparent direct interactions, such as hydrogen bonding, between the loop in this region and N(5) of the flavin in the oxidized state (*10*−*12*). Amino acid replacements at Gly57, which were specifically designed to alter interactions at the N(5) position, result in only small losses in the binding free energy of FMN_{OX} (but cause large changes in the reduced states) (*28*), and no significant changes in hydrogen bonding interactions at N(3)H based on the X-ray crystal structures (*12*) and the similarities of the N(3)H temperature coefficients of these mutants to wild type (Table 3). So, it is unlikely that the changes in the ^{15}N(5) in the Glu59 mutants contribute to the losses in binding, but may be signaling small localized change(s) in its environment. Second, the chemical shift values for N(10) in the oxidized state for all of the Glu59 mutants have shifted upfield somewhat compared to that for the wild type (Table 2), perhaps reflecting changes in interactions at C(4)O. For these reasons, the observed loss of binding free energy for the Glu59 mutants was primarily attributed to the alterations in the N(3)H and possibly the C(4)O interactions, or from eq 2, $\Delta\Delta G_{obs}^{(ox)} = \Delta\Delta G_{N(3)H/[C(4)O]}^{(ox)}$. Thus, the N(3)H interaction appears to contribute at most about 2 kcal/mol to the binding energy in the oxidized state.

(B) The Semiquinone State. The hydrogen bonding interaction between the backbone carbonyl group of Gly57 and N(5)H of FMN plays a critical role in the stabilization of the neutral semiquinone species (*12*, *28*). However, the anchoring effect of Glu59 also contributes to this stabilization through its effect on the N(5)H interaction (*14*, this work) (Table 2). Any attempt to estimate the magnitude of the change in the N(3)H interaction induced by the substitutions at Glu59 must correct for this effect. This was challenging, but we have done so here by taking advantage of results obtained previously from amino acid replacements for Gly57 (*28*). The temperature coefficients for the N(3)H in the Gly57 mutants in the reduced state, while somewhat larger than for wild type, are all <-1 ppb/K, which is considered to represent a strong hydrogen bond (Table 3) (*38*). This is also evident in the X-ray crystal structures of this group of mutants, where no significant differences in this interaction are noted (*12*). In contrast, the temperature coefficients for the N(5)H in the Gly57 mutants are substantially larger than wild type and most often fall within or above the -3 to -5 ppb/K range that defines the limit for significant intramolecular hydrogen bonding for amide groups in reverse turns (*28*, *38*). In one case (G57T), the temperature coefficient is greater than -6.5 ppb/K, the point at which intramolecular hydrogen bonding is lost and interactions with solvent are formed (*38*). Therefore, the loss of binding free energy for FMN_{SQ} (and FMN_{HQ}) for the Gly57 mutants is attributed primarily to the changes in the hydrogen bonding strength at N(5)H (*28*).

The substantial increases in the temperature coefficients observed for both N(3)H and N(5)H in the Glu59 mutants in the fully reduced state (Table 3) signify that both interactions were weakened. Thus, $\Delta\Delta G_{obs}^{(sq)}$ values [and $\Delta\Delta G_{obs}^{(hq)}$] for the Glu59 mutants are a function of both $\Delta\Delta G_{N(3)H/[C(4)O]}$ and $\Delta\Delta G_{N(5)H}$. To correct for the contribution of $\Delta\Delta G_{N(5)H}^{(sq)}$, we took advantage of the linear correlation that was previously noted between the temperature coefficients for N(5)H in the reduced state (proportional to the strength of the hydrogen bond) and the binding free energy of the cofactor for the Gly57 mutants (Figure 3A) (*28*). The temperature coefficients for the N(5)H cannot be obtained in the semiquinone state due to significant paramagnetic broadening of the resonance cross-correlation peaks and must be derived from the fully reduced state. It has been argued that because of the structural similarity between the semiquinone and hydroquinone states, including the flavin interactions, the temperature coefficients provide reasonable comparisons of the relative N(5)H hydrogen bonding strength of different mutants in the semiquinone as well as the fully reduced state (*28*). Factoring in an estimated average experimental error of ± 0.2 kcal/mol in the $\Delta\Delta G_{obs}^{(sq)}$, the temperature coefficients for the Glu59 mutants were noticed to deviate significantly from the linear correlation for the Gly57 mutants. We reasoned that the additional loss of binding free energy beyond what was predicted by the change in the temperature coefficient based on this correlation can be attributed to the changes in the hydrogen bonding at N(3)H [with possibly some effect on the interaction at C(4)O]. Thus, for the Glu59 mutants, $\Delta\Delta G_{N(3)H/[C(4)O]}^{(sq)} = \Delta\Delta G_{obs}^{(sq)} - \Delta\Delta G_{N(5)H}^{(sq)}$ where $\Delta\Delta G_{N(5)H}$ was derived from the linear regression data for the Gly57 mutants. For example, the actual N(5)H temperature coefficient for the E59A mutant

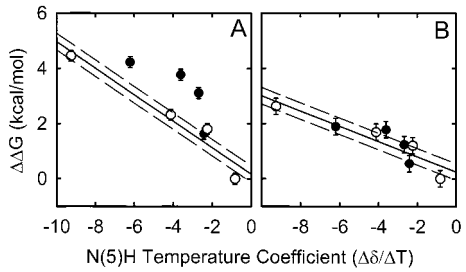

FIGURE 3: Correlation between the N(5)H temperature coefficient values and the changes in free energy associated with the semiquinone state (panel A) and the hydroquinone state (panel B) for the previously characterized Gly57 mutants (open circles) (from ref *28*) and Glu59 mutants (closed circles) from this study. The solid linear regression lines are fit to the Gly57 data only and represent the contribution to the changes in free energy associated primarily with the Gly57···N(5)H interaction. The dashed lines represent an estimated error of 0.2 kcal/mol for the semiquinone and 0.3 kcal/mol for the hydroquinone states, respectively, based on the average experimental error in the determination of the dissociation constants. The significantly larger losses in binding free energy over that predicted for altered N(5)H interactions based on the Gly57 correlation data for the semiquinone state (panel A) but not for the hydroquinone state (panel B) were attributed to the disruption of the N(3)H/[C(4)O] interaction(s) in the semiquinone complex in these mutants as discussed in the text.

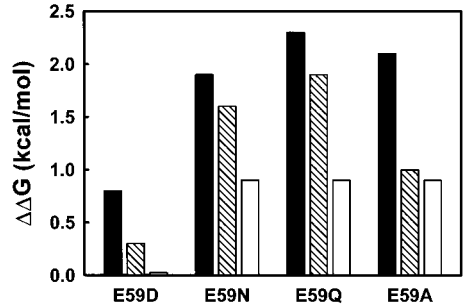

FIGURE 4: Calculated changes in the free energy of binding attributable to the disruption in the N(3)H interaction in the E59D, E59N, E59Q, and E59A flavodoxin mutants in the oxidized (closed bar), semiquinone (shaded bar), and hydroquinone (open bar) redox states. Notice that the E59D mutant was predicted to have little net loss of binding free energy in the fully reduced state.

predicts a loss of binding energy of about 3.2 kcal/mol based on the G57X correlation. However, the experimentally determined loss was 4.2 kcal/mol, suggesting to us that the disruption of the N(3)H/[C(4)O] interaction in this mutant contributes about 1 kcal/mol to the loss of binding.

According to this analysis, the loss in binding free energy attributable to changes in the N(3)H/[C(4)O] interaction ranged from 0.3 to 1.9 kcal/mol among this group of mutants and contributes from approximately 20−50% to the total observed loss in free energy. Just as for the oxidized complex, the Glu59 to aspartate substitution results in the smallest increase. The elimination of the hydrogen bonding between N(3)H and the protein in the E59A mutant resulted in a loss of binding energy of ∼1 kcal/mol. The amide substitutions once again appeared to destabilize the semiquinone state the most, perhaps due to the alternative hydrogen bonding interactions as was discussed earlier. Thus, based primarily on the E59A data, the N(3)H interaction appears to contribute about 1 kcal/mol to the binding energy in the semiquinone state.

(C) The Hydroquinone State. A similar correlation between the temperature coefficient for N(5)H and the $\Delta\Delta G_{obs}^{(hq)}$ was observed for the hydroquinone state (Figure 3B). However, unlike for the semiquinone, the coefficients for each of the Glu59 substitutions did not deviate significantly beyond the estimated error range of ±0.3 kcal/mol from the linear correlation observed for this oxidation state in Gly57 mutants. This observations suggests that the amino acid replacements do not cause any significant additional destabilization of the FMN hydroquinone beyond what can be attributed primarily to alterations in the N(5)H interaction. This phenomenon was the most apparent for the E59D mutant for which the shortened side chain should alter the N(3)H interaction without significantly affecting the electrostatic environment. The absence of any apparent loss of stability attributable to the N(3)H interaction contrasts with the loss of about 0.8 kcal/mol in binding energy for the oxidized cofactor in this mutant. Does this mean that the N(3)H interaction is inherently very weak in the hydroquinone complex? Perhaps, but it is also possible that a longer hydrogen bonding interaction with N(3)H can accommodate the shorter aspartyl side chain. Ab initio calculations have suggested that a longer hydrogen bond is preferred in the reduced state of FMN (*4*). On the other hand, the temperature coefficient for N(3)H in the aspartate mutant was 6-fold greater than for wild type, which does seem to reflect a weaker interaction in this mutant.

The nonacidic replacements tell a slightly different story. These replacements also eliminate a negative charge in the vicinity of the cofactor. It is known that the electrostatic interactions between the anionic FMN hydroquinone and nearby charged residues affect the midpoint potential, especially for the sq/hq couple (*20*, *43*). This effect must be accounted for. Based on the average experimental change observed upon the replacement of acidic residues in the vicinity of the FMN in the flavodoxin (*20*, *44*) and the distance between the side chain carboxylate of Glu59 and the N(1) of the FMN (where the charge is largely localized), the neutralization of Glu59 was calculated to increase $E_{sq/hq}$ by 35−40 mV due to electrostatic effects alone. This represents an increase in the stability of FMN_{HQ} [i.e., $\Delta\Delta G_{obs}^{(hq)}$] of ∼0.9 kcal/mol. The fact that no increase in stability over that contributed by the weaker N(5)H interaction was observed in those mutants (see Figure 3B) suggests that this increase in stability may be offset by a similar loss of stability due to the disruption of the N(3)H hydrogen bonding interaction for the hydroquinone in the E59Q, E59N, and E59A mutants (i.e., $\Delta\Delta G_{N(3)H/[C(4)O]}^{(hq)}$ increases by ∼0.9 kcal/mol). Taken together, the results suggest that the N(3)H interaction contributes at most about 0.9 kcal/mol to the binding energy in the fully reduced state and, based on E59D, may actually contribute very little to the stabilization of this redox state.

The N(3)H Hydrogen Bond Interaction Primarily Stabilizes the Oxidized State and Contributes Only Moderately in Establishing the Midpoint Potentials of the Flavodoxin. Having corrected for the loss of binding energy due primarily to changes in the N(5)H interaction in response to each substitution in each redox state, the changes attributed to alterations in the N(3)H [and possibly C(4)O] interactions (i.e., $\Delta\Delta G_{N(3)H/[C(4)O]}^{(ox,\ sq,\ or\ hq)}$) are depicted in Figure 4. A definite trend was noted. The alteration of the N(3)H interaction consistently affected the stability of the oxidized

state the most and the fully reduced state the least in this group of mutants. This was particularly evident for the most conservative E59D mutant. The weaker hydrogen bonding interaction in the reduced states quite plausibly stems from the declining donor characteristics of the N(3)H as the electron density of the cofactor increases as it becomes reduced.

These differential effects are expected to alter the reduction potentials of the cofactor. For the E59A mutant, which presumably completely disrupts the N(3)H hydrogen bonding interaction between FMN and the protein, a difference of ~1.2 kcal/mol was noted in the change in the binding free energy in comparing the oxidized state to the fully reduced state (Figure 4). The midpoint potential of the bound FMN is directly proportional to the free energy difference between the two relevant oxidation states; thus, the change in the two-electron reduction potential, $\Delta E_{ox/hq}$, is proportional to $\Delta\Delta G_{ox/hq}$ or $\Delta G_{hq} - \Delta G_{ox}$. For E59A, the two-electron reduction potential was calculated to increase by up to ~25 mV as the result of the disruption in the N(3)H interaction. Similar increases were noted for the amide substitutions. Interestingly, these substitutions seem to have their largest effect on the sq/hq couple, whereas complete removal of the side chain through the Glu59 to alanine substitution primarily affects the ox/sq couple (Figure 4). The changes observed for the Glu59 to aspartate substitution, which was expected to only weaken the interaction, fall in between. We attribute these differences to a high degree of sensitivity of the stability of the semiquinone to minor alterations in the N(5)H interactions observed previously. As a result, a somewhat variable pattern for the effects on the one-electron reduction potentials for each couple was noted. However, the increases were no greater than ~45 mV for either couple. Thus, our results suggest that the N(3)H interaction in this flavodoxin contributes only modestly to overall modulation of the midpoint potentials of the FMN cofactor. This conclusion is generally consistent with previous ab initio calculations and small molecule studies (*3*, *4*). Although the magnitude of the N(3)H contribution was concluded to be somewhat smaller than observed in the model studies of Cuello and co-workers, there is agreement that the oxidized state of the flavin is preferentially stabilized by this interaction (*4*). Finally, the results of this study once again highlight the dominant influence of the N(5)H interaction in the stabilization of the reduced states, particularly the neutral semiquinone. Relatively minor alterations introduced at position-59, which does not make a direct contact with this heteroatom, appear to exert their major influence at this position.

Conclusion. Glutamate 59 plays a major role in the redox properties of the *C. beijerinckii* flavodoxin. The carboxylate group of this residue serves a unique function in the flavodoxin family, serving as a dual hydrogen bond acceptor with N(3)H and the amide backbone of Trp95. Disruption of the "bridging" role of this residue results in the semiquinone state becoming differentially destabilized relative to the other two redox states. NMR studies have shown that the Gly57···N(5)H hydrogen bonding interaction has been weakened by these substitutions, which is responsible for the loss of semiquinone stability. When the interactions at Gly57 are corrected out, one is left with the changes in stability for each redox state mainly affecting the interaction at N(3)H. It was observed that this interaction was most important in the stability of the oxidized state, followed by the semiquinone state, and finally the fully reduced state. This interaction provides roughly a 20−25 mV contribution to the lowering of the midpoint potential for each redox couple. Therefore, this residue primarily affects the redox properties of the *C. beijerinckii* flavodoxin through the anchoring of the FMN to the 50's loop, promoting the formation of proper flavin−protein contacts, particularly at N(5)H, that serve to stabilize the semiquinone rather than through effects transmitted directly via the hydrogen bonding interaction with the N(3)H itself.

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