Regulation of Oxidation−Reduction Potentials through Redox-Linked Ionization in the Y98H Mutant of the *Desulfovibrio vulgaris* [Hildenborough] Flavodoxin: Direct Proton Nuclear Magnetic Resonance Spectroscopic Evidence for the Redox-Dependent Shift in the pK_a of Histidine-98[†]

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ABSTRACT: Flavodoxin from *Desulfovibrio vulgaris* is a low molecular weight (15 000 Da) acidic flavoprotein that contains a single flavin mononucleotide (FMN) cofactor. A distinguishing feature of the flavodoxin family is the exceptionally low midpoint potential of the semiquinone/hydroquinone couple. Tyrosine-98, which flanks the outer or *si* face of the FMN, plays an important role in establishing the oxidation−reduction properties of the bound cofactor as demonstrated by the substitution of a number of amino acids at this position [Swenson, R. P., & Krey, G. D. (1994) *Biochemistry 33*, 8505−8514]. The midpoint potential for the semiquinone/hydroquinone couple increases substantially when basic residues are introduced at this position. The pH dependency in the Y98H mutant is consistent with a redox-linked ionization model in which the favorable electrostatic coupling between the imidazolium cation and the flavin hydroquinone anion is responsible for the higher potential. Such a model predicts an increase in the pK_a of 1.5 units for His98 upon complete reduction of the FMN. In this study, proton nuclear magnetic resonance spectroscopy was used to directly determine the intrinsic pK_a of His98 as a function of the redox state of the cofactor in this flavodoxin. Values for the pK_a of His98 in the oxidized and fully reduced flavodoxin are 7.02 ± 0.08 and 8.43 ± 0.11, respectively, an increase in the pK_a by 1.41 units, which conforms with the previous prediction. These results provide direct experimental proof of the redox-linked ionization of this residue and provides further evidence of the crucial role of electrostatic interactions, in this case, in the stabilization of the flavin hydroquinone anion. This phenomenon may represent a general mechanism in the modulation of the reduction potential of the flavin cofactor within flavoenzymes in which ionizable groups such as histidine in the active center change ionization states during the catalytic cycle.

The structural basis for the modulation of the oxidation−reduction properties and potentials of redox centers in electron-transferring and other redox proteins remains a central question in biochemistry and biophysics. The proper poising of the redox potential of these centers is critical not only in establishing transfer kinetics in many instances but also in conserving the energy inherent in the potential gradients associated with electron transport pathways. We have been utilizing the bacterial flavodoxin as one model system in which to study the regulation of one-electron reduction potentials of the noncovalently bound flavin mononucleotide (FMN)[1] cofactor. Shuttling between the one-electron (semiquinone) and fully reduced (hydroquinone) states, flavodoxins play an important role in mediating low-potential electron transfer between other redox proteins in such notable physiological processes as photosynthesis, nitrogen fixation, and microbial respiration, often substituting for the low-potential ferredoxin in these metabolic pathways (Mayhew & Tollin, 1992). These proteins exhibit the lowest reduction potentials among the flavoprotein family, with values recorded as low as −520 mV (Mayhew & Tollin, 1992). Thus, a characteristic and essential role of the flavodoxin protein is to dramatically lower the midpoint potential of the semiquinone/hydroquinone (sq/hq) couple. In the case of the *Desulfovibrio vulgaris* flavodoxin, the one-electron reduction potential for this couple of the flavin shifts from −124 mV observed for FMN in aqueous solution (Anderson, 1983) to approximately −450 mV (at pH 7, 25 °C vs SHE) (Curley *et al.*, 1991; Swenson & Krey, 1994). These proteins seem to accomplish this dramatic shift in potential by thermodynamically stabilizing the neutral semiquinone state while in turn destabilizing the anionic hydroquinone species. How this is done has been the subject of several studies in our laboratory and others. X-ray crystallographic analyses of flavodoxin from *D. vulgaris* in its three redox states indicate that the semiquinone and fully reduced states are nearly identical in structure (Watenpaugh *et al.*, 1973, 1976; Watt *et al.*, 1991). Therefore, conformational differences are unlikely to account for the large shift in the reduction potential of the sq/hq couple. More likely to affect the redox potentials are the differential stabilization/destabilization of each redox state by common features of

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[1] Abbreviations: FMN, flavin mononucleotide; COSY, homonuclear 2D correlated nuclear magnetic resonance spectroscopy; sq/hq, semiquinone/hydroquinone couple; SHE, standard hydrogen electrode; TMSP, sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$.

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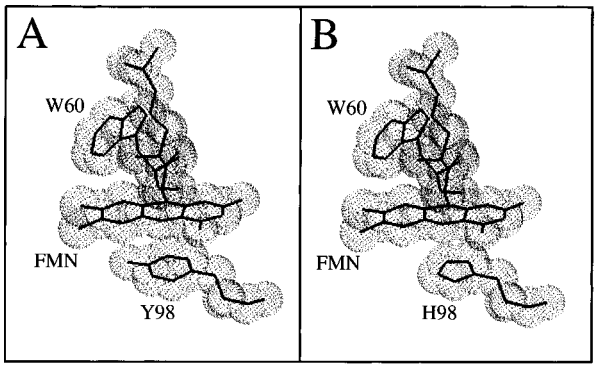


FIGURE 1: Partial view of the structure of the flavin mononucleotide binding site in the flavodoxin from *Desulfovibrio vulgaris* highlighting the aromatic residues flanking the flavin isoalloxazine ring. (Panel A) Wild-type structure is adapted from X-ray crystal structural data (Watt *et al*., 1991). (Panel B) The Y98H mutant structure is a geometry-optimized model based on the NMR solution structure of this protein (Stockman *et al.*, 1994). Dots represent the van der Waals surfaces of each residue. Hydrogen atoms have been omitted for clarity. The side chain of the amino acid at position 98 flanks in a coplanar manner the *si* face of the flavin ring.

the cofactor binding site such as long-range electrostatic, $\pi-\pi$ aromatic stacking, and hydrogen-bonding interactions as has been directly demonstrated recently (Ludwig *et al*., 1990, 1997; Swenson & Krey, 1994; Zhou & Swenson, 1995, 1996).

An important physical property that seems to be of critical importance in the regulation of the one-electron reduction potentials of flavodoxins is the formation of the *anionic* form of the FMN hydroquinone when the flavodoxin is fully reduced. Optical and ^{15}N NMR spectroscopic data suggest that the pK_a of the hydroquinone anion has been shifted substantially from 6.7 found in solution to <4 when bound by the protein (Vervoort *et al.*, 1985, 1986; Ludwig *et al.*, 1990). Structural studies with a flavodoxin reconstituted with 1-deaza-FMN suggest structural reasons for the lower pK_a value (Ludwig *et al.*, 1990). This situation seems somewhat paradoxical in that the anionic form of the hydroquinone selectively accumulates in an environment provided by the protein in which its formation is energetically unfavorable. The importance of unfavorable electrostatic interactions has been demonstrated through the manipulation of the immediate environment of the flavin cofactor by site-directed mutagenesis. For example, systematic substitution of the six acidic residues surrounding the cofactor binding site in the flavodoxin from *D. vulgaris* indicates that the unfavorable electrostatic environment destabilizes the flavin hydroquinone anion, contributing about one-third of the large redox potential shift for the sq/hq couple (Zhou & Swenson, 1995).

However, flavoproteins may not only utilize unfavorable electrostatic interactions to modulate redox potentials. Recent studies have demonstrated that the introduction of a basic residue such as a histidine or an arginine near the flavin isoalloxazine ring can significantly increase the redox potential for the sq/hq couple (Helms, 1990; Swenson & Krey, 1994). For example, Tyr98 is one of two aromatic residues that flanks the flavin in the flavodoxin from *D. vulgaris*, the other being Trp60 (Watenpaugh *et al*., 1973, 1976). The phenolic side chain of Tyr98 is nearly coplanar with the outer or *si* face of the isoalloxazine FMN ring, making extensive van der Waals contacts with it (Figure 1A). Substitution of a histidine or an arginine for this residue significantly increases (by 180 mV) the midpoint potential of the sq/hq couple (Swenson & Krey, 1994). Such large increases do not seem to be the result of significant structural changes in these mutants (Stockman *et al*., 1993, 1994). However, the general pH dependency of the one-electron reduction potential for the sq/hq couple in the Y98H mutant suggests that the reduction of the semiquinone to the hydroquinone is closely coupled to the ionization of His98 and is consistent with the electrostatic stabilization of the flavin hydroquinone anion by the flanking cationic imidazolium side chain of His98 (Figure 1B). As a necessary consequence of the redox-linked ionization model, the pK_a of His98 was predicted to shift from 7.0 in the semiquinone state to approximately 8.5 in the fully reduced state as a consequence of the electrostatic coupling (Swenson & Krey, 1994). However, direct experimental evidence of this pK_a shift was not available at the time and the analysis was compromised somewhat by the inability to extend the redox titrations beyond pH 8.5.

Thus, an important aspect of the redox-linked ionization scheme is that the ionization of the coupled amino acid residue is directly influenced by the redox state of the flavin cofactor and *vice versa*. In this study, NMR spectroscopy was used to directly determine the pK_a of His98 in oxidized and fully reduced states in order to provide direct experimental proof for this linkage and to support and confirm the redox-linked ionization model. The close agreement between the theoretical and experimental results obtained in this and the previous study for the linked equilibria involved provides convincing evidence for this type of control of the oxidation−reduction potentials of the flavin cofactor in flavoproteins. Redox-linked ionization may provide an important means through which the change in the ionization state of an amino acid residue in the active site during catalysis and/or the alteration of its pK_a by environmental effects may indirectly alter the redox state of the flavin during catalytic turnover, perhaps facilitating electron transfer. Such mechanisms will be discussed.

EXPERIMENTAL PROCEDURES

Materials. Deuterium oxide (D_2O) was obtained from Fluka Chemicals. Sodium deuteroxide (NaOD), deuterium chloride (DCl), and sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TMSP) were purchased from Cambridge Isotope Laboratories. Sodium dithionite was from Aldrich Chemical Co. All other chemicals were of analytical reagent grade.

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

NMR Sample Preparation. All NMR samples contained approximately 1.0 mM flavodoxin. Exchangeable protons

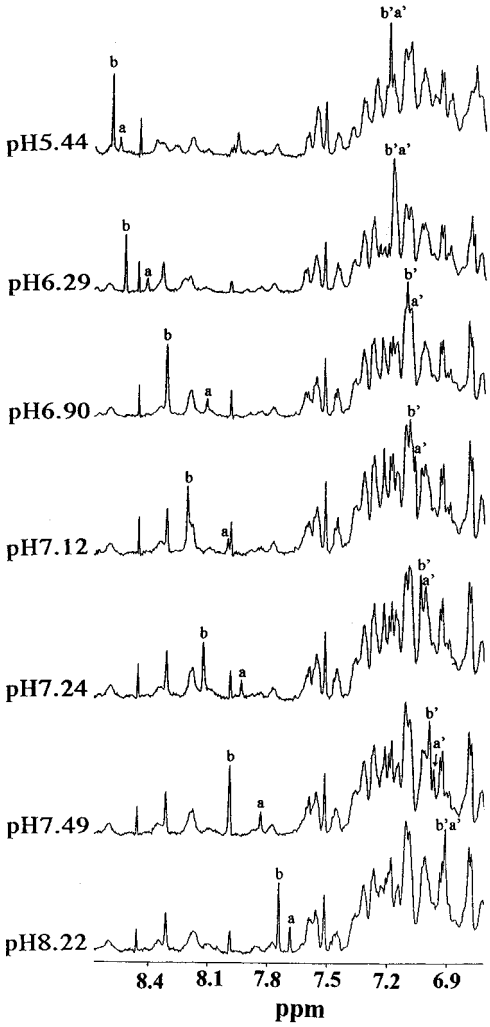


FIGURE 2: ^{1}H NMR spectra (600 MHz) in the aromatic region for the oxidized Y98H mutant flavodoxin as a function of pH. Peaks *a* and *a′* were assigned to the C2 and C4 protons, respectively, of the imidazole side chain of His98. Similarly, peaks *b* and *b′* were assigned to the C2 and C4 protons of His142.

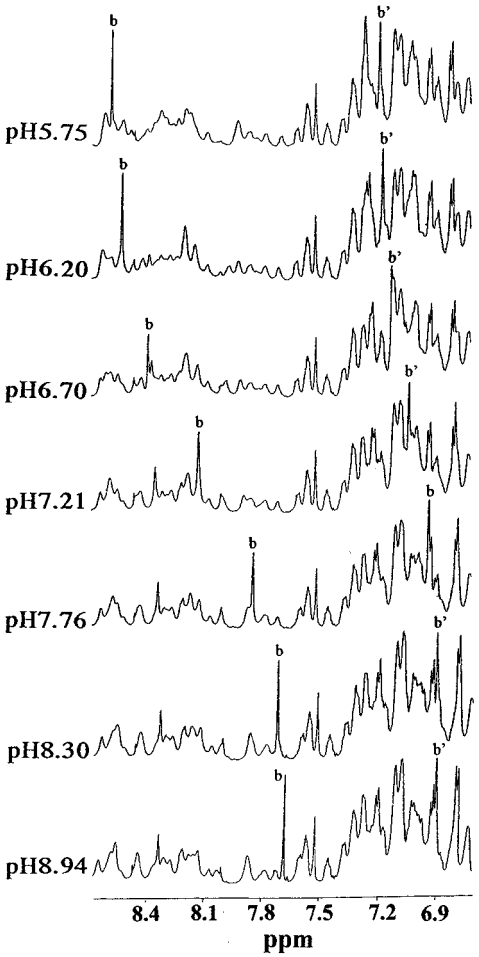


FIGURE 3: ^{1}H NMR spectra (600 MHz) in the low-field region for the oxidized wild-type flavodoxin as a function of pH. Peaks *b* and *b′* were assigned to the C2 and C4 protons, respectively, of His142.

were replaced with deuterons by incubation of the flavodoxin in D_2O at 42 °C for 3.5 h followed by lyophilization. This procedure was repeated at least three times. NMR samples of flavodoxin in the oxidized state were prepared in 20 mM sodium phosphate buffer and the pH was adjusted by the addition of appropriate amount of 5% (w/w) NaOD or DCl in D_2O during the course of the pH titration. Individual NMR samples of flavodoxin in the reduced state were prepared in 50 mM sodium phosphate buffer for pH values below 8.0 and in 50 mM sodium pyrophosphate buffer for pH values above 8.0. The flavodoxin solutions were made anaerobic by purging with several cycles of a partial vacuum and prepurifed argon in NMR sample tubes equipped with septum seals. The flavodoxin was reduced with the addition of an appropriate volume of a freshly prepared sodium dithionite solution. The pH measurements were performed immediately after the NMR measurements. By convention, the reported pH values (designated pH*) are not corrected for the deuterium isotope effect. In this way, the isotope effect at the glass electrode (~0.4 unit) approximately offsets the isotope effect on the acid dissociation equilibrium of the histidine, generating pK_a values comparable to those in H_2O (Markley, 1975).

^{1}H NMR Spectroscopy. All ^{1}H NMR spectra were recorded at 300 K on a Bruker DMX-600 spectrometer operating at 600.13 MHz. Proton chemical shifts in D_2O were referenced to internal standard of TMSP set at 0.0 ppm. Spectra were processed on a Silicon Graphics Indigo workstation using Felix 95.0 software (Biosym Technologies).

RESULTS

Determination of the pK_a of His98 of the Y98H Mutant in the Oxidized State. The ^{1}H NMR spectra of oxidized Y98H mutant in D_2O at different pH values are shown in Figure 2. This flavodoxin contains two histidine residues: His142, which is also present in wild type, and His98, which has been introduced by site-directed mutagenesis (Swenson & Krey, 1994). Four resonance peaks (designated *a*, *a′*, *b*, and *b′*) were observed in the aromatic region of the ^{1}H NMR spectrum that could be assigned to the C2H (*a* and *b*) and C4H (*a′* and *b′*) of histidine on the basis of their general chemical shift values and titration shifts (Markley, 1975). Peaks *b* and *b′* were present in the ^{1}H NMR spectra of oxidized wild-type protein at different pH values (Figure 3) and were assigned to the C2H and C4H, respectively, of His142. Peaks *a* and *a′* in the spectra of the Y98H mutant (Figure 2) and absent in the wild-type spectra were assigned to the C2H and C4H of His98, respectively. These assignments were further confirmed by homonuclear 2D correlation

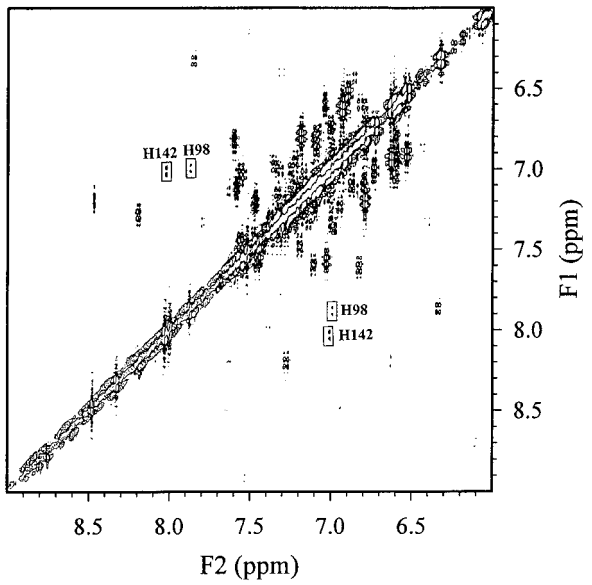


FIGURE 4: Histidine region of a 600 MHz COSY spectrum of oxidized Y98H mutant flavodoxin at pH 7.35, 300 K. The cross-correlations between the C2H and C4H of His98 and of His142 are labeled.

spectroscopy (COSY) analysis in which a correlation or cross-peak between C2H and C4H of histidine can be observed in the ^{1}H COSY spectrum (King & Wright, 1982; Wüthrich, 1986). Figure 4 shows the COSY spectrum of Y98H mutant flavodoxin. The C2H-C4H four-bond connectivities, as manifested by the cross-peaks at 7.86 and 6.98 ppm for His98 and 8.01 and 7.00 for His142 between C2H and C4H, respectively, confirm the previous resonance assignments for the C2H and C4H of these residues.

Both C2H and C4H of His98 and His142 shift upfield as the pH is increased (Figure 5, panels A and B, respectively). The pK_a values of His98 and His142 in the oxidized Y98H mutant were determined by fitting the titration data to the Hill equation (Markley, 1973). The pK_a values of His142 determined from this plot were 7.23 from the C2H signal and 7.22 from the C4H. These values are similar to those obtained from the titration curve for His142 of the wild-type flavodoxin (7.19 from C2H and 7.15 from C4H) (data not shown). The pK_a values of His98 were 6.94 from the C2H and 7.11 from the C4H (Figure 5, panels A and B, respectively), giving an average value of 7.02 ± 0.08.

Determination of the pK_a of His98 of the Y98H Mutant in the Two-Electron Reduced State. Figure 6 shows the aromatic proton resonance region of the NMR spectrum of the two-electron reduced form of Y98H mutant at different pH values. Two narrow resonance peaks (labeled *a* and *b*) corresponding to imidazole C2 hydrogens were observed. Peak *a* was assigned to the C2H of His98 and peak *b* was assigned to the C2H of His142 according to the following two approaches. Resonance assignments were initially based on the comparison of NMR spectrum for the two-electron reduced, one-electron reduced, and oxidized states of this flavodoxin (Figure 7). Due to the paramagnetic effect of the flavin radical generated in the one-electron reduced state, proton resonances close to the isoalloxazine ring will be strongly broadened (Peelen & Vervoort, 1994). Peak *a* is present as a narrow band in the two-electron reduced spectrum and the oxidized spectrum but appears broadened in the one-electron reduced (semiquinone) spectrum. Also,

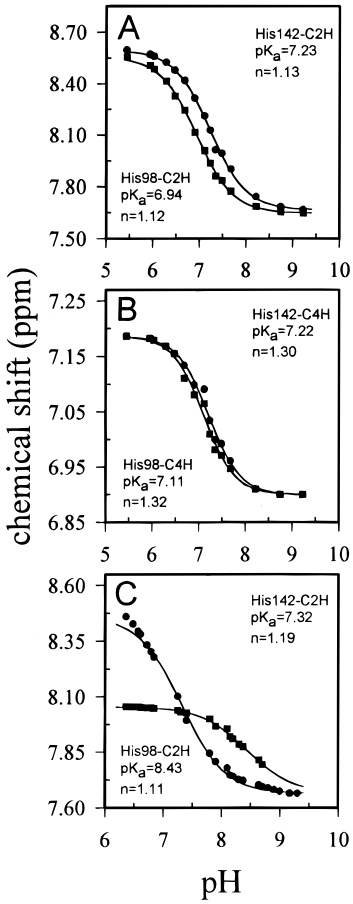


FIGURE 5: pH dependence of the ^{1}H NMR chemical shifts for the histidine C2H (Panel A) and C4H (Panel B) assigned to His98 and His142 in the Y98H mutant flavodoxin in the oxidized state. Panel C represents the pH dependency of the chemical shift for the C2H in the fully reduced Y98H flavodoxin. The chemical shifts assigned to His98 and His142 are represented by the ■ and ● respectively, in each panel.

this peak appears to shift upfield upon full reduction the flavin. Both observations are consistent with the behavior of the C2H of His98 close to the FMN ring. However, peak *b* was observed in the spectra of all three redox states, and its chemical shift did not change significantly, consistent with its assignment to the C2H of His142, which is located on nearly the opposite side of the protein, over 25 Å from the cofactor. It is interesting to note that the doublet at approximately 8.21 ppm appearing in both the oxidized and fully reduced spectra is also broadened in the semiquinone spectrum and may represent other protons near the FMN in this flavodoxin.

A second approach for the assignment of resonance peaks was based on the comparison of the pH dependency of the spectra to the two-electron reduced wild-type flavodoxin (representative spectra are shown in Figure 8). The C2H and C4H (peaks *b* and *b*′) of His142 shift upfield as the pH is increased and the pH shift of C2H correlated very well to that of peak *b* in the fully reduced spectra of the Y98H mutant and to peak *b* in the oxidized spectra (assigned to His142, Figure 3). The pH titration of the C2H resonance of His142 generates a pK_a value of 7.32 (see Figure 5C), which is also very similar to that for the oxidized wild-type protein.

It is quite clear from the pH dependency of the resonance assigned to the C2H of His98 (peak *a*) that the pK_a of this residue has increased substantially in the fully reduced

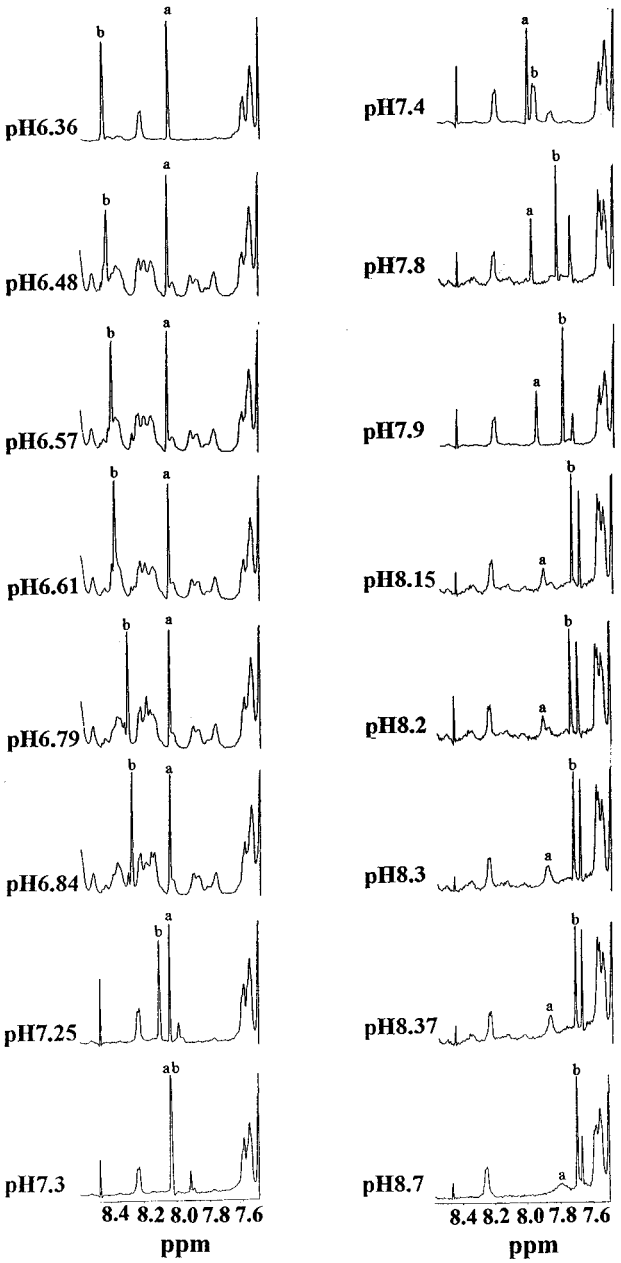


FIGURE 6: Downfield region of the 600 MHz ^{1}H NMR spectra for the two-electron reduced Y98H mutant flavodoxin as a function of pH. Peaks *a* and *b* were assigned to the C2 hydrogens of His98 and His142, respectively.

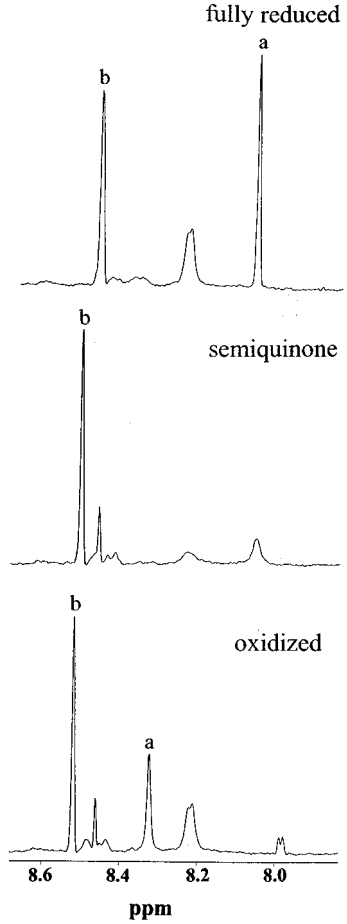


FIGURE 7: Aromatic region of the 600 MHz ^{1}H NMR spectra for the Y98H mutant flavodoxin in each of the three redox states of the FMN cofactor at pH 6.3. Peaks *a* and *b* were assigned to the C2H of His98 and His142, respectively.

flavodoxin. Unlike resonance peak *b* (C2H of His142), peak *a* does not shift upfield to any significant extent below pH 7.5 (Figure 6). This behavior is also in marked contrast to the response of this resonance in the oxidized flavodoxin, in which peak *a* has almost completed its upfield shift by the time pH 7.5 was reached (Figure 2). Above this pH, this peak began to shift upfield, started to broaden noticeably above pH 7.9, and became quite broad at the highest pH tested, pH 8.7 (Figure 6). The reason for the broadening of this resonance peak is not completely understood. This phenomenon was very reproducible, consistently appearing with different samples and on different times of analysis. It may result from a decrease in rotational freedom of the neutral form of the imidazole ring of His98, perhaps as the result of the hydrogen bonding between the neutral form of the imidazole ring and the carboxyl group of either Asp62 or Asp95, which are located nearby. Alternatively, the neutral imidazole may exist in two or more slowly exchanging conformations or environments. The pK_a value of His98 in the two-electron reduced state of the Y98H flavodoxin was obtained from the fit of the titration curve of peak *a* to the Hill equation. The pK_a was determined to be 8.43 ± 0.11 (Figure 5C).

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

$$E_m = E_0 + (RT/nF) \ln \{([H^+] + K_a^{HQ})/([H^+] + K_a^{SQ})\}$$

Therefore, in order to calculate the pH dependency of the midpoint potential of the sq/hq couple, it is necessary to know the acid dissociation constant for the redox-linked ionizable group, in this case His98, in both the semiquinone and hydroquinone reduced states. However, the determination of the pK_a of His98 by NMR when the FMN in the semiquinone state was not possible because the C2H and C4H resonances could not be identified, undoubtedly due

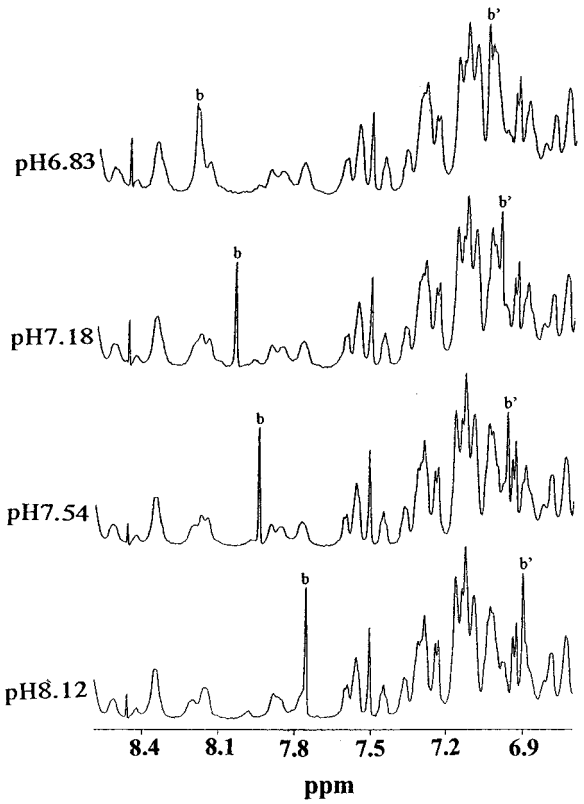


FIGURE 8: ^{1}H NMR spectra (600 MHz) in the region containing the histidine C2H and C4H resonances for the fully reduced wild-type flavodoxin at several different pH values. Peaks *b* and *b′* were assigned to the C2 and C4 protons of His142, respectively.

to extensive line broadening through the paramagnetic shielding effect of the nearby unpaired electron in the flavin radical (for example, see Figure 7). It was necessary, therefore, to assume that the pK_a of His98 in the semiquinone state is very similar to that in the oxidized state. This assumption seems reasonable because the redox-linked ionization effect described here is most likely propagated through electrostatic interactions between the flavin and His98. Because the FMN in the blue neutral semiquinone form, like the oxidized state, does not carry a net charge, we suggest that the pK_a values will not differ appreciably between these two redox states. Also, it was established spectrophotometrically that the semiquinone is maintained in its blue neutral form under the conditions and throughout the pH range used in this study. Given this assumption, the experimental data conform very well to the theoretical curve generated by the redox-linked ionization model using the experimental values for the pK_a^{SQ} (7.02) and for the pK_a^{HQ} (8.43) for His98 generated in this study and an E_o value of −242 mV, where E_o represents the midpoint potential when the ionizable group is in the fully protonated state (Figure 9). In our previous report, similar pK_a values were assigned to His98 through the redox-linked model; however, direct experimental proof of this assignment was not available (Swenson & Krey, 1994).

DISCUSSION

The differential stabilization/destabilization of the various redox states of the flavin cofactor through both short- and long-range electrostatic interactions with ionizable amino acid residues within and around the cofactor binding site represents an important mechanism in the modulation of the one-

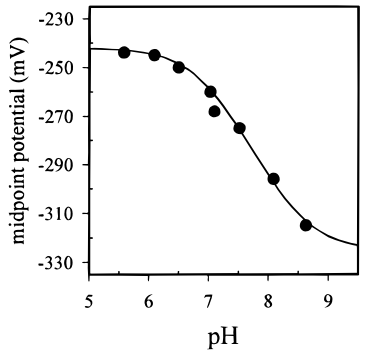
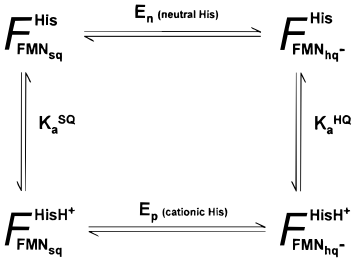


FIGURE 9: Experimental values for the midpoint potential for the sq/hq couple of the Y98H mutant (●) as a function of pH [data from Swenson & Krey (1994)] and the fit (solid line) to the redox-linked ionization model described under Results using the experimental values for the pK_a of His98 of 7.02 ± 0.08 and 8.43 ± 0.11 in the semiquinone (pK_a^{SQ}) and hydroquinone states (pK_a^{HQ}), respectively, from this study and a value of −242 mV for E_0.

Scheme 1: Equilibria Linking the Ionization of His98 to Shifts in the Midpoint Potential for the sq/hq Couple in the Y98H Flavodoxin Mutant[a]



[a] E_n and E_p refer to the midpoint potentials for the sq/hq couple when His98 is either neutral or protonated, respectively. K_a^{SQ} and K_a^{HQ} represent the ionization constants for His98 in the one-electron (semiquinone) and fully reduced (hydroquinone) states, respectively.

electron reduction potentials of the cofactor. Both favorable and unfavorable electrostatic interactions have been demonstrated to have significant effects on the midpoint potentials of the FMN cofactor in the flavodoxin system. The placement of a basic residue immediately adjacent to the flavin by the substitution of Tyr98 with either a histidine or an arginine was found to substantially increase the one-electron reduction potential for this couple (Swenson & Krey, 1994).

In this study, we have experimentally established the pK_a of His98 in the oxidized and reduced states of the Y98H flavodoxin mutant in an effort to more directly support a general redox-linked ionization model for the regulation of the oxidation−reduction potentials of the flavin cofactor in flavoproteins. In such a model, the ionization properties of His98, if coupled to the reduction of the neutral FMN semiquinone to the hydroquinone anion, should be described by the linked equilibria shown in Scheme 1. This linkage predicts that the pK_a of His98 should be directly dependent on the redox state of the flavin and, concurrently, the pH-dependent shift in the midpoint potential of the sq/hq couple (ΔE_m) is determined by the difference in the pK_a values for this residue in each redox state according to

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

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

The pK_a of His98 established by NMR spectroscopy in this study was quite clearly dependent on the redox state of the FMN cofactor, providing direct experimental proof of the redox-linked ionization phenomenon. The pK_a shifted from a value of 7.02 ± 0.08 in the oxidized state to 8.43 ± 0.11 in the fully reduced state. Because the pK_a of His98 in the semiquinone state could not be determined directly by NMR due to paramagnetic line broadening, we were forced to assume that the pK_a is similar to that in the oxidized state, in our opinion, a reasonable assumption (see Results for justification). Given this assumption, the experimental pK_a values determined in this work correlate very well with the previous theoretical values and accurately predict the observed pH dependency of the midpoint potential of the sq/hq couple in this mutant. These results establish with greater certainty that it is the redox-linked ionization of His98 in this flavodoxin mutant that is responsible for the pH-dependent changes in the midpoint potential.

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

$$\Delta G = 2.303RT(\mathrm{p}K_a^{\mathrm{HQ}} - \mathrm{p}K_a^{\mathrm{SQ}})$$

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

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

It is of importance to note that the underlying mechanism of the redox-linked ionization model in which His98 modulates the oxidation−reduction potential of the FMN in the Y98H flavodoxin (Scheme 1) is conceptually different from that reported for the covalent flavin analog 8-α-(*N*-imidazolyl)riboflavin. This derivative is of physiological importance as it represents a model for an important class of flavoproteins with covalently bound flavin cofactors attached through either 8-α-($N^{\delta 1}$-histidinyl) or 8-α-($N^{\epsilon 2}$-histidinyl)flavin linkages. The pK_a values for the imidazole group of 8-α-(*N*-imidazolyl)riboflavin in all three redox states have been established through the analysis of the pH dependency of its oxidation−reduction potential and more directly by ^{1}H NMR spectroscopy of the oxidized and fully reduced states (6.0 ± 0.1 and 7.0 ± 0.1, respectively) (Williamson & Edmondson, 1985a,b). The increase in the pK_a is attributed to the change in the more direct through-bond inductive effect in going from the electron-withdrawing characteristics of the oxidized flavin to that of an electron-donating properties of the fully reduced flavin in this covalent analog. The pK_a of the imidazole in the semiquinone form is estimated to be intermediate between that of the oxidized and fully reduced states (Williamson & Edmondson, 1985a,b). Thus, the ionization of the imidazolyl group in such flavin adducts may represent an important means by which redox potentials are regulated in this class of covalently linked flavoproteins (Williamson & Edmondson, 1985a). For example, in the *E. coli* fumarate reductase, which contains an 8-α-($N^{\epsilon 2}$-histidinyl)FAD with an elevated midpoint potential, substitution of His44 (to which the FAD is attached) with other amino acids results in an enzyme that still retains the ability to bind FAD tightly but noncovalently yet is unable to oxidize succinate, presumably because the redox potential of the flavin has now become too negative, although no direct evidence was given (Blaut *et al.*, 1989).

The situation presented in this study is fundamentally different from that of the 8-α-(*N*-histidinyl)linked flavoproteins. In our case, the effect of the ionization of His98 are necessarily indirect, involving through-space electrostatic interactions rather than more direct through-bond inductive effects. Thus, the Y98H flavodoxin mutant provides a good example of perhaps the more general redox-linked ionization phenomenon in which ionizable groups adjacent to the flavin cofactor in flavoproteins indirectly modulate the reduction potential of the flavin. This is likely to be a relatively common situation in flavoproteins. Such groups could represent catalytically essential amino acid residues in the active site of the flavoenzyme that as a consequence of their participation in acid−base catalysis change ionization state during the catalytic cycle. A good example is found in flavocytochrome b_2 (yeast L-lactate dehydrogenase) and the structurally (and perhaps mechanistically) related enzymes glycolate oxidase and L-lactate oxidase (Ghisla & Massey, 1991). The active-site histidine [His373, His254, and His290 in flavocytochrome b_2, glycolate oxidase, and L-lactate oxidase (by homology), respectively] in these flavoenzymes is stacked in a coplanar fashion over the pyrimidine ring of the *si* face of the FMN in a manner not unlike that in the Y98H flavodoxin (Lindqvist & Brändén, 1989; Xia & Mathews, 1990; Ghisla & Massey, 1991; Stockman *et al.*, 1994). The replacement of His373 by a glutamine residue

in flavocytochrome b_2 by site-directed mutagenesis provides evidence that this residue is essential for activity, serving as a general base and perhaps influencing electron transfer between substrate and the flavin (Gaume *et al*, 1995). Various experiments suggest that the pK_a of this histidine is dramatically increased upon reduction of the FMN such that proton exchange from the imidazolium cation is very slow, leading to estimates of the pK_a as high as 10 or greater. Such a large shift is very unusual and needs to be confirmed; however, it apparently has not been possible to measure its pK_a directly by NMR spectroscopy because of the size of this protein (Lederer, 1992; Balme & Lederer, 1994). These observations have led to the conclusion that His373, after assisting proton abstraction from substrate, facilitates the reduction of the FMN through favorable ion-pairing interaction with the flavin hydroquinone.

The studies reported for the Y98H flavodoxin mutant seem to provide a good model for certain features of this type of mechanism, providing direct experimental evidence for the redox-linked ionization aspect and a measure of the magnitude of such effects (Swenson & Krey, 1994; this work). In this protein, it has been possible to directly determine the extent of the substantial shift in the pK_a of His98 upon reduction of the flavin and to attribute the substantial pH-dependent increase in the midpoint potential of the bound FMN of nearly 100 mV to the favorable ion-pairing interactions between the protonated His98 and the flavin hydroquinone anion as required by a redox-linked ionization mechanism. Such an increase should significantly facilitate the entry of electrons into the flavin. One important structural difference in the Y98H flavodoxin mutant must be considered, however. Located on the surface of the Y98H flavodoxin, His98 is much more exposed to solvent than is likely to be the situation in the active center of a flavoenzyme (Stockman *et al*., 1994). Therefore, the magnitude of the pK_a and midpoint potential shifts observed in the Y98H flavodoxin, while substantial, may actually be more modest than could occur within an active-site cavity. The more restricted solvent exposure of His373 in reduced flavocytochrome b_2 could explain its substantially higher pK_a; however, hydrogen bonding to Asp282 and perhaps its closer proximity to the N1/C2O region of the FMN, which carries the formal charge of the hydroquinone anion, may also contribute (Balme & Lederer, 1994). Whether these and other interactions are significant enough to raise the pK_a of His373 to values greater than 10 when the flavin is reduced remains to be established.

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

Thus, both short- and long-range electrostatic interactions must be considered as part of an important general mechanism for the modulation of reduction potentials in flavoproteins and other redox systems. The deviation of the pH dependency of the midpoint potentials from that expected for the direct protonation of the flavin upon reduction in various flavoproteins has been reported and generally explained by the influence of ionizable groups in the flavin binding site. It is likely, therefore, that the redox-linked ionization mechanism is of general significance in flavoenzymes, and the thorough characterization of this phenomenon is of importance.

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