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[†]

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ABSTRACT: In the *Clostridium beijerinckii* flavodoxin, the reduction of the flavin mononucleotide (FMN) cofactor is accompanied by a local conformation change in which the Gly57−Asp58 peptide bond "flips" from primarily the unusual cis O-down conformation in the oxidized state to the trans O-up conformation 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 [Ludwig, M. L., Pattridge, K. A., Metzger, A. L., Dixon, M. M., Eren, M., Feng, Y., and Swenson, R. P. (1997) *Biochemistry 36,* 1259−1280]. This interaction is thought to contribute to the relative stabilization of the flavin semiquinone and may be at least partially responsible for the substantial separation of the midpoint potentials of the two one-electron reduction steps. Through a series of amino acid substitutions, the above cited study demonstrated the critical role of the often conserved glycine residue in this process. However, it has not been directly established experimentally as to whether these substitutions brought about the changes in the midpoint potentials by altering the strength of this hydrogen-bonding interaction as proposed. In this study, the relative strengths of the FMN N(5)H···O57 hydrogen bond in wild type and the G57A, G57N, and G57T mutants were evaluated by measuring the temperature dependency of the chemical shift for the proton on N(5) of the fully reduced cofactor by $^{1}H-^{15}N$ HSQC nuclear magnetic resonance spectroscopy. Based on the established correlation between the temperature coefficient of amide protons and the strength of hydrogen bonding in small peptides, the apparent strength of the N(5)H···O57 interaction was found to depend on the properties of the side chain at position 57. The glycine residue found in the wild-type flavodoxin appears to provide the strongest interaction while the β-branched side chain in the G57T mutant provides the weakest. A good correlation was noted between the temperature coefficients of N(5)H and the one-electron reduction potential for the ox/sq couple as well as the binding free energy of the FMN semiquinone in this group of mutants. These results provide more direct quantitative evidence that support the previous hypothesis that this conformation change and the associated formation of the hydrogen bonding interaction with N(5)H of the reduced FMN represent an important means of stabilizing the neutral semiquinone and in modulating the oxidation−reduction potentials of the flavin cofactor in this and perhaps other flavodoxins.

The relationship between the oxidation−reduction properties of the cofactor and its interactions with the apoprotein in various electron transfer proteins has been an important area of study in biochemistry and biophysics. Flavodoxins have served as an excellent system in which to investigate this issue in flavoproteins. The flavodoxin represents an important class of electron transfer proteins that utilizes a single, noncovalently bound flavin mononucleotide (FMN)[1] cofactor as its only redox center. These proteins are typified by the thermodynamic stabilization of the blue neutral form of the flavin semiquinone radical while the anionic hydroquinone state is substantially destabilized (for recent reviews, see references *1* and *2*). These phenomena, in large part, are responsible for establishing the very low one-electron reduction potential for the semiquinone−hydroquinone (sq/hq) couple in these proteins. 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 (*3, 4*). The structural basis of these shifts has been the subject of numerous studies.

The early X-ray crystal structures of flavodoxins from *Desulfovibrio vulgaris* and *C. beijerinckii* revealed a con-

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[1] Abbreviations: FMN, flavin mononucleotide; HSQC, heteronuclear single-quantum coherence; ox/sq, oxidized−semiquinone couple; sq/hq, semiquinone−hydroquinone couple.

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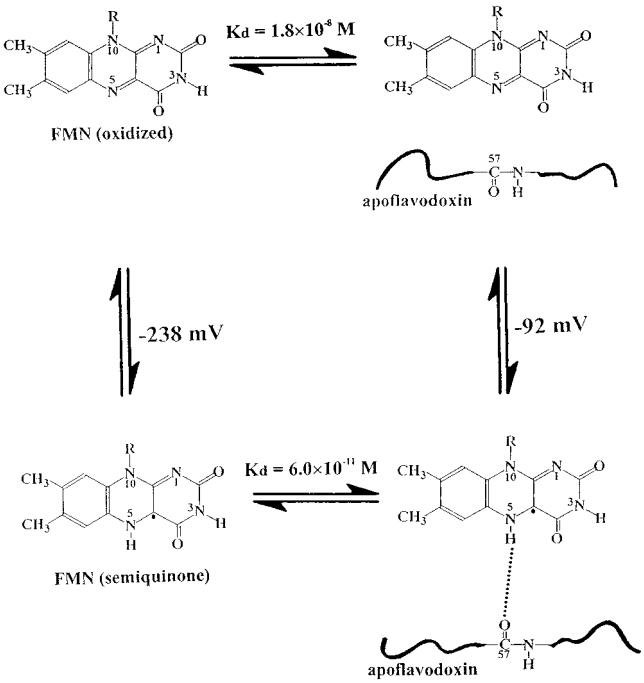
Scheme 1: Depiction of the Linked Equilibria Relating the Midpoint Potentials, Dissociation Constants, and Conformational Changes for *C. beijerinckii* Flavodoxin As Adapted from Ludwig and Luschinsky (*2*)[a]



[a] The value of the midpoint potential for the ox/sq couple for free FMN used here was taken from Draper and Ingraham (*3*). Druhan and Swenson (*20*) determined the K_d value for the oxidized state of the recombinant wild-type flavodoxin. The K_d 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.

formational difference between the oxidized and reduced states of these proteins (*2, 5*−*9*). 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 1) (*2, 8*). A similar rotation involving Gly61 and Asn58 is evident in the *D. vulgaris* and *Anacystis nidulans* flavodoxins, respectively (*7, 10*). This conformation change and the formation of the new hydrogen bond are generally thought to be important for the stabilization of the neutral flavin semiquinone and in establishing the one-electron reduction potentials of the FMN in the flavodoxin (*2*).

The unique structural properties of the frequently conserved glycine residue involved in this conformational change are critical for the unique structure of this loop (*9*). 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 (*9, 11, 12*). 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 (*9*). 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 the ox/sq couple than for the wild type (*13*). 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 mV, a value significantly lower than for the above mentioned flavodoxins (*14*). Interestingly, the N58G mutation in this flavodoxin results in an *increase* in the reduction potential (*2*). 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 (*9, 12*). 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 conformational equilibrium of this loop (*9*). Evaluation of the geometries of flavin−protein interactions and the pK_a 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 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 (*15*−*19*). In the study reported here, a similar approach was used. The $^1H-^{15}N$ 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 (*2*, *8*, *9*).

EXPERIMENTAL PROCEDURES

Materials. Deuterium oxide (D_2O) was obtained from Fluka Chemicals. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate and $^{15}NH_4Cl$ (99%) were purchased from Cambridge Isotope Laboratories. Sodium dithionite was from Aldrich Chemical Co. Isopropylthio-β-D-galactoside (IPTG) was acquired from Gibco BPL. 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 $^{15}NH_4Cl$ as the sole nitrogen source. Each liter of minimal medium contained 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of $^{15}NH_4Cl$, 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 (*20*) and dialyzed against 10 mM NH_4HCO_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_4HCO_3 using a step gradient from 50 to 250 mM NH_4HCO_3, repeatedly lyophilized to remove the NH_4HCO_3, and redissolved in H_2O.

Reconstitution of the Apoflavodoxin with ^{15}N-Labeled FMN. Recombinant flavodoxins were purified by established procedures (*9*, *21*, *22*). Apoflavodoxin was prepared by the procedure of Wassink and Mayhew (*23*), 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_2O. 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 (*24*) with a recycle time of 2.00 s. ^{15}N chemical shifts[2] are referenced to an external standard of 1.5 M $^{15}NH_4NO_3$ in 1 M HNO_3 (21.6 ppm relative to liquid ammonia set by convention to 0.0 ppm) (*25*). 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% D_2O. 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 (*26*) were acquired on a Bruker DMX-600 spectrometer using the water flip-back versions (*27*) with ^{1}H and ^{15}N sweep widths of 8802 and 7298 Hz, respectively. Sixteen scans were recorded for each of 128 t_1 values. Quadrature in t_1 was accomplished by using the TPPI−States method (*28*). GARP decoupling (*29*) was used during acquisition to decouple the ^{15}N. Temperature calibration was performed using methanol and ethylene glycol (*30*). 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 (K_d) 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 (*4*). 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 K_d values from two separate titrations are reported.

RESULTS AND DISCUSSION

^{15}N NMR of FMN, both Free and Bound to Wild-Type and Mutant C. beijerinckii Apoflavodoxin in the Oxidized and Fully Reduced States. X-ray crystallographic and ^{15}N NMR analyses of *C. beijerinckii* flavodoxin indicate that N(1) and N(3) atoms, but not N(5), have hydrogen-bonding contacts with apoflavodoxin in the oxidized state (*2*, *31*, *32*). 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 (*2*). These changes were evaluated by both one- and two-dimensional ^{1}H−^{15}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}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}N chemical shifts of free FMN and recombinant wild-type *C. beijerinckii* flavodoxin in the oxidized state determined in this work (Table 1) agree well with those reported by Vervoort et al. (*32*) except we were unable to

[2] The ^{15}N chemical shift values are not corrected for magnetic susceptibility effects.

Table 1: ^{15}N Chemical Shifts of Free and Flavodoxin-Bound Oxidized FMN at pH 7, 300 K[d]

	^{15}N chemical shifts (ppm) in								
atom	FMN[a]	FMN[b]	TARF[b]	r*C. b.*[a]	*C. MP*[b]	G57A[a]	G57N[a]	G57T[a]	D58P[a]
N(1)	190.3	190.8	199.9	183.7	184.5	185.3	185.5	187.2	185.7
N(3)	−[c]	160.5	159.8	160.3	161.1	−[c]	160.4	161.9	161.2
N(5)	333.6	334.7	344.3	350.9	351.5	343.1	343.6	344.0	342.8
N(10)	163.1	164.6	150.2	163.9	164.8	163.4	163.3	164.3	163.8

[a] This work. [b] From Vervoort et al. (ref *32*). [c] Chemical shift values could not be assigned from this work. [d] Abbreviations: r*C. b.*, recombinant *Clostridium beijerinckii* flavodoxin; *C. MP*, *Clostridium MP (beijerinckii)* flavodoxin; TARF, tetraacetylriboflavin in $CHCl_3$.

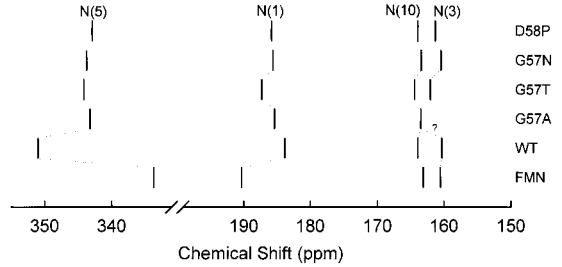


FIGURE 1: Correlation diagram of ^{15}N chemical shifts of free FMN and FMN bound to wild-type and mutant apoproteins from *C. beijerinckii* flavodoxin in the oxidized state. The value for N(3) of FMN was not assigned in this work, but was taken from Vervoort et al. (*32*). A resonance peak for the FMN 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.

make an assignment for N(3) of the unbound FMN. As this is a uniformly labeled ^{15}N derivative and this signal clearly appears in our protein samples, it may be that the N(3) 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 (*33*−*35*). The ^{15}N chemical shift of the N(5) atom of wild-type flavodoxin is shifted downfield compared to that of tetracetylriboflavin (TARF) 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 (*32*). 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 TARF 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 (*9*). 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 (*32*). The N(1) chemical shifts of the mutants appear further downfield than for the wild-type protein (Figure 1 and Table 1), which might imply that the hydrogen-bonding interactions of N(1) are weaker in these proteins.

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 (data not shown). 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 (all nitrogens of the fully reduced FMN) are relatively insensitive to hydrogen-bonding interactions (*32*), making it difficult to accurately evaluate hydrogen bond strengths based on the chemical shift data alone.

Temperature-Dependent ^{1}H−*^{15}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 (*15*, *36*, *37*). 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 non-hydrogen-bonded species (*38*, *39*). The most studied temperature coefficients in peptides and proteins are the backbone amide protons (*15*−*19*). 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 (*16*). Backbone amide protons having temperature coefficient magnitudes less than from −3 to −5 ppb/K are considered to be involved in intramolecular hydrogen bonding (*18*). By comparison of the average value for D/H fractionation factors for backbone amides, the strength of intramolecular hydrogen

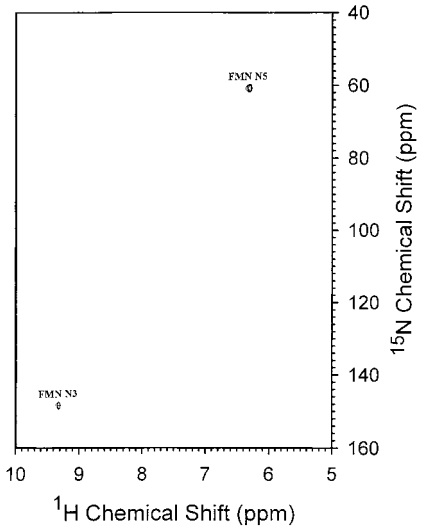


FIGURE 2: Contour plot of the $^{1}H-^{15}N$ HSQC nuclear magnetic resonance spectrum of the G57A mutant flavodoxin reconstituted with ^{15}N-labeled FMN in the fully reduced state at 296 K. The FMN $^{15}N^{3}-^{1}H^{N3}$ and $^{15}N^{5}-^{1}H^{N5}$ cross-correlation peaks are labeled 'FMN N3' and 'FMN N5'. No other cross-correlation peaks, such as for N(1), were observed.

bonding within the staphylococcal nuclease is thought to be stronger than protein−solvent and solvent−solvent interactions (*37*). 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 (*2, 9*). 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 (*40, 41*). However, the X-ray crystal structures of this flavodoxin indicate that the semiquinone and hydroquinone states are nearly identical in structure (*2, 8, 9*). 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 (*9, 42*).

The two-dimensional $^{1}H-^{15}N$ HSQC spectrum of fully reduced G57A mutant flavodoxin at pH 7.0, 296 K shown in Figure 2 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 remains unprotonated at this pH (*32*). The persistent HSQC signal for the protons on

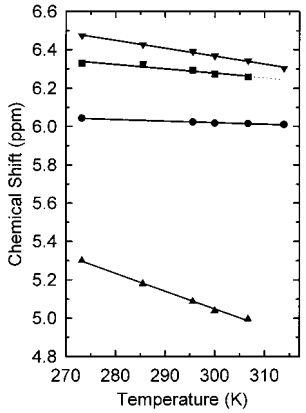


FIGURE 3: Temperature dependence of the chemical shift for the N(5)H of the bound ^{15}N-labeled FMN for the wild-type (circles) and the G57A (squares), G57N (inverted triangles), and G57T (triangles) mutant flavodoxins. A value at 314 K was not obtained for the G57A mutant because of reoxidation of 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 (see Discussion).

Table 2: Comparison of the Temperature Coefficients for the Wild-Type and Mutant *Clostridium beijerinckii* Flavodoxins

	temperature coefficient[a] ($\Delta\delta/\Delta T$) (ppb/K)	
flavodoxin	N(5)H	N(3)H
wild type	−0.8237 (1)	−0.07184 (0.087)
G57A	−2.244 (2.72)	−0.827 (1.00)
G57N	−4.129 (5.01)	−0.512 (0.62)
G57T	−9.264 (11.25)	−0.681 (0.83)

[a] Numbers in parentheses are the temperature coefficients relative to that of the N(5)H of the wild-type flavodoxin.

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 (*2, 8, 9*).

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 3. 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 ($\Delta\delta/\Delta T$) 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 (*15*), suggesting that a rather strong hydrogen bond is formed with the N(5)H atom in this protein as has been proposed previously (*2, 31, 32*).

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 (*16*). 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 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 (*9*). 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 (*2, 8*). 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 (*9, 12*).

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 (*9, 11, 12*). 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 4A. 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 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.

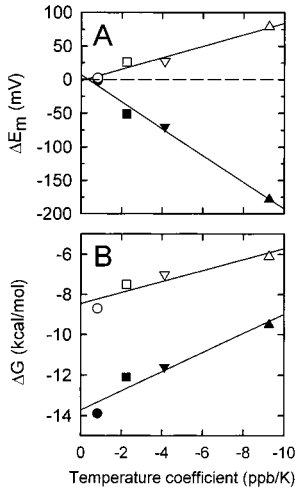


FIGURE 4: Correlation between the changes in midpoint potential (relative to wild type) for the ox/sq couple (closed symbols) and the sq/hq couple (open symbols) (panel A) and the free energy of binding of the FMN semiquinone (closed symbols) and FMN hydroquinone (open symbols) (panel B) with the temperature 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. (*9*).

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 (K_d) for each redox state of the FMN for each mutant. The K_d 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. The K_d values for the FMN semiquinone and hydroquinone for each apoprotein were calculated from the linked equilibria described by Dubourdieu et al. (*43*). The K_d 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 K_d values about 20-, 40-, 50-, and 1800-fold greater than for wild type, respectively. The K_d 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 K_d values also correlates to the temperature coefficient for N(5)H of the reduced FMN (Figure 4B), with the free energy values

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*

			K_d (μM)							
flavodoxin	$E_{ox/sq}$ (mV)	$E_{sq/hq}$ (mV)	ox[d]	sq[e]	hq[e]	ΔG(ox)[f]	ΔG(sq)[f]	ΔG(hq)[f]	$\Delta\Delta G$(sq−ox)	$\Delta\Delta G$(hq−sq)
wild type	−92[a]	−399[a]	0.018[c]	0.000060	0.42	−10.6	−13.9	−8.7	−3.4	5.3
G57A	−143[a]	−373[a]	0.052	0.0013	3.2	−9.9	−12.1	−7.5	−2.2	4.6
D58P	−155[a]	−360[a]	0.059	0.0023	3.6	−9.9	−11.8	−7.4	−1.9	4.3
G57N	−162[a]	−372[a]	0.059	0.0030	7.4	−9.9	−11.6	−7.0	−1.8	4.6
G57T	−270[a]	−320[a]	0.032	0.11	36	−10.2	−9.5	−6.1	0.7	3.4
FMN	−238[b]	−172[b]								

[a] From Ludwig et al. (ref *9*). [b] From Draper and Ingraham (ref *3*). [c] From Druhan and Swenson (ref *20*). [d] Determined directly by spectrofluorometric titration of FMN with apoflavodoxin. [e] The K_d values for the semiquinone and the hydroquinone states of the FMN were calculated from the observed shifts in the midpoint potentials of the cofactor upon binding to flavodoxin (see ref *43* for details). [f] Free energy change of binding, ΔG (kcal/mol) $= -RT \ln(1/K_d)$.

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 4B may be understated relative to that of the hydroquinone. It is also important to note 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 (*44*, *45*).

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 the FMN semiquinone through hydrogen bonding was an early postulate of the pioneering studies by Ludwig and co-workers on the flavodoxin from *C. beijerinckii* (*2*, *8*). 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 (*9*). 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 the C_β of position $i+1$ and the NH of position $i+2$, glycine is strongly preferred in position $i+1$ in such turns (*46*). 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. (*9*), 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 ($\Delta G_c^{ox/sq}$) and of the molecular interactions, including hydrogen bonding, between the FMN cofactor in each oxidation state and the protein (ΔG_i). These two aspects are obviously interrelated, but have been formally separated to assist in the functional interpretation of this phenomenon (*9*). 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 $\Delta G_c^{ox/sq}$ induced by the presence of the side chain rather than in ΔG_i (*9*). 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 (*9*). It was therefore suggested that the ΔG_c 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 ΔG_i 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 (*9*). Also, the apparent pK_a value for N(5)H appears to be lowered by approximately 2 pH units in this mutant (*9*). However, it was noted that the pK_a is still higher than for free FMN. It is important to note that this study cannot truly distinguish between a conformational effect (changing ΔG_c) 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.

In conclusion, the results of this study provide direct experimental evidence that strongly supports several important long-standing hypotheses of Ludwig and co-workers (*2*, *8*). 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 (*2*, *8*, *9*). This study, in conjunction with our previous characterization of this group of flavodoxin mutants (*9*, *11*, *12*), 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.

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