Influence of the Protein Environment on the Redox Potentials of Flavodoxins from Clostridium beijerinckii

Received for publication, April 2, 2007, and in revised form, June 29, 2007. Published, JBC Papers in Press, June 30, 2007, DOI 10.1074/jbc.M702788200

Hiroshi Ishikita

From the Department of Chemistry, University of Southern California, Los Angeles, California 90089

The flavin mononucleotide (FMN) quinones in flavodoxin have two characteristic redox potentials, namely, $E_m$(FMNH$^-$/FMNH$^+$) for the one-electron reduction of the protonated FMN ($E_1$) and $E_m$(FMN/FMN$^+$) for the proton-coupled one-electron reduction ($E_2$). These redox potentials in native and mutant flavodoxins obtained from Clostridium beijerinckii were calculated by considering the protonation states of all titratable sites as well as the energy contributed at the pKa values of FMN during protonation at the N5 nitrogen (pKa(N5)). $E_1$ is sensitive to the subtle differences in the protein environments in the proximity of FMN. The protein dielectric volume that prevents the solvation of charged FMN quinones is responsible for the downshift of 130–160 mV of the $E_1$ values with respect to that in an aqueous solution. The influence of the negatively charged 5′-phosphate group of FMN quinone on $E_1$ could result in a maximum shift of 90 mV. A dramatic difference of 130–160 mV of the $E_2$ values of FMN quinone of the native and G57T mutant flavodoxins is due to the difference in the pKa(N5) values. This is due to the difference in the influence exerted by the carbonyl group of the protein backbone at residue 57.

Crystal structures of native and mutant flavodoxins obtained from Clostridium beijerinckii are available in the following forms: oxidized FMN (OX structure), semiquinone (SQ structure), and hydroquinone (HQ structure) (3). A remarkable feature is that the conformation of residues 56–60 near the binding site of FMN differs among native and mutant flavodoxins.

Only G57T mutants possess HQ structures that contain a mixture of two conformers, i.e. the proximal CO conformer (trans O-up conformation in Ref. 3) and the distal CO conformer (trans O-down conformation in Ref. 3); these differ with respect to the orientation of the backbone carbonyl (CO) group at residue 57 toward the N5 nitrogen atom of the FMN quinone. In the proximal CO conformer (Fig. 2, left), the CO group is oriented toward the FMN quinone so that the oxygen atom of CO can accept an H-bond from the protonated N5 nitrogen atom of the FMN quinone (O57–NFMN distance = 3.0 Å). On the other hand, in the distal CO conformer (Fig. 2, right), the orientation of the CO group is almost opposite to that in the proximal CO conformer, and no H-bond can form between the CO group and the protonated N5 nitrogen atom (O57–NFMN distance = 5.7 Å). Note that HQ structures, except for those in the G57T mutant, possess only proximal CO conformers.

Different conformations may be responsible for difference in the influence exerted on the energy of FMN quinones, and may vary the $E_1$ and $E_2$ values of flavodoxins (3), as indicated by the measured $E_m$ of the G57T mutants. These values of the G57T mutants vary considerably from those of other flavodoxins (Table 1).

The structural geometries of flavodoxins investigated in this study are considerably simple. Nevertheless, factors that determine the $E_1$ and $E_2$ values have not yet been fully clarified. Some earlier studies have not focused on the fact that $E_1$ is the potential for one-electron reduction, whereas $E_2$ is the potential for proton-coupled one-electron reduction. Hence, to analyze $E_2$, it is necessary to consider both redox and protonation events.

By using crystal structures and considering the protonation states of all titratable sites (including the FMN 5′-phosphate group) in the protein, here the calculated $E_1$ and $E_2$ values of flavodoxins obtained from C. beijerinckii are presented. To evaluate the $E_2$ value, the energy contributed by FMN quinone protonation at the N5 nitrogen (pKa(N5)) has been taken into account. The present study clearly demonstrates that the $E_m$ and pKa values of flavodoxins are determined solely by the protein environment as in other proteins (4–7).
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Experimental Procedures

Atomic Coordinates and Charges—For performing computational studies, crystal structures of the native and mutant forms of *C. beijerinckii* flavodoxin were used, whose information is available in the protein data bank (PDB): the native form used was 5NLL/5ULL for oxidized/hydroquinone forms and the mutant forms were G57D (4NLL/1FLA for oxidized/hydroquinone forms) and G57T (1FLD/2FVX for oxidized/hydroquinone forms at 277 K) (3). The calculations to obtain the $E_m$ ($\text{FMNH}^*/\text{FMNH}^-$) values of flavodoxin were performed using the OX crystal structures. Calculations for obtaining both $E_m$ ($\text{FMN}^*/\text{FMNH}^-$) and $pK_a$ values at the N5 nitrogen for $\text{FMN}^*/\text{FMNH}^-$ ($pK_a(N5)$) were performed using the HQ structures, if not otherwise specified (also see Fig. 1).

In the original atomic coordinates of the G57T mutant possessing the HQ structures (PDB: 2FVX), there were two conformers labeled conformer A and B (the proximal CO conformer (trans O-up conformation) and the distal CO conformer (trans O-down conformation), respectively) (3). However, in the original atomic coordinates of conformer B there exist two backbone O atoms at residue 58 (atom numbers 450 and 451). In the present study, the O atom with atom number 450 was regarded as that of the conformer A. The PDB file with information on this conformer shows that it lacks a backbone N atom that is present at residue 60 in conformer B. In the present study, the atomic coordinate of the N atom not only for the A conformer but also for the B conformer was used.

Atomic coordinates of the E59Q mutant (8, 9) were modeled based on the HQ structures of the native flavodoxin. The side chain at 59 was replaced with Gln, and only the side chain was energetically optimized with CHARMM (10). Resulting atomic coordinates of the Gln-59 side chain were listed in supplemental Table S1.

The atomic coordinates were prepared using the same procedures as those used in previous applications (for instance, Refs. 4–7). The position of H atoms were energetically optimized with CHARMM (10) by using the CHARMM22 force field. While carrying out this procedure, the positions of all non-H atoms were fixed, and the standard charge states of all the titratable groups were maintained, i.e. the basic and acidic groups were considered protonated and ionized, respectively. All the other atoms whose coordinates were available in the crystal structure were not geometrically optimized.

Atomic partial charges of the amino acids were adopted from the all-atom CHARMM22 (10) parameter set. Atomic charges of the 5'-phosphate group of FMN quinone ($-\text{H}_2\text{PO}_4^-, -\text{H}_2\text{PO}_5^-, \text{and } -\text{HPO}_3^{2-}$) were adopted from those of the methylphosphate CHARMM22 parameter set (labeled using MP_0, MP_1, and MP_2, respectively). The charges of FMN, $\text{FMN}^+$, $\text{FMNH}^-$, and $\text{FMNH}^-$ were determined from the electronic wave functions by fitting the resulting electrostatic potential in the neighborhood of these molecules by using the restrained electrostatic potential procedure (11). In the restrained electrostatic potential procedure atomic charges can be calculated, keeping the dipole and quadrupole moment constant. The electronic wave functions were calculated with the density functional module in JAGUAR$^3$ by using the B3LYP functional with the 6–31G** basis set (supplemental Table S2). Although in some systems density functional methods may not be appropriate to calculate energies (for instance, see Ref. 13), in the present study this method was used to obtain only atomic charges, not other properties (e.g. energies). Note that the energy value of the redox active group is related to the $E_m$ value. In particular, the highest occupied molecular orbital energy is closely related to the $E_m$ for one-electron reduction (14–16). In the present study, energies obtained in the density functional calculation were not used for subsequent calculations. It is widely known that calculations of atomic charges of the molecules are not sensitive to choice of the density functional method or the Hartree-Fock method (i.e. definition of atomic charges is more arbitrary than that of energy). Indeed, the obtained atomic charges of FMN in the present study are essentially the same as those calculated with the Hartree-Fock method (17).

Dielectric Volume—The dielectric volume of a protein complex is the spatial area and shape of molecular components of the protein and includes the polypeptide backbone, side chains, and cofactors, but not the water molecules. To facilitate a direct comparison with past computational results of other systems with the same method, the identical computational conditions and parameters such as atomic partial charges and dielectric constants were uniformly used. As a general and uniform strategy, all the crystallized water molecules were removed for our computations (4–7) because of the lack of experimental information regarding the H atom positions. Cavities that formed after removal of the crystallized water molecules were uniformly filled with a dielectric solvent ($\varepsilon = 80$). Accordingly, the effect of the removed water molecules was considered implicitly and a high value of the dielectric constant was assigned to these cavities. A discussion on the appropriate value of the dielectric constant of proteins has been published (18–21).

Protonation Pattern, Redox Potential, and $pK_a$—The present computation is based on the electrostatic continuum model created by solving the linear Poisson-Boltzmann equation with the MEAD program (22). To facilitate a direct comparison with previous computational results, we uniformly used identical computational conditions and parameters such as atomic partial charges and dielectric constants (for instance, Refs. 4–7). To obtain absolute $E_m$ values of the protein, we calculated the electrostatic energy difference between the two redox states in a reference model system using a known experimental $E_m$ value. The difference in the $E_m$ value of the protein relative to the reference system was added to the known $E_m$. As a reference model system, the following values for $E_m$ versus the normal hydrogen electrode were used: $E_m$($\text{FMNH}^*/\text{FMNH}^-$) = −172 mV at pH 7 for one-electron reduction (−172 mV = $E$1 or $E_m$($\text{SQ/HQ}$)) measured by Draper and Ingraham with potentiometry (2) (see also Fig. 1). All the other titratable sites, including the 5'-phosphate group, were fully equilibrated to the redox state of FMN during the titration. The ensemble of the protonation patterns was sampled by a Monte Carlo method with

$^3$Jaguar 4.2 (1991–2000), Schrödinger, Inc., Portland, OR.
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Karlsberg. All computations were performed at 300 K, pH 7.0, and an ionic strength of 100 mM. The linear Poisson-Boltzmann equation was solved using a three-step grid-focusing procedure at the resolutions 2.5 Å, 1.0 Å, and 0.3 Å. The Monte Carlo sampling yielded the probabilities [Acox] and [Ared] of the two redox states of molecule A. The Eₘ was evaluated using the Nernst equation. A bias potential was applied to obtain an equal amount of both redox states ([Acox] = [Ared]); thus, yielding the redox midpoint potential Eₘ as the resulting bias potential. For convenience, the computed Eₘ value was given with mV accuracy, without implying that the last digit was significant. In general, an Eₘ value of ~10 mV is a sufficiently reproducible range for the computational method used (for instance, see Refs. 4–7). By using the Henderson-Hasselbalch equation, the pKₐ value can be calculated as the pH, where the concentrations, [A⁻] and [AH⁺], are equal. FMN⁺ forms FMNH⁺ upon protonation at the N5 nitrogen in FMN quinone (Fig. 1). The value of 8.6 was considered as the pKₐ(N5) value (2) in the reference model system of FMN⁺/FMNH⁺ equilibrium in aqueous solution (see also Fig. 1). The value of 6.4 (23) was considered as the pKₐ value in the 5’-phosphate group of −H₂PO₄⁻/−HPO₄²⁻. Note that the 5’-phosphate was permanently deprotonated in the −H₃PO₄/−H₂PO₄⁻ equilibrium (pKₐ = 1.4 (23)) in all the crystal structures that were investigated. Thus, in the present study, the −H₂PO₄⁻/−HPO₄²⁻ equilibrium (pKₐ = 6.4 (23)) was investigated, if not otherwise specified.

**Calculation of E₂[Eₘ(FMN/FMNH)] in Flavodoxin**—The reaction involving FMN yielding FMNH⁺ that has an Eₘ(FMN/FMNH) is the process in which one-electron reduction of FMN to FMNH⁺ is coupled with protonation at the N5 nitrogen atom (Fig. 1). Therefore, one has to consider the energetics of both “one-electron redox process” and “one-proton protonation process” to accurately estimate the net energetics.

The potential of proton-coupled one-electron reduction can be written as follows:

\[
Eₘ(FMN/FMNH) = Eₘ(FMN/FMN⁺) + \frac{RT}{F} \ln 10 \log(1 + 10^{-pKₐ(N5)}) \tag{Eq. 1}
\]

where R is the gas constant, T is the temperature, and F is the Faraday constant (see Ref. 24).

At pH 7, \(\log(1 + 10^{-pKₐ(N5)}) \approx pKₐ(N5) - \text{pH} \tag{Eq. 2}\)

Thus, in the present study the following equation was used to calculate Eₘ(FMN/FMNH),

\[
Eₘ(FMN/FMNH) = Eₘ(FMN/FMN⁺) + \frac{RT}{F} \ln 10 \times (pKₐ(N5) - 7) \tag{Eq. 3}
\]

To use Equation 3, the Eₘ(FMN/FMN⁺) value in aqueous solution as the reference model system is needed. By maintaining Eₘ(FMN/FMNH) = −238 mV at pH 7 and pKₐ(N5) = 8.6 in aqueous solution measured by Draper and Ingraham (2) (summarized in Ref. 25), Eₘ(FMN/FMN⁺) = −333 mV was obtained in the present study. This value was used as the reference model system of Eₘ(FMN/FMN⁺) in aqueous solution.

**Reference Eₘ Values in the Reference Model System**—In the present study, the E₁ and E₂ values of −172 mV and −238 mV for free FMN measured by Draper and Ingraham with potentiometry (2) were used. On the other hand, the corresponding values measured by Anderson with pulse radiolysis (26) were −124 mV and −314 mV, respectively. More recently, by performing plot-fitting analysis of pH dependence on E₁ and E₂ obtained from former studies (2, 26), Mayhew (27) observed the larger deviation of data measured by Draper and Ingraham (2) from those anticipated by the Michaelis equation (28). He concluded that the latter was more reliable. Measurements of the E₁ and E₂ values by Williamson and Edmondson (29) also show the deviation from those anticipated by the Michaelis equation. In aqueous solution, redox reactions of quinones are often coupled with the protonation events, whereas in protein the corresponding reaction may be solely a redox process. This difference can make it difficult to obtain the appropriate Eₘ value in the reference system (reviewed in Ref. 30).

In the present calculations, using the HQ (for E₁ and pKₐ(N5)) and OX (for Eₘ(FMN/FMN⁺)) crystal structures, the measured E₁ and E₂ values of flavodoxins were reproduced only when the values of free FMN measured by Draper and Ingraham (2) were used as the reference value. Even if other forms of crystal structures were used (e.g. the OX structures for the E₁ calculations) along with the values measured by Anderson (26), the calculated values were significantly far from the measured values (supplemental Table S3). In using the value obtained by Anderson (26), the discrepancy between the calculated and measured values is remarkably large in the calculation of E₂. From these comparisons, the values measured by Draper and Ingraham (2) that can reproduce a series of the measured values were chosen as reference values.

**RESULTS AND DISCUSSION**

**Calculated Values of E₁ in Flavodoxin**—By using HQ structures, the calculated E₁ (Eₘ(FMN/FMN⁺)) values were found to be −400 mV and −381 mV for the native and G57D flavodoxins, respectively (Table 1). These results were in excellent agreement with the values that were measured earlier, i.e. −399 mV and −378 mV, respectively (3) (Table 1).

Only G57T mutants possess HQ structures that comprise a mixture of two conformers, namely, the proximal CO conformer and the distal CO conformer (Fig. 2). For the G57T mutants, the calculated E₁ values were −378 mV and −339 mV in the proximal and distal CO conformers, respectively (Table 1). The latter value is considerably closer to the value of −320 mV obtained in an earlier measurement (3), implying that the distal CO conformer is more relevant with regard to experimental measurement of E₁ values of G57T mutants.

It has been suggested that the existence of the distal CO conformer in G57T mutants may not be caused by the contamination of incompletely reduced quinones and that it is probably a relevant conformer in G57T mutants (3). To explain the notable difference in the E₁ values between G57T mutants and others (Table 1), the present result strongly suggests that G57T...
mutants must be in the distal CO conformer. It is likely that the "distal CO conformer is a more relevant structure to G57T mutants and that the difference in the \( E_m \) values between the two conformers is mainly attributable to the geometry of residue 57, i.e. at the CO orientation.

**Influence of Protonation States of the FMN Phosphate Group on \( E_1 \) Values**—Protonation states of titratable residues often play an important role in determining the \( E_m \) values of the protein, because the difference in protonation states of residues result in different net charges of proteins.

In this study, the calculated protonation states of the 5'-phosphate group of FMN quinone were essentially found to be identical in all the flavodoxins when \( E_1 \) values were determined (i.e. at pH 7). In \(-\text{H}_2\text{PO}_3^- / \text{HPO}_3^{2-}\) equilibrium (\( pK_a = 6.4 \) (23)), the phosphate group is mostly ionized as \(-\text{HPO}_3^{2-}\). In the corresponding protonation state, the contribution of the atomic charge of the 5'-phosphate group to \( E_1 \) is \(-90 \) mV in all the investigated flavodoxins (Table 2). This calculated value is larger than the experimentally estimated values of 15–28 mV obtained in studies on the *Desulfovibrio vulgaris* mutants T12H and N14H (31). The smaller values derived from the mutants are probably due to partial compensation by changes in protonation states and the possible reorientation of the other titratable side chains accompanied by mutations.

In former theoretical studies by Moonen *et al.* (32), it was proposed that the negatively charged phosphate group was responsible for the \(-180 \) mV downshift in flavodoxin \( E_1 \) values. Coincidently, the measured \( E_1 \) values of the apoflavodoxin-riboflavin complex obtained from *D. vulgaris* (that lacks the negatively charged 5'-phosphate group) was lower by 180 mV than that in the native flavodoxin (33). Thus, the negatively charged 5'-phosphate group was speculated to be a major factor in determining the \( E_1 \) value (for instance, Ref. 34). However, studies on the FMN binding property revealed that the 180-mV downshift in \( E_1 \) values of the apoflavodoxin-riboflavin complex was a consequence of the lowered FMN binding in the SQ form (31).

As compared with the calculated value obtained in the present study, the \( E_1 \) shift of 180 mV calculated by Moonen *et al.* (32) is unreasonably large. In conclusion, the \( E_1 \) shift of 180 mV measured in the apoflavodoxin-riboflavin complex may have been a result of other factors, namely, the lower binding of SQ (31).

**Contribution of Acidic Residues to the Decrease in the \( E_1 \) Value**—Ludwig *et al.* (25) proposed that nega-
nature of protein structures that avoid or minimize unnecessary electrostatic repulsions inside the protein. Note that the influence of acidic residues on $E_1$ exerted by the protein backbone charge should be essentially constant among mutants unless the protein backbone conformations differ dramatically from those of the native flavodoxin, i.e. additional mutations of acidic residues in the proximity of the FMN binding site to uncharged residues will upshift the $E_1$ value, as observed in studies by Zhou and Swenson (35), by changing the contribution from the protein side chains while essentially keeping the contribution from the protein backbone constant. If those acidic residues were not present in flavodoxin, then the $E_1$ value would be much larger.

It has been revealed that in the inner core of the proteins, the absence of the availability of solvation (rather than repulsive interactions) is the major contributor toward destabilizing the charged groups (7, 36). Likewise, the present study also demonstrates that the low $E_1$ value in the C. beijerinckii flavodoxin is a result of the absence of solvation in the protein dielectric environment rather than any specific negatively charged groups (Table 2). This may also hold true for the significant increase in the $E_1$ value (140 mV) upon mutation of Tyr-98 with Ala in the D. vulgaris flavodoxin (37). The bulky residue Tyr-98 has a van der Waals contact with the FMN ring (38). Upon replacement of Tyr-98 with Ala, large van der Waals volume of Tyr-98 is eliminated, exposing the FMN to the bulk solvent and increasing the $E_1$ value.

**Residues in Coupled FMNH/FMNH$^-$ Redox Reaction**—Although most of the acidic residues are permanently in the deprotonated states, Glu-59 changes its protonation state dramatically in response to the changes in redox states of FMNH/FMNH$^-$ (supplemental Table S4). This acidic residue changes its protonation state by nearly 1 H$^+$ upon reduction of FMNH$^-$ (Fig. 3).

Based on the observation of the crystal structures of flavodoxins, Ludwig et al. (25) proposed that Glu-59 is the most plausible candidate for a residue that protonates/deprotonates in response to the FMNH/FMNH$^-$ redox state change. Later, Bradley and Swenson (8) replaced Glu-59 with Gln and observed a significant increase of 86 mV in the $E_1$ value ($E_1 = -313$ mV). Upon the mutation, pH dependence of the $E_1$ value remained unchanged, implying that Glu-59 is not the residue that is responsible for the pH dependence (8).

The calculated $E_1$ value is $-318$ mV for E59Q mutant, using the model structure obtained from the native structure. The resulting upshift of 82 mV in the $E_1$ value is in agreement with the shift of 86 mV observed by Bradley and Swenson (8). In the present study, this upshift is a result of the elimination of negative charge on Glu-59. Indeed, when Glu-59 is forced to be protonated in the native flavodoxin structure, the $E_1$ value is

![Figure 2. The proximal (left) and distal (right) CO conformers of G57T flavodoxin in the hydroquinone form (PDB: 2FVX). The ball-and-stick model represents the nitrogen atoms and carbonyl groups (carbon atoms in gray and oxygen atoms in black), respectively. For enhanced clarity, the protein side chains and the 5'-phosphate group of FMN are omitted.](image-url)

| TABLE 2 | Influence of the protein environment on $E_1$ ($\Delta$) in millivolt units |
|---------|---------------------|---------------------|
| Flavodoxin | Native | G57D | G57T |
| CO-57 in HQ structure | Proximal* | Proximal* | Proximal* | Distal* |
| $E_1$: charged protein | -400 | -381 | -378 | -339 |
| $E_1$: uncharged protein | -328 | -318 | -318 | -305 |
| $E_1$: reference | -172 | -172 | -172 | -172 |
| $\Delta$Dielectric | -156 | -146 | -146 | -133 |
| $\Delta$Charge | -72 | -63 | -60 | -34 |
| $\Delta$Backbone | 257 | 276 | 306 | 318 |
| $\Delta$Side chain | -238 | -243 | -270 | -258 |
| $\Delta$Phosphate | -91 | -96 | -96 | -94 |

* trans O-up form with 57–58 orientation.
† values were calculated in the presence of protein atomic charges.
‡ values were calculated in the absence of protein atomic charges.
§ measured values are for the isolated FMN molecule in aqueous solution (2).
¶ contribution of protein dielectric volume to $E_1$: (charged protein) − (reference).
** contribution of protein atomic charges to $E_1$: (charged protein) − (uncharged protein).
calculated to be $-300$ mV, which is relatively close to the value of the E59Q mutant.

The carboxyl oxygen atom of Glu-59 is at a H-bond distance from the N3 atom of the FMN (O−O distance $= 3.0$ Å) (3). The replacement of Glu-59 with Gln indirectly affects $pK_{a}(N5)$, yielding the value of 12.5, mainly due to change of the net charge at the position of 59. Note that, in the present calculation of the $E1$ value, the only FMNH/FMNH$^-$ redox pair has been considered, i.e. the N3 site is always protonated. The present result suggests that the charge state at residue 59 plays an important role in determining $E1$ by affecting the stability of the FMNH$^-$ state. These results are in agreement with mutational studies by Bradley and Swenson that has demonstrated the H-bond with the N3 atom can affect the stability of the FMNH$^-$ state (8, 9).

Calculated Values of $E2_{em}(FMN/FMNH^+)$ in Flavodoxin—
The calculated values of $E_{em}(FMN/FMNH^+)$ in flavodoxin are in agreement with the measured values (Table 1). The calculated value of $E_{em}(FMN/FMNH^+)$ is influenced by two independent factors, $pK_{a}(N5)$ and $E_{m}(FMN/FMN^+)$ (see Equation 3).

In the case of the G57T mutants, the calculated value of $pK_{a}(N5)$ in the CO distal form is in good agreement with that estimated from experiments (3) (Table 1). In contrast, the corresponding value in the CO proximal form is similar to that estimated for native flavodoxin (Table 1). Thus, the CO distal form is necessary to yield the low $pK_{a}(N5)$ values of $>11$ in G57T mutants. The present result indicates that the CO distal form is the relevant conformer to the HQ structure of G57T mutants under conditions where the $E_{m}$ has been measured.

On the other hand, the $E_{m}(FMN/FMN^+)$ value cannot be measured experimentally due to the inevitable protonation of the FMN$^+$ state in aqueous solution. The calculated values of $E_{m}(FMN/FMN^+)$ vary only by 20 mV among native and mutant flavodoxins (Tables 1 and 3), and these are apparently not sensitive to different protein environments at residue 57.

Thus, the significant difference in the calculated $E2$ values among the C. beijerinckii flavodoxins is predominantly a result of the difference in the $pK_{a}(N5)$ values and that stability of the FMN$^+$ state with respect to the FMN state does not strongly contribute to the difference in the $E2$ values (Table 3). It has been widely observed that, in contrast to the $E1$ values, mutation resulting in replacement of charged residues to uncharged ones in flavodoxins have little influence on the $E2$ value (35, 39). According to the present result, the main reason for this is the predominant effect of $pK_{a}(N5)$, and not $E_{m}(FMN/FMN^+)$, on $E2$. Because $pK_{a}(N5)$ is strongly affected by the protein backbone conformation near the FMN binding site (Table 3), the atomic charges of side chains have very little influence on $E2$, unless mutations lead to a dramatic change in the protein backbone conformation.

The present study revealed that a majority of the differences in $pK_{a}(N5)$ among native and mutant flavodoxins can be explained by the different influences of atomic charges of the protein backbone, namely, at residue 57 (Table 4). In the proximal CO conformer of flavodoxin, a protonated N5 nitrogen atom of quinone in the protonated form can form an H-bond with the backbone carbonyl group-57, although the H-bond is absent in the distal CO conformer (40) (Fig. 2). The presence or absence of an H-bond in the N5 site has a direct influence on the stability of the charged form of the FMN quinone. Thus, the

### Table 3

| Infl uence of protein environment on $E2$ ($\Delta$) in millivolts [$pK_{a}$] units |
|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| CO-57 in HQ structure$^a$       | Proximal$^b$                     | Proximal$^b$                     | Distal$^a$                       |
| From $pK_{a}(N5)$               | -129                            | -167                            | -164                            |
| $pK_{a}(N5)$                   | [13.9]                          | [13.6]                          | [13.3]                          |
| $pK_{a}(N5)$                   | [11.1]                          | [11.0]                          | [11.0]                          |
| $\Delta$Dielectric$^d$          | [6.4]                           | [6.4]                           | [6.4]                           |
| $\Delta$Charge$^e$              | [2.8]                           | [2.6]                           | [2.3]                           |
| $\Delta$Backbone               | [-2.5]                          | [-2.8]                          | [-3.6]                          |
| $\Delta$Side chain             | [4.9]                           | [4.4]                           | [4.2]                           |
| $\Delta$Phosphate              | [1.4]                           | [1.4]                           | [1.4]                           |
| From $E_{m}(FMN/FMN^+)$         | [537]                           | [557]                           | [557]                           |
| $E_{m}(FMN/FMN^+)$              | [537]                           | [557]                           | [557]                           |
| $E_{m}(FMN/FMN^+)$              | [537]                           | [557]                           | [557]                           |
| $\Delta$Dielectric$^d$          | [-141]                          | [-159]                          | [-130]                          |
| $\Delta$Charge$^e$              | [-63]                           | [-65]                           | [-94]                           |
| $\Delta$Backbone               | [3.5]                           | [2.9]                           | [2.5]                           |
| $\Delta$Side chain             | [-245]                          | [-266]                          | [-257]                          |
| $\Delta$Phosphate              | [-93]                           | [-90]                           | [-95]                           |

$^a$ Used for only $pK_{a}(N5)$ calculation.
$^b$ trans O-up form with 57–58 orientation.
$^c$ trans O-down form with 57–58 orientation.
$^d$ Atomic charges of side chains have very little influence on $E2$.
$^e$ Measured values for isolated FMN molecule in aqueous solution (2).
$^f$ Contribution of protein electric volume to $pK_{a}(N5)/E_{m}(FMN/FMN^+)$: (charged protein) − (reference).
$^g$ Contribution of protein atomic charges to $pK_{a}(N5)/E_{m}(FMN/FMN^+)$: (charged protein) − (uncharged protein).

### Table 4

| Infl uence of atomic charges of protein backbone on $pK_{a}(N5)$ in $pK_{a}$ units |
|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| CO-57 in HQ structure$^a$       | Proximal$^b$                     | Proximal$^b$                     | Distal$^a$                       |
| $pK_{a}(N5)$                   | 13.9                            | 13.6                            | 13.3                            |
| $\Delta$Backbone               | [60]                            | [60]                            | [60]                            |
| $\Delta$Side chain             | [2.8]                           | [2.8]                           | [2.8]                           |
| Whole protein                   | [2.8]                           | [2.8]                           | [2.8]                           |

$^a$ Used for only $pK_{a}(N5)$ calculation.
$^b$ trans O-up form with 57–58 orientation.
$^c$ trans O-down form with 57–58 orientation.
conformation of the protein backbone at residue 57 is the main factor in determining p$K_a$(N5), and consequently, $E_2$. Indeed, in mutational studies where residues 57 and 58 were replaced with Ala or Gly, a notable correlation was observed between the measured $E_2$ values and the backbone conformations (12). The results of the mutational studies (12) could indicate that mutational changes in the protein backbone conformation around residue 57 may directly affect p$K_a$(N5), leading to a corresponding change in $E_2$.

Acknowledgments—I thank Dr. Donald Bashford for providing the MEAD program. I am grateful to Dr. Arieh Warshel and Dr. Ernst-Walter Knapp for useful discussions.

REFERENCES

1. Niemz, A., and Rotello, V. M. (1999) Acc. Chem. Res. 32, 44–52
2. Draper, R. D., and Ingraham, L. L. (1968) Arch. Biochem. Biophys. 125, 802–808
3. Ludwig, M. L., Pattridge, K. A., Metzger, A. L., Dixon, M. M., Eren, M., Feng, Y., and Swenson, R. P. (1999) Biochemistry 38, 8686–8695
4. Brooks, B. R., Bruccoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S., and Karplus, M. (1983) J. Comp. Chem. 4, 187–217
5. Bayly, C. I., Cieplak, P., Cornell, W. D., and Kollman, P. A. (1993) J. Phys. Chem. 97, 10269–10280
6. Kasim, M., and Swenson, R. P. (2000) Biochemistry 39, 15322–15332
7. Wodrich, M. D., Corminboeuf, C., Schreiner, P. R., Fokin, A. V., and Schleyer, P. v. R. (2006) Chem. Eur. J. 12, 6344–6351
8. Ishikita, H., and Knapp, E.-W. (2005) Biochemistry 44, 10950–10962
9. Smith, W. W., Burnett, R. M., Darling, G. D., and Ludwig, M. L. (1977) J. Mol. Biol. 117, 195–225