The Cumulative Electrostatic Effect of Aromatic Stacking Interactions and the Negative Electrostatic Environment of the Flavin Mononucleotide Binding Site Is a Major Determinant of the Reduction Potential for the Flavodoxin from *Desulfovibrio vulgaris* [Hildenborough][†]

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ABSTRACT: Flavodoxins are typified by the very low one-electron reduction potential for the semiquinone/hydroquinone couple ($E_{sq/hq}$) of the flavin mononucleotide (FMN) cofactor. In the *Desulfovibrio vulgaris* flavodoxin, the elimination of the side chain of Tyr98, which flanks the outer or *si* face of the flavin, through the Y98A mutation results in a substantial increase in $E_{sq/hq}$ of 139 mV, representing about one-half of the total shift in $E_{sq/hq}$ in this flavodoxin [Swenson, R. P., & Krey, G. D. (1994) *Biochemistry 33*, 8505−8514]. The extent to which this large effect was the result of the elimination of unfavorable coplanar aromatic stacking interactions or to the greater solvent exposure of the flavin ring was not known. The significance of the latter effect was heightened by the characterization of the Fld^{+6} mutant which demonstrated that the unfavorable interaction between the negative electrostatic environment provided by the asymmetric clustering of acidic residues surrounding the cofactor and the FMN hydroquinone anion is responsible for about one-third of the total decrease in $E_{sq/hq}$ in this flavodoxin [Zhou, Z., & Swenson, R. P. (1995) *Biochemistry 34*, 3183−3192]. In this study, a flavodoxin mutant was generated in which an alanine was substituted for Tyr98 while at the same time the negative electrostatic surface was partially neutralized by the substitution of the six acidic amino acid residues with their amide equivalents. The $E_{sq/hq}$ value of this mutant was found to have increased by 221 mV relative to wild type, which accounts for 70−80% of the total shift in $E_{sq/hq}$ in this flavodoxin. This increase is very similar to the sum of the individual changes in $E_{sq/hq}$ introduced independently in the Y98A and Fld^{+6} mutants. The similarity in the magnitude of the effect of the neutralization of the six acidic residues in the context of an alanine residue at position 98 (Y98A) relative to an aromatic tyrosine residue (wild type) suggests that the increase in $E_{sq/hq}$ observed for the Y98A mutant is more likely due to the elimination of unfavorable $\pi-\pi$ interactions between Tyr98 and the FMN hydroquinone rather than to the increased solvent exposure of the flavin. This study provides further support for the concept that the cumulative effect of the unfavorable electrostatic interactions introduced by coplanar aromatic or $\pi-\pi$ stacking interactions and the negative electrostatic environment of the FMN binding site is a major determinant of the low one-electron reduction potential of the flavodoxin.

Flavodoxins are important members of the flavoprotein family. They contain a single, noncovalently bound flavin mononucleotide (FMN)[1] prosthetic group and function as one-electron carriers *in vivo* [for recent reviews, see Ludwig and Luschinsky (1992) and Mayhew and Tollin (1992)]. These proteins may represent a paradigm for the FMN binding domains found in other more complex flavoproteins such as cytochrome P450 reductase and nitric oxide synthase (Degtyarenko, 1995). Among the distinguishing features of the flavodoxin family is the very low one-electron reduction potential for the semiquinone/hydroquinone couple ($E_{sq/hq}$) of the protein bound FMN cofactor. In the flavodoxin from *Desulfovibrio vulgaris*, the $E_{sq/hq}$ value of the FMN is shifted from −172 mV [Draper & Ingraham, 1968; or alternatively −124 mV as determined by Anderson (1983)] for free FMN in aqueous solution to −443 mV, representing a destabilization of the hydroquinone relative to the semiquinone of greater than 6 kcal/mol (Dubourdieu *et al.*, 1975; Curley *et al.*, 1991; Swenson & Krey, 1994). This substantial decrease in the one-electron reduction potential is thought to be essential for the physiological role of flavodoxins as low-potential electron transferases in various essential biological processes such as nitrogen fixation and photosynthesis where they often can replace the low-potential ferredoxin both *in vivo* and *in vitro* (Deistung & Thornely, 1988; Mayhew & Tollin, 1992; Nieva-Gomez *et al.*, 1980).

The elucidation of the structural determinants responsible for the establishment of the oxidation−reduction potentials of flavoproteins and other redox proteins remains a central

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[1] Abbreviations: FMN, flavin mononucleotide; EDTA, ethylenediaminetetraacetic acid; Fld^{+6}, flavodoxin mutant with six acidic residues (Asp62, Asp63, Glu66, Asp95, Glu99, and Asp106) neutralized by mutation to the corresponding amide; $E_{ox/sq}$, midpoint potential for the oxidized/semiquinone couple; $E_{sq/hq}$, midpoint potential for the semiquinone/hydroquinone couple.

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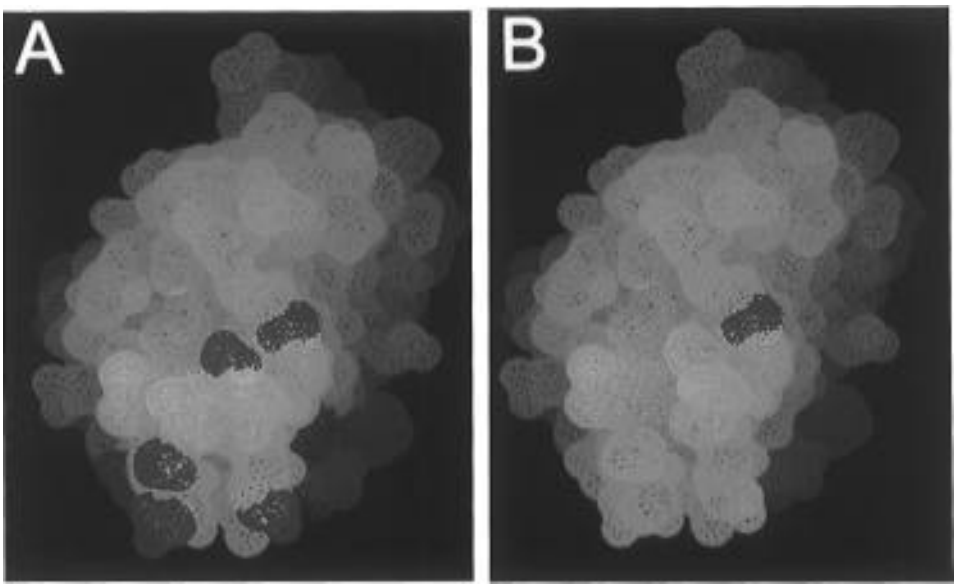

FIGURE 1: Surface representation of the tertiary structures of the wild-type *D. vulgaris* flavodoxin (panel A) and the Y98A/Fld^{+6} mutant (panel B). The flavin mononucleotide is represented by the yellow structure. The side-chain carboxylate groups of the seven amino acids asymmetrically clustered around the cofactor binding site within 13 Å of the N(1) atom of FMN in the wild-type flavodoxin are highlighted in red. The absence of the Tyr98 side chain and greater solvent exposure of the FMN isoalloxazine ring as well as the absence of six of the seven negatively charged side-chain carboxylate groups (through their respective acid to amide amino acid substitutions) are evident in the structural representation of the Y98A/Fld^{+6} mutant.

problem in biochemistry and biophysics. Several studies from this laboratory have focused on the roles of conserved structural features common to most flavodoxins in the modulation of the midpoint potentials of the bound FMN cofactor. In the flavodoxin, the flavin isoalloxazine ring system is invariantly flanked by at least one aromatic amino acid residue which shields the *si* face of the cofactor from solvent (Ludwig & Luschinsky, 1992). The critical role of this residue, as represented by Tyr98 in the *D. vulgaris* flavodoxin (Figure 1, panel A), has been directly demonstrated by the substantial changes in the one-electron reduction potentials, particularly for the sq/hq couple, that result from the substitution at this position of amino acid residues with differing side-chain chemical characteristics (Swenson *et al.*, 1991; Swenson & Krey, 1994). By comparison of the Y98W and Y98F mutants with the wild-type flavodoxin, the significance of aromatic stacking or $\pi-\pi$ interactions seems evident in the variation of $E_{sq/hq}$ with the π-electron density of the flanking aromatic residue. The elimination of the aromatic side chain altogether, as represented by the Y98A mutant, results in a substantial increase in $E_{sq/hq}$ of 139 mV. Thus, the structural consequences of this single substitution account for approximately one-half of the total midpoint potential shift induced when the FMN cofactor binds to this flavodoxin protein, demonstrating the critical role of this tyrosine residue in the modulation of the reduction potential in this flavodoxin (Swenson & Krey, 1994). This increase may reflect not only the loss of important aromatic stacking interactions but, perhaps, also the loss of the solvent shielding characteristics of the residue at this position. It was not possible in that study to determine the relative contribution of each property toward the modulation of the one-electron reduction potential.

Electrostatic interactions, both favorable and unfavorable, have also been demonstrated to be another important class of regulatory factors. The redox-linked ionization of His98 in the Y98H mutant, together with the substantial increases in $E_{sq/hq}$ noted in the related Y98R mutant, illustrates the importance of the coupling of favorable electrostatic interactions with the reduction of the flavin generating the hydroquinone anion (Swenson & Krey, 1994). The significance of the negative electrostatic environment around the FMN binding site generated by the conspicuous asymmetric distribution of charged residues on the surface of the flavodoxin protein (Figure 1, panel A) was demonstrated directly by the systematic substitution of six acidic amino acid residues clustered within 13 Å of N(1) of the flavin with their amide equivalents (*i.e.*, Asp to Asn, Glu to Gln) in various combinations and multiples (Zhou & Swenson, 1995). While there seemed to be a poor correlation between the number of acid residues neutralized and $E_{ox/sq}$, the one-electron reduction potential for the sq/hq couple for each of the mutants was less negative than for wild type. The neutralization of all six acidic residues resulted in an increase in $E_{sq/hq}$ of 93 mV, suggesting that the collective electrostatic effects of these charged residues contribute at least one-third of the total redox potential shift for this flavin couple when bound to the wild-type protein.

The combined effects of both factors, *i.e.*, the aromatic stacking and/or solvent shielding effects of the flanking aromatic amino acid residue and the generally unfavorable electrostatic environment provided by the clustered acidic residues, could be responsible for as much as 85% of the total shift in reduction potential if they act independently and the effects are additive. It also seems plausible that these two general structural features common to most flavodoxins may function synergistically in that the solvent shielding properties of the flanking aromatic residue may also contribute to the apolar environment, decreasing the effective dielectric properties of the FMN binding site. In this way, the electrostatic interactions between the acidic residues and the flavin hydroquinone anion could be enhanced. If this is true, the substantial portion of the increase in the midpoint potential of 139 mV observed in the more solvent-exposed Y98A mutant of the *D. vulgaris* flavodoxin could be at least partially the result of the weakening of this electrostatic

interaction. On the other hand, the substitution of an alanine for Tyr98 also eliminates important aromatic stacking or $\pi-\pi$ interactions. How much does each contribute to the potential shift induced by this residue? Do these two factors act independently or do they act cooperatively in the modulation of the redox potential in this and other flavodoxins?

In this study a multiply mutated flavodoxin protein (designated Y98A/Fld^{+6}) was generated in which six of the acidic amino acid residues clustered within 13 Å of N(1) of the FMN (Asp62, Asp63, Glu66, Asp95, Glu99, and Asp106) were neutralized by substitution with their amide analogs (Fld^{+6}) and the phenol side chain of Tyr98 was eliminated by substitution with alanine (Y98A) (Figure 1, panel B). Therefore, the electrostatic effects of these acidic residues and the apolar aromatic/solvent shielding environment provided by Tyr98 were concurrently eliminated in this mutant. The oxidation−reduction and FMN cofactor binding properties of this mutant have been characterized and compared with those of the wild type, Y98A, and Fld^{+6} mutant flavodoxins in this study.

EXPERIMENTAL PROCEDURES

Materials. Anthraquinone-2,6-disulfonate was purchased from Fluka Chemicals. The Sequenase version 2.0 DNA sequencing kit and phosphatase were obtained from United States Biochemical Corp. The Muta-Gene *in vitro* mutagenesis kit was from Bio-Rad Laboratories. The restriction enzymes *Hin*dIII, *Kpn*I, *Nco*I, *Sal*I, and *Rsa*I were obtained from GIBCO BRL. FMN used for the determination of dissociation constants was extracted from flavodoxin samples using the trichloroacetic acid precipitation method (Wassink & Mayhew, 1975) and purified by anion-exchange chromatography. All other chemicals were of analytical reagent grade.

Bacterial Strain and Plasmids. The pseudo-wild-type flavodoxin gene (P2A) from *D. vulgaris* [Hildenborough] (NCIB 8303/ATCC 29579) has been previously cloned into *p*BUCtac plasmid and characterized (Krey *et al.*, 1988). For this work, the P2A gene was subcloned into the *p*Bluescript KS plasmid for oligonucleotide-directed mutagenesis and overexpression (Zhou & Swenson, 1995). The pseudo-wild-type flavodoxin produced from this plasmid construction differs from the wild-type protein in that an alanine residue replaces proline at position 2 for increased efficiency of expression in *Escherichia coli*. This flavodoxin has properties identical to those of the true wild-type protein in every way tested.

Site-Directed Mutagenesis. Oligonucleotide-directed mutagenesis was carried out using the Kunkel method for selection biased toward the mutant constructions (Kunkel, 1985). The Y98A/Fld $^{+6}$ mutant was generated using the Fld^{+6} mutant gene as the template for mutagenesis. The Fld^{+6} construction which neutralizes six acidic residues surrounding the FMN cofactor by substitutions with their respective amides had been generated previously (Zhou & Swenson, 1995). The oligonucleotide 5′-CGCAGAAGTA-(C)TGGGCGGAACTGTTTCC-3′ was used for the mutagenesis. The underlined nucleotides are different from the wild-type gene sequence. The nucleotide enclosed in parentheses is a "silent" mutation introduced to create a new *Rsa*I site for screening. Mutations as well as the sequence integrity of the entire structural gene were confirmed by the Sanger dideoxy termination DNA sequencing procedure using the Sequenase protocol (Sanger *et al.*, 1977).

Purification of Expressed Mutant Flavodoxin Proteins. Transformed *E. coli* AG-1 cells were cultured for up to 48 h at 37 °C in NZY medium containing 100 μg/mL ampicillin. Flavodoxin was purified by procedures described previously (Zhou & Swenson, 1995) except that the NaCl concentration of the wash and elution solutions used during anion-exchange chromatography was adjusted to accommodate the substantial change in the net charge of this mutant. The purity of the final flavodoxin preparations is routinely established by the ratio of the absorbances at 274 and 456 nm and by SDS−polyacrylamide gel electrophoresis.

One-Electron Reduction Potential Determinations. The oxidation−reduction potentials of both oxidized/semiquinone ($E_{ox/sq}$) and semiquinone/hydroquinone ($E_{sq/hq}$) couples were determined by procedures described previously (Zhou & Swenson, 1995). The $E_{sq/hq}$ value of Y98A/Fld^{+6} was also determined from the formation constant of the semiquinone according to the equilibrium ox + hq $\rightleftharpoons$ 2sq and the equation $E_{ox/sq} - E_{sq/hq} = (RT/nF) \ln(K_f)$, where $K_f = [sq]^2/([ox][hq])$. To establish this equilibrium, the flavodoxin was reduced under anaerobic conditions (prepurified argon) either by titration with sodium dithionite or by light irradiation in 50 mM phosphate buffer containing 50 mM EDTA, pH 7.0 at 25 °C. The concentration of each redox state was estimated by multicomponent analysis based on standard ultraviolet−visible absorbance spectra for each of the three oxidation states of that particular flavodoxin mutant. The formation constant was determined by averaging 9−10 different spectra taken during the course of the reduction.

Determination of the Dissociation Constant for the Oxidized Form of Mutant Flavodoxins. The dissociation constants (K_d) for the oxidized form of the FMN cofactor were determined by either spectrophotometric or spectrofluorometric titration of a FMN solution with the appropriate apoflavodoxin. In each case, the apoflavodoxin was prepared by precipitation by trichloroacetic acid and purified by established procedures (Wassink & Mayhew, 1975). For the spectrophotometric titration, the visible absorbance changes of the flavin were recorded on a Hewlett-Packard Model 8452A diode array spectrophotometer at 25 °C. In a typical titration, a solution of 5.0 μM purified FMN in 50 mM phosphate buffer, pH 7.0, was titrated with the addition of substoichiometric levels of the apoflavodoxin from a 200 μM stock solution. After equilibrium had been reached as indicated by stable absorbance readings, ultraviolet−visible absorbance spectra were recorded and corrected for dilution. Difference spectra were generated by subtracting the starting FMN spectrum from each spectrum obtained during the titration. The fraction of bound FMN was calculated from the absorbance changes at 442 and 492 nm. The K_d was determined by averaging at least five values near the equivalence point.

In some cases, such as for the Y98A and Fld^{+6} mutants, the lower K_d values could not be determined spectrophotometrically. Instead, cofactor binding was monitored by the quenching of the FMN fluorescence upon binding to the apoprotein (Mayhew, 1971). Fluorescence measurements were made with a Perkin-Elmer LS50B luminescence spectrometer equipped for thermostatic control. In a typical titration, a 0.10 μM solution of purified FMN in 50 mM

phosphate buffer, pH 7.0, was progressively titrated at 25 °C with substoichiometric quantities of 10−15 μM apoflavodoxin stock solutions. After equilibration, the fluorescence emission at 520 nm upon excitation at 445 nm was recorded. The fraction of bound FMN was determined from the extent of quenching of the FMN fluorescence at each point in the titration. The average K_d values from three separate titrations are reported.

Solvent Accessibility Analysis. FMN solvent accessibility was calculated in units of Å^2 using the QUANTA molecular graphics software package (Molecular Simulations Inc.). The solvent accessibility of an individual atom is probed using a sphere with a 1.4 Å radius which represents a water molecule (Connolly, 1983). The default values for all other parameters were used in this analysis.

RESULTS AND DISCUSSION

Generation, Expression, and Characterization of the Y98A/Fld^{+6} Mutant. Despite the seven amino acid substitutions introduced into the *D. vulgaris* flavodoxin, the generation, expression, and purification of the Y98A/Fld^{+6} mutant proceeded in an unremarkable manner. The mutant flavodoxin was overproduced in *E. coli* AG-1 cells and accumulated in the bacterial cell as the partially reduced holoprotein, just as with wild type (Swenson & Krey, 1994). The protein was purified to homogeneity as the fully oxidized holoprotein.

The near-ultraviolet−visible absorbance spectra of mutant flavodoxin Fld^{+6} in its three oxidation states are essentially identical to those of the wild-type flavodoxin, suggesting that the structure of the FMN binding site has not been greatly affected in this mutant (Zhou & Swenson, 1995). In this study, similar comparisons were made between Y98A/Fld^{+6} and the previously characterized Y98A mutant (Swenson & Krey, 1994). The near-ultraviolet−visible absorbances of these two mutant flavodoxins are nearly identical (Figure 2). Both display wavelength maxima at 384 and 455 (±2) nm for the major flavin absorbance transitions in the near-ultraviolet−visible region. As expected, the spectral changes induced by the substitutions introduced in the Y98A/Fld^{+6} mutant relative to the wild-type flavodoxin show the characteristics of the elimination of the tyrosine side chain on the substitution with alanine (Swenson & Krey, 1994). The major transition in the 450 nm region in the oxidized state has been blue shifted by *ca*. 5 nm, and the shoulder at about 490 nm is less pronounced. The extinction of the second major transition in the 380 nm region has increased. Both are consistent with the greater solvent exposure of the flavin isoalloxazine ring associated with the mutation (Müller, 1992). In addition to the characteristic absorbance peak at 580 nm, the spectrum of the semiquinone species of the Y98A/Fld^{+6} mutant shows a more pronounced absorbance transition at 515 nm relative to wild type, just as for the Y98A mutant (Swenson & Krey, 1994). The extinction at 580 nm of both Y98A and Y98A/Fld^{+6} are similar to wild type. The reader should note that the semiquinone spectrum for the Y98A/Fld^{+6} mutant shown in Figure 2, panel A, has been calculated on the basis of stoichiometric accumulation of this species during reduction. However, as indicated by the absence of characteristic isosbestic points throughout most of the reductive titration (for example, see Figure 4), only a partial accumulation of the semiquinone actually occurs because the midpoint potentials of the two couples are much closer to one another than in the wild type (see below). The semiquinone spectrum for Y98A shown has not been corrected and represents the actual spectrum obtained just past the midpoint of the reductive titration. Stoichiometric accumulation does not occur in that mutant either (Swenson & Krey, 1994). The spectra for the hydroquinone species of both mutant flavodoxins are similar to that of wild type with the exception that the shoulder in the 350 nm region is somewhat more pronounced in the Y98A mutant. These comparisons suggest that the six acid to amide substitutions within the Y98A mutant flavodoxin have not significantly perturbed the FMN binding site within the Y98A/Fld^{+6} mutant beyond those already introduced by the Y98A substitution, which is likely to simplify and strengthen the interpretation of the observed redox potential changes.

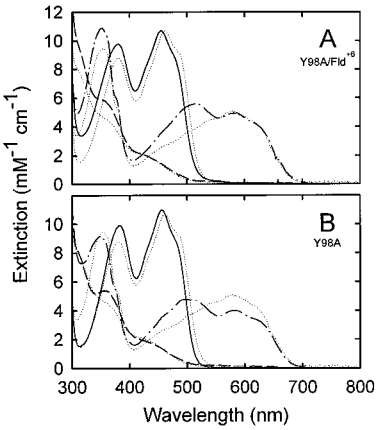

FIGURE 2: Ultraviolet−visible absorbance spectra of the Y98A/Fld^{+6} (panel A) and the Y98A (panel B) mutant flavodoxins during reduction with sodium dithionite under anaerobic conditions (solid line, oxidized; dot-dashed line, partially reduced or semiquinone; dashed line, fully reduced). The spectra for each oxidation state of the recombinant pseudo-wild-type (P2A) *D. vulgaris* flavodoxin are shown for comparison in each case (dotted line). Flavodoxin (*ca.* 30 μM) in 50 mM sodium phosphate buffer, pH 7.0, was titrated under prepurified argon gas in a sealed titration cuvette at 25 °C. For ease in comparisons, a spectrum of the fully formed semiquinone species of the Y98A/Fld^{+6} mutant (panel A) was generated by the subtraction of the contribution of the small amount of oxidized and reduced species present near the midpoint of the titration and rescaled to 100%. It should be emphasized that the semiquinone species only accumulates to ∼40% of stoichiometric level during the titration (see Figure 4). The semiquinone spectrum for the Y98A (panel B) [taken from Swenson and Krey (1994)] has not been normalized and represents the actual level of semiquinone present at approximately midway through the titration. The hydroquinone spectrum in each case was corrected for the presence of a small amount of semiquinone remaining at the end of the titration.

Oxidation−Reduction Potentials. The midpoint potentials for the ox/sq couple and the sq/hq couple of the Y98A/Fld^{+6} mutant were determined from the same titration by equilibration with the indicator dye anthraquinone-2,6-disulfonate ($E_{m,7}$ = −184 mV; Dutton & Baltscheffsky, 1972) during reduction with sodium dithionite. Titration data were evaluated using the Nernst plot in which the system potential, as determined from the dye, was plotted *versus* the logarithm of the ratio of the concentrations of the oxidized and semiquinone species for the ox/sq couple (Figure 3, panel A) or the semiquinone and hydroquinone for the sq/hq couple (Figure 3, panel B). The redox potential for the ox/sq couple of Y98A/Fld^{+6} (−183 mV) is nearly identical to that of Y98A (−186 mV). This result is consistent with a previous

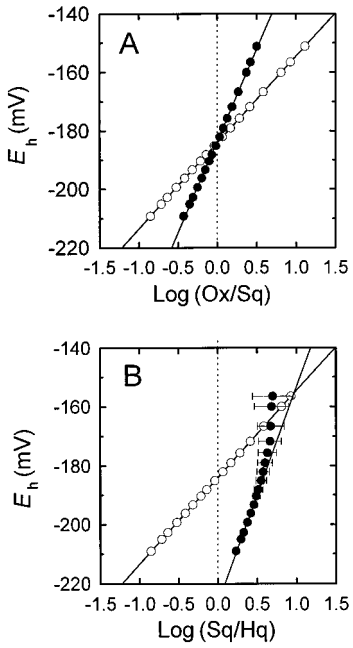

FIGURE 3: Determination of the midpoint potential for the ox/sq couple (panel A) and the sq/hq couple (panel B) of the Y98A/Fld^{+6} mutant in 50 mM sodium phosphate buffer, pH 7.0 at 25 °C. Reduction potentials were determined during titration with sodium dithionite under strict anaerobic conditions by equilibrium of the flavodoxin (*ca.* 30 μM) (●) with 30 μM anthraquinone-2,6-disulfonate (○) as the indicator dye ($E_{m,7} = -184$ mV; Dutton & Baltscheffsky, 1972). Data were plotted according to the Nernst equation and midpoint potentials established as described in Zhou and Swenson (1995).

study in which a discernible trend between charge neutralization and $E_{ox/sq}$ was also not noted (Zhou & Swenson, 1995). Both values are somewhat more negative than the midpoint potential for the recombinant pseudo-wild-type flavodoxin established using 2-hydroxy-1,4-naphthoquinone ($E_{m,7} = -145$ mV; Cammack, *et al.*, 1977) as the indicator dye (Swenson & Krey, 1994).

It was also possible to obtain a value for the midpoint potential for the sq/hq couple of Y98A/Fld^{+6} during the titration with anthraquinone-2,6-disulfonate (Figure 3, panel B). By this method, $E_{sq/hq}$ was estimated to be -224 mV. However, the reliability of this value is somewhat questionable because it is nearly out of the range of this dye. It was possible for this mutant to determine $E_{sq/hq}$ from the formation constant (K_f) of the semiquinone, where $K_f = [sq]^2/([ox][hq])$, because all three redox species simultaneously accumulate to significant levels near the midpoint of reductive titrations. Very comparable values for K_f of 4.73 ± 1.18 and 4.53 ± 0.24 were calculated on the basis of concentrations of the oxidized, semiquinone, and fully reduced species present in equilibrium during reduction of the Y98A/Fld^{+6} flavodoxin either by sodium dithionite in 50 mM phosphate buffer, pH 7.0 at 25 °C (Figure 4), or by photoreduction in the same buffer containing 50 mM EDTA (data not shown), respectively. An $E_{sq/hq}$ value of -223 ± 4 mV was calculated using the equation $E_{ox/sq} - E_{sq/hq} = (RT/nF)\ln(K_f)$, a value in good agreement with that obtained with the indicator dye. Thus, the $E_{sq/hq}$ value of the Y98A/Fld^{+6} mutant was seen to have increased substantially compared to the wild-type and the Y98A or Fld^{+6} mutant flavodoxins. The experimental values for the $E_{sq/hq}$ and $E_{ox/sq}$ values for wild type and relevant mutants are summarized in Table 1 for comparison.

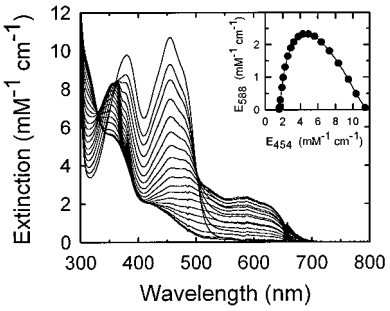

FIGURE 4: Determination of the midpoint potential for the sq/hq couple of the Y98A/Fld^{+6} mutant through the evaluation of the equilibrium constant during the reductive titration of the flavodoxin (*ca.* 30 μM) with sodium dithionite in 50 mM phosphate buffer, pH 7.0 at 25 °C. The absorbance spectrum of the titration mixture was recorded upon establishment of equilibrium at each point in the reduction. Inset: Relationship of the spectral changes (extinction coefficient) at 580 nm (reflecting the formation of the semiquinone) *versus* those at 460 nm during the course of the titration (values were corrected for dilution). The concentrations of the oxidized, semiquinone, and fully reduced species of the flavodoxin in equilibrium at each step of the titration were determined by multicomponent analysis of the visible absorbance spectra. In the example shown, an average value of 4.73 ± 1.18 was determined for K_f, where $K_f = [sq]^2/([ox][hq])$, using data from nine different points near the midpoint of the titration where the mixture contained at least 10% of each redox species. A midpoint potential for the sq/hq couple of -223 mV was calculated on the basis of relationship $E_{ox/sq} - E_{sq/hq} = 59 \log(K_f)$ and an $E_{ox/sq}$ value of -183 mV.

Table 1: Comparison of One-Electron Reduction Potentials and Dissociation Constants for Each Oxidation State of the FMN Cofactor in the Wild-Type and Mutant Flavodoxins from *D. vulgaris*

			K_d (nM)		
flavodoxin	$E_{ox/sq}$ (mV)	$E_{sq/hq}$ (mV)	ox[f]	sq[g]	hq[g]
wild type	−148[a]	−443[a]	0.24[h]	0.0072	280
Fld^{+6}	−162[b]	−350[b]	0.60	0.031	32
Y98A	−186[a]	−304[a]	3.2	0.42	73
Y98A/Fld^{+6}	−183[c]	−223[c]	15	1.8	13
FMN	−238,[d] −314[e]	−172,[d] −124[e]			

[a] From Swenson and Krey (1994). [b] From Zhou and Swenson (1995). [c] This work. [d] From Draper and Ingraham (1968). [e] From Anderson (1983). [f] Determined directly by spectophotometric or spectrofluorometric titration of FMN with apoflavodoxin. Approximate error in the K_d values reported in this work is ±40%. [g] The K_d values for the semiquinone and the hydroquinone states were calculated from the observed shifts in the midpoint potentials of the FMN upon binding to flavodoxin [see Dubourdieu *et al.* (1975) for details]. Values are based on the one-electron potentials for unbound FMN of Draper and Ingraham (1968) as reported here. If the reduction potentials for FMN of Anderson (1983) are used, the K_d values for the semiquinone and hydroquinone in all cases are decreased by approximately 20-fold and 3-fold, respectively. [h] From Curley *et al.* (1991).

The one-electron reduction potentials for the sq/hq couple of the Y98A, Fld^{+6}, and Y98A/Fld^{+6} mutants relative to that of the unbound FMN cofactor and to the shift in its potential when bound to the recombinant wild-type flavodoxin are represented graphically in Figure 5. Upon binding to the wild-type *D. vulgaris* flavodoxin protein, the midpoint potential for the sq/hq couple of the FMN is dramatically lowered by 271−319 mV, depending on the value used for the free FMN [−443 mV for the flavodoxin *versus* either −172 mV or −124 mV for free FMN based on the Draper and Ingraham (1968) or Anderson (1983) one-electron reduction potentials, respectively]. This corresponds to a substantial increase in the Gibbs free energy change of over 6 kcal/mol. This shift is significantly reduced in the case

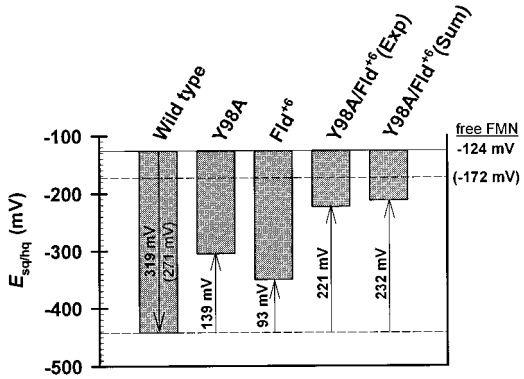

FIGURE 5: Comparison of the shifts in the one-electron reduction potential for the sq/hq couple of the various mutant flavodoxins described in this study relative to the two different reported values for this couple for unbound (free) FMN: −172 mV (Draper & Ingraham, 1968) and −124 mV (Anderson, 1983). The bars represent the actual decrease in $E_{sq/hq}$ when the FMN is bound to the flavodoxin. For example, $E_{sq/hq}$ is lowered by 319 mV [relative to the Anderson (1983) value] when bound to the wild-type flavodoxin [or by 271 mV relative to the Draper and Ingraham (1968) value]. For each of the mutant flavodoxins, the arrows and associated values indicate the increase in $E_{sq/hq}$ relative to the wild type that result from the amino acid substitutions introduced in each case. The bar labeled Y98A/Fld^{+6} (Sum) is a calculated or predicted value based the independent or additive effects of both the Y98A and Fld^{+6} mutations (the sum of 139 and 93 mV, respectively). The bar designated Y98A/Fld^{+6} (Exp) represents the actual experimentally determined value for the Y98A/Fld^{+6} mutant.

of the Y98A/Fld^{+6} mutant, however. Relative to the wild-type potential, the $E_{sq/hq}$ has increased by 221 mV. This value can be compared to increases of 139 and 93 mV for the Y98A and Fld^{+6} mutants, respectively, mutants which represent the individual contributions of the elimination of the aromatic side chain flanking the *si* face of the flavin and the partial neutralization of the negative electrostatic surface surrounding the FMN (Swenson & Krey, 1994; Zhou & Swenson, 1995). If the latter two classes of amino acid substitutions are acting independently, the predicted increase for the Y98A/Fld+6 mutant would be the arithmetic sum of these individual contributions for a calculated increase of 232 mV. As can be seen, this predicted value is slightly larger yet very close to the actual increase observed for the Y98A/Fld^{+6} mutant.

Thus, it would appear that apolar aromatic interactions and the negative electrostatic environment surrounding the cofactor act more or less independently in the modulation of the midpoint potential for the sq/hq couple of this flavodoxin. Collectively, these two structural features, which are found in most flavodoxins, are responsible for approximately 70−80% (depending on the value for FMN used) of the total shift in the one-electron potential for the sq/hq couple when the FMN is bound to this flavodoxin, accounting for over 5 kcal/mol of the change in free energy associated with the reduction of the FMN cofactor to the hydroquinone state when bound to this flavodoxin *versus* when it is free in solution.

Comparison of the FMN Binding Affinities of the Mutant Flavodoxins in Each Oxidation State. Because the one-electron reduction potentials of the bound FMN cofactor are established by the relative binding affinity of the protein for each oxidation state of the flavin, an analysis of the binding strengths was performed. The dissociation constant (K_d) for the oxidized FMN complex of the Y98A, Fld^{+6}, and Y98A/Fld^{+6} mutant flavodoxins was determined by direct titration of FMN solutions with freshly prepared apoprotein while monitoring the visible absorbance and/or fluorescence changes associated with cofactor binding. The K_d values for the semiquinone and hydroquinone forms were calculated on the basis of observed differences in the midpoint potential of the FMN cofactor when bound relative to the reported values of FMN in solution (Draper & Ingraham, 1968; Anderson, 1983) based on the linked equilibria and free energy relationships described by Dubourdieu *et al.* (1975). The K_d values of all three oxidation states for the mutant and wild-type flavodoxins are listed in Table 1. Compared with the wild-type protein, the K_d values for the Fld^{+6}, Y98A, and Y98A/Fld^{+6} complexes in the oxidized state have increased by approximately 2-, 13-, and 60-fold, respectively. The relatively small increase in K_d for the Fld^{+6} mutant is consistent with the very conservative nature of the acid to amide substitutions introduced. The alanine for tyrosine substitution within the context of the Fld^{+6} protein (generating Y98A/Fld^{+6}) resulted in a 25-fold increase in the K_d for the oxidized flavin complex relative to Fld^{+6}, which is slightly higher than the 13-fold increase observed for the Y98A mutant relative to wild type. This difference could be due to the collective effects of all seven amino acid substitutions in the Y98A/Fld^{+6} mutant that may be altering the structure of the binding site and/or the interactions with the cofactor to a slightly greater extent than for the individual substitutions. Nevertheless, the alanine substitution appears to have the greater effect on the binding of the oxidized FMN. This observation is not surprising given the extensive van der Waals contacts between the flavin isoalloxazine ring and the side chain of Tyr98 in the wild-type holoprotein (Watenpaugh *et al.*, 1973; Watt *et al.*, 1991). In fact, one might expect that elimination of such an interaction might affect the binding of the cofactor to a much larger extent. However, on the basis of approximately 3000-fold higher dissociation constant for riboflavin compared to FMN (D'Anna & Tollin, 1972), a substantial portion of the binding energy seems to be contributed by the many hydrogen-bonding interactions between the protein and the 5′-phosphate group of the cofactor so alterations within the isoalloxazine subsite may be of lesser consequence. Also, coplanar aromatic stacking interactions are apparently not always energetically favorable (see discussion below).

In the wild-type *D. vulgaris* flavodoxin, the neutral FMN semiquinone is bound much more tightly than the other redox states, resulting in the significant thermodynamic stabilization of the blue neutral radical species so characteristic of the flavodoxin family and generating $E_{ox/sq}$ values that are less negative than that of the unbound cofactor. This was also true of the Y98A, Fld^{+6}, and Y98A/Fld^{+6} mutants. Only relatively small differences in the K_d (sq) to K_d (ox) ratio are noted among these proteins (Figure 6). These results suggest that these mutations only marginally affect the stability of the semiquinone species, a conclusion previously reached for the Y98A and Fld^{+6} mutants (Swenson & Krey, 1994; Zhou & Swenson, 1995).

On the other hand, the degree of destabilization of the FMN hydroquinone anion relative to the other oxidation states is substantially reduced in all three mutants in comparison to the wild-type protein. This phenomenon is clearly illustrated by the ratio of the dissociation constants of the hydroquinone relative to the oxidized and semiquinone

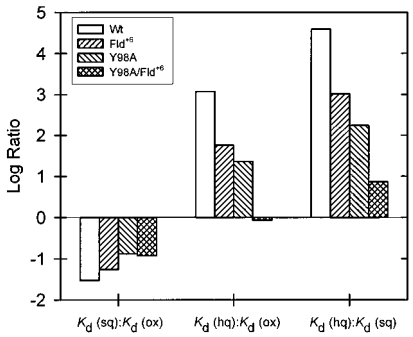

FIGURE 6: Comparison of the ratios of the dissociation constants for each oxidation state of the FMN cofactor for wild type and each mutant flavodoxin. The dissociation constant for the oxidized FMN was determined experimentally while those for the semiquinone and hydroquinone species were derived from the one-electron reduction potentials for each mutant relative to that for unbound FMN according to the linked equilibria and free energy relationships of Dubourdieu *et al.* (1975). The logarithmic values of the ratios are represented for greater clarity. Each group of bars represents different comparisons as indicated by labels on the horizontal axis.

species shown in Figure 6. Keeping in mind the logarithmic scale, the wild-type flavodoxin binds the hydroquinone over 3 orders of magnitude less well than the oxidized species and approximately 40 000-fold less well than the semiquinone. This large binding differential is manifested in the very low reduction potential of the sq/hq couple in wild type. Both the Y98A and Fld^{+6} mutants show substantially improved binding of the hydroquinone anion relative to the oxidized species. While in wild type the K_d for the flavin hydroquinone is nearly 1200-fold higher than that for the oxidized state, in the Y98A/Fld^{+6} mutant the binding of the FMN hydroquinone has been returned to nearly that of the oxidized form. This change is the result of both an increase in the dissociation constant of the oxidized flavin complex and the decrease in the value for the hydroquinone. These observations clearly demonstrate that the elimination of the aromatic interactions and the reduction in the negative charge surrounding the flavin cofactor as represented in the Y98A/Fld^{+6} mutant are both major determinants of the very low one-electron reduction potentials for the sq/hq couple in this flavodoxin. These results are entirely consistent with previous conclusions that the large redox potential shift observed in the wild-type *D. vulgaris* flavodoxin is in large part due to the destabilization of the FMN hydroquinone anion through unfavorable interactions with its aromatic neighbors and the negative electrostatic environment provided by the asymmetric distribution of the charged residues in the vicinity of the cofactor (Swenson & Krey, 1994; Zhou & Swenson, 1995). Because the semiquinone is formally neutral in charge, alterations in the electrostatic environment provided by the protein are seen to have only minimal effects on the strength of this complex, especially when compared to its effect on the oxidized flavin complex. Thus, the decrease in $E_{sq/hq}$ is further accentuated by the thermodynamic stabilization of the semiquinone state relative to the fully reduced species by the flavodoxin protein.

It should be noted that in these comparisons the midpoint potentials for free FMN of Draper and Ingraham (1968) have been used. Using the alternative one-electron potentials reported more recently by Anderson (1983), one finds that the calculated dissociation constants for the semiquinone for all flavodoxin species in the study are smaller by approximately 20-fold than those listed in Table 1 and those for the hydroquinone somewhat lower. However, the major conclusions of this study are not changed using either set of values. Also, because the K_d (hq) to K_d (ox) ratio is dependent on the two-electron midpoint potential for FMN, which is not in dispute, the magnitude of the effects on the binding of the FMN hydroquinone anion holds in either case (Ludwig & Luschinsky, 1992).

The Role of Tyr98 in Lowering $E_{sq/hq}$ May Be Primarily Due to Unfavorable $\pi-\pi$ Interactions with the FMN Hydroquinone. The original intent underlying the design and generation of the Y98A/Fld^{+6} mutant was not only to test the cumulative effects of both the aromatic interactions and the negative electrostatic environment on the modulation of the potentials of the FMN cofactor but also to assess in greater detail the basis for the large increase in $E_{sq/hq}$ observed for the Y98A mutant (Swenson & Krey, 1994). The substitution of alanine for Tyr98 could manifest itself structurally in two ways. Such a substitution obviously eliminates the coplanar aromatic interactions between the side chain of Tyr98 and the FMN isoalloxazine ring. However, the elimination of the bulky tyrosine side chain also increases the solvent accessibility of the flavin ring, thereby possibly increasing the polarity or effective dielectric of the cofactor environment. On the basis of Coulomb's law, such an increase should weaken the electrostatic interactions between the acidic residues and the flavin hydroquinone anion, reducing the effect of the negative electrostatic surface potential in lowering $E_{sq/hq}$. However, the results obtained from the production and characterization of the Y98A/Fld^{+6} mutant seem to suggest that this may only marginally be the case. The charge neutralizations in the more solvent exposed context of the Y98A mutant (*i.e.*, the Y98A/Fld^{+6} mutant) increased $E_{sq/hq}$, but by only about 10 mV less than the same neutralizations in the wild-type context (*i.e.*, the Fld^{+6} mutant) (an increase of 82 mV *vs* 93 mV, respectively; see Figure 5). If one compares the difference in the Gibbs free energy changes ($\Delta\Delta G$) of binding of the FMN hydroquinone to the charged−neutralized flavodoxin in each context, a similar pattern is observed. Neutralization of the six acidic amino acid residues with Tyr98 in place (*i.e.*, the Fld^{+6} mutant) decreases the ΔG of binding relative to wild type by approximately 1.3 kcal/mol or, on average, 0.21 kcal/mol per acidic residue neutralized [see also Zhou and Swenson (1995)]. In the absence of the Tyr98 side chain, the ΔG of binding decreases by about 1.0 kcal/mol or an average of 0.17 kcal/mol per acidic residue neutralized (Y98A/Fld^{+6} *vs* Y98A). So, the effect of charge neutralization on the stability of the hydroquinone anion complex is only slightly smaller in the more solvent exposed situation. These differences, if attributable only to changes in solvent exposure, translate to a rather modest increase in the calculated effective dielectric, too small to account for the large increase observed for the Y98A mutant. These results initially were somewhat surprising to us. Can they be rationalized?

The solvent accessibility of the individual atoms of the FMN isoalloxazine ring within the wild-type or mutant flavodoxin structures was calculated using standard methods invoking a rolling sphere of 1.4 Å radius (Connolly, 1983). In the wild-type *D. vulgaris* flavodoxin approximately 95 Å^2 of the isoalloxazine ring system is exposed (Figure 1, panel A). However, solvent exposure is confined exclusively

to the C-7 and C-8 methyl groups of the FMN which account only for about 15% of the total 650 Å^2 surface area of the FMN isoalloxazine ring. Therefore, all three ring systems of the flavin are essentially solvent inaccessible.

The solution conformation of the Y98A mutant (as well as wild type) has been determined by using heteronuclear three-dimensional NMR spectroscopy recently (Stockman *et al.*, 1993, 1994). It is concluded from those studies that the Y98A substitution does not result in any gross conformational changes in the flavin binding site. The changes that do occur are minor and result from the slightly different packing interactions required to accommodate the new side chain at the position 98 (Stockman *et al*., 1994). The solvent accessibility of the isoalloxazine ring in the restrained-energy-minimized solution structure of the Y98A mutant, again as evaluated by the rolling sphere method, has apparently increased to 139 Å^2, of which 95 Å^2 is due to the methyl groups (Figure 1, panel B). Therefore, only a relatively small percentage of the available surface area of the isoalloxazine ring system becomes solvent accessible. The accessible portions include one face of the *o*-xylene ring and part of the pyrazine ring (see Figure 1, panel B). However, it appears that the negative charge that develops upon reduction of the flavin to the hydroquinone, which is localized largely on the pyrimidine subnucleus (Müller, 1992), is still well protected from solvent. It should be noted that, on the basis of near-ultraviolet−visible absorbance spectrum of the fully reduced Y98A and Y98A/Fld^{+6} mutants, it is likely that the flavin hydroquinone remains in its anionic form in these proteins (Müller, 1992; Swenson & Krey, 1994). Because the spectral differences between the neutral and anionic hydroquinone are small and also a function of environment, this assignment is not entirely unambiguous, however.

In addition, the polarity of the FMN binding site may actually be relatively high despite the fact that the flavin cofactor is largely buried in the protein. For example, the pyrimidine subnucleus is extensively hydrogen bonded to various polar groups on the protein backbone, and an additional hydrogen-bonding interaction with N(5)H occurs in the reduced states (Watt *et al.*, 1991). In fact, the effective dielectric constant has been estimated to range from 50 to 100 on the basis of interaction energies calculated for the surface acidic residues and the anionic FMN hydroquinone (Zhou & Swenson, 1995). Given these relatively high values, one might not expect the small increase in solvent exposure associated with the Y98A substitution to substantially affect the influence of the negative electrostatic environment as seems to be supported by the results of this study. Because this interaction spans the interface between solvent and protein, estimations of the effective dielectric through such discontinuities are problematic, however.

So, it would seem that the increase in potential for the sq/hq couple that resulted from the Y98A mutation cannot be adequately explained by just the increase in solvent exposure and its influence on the unfavorable electrostatic effects. What then is responsible for this potential increase? The nearly coplanar orientation of this tyrosine side chain and the flavin isoalloxazine ring could promote potentially important van der Waals contacts and various aromatic ring stacking interactions such as $\pi-\pi$ and quadrupole interactions and dipole−dipole coupling. The role of aromatic interactions in the control of a number of binding processes has been described (Hunter & Sanders, 1990, and references therein). Contrary to popular intuition, the coplanar stacking of aromatic systems, which maximizes favorable van der Waals and hydrophobic interactions, is not always energetically favorable. This is apparently the consequence of the electrostatic repulsion between the two partially negatively charged faces created by the π-electron clouds of each aromatic in this configuration (Hunter & Sanders, 1990). More favorable electrostatic interactions exist when the aromatic rings are offset such that edge-to-face interactions are possible, as is generally observed in the aromatic−aromatic interactions between phenylalanine residues in proteins, for example (Hunter *et al.*, 1991). In these situations slightly favorable electrostatic interactions are possible because of the small positive charge associated with the hydrogen atoms of the ring edge. According to a recent model developed by Hunter and Sanders (1990), the strength of coplanar aromatic interactions is strongly influenced by π-polarizing heteroatom substituents. Electron-donating moieties, such as the phenolic hydroxyl group on tyrosine, increase the π-electron density of the aromatic ring and accentuate its electrostatic repulsion with the adjacent aromatic system, particularly if it also is electron rich. The results of this study are consistent with this model. In the oxidized state, the coplanar configuration of the π-rich Tyr98 side chain and the FMN isoalloxazine ring is expected to be energetically neutral or perhaps even somewhat favorable with regard to the $\pi-\pi$ interactions given that in the oxidized state the flavin ring is relatively electron deficient. It is well-known that in polar solvents the flavin in its oxidized state tends to associate or stack with electron rich organic molecules, often forming charge-transfer complexes (Müller, 1992). Indeed, the elimination of the Tyr98 side chain in the Y98A mutant was seen to have increased the dissociation constant for the oxidized FMN complex somewhat (Table 1). When the flavin becomes fully reduced, the electron density of the flavin hydroquinone not only increases substantially but also develops a formal negative charge in the flavodoxin (Ludwig & Luschinsky, 1992). This situation should significantly increase the electrostatic repulsion with the electron-rich tyrosine side chain. This is in part reflected in the much higher dissociation constant for the flavin hydroquinone relative to either the oxidized and semiquinone states as is noted experimentally. Also consistent with this conclusion are the redox properties of the previously characterized Y98F mutant of this flavodoxin. In this case, $E_{sq/hq}$ is about 30 mV less negative than either wild type or the Y98W mutant (Swenson & Krey, 1994). The phenylalanine side chain, lacking a heteroatom substituent, is less π-rich than either the phenol or indole ring systems. The absence of the additional π-electron repulsion introduced by those ring systems diminishes the destabilization of the FMN hydroquinone anion complex in Y98F by approximately 1 kcal/mol relative to wild type. Of course, the asymmetric addition of heteroatoms into aromatic structures also induces or alters the overall dipole moment of the system. The introduction of dipole−dipole coupling between the aromatic systems, if favorable, could overcome some of the repulsive effects of $\pi-\pi$ interactions. In the case of the *D. vulgaris* flavodoxin, the dipole moments of Tyr98 and the FMN isoalloxazine ring are not favorably coupled, however.

Recent studies using diaminopyridine-based synthetic receptors to model the flavin binding sites of flavoproteins have

also demonstrated the importance of aromatic π-stacking interactions in the control of flavin redox potentials (Breinlinger *et al.*, 1997). Electrochemical analyses suggest that such interactions can modulate the oxidation−reduction potential of bound flavin derivatives over a 100 mV range, consistent with the magnitude of the effects observed in this study.

CONCLUSIONS

The results of this study lead us to the conclusion that the majority of the increase in the one-electron reduction potential for the sq/hq couple associated with the elimination of the Tyr98 side chain in the Y98A mutant is not principally the consequence of the increased solvent exposure of the flavin isoalloxazine ring. These results support and extend our earlier conclusion that the unfavorable coplanar aromatic stacking interactions between the π-electron-rich Tyr98 residue flanking the *si* face of the isoalloxazine ring and the flavin hydroquinone anion substantially lower the midpoint potential of the sq/hq couple (Swenson & Krey, 1994). Furthermore, the Y98A/Fld^{+6} mutant produced and characterized here clearly demonstrates that the unfavorable aromatic stacking or $\pi-\pi$ interactions *and* the unfavorable general negative electrostatic environment provided by the FMN binding site together represent two major cumulative contributing factors in the modulation of the very low one-electron reduction potential of the flavodoxin, jointly accounting for over 70−80% of the total shift in the midpoint potential for the sq/hq couple of the FMN when bound to this flavodoxin protein. Because these two general structural features are commonly found in the flavin binding site in other flavodoxins, one can conclude that together they represent principal controlling features in the generation of the low one-electron reduction potentials that typify this family of flavoproteins.

REFERENCES

Anderson, R. F. (1983) *Biochim. Biophys. Acta 722*, 158−162.

Breinlinger, E., Niemz, A., & Rotello, V. (1997) in *Flavins and Flavoproteins 1996* (Stevenson, K. J., Massey, V., & Williams, C. H., Jr., Eds.) University of Calgary Press, Calgary, Alberta, (in press).

Cammack, R., Rao, K., Hall, D., Moura, J., Xavier, A., Bruschi, M., LeGall, J., Deville, A., & Gayda, J. (1977) *Biochim. Biophys. Acta 490*, 311−317.

Connolly, M. L. (1983) *Science 221*, 709−712.

Curley, G. P., Carr, M. C., Mayhew, S. G., & Voordouw, G. (1991) *Eur. J. Biochem. 202*, 1091−1100.

D'Anna, J. R., Jr., & Tollin, G. (1972) *Biochemistry 11*, 1073−1080.

Degtyarenko, K. N. (1995) *Protein Eng. 8*, 737−747.

Deistung, J., & Thornely, R. N. F. (1988) *Biochem. J. 253*, 587−595.

Draper, R. D., & Ingraham, L. L. (1968) *Arch. Biochem. Biophys. 125*, 802−808.

Dubourdieu, M., LeGall, J., & Favaudon, V. (1975) *Biochim. Biophys. Acta 376*, 519−532.

Dutton, P., & Baltscheffsky, M. (1972) *Biochim. Biophys. Acta 267*, 172−178.

Hunter, C. A., & Sanders, J. K. M. (1990) *J. Am. Chem. Soc. 112*, 5525−5534.

Hunter, C. A., Singh, J., & Thornton, J. M. (1991) *J. Mol. Biol. 218*, 837−846.

Krey, G. D., Vanin, E. F., & Swenson, R. P. (1988) *J. Biol. Chem. 244*, 15436−15443.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.

Ludwig, M. L., & Luschinsky, C. L. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) Vol. III, pp 427−466, CRC Press, Boca Raton, FL.

Mayhew, S. G. (1971) *Biochim. Biophys. Acta 235*, 276−288.

Mayhew, S. G., & Tollin, G. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) Vol. III, pp 389−426, CRC Press, Boca Raton, FL.

Müller, F. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) Vol. I, pp 1−71, CRC Press, Boca Raton, FL.

Nieva-Gomez, D., Roberts, G. P., Klevickis, S., & Brill, W. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2555−2558.

Sanger, F., Nicklen, S., & Coulson (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.

Stockman, B. J., Euvrard, A., Kloosterman, D. A., Scahill, T. A., & Swenson, R. P. (1993) *J. Biomol. NMR 3*, 133−149.

Stockman, B. J., Richardson, T. E., & Swenson R. P. (1994) *Biochemistry 33*, 15298−15308.

Swenson, R. P., & Krey, G. D. (1994) *Biochemistry 33*, 8505−8514.

Swenson, R. P., Krey, G. D., & Eren, M. (1991) in *Flavins and Flavoproteins* (Curti, B., Ronchi, S., & Zanetti, G., Eds.) pp 415−422, Walter de Gruyter & Co., Berlin and New York.

Wassink, J. H., & Mayhew, S. G. (1975) *Anal. Biochem. 68*, 609−616.

Watenpaugh, K. D., Sieker, L. C., & Jensen, L. H. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3857−3860.

Watt, W., Tulinsky, A., Swenson, R. P., & Watenpauph, K. D. (1991) *J. Mol. Biol. 218*, 195−208.

Zhou, Z., & Swenson, R. P. (1995) *Biochemistry 34*, 3183−3192.

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