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Structure and Oxidation–Reduction Behavior of 1-Deaza-FMN Flavodoxins: Modulation of Redox Potentials in Flavodoxins†

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Received March 5, 1990; Revised Manuscript Received June 27, 1990

ABSTRACT: Flavodoxins from *Clostridium beijerinckii* and from *Megasphaera elsdenii* with 1-carba-1-deaza-FMN substituted for FMN have been used to study flavin–protein interactions in flavodoxins. The oxidized 1-deaza analogue of FMN binds to apoflavodoxins from *M. elsdenii* and *C. beijerinckii* (a.k.a. *Clostridium MP*) with association constants (K_a) of 1.0×10^7 M^{-1} and 3.1×10^6 M^{-1}, values about 10^2 less than the corresponding K_a values for FMN. X-ray structure analysis of oxidized 1-deaza-FMN flavodoxin from *C. beijerinckii* at 2.5-Å resolution shows that the analogue binds with the flavin atoms in the same locations as their equivalents in FMN but that the protein moves in the vicinity of Gly 89 to accommodate the 1-CH group, undergoing displacements which increase the distance between position 1 of the flavin ring and the main-chain atoms of Gly 89 and move the peptide hydrogen of Gly 89 by about 0.6 Å. The X-ray analysis implies that protonation of normal flavin at N(1), as would occur in formation of the neutral fully reduced species, would result in a similar structural perturbation. The oxidation–reduction potentials of 1-deaza-FMN flavodoxin from *M. elsdenii* have been determined in the pH range 4.5–9.2. The oxidized/semiquinone equilibrium (E'_0 = −160 mV at pH 7.0) displays a pH dependence of −60 mV per pH unit; the semiquinone/reduced equilibrium (E'_0 = −400 mV at pH 7.0) displays a pH dependence of −60 mV per pH unit at low pH and is pH independent at high pH, with a redox-linked pK of 7.4. Spectral changes of fully reduced 1-deaza-FMN flavodoxin with pH suggest that this latter pK corresponds to protonation of the flavin ring system (the pK of free reduced 1-deaza-FMN is 5.6 [Spencer, R., Fisher, J., & Walsh, C. (1977) *Biochemistry 16*, 3586–3593]. The pK of reduced 1-deaza-FMN flavodoxin provides an estimate of the electrostatic interaction between the protein and the bound prosthetic group; the free energy of binding neutral reduced 1-deaza-FMN is more negative than that for binding the anionic reduced 1-deaza-FMN by 2.4 kcal. In contrast, the redox-linked pK of native *M. elsdenii* flavodoxin, which is 5.8, is shown not to be associated with changes in the flavin absorbance, indicating that in the normal FMN flavodoxin structure, the pK determined in studies of the semiquinone/reduced equilibrium must be assigned to the protein rather than to FMN. These data are in agreement with the conclusion from NMR studies [Franken, H.-D., Rüterjans, H., & Müller, F. (1984) *Eur. J. Biochem. 138*, 481–489] that flavodoxins bind reduced FMN as the anionic species and suggest a mechanism in which unfavorable electrostatic interactions between the negatively charged ring and its protein neighbors play a major role in determining the redox potential of the semiquinone/reduced flavodoxin couple.

Flavodoxins typically perturb the oxidation–reduction potentials of FMN and display exceptionally low potentials for the one-electron equilibrium between semiquinone and reduced species (Mayhew & Ludwig, 1975). The sq/red[1] potentials are shifted from the free FMN value of −175 mV at pH 7 (Draper & Ingraham, 1968) to about −400 mV. Thermodynamics dictates that the association constant for binding of reduced FMN is therefore smaller than that for FMN semiquinone by a factor of 10^3–10^4, corresponding to a $\delta\Delta G$ of about 5 kcal. The effects of the protein on the potential for the ox/sq equilibrium are more dependent on the species of flavodoxin, ranging from almost no change, relative to free FMN, to increases of about 150 mV. As a consequence, the shifts of the two-electron midpoint potentials, produced by binding of FMN to protein, also vary appreciably with species.

The flavin–protein interactions that are responsible for lowering the sq/red potential have not been easy to identify. The structures of semiquinoid and reduced forms of *Clostridium beijerinckii* flavodoxins are very similar (Smith et al., 1978), indicating that conformation changes do not play a dominant role in the change in affinity for FMN. When the structure of a reduced flavodoxin was first determined (Ludwig et al., 1976) and the flavin ring found to be almost planar, free reduced flavins were thought to be nonplanar (Kierkegaard et al., 1971). It was presumed that the protein forced the cofactor to adopt a high-energy conformation (Ludwig et al.,

† This work was supported by U.S. Public Health Service Grants GM 11106 (V.M.) and GM 16429 (M.L.L.).

[1] Abbreviations: ox, oxidized flavin; sq, one-electron reduced (semiquinone) flavin; red, two-electron reduced flavin; flv, native flavodoxin; dflv, 1-deaza-FMN flavodoxin. Main-chain atoms are denoted as C_α, α-carbon; C, carbonyl carbon; O, carbonyl oxygen; N, peptide nitrogen.

0006-2960/90/0429-10364$02.50/0

1976), and ring strain in the protein-bound reduced FMN was invoked to explain the low sq/red potential. This rationale for the relatively small K_a and low potential had to be abandoned when NMR measurements (Moonen et al., 1984) demonstrated that reduced free flavins are essentially planar. Theoretical calculations (Hall et al., 1987) support the interpretations of the NMR data and suggest that both oxidized and reduced isoalloxazines are flexible, with rather small energy barriers to formation of planar conformations.

More recently, attention has focused on electrostatic effects on K_a (and E'_0). Chemical shifts of ^{15}N-labeled reduced FMN bound to flavodoxins from *Megasphaera elsdenii* (Franken et al., 1984; Vervoort et al., 1986a), *C. beijerinckii* (Vervoort et al., 1986a), *Desulfovibrio vulgaris* (Vervoort et al., 1985), *Azotobacter vinelandii* (Veervoort et al., 1986a), and *Anabaena 7120* (Stockman et al., 1988) all have values characteristic for anionic species, ionized at N(1). In reduced *M. elsdenii* flavodoxin this chemical shift persists even at pH 5.5, which is 1 pH unit below the p*K* of 6.5 for free FMN (Draper & Ingraham, 1968). Computations show that the negative charge on reduced isoalloxazines is delocalized (Dixon et al., 1979; Teitell & Fox, 1982; Hall et al., 1987), primarily onto the heteratoms N(1), O(2), O(4), and N(5). The surroundings of these atoms, as visualized in crystal structures (Smith et al., 1977, 1978), include several N–H dipoles from the peptide backbone but no formal positive charges. Hydrophobic side chains contribute significantly to isoalloxazine–protein contacts, suggesting that the charge on the reduced flavin is in an environment with a low dielectric constant. It has therefore been tempting to postulate that addition of an electron to flavodoxin semiquinone to form anionic reduced flavin is difficult because of unfavorable electrostatic interactions. Moonen (1983) has tried to account quantitatively for the low sq/red potential on this basis.

Electrostatic interactions are expected to perturb p*K*s of titratable groups in proteins. In fact, when oxidized forms of 6-OH-FMN, 8-OH-FMN, or 8-mercapto-FMN are bound to apoflavodoxin from *M. elsdenii*, the p*K*s of the 6- or 8-substituents are increased by 0.5–2.0 units (Mayhew et al., 1974; Ghisla & Mayhew, 1976; Massey et al., 1986). Spectral and redox measurements on 1-deaza-FMN flavodoxin, reported here, show that the p*K* for deprotonation of the reduced form increases by 1.8 units on binding to apoflavodoxin. All of these observations are consistent with the notion that binding of anionic species is unfavorable. It has thus been puzzling that the p*K*s determined from the pH dependence of the sq/red potentials of native flavodoxin are close to or below the value of 6.5 (Draper & Ingraham, 1968) reported for free reduced FMN: measured p*K*s for *M. elsdenii* and *C. beijerinckii* flavodoxin are 5.8 and 6.7, respectively (Mayhew, 1971a). From further analysis of the properties of reduced FMN flavodoxins we now conclude that in native *M. elsdenii* flavodoxin the p*K* for addition of a proton at N(1) is actually below 5.0 and that another group must be the proton acceptor which determines the pH dependence of the redox potentials.

In view of the apparently anomalous behavior of reduced FMN flavodoxin, it is important to understand why N(1) is not protonated at neutral pH. From the known crystal structures we suspected that addition of a proton would disturb the internal packing of the protein around the flavin ring. The distance between the flavin N(1) and the peptide NH of Gly 89 is only 3.0_6 Å in reduced clostridial flavodoxin, and the peptide NH points toward N(1) (Figures 5 and 8). Protonation of N(1) seems to be precluded unless the distance between the peptide and N(1) hydrogens can be increased. To test this idea, we have substituted 1-deaza-FMN, which carries a nonlabile hydrogen at C(1) (Spencer et al., 1977), for FMN and determined the crystal structure of the resulting holoprotein. In the analogue, atoms of the protein move to accommodate the CH group of 1-deaza-FMN, and the affinity for oxidized flavin is reduced by a factor of 200.

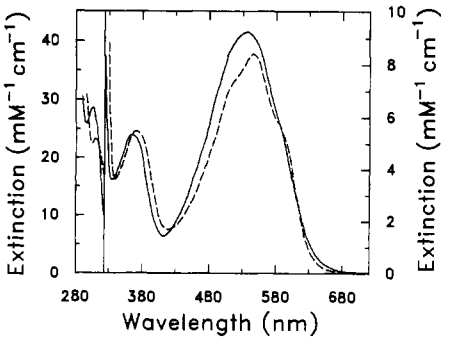


FIGURE 1: Spectra of 1-deaza-FMN (—) and 1-deaza-FMN flavodoxin from *M. elsdenii* (---). See Experimental Procedures for details.

In this paper, observations from the crystal structures of *C. beijerinckii* flavodoxins are correlated with thermodynamic data obtained mostly from the homologous flavodoxin from *M. elsdenii*. Our primary observations, that protonation of the reduced isoalloxazine ring at N(1) requires structural changes and is thus suppressed by steric hindrance, and that a proton acceptor on the protein has a role in its redox chemistry, serve as the basis for a model in which electrostatic repulsion plays a major part in lowering the sq/red potential. The structure seems designed to favor binding of the anionic reduced flavin despite the absence of countercharges and achieves only partial compensation by increasing the p*K* of an adjoining group.

Experimental Procedures

Extinction Coefficient of 1-Deazaflavins. 1-Deaza-FMN (~20 μM) was titrated with standardized apoflavodoxin in 0.1 M potassium phosphate buffer, pH 7.0, at 20 °C. At this concentration of flavin, the protein bound the 1-deaza-FMN stoichiometrically. The quantity of protein needed to reach saturation, together with the initial flavin absorbance, was used to calculate peak extinction coefficients: for free 1-deaza-FMN, 9.23 mM^{-1} cm^{-1} at 536 nm and 5.36 mM^{-1} cm^{-1} at 365 nm, and for 1-deaza-FMN flavodoxin, 8.42 mM^{-1} cm^{-1} at 546 nm and 5.51 mM^{-1} cm^{-1} at 372 nm (Figure 1). Extinction values obtained for 1-deazariboflavin by titration with standardized apo-riboflavin binding protein were essentially identical with those of 1-deaza-FMN. These values for free 1-deazaflavin are substantially larger than those previously reported (Spencer et al., 1977). The peak extinction coefficient for 1-deaza-FAD (8.7 mM^{-1} cm^{-1} at 544 nm) was determined by comparison with 1-deaza-FMN after digestion of the former with *Naja naja* phosphodiesterase.

Determination of Association Constants. Equal volumes of 1-deaza-FMN (5 μM in 0.1 M potassium phosphate buffer, pH 7.0 at 25 °C) were placed in both the sample and reference cuvettes of a Cary 219 double-beam spectrophotometer. The sample cuvette was titrated with apoflavodoxins (0.132 mM *M. elsdenii* or 0.284 mM *C. beijerinckii* standardized by titration against FMN) while the reference cuvette was titrated with equal volumes of buffer. Difference spectra were recorded after each addition (Figure 2). The fraction of bound 1-deaza-FMN was determined from the absorbance difference, and K_a was calculated from the average of 4–6 points around the equivalence point. Displacement of 1-deaza-FMN from 1-deaza-FMN flavodoxin by FMN was used to verify the

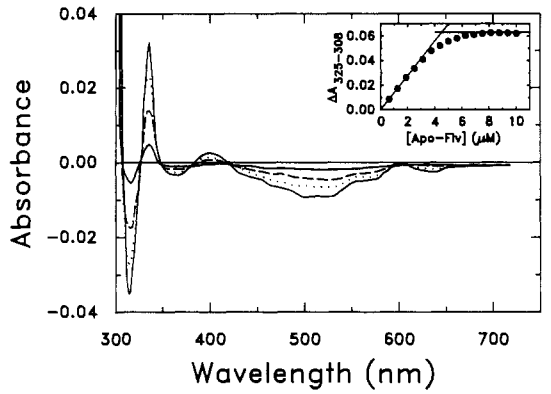


FIGURE 2: Difference titration of 1-deaza-FMN with *M. elsdenii* apoflavodoxin. Conditions are described under Experimental Procedures. Spectra are the difference, 1-deaza-FMN flavodoxin minus 1-deaza-FMN, for mixtures containing apoflavodoxin at 0.65 μM (—), 1.95 μM (---), 3.22 μM (···), and 5.08 μM (—). Some traces have been omitted for clarity. Inset: Plot of the Δ absorbance at 325 nm minus that at 308 nm versus the concentration of apoflavodoxin (all corrected for dilution).

values determined by direct titration.

Determination of Oxidation–Reduction Potentials. The potentials for both the ox/sq and sq/red couples of 1-deaza-FMN flavodoxin were determined by anaerobic titration (25 °C) of mixtures of protein and redox dyes with sodium dithionite as described by Mayhew et al. (1969). Samples were made anaerobic by evacuating and flushing the cell with argon, purified free of oxygen by passage through an R&D oxygen trap, Model OT3-4 (R&D Separations). The anaerobic titrator was similar to that described by Foust et al. (1969). Buffers used for different pH ranges included 0.1 M sodium acetate (pH 4.5–5.7), 0.1 M potassium phosphate (pH 6.2–7.5), 0.1 M sodium pyrophosphate (pH 8.5–8.7), and 0.1 M glycine (pH 9.1). The pH of each reaction mixture was measured after opening to air. The dyes used and their potentials are given in Table I. Concentrations of the dyes were determined from their oxidized absorbance (see Table I). The absorbance of benzyl viologen was followed at 560 nm, an isosbestic point between the reduced monomer and dimer, to give the total concentration of reduced dye (Mayhew & Müller, 1982). Then the data were plotted in terms of the concentration of reduced monomer.

Alternatively, the potentials were estimated from the maximum fraction of semiquinone observed during reduction (Mayhew, 1971a), by using

$$K_{eq} = \left[\frac{2[\text{Flv sq}]}{[\text{Flv tot}]} \Big/ \left(1 - \frac{[\text{Flv sq}]}{[\text{Flv tot}]} \right) \right]^2$$

and

$$\Delta E = (RT/nF) \ln K_{eq}$$

where K_{eq} is the equilibrium constant; [Flv tot] and [Flv sq] are the concentrations of total flavodoxin and of maximum semiquinone, respectively; ΔE is the difference in potential (ox/sq minus sq/red); and R is the gas constant, T the temperature in kelvin, F the Faraday constant, and n the number of electrons (RT/nF = 25.6 mV for one electron) [see Clark (1960) for a derivation of these expressions). The 1-deaza-FMN flavodoxin was photoreduced (Massey & Hemmerich, 1978) by using 5-deazaflavin and 20 μM EDTA while buffered at various pH values. The same range of buffers was used as described previously, except that 0.1 M sodium pyrophosphate was used up to pH 9.2. The data from this method showed more scatter than those from the dye equilibration method (see Figure 3A). This can be attributed to the high percentage of semiquinone found at maximum, typically 92–95% below pH 7.5. At 94% semiquinone, a 1% measurement error translates into an error of 10 mV in potential. The measurement of maximum semiquinone is accurate to no better than 2%.

Table I: Potentials for 1-Deaza-FMN Flavodoxin Measured by Dye Equilibration

dye	extinction of oxidized dye[a] (mM^{-1} cm^{-1})	pH	dye potential (mV)	measured 1-deaza-Flv potential (mV)
Oxidized/Semiquinone Couple				
1-OH-phenazine	1.85 (416)[b]	5.3	−70[g]	−58
1-OH-phenazine	1.85 (416)[b]	8.6	−267[g]	−243
8-NAcCys-riboflavin	28.0 (475)[c]	7.0	−180[c]	−150
riboflavin	12.5 (445)[d]	7.0	−208[h]	−171
2-OH-1,4-naphtha-quinone	2.50 (452)[b]	7.2	−170[i]	−189
2-OH-1,4-naphtha-quinone	2.50 (452)[b]	8.7	−295[i]	−237
Semiquinone/Reduced Couple				
benzyl viologen	27.0 (260)[e]	6.2	−359[j]	−350
benzyl viologen	27.0 (260)[e]	7.0	−359[j]	−400
methyl viologen	21.5 (258)[f]	8.5	−446[j]	−423
methyl viologen	21.5 (258)[f]	9.1	−446[j]	−426

[a] The wavelength (nm) of the peak corresponding to the extinction coefficient is given in parentheses. [b] By weight. [c] Moore et al., 1979. [d] Whitby, 1954. [e] Mayhew & Müller, 1982. [f] Mayhew, 1978. [g] Müller, 1942. [h] Draper & Ingraham, 1968. [i] Louis & Fieser, 1934. [j] Michaelis & Hill, 1933.

Determination of Acid Dissociation Constants (pK). The pK of reduced 1-deaza-FMN flavodoxin was taken from the change in its absorbance spectrum with pH. The extinction coefficient at 560 nm for the fully reduced protein was measured relative to the oxidized peak at 544 nm and plotted versus pH. Reduced spectra were taken from the photoreductions described in the previous section. A Radiometer pH meter (Model 25) with combination electrode GK2321C (Radiometer), standardized from pH 4 to 10, was used for the pH measurements.

The pK values for native flavodoxin, oxidized and semiquinone, were measured in a scanning stopped-flow spectrophotometer, interfaced to a Nova II (Data General) computer (Beaty & Ballou, 1981). Oxidized flavodoxin in 10 mM potassium phosphate buffer, pH 7.0, was mixed rapidly (25 °C) with 0.2 M buffers at various pH values (potassium phosphate for pH 7.0–8.0 and 10.5–12.5, glycine for pH 9.0–10.0, and 0.1–0.3 M KOH for pH >12.5). A spectrum was scanned immediately (elapsed time was <10 s).

Flavodoxin semiquinone was prepared by photoreduction using 5-deazaflavin and 20 mM EDTA and mixed, under anaerobic conditions, with the same set of buffers as was used for oxidized flavodoxin. Buffers were made anaerobic by bubbling for 15 min with oxygen-free argon.

Free Energy Calculations. The following standard expressions were used for calculating free energy:

$$\delta\Delta G = -RT\delta \ln K$$

$$RT = 0.59 \text{ kcal at } 25\ ^\circ\text{C}$$

$$\delta\Delta G = -nF\delta E$$

$$nF = 23.06 \text{ (kcal/V) for a one-electron reaction}$$

where K is the association constant and E is potential [Clark (1960), pp 60–135].

Crystallization and Structure Analysis of 1-Deaza-FMN Flavodoxin from C. beijerinckii. Apoprotein was added to excess 1-deaza-FMN. After separation of free 1-deaza-FMN

and concentration of the protein by centrifugation through a Centricon 10 microconcentrator (Amicon), samples were prepared for crystallization by mixing protein (final concentration 5 mg/mL) with varying amounts of 2.6 M ammonium sulfate and 0.2 M phosphate at pH 6.8 (Ludwig et al., 1969). Crystallization droplets were equilibrated with ammonium sulfate at concentrations of 2.0–2.4 M. The resulting crystals displayed a space group which was identical with that of native flavodoxin, $P3_121$, with slightly different cell dimensions: $a = b = 61.47$, $c = 70.08$ Å, versus $a = b = 61.56$, $c = 70.36$ Å for FMN flavodoxin.

Diffraction data were collected from one crystal at 4 °C by using a Xuong-Hamlin dual area detector system, with crystal–detector distances of 607 and 551 mm. Of the 5554 expected reflections to 2.5 Å, 5077 were measured with an average redundancy of 3.31 and an $R_{sym} = 0.049$.

A difference Fourier map with amplitudes ($|F_o|_{dflv} - |F_o|_{flv}$) was computed with phases determined from the oxidized FMN flavodoxin model (Smith et al., 1977) for data from 2.5 to 5.0 Å and with MIR phases for data inside 5.0 Å. Inspection of this map suggested a structural change in the protein near residue 89 (Figure 4). A Fourier map (10.0–2.5 Å) was then computed with observed amplitudes from 1-deaza-FMN flavodoxin and phases from the model of oxidized flavodoxin deposited in the Protein Data Bank, omitting the atoms of residues 88 and 89 and the N–C_α of Trp 90. With this map as a guide, we rebuilt the protein model in the vicinity of Gly 89, moving Gly 89 away from the flavin C(1) and rotating the 88–89 peptide unit to minimize steric overlap.

Restrained least-squares refinement (Hendrickson, 1985) was employed to adjust the positions of residues 88–90 in the 1-deaza-FMN flavodoxin structure. These computations were conducted at the San Diego Supercomputer Center, using a version of the programs that permits corrections for the effects of solvent on low-order reflections (Bolin et al., 1982). Changes in the parameters for oxidized FMN flavodoxin, resulting from recent refinement of the native protein ($R = 0.181$ for 12 556 observed reflections between 10.0 and 1.8 Å), were incorporated in the model for 1-deaza-FMN flavodoxin. van der Waals restraints for nitrogen at position 1 were replaced by those for carbon so that the ideal C(1) to N 89 distance was 3.3 Å and this atom pair was no longer considered able to form a hydrogen bond. Eight cycles of refinement of the rebuilt model reduced R from 0.199 to 0.164 (for 4980 reflections between 10.0 and 2.5 Å, 82 solvents, individual atom B values). Part of the change in R is the result of accommodating the model to a slightly different unit cell. At the end of these computations the distance between C(1) and the peptide N of Gly 89 was 3.2_9 Å, and the distance between C_α 89 and C(1) was also about 0.2 Å greater than in the corresponding FMN flavodoxin structure. These coordinates were positioned in a Fourier map computed with calculated phases derived from four cycles of refinement in which the atoms C 88 through C_α 90 had been removed from the model. The C_α of Gly 89 was well placed in the density, but N 89 was still not correctly centered. An additional 12 cycles of refinement ($R = 0.158$) produced the coordinates shown in Figure 5a, with an N 89–C(1) distance of 3.2_0 Å. Overall, the rebuilding and refinement resulted in Cartesian coordinate shifts for main-chain atoms that ranged from 0.3 Å for O 88 to 0.8 Å for O 89.

In control computations we found that refinement without rebuilding would not readily move the atoms of residues 88–90 away from the flavin; the temperature factors shifted to account for differences in the observed $|F|$s. However, when B values were not allowed to refine, atom displacements were observed. After nine cycles starting from the FMN flavodoxin model described above, the C(1)–N 89 distance was 3.2_5 Å but the remaining main-chain atoms had not yet reached the positions determined by more prolonged refinement of a rebuilt model. The results of different refinement strategies are compared in Figure 5a.

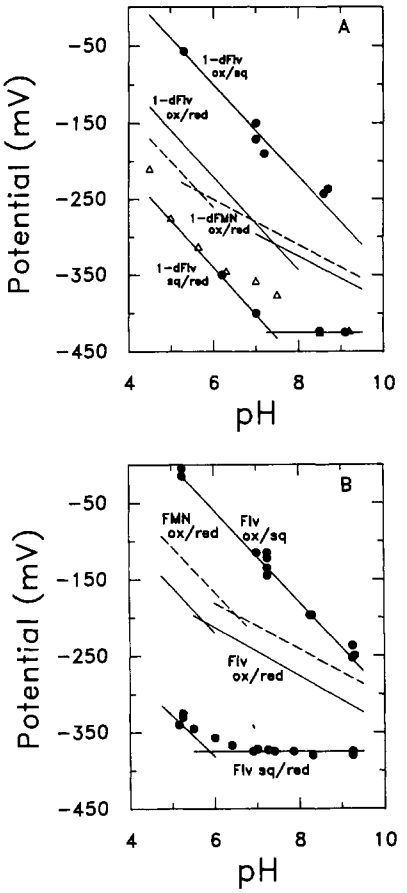


FIGURE 3: Potential versus pH profiles. Panel A gives the potential for free 1-deaza-FMN and 1-deazaFMN flavodoxin from *M. elsdenii*. The one-electron potentials for flavodoxin (closed circles) were measured by the dye equilibration method (see Experimental Procedures). Additional values (open triangles) were calculated for the sq/red couple by adding the difference between the ox/sq and sq/red potentials (taken from the maximum fraction of semiquinone observed in a titration) to the ox/sq values. The two-electron potentials for 1-deaza-FMN were determined from the two-electron potential at pH 7.0 and the pK of 5.6 for the reduced form (Spencer et al., 1977). Panel B gives the potentials for FMN and FMN flavodoxin from *M. elsdenii* which are shown for comparison. The one-electron potentials for FMN flavodoxin (closed circles) were taken from Mayhew (1971a) and Stankovich (1980). The two-electron potentials for free FMN are taken from Draper and Ingraham (1968). ($E'_0 = -208$ at pH 7.0 with a pK of 6.5 for the reduced form.)

Materials. Flavodoxin was purified as previously described (Mayhew & Massey, 1969) and apoflavodoxin prepared by dialysis against KBr (Mayhew, 1971b). 1-Deaza-riboflavin (a generous gift from Merck, Sharp and Dohme Research Laboratories) was converted to 1-deaza-FAD by using partially purified FAD synthetase from *Brevibacterium ammoniagenes* as described by Spencer et al. (1976) and then hydrolyzed to 1-deaza-FMN with the phosphodiesterase from *N. naja* venom (Sigma).

5-Deazaflavin (3-methyl-7,8-didemethyl-5-deazalumiflavin) was a generous gift from the late Dr. P. Hemmerich, University of Konstanz, West Germany. Sodium dithionite was a generous gift from the Virginia Chemical Co., Portsmouth, VA. 8-(*N*-Acetylcysteinyl)riboflavin was prepared from 8-

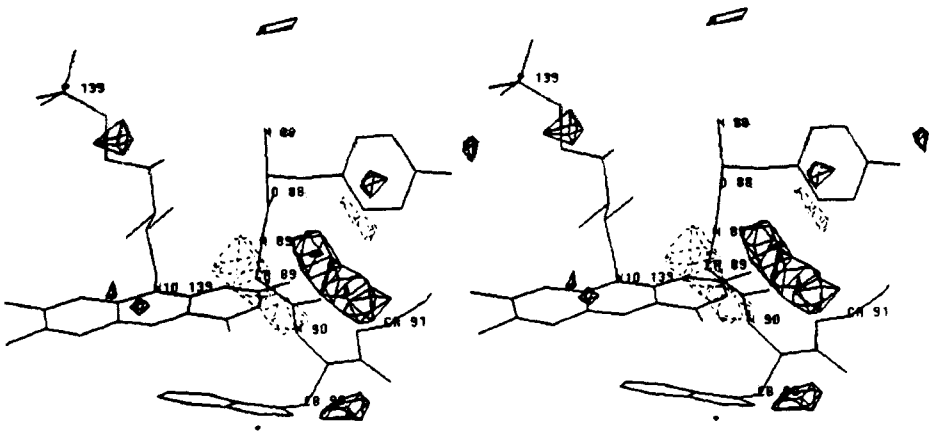


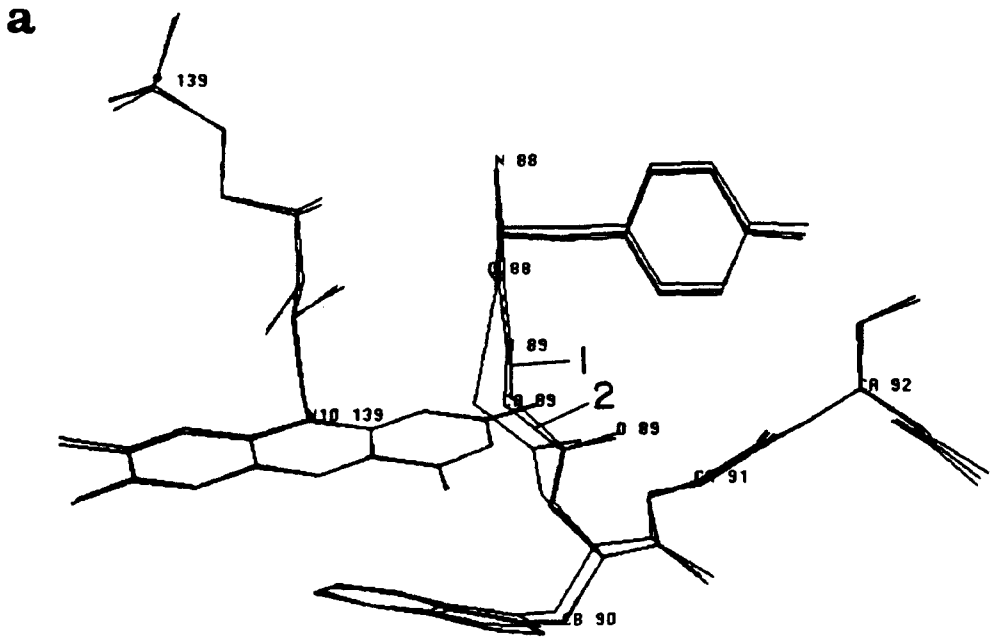
FIGURE 4: Difference Fourier map computed with amplitudes ($|F_o|_{dflv} - |F_o|_{flv}$) and phases from oxidized FMN flavodoxin, superimposed on the refined model for oxidized FMN flavodoxin (see Experimental Procedures). Terms corresponding to d spacings between 10.0 and 2.5 Å have been included in the Fourier summation. Positive and negative features at 3σ (filled and dotted lines) adjoining the main chain at positions 88–90 are the strongest peaks in the map. Figure 5 portrays the displacement of the backbone in 1-deaza-FMN flavodoxin.

chlororiboflavin and *N*-acetylcysteine by Dr. E. Moore in this laboratory (Moore et al., 1979); 6-amino-FAD was prepared from 6-aminoriboflavin by using the FAD synthetase described above. Both 8-chlororiboflavin and 6-aminoriboflavin were generous gifts from Dr. S. Ghisla, University of Konstanz, West Germany. Methyl viologen and benzyl viologen were from Aldrich, riboflavin and 2-hydroxy-1,4-naphthaquinone were from Sigma, and 1-hydroxyphenazine was from the American Tokyo Kasei. All other chemicals were of reagent grade and were used without further purification.

RESULTS

Association Constants for 1-Deaza-FMN. Binding of 1-deaza-FMN to apoflavodoxin was measured by titrating a concentrated solution of the apoprotein into a solution of the flavin, as described under Experimental Procedures. Association with apoprotein resulted in a shift of λ_{max} of free 1-deaza-FMN from 536 (ϵ = 9.23 mM^{-1} cm^{-1}) to 546 nm (ϵ = 8.42 mM^{-1} cm^{-1}) and very pronounced perturbation of the spectrum in the region 300–340 nm. Figure 1 shows the spectra of the free and protein-bound flavin at the end of a titration employing a relatively high concentration of 1-deaza-FMN in order to obtain an accurate end point for determination of extinction coefficients. Figure 2 shows the difference spectra for bound minus free 1-deaza-FMN from a titration using apoflavodoxin from *M. elsdenii*. The progress of complex formation was followed by the change in absorbance ($A_{325} - A_{308}$) as seen in Figure 2, inset. An association constant of 1.0×10^7 M^{-1} was calculated from the points near saturation which deviate from linearity (Heyn & Weischet, 1975). A similar titration using apoflavodoxin from *C. beijerinckii* yielded a K_a of 3.1×10^6 M^{-1}.

The K_a value for *M. elsdenii* flavodoxin is 2 orders of magnitude smaller than the value reported for native flavodoxin, 2.3×10^9 M^{-1} (Mayhew, 1971b), under similar conditions. The much tighter binding of FMN to apoflavodoxin predicts that addition of an equimolar concentration of FMN to a preparation of 1-deaza-FMN flavodoxin should result in complete displacement of 1-deaza-FMN from the protein. Binding of FMN to apoflavodoxin can be followed by the loss of fluorescence since the FMN–protein complex quenches the fluorescence of FMN (Mayhew, 1971b) and free 1-deaza-FMN is nonfluorescent (Spencer et al., 1977). Mixing FMN (2.4 μM, final concentration) and 1-deaza-FMN flavodoxin (2.8 μM, final concentration) resulted in complete loss of FMN fluorescence ($t_{1/2}$ approximately 0.7 min). When the final mixture was concentrated on a Centricon-10 microconcentrator (Amicon), free flavin that separated from the protein-bound



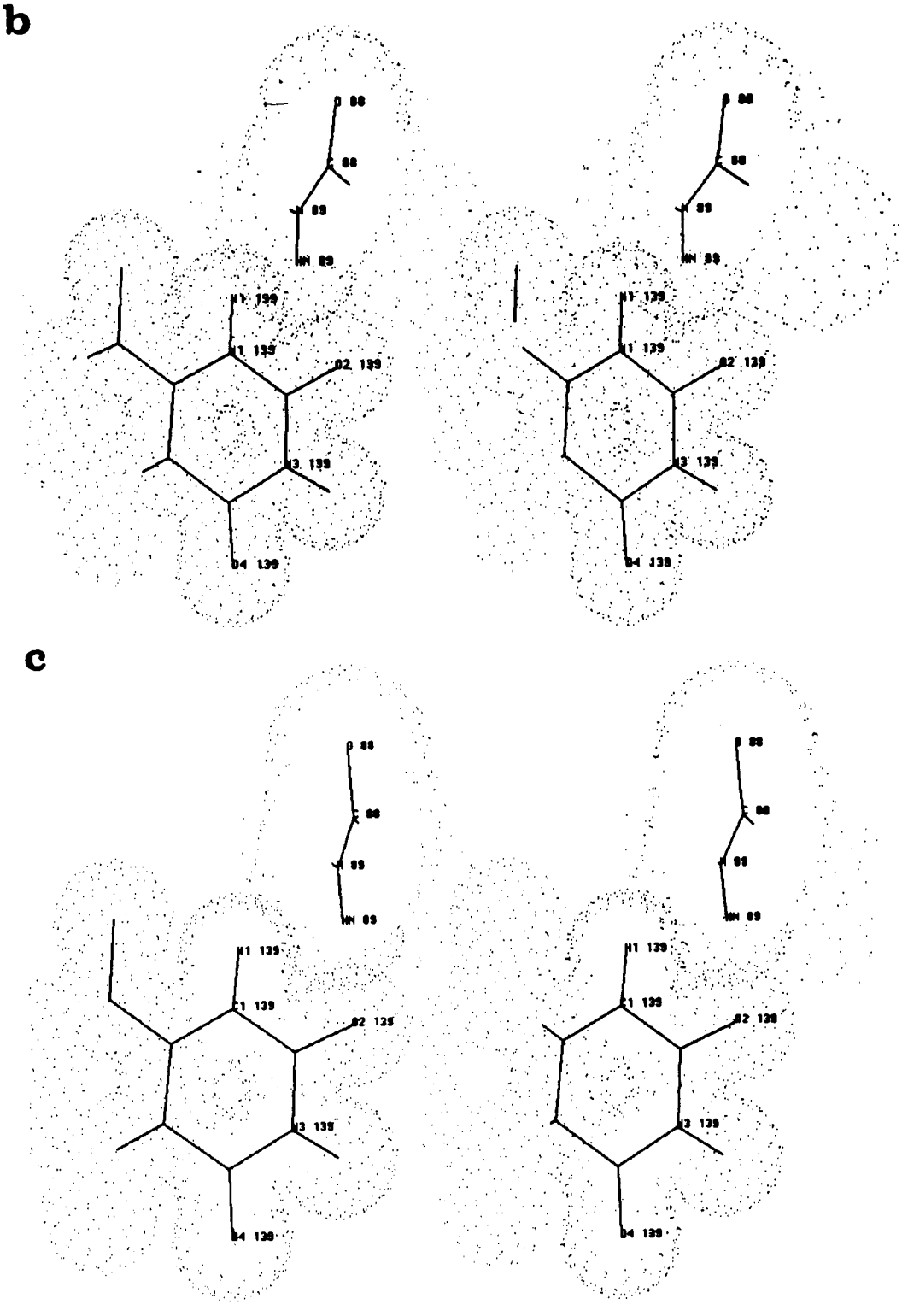


FIGURE 5: (a) Coordinates for 1-deaza-FMN flavodoxin compared with those for FMN flavodoxin. The view is approximately parallel to the plane of the peptide unit connecting Tyr 88 with Gly 89. The coordinates for residues 88 and 89 of FMN flavodoxin are represented by the leftmost bonds; structure 2 is the result of 20 cycles of refinement of an FMN flavodoxin model, starting with residues 88–90 rebuilt and varying temperature factors (R = 0.158); structure 1 is the result of 9 cycles of refinement from a rebuilt model, with temperature factors not varied. Distances for the FMN flavodoxin model and for 1-deaza-FMN flavodoxin (structure 1) are N(1)–N 89, 3.0_8 Å, and C(1)–N 89, 3.2_0 Å. The distance between O(2) and the peptide N of Gly 89 decreases from 2.9_9 Å in FMN flavodoxin to 2.8_3 Å in 1-deaza-FMN flavodoxin (structure 1). The FMN of FMN flavodoxin is not included in the drawing. (b and c) Stereo figures showing expanded views of the contacts between the peptide connecting Tyr 88 with Gly 89 and the "pyrimidine" moieties of the flavin or the deazaflavin rings. Hydrogens were added by model building; the hydrogens attached to flavin atoms were placed at bond distances of 1.0 Å and in the plane of the isoalloxazine ring. van der Waals radii, represented by the dot surfaces, are 1.1 Å for flavin atoms and 1.7 Å for atoms of residues 88 and 89. Panel b shows the large overlap between hydrogens in a hypothetical structure of N(1)-protonated reduced flavodoxin, generated from the refined coordinates by simply adding a hydrogen at N(1). Panel c shows the packing observed in the model of oxidized 1-deaza-FMN flavodoxin. Coordinates for non-hydrogen atoms of 1-deaza-FMN flavodoxin are from model 2 of panel a. These views illustrate how the altered orientation of the 88–89 peptide unit helps to accommodate C(1)H and directs hydrogen-bonding interactions toward O(2) of the flavin.

flavin had the characteristic spectrum of 1-deaza-FMN. Complete displacement of 1-deaza-FMN by FMN indicates a K_a for 1-deaza-FMN at least 2 orders of magnitude smaller than that for FMN and confirms the K_a values measured from the titration data.

Oxidation–Reduction Potentials for 1-Deaza-FMN Flavodoxin. The two-electron reduction potential (E_{mid}) of 1-deazariboflavin has been reported to be −280 mV at 25 °C, pH 7.0 (Spencer, 1977). We measured a similar value (−270 mV) by reducing 1-deazariboflavin in the presence of 6-amino-FAD [E = −293 mV at pH 7.0, 25 °C (Ghisla et al., 1980)]. Reduction of 1-deazariboflavin alone (40–90 μM) occurs with the transient formation of a small amount of long-wavelength absorbance. Close examination of this absorbance at pH 7.0 showed that its formation and decay were reminiscent of that described by Gibson et al. (1962) for FMN, a process involving an elaborate dimerization scheme. We have not pursued these investigations further, though it appears that as with riboflavin, free 1-deaza-riboflavin semiquinone accounts for only a few percent of the total flavin concentration at half-reduction. Values for the individual one-electron potentials of 1-deazariboflavin have not been estimated.

In contrast, during reduction of 1-deaza-FMN flavodoxin (*M. elsdenii*) at pH values <9, more than 90% of the total flavin is stabilized as the semiquinone at half-reduction [cf. Entsch et al. (1980) for spectral properties of the semiquinone]. Such a large separation of the two one-electron potentials allows accurate measurement of each equilibrium. A plot of the measured potentials versus pH is given in Figure 3A. The data for 1-deaza-FMN flavodoxin are qualitatively similar to those for native flavodoxin (Mayhew, 1971a; Stankovich, 1980) (Figure 3B). Potentials of the ox/sq couple lie on a straight line with a −60 mV slope (E'_0 = −160 mV at pH 7.0, 25 °C) which parallels that for native flavodoxin but is 40 mV more negative. The sq/red data can be fitted to a −60 mV slope at low pH and are pH independent at high pH, defining a pK of 7.4 for reduced 1-deaza-FMN flavodoxin. Above the pK, the 1-deaza-FMN flavodoxin potentials are about 45 mV more negative than those for native flavodoxin. Below pH 6.0, the 1-deaza-FMN flavodoxin potentials are more positive (~45 mV) than those for native flavodoxin. This inversion in the relative potentials for the two proteins is a reflection of the difference of 1.6 units in their pK values. As will be shown below, each pK represents the ionization of a different group.

Structural Perturbations in 1-Deaza-FMN Flavodoxin and the K_a for 1-Deaza-FMN. Difference Fourier maps (Figure 4) displayed significant peaks adjacent to the main chain at residues 88–90, near the position of CH(1) of 1-deazaflavin, but no features suggesting shifts in the position of the isoalloxazine ring itself. The differences have been interpreted as displacements of main-chain atoms from the carbonyl group of Tyr 88 to the C_α of Trp 90. Although the calculated distances between C(1) and the atoms of Gly 89 depend somewhat on the strategy of refinement, it is clear from Figure 5 that in 1-deaza-FMN flavodoxin the 88–89 peptide has rotated to align with O(2) of 1-deaza-FMN. Some of the structural perturbation is propagated to the side chains of Tyr 88 and Trp 90. The atomic displacements determined by model building and refinement (Figure 5) allow packing of CH(1) against the peptide NH of Gly 89 and relieve the overlap that would otherwise occur. In particular, hydrogens at the flavin C(1) and Gly 89 N are separated by 2.0–2.2 Å in the 1-deaza-FMN flavodoxin structure whereas in the absence of structural changes, they would have been only 1.4$_6$ Å apart (Figure 5b). Since 1-deaza-FMN is the steric analogue of reduced FMN protonated at N(1), the structure of 1-deaza-FMN flavodoxin substantiates our hypothesis that protonation of N(1) would necessitate some perturbation of the structure.

The association constant for oxidized 1-deaza-FMN binding to apoflavodoxin from *M. elsdenii* is less than that for FMN by a factor of approximately 200: $K_a = 2.3 \times 10^9$ for FMN and 1.0×10^7 for 1-deaza-FMN. The association constant for the reduced anionic form of 1-deaza-FMN is less than that for reduced anionic FMN by a factor of about 40 (data from Figure 3 and Scheme I). Included in the differences in K_a, between oxidized FMN flavodoxin and oxidized 1-deaza-FMN flavodoxin, are the loss of a hydrogen bond to N(1) (Burnett et al., 1974; Franken et al., 1984; Vervoort et al., 1985), changes in the strength of interactions at other flavin atoms that result from the differences in electronic structures of FMN and its 1-deaza analogue, perturbation of the protein structure, and differences in the energy of desolvation of FMN and 1-deaza-FMN. The K_a differences thus include more terms than the "intrinsic" free energy of interaction, as defined by Jencks (1981). Differences in desolvation energies, computed by Bash et al. (1987) for charged phosphonamides and their corresponding esters, were 3–4 kcal in magnitude [cf. Gao et al. (1989)]. We have no comparable estimates for desolvation of flavins, but one might expect desolvation to be less difficult for both the oxidized and reduced 1-CH analogues than for the corresponding FMN species, favoring binding of the 1-deaza analogues. The sum of structural perturbations and changes in hydrogen bonding or other interactions is thus envisaged to be considerably larger than the difference of 3.2 kcal derived from the K_a measurements for oxidized flavodoxins, or the difference of about 2.3 kcal calculated for reduced flavodoxins. Although the contributions of differential hydrogen bonding and deformation of structure to the relative affinities for FMN and 1-deaza-FMN cannot readily be dissected, we assume that perturbation of the structure requires energy and thus decreases the affinity for 1-deaza-FMN. This assumption has been incorporated in our model for control of the redox potential, as a basis for the low pK of N(1) in the protein-bound reduced flavin. It is difficult to estimate the energies associated with the structural change, but repulsion energies for close interatomic contacts, which are avoided by perturbing the structure, can be large.

Spectral Measurements of pKs. Differences in pKs between free and protein-bound flavin provide estimates of the magnitudes of the interactions between the protein and ionizable groups on the flavin ring. We have examined the titration behavior of reduced 1-deaza-FMN flavodoxin, of reduced FMN flavodoxin at N(1)H, of FMN flavodoxin semiquinone at N(5)H, and of oxidized FMN flavodoxin at N(3)H.

As can be seen from Figure 6, where spectra of reduced 1-deaza-FMN flavodoxin at various pH values are displayed, the protonated, reduced form has an absorbance maximum at 560 nm which disappears on deprotonation. Plotting the extinction coefficient at 560 nm versus pH (inset, Figure 6) gives a characteristic pK profile with a midpoint at pH 7.6. Thus the pK found for ionization of the flavin is very close to the pK of 7.4 determined from the redox potential measurements. Similar spectral changes occur upon protonation of free reduced 1-deazariboflavin (Spencer et al., 1977) except that the absorbance maximum is shifted to 480 nm. The pK of reduced 1-deazariboflavin [5.6 (Spencer et al., 1977)] is 2 units below that of 1-deaza-FMN flavodoxin.

For native reduced *M. elsdenii* flavodoxin, the pK = 5.8 observed in the potential versus pH profile (Mayhew, 1971a)

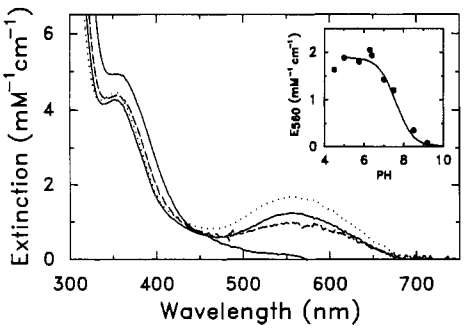


FIGURE 6: pH dependence of the reduced 1-deaza-FMN flavodoxin (*M. elsdenii*) absorbance spectrum. Spectra were obtained from separate photoreduction experiments (see Experimental Procedures) at various pH values: pH 9.2 (—), pH 7.5 (- - -); pH 7.0 (—); and pH 6.4 (···). Some spectra have been omitted for clarity. Inset: Plot of extinction at 560 nm versus pH. The line is a theoretical curve, calculated for a pK of 7.6 with a maximum extinction of 1.9 mM^{-1} cm^{-1} at low pH and a minimum extinction of zero at high pH. The same line is obtained in a simulation of the titration of 1-deaza-FMN in flavodoxin, interacting with group X, in a thermodynamic cycle with the following microscopic pK values: $dFMNH_2$ in the presence of XH, 5.6; $dFMNH_2$ in the presence of X^-, 7.6; XH in the presence of $dFMNH_2$, 4.0; and XH in the presence of $dFMNH^-$, 5.8.

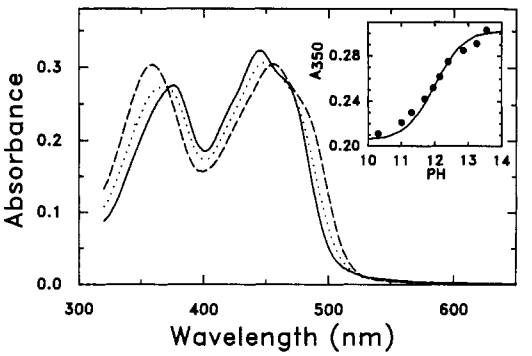


FIGURE 7: Ionization of oxidized flavodoxin (native, *M. elsdenii*). The 445-nm maximum is red shifted to 456 nm with loss of extinction to 9.75 mM^{-1} cm^{-1}. The 376-nm maximum is blue shifted to 357 nm with an increase in extinction to 9.80 mM^{-1} cm^{-1}. Spectra were measured with a scanning stopped-flow spectrophotometer as described under Experimental Procedures: pH 8.0 (—); pH 11.8 (···); pH 12.9 (- - -). Some spectra have been omitted for clarity. Inset: Absorbance at 350 nm plotted versus the measured pH. The line is a theoretical curve calculated for a pK = 12.0 and a total ΔA_{350} = 0.098.

does not coincide with an ionization of the reduced isoalloxazine ring. Reduced flavodoxin spectra are essentially identical at pH values from 4.6 to 8.5 (results not shown). The pK is thus shifted beyond the range of our experiments from its free solution value of about 6.5 (Draper & Ingraham, 1968).

The pK of the N(3)H in free oxidized flavins is 10.0–10.4 (Draper & Ingraham, 1968; Theorell & Nygaard, 1954). The crystal structure of *C. beijerinckii* flavodoxin indicates a hydrogen bond between N(3)H and the carboxylate of Glu 59, suggesting that the pK for the N(3)H of the bound flavin should be greater than 10.2. In order to make measurements at the required pH values without interference from protein denaturation, we employed a scanning stopped-flow spectrophotometer. This allowed us to measure the spectrum within 10 s after a pH jump. Spectral changes associated with ionization of the protein-bound flavin were complete within the 3-ms mixing time of the instrument. Denaturation of oxidized flavodoxin at pH >12.5 could be monitored by the spectral change which occurred as flavin was released. First-order rates for denaturation ($t_{1/2}$ = 5 min at pH 12.8, $t_{1/2}$ = 1 min at pH 13.3) were sufficiently slow that no corrections to the 10-s spectra were necessary up to pH 13.3. The pH of the reaction mixture was measured after the spectra were recorded. Figure 7 shows the spectral changes associated with ionization of oxidized flavodoxin, while the inset gives the dependence of the 350-nm absorbance on pH. A pK of 12 is determined from these data.

The N(5)H in flavodoxin semiquinone is also expected to titrate at a higher pH than its counterpart in free FMN [pK = 8.6 (Draper & Ingraham, 1968)], due to hydrogen bonding between N(5)H and the carbonyl oxygen of Gly 57, which is evident in the crystal structure (Smith et al., 1978). For measurements of this pK we used the scanning stopped-flow spectrophotometer with anaerobic mixing. At pH >11, a first-order loss of semiquinone and concomitant appearance of oxidized flavodoxin was seen, but loss of semiquinone exceeded formation of oxidized flavodoxin. Semiquinone did not disappear completely, and the extent of the reaction increased with pH, indicating an approach to equilibrium. These observations suggested disproportionation rather than oxidation; disproportionation is predicted since the separation between the two 1-electron potentials decreases as the pH is raised (see Figure 3B), lowering the maximum fraction of semiquinone at equilibrium. Extrapolation of the spectral changes back to zero time showed that no ionization of the neutral semiquinone had occurred up to the highest pH used, pH 12.5. Therefore, the pK for N(5)H of the bound flavin semiquinone must be >13, a shift of more than 4.5 pH units from its value for free FMN.

Table II: pK Values for Flavins: Free and Bound to Flavodoxin

flavin	pK (free)	pK (bound to flavodoxin)
FMN		
oxidized	10.0–10.4[a,b]	12.0[c]
semiquinone	8.6[a]	>13[c]
fully reduced	6.5[a]	<4[c]
1-dFMN		
oxidized	ND[d]	ND[d]
semiquinone	ND[d]	ND[d]
fully reduced	5.6[e]	7.4[c]
6-mercapto-FMN[f]	5.9[g]	6.4[h]
6-hydroxy-FMN[f]	7.1[i]	≈9.0[i]
8-hydroxy-FMN[f]	4.8[j]	6.1[j]
2-thio-FMN[f]	9.8[k]	11.0[k]

[a] Draper & Ingraham, 1968. [b] Theorell & Nygaard, 1954. [c] This work. [d] ND stands for not determined. [e] Spencer et al., 1977. [f] Oxidized form of the flavin. [g] Ghisla et al., 1986. [h] Massey et al., 1986. [i] Mayhew et al., 1974. [j] Ghisla & Mayhew, 1976. [k] Claiborne et al., 1982.

To summarize the experimental findings, Table II lists the pK values of the three oxidation states of FMN and 1-deaza-FMN, free and bound to apoflavodoxin, and the pK values of the oxidized forms of various artificial flavins with ionizable substituents, free and protein bound. The neutral form of the flavin is stabilized, i.e., the pK is raised, for ionizations at flavin positions 2, 3 (oxidized FMN flavodoxin), 5 (semiquinone FMN flavodoxin), 6, and 8. Only at position 1 (in reduced FMN flavodoxin) is the negatively charged form of the flavin stabilized. It is apparent that the flavin–protein interactions which result in a preference for the binding of most species in the neutral form must be overwhelmed by other factors which dictate the preferential binding of the reduced native FMN anion.

Discussion

It has been difficult to establish the basis for the very low sq/red potentials of flavodoxins, especially since there are no significant structural changes accompanying reduction of the

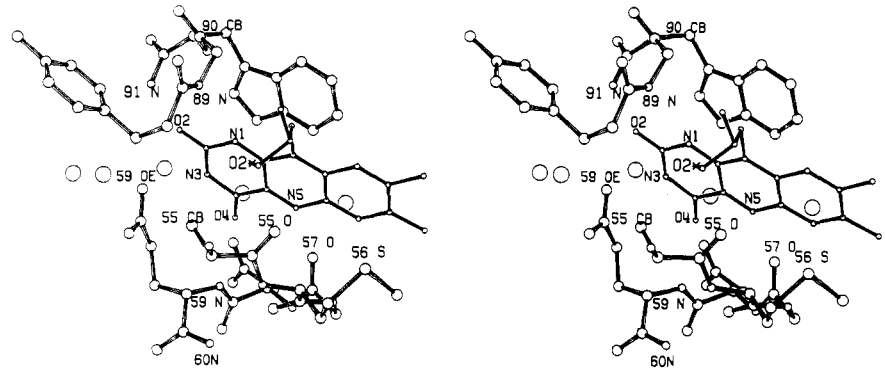


FIGURE 8: The surroundings of the isoalloxazine ring in the crystal structure of reduced flavodoxin from *C. beijerinckii*. The view is of the *re* face of the flavin ring, seen from the interior of the protein. The FMN (thinner bonds) has been truncated at C3 of the ribityl side chain. Protein sequences shown are ^{55}Ala-Met-Gly-Asp-Glu and ^{88}Tyr-Gly-Trp-Gly. Calculations on model flavins (Hall et al., 1987) have shown that in the anionic species much of the net charge of −1 is distributed on the heteroatoms of the isoalloxazine ring. As can be seen in this figure, these atoms have several protein neighbors, some forming favorable, and some unfavorable interactions. For example, partial negative charges on N(1) and O(2) can be stabilized by the NH dipoles at Gly 89 and Gly 91, but each of these flavin atoms is also in contact with aliphatic or with aromatic CH groups; the carbonyl oxygen of Ala 55 contacts C(10a) and N(10) and is 4.2 Å from N(1). O(4) hydrogen bonds to NH 59 but is only 3.1 Å from O 57. A hydrogen bond connects N(5) and O 57, which are separated by 3.1 Å. Bound water molecules (large unfilled spheres) occupy sites near O(2), O(4), O 57, and the carboxylate oxygens of Glu 59. Coordinates are from least-squares refinement (Hendrickson, 1985); R = 0.181 for data between 10.0 and 1.8 Å.

semiquinone species (Ludwig et al., 1976; Smith et al., 1978). The experiments described here were designed to test the idea that neutral reduced flavin, protonated at N(1), is not readily bound by apoflavodoxins from *M. elsdenii* or *C. beijerinckii* and to estimate the magnitude of the electrostatic interactions that result from binding of the negatively charged anionic species.

Ionization State of FMN in Reduced Flavodoxin. ^{15}N NMR spectra have been measured for reduced flavodoxins from *M. elsdenii* (Franken et al., 1984), *C. beijerinckii* (Vervoort et al., 1986a), *A. vinelandii* (Vervoort et al., 1986a), *D. vulgaris* (Vervoort et al., 1985), and *Anabaena 7120* (Stockman et al., 1988) at pH values ranging from 5.5 to 8.0. In these proteins the N(1) chemical shifts vary from 182.0 to 186.6 ppm versus NH_3. Comparison with reduced FMN, which displays a shift of 181.3 ppm at pH 8.5 and 128.0 ppm at pH 5.0 (Vervoort et al., 1986b), leads to the conclusion that the reduced flavodoxins are unprotonated (anionic). Measurements for *M. elsdenii* flavodoxin (Franken et al., 1984) were carried out from pH 7.6 to 5.5, just below the redox-linked pK of 5.8. Optical spectra reported here extend the pH range of observation to 4.6. The absence of any change in chemical shift at pH 5.5 or in the optical spectrum at pH 4.6 implies that the pK for protonation of N(1) must be less than 4.0, more than 2 units below the pK for free reduced FMN. The crystal structure of 1-deaza-FMN flavodoxin from *C. beijerinckii* suggests that steric effects can suppress protonation at N(1). Taken together, these results establish that the pK = 5.8 observed in measurements of the sq/red potential of flavodoxins (Mayhew, 1971a) must be attributed to some other proton acceptor (see below). Further, the unchanged visible spectra of reduced flavodoxin at high and low pH suggest that no other atom on the flavin ring acts as an alternative proton acceptor.

The Redox-Linked Proton Acceptor. The observed pK of 5.8 in *M. elsdenii* flavodoxin must be assigned to a group that interacts with the flavin. Because the structure of the closely related *C. beijerinckii* flavodoxin does not undergo any significant conformation change on reduction of the semiquinone that might induce pK changes far from the FMN, efforts to identify the linked group have been confined to residues in the immediate neighborhood which could be strongly influenced by the changes in charge and electronic structure of the flavin ring. In principle, the ionizable residue could be either XH^+ or XH, but since *M. elsdenii* and *C. beijerinckii* flavodoxins contain no histidines (Tanaka et al., 1973, 1974), acidic groups in the vicinity of the FMN or the FMN phosphate are the most likely candidates. Therefore, we have formulated the reduction below pH 5.8 as

$$FlvH^{\bullet}X^- + H^+ + e \rightarrow FlvH^-XH$$

Addition of a proton to X^- implies that the linked group undergoes a significant change in pK when the semiquinone is reduced.

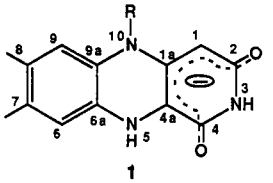
Asp 58 and Glu 59 in *C. beijerinckii* flavodoxin are the acidic groups closest to the flavin ring; FMN phosphate, Glu 63, and Asp 92 are farther away [9.0, 12.3, and 9.9 Å from N(1), respectively]. Because Asp 58 is more exposed to solvent, whereas Glu 59 is hydrogen-bonded to N(3) (Figure 8), Glu 59 may be more affected by changes in the flavin ring. In *M. elsdenii* flavodoxin serine and glutamate occur in the positions homologous to Asp 58 and Glu 59 in *C. beijerinckii*. We believe that glutamate (Glu 60 in *M. elsdenii* flavodoxin or Glu 59 in *C. beijerinnckii* flavodoxin) is the most logical candidate for the redox-linked group X^-.

The pK of this glutamate in oxidized flavodoxin would be lower than normal because of hydrogen bonding to N(3)H. To assess the effect of this hydrogen bond, we determined the pK of N(3)H in oxidized flavodoxin from *M. elsdenii* and found it to be about 1.8 units higher than for free FMN. A simple treatment (Laskowski & Scheraga, 1954) of the interaction of Glu 60 with N(3)H, assuming a value of 10.2 in the absence of the neighboring charge, would yield an equivalent decrease in the glutamate pK to 3.0 from the expected value of 4.8 (Hill, 1944). This estimate ignores the effects of close neighbors and of the net charge on the protein. Nevertheless, the glutamate pK may increase by as much as 3 units, from about 3.0 in the oxidized form to 5.8 in the reduced species, due to introduction of negative charge in the pyrimidine ring of the reduced flavin. An increase of 2.8 units is equivalent to a free energy of 3.8 kcal, about 80% of the energy associated with the shift in the sq/red potential from −175 to −375 mV. Electrostatic effects of this magnitude are well documented in other proteins: ΔpK = −4.2 for cysteine in papain (Lewis et al., 1981) and ΔpK = −4.7 for lysine in acetoacetate decarboxylase (Kokesh & Westheimer, 1971). The presence of this negatively charged residue near the pyrimidine moiety of the flavin would also account for the in-

creased pK values of the artificial flavins listed in Table II, which in their oxidized forms carry an ionizable constituent, and which in the anionic state have appreciable negative charge delocalized in the pyrimidine ring (Ghisla & Massey, 1986).

Determination of the pK of Glu 60 in *M. elsdenii* flavodoxin would test the role of this residue as proton acceptor and may be feasible if appropriate resonances can be assigned in NMR spectra of reduced flavodoxin. It is unlikely that meaningful titration data can be obtained for the oxidized protein, where the pK of XH is expected to be below 4.0. If N(1)H, in the presence of X^-, and XH, in the presence of $N(1)^-$, have similar pKs in reduced flavodoxin, then the titration curves for each should be complex, but our current model postulates a difference of about 1.8 pH units in these pK values and predicts simple titration behavior. The properties of a mutant in which Glu is replaced by Gln may also furnish support for our suggestion that glutamate is the redox-linked group. ^{31}P NMR spectra indicate that the FMN phosphate is a dianion in oxidized *M. elsdenii* flavodoxin (pH 5.5–8.0) and in reduced flavodoxin at pH 8.2 (Moonen & Müller, 1982). Further ^{31}P NMR studies of the reduced protein could determine whether a phosphate pK is linked to reduction of the flavin.

Analysis of the Potentials and Binding Constants of 1-Deaza-FMN Flavodoxin. In choosing the 1-deaza-FMN analogue for detailed studies, we hoped to exploit both its steric and electronic properties. The negative charge on anionic reduced 1-deaza-FMN is distributed into the pyrimidine moiety (Hall et al., 1987), as is the charge on reduced FMN, making 1-deaza-FMN a good analogue with which to probe the role of electrostatic interactions in native reduced flavodoxins (cf. formula **1**). Unlike FMN, neutral reduced 1-



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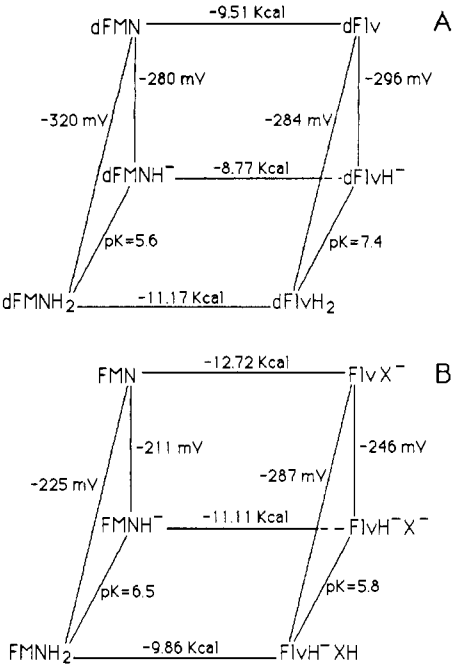
deaza-FMN is protonated at O(2) or O(4) (Spencer et al., 1977). These positions appear to be accessible for protonation in *C. beijerinckii* flavodoxin (Figure 8).

Since the pK value found from spectral titration of reduced 1-deaza-FMN flavodoxin coincides with the redox-linked pK, we assume that the flavin itself is the redox-linked proton acceptor in this case. We see no evidence, down to pH 4.5, for addition of a second proton, and conclude than when the reduced isoalloxazine has no net charge, the pK of group XH remains low. Nevertheless, X^- is still in a position to interact with the flavin ring and to affect differentially the affinities of neutral and anionic 1-deaza-FMN for apoflavodoxin. As shown in Figure 6, the interaction of the ionization of $dFMNH_2$ with ionization of XH will not be evident in the shape of the titration curve unless the pK values for these species are rather close to one another.

Because the pK of the protein-bound 1-deaza-FMN is at an accessible pH, we have been able to determine the affinity of apoflavodoxin for both the anionic and neutral forms of reduced 1-deaza-FMN. The potentials and association constants are connected by thermodynamic cycles as shown in Scheme IA, so that K_a values for lower oxidation states can be calculated from K_a for the oxidized form and the redox potentials for free and bound flavin species. At pH 7, free reduced 1-deaza-FMN is anionic, while the protein is a mixture of neutral and anionic species. A general treatment of effects of ionizations on potentials is presented by Clark (1960; pp 60–135), but when pKs are well separated as is the case

Scheme I: Interactions of Free Energies of Binding, Redox Potentials, and pKs for Free Flavins and Flavodoxins[a]



[a] The relevant two-electron equilibria between fully oxidized and reduced forms are represented on three-dimensional figures (pH 7.0 and 25 °C). (A) 1-Deaza-FMN and 1-deaza-FMN flavodoxin. The front face of the figure describes a two-electron reduction to the neutral species ($2e^- + 2H^+$); the back face describes a two-electron reduction to anionic forms ($2e^- + H^+$). The third (bottom) face relates pK values to the free energies of association. The K_a for oxidized 1-deaza-FMN, the protein potentials, and the protein pK values are reported in this paper. All the potentials were taken from Figure 3A. The potential of −320 mV for the free 1-deaza-FMN/1-deaza-$FMNH_2$ couple was obtained by extrapolating to pH 7 the low-pH limb of the graph of free 1-deaza-FMN potentials versus pH. The corresponding potential of −296 mV for the 1-deaza-FMN flavodoxin/reduced 1-deaza-FMN flavodoxin (anion) couple was obtained by extrapolating to pH 7 the high-pH limb of the graph for the two-electron equilibrium (Figure 3A). (B) Two-electron reduction and associated equilibria for FMN flavodoxin at pH 7.0. K_a for oxidized FMN, 2.3×10^9 M^{-1}, is from Mayhew (1971b), and the potentials are from Figure 3B. The diagram is the analogue of that in panel A. It differs from (A) by explicit inclusion of the redox-linked group X as a proton acceptor; the complex $FMNH_2X^-$ is not observed. Values at pH 7.0 for the FMN/$FMNH_2$ couple (−225 mV) and the Flv/FlvH XH flavodoxin couple (−287 mV) were obtained by extrapolation to pH 7 of the low-pH limbs of the FMN and flavodoxin traces, respectively.

for the flavodoxin data, potentials for equilibria involving single species can be obtained for any pH by extrapolation of the linear portions of E_0 versus pH curves (See Figure 3A). The free energies of association calculated in this way for 1-deaza-$FMNH^-$ and for 1-deaza-$FMNH_2$ show that the protonated form is bound more tightly ($|\delta\Delta G| = 2.4$ kcal). Reduced affinity for the negatively charged flavin is consistent with the presence of negatively charged group (X^-) in the flavin binding site. The differences in binding constants can equally well be obtained from the cycle in the lower part of Scheme IA, which emphasizes that they depend upon the relative pKs of the bound and free species.

A Model for Modulation of the Semiquinone/Reduced Potential of Native Flavodoxin. Analysis of the reduction and protonation of FMN flavodoxin shows properties distinctly different from those of 1-deaza-FMN flavodoxin (cf. Scheme I). Because the pK of reduced FMN decreases on protein binding, combination of $FMNH_2$ with apoprotein is not observed, and experimental comparison of affinities of the protein for the neutral and anionic species of reduced FMN is pre-

cluded. Electrostatic interactions in reduced FMN flavodoxin can instead be estimated from the ionization behavior of group XH, tentatively identified as Glu 60 in *M. elsdenii* flavodoxin, whose pK is estimated to increase by about 3 units, corresponding to a $|\Delta G|$ of about 4 kcal, upon reduction of FMN to the anionic form.

The present examination of *M. elsdenii* and *C. beijerinckii* flavodoxins and their 1-deaza-FMN analogues leads to a model for the reduced FMN flavodoxins that represents a compromise in energetics. Perturbation of the protein structure and loss of the hydrogen bond between N(1) and the peptide NH of Gly 89, both required for protonation of N(1), combine to lower the pK of N(1). At the same time, the protein environment, particularly the linked group X^- (probably glutamate), provides charge repulsion that decreases the binding affinity of $FMNH^-$ and thereby lowers the sq/red potential.

The structural elements providing electrostatic repulsion, e.g., the hydrogen bond from N(3)H that lowers the pK of glutamate, and the steric hindrance that lowers the reduced flavin pK, are in place in the oxidized and semiquinone flavodoxins. What is required to trigger electrostatic repulsion is introduction of the second electron into the flavin. Our experiments on the binding of reduced 1-deaza-FMN indicate a magnitude of about 3 kcal for the electrostatic repulsion associated with charging of the flavin ring, but we do not mean to dismiss entirely the effects of other factors, such as ring distortion (Hall et al., 1987; Moonen et al., 1984), on the redox potentials.

The general features of this model may apply to other species of flavodoxins, despite differences in sequence and three-dimensional structure (Laudenbach et al., 1987). The arrangement of the main chain, relative to N(1) and O(2), is very similar in the structures of *C. beijerinckii*, *D. vulgaris* (Watenpaugh et al., 1973), and *Anacystis nidulans* (Smith et al., 1983) flavodoxins. In the latter structure, the distance between N(1) and the N of Asp 90 is 3.1 Å in the oxidized form, very close to the value of 3.0_8 found in *C. beijinckerii* flavodoxin for the spacing between N(1) and the N of Gly 89. However, the counterpart of Glu 59 in *C. beijerinckii* is not found in *D. vulgaris* or in longer chain flavodoxins such as *A. nidulans*. In these molecules a peptide carbonyl oxygen hydrogen bonds to N(3)H. The flavin ring is also less accessible to solvent, and conserved acidic residues near the FMN ring may assume the role we assign to Glu 60 in *M. elsdenii* or Glu 59 in *C. beijerinckii* flavodoxin.

In our discussion of the redox potentials of flavodoxin, we have focused on the pK values of the flavin and its protein neighbors. It is our contention that the flavin potentials are manipulated, in large part, by altering these pK values. Since the redox potential of the flavin depends on its ionization state, altering the flavin pK values necessarily affects the potentials. The reader can examine this point by shifting the pK values on the potential versus pH plot for free flavin [see Draper and Ingraham (1968)], or by changing the pK values for plots like those shown in Figure 3. For each oxidation state of FMN there are ionizations that affect the potentials observed at physiological pH: pK = 10.2 for oxidized, pK = 8.6 for semiquinone, and pK = 6.5 for reduced. Furthermore, the locus of ionization (protonation) is different for each level of reduction: N(3) for oxidized, N(5) for semiquinone, and N(1) for reduced. This makes it possible for protein–flavin interactions to raise the pK of oxidized flavin while lowering the pK of reduced flavin. When one adds the capability of selectively stabilizing or destabilizing an oxidation state by introducing or removing hydrogen bonds, it is possible to generate a variety of potential versus pH profiles. Flavodoxin from *M. elsdenii* provides a specific illustration of these factors in operation. We would like to suggest that combinations of these same factors will be involved in determining the corresponding profiles for other flavoproteins. Thus the pK values for protein-bound flavins become important determinants in understanding the oxidation–reduction behavior of flavoproteins.

Acknowledgments

We thank Carl C. Correll for assistance with collection and processing of the X-ray data and Z. Song for assistance with measurements of the association constants. Comments by a reviewer, on the treatment of interacting pKs, were very helpful.

Registry No. FMN oxidized, 146-17-8; FMN semiquinone, 34469-63-1; FMN reduced, 5666-16-0; 1-dFMN oxidized, 70805-82-2; 1-dFMN semiquinone, 129443-15-8; 1-dFMN reduced, 77289-04-4.

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