Electrostatic Effects of Surface Acidic Amino Acid Residues on the Oxidation−Reduction Potentials of the Flavodoxin from *Desulfovibrio vulgaris* (Hildenborough)†

Zhimin Zhou and Richard P. Swenson*

Department of Biochemistry, The Ohio State University, Columbus, Ohio 43210

Received September 6, 1994; Revised Manuscript Received December 19, 1994⊗

ABSTRACT: The flavodoxin from *Desulfovibrio vulgaris* (Hildenborough) is a member of a family of small, acidic proteins that contain a single noncovalently bound flavin mononucleotide (FMN) cofactor. These proteins function as low-potential one-electron transferases in bacteria. A distinguishing feature of these flavoproteins is the dramatic decrease in the midpoint potential of the semiquinone/hydroquinone couple of the FMN upon binding to the apoprotein (−172 mV for FMN free in solution *versus* −443 mV when bound), a perturbation thought to be essential for physiological function. The structural basis of this phenomenon is not yet thoroughly understood. In this study, the contribution of six acidic residues (Asp62, Asp63, Glu66, Asp95, Glu99, and Asp106) to the perturbation of the redox properties of the cofactor has been investigated. These residues are clustered about the FMN binding site within 13 Å of the N(1) atom of the cofactor. Using oligonucleotide-directed mutagenesis, these residues were neutralized in various combinations through the substitution of asparagine for aspartate and glutamine for glutamate. Seventeen mutant flavodoxins were generated in which one to all six acidic residues were systematically neutralized, often in various spatial configurations. There was no obvious correlation between the midpoint potentials for the oxidized/semiquinone couple and general electrostatic environment, although some differences were noted. However, the midpoint potential for the semiquinone/hydroquinone couple for each of the mutants was less negative than that of the wild type. These increases are strongly correlated with the number of acid to amide substitutions, with an average contribution of about 15 mV per substitution. Collectively, the unfavorable electrostatic environment provided by these acidic residues accounts for approximately one-third of the large midpoint potential shift for the semiquinone/hydroquinone couple that typifies the flavodoxin family, apparently through the destabilization of the flavin hydroquinone anion.

Flavodoxins represent an important family of electron-transferring flavoproteins, containing a single noncovalently bound flavin mononucleotide (FMN)[1] prosthetic group. These acidic proteins have been isolated from a variety of sources, and their molecular masses range from 14 to 18 kDa [for recent reviews, see Mayhew and Tollin (1992) and Ludwig and Luschinsky (1992)]. A distinguishing feature of the flavodoxin family is the very negative reduction potential for the semiquinone/hydroquinone (sq/hq) couple of the bound FMN cofactor, placing these proteins in the potential range of ferredoxins for which they can substitute *in vitro* and, apparently, *in vivo* (Mayhew & Ludwig, 1975). For the flavodoxin from *Desulfovibrio vulgaris*, the midpoint potential of the cofactor is shifted from −172 mV (Draper & Ingraham, 1968) to approximately −445 mV when associated with the apoprotein, repesenting a Gibb's free energy change of over 6 kcal/mol (Dubourdieu *et al.*, 1975; Curley *et al.*, 1990; Swenson *et al.*, 1991; Swenson & Krey, 1994). X-ray crystallographic analyses of several different flavodoxin proteins have assisted in the development of structural explanations for this rather large shift in potential. A general observation emerging from the comparison of the three-dimensional structures of all three oxidation states of the flavodoxins from *Clostridium beijerinckii*, *D. vulgaris*, and *Anacystis nidulans* is that the semiquinone form differs from the oxidized state in the conformation of the polypeptide backbone within equivalent regions in each protein that is situated adjacent to the isoalloxazine ring of the cofactor. This conformational change is thought to contribute to the stability of the flavin semiquinone through the formation of an additional hydrogen bond between the protein and the N(5)H of the cofactor (Ludwig & Luschinsky, 1992). On the other hand, the semiquinone and fully reduced forms of the flavodoxins are virtually identical in structure (Ludwig *et al.*, 1976; Smith *et al.*, 1978; Watenpaugh *et al.*, 1976; Watt *et al.*, 1991). Therefore, the dramatic shift in the reduction potential of the sq/hq couple is unlikely to result from conformational differences between the two reduced states. Instead, the influences of the specific conserved features of the FMN binding site in these proteins have been invoked as possible explanations.

Three prominent features of the FMN binding site (Mayhew & Ludwig, 1975) have been noted in all flavodoxins. The isoalloxazine ring of the cofactor is bound in a largely apolar environment and is invariably flanked by at least one aromatic amino acid residue. A tryptophan residue resides on the "outer" face of the flavin in the flavodoxin from *C.*

† This study was supported by Grant GM36490 from the National Institutes of Health.

* To whom correspondence should be addressed at the Department of Biochemistry, 776 Biological Sciences Building, The Ohio State University, 484 West 12th Ave., Columbus, OH 43210-1292 (telephone, 614-292-9428; Fax, 614-292-6773).

⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1995.

[1] Abbreviations: FMN, flavin mononucleotide; Fld^{+n}, flavodoxin mutant with *n* acid to amide substitutions; BV, benzyl viologen; MV, methyl viologen; ϵ_{eff}, effective dielectric constant.

0006-2960/95/0434-3183$09.00/0

beijerinckii (Smith *et al.*, 1983). The isoalloxazine ring is situated between a tyrosine and a tryptophan residue in the flavodoxins from several members of the *Desulfovibrio* genus as well as other bacteria (Helms & Swenson, 1991, 1992; Watenpaugh *et al.*, 1973; Stockman *et al.*, 1990; Fukuyama *et al.*, 1990). The tyrosine residue is positioned in a nearly coplanar orientation with the outer face of the FMN isoalloxazine ring while the tryptophan forms a large portion of the inner surface of the flavin binding pocket. The functional significance of this highly conserved theme has been demonstrated recently (Swenson & Krey, 1994).

A second distinctive structural feature within the flavodoxin family is a polypeptide loop near the NH_2 terminus which is comprised principally of hydroxyamino acid residues. This loop forms a unique binding site for the terminal phosphate group of the FMN cofactor (Burnett *et al.*, 1974; Watenpaugh *et al.*, 1973; Watt *et al.*, 1991; Smith *et al.*, 1983). Interactions between the phosphate moiety and this loop are primarily through hydrogen bonds. A notable absence of basic residues that might ion pair with the phosphate dianion leaves this charged group uncompensated in these proteins. The unfavorable electrostatic interaction between the phosphate dianion and the negatively charged flavin hydroquinone has been proposed as a partial explanation for the low reduction potentials of these proteins (Moonen *et al.*, 1984).

A third characteristic feature of the flavodoxin family is the asymmetric distribution of the charged amino acid residues of these acidic proteins. Several acidic residues are clustered around the cofactor binding site (Watenpaugh *et al.*, 1973; Burnett *et al.*, 1974; Smith *et al.*, 1983). The absence of positively charged amino acid residues in the vicinity of the isoalloxazine ring may be notable. In the flavodoxins from the *Desulfovibrio* family, seven acidic residues are distributed most closely around the N(1) atom of the FMN cofactor. Despite their locations on the surface of the protein, nearly all of these residues are conserved (Helms & Swenson, 1992). These residues seem to play a role in the formation of electron-transfer complexes between the flavodoxin and its redox partners (Weber & Tollin, 1985; Stewart *et al.*, 1988; Palma *et al.*, 1994). However, because electrostatic interactions may play a crucial role in poising the oxidation−reduction potentials of various electron-transfer proteins, it is important to establish the extent, if any, that these acidic residues contribute in this capacity in the flavodoxin protein system.

In this study, the contributions by six acidic residues—Asp62, Asp63, Glu66, Asp95, Glu99, and Asp106—in establishing the redox properties of the FMN cofactor in the *D. vulgaris* flavodoxin are investigated. These residues (except Asp106) are located on both 60's and 90's loops that form a major portion of FMN binding site and are within approximately 13 Å distance of the FMN N(1) atom (Figure 1). The electrostatic effects of these residues were studied by the neutralization of each acidic residue by the substitution of its respective amide (*i.e.*, Asn for Asp and Gln for Glu) by oligonucleotide-directed mutagenesis. Seventeen mutant flavodoxins were generated, purified, and characterized in this study in which one to six acidic residues were systematically neutralized in various spatial configurations. The effects of alterations in the electrostatic surface on the oxidation−reduction properties of the bound FMN cofactor are correlated and reported.

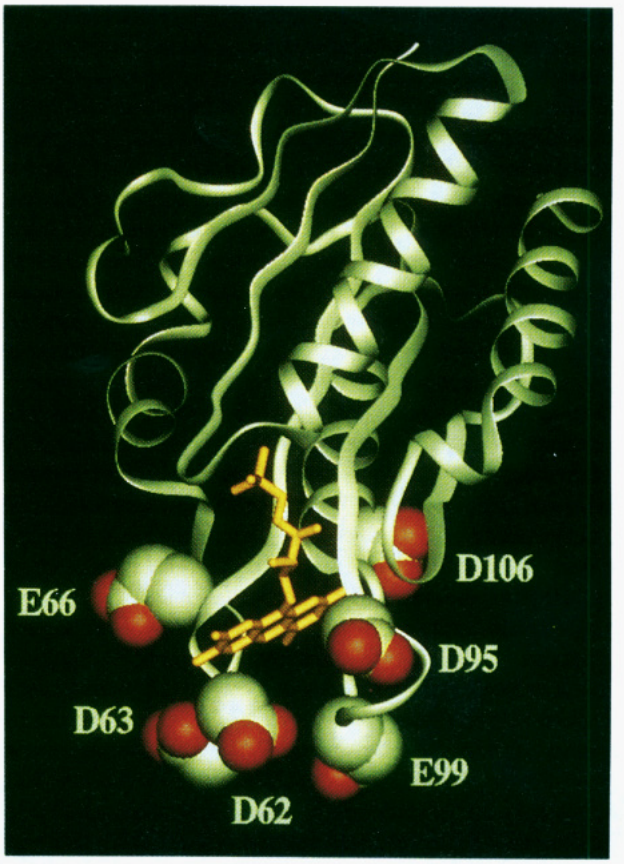

FIGURE 1: Representation of the six acidic amino acid residues clustered around the flavin mononucleotide binding site in the *D. vulgaris* flavodoxin. The acidic residues are depicted as the van der Waals surfaces. The FMN cofactor is highlighted in yellow. The structure is adapted from the coordinates published by Watt *et al.* (1991).

MATERIALS AND METHODS

Materials. Anthraquinone-2,6-disulfonate and methyl viologen were purchased from Fluka Chemicals. Benzyl viologen was obtained from Serva Chemicals. Hydroxyethyl viologen [*N*,*N*′-bis(β-hydroxyethyl)-4,4′-bipyridinium dichloride] was synthesized as described previously (Kazarinova *et al.*, 1967) and recrystallized from methanol. All other chemicals were of analytical reagent grade.

Bacterial Strains and Plasmids. All mutations were introduced into a modified version of the previously cloned and characterized structural gene encoding the wild-type flavodoxin from *D. vulgaris* (Hildenborough) (NCIB 8303, ATCC 29579) (Krey *et al.*, 1988). The modification to the second codon converting Pro2 to an alanine greatly increases the level of expression in *Escherichia coli* (Krey *et al.*, 1988). This P2A or "pseudo-wild-type" flavodoxin is structurally isomorphous to the true wild-type protein and is similar in all other respects (Watt *et al.*, 1991). In this study, the *Kpn*I/*Hin*dIII fragment containing the coding region from that construct was subcloned in the *Kpn*I and *Hin*dIII sites of the pBluescript KS plasmid for oligonucleotide-directed mutagenesis. Although it was planned to shuttle the structural gene back into the expression vector, it was noted that expression of the flavodoxin holoprotein in *E. coli* takes place from this construct, presumably utilizing either the *lac* or T3 promoters contained within the pBluescript KS vector just 5′ to the flavodoxin coding region.

Site-Directed Mutagenesis. Three degenerate oligonucleotide sequences (Table 1) were designed to introduce acid

Table 1: Oligonucleotide Sequences for Site-Directed Mutagenesis

mutation	oligonucleotide sequence[a]	restriction site[b]
D62N, D63N, E66Q, D62N/D63N, D62N/E66Q, D63N/E66Q, D62N/D63N/E66Q	5′-CCTGCAGCT(G/C)GATGGAGT(C/T)GT(C/T)ACCCC-3′	*HphI*⁻ (D62N) *HinfI*⁻ (D63N) *PvuII*⁺ (E66Q)
D95N, D106N, D95N/D106N	5′-CGATGGCGT(C/T)GACAGCCCCGCAGAAGTACTCGTAGGAACTGT(C/T)TCCGCAGCC-3′	*XmnI*⁺ (D95N) *SalI*⁻ (D106N)
E99Q, D95N/E99Q, E99Q/D106N, D95N/E99Q/D106N	5′-CGATGGCGT(C/T)GACAGCCCCGCAGAAGTATTGGTAGGAACTGT(C/T)TCCGCAGCC-3′	*XmnI*⁺ (D95N) *RsaI*⁻ (E99Q) *SalI*⁻ (D106N)

[a] The underlined nucleotides are different from the wild type, and those indicated by asterisks are "silent" mutations introduced to create convenient restriction sites. [b] Restriction sites either created (+) or eliminated (−) as the consequence of the mutation introduced as indicated.

to amide substitutions (*i.e.*, Asp to Asn and Glu to Gln) at each of the three acid residues in either the 60's or the 90's loop of the flavodoxin protein (Asp106 has been grouped into the 90's loop for the sake of convenient statement) as well as multiple substitutions in various combinations of these residues. The design and degeneracy incorporated into these oligonucleotides made it possible to produce, in a single oligonucleotide-directed mutagenesis experiment, all three mutants containing a single acid to amide substitution at each of three acidic amino acid residues, the three mutants containing all possible combinations of double mutations involving these three positions, and the one mutant containing all three substitutions in the 60's loop. To simplify selection, two oligonucleotides were used separately to introduce the corresponding combinations of single, double, and triple acid to amide substitutions in the 90's loop. In all cases, the nucleotide sequences of each oligonucleotide were designed in such a way so as to either create or eliminate a unique restriction site as the consequence of the mutation at each of the desired codons as indicated in Table 1. This strategy greatly simplified the screening of transformants and the identification of the various mutants and combinations required for this study. The oligonucleotides were synthesized on an Applied Biosystems Model 380B instrument using β-cyanoethyl phosphoramidite chemistry by The Ohio State University Biochemical Instrument Center and used without further purification. Oligonucleotide-directed mutagenesis was carried out using the Kunkel method for selection biased toward the mutant constructions (Kunkel *et al.*, 1985).

Flavodoxin mutants in which four, five, or all six acidic residues had been neutralized were generated in the following manner. The plasmid construction containing the triple mutant (D62N/D63N/E66Q) was digested with *Pst*I, and the large fragment comprised of the vector plus the 5′-end of the mutant gene coding for the amino-terminal portion of the flavodoxin protein (spanning residues 1−68) was isolated by 0.8% agarose gel electrophoresis. Plasmids containing the single (E99Q), double (E99Q/D106N), and triple (D95N/E99Q/D106N) mutants were also digested with *Pst*I, and in each case, the smaller fragment containing the 3′-end of the mutant gene encoding the carboxyl-terminal region (spanning residues 69−148) was isolated by 1.2% agarose gel electrophoresis. The large fragment and each of the different smaller fragments were ligated with T4 DNA ligase, and each recombinant preparation was used to transform *E. coli* AG-1 cells. All mutations and 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 proteins were purified by procedures described previously (Krey *et al.*, 1988) except that the NaCl concentrations of the wash and elution solutions used during ion-exchange chromatography were adjusted to accommodate the change in the net charge of the mutant. Fractions containing flavodoxin solutions having an A_{274}:A_{458} ratio $\leq$5.2 were pooled, concentrated by ultrafiltration, and rechromatographed over a Sephadex G-50 column (2.5 × 50 cm). Fractions having an A_{274}:A_{458} ratio $\leq$4.3 were pooled and concentrated. These preparations ran as a single band on SDS−PAGE.

Midpoint Potential Determinations. Oxidation−reduction potentials were determined spectrophotometrically at 25 °C in 50 mM phosphate buffer, pH 7.0, by equilibration of the flavodoxin and an appropriate redox indicator dye, both at concentrations of *ca.* 30 μM. Protein and dye solutions were made anaerobic by several cycles of vacuum and prepurified argon gas in a special closed titrator cuvette apparatus. Ultraviolet−visible absorption spectra were recorded on a Hewlett-Packard Model 8452A diode array spectrophotometer during the reductive titration with sodium dithionite, with special attention paid to ensuring that equilibrium between all species in solution had been reached. Anthraquinone-2,6-disulfonate ($E_{m,7}$ = −184 mV; Dutton & Baltscheffsky, 1972) was used as the redox indicator in establishing the reduction potentials of the ox/sq couple. The reduction potentials for the sq/hq couple were established using either benzyl viologen ($E_{m,7}$ = −359 mV; Michaelis & Hill, 1933), hydroxyethyl viologen ($E_{m,7}$ = −408 mV; Homer *et al.*, 1960), and/or methyl viologen ($E_{m,7}$ = −446 mV; Michaelis & Hill, 1933) as the redox indicator. The midpoint potential for the sq/hq couple was determined from the equilibrium constant according to the relationship

$$E_{Fld} - E_{dye} = \frac{RT}{nF} \ln K_{eq}$$

where E_{Fld} and E_{dye} are the midpoint potentials of the sq/hq

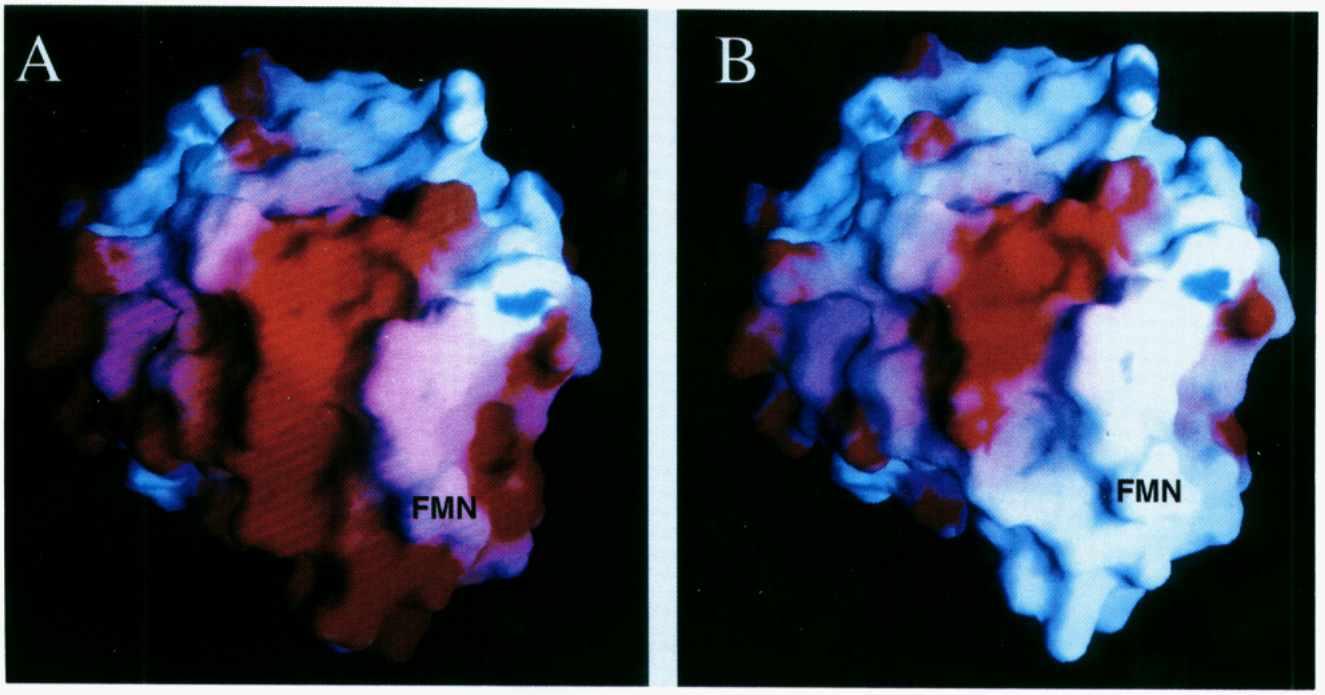

FIGURE 2: Representation of electrostatic surface potentials for wild-type flavodoxin (panel A) and the Fld^{+6} mutant (panel B) as calculated using the computer program GRASP (Nicholls & Honig, 1991). The Fld^{+6} structure was simulated using the wild-type structure (Watt *et al.*, 1991). The surfaces in red represent acidic regions; those in blue are basic. The location of the 7- and 8-methyl groups of the FMN cofactor that protrude onto the surface of the protein is indicated by the label FMN. It should be noted that the majority of the flavin isoalloxazine ring system is buried within the protein.

couple for the mutant flavodoxin and the redox indicator dye, respectively, R is the gas constant, T is the temperature in kelvin, F is the Faraday constant, n is the number of electrons involved in the reduction, and K_{eq} is the equilibrium constant. For redox titrations utilizing either hydroxyethyl or methyl viologen, the equilibrium constant was determined by linear regression analysis of the concentration data expressed in a linearized form as

$$\frac{1}{[V_R]} = \frac{K_{eq}}{[V_T]}\frac{[SQ]}{[HQ]} + \frac{1}{[V_T]}$$

where $[V_R]$ is the concentration of the reduced viologen, $[V_T]$ is the total viologen concentration, and [SQ] and [HQ] are the concentrations of the flavodoxin semiquinone and hydroquinone oxidation states, respectively. Because benzyl viologen dimerizes in the reduced state (Bird & Kuhn, 1981), a modification of the linear equation is required:

$$[BV_R] + 2[dBV_R] = -K_{eq}[BV_R]\frac{[SQ]}{[HQ]} + [BV_T]$$

where $[BV_R]$ and $[dBV_R]$ are the concentrations of the reduced benzyl viologen monomer and dimer, respectively, and $[BV_T]$ is the total concentration of all benzyl viologen species. The concentrations of each species at various points during the reductive titration were determined by a least squares multicomponent analysis of the ultraviolet−visible absorbance spectrum of the equilibrium mixture using standard reference absorbance spectra for each component in its various oxidation states generated under identical conditions.

Calculations. Free energy changes were calculated by using the relationship

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

where n, F, and E are the number of electrons, the Faraday constant, and the redox potential, respectively. The effective dielectric constant for the individual monopole−monopole interaction between each of the six acidic residues and the negatively charged N(1) atom on the FMN hydroquinone was calculated using Coulomb's law:

$$\epsilon_{eff} = q_F q_i/(4\pi\epsilon_0 r\delta E)$$

where ϵ_0 is the permittivity of vacuum, q_F is the unit charge on N(1) of the FMN hydroquinone anion, q_i is the unit charge on the acidic residue, r is the average distance from the oxygen atoms of the side-chain carboxylate group of Asp or Glu to the N(1) atom of the FMN, and ΔE is the redox potential change observed upon neutralization.

RESULTS

Generation, Expression, and Characterization of Mutants. Six of the seven acidic residues clustered within 13 Å of the N(1) atom of the FMN cofactor were the subject of this investigation. These residues introduce a ring of electronegative surface potential surrounding the FMN cofactor (Figure 2A). Each acidic residue was neutralized by its substitution with its respective amide analog using the oligonucleotide-directed mutagenesis technique to alter the previously cloned structural gene for this flavodoxin (Krey *et al.*, 1988). Substitutions were introduced individually, in all three possible combinations of two within each loop, in combinations of three within each loop, in one combination of four, one combination of five, and, finally, in which all six residues had been neutralized. In all, 17 individual mutants with from one to six of the acidic residues neutralized were generated. Not all combinations at each neutralization level could be introduced due to the prohibitive number of mutants involved; however, it was concluded that

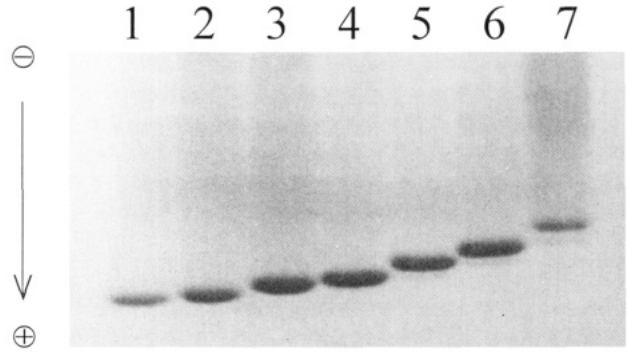

FIGURE 3: Comparison of the electrophoretic mobilities of wild-type and mutant flavodoxins on nondenaturing polyacrylamide gel (21%). Lanes 1 through 7 are wild-type, Fld^{+1} (D106N), Fld^{+2} (D95N/E99Q), Fld^{+3} (D62N/D63N/E66Q), Fld^{+4} (D62N/D63N/E66Q/E99Q), Fld^{+5} (D62N/D63N/E66Q/E99Q/D106N), and Fld^{+6} (D62N/D63N/E66Q/D95N/E99Q/D106N), respectively.

the mutations studied are representative. Neutralization of all six acidic residues can be seen to substantially reduce the electronegative environment of the flavin cofactor (Figure 2B).

All 17 individual mutant flavodoxin proteins were expressed, purified, and characterized in this study. Each protein was overproduced in *E. coli* AG-1 cells as the holoprotein, accumulating in substantial amounts within the cell in the partially reduced state as indicated by the dark bluish color of the bacterial pellet and the crude cellular extract. When purified to homogeneity, the fully oxidized forms of the flavodoxin mutants all displayed similar A_{274}:A_{458} ratios of 4.3 ± 0.1, suggesting that all 17 proteins bind the FMN cofactor in a stoichiometry similar to that of wild type and that cofactor binding was not grossly affected by the neutralization of up to six acid residues in the vicinity of the cofactor binding site. The decrease in the net negative charge of each mutant flavodoxin was confirmed by electrophoresis on nondenaturing polyacrylamide gels. The relative mobilities of representative mutant proteins having from one to six acidic residues neutralized are shown in Figure 3. Within experimental error, a linear dependency of the electrophoretic mobility *versus* the total number of acid residues neutralized was apparent, suggesting that an equivalent change in net charge results from each acid to amide mutation. With the exception of the mutant flavodoxins containing the D95N substitution, all of which tended to migrate slightly slower than the general trend, the electrophoretic mobilities did not seem to be dependent on the location or the combination of acidic residues neutralized (data not shown). The electrophoretic behavior of the D95N-containing mutants is not yet understood.

The ultraviolet−visible absorbance spectra of the oxidized forms of all 17 mutants were essentially identical to that of the wild-type holoprotein, exhibiting absorbance maxima at 378 and 458 nm in the visible region (Figure 4). Just as for the wild-type flavodoxin, the blue neutral form of the FMN semiquinone was observed to accumulate during the reduction of each mutant by titration with sodium dithionite. There was no spectral evidence for the formation and/or accumulation of the red anionic flavosemiquinone species. A representative example of the spectral changes that occur during titration is shown in Figure 4, panel B, for the mutant in which each of the six acidic residues involved in this study had been substituted with its respective amide (designated

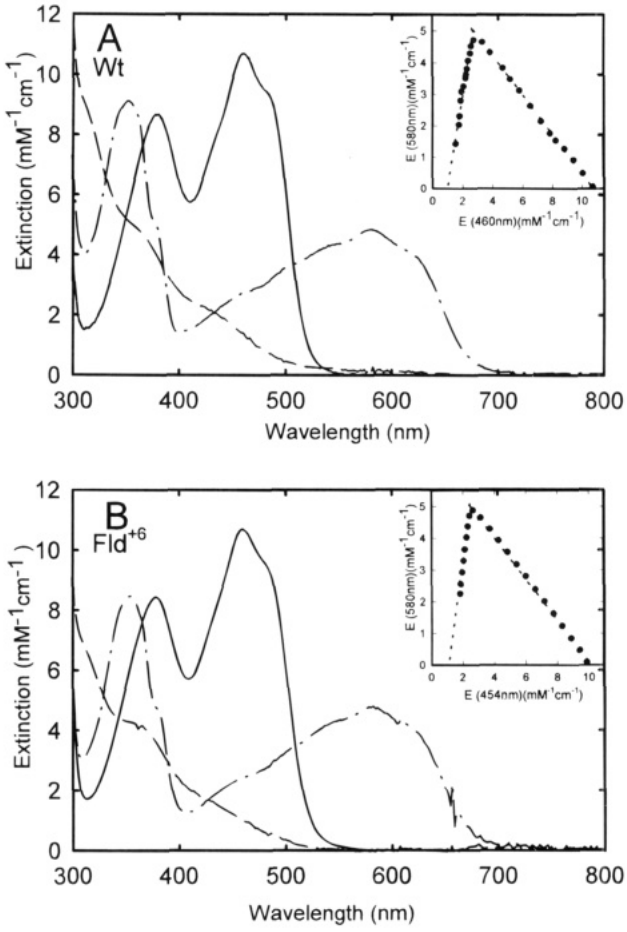

FIGURE 4: Ultraviolet−visible absorbance spectra of the wild-type (panel A) and the Fld^{+6} mutant (panel B) flavodoxins during reduction with sodium dithionite under anaerobic conditions. Flavodoxin (*ca.* 30 μM) in 50 mM phosphate buffer, pH 7.0, was titrated under argon in a sealed titration cuvette at 25 °C. Spectra: solid line, oxidized; dot-dashed line, semiquinone; dashed line, fully reduced. In each case, the spectrum of the fully formed semiquinone was generated by the subtraction of the contribution of the small amount of oxidized species present near the midpoint of the titration and rescaled to 100%. Similarly, the hydroquinone spectrum was corrected for the presence of a small amount of semiquinone remaining at the end of the titration. Insets: Plot of the absorbance changes at 580 nm *versus* those at 454 nm during the course of the titration (values were corrected for dilution).

Fld^{+6}). The ultraviolet−visible absorbance properties of the semiquinone and hydroquinone states were similar to those of wild type (Figure 4, panel A). These observations suggest that the mutations have not substantially affected the structure of the FMN binding site or the oxidation−reduction properties of the bound cofactor. Although not a very sensitive measure of the ionization state of the hydroquinone, the similarity in the hydroquinone spectra also implies that the anionic form was present in all 17 of the mutants under these conditions.

Oxidation−Reduction Potentials. The midpoint potential for the ox/sq couple (E_2) for each of the mutants was determined by equilibration with the redox indicator dye anthraquinone-2,6-disulfonate ($E_{m,7}$ = −184 mV) during reduction with dithionite. Titration data were evaluated using the Nernst plot in which the system potentials, as determined by the dye, were plotted *versus* the logarithm of the ratio of the concentrations of the oxidized and semiquinone species of the flavodoxin present at equilibrium during the reduction. The results obtained for the Fld^{+6} mutant shown in Figure

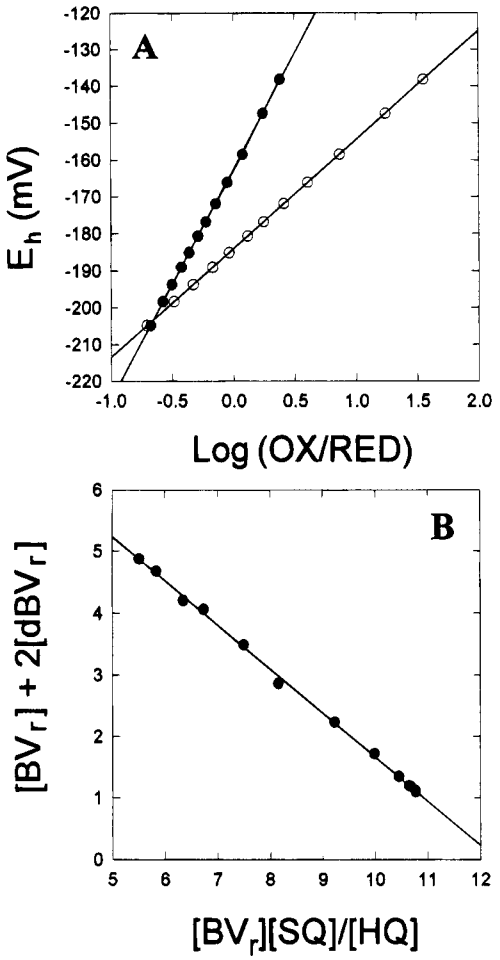

FIGURE 5: Determination of the midpoint potential for the ox/sq couple (panel A) and sq/hq couple (panel B) of the Fld^{+6} mutant. Reduction potentials were determined by the equilibration of the flavodoxin (*ca.* 30 μM) with either of the redox indicators anthraquinone-2,6-disulfonate (30 μM) (panel A) or benzyl viologen (12 μM) (panel B) during reduction with sodium dithionite under strict anaerobic conditions. For the determination of the ox/sq couple, the system potentials were determined from the ratio of the concentrations of the oxidized and reduced forms of the indicator dye (○). Data used in the determination of the midpoint potential for each flavodoxin couple (●) were plotted and analyzed as described in Materials and Methods.

5A were typical for the group of mutants. The E_2 values for the 17 mutants, summarized in Table 2, ranged from −152 mV for the D95N/E99Q/D106N mutant to −191 mV for D63N/E66Q, with a trend toward most being more negative than the wild-type value ($E_{m,7}$ = 149 ± 5 mV; Dubourdieu *et al.*, 1975; Curley *et al.*, 1990; Swenson *et al.*, 1991; Swenson & Krey, 1994).

The midpoint potential for the sq/hq couple (E_1) was also determined for each of the 17 mutants by equilibration with one of several viologen derivatives. A representative example of the titration data used to determine these potentials is included in Figure 5B. The E_1 values obtained are summarized in Table 2. All 17 mutant flavodoxins exhibit midpoint potentials that were less negative than that of wild type, ranging from −432 mV for the double mutant E99Q/D106N to −350 mV for the mutant in which all six of the acidic residues were neutralized (D62N/D63N/E66Q/D95N/E99Q/D106N). With the exception of the D95N mutant, the E_1 values for the six individual single mutants were quite similar to one another, ranging from −425 mV

Table 2: Oxidation−Reduction Midpoint Potentials[a] Determined for the Flavodoxin Mutants

flavodoxin	net neutralized charge	E_2 (ox/sq couple)[c]	E_1 (sq/hq couple)
wild type	0	−148[b]	−443[b,d]
D62N	1	−171	−426[e]
D63N	1	−189	−431[e]
E66Q	1	−182	−425[e]
D95N	1	−176	−397[e]
E99Q	1	−158	−425[e]
D106N	1	−185	−429[e]
D62N/D63N	2	−177	−419[e]
D62N/E66Q	2	−167	−419[e]
D63N/E66Q	2	−191	−414[e]
D95N/E99Q	2	−153	−400[e]
D95N/D106N	2	−176	−398[e]
E99Q/D106N	2	−161	−432[e]
D62N/D63N/E66Q	3	−185	−398[e]
D95N/E99Q/D106N	3	−152	−392[e]
D62N/D63N/E66Q/E99Q	4	−166	−377[f]
D62N/D63N/E66Q/E99Q/D106N	5	−175	−368[f]
D62N/D63N/E66Q/D95N/E99Q/D106N	6	−162	−350[f]

[a] Values are reported in millivolts *versus* SHE, pH 7.0, 25 °C. [b] From Swenson and Krey (1994). [c−f] Potentials determined using anthraquinone-2,6-disulfonate, methyl viologen, hydroxyethyl viologen, and benzyl viologen as the redox indicator, respectively.

Table 3: Comparison of Electrostatic Interactions of the Six Acidic Residues

residue	distance (Å)[a]	ΔE_1 (mV)[b]	$\Delta\Delta G$ (kcal/mol)[c]	ϵ_{eff}[c]
Asp62	8.6	17	0.39	99
Asp63	12.3	12	0.28	98
Glu66	13.0	18	0.42	62
Asp95	6.3	46	1.06	50
Glu99	12.9	18	0.42	62
Asp106	10.9	14	0.32	95

[a] Average distance from the oxygen atoms of the side-chain carboxyl group of Asp or Glu to the N(1) atom of FMN (Watt *et al.*, 1991). [b] Potential differences relative to the reported wild-type E_1 value (Swenson *et al.*, 1991). [c] Calculation of $\Delta\Delta G$ and ϵ_{eff} are described in Materials and Methods.

−431 mV and differing from wild type by +12 to +18 mV (all E_1 shifts here and thereafter are referenced to the E_1 value of −443 mV for the wild-type flavodoxin (Swenson & Krey, 1994)) (Table 3). The E_1 value for the D95N mutant was substantially less negative than the other single mutants, being −397 or 46 mV less negative than wild type. The strength of the monopole−monopole interaction is, among other factors, inversely related to the distance between the charges. Referring to the reported X-ray crystallographic data for the wild-type protein (Watt *et al.*, 1991), some differences in the distances between the targeted acidic residue side chains and the N(1) position of the bound FMN were noted (Table 3). The β-carboxyl group of Asp95 was closest, within 6.3 Å of N(1), Asp62 was at an intermediate distance, and the rest are at a similar distance of about 12 Å. In general, a weak correlation between the observed potential shifts and these distances was noted, however,

Of the six double mutants, those in which Asp95 was neutralized display E_1 values that have increased the most, −400 and −398 mV for D95N/E99Q and D95N/D106N, respectively. In contrast, the value for the E99Q/D106N double mutant was significantly more negative than the others despite the fact that single mutants involving these

residues were similar to others in that group. The two triple mutants within the 60's and 90's loops showed very similar E_1 values of −398 and −392 mV, respectively, implying a similar overall effect of three acid to amide substitutions from both loops on the E_1 value. The largest shift in potential occurred in the mutant with all six acidic residues neutralized, displaying an accumulative effect on this couple of +93 mV.

The mutants containing the D95N mutations require further comment. As noted above, the neutralization of Asp95 often resulted in midpoint potential shifts that were significantly larger than the others. Of the six mutants involving a single acid to amide substitution, the D95N mutant exhibited the largest increase of +46 mV. This shift is very similar to that reported for the analogous D90N mutation in the *A. nidulans* flavodoxin (Luschinksy *et al.*, 1991). Asp90 is the structural equivalent to Asp95 in *D. vulgaris* flavodoxin (Ludwig & Luschinsky *et al.*, 1992). Both are located close to the N(1) atom of the flavin. The carboxylate of Asp95, located within 6.3 Å of N(1) of the FMN, represents the nearest formally charged group to the cofactor in the *D. vulgaris* flavodoxin (see Table 3). This close proximity may account for a large part of the greater potential shift observed in the D95N mutant. However, this effect does not seem to be consistently additive in the mutants containing multiple neutralizations. The double mutants involving Asp95 have midpoint potential shifts of +43 and +45 mV for D95N/E99Q and D95N/D106N, respectively, approximately 15 mV larger than the average of +29 mV for the other members of this group. However, the mutant in which all three acidic residues in the 90's loop are neutralized (including Asp95) displays a potential shift very similar to the Fld^{+3} mutant involving only those acidic residues found in the 60's loop. Also, the Fld^{+6} mutant has a potential shift that agrees well with the general trend evident in Figure 6A.

An adequate explanation for the nonadditive nature of the Asp95 mutations is not apparent at this time. One could argue that our assumption that the substitution of each acidic residue by its respective amide equivalent increases the net charge by one electrostatic unit under the conditions studied is not valid. This assumption seemed reasonable given that the pK_a for the side-chain carboxylic acid group of aspartate and glutamate is approximately 4.5 (Tanford, 1962). However, because of electrostatic interactions among the acidic groups themselves, the neutralization of one or more of the acidic residues could affect the ionization of the others, potentially altering the net charge to an extent substantially different from that assumed. For this to manifest itself under the conditions of this study, the pK_a value for one or more of these acidic residues would have to be shifted to an unusually high value for such effects to be noticeable at pH 7.0. Several counter arguments can be poised. On the basis of the results of this study, changes in the ionization state of one or more of these acidic residues should affect the stability of the FMN hydroquinone anion. However, the reduction potential of the sq/hq couple for several wild-type flavodoxins is essentially independent of pH at values greater than 6.0 (Ludwig & Luschinsky, 1992). Further support for our assumption arises from the observation that the electrophoretic migrations of the mutants on polyacrylamide gels under nondenaturing conditions at pH 7.0 appeared to be directly proportional to the number of acidic residues neutralized in each case (representative results are included in Figure 3), consistent with the equivalent decrease in net

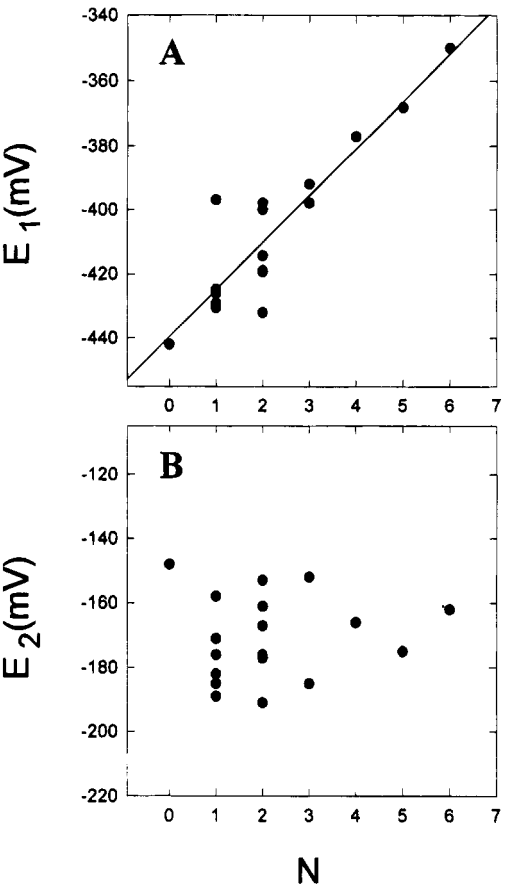

FIGURE 6: Relationship between the midpoint potentials for the sq/hq couple (panel A) and the ox/sq couple (panel B) and the number of acid to amide substitutions (*N*). The line in panel A represents the linear correlation as determined by the least squares linear regression analysis (slope, 15 mV/substitution; correlation coefficient, 0.91).

negative charge upon neutralization of each acidic residue. The slightly slower migration of mutants involving the neutralization of Asp95 was also evident in the Fld^{+3} and Fld^{+6} mutants, both of which display potential shifts that are consistent with the average of 15 mV/unit charge relationship observed in this study. Irrespective of the special circumstances surrounding the Asp95 neutralizations, the compelling dependency of the oxidation−reduction potential for the sq/hq couple on the surrounding negative electrostatic surface potential remains.

Determination of the Effective Dielectric Constants of Individual Monopole−Monopole Interactions. The Gibb's free energy associated with the monopole−monopole interaction between each individual acidic residue and the negative charge on N(1) of the flavin hydroquinone was calculated using the observed difference between the midpoint potential for the sq/hq couple for each single charge neutralization mutant compared to the wild-type protein. These calculations assume that each acid to amide conversion principally affects electrostatic interactions and does not cause significant structural changes. Also, the effective dielectric constant (ϵ_{eff}) for each of the monopole−monopole interactions was estimated using Coulomb's law under the conditions that the redox potentials were measured. Both the interaction energy and the calculated effective dielectric constant associated with each of the six acidic residues are included in Table 3. The ϵ_{eff} values ranged from 50 to 99, with an average of 78. These values can be considered

appropriate for electrostatic interaction involving unpaired solvent-accessible charges and can be compared with ϵ_{eff} values determined either by indirect determinations such as from shifts in the pK_a of the ionizable groups involved (Rees, 1980; Russell & Fersht, 1987; Bashfold & Karplus, 1990) or from the more direct measurement of the electric field (Lockhart & Kim, 1992, 1993; Steffen *et al.*, 1994). In those studies, ϵ_{eff} values were observed to range from 40 to 100 for charged groups on the surface and from 1.5 to 10 for the interior protein environment.

DISCUSSION

Flavodoxins are typified within the flavoprotein family by the very negative reduction potentials of the sq/hq couple of the bound FMN cofactor. The factors responsible for the control of the oxidation−reduction potentials of riboflavin-based cofactors have been studied for many years. It is likely that several different factors contribute in different ways to the control of the flavin potentials. One such factor, the electrostatic interaction, has been implicated in several studies. An attempt to correlate the reduction potential with the total net charge of various redox proteins has been reported (Rees, 1985). The conclusions from that study are limited in that other effects cannot be excluded given the number of different types of redox proteins included in the analysis. Calculations of the absolute midpoint potential in the cytochrome subunit of the *Rhodopseudomonas viridis* reaction center are found to be in good but not exact agreement with experiment (Gunner & Honig, 1991). Redox potential calculations have also been successfully applied to iron−sulfur proteins such as ferredoxins and have led to the conclusion that cluster-charged residue interactions are not of major significance, although the explicit incorporation of formally charged residues has not always been included in the calculations (Jensen *et al.*, 1994). Direct experimental evidence based on the mutation of several charged residues within the *Azotobacter vinlandii* ferredoxin has provided mixed results in this regard; however, in some cases the midpoint potential of one or the other of the iron−sulfur clusters was increased by approximately 20 mV (Shen *et al.*, 1993, 1994). For the flavodoxin system, theoretical calculations have estimated a contribution of −4 mV (at an ionic strength of 100 mM) per acidic residue toward the shift in the FMN potential (Moonen *et al.*, 1984). However, the contribution of the general electrostatic environment in the establishment of the oxidation−reduction properties of the bound FMN cofactor has not been directly established by experiment.

The *D. vulgaris* flavodoxin, like other flavodoxins, is highly charged, containing 30 acidic and 12 basic amino acid residues. These residues are asymmetrically distributed over the surface of the protein. Seven of the acidic residues are clustered within 13 Å of the N(1) atom of the FMN cofactor. Asp62, Asp63, Glu66, Asp95, and Glu99 are all located within two loops that form the majority of the flavin isoalloxazine binding site in this flavodoxin (Figure 1). Asp106 and Asp127 are located within an α-helix and a β-sheet, respectively (Watt *et al.*, 1991). The closest basic amino acid residue is Arg125, with its guanidino group lying just outside this region. Despite their surface locations, these seven acidic residues are conserved in most of the flavodoxins thus far characterized from the *Desulfovibrio* family, forming a ring of electronegative surface potential surrounding the cofactor, a dominant feature of this family of proteins (Figure 2A) (Helms *et al.*, 1990; Helms & Swenson, 1991, 1992). The intent of this study was to determine directly the influence, if any, on the oxidation−reduction potentials of the bound FMN cofactor.

The Midpoint Potential of the sq/hq Couple, but Not the ox/sq Couple, Correlates Well with Charge Neutralization. The midpoint potentials for the ox/sq couple were observed to vary over a 40-mV range; however, a very poor correlation was noted between the E_2 values and the number of charged groups neutralized, although most of the mutants seemed to display reduction potentials more negative than the wild-type protein (Figure 6B). This result may not be surprising in that both redox species in this couple are neutral in net charge. The molecular basis for the variation in the E_2 potential among these mutants is not yet fully understood. It may be that the neutralization of these charges is indirectly affecting other important interactions or directly altering the electron distribution within the isoalloxazine ring. There does not seem to be any obvious relationship between the midpoint potentials and the relative location of the acidic residue neutralized, however. In comparing the X-ray crystal structures of the oxidized and the reduced states, a small conformation difference has been noted involving the peptide backbone within the 60's loop (Watt *et al.*, 1991). However, the neutralization of the acidic residues in this loop did not seem to affect the stability of the flavin semiquinone differently than those within the 90's loop.

The midpoint potential data obtained for the sq/hq couple were in striking contrast to the ox/sq couple for this group of 17 mutants. Unlike for the ox/sq couple, a definite trend in the midpoint potential data for the sq/hq couple was noted. In general, as the number of acidic groups that were neutralized increased, the midpoint potential for this couple became substantially less negative. This relationship is apparent in a plot of E_1 *versus* the number of acid to amide substitutions (N) as shown in Figure 6A. The data conform very well (with a correlation coefficient of 0.91) to the linear relationship:

$$E_{m,7}(\text{mV}) = 15N - 440$$

The y-axis intercept of −440 mV, where $N = 0$, agrees very well with the published E_1 value for the wild-type recombinant protein (Swenson & Krey, 1994). Thus, for the various combinations of the six acidic amino acid residues neutralized in this study, the electrostatic interaction effects appear to be largely additive, with an average contribution to the total reduction potential shift of the FMN cofactor of −15 mV per acidic residue. As noted above, the midpoint potential values obtained for the single and double mutants containing the D95N substitution do not conform to this trend very closely. However, even if the E_1 values for these mutants were excluded from the linear regression analysis, the very similar slope of 16 mV per unit charge was obtained (correlation coefficient, 0.97; y-axis intercept, −444 mV).

With the possible exception of Asp95, the contribution of each acidic residue appears to be of a general electrostatic nature rather than through a specific interaction effect. There were no apparent differences in the contributions based on the relative location or spatial orientation of the acidic residue neutralized to the cofactor. For example, the total effect of the triple neutralization was essentially the same whether

the three acidic residues on the 60's loop were neutralized or those on the 90's loop. This was also true for the various combinations of double mutants. This conclusion is also supported by the fact that the ox/sq couple is not affected in any consistent way by the charge neutralizations introduced in this study. Furthermore, the ϵ_{eff} values calculated for each of six single-charge neutralizations based only on the potential shifts observed in this study (Table 3) were found to be within the range expected for solvent-accessible unpaired electrostatic interactions and are also consistent with the general conclusion that the midpoint potential shifts are the result of general electrostatic effects.

The results of this study demonstrate conclusively that the cumulative unfavorable electrostatic field provided by a cluster of six acidic residues within 13 Å of the N(1) of the FMN contributes significantly to the substantial shift in the midpoint potential of the FMN cofactor when associated with the flavodoxin protein, most likely through the destabilization of the negative charge that develops on the N(1)−C(2)O locus of the flavin hydroquinone during reduction. The midpoint potential for the sq/hq couple of the flavin decreases from a solution value of −172 mV to −443 mV when bound to the apoflavodoxin protein, a shift in potential of 271 mV of equivalent to a Gibb's free energy change of 6.2 kcal/mol. On the basis of the potential increase of 93 mV observed for the Fld^{+6} mutant in which all six acidic residues are neutralized, the electrostatic interactions provided by these residues account for approximately one-third of the total change (93 mV of the 271-mV decrease), equivalent to 2.1 kcal/mol in free energy or an average of 0.36 kcal/mol per acidic residue. This contribution may represent a minimum value in that Asp127 represents a seventh acidic residue within 13 Å, but was not neutralized in this study for practical reasons.

It is obvious that additional factors must also contribute to the destabilization of the FMN hydroquinone anion. The apolar environment provided by Tyr98 that flanks the flavin isoalloxazine ring in these flavodoxins has been demonstrated to play a crucial role in decreasing the midpoint potential of the sq/hq couple, contributing up to 140 mV of the potential shift (Swenson & Krey, 1994). Favorable electrostatic interactions introduced by the substitution of basic residues at position 98 substantially increase the midpoint potential, again demonstrating the significance of electrostatic effects in poising the oxidation−reduction potentials in the flavodoxin. It is possible that other unfavorable electrostatic interactions may also play a role. Although charged residues other than the seven clustered near the cofactor are present, it is unlikely that they contribute in a substantial way because of their distance from the cofactor. However, the terminal phosphate group on the FMN cofactor is situated approximately 9 Å from N(1) of the flavin. This phosphate is bound in an unusual binding site that is comprised primarily of several hydroxyamino acids that hydrogen bond with the phosphate (Watenpaugh *et al.*, 1973; Burnett *et al.*, 1974). This binding site does not seem to compensate well for the charge on the phosphate group that, paradoxically, is found in its dianionic ionization state at pH 8.0 (Vervoort *et al.*, 1986a). Calculations suggest that the electrostatic effect of the negative charges on the phosphate group may account for a significant portion of the shift in the midpoint potential of the sq/hq couple in flavodoxins (Moonen *et al.*, 1984). Although the magnitude of this effect is now questionable, there is experimental support for the fundamental premise. For example, the midpoint potential for the sq/hq couple becomes 20 mV more negative when the 3′,5′-bisphosphate analog of FMN is incorporated into the apoflavodoxins from *M. elsdenii* and *D. vulgaris*, presumably as the consequence of the addition of the second anionic phosphate group (Vervoort *et al.*, 1986b). Also, the complex of *D. vulgaris* apoflavodoxin with riboflavin, with spectroscopic properties very similar to those of the wild-type holoprotein, has been reported to have an E_1 value of −354 mV, which is 86 mV more positive than that of the FMN complex (Curley *et al.*, 1990).

Several of the acidic amino acid residues involved in this study may also play an important role in the transfer of electrons between this flavodoxin and its redox partners. Electrostatic interactions have been proposed to facilitate the formation of the electron-transfer complex. Although physiological electron-transfer complexes involving the flavodoxin have not been identified, interactions between flavodoxins and various *c*-type cytochromes have been studied by molecular graphics modeling, computational methods, and electron-transfer kinetics (Weber & Tollin, 1985; Stewart *et al.*, 1988; Palma *et al.*, 1994). In the hypothetical molecular graphics models of the electron-transfer complex, several of the acidic residues surrounding the FMN binding site of flavodoxin form salt bridges with specific basic residues on the surface of the cytochrome protein. These charge−charge interactions together with other interactions at the protein−protein interface are thought to stabilize the complex and assist in the orientation of the two redox centers for efficient electron transfer. The formation of these salt bridges effectively neutralizes several of the charged groups near the redox centers in both the donor and acceptor proteins. It has been proposed that these interactions will also affect the reduction potentials of each redox partner, increasing that for the flavodoxin and lowering that of the cytochrome (Smith *et al.*, 1977). Such shifts effectively reduce the driving force of the transfer reaction. The results presented in this work provide indirect experimental evidence supporting this shifting phenomenon. It also implies that oxidation−reduction properties of these proteins *in situ* may differ from those measured *in vitro*. These mutants should provide the means by which to experimentally test these electron-transfer complex models.

Conclusions. This study directly demonstrates for the first time in a flavoprotein system that the unfavorable electrostatic environment provided by solvent-accessible surface acidic residues clustered about the cofactor is a significant determinant in the poising of the midpoint potential of the sq/hq couple of the cofactor when the flavin hydroquinone anion is generated during reduction. In the flavodoxin studied, the contribution of six acidic residues clustered with 13 Å of the N(1) atom of the cofactor accounts for approximately one-third of the large midpoint potential shift for this couple under the conditions studied. The acidic nature of the flavodoxin proteins in general, the absence of compensating charges within the unusual binding pocket for the terminal phosphate group of the cofactor, and the apolar environment of the binding site for the isoalloxazine ring all seem to collectively produce an unfavorable environment in which to accommodate the developing negative charge on the flavin isoalloxazine ring as it becomes fully reduced,

establishing the very negative reduction potential that typifies the flavodoxin family.

REFERENCES

Bashford, D., & Karplus, M. (1990) *Biochemistry 29*, 10219−10225.

Bird, C. L., & Kuhn, A. T. (1981) *Chem. Soc. Rev. 10*, 49−82.

Burnett, R. M., Darling, G. D., Kendall, D. S., LeQuesne, M. E., Mayhem, S. G., Smith, W. W., & Ludwig, M. L. (1974) *J. Biol. Chem. 249*, 4383−4392.

Curley, G. P., Carr, M. C., O'Farrell, P. A., Mayhew, S. G., & Voordouw, G. (1990) in *Flavins and Flavoproteins 1990* (Curti, B., Zanetti, G., & Ronchi, S., Eds.) pp 429−436, Walter de Gruyter & Co., Berlin.

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.

Fukuyama, K., Wakabayashi, S., Matsubara, H., & Rogers, L. J. (1990) *J. Biol. Chem. 265*, 15804−15812.

Gunner, M. R., & Honig, B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9151−9155.

Helms, L. R., & Swenson, R. P. (1991) *Biochim. Biophys. Acta 1089*, 417−419.

Helms, L. R., & Swenson, R. P. (1992) *Biochim. Biophys. Acta 1131*, 325−328.

Helms, L. R., Krey, G. D., & Swenson, R. P. (1990) *Biochem. Biophys. Res. Commun. 168*, 809−817.

Homer, R. F., Mees, G. C., & Tomlinson, T. E. (1960) *J. Sci. Food Agric. 11*, 309−315.

Jensen, G. M., Warshel, A., & Stephens, P. J. (1994) *Biochemistry 33*, 10911−10924.

Kazarinova, N. F., Solomko, K. A., & Kotelenets, M. N. (1967) *Chem. Abstr. 67*, 2975d.

Krey, G. D., Vanin, E. F., & Swenson, R. P. (1988) *J. Biol. Chem. 263*, 15436−15443.

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

Laudenbach, D. E., Straus, N. A., Pattridge, K. A., & Ludwig, M. L. (1987) in *Flavins and Flavoproteins* (Edmondson, D. E., & McCormick, D. B., Eds.) pp 249−260, Walter de Gruyter & Co., Berlin.

Lockhart, D. J., & Kim, P. S. (1992) *Science 257*, 947−951.

Lockhart, D. J., & Kim, P. S. (1993) *Science 260*, 198−202.

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.

Ludwig, M. L., Burnett, R. M., Darling, G. D., Jordan, S. R., Kendall, D. S., & Smith, W. W. (1976) in *Flavin and Flavoproteins* (Singer, T. P., Ed.) pp 393−404, Elsevier, Amsterdam.

Luschinsky, C. L., Dunham, W. R., Osborne, C., Pattridge, K. A., & Ludwig, M. L. (1991) in *Flavins and Flavoproteins 1990* (Curti, B., Zanetti, G., & Ronchi, S., Eds.) pp 409−413, Walter de Gruyter & Co., Berlin.

Mayhew, S. G., & Ludwig, M. L. (1975) in *The Enzymes* (Boyer, P. D., Ed.) 3rd ed., Vol. 12, pp 57−118, Academic Press, New York.

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.

Michaelis, L., & Hill, E. S. (1933) *J. Gen. Physiol. 16*, 859−873.

Moonen, C. T. W., Vervoort, J., & Müller, F. (1984) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P. C., & Mayhew, S. G., Eds.) pp 493−496, Walter de Gruyter & Co., Berlin.

Nicholls, A., & Honig, B. (1991) *J. Comput. Chem. 12*, 435−445.

Palma, P. N., Moura, I., LeGall, J., Beeumen, J. V., Wampler, J. E., & Moura, J. J. G. (1994) *Biochemistry 33*, 6394−6407.

Rees, D. C. (1980) *J. Mol. Biol. 141*, 323−326.

Rees, D. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3082−3085.

Russell, A. J., & Fersht, A. R. (1987) *Nature 328*, 496−500.

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

Shen, B., Martin, L. L., Butt, J. N., Armstrong, F. A., Stout, C. D., Jensen, G. M., Stephens, P. J., LaMar, G. N., Gorst, C. M., & Burgess, B. K. (1993) *J. Biol. Chem. 268*, 25298−25939.

Shen, B., Jollie, D. R., Stout, C. D., Diller, T. C., Armstrong, F. A., Gorst, C. M., LaMar, G. N., Stephens, P. J., & Burgess, B. K. (1994) *J. Biol. Chem. 269*, 8564−8575.

Smith, W. W., Ludwig, M. L., Pattridge, K. A., Tsernoglou, D., & Petsko, G. A. (1978) in *Frontiers of Biological Energetics: From Electron to Tissues* (Dutton, P. L., Leigh, J. S., & Scarpa, A., Eds.) Vol. 2, pp 957−964, University of Pennsylvania, Philadelphia, PA.

Smith, W. W., Pattridge, K. A., Ludwig, M. L., Petsko, G. A., Tsernoglou, D., Tanaka, M., & Yasanobu, K. T. (1983) *J. Mol. Biol. 165*, 737−755.

Steffen, M. A., Lao, K., & Boxer, S. G. (1994) *Science 264*, 810−816.

Stewart, D. E., LeGall, J., Moura, I., Moura, J. J. G., Peck, H. D., Jr., Xavier, A. V., Weiner, P. K., & Wampler, J. E. (1988) *Biochemistry 27*, 2444−2450.

Stockman, B. J., Krezel, A. M., & Markley, J. L. (1990) *Biochemistry 29*, 9600−9609.

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

Swenson, R. P., Krey, G. D., & Eren, M. (1991) in *Flavins and Flavoproteins 1990* (Curti, B., Zanetti, G., & Ronchi, S., Eds.) pp 415−422, Walter de Gruyter & Co., Berlin.

Tanford, C. (1962) *Adv. Protein Chem. 17*, 69−165.

Taylor, M. F., Boylan, W. H., & Edmondson, D. E. (1990) *Biochemistry 29*, 6911−6918.

Vervoort, J., Müller, F., Mayhew, S. G., van den Berg, W. A. M., Moonen, C. T. W., & Bachner, A. (1986a) *Biochemistry 25*, 6789.

Vervoort, J., van Barkel, W. J. H., Mayhew, S. G., Müller, F., Bacher, A., Nielsen, P., & Le Gall, J. (1986b) *Eur. J. Biochem. 161*, 749−756.

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

Watenpaugh, K. D., Sieker, L. C., & Jensen, L. H. (1976) in *Flavin and Flavoproteins* (Singer T. P., Ed.) pp 405−410, Elsevier, Amsterdam.

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

Weber, P. C., & Tollin, G. (1985) *J. Biol. Chem. 260*, 5568−5573.

BI942111E