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Site-Directed Mutagenesis of Tyrosine-98 in the Flavodoxin from *Desulfovibrio vulgaris* (Hildenborough): Regulation of Oxidation–Reduction Properties of the Bound FMN Cofactor by Aromatic, Solvent, and Electrostatic Interactions†

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ABSTRACT: The contributions made by tyrosine-98 in establishing the redox properties of the flavodoxin from *Desulfovibrio vulgaris* were investigated by substituting a number of amino acids at this position using site-directed mutagenesis. Tyr98, which makes extensive van der Waals contacts with the isoalloxazine ring of the flavin mononucleotide cofactor, is often found in the cofactor binding site of flavodoxins and related flavoproteins. Solution studies suggest that tyrosine may assist in the stabilization of the neutral flavin semiquinone through preferential complex formation relative to the other oxidation states. In this study, the midpoint potentials of the oxidized/semiquinone couple of the Y98W and Y98F mutants were found to be very similar to the wild-type flavodoxin. However, significantly more negative midpoint potentials (by 25–60 mV) were observed in the Y98A, Y98H, and Y98R mutants. These results imply that it is the general apolar environment provided by the aromatic amino acids rather than preferential affinities suggested by solution studies that is at least partially responsible for the thermodynamic stabilization of the neutral flavin semiquinone in this flavodoxin. The midpoint potential of the semiquinone/hydroquinone couple is profoundly dependent on the properties of the amino acid at this position. Compared to phenylalanine, the more electron-rich aromatic side chains of tryptophan and tyrosine decrease the midpoint potential of this couple by 30–40 mV. Greater solvent exposure of the isoalloxazine ring in the Y98A mutant increases the midpoint potential by 140 mV relative to wild type. The positively charged amino acids increase the midpoint potential of this couple by >180 mV, most probably through favorable electrostatic interactions with the flavin hydroquinone anion. These observations strongly support the proposition that the functional role of the electron-rich, apolar aromatic amino acid residues adjacent to the flavin isoalloxazine ring is to substantially destabilize the flavin hydroquinone anion, resulting in the very low oxidation–reduction potentials for the semiquinone/hydroquinone couple that typify the flavodoxin family.

Flavodoxins represent an important class of electron transfer protein that utilizes the noncovalently bound flavin mononucleotide cofactor as the only redox center. Flavodoxins have been isolated from a wide range of sources including strictly anaerobic, facultatively anaerobic, obligately aerobic, and photosynthetic bacteria, as well as from several species of algae (Mayhew & Tollin, 1992). None have been reported in higher plants and animals. Although the exact physiological role is not well understood in every case, flavodoxins seem to function as low-potential electron carriers between other redox proteins, cycling between the semiquinone and the fully reduced states. These proteins can often substitute for ferredoxins *in vitro* and, in some bacteria, may also do so under certain growth conditions *in vivo* (Mayhew & Ludwig, 1975). The physiological role for the flavodoxin from *Klebsiella pneumoniae* has been more convincingly established as the principal electron donor to the nitrogenase complex (Nieva-Gomez *et al.*, 1980).

Flavodoxins have been studied extensively, especially with regard to their redox and electron-transferring properties [for a recent review, see Mayhew and Tollin (1992)]. A characteristic feature of this class of flavoprotein is the rather large perturbation of the reduction potentials of the bound FMN cofactor, particularly for that of the semiquinone/hydroquinone (sq/hq) couple, for which the midpoint potential (at pH 7) is shifted from −172 mV for FMN in solution to about −400 mV when bound. Midpoint potentials as negative as −520 mV have been reported for this couple in some flavodoxins, among the lowest of all flavoproteins. The flavin hydroquinone appears to be present in its anionic form throughout the pH range that can be studied, an observation thought to be of functional importance (Ludwig *et al.*, 1990). The midpoint potential of the oxidized/semiquinone (ox/sq) couple is generally shifted to more positive potentials from that of FMN in aqueous solution, with values reported as high as −50 mV versus −238 mV for free FMN, although the degree of this perturbation is quite dependent on the source of the flavodoxin (Ludwig & Luschinsky, 1992). All flavodoxins seem to stabilize the blue neutral form of the flavin semiquinone, shifting the pK_a of the semiquinone from about 8.3 for FMN in aqueous solution to values greater than 10.5.

Tertiary structures have been established for several different flavodoxins, often in all three redox states, contributing greatly to our understanding of the specific molecular interactions that exist between the cofactor and this class of flavoprotein. Although the general folding pattern of the polypeptide is similar in all flavodoxins, the specific interactions between the FMN cofactor and the protein differ, sometimes substantially. The structures of both the semiquinone and fully reduced states are very nearly identical but differ with regard to the orientation of a carbonyl group of the peptide backbone near the N(5) position of the isoalloxazine ring of

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the cofactor from that noted in the oxidized state. The rearrangement of this carbonyl group during reduction of the flavin is a common feature of several flavodoxins and seems to result in the formation of a new hydrogen bond between the peptide backbone and the N(5)H of the reduced cofactor (Smith *et al.*, 1977; Watt *et al.*, 1991). This hydrogen bond may be at least partially responsible for the thermodynamic stabilization of the semiquinone in the flavodoxins (Smith *et al.*, 1977). However, other structural features of the FMN binding site may also make significant contributions such as aromatic stacking interactions, the apolar environment, steric constraints, solvent exclusion, and electrostatic interactions. Thus, it remains unclear as to which of the flavin–protein interactions are responsible for establishing the oxidation–reduction properties of the bound FMN and to what extent each contributes. Flavodoxins would seem to be a good model system in which to investigate this fundamental aspect of flavoprotein biochemistry.

In this study, the role of the tyrosine residue at position 98 in the flavodoxin from *Desulfovibrio vulgaris* is investigated through the introduction of several amino acid substitutions at this position by the oligonucleotide-directed mutagenesis of the cloned structural gene (Krey *et al.*, 1988). Tyrosine-98 is one of two aromatic amino acid residues (the other being tryptophan-60) that are part of two polypeptide loops (residues 58–67 and residues 92–102) that form a major portion of the FMN binding site in this flavodoxin (Figure 1A) (Watenpaugh *et al.*, 1973; Watt *et al.*, 1991). These two residues are conserved in all flavodoxins thus far characterized from the *Desulfovibrio* genus and several other sources including *Anacystis nidulans*, *Anabaena 7120*, *Azotobacter vinlandii*, and *Chondrus crispus* (Helms *et al.*, 1990; Helms & Swenson, 1991, 1992; Ludwig & Luschinsky, 1992). The phenol side chain of Tyr98 is nearly coplanar with the outer face of the flavin isoalloxazine ring and makes extensive van der Waals contacts with it (Figure 1B). This spatial orientation provides the opportunity for the direct effect of this residue on the regulation of the oxidation–reduction properties of the cofactor in these flavoproteins. Solution studies involving complexes between flavin and aromatic amino acids indicate that nonbonding, aromatic stacking interactions may be important in the stabilization of the various redox states of the flavin (Draper & Ingraham, 1970; McCormick, 1977; Ishida *et al.*, 1986). The solvent shielding effects and the apolar environment provided by this residue may also represent important determinants in the reduction potential of the flavin redox system as lower midpoint potentials are observed for the flavin in aprotic organic solvents (Tatwawadi *et al.*, 1968; Sawyer *et al.*, 1971).

The systematic alteration of the chemical properties of the amino acid side chain at this juxtaposition to the flavin could also lead to the determination of the effects of such properties as charge, polarity, and solvent exposure on the flavin cofactor. This study was designed to test these concepts within a fixed protein structural framework. The location of this residue at the surface of the flavodoxin structure suggests that amino acid substitutions at this position may not introduce significant structural perturbations. Preliminary accounts of this work have been presented previously (Swenson *et al.*, 1991a,b).

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Mutations were generated in a modified structural gene for the flavodoxin from *D. vulgaris* which has been cloned, characterized, and heterologously expressed in *Escherichia coli* (Krey *et al.*, 1988). The structural gene was modified by mutagenesis at the second codon, resulting in a proline to alanine substitution which greatly increases the level of expression in *E. coli* (Krey *et al.*, 1988). Because the amino-terminal methionine residue is completely processed off in *E. coli*, the alanine residue becomes the new amino terminus and is located near the surface of the protein opposite the FMN binding site. The P2A variant or "pseudo wildtype" flavodoxin is structurally isomorphous to the true wild-type protein in all other respects (Watt *et al.*, 1991) and appears to have identical physicochemical properties in all ways tested.

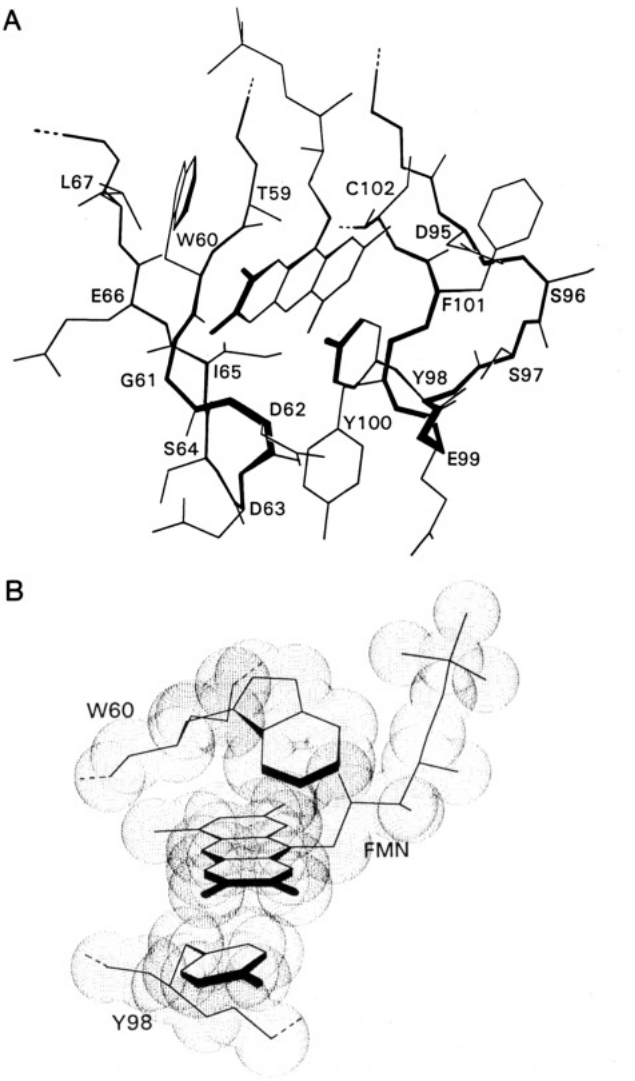


FIGURE 1: Structure of the flavin mononucleotide binding site in the flavodoxin from *Desulfovibrio vulgaris*. Representations are taken from X-ray crystal structural data (Watenpaugh *et al.*, 1973; Watt *et al.*, 1991). *Panel A*: Partial view of the protein structure which includes the two major polypeptide loops that form the majority of the flavin isoalloxazine binding site, residues 59–67 and 94–102. *Panel B*: Orientation of the aromatic amino acids tryptophan-60 and tyrosine-98 which flank either side of the isoalloxazine ring system of the flavin cofactor. Both aromatic amino acid residues are highly conserved in the flavodoxins from the *Desulfovibrio* species and other organisms. Dots represent the van der Waals surfaces of each residue.

Two different phagemid constructions were used for mutagenesis, pBS(+)MAK and pBS(–)DVFD.1. Oligonucleotide-directed mutagenesis was performed using the method described by Kunkel (Kunkel, 1985; Kunkel *et al.*, 1987) utilizing the MUTA-GENE kit by Bio-Rad. Template DNA was produced in *E. coli* strain CJ236 (*dut*$^-$, *ung*$^-$) and single-stranded DNA isolated by established methods. Mutagenic oligonucleotides were synthesized by the Ohio State

University Biochemical Instrument Center on an Applied Biosystems Model 380B synthesizer using β-cyanoethyl phosphoramidite chemistry. Four oligonucleotides (22 nucleotides in length), spanning codon 94 (wobble position only) through codon 101 in the structural gene, were synthesized to be complementary to the "sense" strand [for use with the single-stranded pBS(+)MAK template]. These oligonucleotides contain appropriate base mismatches at codon 98 so as to convert a tyrosine codon (TAC) to either an alanine (GCC), a methionine (ATG), an arginine (CGC), or a tryptophan (TGG). Two additional oligonucleotides (18 nucleotides in length) which are complementary to the "nonsense" strand of the gene [for use with the single-stranded pBS(−)DVFD.1 template] were synthesized to convert the codon for Tyr98 to that for a histidine (CAC) or phenylalanine (TTC). These two oligonucleotides also introduce an additional silent C→A mutation at the third position of codon 97 (serine) so as to allow for a more efficient screening of transformants by direct hybridization of dot blots to the mutagenic oligonucleotides, if so desired.

The mismatched oligonucleotides (2–3 pmol) were annealed to the single-stranded template (0.1 pmol) by heating the mixture to >70 °C followed by cooling to 30 °C over 40 min before elongation with T4 DNA polymerase and ligation by T4 ligase. A portion of this mixture was used to transform *E. coli* AG-1 cells. Plasmids from a representative sample of transformants were screened for mutants by dideoxy DNA sequencing using the Sequenase (Stratagene) protocol.

The 450-bp *Nco*I–*Hin*dIII restriction fragment from the confirmed Y98A, -M, -R, and -W mutants was transferred, in each case, into the pBUCtac expression vector. This vector is a hybrid phagemid which contains the *tac* promoter cloned along with a portion of the polylinker region of pUC19 plasmid into pBluescript phagemid (Krey *et al.*, 1988). In the case of the confirmed Y98F and -H mutants, the 640-bp *Cla*I fragment from the mutated pBS(−)DVFD.1 construction was isolated, digested with *Acc*I, and ligated into pBS(+)MAK which had the internal 295-bp *Acc*I fragment containing the wild-type sequence removed. The 450-bp *Nco*I–*Hin*dIII restriction fragment from this construction was transferred to the pBUCtac vector as above. The final pBUCtac vector construction for all six mutants was used to transform *E. coli* XL-1 Blue cells. The nucleotide sequence of the entire coding region for each mutant phagemid construction was confirmed using the Sanger dideoxynucleotide sequencing procedure using the Sequenase protocol.

Expression and Purification of the Flavodoxin Holoprotein Mutants. Transformed *E. coli* XL-1 Blue cells were cultured for 15–24 h at 37 °C with agitation in NZY medium containing 25 μg/mL ampicillin. Cells were harvested by centrifugation at 6000*g*, resuspended in 50 mM Tris-HCl, pH 7.3, and lysed by a single passage through a French press cell at 12 000–15 000 psi. The flavodoxin holoprotein was isolated from the supernatant by a modification of the method described by Mayhew and Massey (1969). The supernatant fraction was applied to a DEAE-cellulose column equilibrated in 50 mM Tris-HCl, pH 7.3 (determined at 25 °C) and washed with 50 mM Tris-HCl, pH 7.3, containing 150 mM NaCl. After the absorbance at 280 nm of the wash solution returns to a value less then 0.1, the flavodoxin protein was eluted with the Tris buffer containing 250 mM NaCl. The fractions containing flavodoxin holoprotein are pooled, diluted with an equal volume of Tris buffer, and loaded on a second smaller column of DEAE-cellulose. The column was washed with 50 mM Tris-HCl, pH 7.3, containing 150 mM NaCl until the absorbance at 280 nm was less then 0.05 and the flavodoxin eluted with Tris buffer containing 225 mM NaCl. Fractions having A_{274}:A_{457} ratios ≤4.6 were pooled and concentrated by ultrafiltration. The purity of each flavodoxin preparation was confirmed by SDS–polyacrylamide gel electrophoresis.

Extinction Coefficients. Extinction coefficients of the mutant flavodoxins in the fully oxidized state were determined as described by Mayhew and Massey (1969) as follows. Visible absorbance spectra are recorded for the purified flavodoxin mutants in 50 mM Tris-HCl, pH 7.3. The FMN cofactor was released and extracted from the apoprotein by incubation in 5% trichloroacetic acid for 5 min at room temperature followed by centrifugation at 4 °C. The protein pellet was washed with 5% trichloroacetic acid, the two supernatant fractions were combined on ice, neutralized with 2 M K_2HPO_4, and adjusted for volume, and the absorbance at 445 nm was recorded. An extinction coefficient of 12 500 M^{-1} cm^{-1} at 445 nm for the released FMN was used in the calculations (Whitby, 1953). All extinction coefficient values were determined in triplicate experiments. As a control, pure FMN was treated identically, with yields exceeding 97%. For all determinations, the A_{445}:A_{373} ratio was between 1.17 and 1.20, indicating that the released FMN had not undergone chemical degradation during the procedure (Beinert, 1960).

The extinction coefficient at 580 nm for the semiquinone of each mutant flavodoxin was determined during the anaerobic reduction in 60 mM sodium phosphate, pH 7.0, with either dithionite or NADPH in the presence of catalytic amounts of ferredoxin/$NADP^+$ reductase. The absorbance at 580 nm of fully formed semiquinone was determined at the intercept of the extrapolation of the linear portions of plots of the absorbance maximum of the oxidized protein in the 450 nm region versus the absorbance at 580 nm.

Fluorescence Measurements. Fluorescence excitation and emission spectra were recorded in 50 mM Tris-HCl, pH 7.3, at ambient temperature in an SLM Aminco spectrofluorometer. Emission values at 530 nm obtained for each flavodoxin are reported relative to that for free FMN under identical conditions and concentrations.

Midpoint Potential Determinations. The midpoint potential of the ox/sq couple of each flavodoxin was determined spectrophotometrically at 25 °C by equilibration with standard redox dyes in 60 mM sodium phosphate, pH 7.0, during anaerobic titration with dithionite. The indicator dyes utilized include indigo disulfonate ($E_{m,7}$ = −116 mV), 2-hydroxy-1,4-naphthoquinone ($E_{m,7}$ = −145 mV), anthraquinone-2,6-disulfonate ($E_{m,7}$ = −184 mV), and anthraquinone-2-sulfonate ($E_{m,7}$ = −225 mV). The reduction potential of the sq/hq couple was determined as above using safranine T ($E_{m,7}$ = −276 mV) or benzyl viologen ($E_{m,7}$ = −359 mV) as indicator dyes and/or by redox potentiometry versus a standard Ag/AgCl reference electrode which was calibrated after each experiment using the equimolar ferro–ferricyanide redox couple in 20 mM sodium phosphate, pH 7.0, and corrected for temperature (O'Reilly, 1973; Stankovich, 1980). Methyl and/or benzyl viologen were included as mediator dyes. Concentrations of the various oxidized and reduced species in equilibrium in solution were determined using multicomponent linear regression analysis of the near-ultraviolet/visible absorbance spectrum. Midpoint potentials for both couples were calculated by linear regression analysis of the plots of the system potential determined at each point in the titration *versus* the logarithm of the ratio of the concentrations of the oxidized and reduced forms of the flavodoxin according to the Nernst equation and are reported relative to the potential of

Table 1: UV/Visible Spectral Properties of Mutant Flavodoxins in the Oxidized Form

	λ_{max}[a] (nm)			ϵ_{ox}[a] (M^{-1} cm^{-1})			
flavodoxin	I	II	λ_{min} (nm)	I	II	ϵ_I/ϵ_{II}	$\epsilon_{274}/\epsilon_I$[b]
wt (P2A)	460	378	408, 310	10700	8500	1.26	4.36
Y98F	458	388	412, 310	10600	8100	1.31	4.20
Y98W	454	382	408, 318	9900	7700	1.29	4.50
Y98H	456	380	410, 312	11000	8500	1.29	4.40
Y98R	456	384	412, 314	11100	10000	1.11	4.60
Y98A	455	384	408, 312	11400	10100	1.13	4.54
Y98M	455	376	408, 312	11000	9500	1.16	4.24
FMN	445	373	400, 304	12500	10400	1.20	N/A[c]

[a] The λ_{max} and molar extinction coefficient of each major absorption band (designated I and II) for each flavodoxin variant are given. [b] Ratio of extinction at 274 nm to the maximal extinction for transition I. [c] Not applicable.

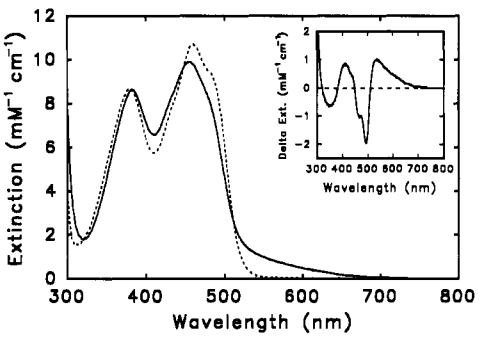


FIGURE 2: Ultraviolet/visible absorption spectra of the oxidized form of wild-type and Y98W mutant flavodoxins in 50 mM NaP_i, pH 7.0, 25 °C. *Dashed line*, recombinant wild-type *D. vulgaris* flavodoxin; *solid line*, Y98W mutant. *Inset*: Y98W minus wild-type difference spectrum.

the standard hydrogen electrode (25 °C). In the investigation of the pH dependencies of the reduction potentials, the E_m values for anthraquinone-2,6-disulfonate and safranine T were calculated according to the pH dependencies reported by Clark (1972).

RESULTS AND DISCUSSION

UV/Visible Absorption Characteristics. The near-ultraviolet and visible absorbance properties of the oxidized form of each of the mutant flavodoxins are summarized in Table 1. Altering the physical properties of the amino acid at position 98 results in small but significant changes in the visible absorption characteristics of the flavin cofactor. In all cases, the absorbance maximum of transition I of the flavin (in the region of 460 nm) is blue-shifted relative to wild type; however, none approach that of FMN free in solution (445 nm). In contrast, the absorption maximum of transition II (in the 380 nm region) has been red-shifted in every case except Y98M. In general, the extinction coefficients of both transitions for all mutants lacking an aromatic residue at position 98 are higher then wild type. Also, the ratio of the extinction coefficient of transition I to that of transition II decreases in the nonaromatic mutants. These observations are consistent with a more polar environment for the flavin in those mutants.

Particularly noteworthy is the visible absorption spectrum of the Y98W mutant (Figure 2). The flavin spectrum is broadened and more featureless than wild type or the other mutants, and the extinction coefficients of both major transitions are substantially lower. Although the absorption spectrum of Y98W remains red-shifted relative to free FMN, the differences between this spectrum and that of the wild-type protein contrast with the red shift in the flavin absorption in model studies of the flavinyl tryptophan complex (Forey *et al.*, 1968). An even more distinctive feature of the Y98W absorbance spectrum is the presence of a long-wavelength band extending from 500 to 700 nm. Similar spectral features have also been observed with the flavoprotein Old Yellow Enzyme when complexed with the phenolate anion (Stewart & Massey, 1985) and are also characteristic of the spectral properties of solution mixtures of flavin and indoles (Pereira & Tollin, 1967). This long-wavelength absorbance transition has been attributed to the formation of a charge-transfer complex in which the aromatic compound rich in π-electrons serves as the electron donor to the more electron-deficient oxidized flavin (Kosower, 1966). The coplanar relationship between Tyr98 and the flavin isoalloxazine ring in the wild-type flavodoxin (Watenpaugh *et al.*, 1973; Watt *et al.*, 1991) is also apparent in the preliminary X-ray crystal structure of the Y98W mutant (Reynolds *et al.*, 1992). Such a configuration should allow for shorter than normal contacts between the aromatic ring systems of this tryptophan residue and the flavin, facilitating charge-transfer complex formation through more efficient overlap of the π-electron systems (Inoue *et al.*, 1983). This stands in contrast to the tryptophan residue (Trp60) which resides next to the inner face of the isoalloxazine ring in wild-type *D. vulgaris* flavodoxin, and Trp90 and Trp57 that flank the outer face of the flavin in the *Clostridium beijerinckii* and *A. nidulans* flavodoxins, respectively, all of which are inclined at an angle of approximately 20–45° relative to the flavin rings (see also Figure 1) (Watenpaugh *et al.*, 1973; Smith *et al.*, 1977; Ludwig *et al.*, 1982). Further biophysical and structural analyses of this unique Y98W flavodoxin mutant should provide additional insight into the nature of flavin–indole charge-transfer complexes within a fixed protein framework.

Upon partial reduction, the blue-neutral flavin semiquinone radical accumulates to varying degrees for each mutant flavodoxin and remains for extended periods of time under anaerobic conditions, consistent with the thermodynamic stabilization of the semiquinone in all cases and with the midpoint potentials observed for each couple (see below). Representative spectral changes observed during the reduction of the wild-type, Y98F, Y98A, and Y98H mutants are shown in Figure 3 for comparison. Differences from the wild-type flavodoxin are noted in the visible absorbance spectrum of the semiquinone for the Y98F, Y98W, Y98M, and Y98A mutants in that each has a more pronounced absorbance band in the 500–520 nm region. The absorption characteristics of the semiquinone generated in all the mutants tested, including extinction coefficients and relevant isosbestic points observed during the generation of the various redox states, are summarized in Table 2.

The red anionic form of the flavin semiquinone does not accumulate under any conditions tested thus far, not even when the pH of the solutions was extended to values approaching 9.5. Only the extent of blue-neutral semiquinone that accumulates is affected, particularly for the Y98H and Y98R mutants. The pK_a of the flavin semiquinone of free FMN is approximately 8.3 (Ehrenberg *et al.*, 1967). In the absence of other interactions, the presence of a basic residue in the vicinity of the pyrimidine subnucleus of the isoalloxazine ring might serve to stabilize the anionic radical (Massey & Hemmerich, 1980). However, the observations presented here suggest that the pK_a of the flavin semiquinone in these mutants must be greater than 10. One could argue that the positive charge on these residues is not optimally located for favorable interaction with the negative charge which resides on the

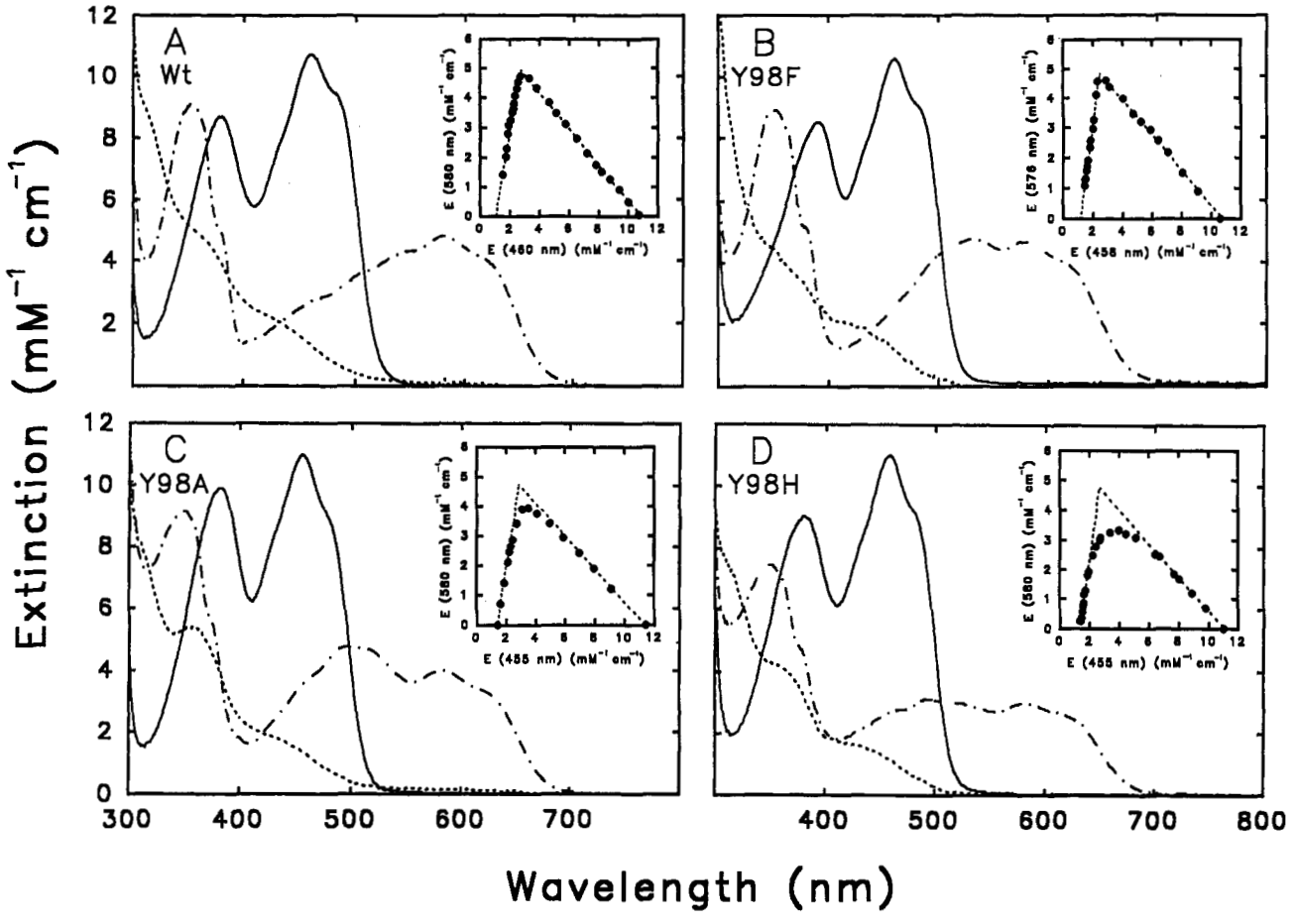


FIGURE 3: Absorbance spectra of representative Tyr98 flavodoxin mutants during reduction in 50 mM NaP_i, pH 7.0, 25 °C. *Panel A*, recombinant wild-type (P2A) flavodoxin; *panel B*, Y98F mutant; *panel C*, Y98A mutant; *panel D*, Y98H mutant. In all panels: *solid line*, fully oxidized; *dot–dashed line*, partially reduced (near-maximal semiquinone formation); *dashed line*, fully reduced. The hydroquinone spectra for wild type and Y98F were corrected for the presence of a small amount of semiquinone remaining at the end of each titration. *Insets*: Plot of the absorbance changes in the 580 nm region *versus* those in the 460 nm region associated with the formation of the neutral flavin semiquinone during reductive titration with dithionite. Dashed lines are linear extrapolations of the initial and final portions of each leg of the titration with the intersection indicative of the maximal absorption at each wavelength of the flavin semiquinone if it were fully formed.

Table 2: UV/Visible Spectral Properties of the Mutant Flavodoxins in the Semiquinone Form

flavodoxin	λ_{max} (nm) I	II	IP[a] (ox→sq)	ϵ_{sq} (580 nm)
wt (P2A)	580	350	508, 366	4700
Y98F	576, 528[b]	352	504, 369	4800
Y98W	580, 494[b]	356	501, 367	4400
Y98H	580	350	504, 363	4800
Y98R	580	350	500, 366	4600
Y98A	580, 515[b]	352	498, 365	4700
Y98M	580, 518[b]	352	498, 364	4300

[a] IP, isosbestic points (in nanometers) observed during conversion of oxidized to semiquinone redox states. [b] Two distinct absorbance peaks are observed in the visible region in these flavodoxins. The λ_{max} for each is reported.

pyrimidine subnucleus of the flavosemiquinone anion. Indeed, molecular modeling places the positively charged guanidino group of Arg98 in the Y98R mutant nearer the *o*-xylene ring of the flavin. On the other hand, while the imidazole side chain in Y98H is more favorably positioned for stabilization, its pK_a may be such that it remains in the neutral form at pH values favoring ionization of the flavin radical (see discussion below). These results seem to support the premise that the hydrogen bond formed between the polypeptide backbone of the protein and the N(5)H of the reduced FMN is a significant factor in the stabilization of the neutral form of the flavin semiquinone in the flavodoxins (Mayhew & Ludwig, 1975; Vervoort *et al.*, 1986; Ludwig *et al.*, 1990) although other factors may also contribute.

Fluorescence. Flavodoxins are known to completely quench the fluorescence of the bound FMN cofactor (Mayhew & Ludwig, 1975). Mutants containing an aromatic residue at position 98 have fluorescent quantum yields less than 1.0% of free FMN. Y98H, Y98R, and Y98A display fluorescent yields of from 4 to 6%. Y98M is intermediate. These results are consistent with model studies with flavinyl amino acids in which histidine and methionine quench flavin fluorescence but less effectively than aromatic residues (Johnson & McCormick, 1973; Falk & McCormick, 1976). Neutral histidine is more effective than its protonated form. The higher level of fluorescence of the Y98A mutant is consistent with the greater solvent accessibility of the flavin likely in this variant. The emission wavelength maximum (530 nm) was similar for all mutants.

Reduction Potentials for the ox/sq Couple. Oxidation–reduction potentials for the ox/sq couple of the FMN cofactor bound to each of the Tyr98 mutants were established by equilibration with appropriate redox indicator dyes having established $E_m(7)$ values and by graphical analysis according to the Nernst equation (Figure 4). The midpoint potentials for each flavodoxin are given in Table 3. Mutant flavodoxins retaining an aromatic residue at position 98 all have reduction potentials very similar to that of the wild-type protein. Solution studies with free flavin or flavinyl amino acids have suggested

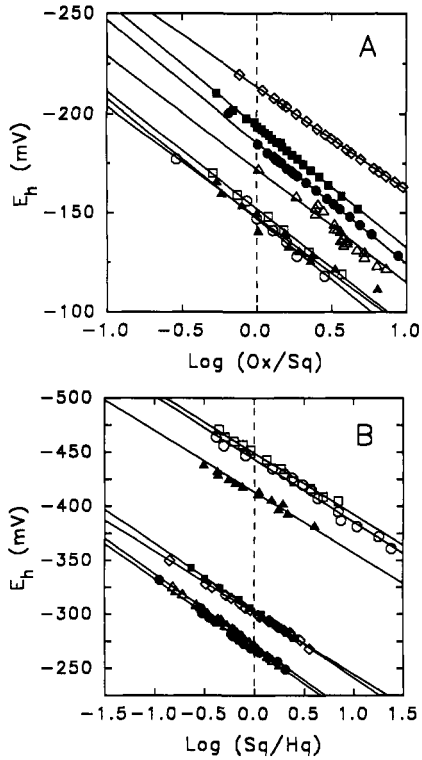


FIGURE 4: Linearized Nernst plot of the redox potentials for the ox/sq couple (*panel A*) and the sq/hq couple (*panel B*) of the flavodoxin mutants at tyrosine-98 in 50 mM NaP_i, pH 7.0, 25 °C, using methods described under Experimental Procedures. The lines represent the best fit by linear regression analysis through each data set. Symbols in both panels represent data obtained for each flavodoxin variant as follows: wild type (○); Y98W (□); Y98F (▲); Y98M (◇); Y98A (■); Y98H (●), and Y98R (△).

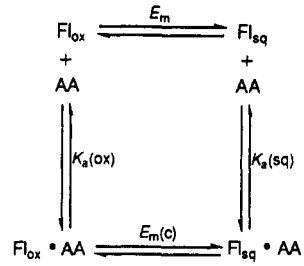
Table 3: Oxidation–Reduction Midpoint Potentials[a] for the *D. vulgaris* Flavodoxins Mutants

flavodoxin	E_2 (ox/sq couple)	E_1 (sq/hq couple)
wt (P2A)	−148	−443
Y98F	−149	−414
Y98W	−152	−452
Y98H	−185	−262
Y98R	−173	−265
Y98A	−186	−304
Y98M	−211	−302
FMN[b]	−238	−172

[a] Values reported in millivolts *versus* SHE, pH 7.0, 25 °C. The error in these determinations is estimated to be ±5 mV. [b] Values for FMN in solution at pH 7 taken from Draper and Ingraham (1968).

that certain aromatic amino acids form complexes and preferentially stabilize individual redox states of the flavin through nonbonding, aromatic stacking interactions (Draper & Ingraham, 1970; Müller *et al.*, 1972; McCormick, 1977; Ishida *et al.*, 1986). In the Draper and Ingraham study, tyrosine was found to form stronger complexes with the FMN semiquinone than either the quinone or the hydroquinone states. The minimal association constants determined in that study for the tyrosine complex of the oxidized and semiquinone forms of FMN are 66 ± 12 and 170 ± 40 M^{-1}, respectively. On the basis of a linked equilibria model shown in Scheme 1 and the ratio of $K_a(sq)/K_a(ox)$, the preferential binding of tyrosine to the FMN semiquinone should result in a positive shift in the midpoint potential for the ox/sq couple of 24 ± 7 mV for the complex.[1] In this study, a similar comparison can be made between the wild-type flavodoxin in which Tyr98 forms a complex with the FMN cofactor and Y98A which lacks such a complex. From the data in Table 3, a midpoint

Scheme 1: Equilibria Linking Aromatic Amino Acid (AA) Binding to Midpoint Potential Perturbations



potential difference of +38 mV is noted between wild type and Y98A, a value that compares relatively well to the solution studies given the differences in the two systems. Thus, the conservation of a tyrosine residue in complex with the flavin in the cofactor binding sites of many flavodoxins seems to be at least partially responsible for the thermodynamic stabilization of the flavin semiquinone in this family of flavoprotein.

The results obtained for the Y98W and Y98F mutants do not seem to follow the same patterns observed in solution studies, however. Although displaying a strong tendency to complex with flavin derivatives, tryptophan seems to show little preference for the oxidation state of FMN. On the other hand, phenylalanine apparently exhibits little affinity for the semiquinone while binding somewhat more weakly than tyrosine to the oxidized and fully reduced forms of FMN in solution (Draper & Ingraham, 1970). These results would predict that the substitution of a phenylalanine for tyrosine in the Y98F mutant should result in more negative reduction potentials for the ox/sq couple for this mutant as compared to either the wild-type or the Y98A flavodoxins. Based only on the differential binding model and the comparable association constants of tryptophan for both the oxidized and semiquinone forms of the FMN, the Y98W mutant should display a reduction potential comparable to Y98A which is more negative than the wild-type protein. However, the midpoint potentials for the Y98F and Y98W mutants are essentially identical to that of the wild-type flavodoxin.

In contrast, the introduction of an imidazole side chain at position 98 in the Y98H mutant results in a midpoint potential for the ox/sq couple that is significantly more negative than the wild-type flavodoxin and very nearly identical to the Y98A mutant (see Table 3). A pH dependency of the midpoint potential for the ox/sq couple of *ca.* −60 mV per pH unit increase (Figure 5, panel A) conforms to a single protonation of the flavin upon one-electron reduction as expected to maintain the blue-neutral flavin radical as observed spectroscopically over the entire pH range testable. If ionization of this histidine occurs in this pH range as expected (see also next section), then this general dependency suggests that there are little or no preferential interactions of either the neutral or the protonated forms of the histidine with the neutral flavin semiquinone in this flavodoxin structure. These results are quite consistent with solution studies which indicate only weak

[1] The shift in the midpoint potential, ΔE_m, can be calculated from the linked equilibria shown in Scheme 1 according to the following relationship: $\Delta E_m = E_m(c) - E_m = (RT/nF) \ln[K_a(ox)/K_a(sq)]$ where $E_m(c)$ and E_m are the midpoint potentials of FMN within and without the complex, respectively, R is the gas constant, T is the absolute temperature, n is the number of electrons, F is the Faraday constant, and $K_a(ox)$ and $K_a(sq)$ are the association constants for the amino acid complex with the oxidized and semiquinone forms of the FMN, respectively.

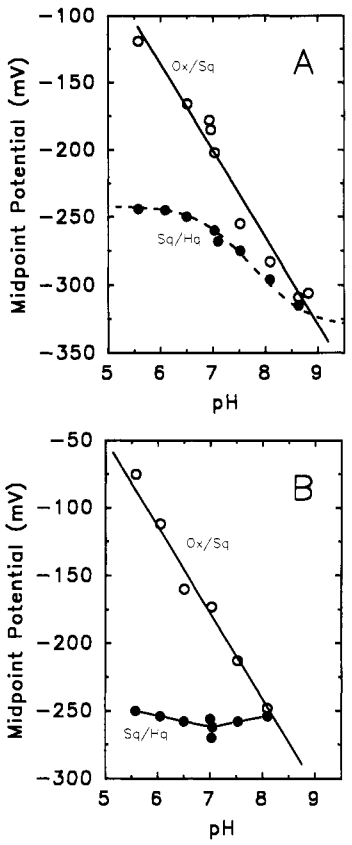


FIGURE 5: pH dependency of the midpoint potentials for the ox/sq couple (○) and sq/hq couple (●) of the Y98H mutant (*panel A*) and of the Y98R mutant (*panel B*), 25 °C. The best fit line through the ox/sq couple data has a slope of −64 mV/pH unit for the Y98H mutant and −63 mV/pH unit for the Y98R mutant. The data for the sq/hq couple in the Y98H mutant were fit to a model involving the redox-linked ionization of His98 as described under Results and Discussion. The parameters as defined in the text which were used in the fit shown (*dashed line*) are as follows: E_o, −242 mV; pK_a^{SQ}, 7.0; pK_a^{HQ}, 8.5.

interactions between the neutral imidazole and flavins, and little association when protonated (Johnson & McCormick, 1973; Draper & Ingraham, 1970).

The other more polar substitutions at position 98 also result in significantly more negative midpoint potentials for the ox/sq couple. The Y98R mutant, with a slightly more positive potential than either Y98A or Y98H, displays the characteristic pH dependency of the midpoint potential of the ox/sq couple of −60 mV per pH unit (Figure 5, panel B), consistent with the stabilization of the neutral flavin radical throughout. Although alanine is nonpolar, greater accessibility of the flavin semiquinone to the polar solvent and solution ions is provided in the Y98A mutant. The Y98M mutation was included in this study to test the effect of a nonaromatic apolar residue at this position. However, the significantly more negative midpoint potential for the ox/sq couple of this mutant relative to the others does not follow the polarity trend, and the results are not easily explained at this point. Perhaps additional localized structural perturbations are introduced by this amino acid.

Taken together, these results support the premise that conservation of a tyrosine residue adjacent the FMN isoalloxazine ring in the cofactor binding site of several flavodoxins and some other flavoproteins is at least partially responsible for the thermodynamic stabilization of the neutral flavin semiquinone in these proteins. However, in contrast to suggestions made from solution studies, tyrosine is not unique in this regard, at least in this system. In fact, a tryptophan residue is found at the equivalent position to Tyr98 in *Clostridium beijerinckii*, which has an even more positive reduction potential for the ox/sq couple than the *D. vulgaris* flavodoxin (Mayhew & Ludwig, 1975). The stabilization to the extent observed in this study seems more dependent on the conservation of an aromatic side chain adjacent to the flavin isoalloxazine ring, perhaps the consequence of the more apolar environment provided by these bulky side chains. The increased accumulation of the neutral form of the flavin semiquinone in apolar, aprotic solvents has been noted (Müller *et al.*, 1972).

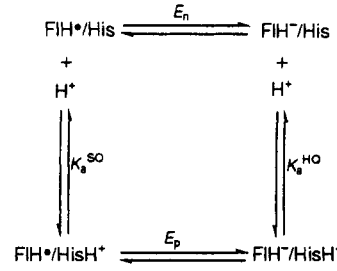
The structural consequences of the amino acid substitutions introduced at position 98 in this study have not been considered in the above discussion and cannot be completely excluded as explanations for the altered properties and/or for differences from the solution studies. The formation of complexes between small aromatic molecules and flavin in solution is not nearly as restricted conformationally as in a protein structure. Perhaps the structural constraints imposed by the tertiary structure of the protein prevent optimal alignment of the two aromatic systems which precludes the effects of differential binding to each redox state of the flavin. Inoue *et al.* (1983) have shown by X-ray crystallographic analyses of complexes between flavin and indole or adenine derivatives that preferred orientations are assumed. Binding conformations seem to prefer those which strongly favor the charge-transfer interaction. However, mutual orientation of the indole and flavin rings does not seem to be driven by dipole–dipole interactions, and, for purine–flavin complexes at least, a variety of conformations are possible, with the principal criterion for stability being maximum overlap of the molecular planes (Song, 1970). X-ray crystallographic and multidimensional NMR analyses on several of these mutants which are underway should establish the structural orientation of the aromatic residues relative to the cofactor and provide a more definitive interpretation of the structural contribution to the observed redox and spectral properties (unpublished results; Reynolds *et al.*, 1992; Stockman *et al.*, 1993).

Finally, it should be noted that the increases of 30–40 mV in the midpoint potentials of the ox/sq couple of the mutants containing an aromatic amino acid at position 98 relative to the Y98A mutant account for only approximately one-third of the 90 mV increase in potential that occurs upon binding of free FMN to the wild-type apoflavodoxin (−238 mV *vs* −148 mV, respectively). Therefore, it would seem that other interactions must contribute from 1 to 1.5 kcal/mol to the stabilization of the flavin semiquinone relative to the oxidized state. One such interaction may be the apparent formation of a hydrogen bond between the carbonyl oxygen of Gly61 and N(5)H of both reduced states of the FMN (Watt *et al.*, 1991), an interaction that is not possible in the oxidized state. Similar observations have been made in other flavodoxins (Ludwig & Luschinsky, 1992). The contribution of 1–2 kcal/mol of binding energy for a hydrogen bond in a relatively apolar environment is reasonable. Thus, the additive effects of an aromatic residue at position 98 flanking the isoalloxazine ring and the additional hydrogen bonding at N(5)H of the FMN semiquinone may account for the majority of the stabilization of the semiquinone relative to the oxidized flavin cofactor in this family of flavodoxins. The contribution of this hydrogen bond to the stabilization of the semiquinone state of the *C. beijerinckii* flavodoxin is currently under investigation (Swenson *et al.*, 1991a).

Midpoint Potentials for the sq/hq Couple. The midpoint potentials for the sq/hq couple, as determined by equilibration with redox indicator dyes and/or potentiometric titration, conform favorably to the Nernst equation as shown in Figure 4 (panel B). The midpoint potentials obtained from these data are included in Table 3. The chemical properties of the amino acid side chain at position 98 were found to have a profound effect on the redox properties of this couple, altering the potential over a nearly 200 mV range. The properties seem to segregate into three general groups. Flavodoxin proteins having aromatic amino acid residues at this position, *i.e.*, tyrosine (wild type), tryptophan (Y98W), and phenylalanine (Y98F), all have midpoint potentials less than −400 mV. Some differences are noted within this group. The Y98W mutant may be slightly more negative than wildtype. The Y98F mutant displays a midpoint potential approximately 30 mV more positive than wild type. These results seem to be consistent with direct electronic effects of the π-electron system on the stability of the flavin hydroquinone anion. Both tyrosine and tryptophan have electron-donating heteroatoms associated with the aromatic ring, making these aromatic systems more electron rich than phenylalanine, leading to an apparent increased destabilization of approximately 1 kcal/mol relative to this residue. It would seem that the formation of an electron-rich flavin hydroquinone is less favored when a more electron-dense aromatic system is associated by nonbonding interactions.

The second group of amino acid substitutions, which includes Y98A and Y98M, displays significantly more positive midpoint potentials than wild type, by approximately 130 mV. In the wild-type structure, the FMN is largely buried below the surface of the protein, with only a portion of the *o*-xylene ring exposed to solvent (Watt *et al.*, 1991). Only a few well-ordered water molecules are found situated near the FMN, primarily hydrogen-bonded to the ribityl side chain; however, a water molecule is consistently observed bridging O(4) of the flavin to O(62) and N(100) of the protein. Molecular modeling suggests that the flavin isoalloxazine ring is much more exposed to solvent in the Y98A mutant. The high dielectric environment provided by water and the possibility of the more direct interaction with cationic counterions present in solution are expected to represent a more favorable situation for the developing flavin hydroquinone anion, consistent with the substantially more positive reduction potential observed for the sq/hq couple in this mutant. Theoretical calculations have estimated that burying an uncompensated charged amino acid side chain in the largely apolar environment of the interior of a protein costs from 10 to 40 kcal/mol (Gilson *et al.*, 1985). A recent study provides evidence that the price is somewhat lower, in the range of 5–6 kcal/mol to bury a lysine or aspartate residue into a largely hydrophobic region in T4 lysozyme (Daopin *et al.*, 1991). Given that position 98 in our flavodoxin system is situated near the surface of the protein, it is expected that the dielectric constant in this region is higher than for the more apolar environment in the T4 lysozyme study. Thus, the differences in free energy of approximately 3.0 kcal/mol of the wild-type, Y98F, and Y98W mutants compared to the Y98A mutant seem consistent with the unfavorable generation of the flavin hydroquinone anion in the apolar environment provided by the aromatic side chain. The similarity between the midpoint potentials of the Y98A and Y98M mutants is less consistent with this trend, however. The increased fluorescence of the Y98M mutant relative to the wild-type flavodoxin may indicate greater solvent accessibility of the flavin in this mutant, perhaps due to localized conformational changes induced by this amino acid.

Scheme 2: Equilibria Linking the Ionization of His98 to Shifts in the Midpoint Potential in the Y98H Mutant



The third group, displaying the least negative midpoint potentials of the mutants studied here, contains those substitutions introducing the basic residues arginine and histidine at this position. Both mutants have reduction potentials that are similar to one another but are approximately 180 mV more positive than wild-type (at pH 7), representing an apparent stabilization of about 4.0 kcal/mol over the wild-type flavodoxin. Because both represent basic amino acid residues, these results suggest that the cationic nature of the side chains may stabilize the flavin hydroquinone anion through favorable electrostatic interactions resulting in the more positive reduction potentials in these two mutants.

The pH dependency of the sq/hq couple for these mutants is consistent with this premise. The midpoint potential of the Y98R mutant remains essentially constant over the entire pH range tested (Figure 5, panel B). With a pK_a of *ca.* 12, the guanidino group of arginine could provide favorable electrostatic interactions with the flavin hydroquinone anion throughout this pH range. In contrast, at pH values above ~6.0, the midpoint potential of the sq/hq couple of the Y98H mutant gradually becomes more negative (Figure 5, panel A). The data were fit to a model in which the reduction of the neutral flavin semiquinone (FlH$^{\cdot}$) to the hydroquinone anion (FlH$^-$) is linked to the ionization of His98 as shown in Scheme 2. In this model, four interdependent species are present during the reduction of the flavodoxin by a second electron equivalent: FlH$^{\cdot}$/His, FlH$^{\cdot}$/HisH$^+$, FlH$^-$/His, and FlH$^-$/HisH$^+$, that is, the two redox states of the flavin in the presence of either the neutral or the protonated form of the histidine with the FMN hydroquinone remaining as the anion throughout the pH range tested. As a consequence of the linked equilibria, the shift in midpoint potential of the sq/hq couple in response to the ionization of His98 requires that the pK_a of the histidine also be dependent on the redox state of the flavin according to the relationship:

$$\Delta E_m = E_p - E_n = (RT/nF)\ln(K_a^{HQ}/K_a^{SQ})$$

where E_p and E_n are the midpoint potentials for the sq/hq couple when His98 is protonated and neutral, respectively, and K_a^{SQ} and K_a^{HQ} are the ionization constants for His98 in the semiquinone and fully reduced states, respectively.

The data can also be analyzed according to the equation systems that involve coupling of electron transfer with proton transfer (Clark, 1972). In this derivation, both the oxidized and reduced species are considered to undergo a single ionization through the coupling to His98 (as in Scheme 2); *i.e.*, for the oxidized species, FlH$^{\cdot}$/His + H$^+$ ⇌ FlH$^{\cdot}$/HisH$^+$; for the reduced species, FlH$^-$/His + H$^+$ ⇌ FlH$^-$/HisH$^+$. For this instance, the relationship of the midpoint potential to pH

in response to these ionizations can be represented by the equation [see also Clark (1972)]:

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

The midpoint potential data at 25 °C for the sq/hq couple for Y98H *vs* pH (Figure 5, panel A) can be fit reasonably well to this equation using the following values: E_o, −242 mV; pK_a^{SQ}, 7.0 ± 0.2; pK_a^{HQ}, 8.5 ± 0.3. The value of 7.0 ± 0.2 for the pK_a of His98 complexed to the neutral semiquinone falls within the expected range for a histidine residue near the surface of a protein. However, the analysis implies that its pK_a in the fully reduced flavodoxin has increased by *ca.* 1.5 units, to an unusually high pK_a value for histidine. However, this value is not unreasonable given the opportunity for strong electrostatic coupling between the protonated imidazole group immediately adjacent to the flavin hydroquinone anion together with perhaps the secondary influence of the cluster of acidic residues nearby (see Figure 1). A pK_a as high as 9.3 has been determined for an ion pair involving histidine and an aspartic acid residue in T4 lysozyme, for example (Anderson *et al.*, 1990).

This model also predicts that the midpoint potential of the sq/hq couple in this mutant shifts from *ca.* −240 mV to *ca.* −330 mV upon deprotonation of His98. The reduction potential did not return to that of the wild-type flavodoxin at the most alkaline pH value tested, but did become more negative than the Y98A mutant. This presumably represents the solvent shielding effect of the imidazole side chain. If so, this would mean that the electrostatic interaction provided by the charge on the imidazole is directly responsible for *ca.* 90 mV of stabilization or about 2 kcal/mol. A note of caution should be expressed with regards to this data fitting analysis. It was not possible in this study to extend the measurements beyond *ca.* pH 9.0. To have done so would have provided greater confidence of the lower limit of the midpoint potential for the flavodoxin having the neutral form of histidine-98 and for the pK_a of the histidine in the fully reduced state. Although technically difficult and complicated by the effects of the paramagnetic flavin radical, more convincing support of this model would come from the direct determination of the pK_a of His98 in each redox state by nuclear magnetic resonance spectroscopy.

Is this degree of stabilization reasonably explained by the favorable electrostatic interactions alone? Several studies have estimated that ion pairing between amino acid side chains can contribute from 1 to 5 kcal/mol to protein stability, varying with sequence and location (Fersht, 1972; Perutz & Raidt, 1975; Anderson *et al.*, 1990). The degree of stabilization of the flavin hydroquinone anion by the favorable electrostatic interaction with the charge side chains introduced in the Y98H and Y98R mutants falls within this range. Modulating influences of the immediate environment surrounding these residues, including solvent and several acidic amino acid residues, may diminish their effects.

It should also be noted that it is unlikely that the pH dependency observed for the Y98H mutant is due to the ionization of the flavin hydroquinone itself for the following reasons (see also discussion below). First, the hydroquinone UV/visible absorption spectra over the entire pH range tested remain essentially identical (data not shown). Although not a particularly sensitive measure of the ionization of 1,5-dihydroflavin at N(1) and somewhat dependent on other factors, the UV/visible absorption spectra of the neutral and anionic flavin hydroquinone do differ (Ghisla, 1980). A more definitive and direct assignment could be provided through the characteristic chemical shift of $^{15}N(1)$ of the flavin by NMR (Franken *et al.*, 1984). Second, the pH dependency does not follow a trend expected for the protonation of the hydroquinone anion at lower pH values in that the dependency actually is diminished or eliminated in the most acidic portion of the pH range tested. The reduction potentials of the flavin sq/hq couple in wild-type flavodoxins are essentially independent of pH above pH 6.5. Below this value, a dependency has been noted in the *Megasphaera elsdenii* flavodoxin but has been ascribed to a redox-linked group on the protein itself (Ludwig *et al.*, 1990). Finally, it is difficult to envision how the Y98H mutant might differ from the Y98R mutant, for which the sq/hq couple is pH independent, with respect to differing ionization potentials for the flavin hydroquinone.

These results support the hypothesis that the very low midpoint potential of the sq/hq couple in these flavodoxins is primarily the result of the energetically unfavorable formation of the flavin hydroquinone anion in an apolar environment provided by electron-rich aromatic amino acids immediately flanking the flavin isoalloxazine ring. The elimination of the aromatic side chain at position 98 or the introduction of favorable electrostatic interactions by placement of positively charged amino acid residues adjacent to the flavin greatly reduces the unfavorable characteristics as reflected in the much more positive midpoint potentials.

However, if the generation of the flavin hydroquinone anion is unfavored in the environment provided by the flavodoxin protein, why does not protonation of the anion serve to minimize this energetically adverse situation in these proteins? Protonation would not seem to be precluded by the inherent pK_a of the flavin hydroquinone which is 6.7 in solution (Franken *et al.*, 1984). However, the characteristic $^{15}N(1)$ chemical shift associated with its ionization state is not observed in the NMR spectra of flavodoxins, even at pH values as low as 5.5 (van Schagen & Müller, 1981; Franken *et al.*, 1984; Vervoort *et al.*, 1985, 1986). There is no evidence of the protonation of reduced 1-deazaflavin bound to apoflavodoxin from *C. beijerinckii* as monitored by its optical spectrum even to pH values extending down to 4.6 (Ludwig *et al.*, 1990). These studies suggest that the pK_a of the N(1) of 1,5-dihydroflavin is actually greatly suppressed to a value below 4.0 when bound to the flavodoxin protein. But why? A recent structural study suggests that protonation of N(1)/C(2)O of the fully reduced flavin is hindered by the close proximity of the polypeptide backbone in *C. beijerinckii* flavodoxin (Ludwig *et al.*, 1990). An energetically unfavorable perturbation of the protein structure and loss of a hydrogen bond between N1 and a peptide NH would be required before protonation could take place. Similar structural arrangements are seen in other flavodoxin structures including that from *D. vulgaris* (Watt *et al.*, 1991; Watenpaugh *et al.*, 1973). Thus, the flavodoxin seems to be faced with a trade-off; either it forces the flavin hydroquinone anion to exist in an unfavorable environment or it must accommodate an energetically unfavorable conformational change to allow protonation (and neutralization) of the flavin as it becomes reduced. It would be particularly intriguing to engineer the flavin binding site in such a way so as to reduce this steric interference and, perhaps, allow for greater accessibility of N(1) to protonation. Would the pK_a of the hydroquinone shift back closer to solution values? Would the midpoint potential of the sq/hq couple move to more positive values as would be predicted from this study?

The results presented in this work are consistent with and support the hypothesis that the flavodoxin protein structure

has evolved to bind the **anionic** form of the flavin hydroquinone in a binding site that makes the formation of this anion energetically unfavorable. The isoalloxazine ring is sandwiched between two electron-dense aromatic systems, at least one of which is nearly coplanar, allowing for considerable π-orbital overlap. Thus, the flavin remains largely shielded from solvent and contained within a particularly electronegative environment. Furthermore, the binding site is surrounded by numerous acidic amino acid residues (e.g., Asp62, Asp63, Glu66, Asp95, Glu99, and others) (Figure 1A) and the dianionic phosphate group of the cofactor itself, bound in an unusual phosphate binding loop comprised exclusively of neutral amino acid side chains which provide no charge compensation (Smith *et al.*, 1977, Watenpaugh *et al.*, 1973; Ludwig & Luschinsky, 1992). Taken together, these unfavorable interactions make the addition of the second electron to the flavin very difficult, resulting in the very low reduction potentials for the sq/hq flavin couple that typify the flavodoxin family.

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