Role of Glutamate-59 Hydrogen Bonded to N(3)H of the Flavin Mononucleotide Cofactor in the Modulation of the Redox Potentials of the *Clostridium beijerinckii* Flavodoxin. Glutamate-59 Is Not Responsible for the pH Dependency but Contributes to the Stabilization of the Flavin Semiquinone†

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ABSTRACT: The midpoint potentials for both redox couples of the noncovalently bound flavin mononucleotide (FMN) cofactor in the flavodoxin are known to be pH dependent. While the pH dependency for the oxidized−semiquinone (ox/sq) couple is consistent with the formation of the blue neutral form of the flavin semiquinone, that of the semiquinone−hydroquinone (sq/hq) couple is more enigmatic. The apparent pK_a of 6.7 for this couple in the flavodoxin from *Clostridium beijerinckii* has been attributed to the ionization of the FMN_{HQ}; however, nuclear magnetic resonance data strongly suggest the FMN_{HQ} remains anionic over the entire pH range testable. As an alternative explanation, a specific glutamate residue (Glu59 in this flavodoxin), which is hydrogen-bonded to N(3)H of the FMN, has been postulated to be the primary redox-linked proton acceptor responsible for the pH effect in some flavodoxins. This model was directly tested in this study by permanently neutralizing Glu59 by its replacement with glutamine. This conservative substitution resulted in an increase of 86 mV (at pH 7) in midpoint potential of the sq/hq couple; however, the pH dependency of this couple was not altered. Thus, the redox-linked protonation of Glu59 clearly cannot be responsible for this effect as proposed. The pH dependency of the ox/sq couple was also similar to wild type, but the midpoint potential has decreased by 65 mV (pH 7). The K_d values for the oxidized, semiquinone, and hydroquinone complexes increased by 43-, 590-, and 20-fold, respectively, relative to the wild type. Thus, the Glu59 to glutamine substitution substantially effects the stability of the semiquinone but, on a relative basis, slightly favors the formation of the hydroquinone. On the basis of $^1H-^{15}N$ HSQC nuclear magnetic resonance spectroscopic studies, the increased temperature coefficients for the protons on N(3) and N(5) of the reduced FMN in E59Q suggest that the hydrogen-bonding interactions at these positions are significantly weakened in this mutant. The increase for N(5)H correlates with the reduced stability of the FMN_{SQ} and the more negative midpoint potential for the ox/sq couple. On the basis of the X-ray structure, an "anchoring" role is proposed for the side chain carboxylate of Glu59 that stabilizes the structure of the 50's loop in such a way so as to promote the crucial hydrogen-bonding interaction that stabilizes the flavin semiquinone, contributing to the low potential of this flavodoxin.

Flavodoxins are small, acidic flavoproteins that contain a single redox center of a noncovalently bound flavin mononucleotide cofactor (FMN[1]) and serve as low-potential electron-transfer proteins (for a recent review, see ref *1*). The flavodoxin has proved to be an excellent model system in which to investigate the role of flavin−protein interactions in the control the oxidation−reduction properties of FMN. The flavodoxin protein profoundly perturbs the oxidation−reduction potentials of the bound FMN cofactor, substantially lowering the midpoint potential of the semiquinone−hydroquinone couple ($E_{sq/hq}$) from −172 mV for unbound FMN to less than −400 mV at pH 7 in the flavodoxin (*2*). Depending on the flavodoxin, the midpoint potential for the oxidized−semiquinone couple ($E_{ox/sq}$) is generally increased (*2*). These perturbations of the one-electron reduction potentials of the bound FMN cofactor result in the separation of the two redox couples so that the flavodoxin might function as a low-potential one-electron carrier in vivo (*3*).

The midpoint potentials for both redox couples of the flavodoxin are pH dependent (refs *2*, *4*, *5*, and this work). The one-electron reduction potential for the ox/sq couple decreases by about 60 mV/pH unit increase, a relationship consistent with the uptake of one electron and one proton during the formation of the blue neutral FMN_{SQ} species over the entire pH range testable. Other than the substantial apparent increase in the pK_a of the SQ to values >12, this is not an unusual characteristic. However, the pH dependency of $E_{sq/hq}$ is somewhat more enigmatic. Above approximately

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

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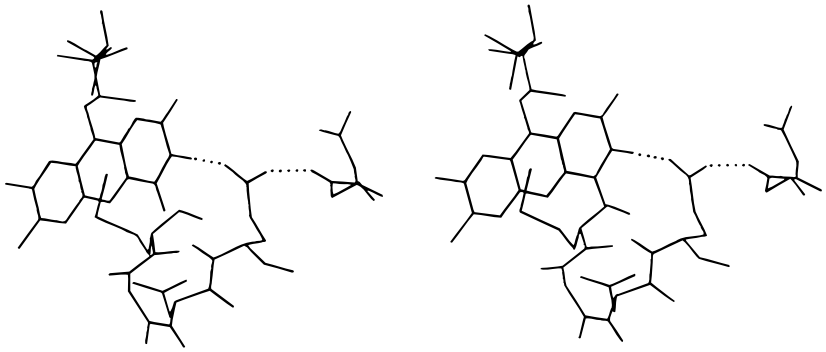

FIGURE 1: Stereodiagram of the structure of a portion of the FMN binding site in the *Clostridium beijerinckii* flavodoxin in the oxidized state featuring the peptide loop involving residues 56−59 as well as the backbone atoms of residues 94 and 95 to the right. This view focuses on the apparent role of side chain carboxylate of Glu59 to serve as a hydrogen bond acceptor for both N(3)H of FMN and the amide hydrogen of Trp95 (*28*, *50*).

pH 7, the midpoint potential is independent of pH. Below pH 7, the $E_{sq/hq}$ value gradually becomes less negative. Because the reported pK_a of the unbound FMN_{HQ} is 6.7 (*6*), it was initially assumed that the observed pH dependency was due to the ionization of FMN_{HQ} itself. However, ^{15}N NMR studies of reduced flavodoxins seems to unambiguously establish that FMN_{HQ} remains in the anionic form throughout the testable pH range, suggesting that the pK_a has been shifted to values <4 (*7*−*11*). This shift has been attributed to the prevention of the protonation of the N(1) atom by steric blockage by the adjacent polypeptide backbone (*2*). These observations require an alternative explanation for the pH dependency of $E_{sq/hq}$. Citing pH-dependent changes to the UV/visible spectrum of the fully reduced flavodoxins form *Desulfovibrio vulgaris*, *Megasphaera elsdenii*, and *Anabaena* PCC 7119, Yalloway et al. (*12*) once again argue for the ionization of the HQ, perhaps at C(4)O rather than N(1). However, the evidence is rather indirect and other causes for the spectral changes such as changes in the polarization, conformation, or hydrogen-bonding interactions of the FMN_{HQ} could not be excluded by these authors. Thus, the molecular basis for the pH dependency of the sq/hq couple still remains somewhat of a mystery. Another reasonable suggestion is that the pH effect is the consequence of the protonation of one or more charged groups on the protein rather than the bound FMN cofactor itself (*11*, *13*). But, what is the identity of this (these) proton acceptor group(s)?

It has long been suspected that unfavorable electrostatic interactions between negatively charged groups and the anionic FMN_{HQ} may contribute to the unusually low potential for the sq/hq couple in the flavodoxin and could be responsible for this pH dependency (*2*, *13*, *14*). One or more of the acidic amino acid residues on the surface of the protein and/or the 5′-phosphate of the FMN cofactor itself have been implicated. Recent studies have, in fact, directly demonstrated the importance of seven acidic residues clustered with 13 Å of N(1) of the FMN in establishing $E_{sq/hq}$ in the flavodoxin from *D. vulgaris* (*15*−*17*). The involvement of individual acidic amino acids in other flavodoxins has been investigated (*11*, *18*). The influence of the 5′-phosphate group appears to be rather modest, however, contributing about as much to the destabilization of the FMN_{HQ} as the average surface acidic amino acid surrounding the cofactor (*19*).

The X-ray crystal structures of the homologous flavodoxins from *Clostridium beijerinckii* (MP) and *M. elsdenii* provided a particularly intriguing possibility in that an acidic residue in each flavodoxin, Glu59 and Glu60, respectively, is located immediately adjacent to the pyrimidine ring of the cofactor. In fact, the side chain carboxylate groups appear to form a hydrogen bond with hydrogen atom on N(3) of the flavin as shown for the clostridial flavodoxin in Figure 1 (*20*). This observation lead to the quite logical and reasonable hypothesis that it is the redox-linked protonation of this glutamate residue that is responsible for the observed pH dependency (*11*, *13*). In this model, the formation of the negative charge on the anionic FMN_{HQ} during reduction of the flavodoxin next to the ionized and somewhat buried Glu59 (or Glu60) generates a destabilizing charge repulsion in the reduced flavodoxin which contributes to the lower $E_{sq/hq}$ at higher pH values. A consequence of this mechanism is the redox-linked increase the pK_a of this carboxylate group to the apparent pK_a observed from the pH dependency of the sq/hq couple of about 6.7 (or 5.8 in the *M. elsdenii* flavodoxin) (*4*, *11*). Therefore, the pH dependency observed for the sq/hq couple was proposed to be primarily due to the ionization state of the proximal Glu59 or Glu60 residue of *C. beijerinckii* or *M. elsdenii* flavodoxin, respectively, although it has been suggested that other acidic residues could contribute (*13*).

Another ambiguity in this model arises from the observation that the direct interaction of the side chain carboxylate with the isoalloxazine ring in these flavodoxins is not found in all flavodoxins. More typically, hydrogen-bonding interactions occur between N(3)H and main chain acceptor groups such as the nonionizable carbonyl group of the peptide bond, e.g., the flavodoxins from *D. vulgaris* (*21*) and *Anacystis nidulans* (*22*). However, as pointed out by Ludwig et al. (*11*), the general features of this model could apply to other flavodoxins if conserved acidic residues near the FMN ring assume the same role as Glu59 or Glu60.

Thus, important questions remain unanswered. Is the redox-linked protonation/ deprotonation of a specific acid residue near N(3)H of the FMN solely or primarily responsible for the pH dependency of $E_{sq/hq}$ in certain flavodoxins as previously proposed? If so, what are the structural features responsible for the similar pH dependencies observed in flavodoxins lacking such an interaction? If not, what is the nature of the proton acceptor? Does the hydrogen bonding of an ionizable group directly to N(3)H influence other properties of the FMN cofactor? In this investigation, the specific role(s) of Glu59 in the *C. beijerinckii* flavodoxin in

establishing the redox properties of FMN and pH dependency of $E_{sq/hq}$ are investigated. The electrostatic and putative redox-linked ionization effects of this residue were studied by the conservative substitution with glutamine.

MATERIALS AND METHODS

Materials. Anthraquinone-2, 6-disulfonate, safranine T, and D_2O were purchased from Fluka Chemicals. Safranine T was recrystallized from ethanol before use. All other chemicals were of analytical reagent grade.

Oligonucleotide-Directed Mutagenesis. The Glu59 to glutamine substitution was introduced by cassette mutagenesis of the chemically synthesized artificial gene encoding the flavodoxin from *Clostridium beijerinckii* (MP) which was previously constructed and cloned into the *p*KK223-3 expression vector (designated *p*KKFlasy) (*23*). The mutagenic cassette containing the GAA (Glu) to CAG (Gln) codon replacement was ligated into the *Nco*I and *Xho*I restriction sites within the coding region of the plasmid *p*KKFlasy using standard protocols (*24*). This mutation also destroys an existing *Sau*3A restriction site, which was useful for screening. The mutation and the sequence integrity of the entire coding region were confirmed by dideoxy termination DNA sequencing using the Sequenase protocol (*25*).

Purification of the E59Q Flavodoxin Mutant. Transformed *Escherichia coli* XL-1 cells were cultured in NZY medium containing 100 μg/mL ampicillin at 37 °C with agitation. After 24 h, ampicillin was added to a final concentration of 200 μg/mL along with isopropylthiol-β-D-galactoside (IPTG) to a concentration of 100 μg/mL. The flavodoxin holoprotein was purified as described previously (*26*) except that prior to chromatography the flavodoxin was efficiently precipitated along with nucleic acids by polyethylenimine (final concentration, 0.1% w/v). Flavodoxin was resolubilized from the precipitate in 50 mM Tris-HCl, pH 7.3 (at 25 °C), containing 0.5 M NaCl and centrifuged at 18 000 *g* for 30 min. The supernatant fraction was diluted with the Tris buffer to adjust the NaCl to a final concentration of 0.1 M and subjected to anion exchange chromatography on DEAE-cellulose as previously described (*26*). Gel filtration chromatography was not performed due to the slight tendency for the FMN to dissociate under these conditions in this mutant. SDS−PAGE analysis indicated that the flavodoxin preparation was >90% pure.

Determination of Far-UV Circular Dichroism Spectra. Circular dichroism spectra were measured from 195 to 250 nm using a Aviv 62DS spectropolarimeter. All measurements were made using 2-mm quartz cuvettes. Baseline measurements of 50 mM phosphate buffer pH 7 were subtracted from the experimental data. The far-UV CD spectrum for each sample is the average of 20 scans.

Midpoint Potential Determinations. The midpoint potentials for both the ox/sq and sq/hq couples were determined at 25 °C by anaerobic reduction by sodium dithionite of mixtures of flavodoxin (generally 30 μM) and redox indicator dyes as described previously (*16*, *27*, *28*). Protein and dye solutions were made anaerobic by purging the solution with several cycles of a partial vacuum and argon gas in a closed titration cuvette. Ultraviolet−visible absorption spectra were recorded on a Hewlett-Packard model 8452A diode array spectrophotometer during the reductive titration with sodium dithionite. Anthraquinone-2,6-disulfonate ($E_{m,7} = -185$ mV; *29*) was used as an indicator dye for establishing $E_{ox/sq}$ while safranine T ($E_{m,7} = -280$ mV; *29*) was used in establishing $E_{sq/hq}$. Both were used over the entire pH range of 5.5−8.5 used in this study. The midpoint potentials for each dye at each of the pH values were calculated on the basis of the equations described in Clark (*29*). The following buffer systems were used for the reductive titrations: 50 mM sodium acetate buffer (pH 5.5−6.0); 50 mM Na_2PO_4 (pH 6.5−7.5); 15 mM sodium pyrophosphate (pH 8.0−8.5). The ionic strength was maintained at 50−120 mM throughout this study. Concentrations of the various oxidized and reduced species in equilibrium in solution were determined using multicomponent linear regression analysis of the absorbance spectrum of the equilibrium mixture using standard reference absorbance spectra for each component in its various oxidation states generated under identical conditions. Midpoint potentials for both couples were calculated by fitting the plot of the FMN_{SQ} concentration versus the system potential that were determined at each point in the titration.

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 by spectrophotometric titration. Apoflavodoxin was prepared as described previously (*30*). A 5 μM solution of FMN in 50 mM sodium phosphate buffer (pH 7.0) was titrated by the addition of a substoichiometric amount of a 200 μM stock solution of freshly prepared apoflavodoxin. Upon the sample reaching equilibrium, the visible absorbance spectrum was recorded on a Hewlett-Packard model 8452A photodiode array spectrophotometer at 25 °C and corrected for dilution. The titration was continued until no further absorbance changes were observed. Subtracting the starting FMN spectrum from each spectrum recorded during the titration generated difference spectra. The fraction of bound FMN was determined from the absorbance changes at 498 and 442 nm in the difference spectrum and plotted versus the amount of apoflavodoxin added to the FMN solution. K_d values for FMN_{OX} were obtained by computer fitting the titration data to the quadratic equation describing a single-site binding isotherm. The K_d values for FMN_{SQ} and FMN_{HQ}, which cannot be determined directly, were calculated from the differences in the midpoint potentials for each couple of the FMN bound to the flavodoxin versus free FMN according to the relevant thermodynamic cycles involved (*31*). The midpoint potentials for FMN in solution of Draper and Ingraham (*32*) were used.

^{15}N and $^1H-^{15}N$ HSQC NMR Spectroscopy. Greater than 95% enriched ^{15}N-FMN was prepared by extraction of the cofactor from recombinant flavodoxin partially purified from *E. coli* cultured in minimal media containing $^{15}NH_4Cl$ as the only nitrogen source (*33*, *34*). The wild-type and E59Q flavodoxins reconstituted with ^{15}N-FMN were prepared by dissolving lyophilized samples of apoflavodoxin in buffered solutions of ^{15}N-FMN. Apoflavodoxin was prepared as described above but was dissolved in a small volume of 10 mM sodium phosphate buffer, pH 7, and dialyzed before lyophylization. Samples for the ^{15}N NMR analyses contained approximately 1 mM oxidized E59Q in 50 mM sodium phosphate buffer (pH 7.0) containing 10% D_2O. The ^{15}N NMR spectra were recorded at 298 K on a Bruker AM-500

MHz spectrometer operating at 500.13 MHz. The ^{15}N chemical shifts were referenced to an external standard of 1.5M $^{15}NH_4NO_3$ in 1 M HNO_3. Samples for the $^1H-^{15}N$ HSQC analyses contained approximately 1 mM oxidized or fully reduced flavodoxin in 50 mM sodium phosphate buffer (pH 7.0) containing 10% D_2O as described previously (*33*, *34*). Flavodoxin was reduced by adding an appropriate amount of freshly prepared sodium dithionite solution to an anaerobic solution of the flavodoxin. Anaerobic conditions were obtained by flushing the solution with argon for 30 min, and the tube was sealed with a rubber septum. The $^1H-^{15}N$ HSQC NMR spectra (*35*) were recorded on a Bruker DMX 600 MHz spectrometer. Sample temperatures were calibrated using methanol and ethylene glycol (*36*). Proton chemical shifts were referenced to an internal standard of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) set at 0.0 ppm.

RESULTS

This study was initially designed to specifically address the question of whether the redox-linked ionization of glutamate-59 in the *Clostridium beijerinckii* flavodoxin is primarily responsible for the pH dependency of $E_{sq/hq}$ as previously proposed (*11*, *13*). Because the side chain of this residue hydrogen bonds to N(3)H of the FMN, the functional role of this residue in the stabilization/destabilization of specific redox states of the FMN and the modulation the midpoint potentials of the cofactor were also investigated. Glutamate-59 was replaced by glutamine by the site-directed mutagenesis. This conservative isosteric substitution eliminates the ionizable group at position 59 while possibly retaining a hydrogen-bonding interaction between N(3)H of FMN and the oxygen atom of the neutral γ-amide group of the glutamine residue, although alternative interaction modes are conceivable (see below).

Physical and Spectral Properties of the E59Q Mutant. The E59Q mutant was heterologously expressed in *E. coli* and isolated in good yields as the holoprotein just as for the wild-type recombinant protein (*23*). The polypeptide migrates just as the wild type during SDS−PAGE electrophoresis. The far-ultraviolet circular dichroism spectrum of the purified holoprotein exhibits a strong band of negative elipticity in the 195−250 nm range, consistent with an α/β-type protein, and was very similar to the wild-type protein (data not shown). Small differences that were noted are very similar to those noted between the *Megasphera elsdenii* flavodoxin and its apoprotein reconstituted with FMN, which were attributed to the presence of small amounts of apoprotein remaining in the reconstituted sample (*37*). The addition of a large excess of FMN to our E59Q holoprotein preparation did not significantly change the CD spectrum, suggesting that our E59Q preparations may contain small amounts of irreversibly denatured apoprotein. However, the CD data clearly indicate that this conservative substitution is not substantially altering the structure of the protein.

The UV/visible absorbance spectrum of the oxidized form of this mutant resembles the wild-type spectra showing absorbance maxima at 370 and 454 nm (Figure 2). The extinction coefficient at 454 nm of 10.4 ± 0.2 mM^{-1} cm^{-1} is comparable to wild type, although the A_{454}:A_{370} ratio for E59Q is somewhat smaller. Just as for the wild type, the blue neutral form of the FMN_{SQ} was observed to accumulate over the entire pH range tested during anaerobic reduction with sodium dithionite (Figure 2). An extinction coefficient for the SQ at 586 nm of 4.4 ± 0.1 mM^{-1} cm^{-1} (pH 7.0) is similar to the wild type. The spectrum of the HQ species was also similar to that of the wild type, displaying a noticeable shoulder at about 350 nm which has been interpreted as evidence for the anionic form of the HQ (Figure 2) (*38*). Thus, this amino acid substitution has not appreciably affected the spectral properties of the bound FMN.

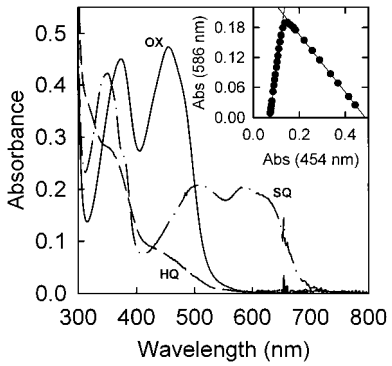

FIGURE 2: Standard absorbance spectra of the E59Q flavodoxin mutant during reduction in 50 mM sodium phosphate, pH 7.0, at 25 °C: *Solid line*, fully oxidized; *dot−dashed line*, semiquinone; *dashed line*, hydroquinone. *Inset*: Plot of absorbance changes at 586 nm versus changes at 454 nm associated with the formation and subsequent reduction of the blue neutral semiquinone during reduction with sodium dithionite. The intersection of the linear extrapolation of the initial and final portions of the reductive titration represents the maximal absorbance at 454 and 586 nm for the fully formed flavin semiquinone. All experimental points were corrected for dilution.

Table 1: One-Electron Reduction Potentials and Dissociation Constants for Each Oxidation State for the FMN Cofactor in the *C. beijerinckii* Wild-Type and E59Q Flavodoxins at pH 7.0

			K_d (μM)		
flavodoxin	$E_{ox/sq}$ (mV)	$E_{sq/hq}$ (mV)	OX[d]	SQ[e]	HQ[e]
wild type	−92[a]	−399[a]	0.018[c]	0.000 060	0.42
E59Q	−159	−313	0.77	0.035	8.3
FMN	−238[b]	−172[b]			

[a] From ref *28*. [b] From ref *32*. [c] From ref *39*. [d] Determined by spectrophotometric titration of FMN with apoflavodoxin. [e] Calculated from the observed shifts in the midpoint potentials of the FMN upon binding to apoflavodoxin according to Dubourdieu et al. (*31*).

Midpoint Potentials of the Ox/Sq and Sq/Hq Couples and Their pH Dependencies. The $E_{ox/sq}$ at pH 7 was determined to be 67 mV more negative than that for the wild type (−159 mV versus −92 mV) (Table 1). $E_{ox/sq}$ exhibits a −60 mV/pH unit dependency (Figure 3) that is characteristic of a one electron reduction of the FMN accompanied by the uptake of one proton and is consistent with the generation of the blue neutral flavin radical over the entire pH range studied. The $E_{sq/hq}$ values for E59Q were significantly less negative than for the wild-type flavodoxin at all pH values tested. At pH 7, $E_{sq/hq}$ for E59Q was −313 mV as compared to −399 mV for the wild type (Table 1 and Figure 3). This increase in $E_{sq/hq}$ of 86 mV was approximately maintained over the entire pH range of the study and is significantly larger than the average 15 mV increase observed upon neutralization of a single general acidic residue within 13 Å of N(1) of the

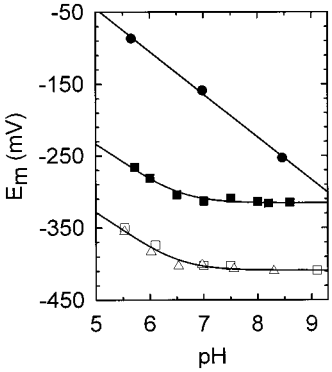

FIGURE 3: pH dependencies of the midpoint potentials for the ox/sq and sq/hq couple for the E59Q mutant (●, ■) and wild-type *C. beijerinckii* (□, △) flavodoxin at 25 °C. Data for the sq/hq couple for the wild type were replotted from Mayhew (*4*) (□) and Druhan and Swenson (*39*) (△) for comparison to E59Q. The $E_{ox/sq}$ data for E59Q (●) were fit to a line with a slope of −61 mV/pH unit. The $E_{sq/hq}$ data were fit to a hypothetical model involving the ionization of a single group with a pK_a of 6.4 for both the wild-type (□, △) and E59Q (■) data sets.

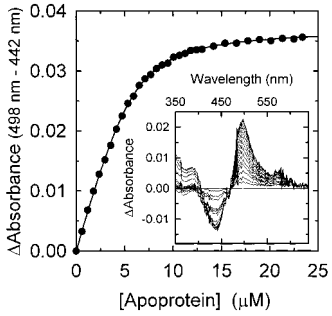

FIGURE 4: Determination of the dissociation constant for the FMN_{OX} complex with E59Q apoflavodoxin. A 5 μM solution of FMN in 1 mL of 50 mM sodium phosphate, pH 7.0, was titrated with substoichiometric amounts of freshly prepared apoflavodoxin at 25 °C. After correction for dilution, the relative concentrations of bound FMN_{OX} were determined from the absorbance changes at 498 and 442 nm associated with binding (*inset*) and plotted versus the final apoprotein concentration in the cuvette. A K_d value of 0.77 ± 0.08 μM was determined for the E59Q mutant by the best fit of the data to a single-site binding isotherm (*solid line*).

FMN in the *D. vulgaris* flavodoxin (*15*). Although shifted to less negative values, the $E_{sq/hq}$ for E59Q exhibited a pH dependency very similar to the wild type over the pH range of 5.7−8.5, remaining largely independent of pH above pH 7.0 and gradually increasing at lower pH values (Figure 3). An apparent pK_a value of 6.4 obtained from curve fitting to a single ionizable species is very comparable to the value obtained for the wild type both in this study and previous work (*4*).

Dissociation Constants for the Oxidized, Semiquinone, and Hydroquinone Species. The midpoint potential for either couple of the flavin in a flavoprotein is fixed by the relative binding energy of relevant redox forms of the cofactor to the protein. The dissociation constant for the oxidized holoflavodoxin complex, as determined by monitoring the spectral changes occurring upon addition of freshly prepared apoprotein to a solution of FMN of known concentration (Figure 4), has a value of 0.77 ± 0.08 μM for E59Q (Table 1). This value is about 43-fold larger than the K_d value of 0.018 μM obtained under identical conditions for the recombinant wild-type flavodoxin (*39*). Since the free energy is path-independent, the dissociation constants for the two reduced states of the FMN cofactor can be determined from the shift in midpoint potentials of the two couples relative to those of the unbound cofactor (*31*). Values of 0.035 and 8.3 μM were calculated for the dissociation constants for the SQ and HQ complexes, respectively (Table 2). Both are significantly higher than those of the recombinant wild-type flavodoxin determined under identical conditions (0.06 and 420 nM for the SQ and HQ complex, respectively) (*39*). Therefore, while all three oxidation states are less stable in E59Q, the binding of FMN_{SQ} was disproportionally affected, with the apparent K_d increasing 590-fold over wild type versus 43- and 20-fold for the FMN_{OX} and FMN_{HQ}, respectively. Because of the relatively high K_d values displayed by E59Q, particularly for the FMN_{HQ}, one-electron reduction potentials were reevaluated in the presence of at least a 5-fold excess of apoprotein to minimize any effects of the dissociation of the FMN during the titration. No significant differences in experimental values of the midpoint potentials of either couple were noted, however.

Table 2: Gibbs Free Energy Change Associated with Binding of Each Oxidation State of the FMN Cofactor to the *C. beijerinckii* Wild-Type and E59Q Flavodoxins at pH 7.0[a]

flavodoxin	ΔG_{OX}	ΔG_{SQ}	ΔG_{HQ}	$\Delta G_{ox/sq}$	$\Delta G_{sq/hq}$
FMN				5.5	4.0
wild type	−10.6	−13.9	−8.7	2.1	9.2
E59Q	−8.3	−10.2	−6.9	3.7	7.2
$\Delta\Delta G_{E59Q-WT}$[b]	+2.3	+3.7	+1.8	+1.6	−2.0

[a] Units in kcal/mol. [b] Difference in free energy changes between the wild type and E59Q.

Table 3: ^{15}N Chemical Shifts of Oxidized FMN, Free and Bound to Flavodoxin at pH 7 and 300 K[a]

	^{15}N chemical shift (ppm)				
atom	FMN[b]	TARF[b]	rC.b.[c]	C.MP[b]	E59Q
N(1)	190.8	199.9	183.7	184.5	185.7
N(3)	160.5	159.8	160.3	161.1	156.9
N(5)	334.7	344.3	350.9	351.5	336.7
N(10)	164.6	150.2	163.9	164.8	159.3

[a] Abbreviations: *r*C.b., recombinant *C. beijerinckii* flavodoxin; C.MP, *Clostridium MP* (*beijerinckii*) flavodoxin; TARF, tetraacetylriboflavin. [b] From Vervoort et al. (*9*). [c] From Chang and Swenson (*34*).

^{15}N NMR of FMN Bound to E59Q C. beijerinckii Flavodoxin. The reduced binding affinities reflected by the K_d values displayed by E59Q, particularly for the SQ, prompted a more comprehensive evaluation of the FMN environment and hydrogen-bonding interactions using ^{15}N NMR spectroscopy. For these studies, the cofactor in E59Q was replaced with uniformly enriched ^{15}N-FMN for ease in monitoring the chemical shifts of each of the nitrogen atoms of the flavin (*34*). Four well-resolved resonance peaks were observed for the FMN in E59Q in the oxidized state (data not shown). The ^{15}N-chemical shifts for each flavin nitrogen atom could be assigned (Table 3). The ^{15}N-chemical shifts of the pyridine-like N(1) and N(5) atoms in the FMN_{OX} are typically sensitive to hydrogen bonding, shifting upfield when involved in such interactions (*40*). The ^{15}N-chemical shift of the N(1) atom in E59Q is shifted upfield compared to that of FMN in aqueous solvent but is somewhat farther downfield relative to the wild type (Table 3), consistent with hydrogen bond formation between the protein and N(1) as in the wild type. This interaction may be somewhat weaker

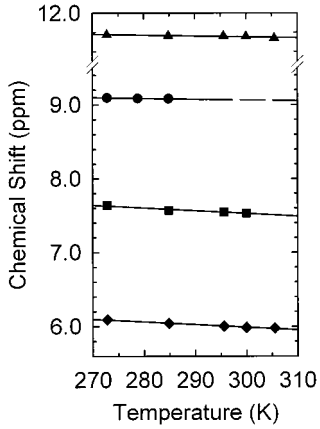

FIGURE 5: Temperature dependence of the chemical shift for N(3)H of the bound ^{15}N-enriched FMN for oxidized wild type (*triangles*), oxidized E59Q (*circles*), and reduced E59Q (*squares*) and the temperature dependence of the chemical shift for N(5)H of the ^{15}N-labeled FMN for E59Q (*diamonds*). The N(3)H cross correlation peak for the E59Q oxidized mutant disappeared at 285 K, suggesting loss of hydrogen bonding and/or rapid exchange with solvent.

in E59Q, however. The ^{15}N-chemical shift for the N(5) atom in E59Q is shifted upfield relative to tetraacetylriboflavin (TARF) in chloroform and to the wild-type flavodoxin (Table 3). This upfield shift is greater than those observed for the Gly57 mutants observed by Chang and Swenson (*34*). This upfield shift could be attributed to a hydrogen bond formation with N(5) of FMN with the mutant apoflavodoxin. The chemical shift of N(5) in the mutant flavodoxin, however, is much closer to the FMN in an aqueous environment (Table 3), suggesting that the glutamine substitution has led to an increase in polarity near N(5) and/or has altered the loop region to allow a hydrogen bonding interaction to occur (likely with solvent).

The ^{15}N-chemical shifts for the pyrrole-like N(3) and N(10) atoms of FMN_{OX}, while typically less sensitive to hydrogen bonding interactions than the pyridine-type nitrogen atoms, are expected to shift slightly downfield on hydrogen bonding (*40*), as is observed for both the native and recombinant wild-type flavodoxins (Table 3). However, the ^{15}N(3) chemical shift in E59Q is shifted upfield relative to FMN in aqueous and in apolar solutions and to the wild-type flavodoxin. This upfield shift could be attributed to the absence of a strong hydrogen-bonding interaction at N(3) of FMN with the E59Q apoflavodoxin. This apparent loss in hydrogen bonding was subsequently confirmed during the temperature-dependent ^{1}H$-^{15}$N HSQC studies of the oxidized mutant flavodoxin in which the cross correlation peak of the N(3)H signal was lost at temperatures greater than 285 K (Figure 5) (see also the next section). This change is perhaps not surprising given the direct interaction between the side chain carboxylate of Glu59 and the N(3)H evident in the crystal structure of the wild-type flavodoxin. Other possible structural effects will be considered in the Discussion.

The ^{15}N-chemical shift of the N(10) atom in oxidized E59Q is shifted upfield from FMN in aqueous medium but is shifted downfield from TARF in chloroform (Table 3). A downfield shift in the N(10) resonance in the transfer of FMN from apolar to polar solvents (and wild-type flavodoxin) has been previously explained as an increase in sp^2 hybridization at this atom as a result of stabilizing the isoalloxazine polarization with hydrogen-bonding interactions at C(2)O and C(4)O (*9*). Data obtained by ^{13}C NMR and Raman spectroscopy 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) (*41*, *42*). In the *C. beijerinckii* flavodoxin, the hydrogen bond between the backbone NH of Glu59 and C(4)O (2) may contribute to the large downfield shift of the N(10) resonance. If this is the case, small local structural perturbations in the 50's loop in E59Q that affect this interaction may be responsible for the upfield shift of the N(10) resonance in this mutant. The significance of this observation will be discussed later.

Table 4: Temperature Coefficients for the *C. beijerinckii* Wild-Type and E59Q Flavodoxins

	temp coeff ($\Delta\delta/\Delta T$) (ppb/K)	
proton	wild type	E59Q
N(3)H (oxidized FMN)	−0.889	−1.000
N(3)H (reduced FMN)	−0.0718[a]	−3.756
N(5)H (reduced FMN)	−0.824[a]	−3.615

[a] From Chang and Swenson (*34*).

^{1}H$-^{15}$N HSQC Temperature Dependency of the Hydrogen-Bonding Interactions of the E59Q Flavodoxin with FMN in both Oxidized and Reduced States. The temperature dependency 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 (*43*−*45*). The weakening of hydrogen-bonding interactions at elevated temperature alters the distribution between the hydrogen bonded and nonbonded species, resulting in a temperature-dependent upfield shift of the resonance of the proton involved (*46*, *47*). 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 (*48*, *49*).

This technique has been applied recently to the *C. beijerinckii* flavodoxin in an effort to evaluate the effects of amino acid replacements for Gly57 on the hydrogen-bonding interactions with N(5)H and N(3)H of the reduced FMN (*34*). As in that study, the cofactor in E59Q was replaced with uniformly enriched (>95%) ^{15}N FMN and subjected to ^{1}H$-^{15}$N HSQC NMR spectroscopy so that the cross correlation peaks for the protons on the FMN nitrogen atoms can be easily identified. In the oxidized flavodoxins, only a single HSQC cross correlation peak was observed, which was assigned to the proton on the N(3) of the FMN (data not shown). At 280 K, the chemical shift of this proton in oxidized E59Q was 9.081 versus 11.765 for wild type (Figure 5). This upfield shift of ∼2.7 ppm for the N(3)H signal can be attributed to the elimination of the negative charge that resided at Glu59, allowing the proton on N(3) to become more shielded.

The temperature coefficient for the N(3)H in the oxidized state of E59Q was similar to that of wild type (Figure 5 and Table 4). The N(3)H cross correlation peak for E59Q disappeared above 285 K but was fully reversible in that the signal reappeared when the temperature was lowered below this temperature. These observations suggest that the hydrogen-bonding interaction with N(3)H in E59Q has been weakened in the oxidized state relative to the wild type. The

N(3)H temperature coefficient of E59Q in the reduced state was 52-fold greater than that for the wild-type flavodoxin and from 5- to 10-fold greater than the Gly57 mutants in the fully reduced state (*34*) (Figure 5 and Table 4). Again, these results are indicative of a weaker hydrogen-bonding interaction in E59Q and are consistent with the ^{15}N-chemical shift data discussed above.

It was not surprising that the glutamine substitution affects interactions at N(3)H in both the oxidized and reduced states. However, the NMR data suggests that the hydrogen-bonding interactions at N(5)H were also altered in this mutant (Figure 5). The temperature coefficient for N(5)H in E59Q was 4.4-fold greater than that of the wild type (Table 4). This increase is similar in magnitude to the those observed for the Gly57 mutants that were intentionally designed to primarily affect the interactions at N(5)H (*34*). These results were somewhat surprising because the side chain of Glu59 does not interact with this part of the cofactor. These results, like the chemical shift data, again imply that the effects of the glutamine substitution are propagated in some manner down the loop to N(5)H of the reduced cofactor.

DISCUSSION

Redox-Linked Ionization of Glu59 Is Not Responsible for the pH Dependency of the Midpoint Potential of the Sq/Hq Couple of the C. beijerinckii Flavodoxin. The side chain carboxylate group of Glu59 in the *C. beijerinckii* flavodoxin seems to serve as a hydrogen bond acceptor for N(3)H of the cofactor (Figure 1) (*20*, *28*, *50*, *51*). Because of this proximity, the redox-linked ionization of this carboxylate group was quite logically proposed to represent the group responsible for the pH dependency of the midpoint potential of sq/hq couple in this flavodoxin (*11*, *13*). In this study, Glu59 was conservatively replaced by a glutamine residue by site-directed mutagenesis, thus, permanently and irreversibly neutralizing this residue. If the redox-linked ionization of Glu59 is *by itself* responsible as proposed, then E59Q should not display such a pH dependency. However, as can clearly be seen in Figure 3, the $E_{sq/hq}$ for E59Q exhibited a pH dependency very similar to the wild type over the pH range of 5.7−8.5, although the curve is shifted to less negative values throughout. Just as for the wild type, the data can be fit to a single ionizable group exhibiting an apparent pK_a value of 6.4, a value similar to those obtained for the wild type in this study and in previous work (*4*). Thus, the removal of the proposed redox-linked carboxylate group of Glu59 of *C. beijerinckii* flavodoxin by substitution with glutamine did not eliminate, or even substantially alter, the pH dependency of the sq/hq couple. Thus, it seems quite implausible that the pH dependency of the reduction potentials of the sq/hq couple is due solely to ionization of the Glu59 residue of the *C. beijerinckii* flavodoxin (and, by analogy, the Glu60 in the *M. elsdenii* flavodoxin). It is likely that the pH effect cannot be attributed to any *individual* redox-linked amino acid residue in the flavodoxin, for that matter. It should be noted, however, that the role of the redox-linked ionization of amino acid residue(s) adjacent the flavin cofactor has been shown to be a valid means of modulating the redox potentials in the flavodoxin. A direct linkage between $E_{sq/hq}$ and the pK_a of a histidine residue introduced into position 98 of the *D. vulgaris* flavodoxin by site-directed mutagenesis has been well documented (*26*, *33*). But, basic residues are not present near the cofactor in wild-type flavodoxins.

What, then, is responsible for the enigmatic pH dependency of $E_{sq/hq}$ in flavodoxins? As stated previously, Yalloway et al. (*12*) have once again raised the possibility that the FMN_{HQ} in several flavodoxins may actually be undergoing ionization. This conclusion is based on the interpretation of changes in the UV/visible spectra of the fully reduced flavodoxins in response to changes in pH. While the *C. beijerinckii* flavodoxin was not the subject of that study, the highly homologous protein from *M. elsdenii* was included. Their conclusions appear to directly conflict with previous NMR measurements that indicate that N(1) of the HQ remains unprotonated due to steric constraints introduced by the peptide backbone (*7*−*11*). However, the authors did raise the interesting possibility that the cofactor may undergo some type of ionization, perhaps involving C(4)O (12). The results provided by this present study are consistent with and, therefore, do not exclude this possibility in the *C. beijerinckii* flavodoxin. It should be noted again that the experimental evidence provided by Yalloway et al. (*12*) is rather indirect and changes in the polarization, conformation, and/or hydrogen bonding of the FMN_{HQ} could not be entirely excluded. Such events, if they occur, need to be confirmed by other independent and more direct measurements. Thus, the pH dependency of $E_{sq/hq}$ still requires further clarification.

A credible explanation has been suggested previously on the basis of experimental support provided by this laboratory. It is possible that one or more acidic residues not directly in contact with the flavin isoalloxazine ring may be responsible for the pH dependency as well as contribute to the low potential for the sq/hq couple when ionized (*11*, *13*). Flavodoxins are notable in the clustering of acidic residues near the cofactor (*2*, *50*, *52*). The general negative electrostatic environment generated by at least seven of the acidic residues clustered within 13 Å of N(1) of the FMN is known to contribute substantially to the negative $E_{sq/hq}$ in flavodoxins (*15*, *17*). In those studies, $E_{sq/hq}$ was seen to increase proportionally (by an average of about 15 mV per residue) with the number of acid residues irreversibly neutralized by conservative acid-to-amide amino acid substitutions. While each individual contribution is rather modest, the cumulative effect of the entire cluster contributes over one-third of the shift in $E_{sq/hq}$ of the FMN when bound to this protein (*15*). This negative shift in midpoint potential appears to be the consequence of the destabilization of the anionic FMN_{HQ} through the unfavorable negative electrostatic field provided by these residues (*15*, *53*). Individual acid-to-amide substitutions do not appear to eliminate the pH dependency of $E_{sq/hq}$ that is observed for the wild-type flavodoxin (refs *18* and *53* and unpublished results). However, a definite trend was noted in that as the total number of acidic residues neutralized by the amide substitution was increased, $E_{sq/hq}$ becomes progressively less pH dependent (*53*). When six of the seven acidic residues were neutralized, almost no pH dependency was observed above pH 5.5. It seems quite plausible, then, that the pH dependency of the sq/hq couple could be the direct result of the *collective* effect of the protonation/deprotonation of several acidic residues surrounding the FMN binding site as proposed recently by Swenson and Zhou (*53*). It should also be noted that the *D. vulgaris* flavodoxin does not have the equivalent of Glu59, with hydrogen-bonding

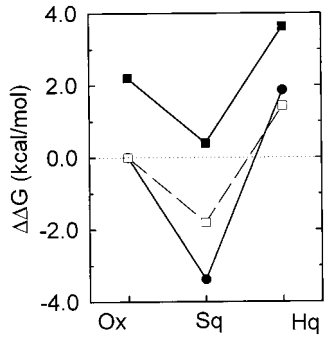

FIGURE 6: Free energy plots comparing the relative changes in the free energy of binding for the various oxidation states of the FMN cofactor by wild-type (*closed circles*) and the E59Q (*closed squares*) flavodoxins. The data points represent the changes in the free energies relative to the oxidized wild-type holoflavodoxin complex. The free energy plot depicted by the *open squares* and the *dashed lines* represent the free energies changes for the E59Q mutant relative to its own oxidized complex (see Discussion).

interactions with the N(3)H occurring with peptide backbone atoms (*21*). Because the pH dependency of the sq/hq couple is apparently a general phenomenon in the flavodoxin, a general mechanism such as the collective ionization scheme described could apply in all cases despite observed differences in the immediate FMN binding pocket.

Glutamate-59 Is Important in the Thermodynamic Stabilization of the FMN Semiquinone by Indirectly Contributing to the Strength of Hydrogen Bonding at N(5)H. An unexpected observation from this study was that although E59Q maintains pH dependencies similar to wild type, the midpoint potentials for both couples of the FMN cofactor are shifted by about 80 mV, each in opposite directions, by this rather conservative substitution. Because $E_{ox/sq}$ becomes more negative while $E_{sq/hq}$ increases, this substitution seems to primarily affect the stability of the FMN_{SQ}. Indeed, the K_d for the FMN_{SQ} was observed to increase much more than the other two oxidation states in response to the glutamine substitution (Table 2). These changes are more clearly represented by the associated Gibb's free energy diagram shown in Figure 6 (see also Table 2). The binding free energy for FMN_{OX} has increased by about 2.3 kcal/mol for E59Q. This loss in binding energy was confirmed by the ^{15}N NMR data. The upfield shifts observed for the $^{15}N(3)$ resonance and the increased temperature coefficients for the N(3)H from the $^{1}H-^{15}N$ HSQC experiments in both the oxidized and fully reduced states suggest that the hydrogen-bonding interactions at this location have been significantly weakened in E59Q. This effect is not difficult to rationalize given the direct hydrogen bonding of the carboxylate of Glu59 with the N(3)H. The substitution of this residue by glutamine residue is expected to introduce two important changes. The neutral amide group, if retained as an acceptor for N(3)H, should form a weaker hydrogen bond than the anionic carboxylate (*54*). In addition, the crystal structure of the wild-type *C. beijerinckii* flavodoxin suggests that the carboxylate group of Glu59 may serve as a "bridge" between the FMN and the adjacent peptide backbone, with each carboxylate oxygen atom serving as a hydrogen-bond acceptor for N(3)H of the FMN or the amide hydrogen of Trp95 of the adjacent peptide backbone (Figure 1) (*20*, *28*, *50*). Because the amide group of glutamine provides only one acceptor group, one or both of these interactions may be disrupted. Because this interaction is common to all three oxidation states (*11*, *51*), the stability of each should be affected. If the binding free energies are uniformly corrected for this loss of stability, a profile shown by the open symbols and *dashed* lines in Figure 6 is obtained. It becomes much more apparent that the SQ is destabilized to a greater extent by this substitution. Also, on this relative basis, the HQ complex is now slightly more stable than for the wild type. It should be emphasized that this adjustment assumes that the reduced binding is primarily the result of changes in hydrogen bonding and that the hydrogen-bonding strength remains uniform for all three oxidation states. This may not be the case given the changes in electronic distribution and charge of the FMN as it becomes reduced (*38*), so appropriate caution in the over-interpretation of this correction is advised.

What is the structural basis of the preferential destabilization of the SQ state of the flavin? Is this effect the direct consequence of charge neutralization or the result of alterations in hydrogen bonding at N(3)H? Or, does this substitution indirectly affect the stability of the SQ by altering other interactions within the 50's loop? It is quite likely that this destabilization is not the direct consequence of changes in the electrostatic environment of the FMN_{SQ}. Changes in $E_{ox/sq}$ have been observed upon similar acid-to-amide substitutions of several acidic residues clustered around the FMN binding site in the *D. vulgaris* flavodoxin (*15*). However, a correlation between the change in $E_{ox/sq}$ and either the location or the number of acidic residues neutralized was not apparent in that study.

The ^{15}N NMR chemical shift data and the $^{1}H-^{15}N$ HSQC temperature-dependency studies provide a more plausible explanation. The ^{15}N-chemical shift of the N(5) atom of the oxidized FMN in E59Q has shifted upfield relative to the wild-type flavodoxin, similar to that of FMN in aqueous solution, suggesting greater solvent accessibility and/or participation in hydrogen bonding (Table 3). Also, the temperature coefficient for N(5)H is 4.4-fold greater than that of the wild type (Table 4). This increase in N(5)H temperature coefficient is similar in magnitude to those for the Gly57 mutants that were specifically constructed to affect the interactions primarily at N(5)H (*34*). In that study, experimental evidence was provided that more conclusively establishes that that this hydrogen-bonding interaction is critical for the stabilization of SQ. The decreased strength of this hydrogen bond in the various G57 mutants as reflected by the temperature coefficients of the N(5)H correlates very well with decreases in the $E_{ox/sq}$ and to elevated K_d values for the SQ, suggesting that the N(5)H···O57 interaction primarily favors the stability of the blue-neutral SQ formation (*34*). That the temperature coefficient and $E_{ox/sq}$ for E59Q follow the same trend (Figure 7) suggests a similar mechanism of preferential destabilization of the SQ, namely, the weakening of this critical hydrogen-bonding interaction between N(5)H of the SQ and the backbone of the 50's loop. The correlation with $E_{sq/hq}$ was not evident, which may reflect that this substitution, unlike the Gly57 mutations, is affecting the stability of the HQ by other mechanisms such as electrostatic interactions.

Changes induced in the loop are also reflected in apparent alterations in the interactions at C(4)O, but the evidence is somewhat more indirect. Moonen and coauthors suggested that hydrogen-bonding interactions with C(4)O of FMN are

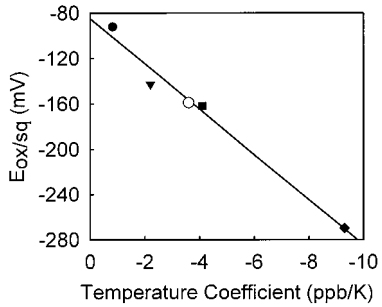

FIGURE 7: Correlation between the change in the midpoint potential for the ox/sq couple with the temperature coefficient of N(5)H of the FMN_{HQ} bound to the E59Q (*open circle*) mutant. For reference, the E59Q data are compared to data previously determined for the wild type (*closed circle*) and the G57A (*square*), G57N (*inverted triangle*), and G57T (*diamond*) mutants designed to disrupt the hydrogen-bonding interactions at N(5)H (*34*).

partly responsible for the downfield $^{15}N(10)$ chemical shift of FMN in polar environments (*41*). The $^{15}N(10)$ chemical shift in E59Q is shifted upfield relative to both FMN in aqueous solution and the wild-type flavodoxin. Crystal structures of the *C. beijerinckii* flavodoxin indicate that the backbone amide group of Glu59 forms a hydrogen bond with C(4)O of FMN (*2*). If the glutamine substitution is indirectly affecting the 50's loop structure in such a way as to weaken the hydrogen bond at C(4)O of the FMN, this carbonyl will be a less effective π-electron sink for the N(10) atom of the polarized oxidized flavin resulting in a relative upfield shift in $^{15}N(10)$. Thus, the NMR data suggest that the glutamine substitution not only weakens the hydrogen bonding interactions with the N(3)H, as might be expected, but also affects the environment of C(4)O and N(5)H of the reduced states of the FMN. This may come about by altering the structure of the 50's loop through the loss of the "anchoring" function provided by the dual hydrogen-bonding role of the Glu59 carboxylate group in this flavodoxin, indirectly affecting the interactions of the loop with C(4)O and N(5)H which appear directly affect the stability of the SQ,

These results provide more direct experimental evidence that clarifies the role of the Glu59 side chain in the flavodoxin from *C. beijerinckii*. The evidence provided (Figure 3) clearly demonstrate that the redox-linked ionization of Glu59 is not by itself responsible for the observed pH dependency of the sq/hq couple as has previously been proposed. This study does point to another important role for this residue, however. The unique dual hydrogen bond acceptor role of the side chain carboxylate bridging N(3)H of the FMN and NH of the adjacent peptide backbone appears to assist in anchoring the FMN to the loop and/or stabilizes the structure of the 50's loop in such a way so as to promote the critical hydrogen bonding interaction that stabilizes the SQ state. The structural configuration and conformational energetics of this loop have been shown to be very sensitive to small changes in the loop (*27*, *28*). For example, the substitution of alanine for Gly57 decreases the $E_{ox/sq}$ by 51 mV. On average, substitutions at positions 57 and 58 increase the free energy associated with the reduction of the FMN to the SQ by about 1.3 kcal/mol relative to the wild type (*28*), a value similar to that observed for E59Q (about 1.6 kcal/mol). Such similarities might suggest a role of Glu59 in maintaining or influencing the conformation of this loop in such a way so as to contribute to the crucial strong hydrogen bond at N(5)H that stabilizes the flavin semiquinone, contributing to the remarkably low potential of this class of flavoprotein.

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REFERENCES

1. Mayhew, S. G., and Tollin, G. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) Vol. III, pp 389−426, CRC Press, Boca Raton, FL.
2. Ludwig, M. L., and Luschinsky, C. L. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (F. Müller, Ed.) Vol. III, p 427−466, CRC Press, Boca Raton, FL.
3. Mayhew, S. G., and Ludwig, M. L. (1975) in *The Enzymes* (Boyer, P. D., Ed.) 3rd ed., Vol. 12, pp 57−118, Academic Press, New York.
4. Mayhew, S. G. (1971) *Biochim. Biophys. Acta 235*, 276−288.
5. Curley G. P., Carr, M. C., Mayhew, S. G., and Voordouw, G. (1991) *Eur. J. Biochem. 202*, 1091−1100.
6. van Schagen, C. G., and Müller, F. (1981) *Eur J. Biochem 120*, 33−39.
7. Franken, H. D., Rüterjans, H., and Müller, F. (1984) *Eur. J. Biochem. 202*, 1091−1100.
8. Vervoort, J., Müller, F., LeGall, J., Bacher, A., and Sedlmaier, H. (1985) *Eur. J. Biochem. 151*, 49−57.
9. Vervoort, J., Müller, F., Mayhew, S. G., van den Berg, W. A. M., Moonen, C. T. W., and Bacher, A. (1986) *Biochemistry 25*, 6789−6799.
10. Stockman, B. J., Westler, W. M., Mooberry, E. S., and Markley, J. L. (1988) *Biochemistry 27*, 136−147.
11. Ludwig, M. L., Schopfer, L. M., Metzger, A. L., Pattridge, K. A., and Massey, V. (1990) *Biochemistry 29*, 10364−10375.
12. Yalloway, G. N., Mayhew, S. G., Malthouse, J. P. G., Gallagher, M. E., and Curley, G. P. (1999) *Biochemistry 38*, 3753−3762.
13. Schopfer, L. M., Ludwig, M. L., and Massey, V. (1991) in *Flavins and Flavoproteins 1990* (Curti, B., Ronchi, S., and Zanetti, G., Eds.) pp 399−404, Walter de Gruyter, Berlin/ New York.
14. Moonen, C. T. W. (1983) Ph.D. Thesis, Landbouwhogeschool, Wageningen, The Netherlands.
15. Zhou, Z., and Swenson, R. P. (1995) *Biochemistry 34*, 3183−3192.
16. Zhou, Z., and Swenson, R. P. (1996) *Biochemistry 35*, 15980−15988.
17. Feng, Y., and Swenson, R. P. (1997) *Biochemistry 36*, 13617−13628.
18. Mayhew, S. G., O'Connell, D. P., O'Farrell, P. A., Yalloway, G. N., and Geoghegan, S. M. (1996) *Biochem. Soc. Trans. 24*, 122−127.
19. Zhou, Z., and Swenson, R. P. (1996) *Biochemistry 35*, 12443−12454.
20. Ludwig, M. L., Burnett, R. M., Darling, G. D., Jordan, S. R., Kendal, D. S., and Smith, W. W. (1976) in *Flavin and Flavoproteins* (Singer, T. P., Ed.) pp 393−404, Elsevier Scientific Publishing Co., Amsterdam.
21. Watt, W., Tulinsky, A., Swenson, R. P., and Watenpaugh, K. D. (1991) *J. Mol. Biol. 218*, 195−208.
22. Smith, W. W., Pattridge, K. A., Ludwig, M. L., Petsko, G. A., Tsernoglou, D., Tanaka, M., and Yasanobu, K. T. (1983) *J. Mol. Biol. 165*, 737−755.

23. Eren, M., and Swenson, R. P. (1989) *J. Biol. Chem. 264*, 14874−14879.
24. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.
25. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci U.S.A. 74*, 5463−5467.
26. Swenson, R. P., and Krey, G. D. (1994) *Biochemistry 33*, 8505−8514.
27. Ludwig, M. L., Pattridge, K. A., Eren, M., and Swenson, R. P. (1991) in *Flavins and Flavoproteins 1990* (Curti, B., Ronchi, S., and Zanetti, G., Eds.) pp 393−404, Walter de Gruyter and Co., Berlin.
28. 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.
29. Clark, W. M. (1972) in *Oxidation-Reduction Potentials of Organic Systems*, Robert E. Krieger Publishing Co., New York.
30. Wassink, J. H., and Mayhew, S. G. (1975) *Anal. Biochem. 68*, 609−616.
31. Dubourdieu, M., LeGall, J., and Favaudon, V. (1975) *Biochim. Biophys. Acta 376*, 519−532.
32. Draper, R. D., and Ingraham, L. L. (1968) *Arch. Biochem. Biophys. 125*, 802−808
33. Chang, F. C., and Swenson, R. P. (1997) *Biochemistry 36*, 9013−9021.
34. Chang, F. C., and Swenson, R. P. (1999) *Biochemistry 38*, 7168−7176.
35. Bodenhausen, G., and Ruben, D. L. (1980) *Chem. Phys. Lett. 69*, 185−188.
36. VanGeet, A. L. (1969) from *Abstracts of the 10th Experimental NMR Conference*, Mar 1969, Mellon Institute, Pittsburgh, PA.
37. D′Anna, J. A., and Tollin, G. (1972) *Biochemistry 11*, 1073−1080.
38. Müller, F. (1991) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) Vol. I, pp 38−45, CRC Press, Boca Raton, FL.
39. Druhan, L., and Swenson, R. P. (1998) *Biochemistry 37*, 9668−9678.
40. Witanowski, M., Stefaniak, L., and Webb, G. A. (1981) *Annu. Rep. NMR Spectrosc. 11B*, 1−493.
41. Moonen, C. T., Vervoort, J., and Müller, F. (1984) *Biochemistry 23*, 4859−4867.
42. Müller, F., Vervoort, J., Lee, J., Horowitz, M., and Carreira, L. A. (1983) *J. Raman Spectrosc. 14*, 106−117.
43. Dyson, H. J., Rance, M., Houghten, R. A., Lerner, R. A., and Wright, P. E. (1988) *J. Mol. Biol. 201*, 161−200.
44. Loh, S. N., and Markley, J. L. (1994) *Biochemistry 33*, 1029−1036.
45. Markley, J. L., and Westler, W. M. (1996) *Biochemistry 35*, 11092−11097.
46. Liddel, V., and Ramsey, N. F. (1951) *J. Chem. Phys. 19*, 1608.
47. Muller, N., and Reiter, R. C. (1965) *J. Chem. Phys. 42*, 3265−3269.
48. Baxter, N. J., and Williamson, M. P. (1997) *J. Biomol. NMR 9*, 359−369.
49. Merutka, G., Dyson, H. J., and Wright, P. E. (1995) *J. Biomol. NMR 5*, 14−24.
50. Burnett, R. M., Darling, G. D., Kendall, D. S., LeQuesne, M. E., Mayhew, S. G., Smith, W. W., and Ludwig, M. L. (1974) *J. Biol. Chem. 249*, 4383−4392.
51. Smith, W. W., Burnett, R. M., Darling, G. D., and Ludwig, M. L. (1977) *J. Mol. Biol. 117*, 195−225.
52. Watenpaugh, K. D., Sieker, L. C., and Jensen, L. H. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 38577−3860.
53. Swenson, R. P., and Zhou, Z. (1997) in *Flavins and Flavoproteins 1996* (Stevenson, K. J., Massey, V., and Williams, C. H., Jr., Eds.) pp 427−436, University of Calgary Press, Calgary, Alberta, Canada.
54. Pauling, L. (1960) in *The Nature of the Chemical Bond*, 3rd ed., pp 449−504, Cornell University Press, Ithaca, NY.

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