pH-Dependent Spectroscopic Changes Associated with the Hydroquinone of FMN in Flavodoxins†

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ABSTRACT: Photoreduction with a 5-deazaflavin as the catalyst was used to convert flavodoxins from *Desulfovibrio vulgaris*, *Megasphaera elsdenii*, *Anabaena* PCC 7119, and *Azotobacter vinelandii* to their hydroquinone forms. The optical spectra of the fully reduced flavodoxins were found to vary with pH in the pH range of 5.0−8.5. The changes correspond to apparent pK_a values of 6.5 and 5.8 for flavodoxins from *D. vulgaris* and *M. elsdenii*, respectively, values that are similar to the apparent pK_a values reported earlier from the effects of pH on the redox potential for the semiquinone−hydroquinone couples of these two proteins (7 and 5.8, respectively). The changes in the spectra resemble those occurring with the free two-electron-reduced flavin for which the pK_a is 6.7, but they are red-shifted compared with those of the free flavin. The optical changes occurring with flavodoxins from *D. vulgaris* and *A. vinelandii* flavodoxins are larger than those of free reduced FMN. The absorbance of the free and bound flavin increases in the region of 370−390 nm ($\Delta\epsilon = 1-1.8$ mM^{-1} cm^{-1}) with increases of pH. Qualitatively similar pH-dependent changes occur when FMN in *D. vulgaris* flavodoxin is replaced by iso-FMN, and in the following mutants of *D. vulgaris* flavodoxin in which the residues mutated are close to the isoalloxazine of the bound flavin: D95A, D95E, D95A/D127A, W60A, Y98S, W60M/Y98W, S96R, and G61A. The ^{13}C NMR spectrum of reduced *D. vulgaris* [2,4a-$^{13}C_2$]FMN flavodoxin shows two peaks. The peak due to C(4a) is unaffected by pH, but the peak due to C(2) broadens with decreasing pH; the apparent pK_a for the change is 6.2. It is concluded that a decrease in pH induces a change in the electronic structure of the reduced flavin due to a change in the ionization state of the flavin, a change in the polarization of the flavin environment, a change in the hydrogen-bonding network around the flavin, and/or possibly a change in the bend along the N(5)−N(10) axis of the flavin. A change in the ionization state of the flavin is the simplest explanation, with the site of protonation differing from that of free FMNH$^-$. The pH effect is unlikely to result from protonation of D95 or D127, the negatively charged amino acids closest to the flavin of *D. vulgaris* flavodoxin, because the optical changes observed with alanine mutants at these positions are similar to those occurring with the wild-type protein.

The flavodoxins (*1*−*3*) are small microbial proteins that contain a molecule of FMN and that function as electron carriers at an oxidation−reduction potential close to that of the hydrogen electrode (−0.413 V at pH 7 and 25 °C). They achieve the low potential by shifting the redox potentials of the two one-electron steps in the reduction of flavin (eqs 1 and 2).[1]

$$\mathrm{FMN} + \mathrm{H^+} + 1\mathrm{e^-} \overset{E_2}{\longleftrightarrow} \mathrm{FMNH^\bullet} \quad (1)$$

$$\mathrm{FMNH^\bullet} + 1\mathrm{e^-} \overset{E_1}{\longleftrightarrow} \mathrm{FMNH^-} \quad (2)$$

The value for E_1 is −0.172 V for free FMN; however, depending on the protein, it varies between −0.372 and −0.518 V when FMN is bound to apoflavodoxin, and it is therefore considered likely that in low-potential reactions the flavodoxins operate by cycling between the semiquinone and hydroquinone forms of the flavin.

The shifts in redox potential can be related to changes in the strength of interaction between the flavin and protein (*1*−*4*). The semiquinone form of the flavin is bound more tightly than the oxidized flavin, while binding of the hydroquinone is relatively weak. The three-dimensional structures of flavodoxins change very little when the bound FMN is reduced from the semiquinone to the hydroquinone (*5*−*10*), and therefore, other explanations have been considered to account for destabilization of the hydroquinone. It was first proposed that since the isoalloxazine moiety of the FMN in flavodoxins from *Clostridium beijerinckii* MP and *Desulfovibrio vulgaris* is almost flat while the first structures of reduced flavins determined by X-ray crystallography were found to be bent along the N(5)−N(10) axis (*11*), the free energy required to hold the flavin flat in the protein might help to destabilize the hydroquinone complex (*12*). This theory was rejected when subsequent NMR measurements with different flavin models suggested that

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[1] Abbreviations: FMNH•, neutral FMN semiquinone; FMNH$^-$, anion of FMN hydroquinone; $FMNH_2$, neutral form of FMN hydroquinone; 5-deazaflavin, 3-methyl-5-deazalumiflavin.

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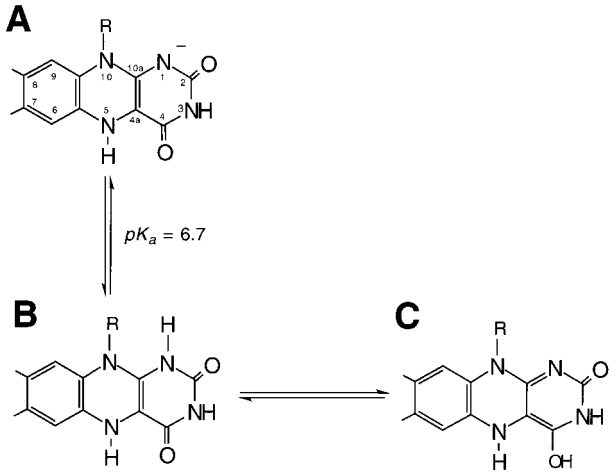

FIGURE 1: Possible structures of FMN hydroquinone. Structures **A** and **B** occur with free FMN in aqueous solvent. R refers to ribityl phosphate.

the hydroquinone of free flavins is also flat (*13*, *14*). However, more recent ab initio calculations for the structures of free flavin have suggested that while the anionic form of the hydroquinone is flat, the neutral form is bent (puckering angle of 27.6°) and in rapid butterfly-like motion because of a low intrinsic barrier to inversion (*15*).

Charge repulsion between the flavin and the protein has also been proposed as a contributory factor in destabilization of the hydroquinone. The side chains of acidic amino acids are close to the FMN, and the flavin itself carries two negative charges on the phosphate group, for which there are no compensating positive charges on the protein. In the anionic form of the hydroquinone, an additional negative charge is associated with the N(1)C(2)O region of the isoalloxazine (Figure 1, structure **A**). Moonen et al. (*16*) suggested that interaction between the charged phosphate group and the charge around N(1) might help to destabilize the hydroquinone complex. It was shown that the redox potential of the complex of apoflavodoxin from *Megasphaera elsdenii* and riboflavin 3′,5′-bisphosphate, an FMN analogue with additional negative charge on the side chain, is more negative than that of native flavodoxin (*17*). However, Zhou and Swenson (*18*) showed subsequently that when a histidine residue is incorporated into flavodoxin from *D. vulgaris* to neutralize negative charge on the phosphate, the resulting positive shift in the redox potential is small and is smaller than might be expected if negative charge on the phosphate has a unique role in destabilizing the hydroquinone.

Ludwig et al. (*19*) suggested that an important destabilizing interaction in flavodoxins from *M. elsdenii* and *C. beijerinckii* involves the charge at the flavin N(1)C(2)O region and the charge on the side chain of the nearest glutamate residue (E60 and E59 in flavodoxins from *M. elsdenii* and *C. beijerinckii*, respectively). The FMN-binding site of flavodoxin from *D. vulgaris* is rather different, and it does not contain a negatively charged side chain at the equivalent structural position. Swenson and colleagues (*18*, *20*−*22*) have suggested that destabilization of the hydroquinone in this flavodoxin results in part from the cumulative effects of charge interactions with several negatively charged amino acids near the flavin and on the surface of the protein, and that the charge interactions are enhanced by the nonpolar nature of the FMN-binding site (*22*). It was further proposed that aromatic stacking interactions between the flavin and the side chain of the tyrosyl residue that flanks the outer face of *D. vulgaris* flavodoxin also help to preferentially destabilize the hydroquinone complex (*22*).

Electrostatic destabilization of the hydroquinone requires that this redox form of the flavin carry a negative charge not carried by the quinone and semiquinone. There is evidence that the pK_a of 6.7 for the ionization of free flavin hydroquinone at N(1), according to structures **A** and **B** in Figure 1 (*23*), is decreased when FMN is bound by apoflavodoxins. Measurements by ^{13}C and ^{15}N NMR spectroscopy have suggested that the isoalloxazine of the fully reduced flavodoxins remains as the anion at pH values below the pK_a of the free flavin (*24*−*27*); measurements of the optical spectrum of the hydroquinone of *M. elsdenii* flavodoxin led to the conclusion that the spectrum remains essentially unchanged down to pH 4.6 (*19*), and crystallographic measurements on flavodoxin from *C. beijerinckii* suggested that protonation at N(1) is unlikely for steric reasons (*19*).

In view of the evidence that the flavin hydroquinone in flavodoxins does not protonate, we were surprised to detect large changes with pH in the optical spectrum of the hydroquinone of *D. vulgaris* flavodoxin (*28*). The optical measurements have now been extended to three other flavodoxins, to mutants of *D. vulgaris* flavodoxin, and to the complex of the apoprotein of this flavodoxin with iso-FMN. We have also examined the effects of pH on the ^{13}C NMR spectra of the complex of apoflavodoxin from *D. vulgaris* and [2,4a-$^{13}C_2$]FMN. In all cases, pH-dependent changes are observed.

MATERIALS AND METHODS

Preparation of Flavodoxins and Apoflavodoxin. Flavodoxin from *D. vulgaris* (Hildenborough) was prepared as the recombinant protein (*29*). Mutants of this flavodoxin were prepared by site-directed mutagenesis of the recombinant gene (*30*−*32*). Flavodoxin from *M. elsdenii* LC1 was prepared as described previously (*33*) except that phosphate buffers at pH 7 were used instead of Tris-HCl buffers at pH 8. Flavodoxin from *Anabaena* PCC 7119 was a gift from M. Fillat. Flavodoxin from *Azotobacter vinelandii* (ATCC 478) was purified from cells grown under nitrogen-fixing conditions (*34*). The apoprotein of flavodoxin from *D. vulgaris* was made by treatment of the holoprotein with 5% (w/v) trichloroacetic acid in the presence of EDTA (*35*).

Photoreduction of Free Flavins and Flavodoxin. FMN and iso-FMN were photoreduced in an all-glass anaerobic spectrophotometer cuvette that was fitted with a combination pH micro electrode through a glass joint, and that allowed the anaerobic addition of acid or base from a syringe (*36*). The cuvette contained in a final volume of 3 mL 30 μM FMN or iso-FMN, 25 mM potassium phosphate, and 25 mM sodium acetate (pH 8.5), 3 mM EDTA, and 1.2 μM 3-methyl-5-deazalumiflavin (a gift from F. Müller). A sidearm attached to the cuvette contained in a final volume of 0.6 mL 500 μM methyl viologen, 3 mM EDTA, 100 mM Tris-HCl buffer (pH 8), and 1.2 μM 3-methyl-5-deazalumiflavin. The cuvette and contents were made anaerobic by evacuation with a vacuum pump and filling with N_2 that had been purified by passage over BASF catalyst (R3-11) at 120 °C and bubbling

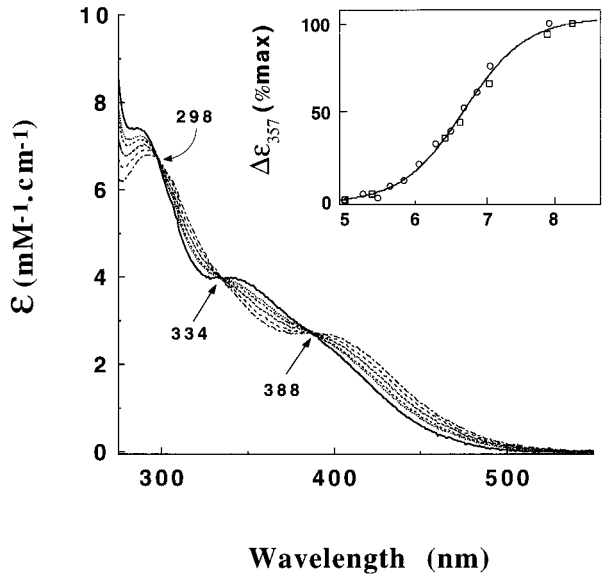

FIGURE 2: Effects on pH on the spectrum of the hydroquinone of FMN at (—) pH 7.85 and (− - −) pH 4.98. Other curves show spectra at intermediate pH values. Some of the spectra from the titration have been omitted for clarity. The inset shows a plot of the change in the extinction coefficient at 357 nm versus pH with (○) additions of acid and (□) additions of base. The line through the data points is for a system with a pK_a of 6.7.

through a solution of photoreduced methyl viologen (*36*). The evacuation and filling with N_2 was repeated eight times over the course of 30 min while gently shaking the cuvette. The pH electrode was then inserted while flushing the cuvette with N_2, followed by the syringe filled with anaerobic 1.7 M acetic acid. The methyl viologen in the sidearm was photoreduced to provide a sink for residual oxygen, before photoreducing the flavin in the main compartment using short periods of exposure of the cuvette to light from a 250 W slide projector. The course of the reduction was monitored spectrophotometrically, and the irradiation was stopped when the flavin had been converted to the hydroquinone. The spectrum was recorded. The pH was then changed stepwise by addition of acid, and the pH and the absorption spectrum were recorded at each step. When the pH reached 4.94, the acid in the syringe was replaced by anaerobic 1 M KOH, and the pH titration continued until the pH was 8.17. A slight reoxidation of the flavin occurred after each addition of acid or base (1−2% of the flavin), especially at high pH values. The flavin was converted back to the hydroquinone by a short exposure to light from the projector. The data were corrected for dilution, and a plot of $\Delta\epsilon_{357}$ versus pH was made. A value for the pK_a was obtained by fitting the data with the Henderson−Hasselbach equation using the program MacCurveFit. The zero point on the ordinate in the plots of Figures 2, 3, 6, and 7 represents the observed extinction coefficient at the lowest pH value used.

Flavodoxin was photoreduced in a manner similar to that of the free flavins except that a different experiment was carried out at each pH value. The cuvette contained in a final volume of 1 mL wild-type flavodoxin or a mutant of flavodoxin (approximately 37 μM), 0.1 M buffer, 1.2 μM 3-methyl-5-deazalumiflavin, and 3 mM EDTA. The buffers used were sodium acetate (pH 5.0−5.7), potassium phosphate (pH 6.0−7.5), and sodium pyrophosphate-HCl buffer (pH 8.0−9.5). It was observed that photoirradiation of flavodoxin hydroquinone results in its slow conversion to semiquinone. The explanation for this secondary reaction is not known. It might result from the slow accumulation of a photoreaction product that forms a redox equilibrium with the two reduced forms of flavodoxin. The rate of the secondary reaction is very low, and its effects were minimized by exposing flavodoxin to only brief periods of light and monitoring the extent of reduction by spectrophotometry. The irradiation was stopped when the semiquinone had just been converted to hydroquinone. The time required for full reduction is about 1 min at pH 8 and about 2 min at pH 5. Most photoreduction experiments were carried out at 25 °C; to increase the stabilities of the flavodoxin mutants, measurements on some of them were taken at 10 °C as indicated.

Flavodoxin was also photoreduced in a 10 mm diameter NMR tube that was fitted with a ground glass socket (standard taper 14/23). The tube contained 1 mM [2,4a-$^{13}C_2$]-FMN (a gift from F. Müller) bound to apoflavodoxin from *D. vulgaris*, 3 mM EDTA, 50−97 μM 3-methyl-5-deazalumiflavin, 20 mM sodium acetate, potassium phosphate, or sodium pyrophosphate-HCl buffer at the indicated pH, and 25% (v/v) ^2H_2O to obtain a deuterium lock signal; the final volume was 2 mL. The 5-deazaflavin was dissolved in methanol so that the samples contained 2.5−8.0% (v/v) methanol. The tube was fitted with a cone and glass extension through which the NMR tube was made anaerobic as described above. After the tube had been filled with N_2 at atmospheric pressure, the tube was sealed by melting the glass extension tube. Photoreduction of the flavin or flavoprotein was then carried out as described above, using optical spectroscopy to monitor the course of reduction.

Preparation of Iso-FMN Flavodoxin. Apoflavodoxin from *D. vulgaris* [0.62 mM in 10 mM sodium acetate buffer (pH 6), 0.2 M NaCl, and 0.3 mM EDTA] was mixed with a slight molar excess of iso-FMN (a gift from P. Hemmerich) that had been purified by treating it with the FMN-specific apoflavodoxin from *M. elsdenii* and extracting the purified flavin with trichloroacetic acid as described above. The complex with *D. vulgaris* apoflavodoxin was treated on a column of Sephadex G25, concentrated by ultrafiltration, and finally dialyzed versus 20 mM potassium phosphate buffer (pH 7) and 0.3 mM EDTA.

NMR Spectroscopy. NMR spectra at 1.88 T were recorded with a Bruker WP80 wide-bore spectrometer operating at 20.115 MHz for ^{13}C nuclei. The pulse sequence of Belton et al. (*37*) was used to minimize acoustic ringing. The spectral conditions were as follows: 4096 time domain data points, spectral width of 240 ppm, acquisition time of 0.426 s, 0.4 s relaxation delay time, and 1 W broad-band ^1H decoupling. All spectra were zero-filled to give 8192 data points. The sample temperature was 27 ± 1 °C.

RESULTS

Reduction of Flavodoxin. The redox potential of the semiquinone−hydroquinone couple (E_1) of flavodoxins varies with pH. It is independent of pH at high pH (>7.5), indicating that the redox reaction does not involve a change in the protonation state of the protein−flavin complex. At low pH, the line approaches a slope of −0.06 V/pH unit,

Table 1: Light Absorption Characteristics and Apparent pK_a Values of Fully Reduced FMN, Iso-FMN, and Flavodoxins[a]

	pH	wavelength maxima (mM^{-1} cm^{-1})	wavelength isosbestic points (mM^{-1} cm^{-1})	pK_a optical	pK_a redox
FMN[b]	7.8	288 (3.79), 342 (3.98), 390 sh (2.68)	298 (6.72), 334 (3.98), 388 (2.69)	6.7 ± 0.02	6.7[d]
	5.0	291.5 (6.77), 398 (2.66)			
iso-FMN[b]	8.2	287 (7.78), 340 sh (3.67), 375 sh (2.31)	299 (6.93), 328 (4.11), 377 (2.28)	6.5 ± 0.02	ND
	5.0	292 (7.02), 397 (2.49)			
flavodoxin					
D. vulgaris					
wild-type[b]	8.5	306 (8.86), 357 (4.86), 422 sh (2.03)	327 (6.22), 348 (4.83), 417 (2.11)	6.5 ± 0.07	7.0[e]
	5.2	315 (6.66), 414 (2.24)			
D95E[c]	8.5	312 sh (8.31), 357 sh (4.82), 425 sh (1.94)	325 (6.50), 351 (4.84), 406 (2.30)	6.3 ± 0.1	ND
	5.2	312 sh (6.88), 416 sh (2.29)			
D95A[c]	8.0	311 sh (8.87), 356 (5.09), 419 sh (2.02)	326 (6.70), 342 (5.21), 405 (2.23)	6.1 ± 0.05	6.5[f]
	5.2	311 (7.53), 412 sh (2.31)			
D95A/D127A[c]	8.5	309 sh (8.90), 358 (5.07), 413 (2.21)	329 (6.11), 344 (4.99), 405 (2.39)	6.2 ± 0.08	6.7[f]
	5.2	310 sh (7.03), 413 (2.38)			
S96R[b]	8.5	314 sh (7.19), 356 (4.52), 417 sh (1.91)	323 (6.20), 349 (4.53), 402 (2.20)	6.0 ± 0.05	ND
	5.2	315 (6.38), 416 (2.20)			
Y98S[b]	8.5	313 sh (8.47), 359 (4.99), 419 sh (1.93)	325 (6.21), 344 (4.69), 390 (2.92)	6.2 ± 0.20	5.6[g]
	5.2	312 sh (7.88), 362 sh (4.36), 416 sh (2.23)			
W60A[b]	8.5	311 sh (8.63), 365 (4.92), 437 sh (1.46)	330 (5.91), 357 (4.79), 412 (1.87)	6.0 ± 0.10	6.1[g]
	5.5	313 sh (7.29), 365 sh (4.55), 437 sh (1.59)			
W60M/Y98W[c]	8.0	314 sh (7.42), 361 (4.78), 426 sh (1.63)	332 (5.25), 345 (4.68), 404 (2.19)	6.8 ± 0.30	6.4[f]
	6.0	364 sh (3.91), 417 sh (1.98)			
G61A[c]	8.5	305 sh (7.28), 3.57 (3.83), 414 sh (1.93)	309 (6.47), 351 (3.81), 395 (2.40)	6.6 ± 0.10	6.9[g]
	5.5	309 sh (6.64), 414 (2.35)			
wild-type[b] (iso-FMN)	9.5	347 sh (3.79), 405 (1.64)	383 (1.71)	6.7 ± 0.10	ND
	5.2	327 sh (4.38), 407 (2.14)			
M. elsdenii[b]	7.5	314 (8.17), 367 (5.24), 463 sh (1.42)	329 (6.37), 370 (5.25), 431 (1.75)	5.8 ± 0.05	5.8[h]
	5.0	316 (7.10), 350 (5.73), 442 (1.77)			
A. vinelandii[b]	7.9	310 sh (10.25), 370 (5.35), 460 sh (1.30)	333 (7.36), 349 (5.23), 417 (2.53)	ND	ND
	6.0	315 sh (8.91), 370 sh (3.56), 435 sh (2.30)			
Anabaena PCC 7119[b]	8.0	307 sh (6.89), 359 (376), 406 sh (2.23)	310 (5.38), 336 (4.43), 423 (1.55)	ND	6.1[i]
	5.3	340 sh (3.36), 414 sh (1.84)			

[a] sh refers to shoulder. ND means not determined. [b] At 25 °C. [c] At 10 °C. [d] Ref *23*. [e] Ref *29*. [f] Ref *32*. [g] Ref *31*. [h] Ref *38*. [i] Ref *49*.

consistent with the net addition of one proton in forming the hydroquinone from the semiquinone (*1*, *2*, *29*, *33*, *38*). The apparent pK_a calculated from this change in slope varies with the flavodoxin in the range of 5.8−7.0 (*1*, *2*). At low pH values, the potential is more negative than that of the hydrogen electrode, and in this pH region, it is difficult to reduce the flavodoxins fully. Dithionite ion was used to obtain the flavodoxin hydroquinone in earlier NMR studies. However, dithionite ion does not fully reduce the bound flavin at low pH (*36*). Massey and Hemmerich (*39*) showed that photochemical reduction using EDTA as the photooxidizable substrate and a 5-deazaflavin as the photocatalyst leads to full reduction of flavodoxins, a reaction that occurs with *D. vulgaris*, *A. vinelandii*, and *M. elsdenii* flavodoxins even at low pH values (*36*). It was the method chosen for the study described here.

Effects of pH on the Optical Spectra of FMN and Native Flavodoxins. The absorption spectra of the four oxidized flavodoxins differ from each other, and they also differ from that of FMN, reflecting differences that are known to exist between the flavin-binding sites of the flavodoxins, and between the polar environment of FMN in free aqueous solution and the hydrophobic environment of the flavin in the protein (*1*, *2*). None of the spectra are appreciably affected by pH in the pH range of 5.0−8.5. The spectra of the semiquinone forms of the flavodoxins also differ from each other, and they are similarly independent of pH. The extent of semiquinone formation at half-reduction decreases with increasing pH in accordance with the decrease in the difference in the redox potentials of the two one-electron reduction steps for flavodoxin. However, even at pH 8.5, the semiquinone formed at half-reduction of flavodoxin from *D. vulgaris* for example is 97% of the maximum (*29*). The absorption spectrum of the fully formed semiquinone of all four flavodoxins indicates that the semiquinone remains in its neutral form to high pH. The extent of semiquinone formation at half-reduction of free FMN is very much smaller than that which occurs with flavodoxins (*23*), and the semiquinone cannot be detected by the optical method used in the work described here; the spectrum of FMN changes smoothly to that of the hydroquinone during photochemical reduction. At high FMN concentrations, the optical changes are more complex due to the formation of various intermediates (*40*). The absorption spectrum of FMN hydroquinone that is formed at full reduction depends on pH (Figure 2) and on the solvent (*41*). In aqueous solution at pH 8.5, the absorption spectrum shows maxima at 288 and 342 nm characteristic of the flavin hydroquinone anion. The stepwise addition of acid to FMN hydroquinone causes the spectrum to change to that characteristic of the neutral form, and the changes correspond to a pK_a value of 6.7 (inset of Figure 2 and Table 1), in agreement with earlier redox (*23*) and NMR (*42*) data.

The spectra of the hydroquinone forms of the four flavodoxins also change with pH. It was not possible to carry out pH titrations of the fully reduced flavodoxins because this form of the protein tends to reoxidize to the semiquinone when acid or base is added under anaerobic conditions, and

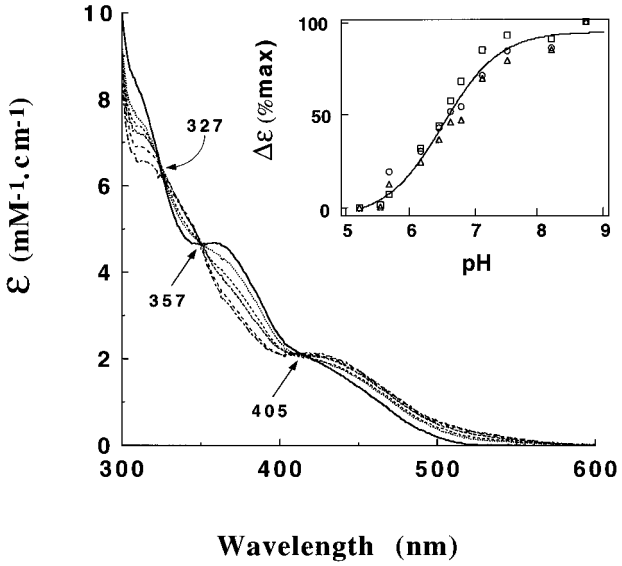

FIGURE 3: Effects of pH on the spectrum of the hydroquinone of flavodoxin from *D. vulgaris* at (—) pH 8.73 and (− - −) pH 5.23. Other curves show spectra at intermediate pH values. The inset shows a plot of the change in the extinction coefficient at (△) 307, (□) 370, and (○) 480 nm versus pH. The data points are fitted with a theoretical line for a system with a pK_a of 6.5.

subsequent light irradiation does not always return the bound flavin to the hydroquinone. Therefore, the spectrum of the hydroquinone at each pH value was obtained after photoreduction of a different sample of protein. Comparison of the spectra of fully reduced flavodoxin from *D. vulgaris* at different pH values shows that while at high values the spectrum is characterized by a maximum at 362 nm and shoulders at 306 and 420 nm, the spectrum at the lowest pH value tested has maxima at 315 and 415 nm (Figure 3). Intermediate spectra pass through three isosbestic points, showing that only two light-absorbing species are present at each pH. The pK_a value calculated by curve fitting of the changes at 307, 370, and 480 nm is 6.5 ± 0.1 (inset of Figure 3 and Table 1). The absolute spectra of the flavodoxin hydroquinone at high and low pH resemble those of the anionic and neutral forms of the hydroquinone of free FMN, but the protein spectra are shifted to longer wavelengths. The differences between the spectra at high and low pH values are clearly shown in spectra obtained by subtraction of the spectrum at the lowest pH value from the spectrum at the highest pH (Figure 4 and Table 1). The spectroscopic changes revealed in the difference spectrum for the protein-bound flavin are again similar in shape to those of the free flavin, but they are shifted to longer wavelengths and have greater intensities.

Photoreduction with EDTA was used to reduce the flavodoxins, and it seemed possible that this method might modify the flavin and/or the apoprotein, and that the modification might result in the pH dependency of the hydroquinone spectrum. However, there is no evidence for such a modification reaction. Addition of air to photoreduced flavodoxins causes rapid regeneration of the flavin semiquinone, followed by slow reformation of the oxidized flavin, as described previously (*29*, *33*). The spectrum of the reoxidized material is identical to that of untreated flavodoxin. In addition, when FMN was extracted from *D. vulgaris* flavodoxin that had been photoreduced at low pH,

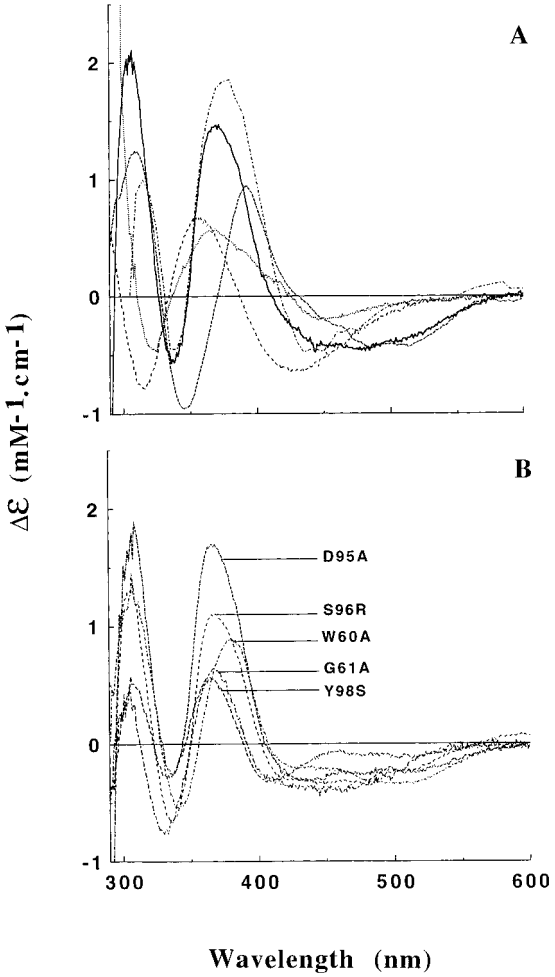

FIGURE 4: Comparison of the difference spectra between the hydroquinone forms of FMN and flavodoxins at high and low pH. Difference spectra for FMN and flavodoxins were generated by subtraction of the spectra at low pH in Figures 2, 3, and 5−7 from the corresponding spectra at high pH: (A) (- - -) FMN, (—) *D. vulgaris*, (-•-) *A. vinelandii*, (- - -) *M. elsdenii*, and (.....) *Anabaena* flavodoxins and (B) mutants of *D. vulgaris* flavodoxin as indicated.

and then added to untreated apoflavodoxin, subsequent photoreduction of the new complex at high pH proceeded exactly as described above. A similar control experiment showed that when untreated FMN is added to apoflavodoxin recovered from flavodoxin that has been subjected to a cycle of photoreduction, subsequent photoreduction of the complex is again as described above. We conclude that the pH-dependent changes in the spectrum are not due to a pH-dependent covalent modification of either the flavin or the protein.

pH-dependent optical changes are also associated with the hydroquinone of flavodoxins from *A. vinelandii* and *Anabaena* (Figure 5). The absolute spectra of the two forms of the hydroquinone of *A. vinelandii* flavodoxin and the differences between them are similar to those observed with *D. vulgaris* flavodoxin. The data for the *A. vinelandii* protein were obtained 20 years ago during the course of other studies (*36*), and they are not sufficient to determine the apparent pK_a for the change; from the pH range over which the changes occur, the pK_a appears to be similar to that of *D. vulgaris* flavodoxin. The hydroquinone of *Anabaena* flavodoxin has weaker absorption bands than the other flavodoxins, and its band at the longest wavelength is shifted

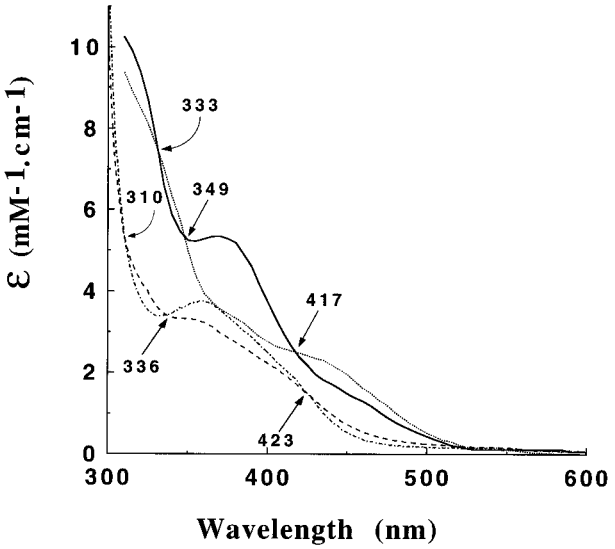

FIGURE 5: Effects of pH on the spectrum of the hydroquinone of flavodoxins from *A. vinelandii* at (—) pH 7.93 and (•••) pH 6.0 and *Anabaena* at (-•-) pH 8.0 and (- - -) pH 5.3.

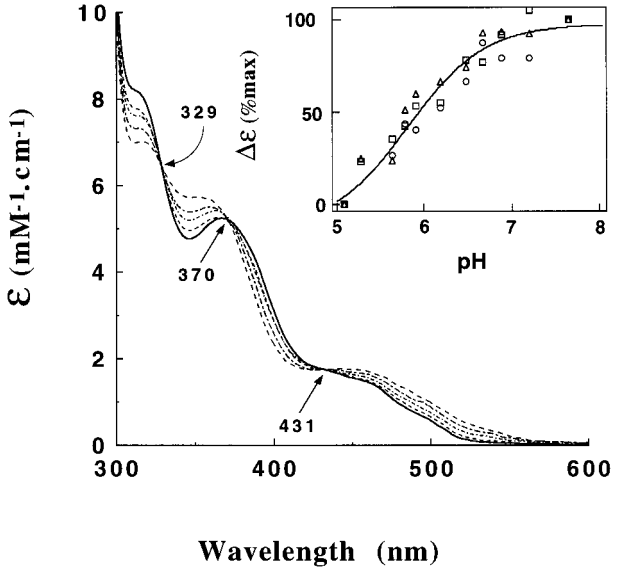

FIGURE 6: Effects of pH on the spectrum of the hydroquinone of flavodoxin from *M. elsdenii* at (—) pH 7.62 and (- - -) pH 5.08. Other curves show spectra at intermediate pH values. The inset shows a plot of the change in the extinction coefficient at (○) 309, (□) 345, and (△) 392 nm versus pH. All of the data points were used in fitting the theoretical line for a system with a pK_a of 5.8.

hypsochromically, as is the difference spectrum between the spectra at high and low pH. The spectrum at high pH is quite similar to that of free $FMNH^-$. A value for the pK_a was not determined for *Anabaena* flavodoxin.

Qualitatively similar changes with pH occur in the optical spectrum of the hydroquinone of flavodoxin from *M. elsdenii*, but the changes span a pH region different from those of *D. vulgaris* flavodoxin. They conform to a single ionization, and the apparent pK_a is 5.8 ± 0.1 (Figure 6). As in the case of the other flavodoxins, the spectra of the oxidized and semiquinone forms of *M. elsdenii* flavodoxin do not change appreciably with pH in this range. However, the spectra of all three redox forms of this flavodoxin differ from those of *D. vulgaris* flavodoxin, reflecting differences in the amino acid side chains in the two proteins that form the flavin-binding site. The spectrum of the hydroquinone of *M. elsdenii* flavodoxin at high pH has a maximum at 367 nm, a shoulder at 314 nm, and several inflections in the longest wavelength band centered at about 450 nm; at low pH, the spectrum shifts to give maxima at 316, 350, and 442 nm, and the structure on the side of the longest wavelength band decreases. The peaks and troughs in the difference spectrum are at longer wavelengths than those in the difference spectra of the other three flavodoxins (Figure 4), as are the three isosbestic points. The observation that the optical spectrum of the hydroquinone of flavodoxin from *M. elsdenii* changes substantially with pH is in contrast to earlier reports (*19*, *41*). The changes that we observe are similar in intensity to those occurring with FMN hydroquinone, although the wavelengths of greatest change are different from those of the free flavin. While the apparent pK_a for the bound flavin is lower than that of free flavin, it is identical to the value reported earlier from measurements on the effects of pH on the oxidation−reduction potential for the semiquinone−hydroquinone couple of *M. elsdenii* flavodoxin (*38*).

The hydroquinone of flavodoxins is weakly fluorescent (*41*). This fluorescence was partly obscured by the fluorescence of the 5-deazaflavin used as the photocatalyst in the experiments described above. However, by measuring the fluorescence emission at 550 nm to minimize this interference, it was possible to observe that the shape of the fluorescence excitation spectrum due to *D. vulgaris* flavodoxin changes with pH as described for the absorption spectrum in Figure 3, with the main change being a decrease in the band around 370 nm (data not shown). Overall, the fluorescence intensity decreases by about 50% at low pH.

Mutants of D. vulgaris Flavodoxin. Changes similar to those described above with wild-type flavodoxin from *D. vulgaris* have been observed with a variety of mutant forms of this flavodoxin. Differences occur between the absolute absorption spectra of the hydroquinone forms of the wild-type flavodoxin and its mutants at high and low pH, in the extent of the change with pH, and in the pK_a (Table 1). However, the shapes of the difference spectra generated with the mutants are not too different from that exhibited by the wild-type protein (Figure 4). Of particular interest are the mutations which either affect the charge close to the flavin (D95A, D95E, and the double mutant D95A/D127A) or change the two aromatic residues whose side chains flank the flavin (Y98S, W60A, and the double mutant W60M/Y98W). The absolute spectra of both forms of the hydroquinone in the first group are similar to those of wild-type flavodoxin. The spectra of the second group at high pH are similar to that of wild-type flavodoxin under similar conditions. However, their spectra at low pH show considerable variation. The extent of the change with pH in the spectrum of the Y98S mutant is smaller than is observed with the other mutants, perhaps reflecting a greater degree of exposure of the flavin in this mutant to solvent. The spectra of the G61A mutant differ from those of wild-type flavodoxin at both extremes of pH, perhaps indicating a substantial change in the protein conformation as a result of this mutation, and as revealed in the oxidized protein by X-ray crystallography (*30*). None of the mutations has a particularly dramatic effect on the pH-dependent changes in the absorption spectrum of the hydroquinone, all of the difference spectra showing peaks and troughs in regions similar to those of wild-type fla-

vodoxin (Figure 4). This suggests that the optical change with pH is not specifically related to the side chains of these amino acids.

Complex of D. vulgaris Apoflavodoxin and Iso-FMN. The two methyl groups at the C(7) and C(8) positions on the benzene ring of FMN in flavodoxin are exposed to solvent. In iso-FMN, the methyl group at position C(8) is replaced by a methyl group at C(6). It was reasoned that this change in the flavin structure might affect the strength of the interaction of the flavin with apoflavodoxin such that the flavin environment in the protein would be changed. The change might alter the polarity of the environment, and this in turn could affect the pH-dependent changes in the absorption spectrum of the hydroquinone. The apoprotein of *D. vulgaris* flavodoxin was found to bind iso-FMN as tightly as FMN, with changes in the absorption spectrum of the flavin and quenching of the flavin fluorescence similar to those reported previously when iso-FMN is bound by apoflavodoxin from *M. elsdenii* (*4*, *43*). Photoirradiation of the complex in the presence of EDTA and 5-deazaflavin leads to the formation of flavin semiquinone followed by reduction of the semiquinone to hydroquinone. The absorption spectrum of the hydroquinone formed at full reduction depends on pH (Figure 7). A large decrease in absorption with a decrease in pH occurs at 351 nm ($\Delta\epsilon = 1.6$ mM^{-1} cm^{-1}). The apparent pK_a determined from the data is 6.7 and is therefore similar to that given by the native form of *D. vulgaris* flavodoxin.

Effects of pH on the ^{13}C NMR Spectrum of the Complex of D. vulgaris Apoflavodoxin and [2,4a-$^{13}C_2$]FMN. The observation that pH affects the optical spectra of the hydroquinone of flavodoxins suggests that protonation occurs either on the isoalloxazine moiety of the bound flavin or sufficiently close to this part of the flavin that it substantially changes the environment of the flavin. It was reasoned that the protonation might occur at N(1) of the flavin, as occurs with FMNH$^-$ in free solution but with different effects on the spectrum in accordance with the different environments provided by the protein. Alternatively, the pH effect might be due to an ionizable group or groups on the protein which cause either a change in the environment of the reduced flavin, a change in the structure of the flavin, such as an increase in the puckering angle at N(5)−N(10), or a change in a hydrogen-bonding interaction, such as those at N(1) and N(5)H (*6*). Changes of this kind should be able to be detected by NMR spectroscopy. ^{15}N NMR and suitable ^{15}N-enriched FMN was not available to us. However, we have carried out preliminary measurements using ^{13}C NMR and FMN that is enriched with ^{13}C at the C(2) carbonyl group of the isoalloxazine and at the C(4a) bridging carbon atom.

Photoreduction of *D. vulgaris* flavodoxin substituted with ^{13}C-enriched FMN results in the upfield shifts of the two peaks due to C(2) and C(4a) expected for reduction of the oxidized flavin to the hydroquinone (Figure 8A; *25*). The chemical shift and line width (102 ppm and 5.8 ± 0.9 Hz, respectively) of the peak due to C(4a) do not change when the pH of the photoreduction is decreased as low as pH 5.2, in agreement with an earlier report (*25*). The apparent increase in line width of this signal as the pH decreases (Figure 8A) is due to the fact that to optimize the signal-to-noise ratio for the C(2) signal the spectra were processed using an exponential weighting factor approximately matched to the line width of the C(2) signal. In contrast, the peak due to C(2) at 156 ppm was found to broaden with decreasing pH. The chemical shift of the peak does not change. A plot of the line width versus pH was found to fit a theoretical curve with a pK_a value of 6.2 (Figure 8B).

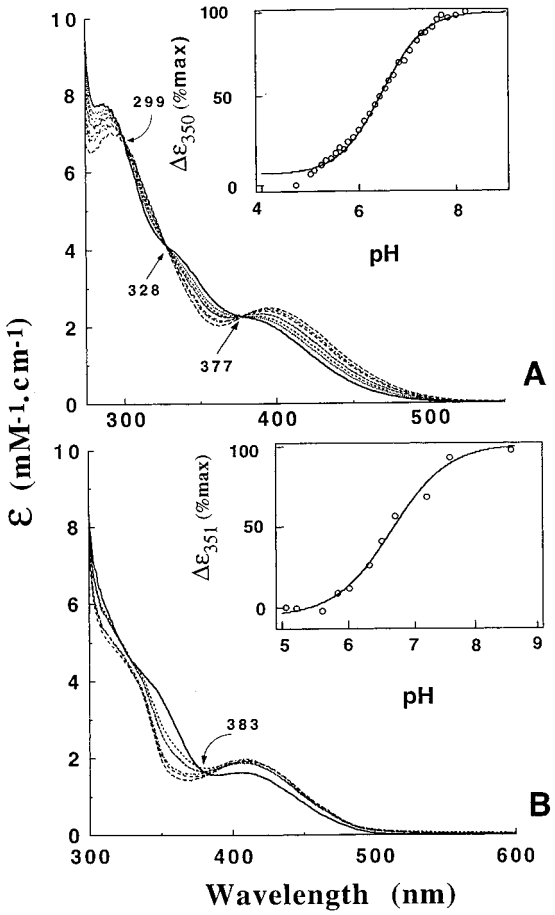

FIGURE 7: Effects of pH on the spectra of the hydroquinone of iso-FMN and the complex of iso-FMN and apoflavodoxin from *D. vulgaris*: (—) spectra at the highest pH and (- - -) spectra at the lowest pH for (A) iso-FMN and (B) the iso-FMN−apoflavodoxin complex. Other curves show some of the spectra obtained at intermediate pH values. The insets show plots of the change in the extinction coefficient at 350 (iso-FMN) or 351 nm (iso-FMN−apoflavodoxin) versus pH. The data points are fitted with theoretical lines for systems with pK_a values of 6.5 and 6.7 for iso-FMN and iso-FMN−apoflavodoxin, respectively.

DISCUSSION

Ghisla et al. (*41*) concluded that the optical spectra of flavin hydroquinones consist of at least three transitions whose intensities and energies change not only with the pH, the polarity, and the rigidity of the solvent but also with substitution in the isoalloxazine system of the flavin, and with the planarity of the flavin ring system, which can vary because of flexing along the axis through N(5) and N(10). In general, the absorption maxima of reduced flavoproteins occur at longer wavelengths than those of model compounds, suggesting that the flavin hydroquinone in flavoproteins is more planar than models in free solution. It was concluded that the fully reduced flavin in flavodoxins from *M. elsdenii* and *C. beijerinckii* is anionic and rather planar, conclusions that were supported by the crystal structures of the hydroquinone forms of flavodoxins from *C. beijerinckii* (*12*) and

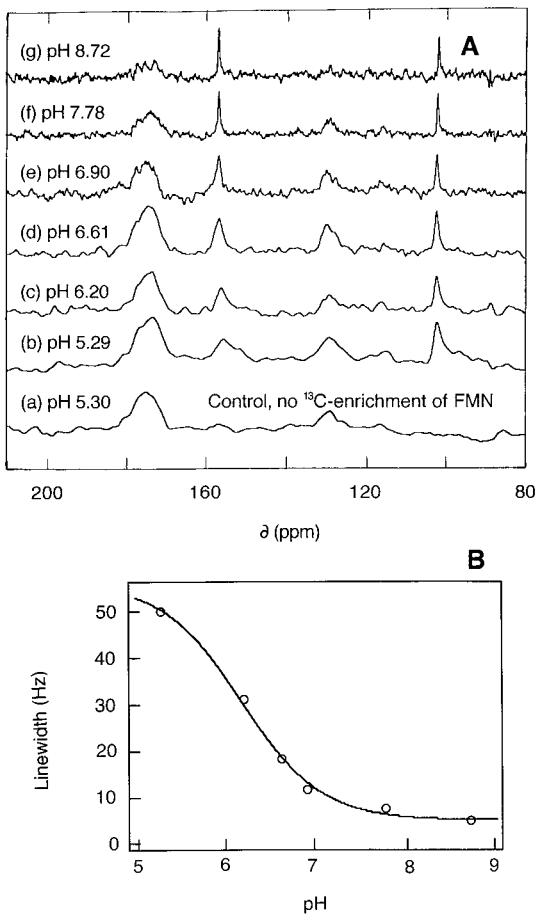

FIGURE 8: (A) Effects of pH on the ^{13}C NMR spectrum of the complex of apoflavodoxin from *D. vulgaris* and [2,4a-$^{13}C_2$]FMN. Acquisition parameters and sample conditions were as described in Materials and Methods. The pH values, buffers, exponential weighting factors used, and the number of transients recorded per spectrum were as follows: (a) 5.30, 20 mM sodium acetate, 40 Hz, and 81 920; (b) 5.29, 20 mM sodium acetate, 40 Hz, and 98 304; (c) 6.20, 20 mM potassium phosphate, 30 Hz, and 81 920; (d) 6.61, 20 mM potassium phosphate, 20 Hz, and 98 304; (e) 6.90, 20 mM potassium phosphate, 10 Hz, and 65 536; (f) 7.78, 20 mM potassium phosphate, 5 Hz, and 98 304; and (g) 6.20, 20 mM sodium pyrophosphate, 5 Hz, and 49 152. The exponential weighting factors used were set to approximately match the observed line widths. Samples were photoreduced as described in Materials and Methods. Spectrum a is for a control sample in which the FMN used was not ^{13}C-enriched. (B) The effect of pH on the line broadening of the ^{13}C NMR signal from C(2) of the reduced FMN complexed with apoflavodoxin from *D. vulgaris*. Chemical shifts and pH values for the signal from C(2) were obtained from the spectra in panel A. The continuous line was calculated using the equation $\delta = [S_1/(1 + K_a/[H^+])] + [S_2/(1 + [H^+]/K_a)]$ and the following fitted parameters: pK_a = 6.18 ± 0.06, S_1 = 56.26 ± 2.05 ppm, and S_2 = 4.97 ± 1.0 ppm.

D. vulgaris (*6*) which show that the flavin is almost flat, and subsequently by NMR measurements which provided further evidence that the flavin is anionic in the pH range of 5.5−7.6 for *M. elsdenii* flavodoxin (*24*) and in the pH range of 6.0−8.5 for *D. vulgaris* flavodoxin (*25*). In contrast to the earlier work which reported that changes in pH in the range of 4.6−8 (*19*, *41*) have very little effect on the optical spectrum of flavodoxin from *M. elsdenii*, the data reported in this paper show that the spectra of four flavodoxins, including that from *M. elsdenii*, do in fact change with pH in this region, and that the changes are at least as great as those occurring with reduced FMN in free solution. The pH-dependent optical changes observed for the *M. elsdenii* protein are not as obvious from inspection of the absolute spectra as those occurring with the flavodoxins from *D. vulgaris* and *A. vinelandii*, probably accounting for the failure to detect them earlier (*19*, *41*).

The absolute spectra of the flavodoxins differ from each other as well as from those of free flavin. The longest wavelength transition is weaker in the proteins, while, with the exception of *Anabaena* flavodoxin, the transition in the 360−370 nm region is stronger. In addition, while the low-pH spectra of $FMNH^-$ and reduced flavodoxins from *D. vulgaris* and *A. vinelandii* have at best only an indistinct shoulder at about 350 nm in addition to the two maxima at shorter and longer wavelengths, the corresponding spectrum of *M. elsdenii* flavodoxin is resolved to give a third maximum (at 350 nm). The changes with pH in the 450−550 nm region of the flavodoxins are smaller than those occurring in the 400−500 nm region of free flavin in aqueous solution. Nevertheless, the spectra of three of the flavodoxins at the low extreme of pH resemble that of free $FMNH_2$ in having a more intense transition in the long wavelength region, in contrast to the broad shoulder observed at high pH. It is notable that when free flavin hydroquinone is dissolved in ethanol or dimethylformamide its longest wavelength band is also relatively insensitive to pH (*41*).

The effects of pH on the flavin hydroquinone have been studied with very few other flavoenzymes (*44*, *45*). Changes with pH have been observed in the optical spectrum of ferredoxin−$NADP^+$ reductase and also in the redox potential of the semiquinone−hydroquinone couple of this protein, and both measurements give a pK_a of 7.5 (*44*). The optical changes, which are quite similar to those observed with flavodoxin from *D. vulgaris*, were attributed to protonation at N(1) of the reduced flavin. In the case of chorismate synthase, the FMN hydroquinone functions in catalysis even though the overall reaction does not involve oxidation−reduction. The hydroquinone in this protein is stabilized as the anion; protonation and a shift of the pK_a to a greater value occur in the presence of substrate (*45*).

The simplest and most likely explanation for the pH-dependent changes in the optical spectra of the flavodoxins is that the isoalloxazine moiety of the bound flavin undergoes a protonation at low pH as occurs with free flavin hydroquinone. The titration curves conform to protonation at a single site, and while the optical changes vary with the protein, they are not too different from those occurring during protonation of free $FMNH^-$. The free flavin hydroquinone protonates in the N(1)C(2) region of the flavin (*24*−*27*). Published NMR data for reduced flavodoxins from *M. elsdenii* and *D. vulgaris*, and the ^{13}C NMR measurements for *D. vulgaris* flavodoxin described above, seem to rule out a protonation in the same region of the protein-bound flavin. In addition, protonation at N(1) of the flavin in flavodoxin from *C. beijerinckii* is sterically restricted (*19*). The broadening of the NMR signal due to the flavin ^{13}C(2) in *D. vulgaris* flavodoxin reported above fits reasonably well to a pK_a at 6.2, quite similar to the pK_a value determined from the optical spectra; however, the failure to observe a change in the chemical shift of the signal with a change in pH is in contrast to the large change in the chemical shift observed when free reduced flavin models protonate in the N(1)C(2)0 region (*25*, *42*), and the cause of the broadening is not known. It is

possible that protonation occurs elsewhere on the isoalloxazine structure, perhaps at the oxygen atom on C(4) (Figure 1, structure **C**). Support for this possibility comes from work which showed that the complex of reduced 1-deaza-FMN and apoflavodoxin from *M. elsdenii* protonates at a pK_a of 7.4 (*19*). Since C(1) of this flavin analogue is already protonated, the additional proton must add elsewhere in the molecule.

An alternative interpretation of the changes in the optical spectra is that while the FMN hydroquinone remains as the anion, protonation occurs on an amino acid side chain(s), with a resultant change in the environment of the $FMNH^-$ arising either from different electrostatic interactions or from a conformational change in the protein or in the flavin. The changes associated with *D. vulgaris* flavodoxin are unlikely to result from protonation of the negatively charged side chains that are closest to the flavin because the optical changes observed with the D95A and D95A/D127A mutant proteins are similar to those occurring with the wild-type protein. In addition, they do not appear to be directly associated with the two aromatic amino acids that sandwich the isoalloxazine moiety in this flavodoxin because pH-dependent spectroscopic changes also occur in mutant proteins in which these residues have been replaced by amino acids with aliphatic side chains (Y98S and W60A). It is known that the two loops of the protein which make contact with the flavin in this flavodoxin are more flexible when the flavin is fully reduced than when it is oxidized (*46*), and therefore, a pH-dependent conformational change at the flavin site that results from protonation of a group some distance from the flavin cannot be ruled out. Such a change might affect one or more of the hydrogen-bonding interactions that occur with the reduced flavin. In particular, the loop of the protein that provides the hydrogen-bonding interaction between N(5)H of the reduced flavin in *D. vulgaris* flavodoxin and the carbonyl oxygen of Gly-61 can move 5−6 Å away from the oxidized flavin when the glycine is mutated (*30*). It is possible that when the bound flavin in the wild-type protein is fully reduced the conformation of this 60-loop changes with pH.

The apparent pK_a values determined from the optical changes for flavodoxins from *M. elsdenii* and *D. vulgaris* are similar to the apparent pK_a values determined previously from the change in the slope of a plot of the redox potential, E_1, of the semiquinone−hydroquinone couples of flavodoxins versus pH (Table 1). The redox-dependent protonation of the wild-type *M. elsdenii* flavodoxin was originally ascribed to protonation at N(1) (*38*), and subsequently to a group on the protein (*19*). Kyte (*47*) and Swenson and Zhou (*48*) have pointed that the E_1 versus pH data of the kind observed with the flavodoxins can also be fitted if negatively charged groups on the protein interact with negative charge on the flavin, and if the values for the microscopic pK_a values change when a second electron is added to the flavin. It is important to note that the changes detected in the optical spectra are associated only with the hydroquinone form, and they are consistent with protonation on a single group.

A description of the ionization state of the hydroquinone of FMN in the flavodoxins, and of the structure of this form of the flavin, is essential to an understanding of the forces that determine the interaction of the flavin and protein, and which regulate the redox properties of the flavin. The difference between the redox potential of the semiquinone−hydroquinone couple of the protein-bound FMN and that of free FMN is greater at low pH, indicating that the hydroquinone complex is more strongly destabilized at pH values below the apparent pK_a. The observation that the optical properties of the hydroquinone change with pH leaves open the possibility that the reduced flavin is neutral in this pH region. A more detailed investigation by NMR spectroscopy with photoreduced flavin−apoflavodoxin complexes that are selectively enriched with ^{13}C or ^{15}N at additional positions in the flavin may elucidate the site of protonation. Comparative NMR studies should show whether the protonation site in mutants of *D. vulgaris* flavodoxin is the same as that in the wild-type protein, and whether the site of protonation is the same in flavodoxins in which the flavin−protein interactions are different from those of *D. vulgaris* flavodoxin.

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