Differential Stabilization of the Three FMN Redox Forms by Tyrosine 94 and Tryptophan 57 in Flavodoxin from *Anabaena* and Its Influence on the Redox Potentials[†]

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ABSTRACT: Flavodoxins are electron transfer proteins that carry a noncovalently bound flavin mononucleotide molecule as the redox-active center. The redox potentials of the flavin nucleotide are profoundly altered upon interaction with the protein. In *Anabaena* flavodoxin, as in many flavodoxins, the flavin is sandwiched between two aromatic residues (Trp57 and Tyr94) thought to be implicated in the alteration of the redox potentials. We have individually replaced these two residues by each of the other aromatic residues, by alanine and by leucine. For each mutant, we have determined the redox potentials and the binding energies of the oxidized FMN−apoflavodoxin complexes. From these data, the binding energies of the semireduced and reduced complexes have been calculated. Comparison of the binding energies of wild-type and mutant flavodoxins at the three redox states suggests that the interaction between Tyr94 and FMN stabilizes the apoflavodoxin−FMN complex in all redox states. The oxidized and semireduced complexes are, however, more strongly stabilized than the reduced complex, making the semiquinone/hydroquinone midpoint potential more negative in flavodoxin than in unbound FMN. Trp57 also stabilizes all redox forms of FMN, thus cooperating with Tyr94 in strong FMN binding. On the other hand, Trp57 seems to slightly destabilize the semireduced complex relative to the oxidized one. Finally, we have observed that reduction of mutants lacking Trp57 is slow relative to that of wild-type or mutants lacking Tyr94, which suggests that Trp57 could play a role in the kinetics of flavodoxin redox reactions.

Flavodoxins are small flavoproteins involved in electron transfer reactions, often replacing ferredoxin under iron stress conditions (Mayhew & Tollin, 1992; Ludwig & Luschinsky, 1992). All flavodoxins of known structure are α/β proteins consisting of a central parallel β-sheet surrounded by α-helices. At the C-terminal end of the β-sheet, apoflavodoxins bind a molecule of flavin mononucleotide that confers redox properties to the protein. On binding to apoflavodoxin, the midpoint redox potentials of the FMN are altered: the midpoint potential of the oxidized/semiquinone couple is moderately shifted from −237 mV to usually less negative values, and that of the semiquinone/hydroquinone couple is drastically shifted from −172 mV to about −400 mV (Ludwig & Luschinsky, 1992). With these redox potentials, the semiquinone of bound FMN becomes much more stable than that of unbound FMN, allowing flavodoxins to transfer electrons one-by-one (Mayhew & Tollin, 1992). Alteration of the redox potentials of FMN is a direct consequence of the different stability of the oxidized, semireduced, and reduced apoflavodoxin−FMN complexes: the apoprotein binds the redox forms of FMN with different affinity, and this modifies the redox potentials of bound FMN. Similar mechanisms are used by other redox proteins to modulate the redox potentials of bound cofactors. Flavodoxins are ideal models to study these mechanisms because they are small, are quite stable, and can be easily converted into the apo forms; the apo forms reversibly bind FMN, allowing the study of the apoflavodoxin−FMN complex energetics; and the tridimensional structures of several flavodoxins are known (Watenpaugh et al., 1973; Burnett et al., 1974; Smith et al., 1983; Fukuyama et al., 1990; van Mierlo et al., 1990; Rao et al., 1993). In most flavodoxins, the redox-active moiety of FMN, the isoalloxazine ring, is sandwiched between two aromatic residues located in loops at the C-terminus of the β-sheet. One of these aromatic residues makes extensive contacts with the isoalloxazine. In 19 out of 23 flavodoxin sequences found in SwissProt, this residue is a tyrosine. The other aromatic residue interacts less tightly with the isoalloxazine, usually contacting its two methyl groups. In 14 of the 23 flavodoxin sequences in SwissProt, this residue is a tryptophan. The proximity of these two aromatic residues to the isoalloxazine ring makes them interesting candidates to play a role in modulating the redox potentials of flavodoxin. We have chosen the flavodoxin from the cyanobacteria *Anabaena* PCC 7119 as a model to study how apoflavoproteins influence the redox potentials of flavin cofactors because its gene has been cloned (Fillat et al., 1991), because the recombinant protein can be expressed in *E. coli* with good yields, thus allowing site-directed mutagenesis, and because it is the only flavodoxin for which the structures of both the apo and holo forms are known (Rao et al., 1993; Burkhart et al., 1995; Genzor et al., 1996a). Apoflavodoxin from *Anabaena* is a well-folded protein, but its conformational stability is low (Genzor et al., 1996b). It can reversibly form a tight complex with FMN, and the resulting redox-active holoflavodoxin is

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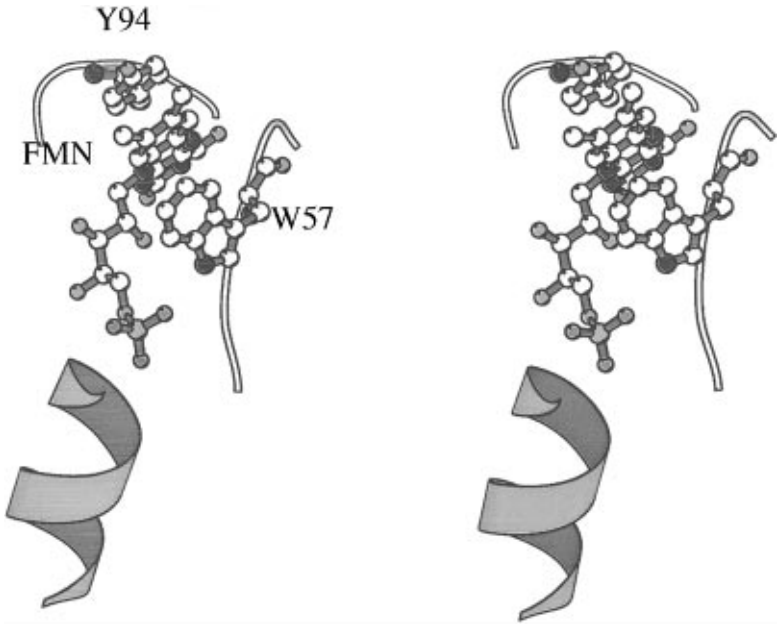

FIGURE 1: Stereo diagram (MOLSCRIPT; Kraulis, 1991) of the FMN binding site in flavodoxin from *Anabaena* PCC 7119.

very stable (C. Genzor, Ph.D. Thesis, 1996). Most of the conformational stability of holoflavodoxin derives from interactions between the apoprotein and the FMN. The phosphate is mainly bound by residues at the N-terminus of helix 1 while the isoalloxazine ring is sandwiched between Trp57 (packed against the methyl groups of the ring) and Tyr94 (that packs directly against the diazine system of the isoalloxazine; see Figure 1). These are the only side chains in contact with the isoalloxazine (Rao et al., 1993). The role of the equivalent Tyr98 from *Desulfovibrio vulgaris* flavodoxin has been studied by mutating it to other residues and determining the effects of mutation on the redox potentials (Swenson & Krey, 1994). These authors proposed that Tyr98 from *D. vulgaris* substantially destabilizes the flavin hydroquinone anion. In this study, we mutate Tyr94 and Trp57 in *Anabaena* flavodoxin to the other aromatic residues and to alanine and leucine. From the redox potentials and the stability of the oxidized apoflavodoxin−FMN complexes, we have determined the stability of the semireduced and reduced complexes. Comparison of the binding energy profiles along reduction of wild-type and mutant apoflavodoxin−FMN complexes clarifies the role of the two aromatic residues in the modulation of flavodoxin redox potentials.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Oligonucleotide-directed mutations were introduced in the gene for the flavodoxin from *Anabaena* PCC 7119 [previously cloned and expressed in *E. coli* by Fillat et al. (1990)] by the method of Deng and Nickoloff (1992) using the Transformer kit (2nd version) from Clontech and oligonucleotides from Pharmacia. Mutagenic oligonucleotides at position 94 were designed to convert the wild-type tyrosine codon (TAC) to either an alanine (GCT), a phenylalanine (TTC), a tryptophan (TGG), or a leucine (TTA) codon. At position 57, the wild-type tryptophan codon (TGG) was replaced by either an alanine (GCT), a phenylalanine (TTC), a tyrosine (TAC), or a leucine (TTG) codon. The selection primer changed a unique *Nde*I restriction site outside the gene into a unique *Sac*II restriction site. Template pTrc99A-AFD DNA was further purified using a Microspin S-300HR minicolumn from Pharmacia Biotech. The DNA required for the second transformation was isolated using the Wizard DNA isolation kit by Promega and was used to transform TG1 *E. coli* cell supplied by Amersham. Plasmids from a representative sample of transformants were screened for mutations by dideoxy DNA sequencing (Sanger et al., 1977) using the Sequenase Version 2.0 DNA sequencing kit from United States Biochemical.

Expression and Purification of the Flavodoxin Holoprotein Mutants. The expression and purification of flavodoxin mutants were done essentially as described by Genzor et al. (1996b). The purity of each flavodoxin preparation was confirmed by SDS−polyacrylamide gel electrophoresis using PhastGel^{R} Gradient 8-25 in a PhastSystem, both by Pharmacia Biotech.

Extinction Coefficients. The extinction coefficients of mutant flavodoxins in the fully oxidized state were determined as described by Mayhew and Massey (1969) using, for released FMN, a corrected extinction coefficient of 12 020 M^{-1} cm^{-1} at 445 nm. All extinction coefficients, as well as the correction factor, were determined in triplicate.

The extinction coefficient at 580 nm for the semiquinone of each mutant flavodoxin was determined in 50 mM potassium phosphate, pH 7.0, from data obtained during anaerobic photoreduction in the presence of 5-deazalumiflavin and EDTA (see below). The absorbance at 580 nm of fully formed semiquinone was determined as the intercept of the extrapolation of the linear portions of a plot of the absorbance at 580 nm versus the absorbance at 460 nm.

UV/Visible Absorption, Fluorescence, and Circular Dichroism Measurements. The UV/Vis spectra of oxidized flavodoxins in 50 mM potassium phosphate, pH 7.0, at room temperature were recorded in a Uvikon 860 UV−Visible Spectrophotometer from Kontron Instruments. Fluorescence spectra of the apo- and holoflavodoxins in 50 mM potassium phosphate, pH 7.0, were recordered at 25 ± 0.1 °C in a Kontron SFM25 fluorometer. Circular dichroism spectra of the apo- and holoflavodoxins in 50 mM potassium phosphate, pH 7.0, were recordered at 25 ± 0.1 °C in a Jasco 710 spectropolarimeter.

Midpoint Potential Determinations. The ox/sq[1] midpoint potential of each flavodoxin was determined by anaerobic photoreduction in the presence of 1 μM 5-deazalumiflavin and 3 mM EDTA using a CARY UV−Visible Spectrophotometer to follow the reduction of the protein. All experiments were performed at 25.0 ± 0.5 °C in 50 mM potassium phosphate, pH 7.0. The following dyes were used as mediators: 1 μM 2-hydroxy-1,4-naphthoquinone ($E_{m,7}$ = −145 mV), anthraquinone-2,6-disulfonate ($E_{m,7}$ = −184 mV), anthraquinone-2-sulfonate ($E_{m,7}$ = −225 mV), and phenosaffranine ($E_{m,7}$ = −252 mV). The sq/hq midpoint potentials were determined in the same way but in this case 1 μM benzyl viologen ($E_{m,7}$ = −359 mV) and methyl viologen ($E_{m,7}$ = −440 mV) were used as mediator dyes. The voltage of the system was measured with gold and calomel electrodes connected to a Ministat 29-500 50 Hz Potentiostat. A Kodak Carousel S-AV 1010 Projector was used to illuminate the cell, allowing the formation of the 5-deazalumiflavin radical. This in turn reduced the FMN in flavodoxin. The calomel electrode was previously calibrated against a quinhydrone solution. The concentrations of the various redox species in equilibrium were determined from the absorbance spectra. During each of the one-electron semireductions, only semiquinone and either oxidized or reduced flavodoxin were considered to be present in significant amounts. The concentration of semiquinone was calculated from the corresponding extinction coefficient, and the concentration of the other species by subtraction of the semiquinone concentration from the total protein concentration. The midpoint potentials for the redox couples were calculated by linear regression analysis of plots of system potential *versus* logarithm of concentration ratio (oxidized/semiquinone or semiquinone/reduced) according to the Nerst equation. Data points in the region of maximal semiquinone accumulation were not included in the regression as the three redox species might be significantly present. All midpoint potentials are reported relative to the potential of the standard hydrogen electrode (25 °C).

Dissociation Constants. The dissociation constants of the apoflavodoxin−FMN complexes in their fully oxidized state were determined fluorometrically in an SMF 25 Spectrofluorimeter (Kontron) at 25 ± 0.1 °C in darkness. Excitation was at 445 nm, and the emission was recorded at 525 nm. The FMN used was >95% pure according to reverse-phase HPLC. In a typical experiment, 1 mL of 0.2 μM FMN in 50 mM potassium phosphate, pH 7.0, was titrated with aliquots of 5−300 μM apoflavodoxin (depending on the mutant) in the same buffer. Binding of the protein to the cofactor strongly quenches the fluorescence emission of FMN. After each protein addition, the system was allowed to reach equilibrium for 2 min. The dissociation constants were calculated using Kaleidagraph by Abelbeck Software by fitting the emission fluorescence to eq 1:

$$F = F_{end} + F_{\delta}\left[dC_F - \frac{(C_A + K_d + dC_F) - \sqrt{(C_A + K_d + dC_F)^2 - 4C_A dC_F}}{2}\right] \quad (1)$$

where F is the observed fluorescence emission intensity after each addition, F_{end} the remaining emission intensity at the end of the titration, F_{δ} the difference in emission intensity between 1 μM free FMN and 1 μM flavodoxin, C_A the total protein concentration after each addition (apo + holo), K_d the dissociation constant of the apo-FMN complex in micromolar units, C_F the starting concentration of FMN, and d the dilution factor of this initial concentration (initial volume/total volume) after each addition. This equation also holds in the presence of traces of fluorescence impurities that do not bind to apoflavodoxin since their emission appears as a fraction of F_{end}. In the absence of impurities, this term represents the total fluorescence of the holoflavodoxin present at the end of the titration. The fitting treats the initial concentration of FMN as an unknown. The robustness of the fits is indicated by the fact that the known initial concentration of FMN was correctly predicted (within 90%).

[1] Abbreviations: ox, oxidized; sq, semiquinone (one-electron reduced); red, reduced (two-electron reduced).

Table 1: UV/Visible Spectral Properties of Wild-Type and Mutant Flavodoxins in the Oxidized Form[a]

	λ_{max} (nm)		λ_{min} (nm)		ϵ_{max} (M^{-1} cm^{-1})		
molecule	I	II	I	II	I	II	ϵ_I/ϵ_{II}
WT	463	374	406	315	9400	8600	1.10
Y94A	456	382	408	314	12200	9700	1.25
Y94F	459	*b*	*b*	313	11800	*b*	*b*
Y94W	451	375	400	320	10400	7700	1.34
Y94L	457[c]	377[c]	407[c]	314[c]	–	–	1.17[c]
W57A	460	373	407	315	9200	8600	1.07
W57F	460	373	404	315	9100	8800	1.03
W57Y	460	369	406	315	9700	9400	1.03
W57L	461	374	403	315	9100	8600	1.06
FMN	445	372	400	303	12200	10200	1.20

[a] Data from spectra recorded in 50 mM potassium phosphate, pH 7.0, at room temperature. [b] Atypical spectral features in this region (see Figure 2). [c] Excess apoflavodoxin present in the solution.

RESULTS

Absorption Spectra of Wild-Type and Mutant Flavodoxins. The main near-ultraviolet and visible absorbance properties of oxidized wild-type and mutant flavodoxins are shown in Table 1. Mutation of Trp57 consistently produced absorption spectra very similar to wild-type in both shape and extinction coefficients. In contrast, when Tyr94 was mutated, much more prominent differences were observed in the absorption spectra relative to wild-type. Replacement of Tyr94 by tryptophan (Figure 2) produced a spectrum with a broad absorption transition extending beyond 600 nm. The same feature has been described in the equivalent mutation of the *D. vulgaris* flavodoxin where it was attributed to a charge-transfer complex between the indole ring of the amino acid residue and the flavin [see Swenson and Krey (1994) and references cited therein]. In contrast to *D. vulgaris* flavodoxin, the Tyr94Trp mutant in *Anabaena* shows an enhanced extinction coefficient of transition I, relative to wild-type. Replacement of Tyr94 by phenylalanine also yielded an unusual flavin spectrum in *Anabaena* (Figure 2), which was not observed in *D. vulgaris* (Swenson & Krey, 1994). As in the case of the Tyr94Trp mutant, the extinction coefficient of transition I in the *Anabaena* Tyr94Phe mutant is enhanced relative to wild-type. The spectrum of Tyr94Leu, obtained in the presence of excess apoflavodoxin to ensure saturation of FMN (see *Dissociation Constants* below), is more featureless than wild-type or the other mutants (not shown). As for the Tyr94Ala mutant, the shape of the

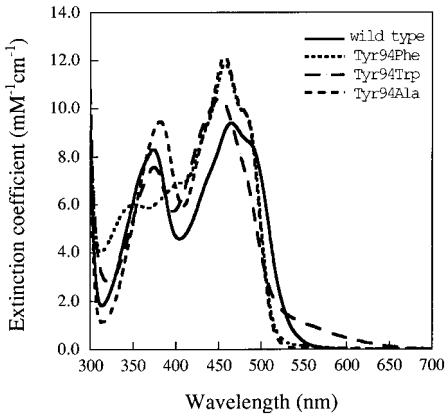

FIGURE 2: UV/Visible spectra of oxidized wild-type (continuous line), Tyr94Trp (long-dash line), Tyr94Ala (medium-dash line), and Tyr94Phe (short-dash line) flavodoxin mutants. The proteins (40 μM) were dissolved in 50 mM potassium phosphate, pH 7.0, and the spectra were recorded at 25.0 ± 0.1 °C.

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

protein	λ_{max} (nm) I	λ_{max} (nm) II	isosbestic points ox-sq (nm)	$\epsilon_{max\ I}$ (M^{-1} cm^{-1})
WT[b]	580	354	514, 351	5100
Y94A	577	353	475, 354	4800
	493			5100
Y94F	565	352	502, 368	5200
Y94W	577	351	500, 361	4500
	499			4500
Y94L	–	–	–	–
W57A	574	353	511, 356	5100
W57F	575	352	506, 356	4400
W57Y	576	353	510, 359	5400
W57L	575	353	511, 358	5300

[a] All spectra recorded in 50 mM potassium phosphate, pH 7.0, at 25.0 ± 0.5 °C. [b] Data from Pueyo et al. (1991). In 50 mM potassium phosphate pH 7.0, at 25 °C.

spectrum is qualitatively closer to wild-type than to free FMN, and yet the extinction coefficients of the two main transitions are clearly higher than in wild-type and very close to free FMN (Figure 2).

On partial reduction, all mutants populated the blue neutral semiquinone, which was stable in the absence of oxygen. The semiquinone visible spectra of all mutants of Trp57 (Table 2) are very similar to wild-type (Pueyo et al., 1991). Mutation of Tyr94, however, enhanced the absorption spectra around 500 nm (Figure 3) as it was described for mutations in *D. vulgaris* flavodoxin (Swenson & Krey, 1994). In fact, the Tyr94Trp and Tyr94Ala mutants display absolute maxima at around 500 nm (see Figure 3) which are not observed in wild-type.

Fluorescence Spectra of Wild-Type and Mutant Flavodoxins. The fluorescence emission of FMN bound to wild-type flavodoxin is very low (around 0.3% that of free FMN) as a result of strong quenching from the apoprotein. As Tyr94 and Trp57 are in contact with the isoalloxazine ring, mutation of these amino acids may change the fluorescence properties of flavodoxin. The emission fluorescence spectra in the visible region of wild-type and selected mutant flavodoxins are shown in Figure 4. Each spectrum has been corrected to subtract the small amount of free FMN that is present according to the dissociation constant (Table 4) of the corresponding mutant flavodoxin. This correction is very important especially for those mutants with weaker FMN binding than wild-type flavodoxin. Our data indicate that quenching of FMN fluorescence at position 57 is similarly exerted by the wild-type Trp residue, by Tyr, Phe, or Leu (not shown). Replacement of Trp57 by Ala, however, doubles the quantum yield of bound FMN relative to wild-type flavodoxin (0.7% of free FMN fluorescence). At position 94, the quenching is similar with the wild-type Tyr and with Phe (not shown), and decreases with Trp (0.9% of free FMN fluorescence) and especially with alanine (4% of free FMN fluorescence). A similar increase in fluorescence emission has been reported for the Tyr to Ala mutation in *D. vulgaris* flavodoxin.

Spectroscopic Characterization of the Mutant Apoflavodoxins. The spectroscopic properties of wild-type apoflavodoxin from *Anabaena* have been reported in detail (Genzor et al., 1996). We have studied the fluorescence emission in the near-UV region and the circular dichroism in the far-UV region of all the mutant apoproteins prepared for this work to check that no gross departure from the structure of wild-type apoflavodoxin has occurred. This is important to further discuss the binding equilibrium between mutant apoflavodoxins and FMN. The fluorescence emission spectra in the near-UV of wild-type and mutant apoflavodoxins are shown in Figure 5. Replacement of Tyr 94 causes minor changes in the spectrum. Since the Tyr94Trp mutant has five tryptophan residues and the wild-type protein only has four, the observed lack of increase in quantum yield in the Tyr94Trp mutant suggests that the introduced tryptophan is quenched in the apoprotein. Replacement of Trp57 consistently yield blue-shifted spectra, indicating, in agreement with the structure (Genzor et al., 1996), that Trp57 is an exposed tryptophan residue. The spectra of all apoflavodoxins with mutations at position 57 are similar in shape with small differences in quantum yield.

The far-UV circular dichroism spectra of wild-type and mutant apoflavodoxins (Figure 6) are almost identical. This indicates that the global fold of the protein is not affected by the mutations at positions 57 and 94. Analysis of the circular dichroism spectrum of wild-type apoflavodoxin in this region (not shown) indicates that it contains, besides the expected contributions from the α-helices and β-sheet in the protein, further contributions from aromatic side chains, as has been described for other proteins (Vuilleumier et al., 1993). The fact that all the mutations studied retained the shape of the spectrum indicates that the contribution from those aromatic side chains is also present in the mutant proteins.

Redox Potentials. Nerst plots corresponding to the redox potential determinations of seven *Anabaena* flavodoxin mutants are shown in Figure 7 (the redox potentials of Tyr94Leu could not be determined because the affinity of the apoprotein for the FMN is very low; see next section). The average slope of plots of E_2 was 62 ± 2 mV, and that of plots of E_1 was 60 ± 2 mV. During the titrations, considerably longer equilibration times were required to achieve stable reading of solution potentials for mutants at position 57 than for mutants at position 94. The midpoint redox potentials calculated from the Nerst plots are shown in Table 3. Two general trends are noticed. First, the alterations caused in E_1 (relative to wild-type flavodoxin) are more pronounced (from −26 to +137 mV) than the changes induced in E_2 (from 0 to +73 mV). Second,

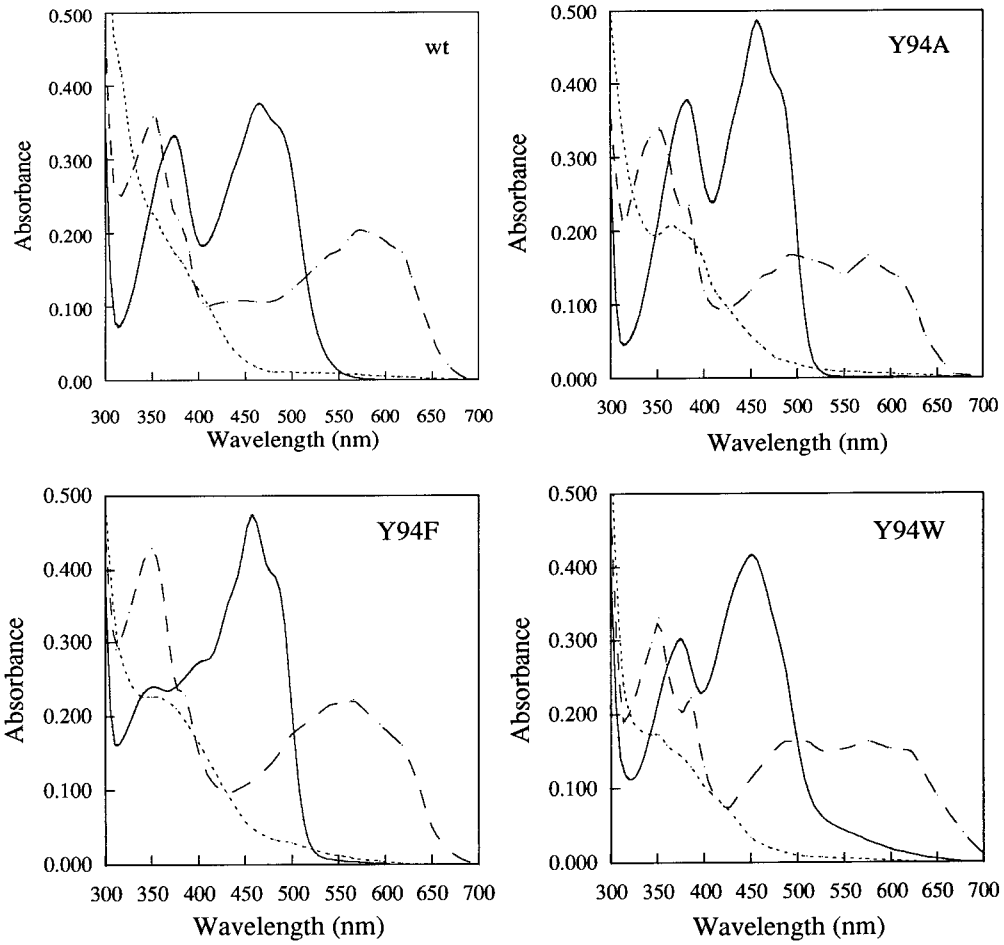

FIGURE 3: UV/Visible spectra of wild-type, Tyr94Ala, Tyr94Phe, and Tyr94Trp at different redox states: oxidized (continuous line), semireduced (long-dash line), and reduced (short-dash line). The proteins (40 μM) were dissolved in 50 mM potassium phosphate, pH 7.0, and the spectra were recorded at 25.0 $\pm$ 0.5 °C.

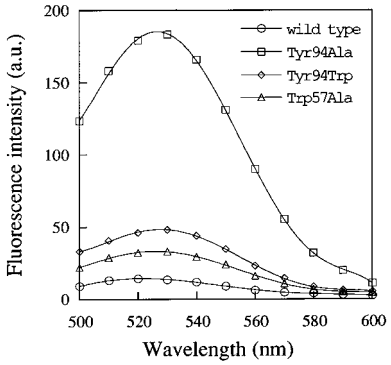

FIGURE 4: Fluorescence emission spectra of wild-type (○), Tyr94Ala (□) Tyr94Trp (◇), and Trp57Ala (△) in the visible region. The proteins (100 μM) were dissolved in 50 mM potassium phosphate, pH 7.0, and the spectra were recorded at 25.0 $\pm$ 0.1 °C. The dissociation constants of the apoflavodoxin−FMN complexes were used to calculate the amount of free FMN present in each solution, which was subtracted.

mutating Tyr94 produces bigger changes in E_1 than in E_2, but the opposite occurs when the mutated residue is Trp57. Mutation of Tyr94 to the more bulky and more electron-rich tryptophan makes E_1 more negative and E_2 more positive. When Tyr94 is replaced by the similarly bulky but less electron-rich phenylalanine, E_1 does not change, and E_2 becomes more positive. The largest change in redox potentials occurs when tyrosine is replaced by alanine. This leads to a much less negative E_1 with a basically unchanged E_2. As for the effects of mutating Trp57, when this residue is replaced by any of the other two aromatic residues (tyrosine and phenylalanine), E_1 varies little and E_2 becomes less negative. Replacement of Trp57 by the bulky apolar leucine makes E_1 less negative, leaving E_2 unaltered. Finally, when the tryptophan is replaced by alanine, both redox potentials became less negative.

Mutational studies of some of the amino acids in the FMN binding site of the homologous flavodoxin from *D. vulgaris* have been reported. Particularly relevant for our study are mutants of Tyr98 (Tyr98Trp, Tyr98Phe, and Tyr98Ala; Swenson & Krey, 1994) that is equivalent to Tyr94 in *Anabaena*, and mutant Trp60Ala (Mayhew et al., 1995), equivalent to Trp57Ala in *Anabaena*. Comparison of the redox potentials of these mutants and the corresponding mutants in *Anabaena* is presented in Table 3. A plot of redox potentials for equivalent mutants in the two species (Figure 8) shows that there is a fairly good correlation ($R = 0.91$, slope $= 0.88$) between the changes induced in E_1 by equivalent mutations. Correlation between the E_2 potentials is much poorer ($R = 0.19$, slope $= 0.18$). The different degree of correlation between the changes observed in E_2 and in E_1 in the two flavodoxins is due to the fact that while most mutations tend to yield less negative E_1 potentials in both flavodoxins (with the noticeable exception of the substitution of tyrosine by tryptophan) most mutations tend to make E_2 more negative in *D. vulgaris* flavodoxin but less negative in *Anabaena* flavodoxin. As a consequence, the

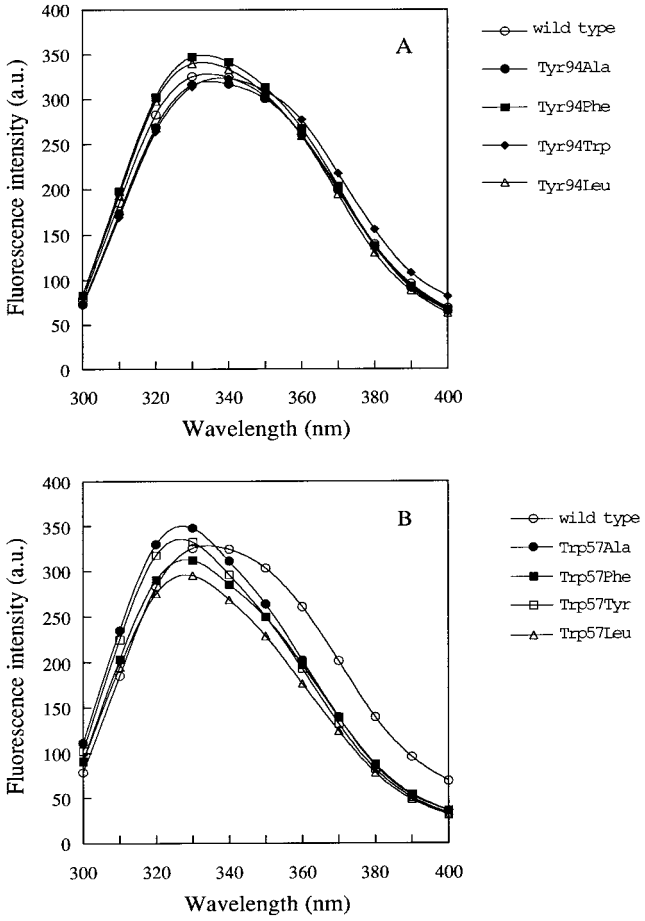

FIGURE 5: Fluorescence emission spectra of wild-type and mutant apoflavodoxins in the near-UV region. Proteins (2.4 μM) were dissolved in 50 mM potassium phosphate, pH 7.0. The spectra were recorded at 25.0 ± 0.1 °C. (A) Wild-type and mutants at position 94: wild-type (○); Tyr94Ala (●); Tyr94Trp (◆); Tyr94Phe (■); Tyr94Leu (△). (B) Wild-type and mutants at position 57: wild-type (○); Trp57Ala (●); Trp57Tyr (□); Trp57Phe (■); Trp57Leu (△).

64 mV difference between the E_2 potentials in wild-type *D. vulgaris* and *Anabaena* flavodoxins is reduced to an average of only 24 mV in the mutants.

Dissociation Constants. The dissociation constant of the oxidized FMN−apoflavodoxin complex has been determined for wild-type flavodoxin and the eight mutants (Table 4). All mutations decrease the affinity of apoflavodoxin for FMN. There is, however, a difference between replacing the tryptophan and replacing the tyrosine. All mutations at the tryptophan site decrease the strength of the complex by 2−3 kcal mol^{-1}, and, as a consequence, the four mutant proteins have the same affinity for FMN within 1 kcal mol^{-1}. In contrast, the effect of mutating the tyrosine on the affinity of the complex depends on the type of residue that is introduced. When tyrosine is mutated to an aromatic residue (either phenylalanine or tryptophan), the complex becomes about 2 kcal mol^{-1} weaker, but when the tyrosine is replaced by either leucine or alanine, marked decreases in the strength of the complex are observed. Replacement of Tyr94 by alanine lowers the affinity by 5 kcal mol^{-1}. This mutant protein is still purified as a holoflavodoxin. Replacement of Tyr94 by leucine lowers the affinity by as much as 8 kcal mol^{-1}. This mutant protein loses the FMN as the purification proceeds and is thus obtained essentially as the apoflavodoxin.

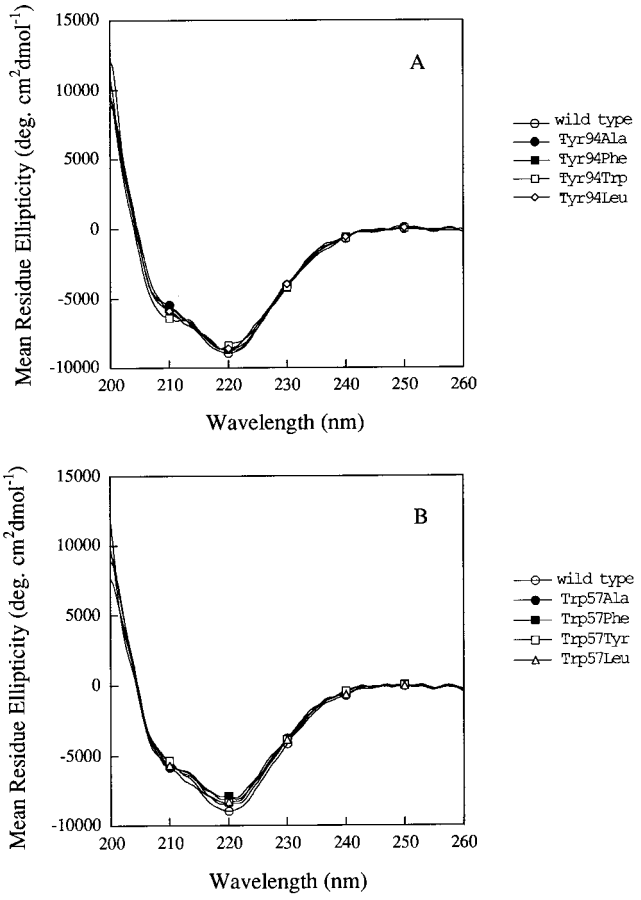

FIGURE 6: Far-UV circular dichroism spectra of wild-type and mutant apoflavodoxins. The proteins (0.9 μM) were dissolved in 50 mM potassium phosphate, pH 7.0, and the spectra were recorded at 25.0 ± 0.1 °C. (A) Wild-type and mutants at position 94: wild-type (○); Tyr94Ala (●); Tyr94Trp (□); Tyr94Phe (■); Tyr94Leu (◇). (B) Wild-type and mutants at position 57: wild-type (○); Trp57Ala (●); Trp57Tyr (□); Trp57Phe (■); Trp57Leu (△).

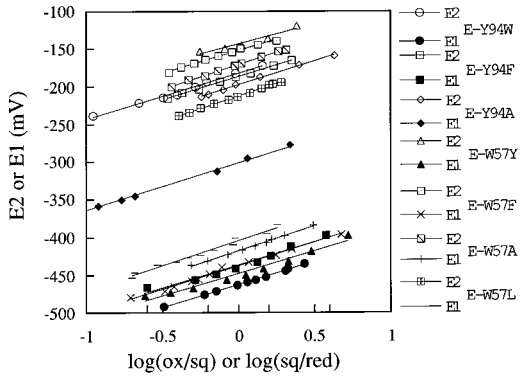

FIGURE 7: Nerst plots for the oxidized/semiquinone and for the semiquinone/reduced one-electron reductions of mutant flavodoxins. Tyr94Ala (◆, E_1; ◇, E_2); Tyr94Trp (●, E_1; ○, E_2); Tyr94Phe (■, E_1; □, E_2); Trp57Ala (+, E_1; slant in open squares, E_2); Trp57Tyr (▲, E_1; △, E_2); Trp57Phe (×, E_1; ⊡, E_2); Trp57Leu (−, E_1; + in open square, E_2).

The redox potentials of apoflavodoxin-bound FMN are linked to the binding affinities of the three FMN redox forms to apoflavodoxin by the thermodynamic cycles in Figure 9. If the redox potentials of wild-type and mutant flavodoxins and the binding constants of the corresponding oxidized FMN−apoflavodoxin complexes are known, the binding constants of the semireduced and reduced complexes can be easily derived.

Table 3: Midpoint Redox Potentials[a] of Wild-Type and Mutant Flavodoxins

protein (*Anabaena*)	E_2 (mV)	E_1 (mV)	protein (*D. vulgaris*)	E_2 (mV)	E_1 (mV)
wild-type[b]	−212	−436	wild-type[d]	−148	−443
Y94A[c]	−203	−299	Y98A[d]	−186	−304
Y94F[c]	−186	−436	Y98F[d]	−149	−414
Y94W[c]	−182	−462	Y98W[d]	−158	−452
W57A[c]	−173	−417	W60A[e]	−157	−357
W57L[c]	−212	−407	−	−	−
W57F[c]	−152	−437	−	−	−
W57Y[c]	−139	−447	−	−	−

[a] The estimated error for these determinations is ±5 mV. [b] Data from Pueyo et al. (1991). In 50 mM potassium phosphate, pH 7.0, at 25 °C. [c] Data from this study. All experiments in 50 mM potassium phosphate, pH 7.0, at 25.0 ± 0.5 °C. [d] Data from Swenson and Krey (1994). All data in 60 mM sodium phosphate, pH 7.0, at 25 °C. [e] Data from Mayhew et al. (1995) obtained in phosphate, pH 7.0.

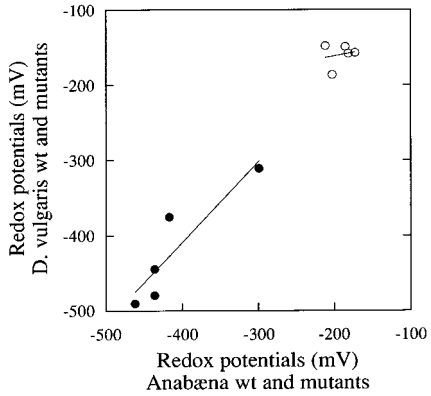

FIGURE 8: Comparison of *Anabaena* and *D. vulgaris* flavodoxin redox potentials. Each data point represents the values of E_1 (●) or E_2 (○) for *D. vulgaris* and *Anabaena* equivalent proteins. The data include wild-type, Tyr94(98)Ala, Tyr94(98)Trp, Tyr94(98)-Phe, and Trp57(60)Ala.

Table 4: Dissociation Constants of Oxidized FMN−Apoflavodoxin Complexes and Free Energy of the Corresponding Oxidized, Semireduced, and Reduced Complexes

protein	K_d (nM)[a]	ΔG_{ox} (kcal mol^{-1})[b]	ΔG_{sq} (kcal mol^{-1})[c]	ΔG_{red} (kcal mol^{-1})[c]
wild-type	0.043 ± 0.029[d]	−14.2 ± 0.4	−14.8 ± 0.5	−8.7 ± 0.6
Y94A	125 ± 35[e]	−9.4 ± 0.2	−10.2 ± 0.3	−7.3 ± 0.4
Y94F	1.9 ± 0.3	−11.9 ± 0.1	−13.1 ± 0.2	−7.0 ± 0.3
Y94W	0.79 ± 0.07	−12.4 ± 0.1	−13.7 ± 0.2	−7.0 ± 0.3
Y94L	26000 ± 3800	−6.2 ± 0.1	−	−
W57A	4.4 ± 0.4[e]	−11.3 ± 0.1	−12.8 ± 0.2	−7.1 ± 0.3
W57F	4.0 ± 0.4	−11.4 ± 0.1	−13.4 ± 0.2	−7.2 ± 0.3
W57Y	2.4 ± 0.3	−11.7 ± 0.1	−14.0 ± 0.2	−7.6 ± 0.3
W57L	9.9 ± 2.7	−10.9 ± 0.2	−11.5 ± 0.3	−6.1 ± 0.4

[a] Determined from fluorometric titrations of FMN with wild-type or mutant apoflavodoxins. All data in 50 mM potassium phosphate, pH 7.0, at 25 ± 0.1 °C. Errors provided by the fitting program unless otherwise indicated. [b] Calculated from data in *a*. [c] Calculated from data in *b* using eqs 2 and 3. The reported errors are those of ΔG_{ox} plus those derived from a ±5 mV uncertainty in the determined values of E_2 and E_1. [d] Mean of three experiments ± SD. [e] Mean of two experiments ± SD.

The free energy of the semireduced FMN−apoflavodoxin complexes (ΔG_{sq}) can be calculated from eq 2:

$$\Delta G_{sq} = \Delta G_{ox} - F(E_2 - E_2^{free}) \quad (2)$$

where ΔG_{ox} is the free energy of the oxidized FMN−apoflavodoxin complex, E_2 and E_2^{free} are the ox/sq midpoint potentials of bound FMN and free FMN, respectively, and F is the Faraday constant. Similarly, the free energy of the reduced FMN−apoflavodoxin complexes (ΔG_{red}) can be calculated from eq 3:

$$\Delta G_{red} = \Delta G_{ox} - F(E_2 + E_1 - E_2^{free} - E_1^{free}) \quad (3)$$

where E_1 and E_1^{free} are the sq/red midpoint potentials of bound FMN and free FMN, respectively, and the other terms have the same meanings as in eq 2.

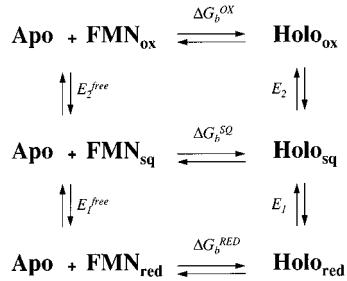

FIGURE 9: Thermodynamic cycles showing the relationship between the redox potentials of free and bound FMN with the affinity constants of the oxidized, semireduced, and fully reduced apoflavodoxin−FMN complexes.

The ΔG_{sq} and ΔG_{red} values calculated for wild-type and seven mutants are shown in Table 4. Six out of the seven mutations analyzed destabilize the oxidized complexes more than the semireduced complexes (the other mutation, Trp57Leu, destabilizes both complexes by the same extent). The most stable semireduced complex is that of the wild-type protein. There are five mutants that exhibit a similar affinity for semireduced FMN (from −14.0 to −12.8 kcal mol^{-1}) and two mutants (Trp57Leu and Tyr94Ala) that form less stable complexes (−11.5 and −10.2 kcal mol^{-1}, respectively). The most stable reduced complex is also that of wild-type apoflavodoxin. As for the mutants, the stabilities of the reduced complexes are quite similar, ranking from −7.0 to −7.6 kcal mol^{-1} (Trp57Leu is a little less stable: −6.1 kcal mol^{-1}). These data show that replacement of Tyr94 by alanine strongly destabilizes the oxidized and semireduced complexes (about 5 kcal mol^{-1}), and moderately destabilizes the reduced complex (about 1.5 kcal mol^{-1}). For the purpose of discussion, we have built, for wild-type and mutant flavodoxins, profiles of binding energy along reduction. These profiles (Figure 10) show how the binding energy of each FMN−apoflavodoxin complex varies as the complex becomes reduced.

DISCUSSION

Role of Tyrosine 94 in FMN Binding. Substitution of Tyr94 in *Anabaena* flavodoxin by either of the two other aromatic amino acids produced a limited change in redox potentials, and the same was found for the equivalent tyrosine residue in *D. vulgaris* flavodoxin (Swenson & Krey, 1994). This indicates that a tyrosine residue is not strictly required at this position for setting the characteristic redox potentials of apoflavodoxin-bound FMN. The fact that tyrosine is well conserved at this position among the different flavodoxins suggests, however, that it might play an additional role in its interaction with the flavin. Comparison of the binding energy profiles of wild-type and mutant flavodoxins (Figure 10) indicates that one such role is to provide tight cofactor binding throughout the catalytic cycle.

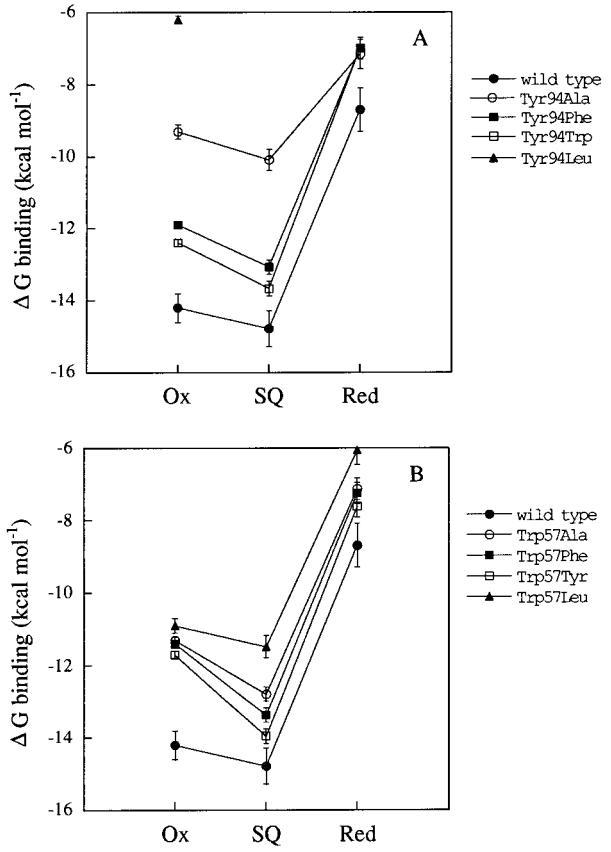

FIGURE 10: Binding energy profiles along reduction for wild-type and mutant FMN−apoflavodoxin complexes. (A) Wild-type and mutants at position 94: wild-type (●); Tyr94Ala (○); Tyr94Trp (□); Tyr94Phe (■); Tyr94Leu (▲). (B) Wild-type and mutants at position 57: wild-type (●); Trp57Ala (○); Trp57Tyr (□); Trp57Phe (■); Trp57Leu (▲). The binding energies of the oxidized complexes have been determined in 50 mM potassium phosphate, pH 7.0, by titration of FMN fluorescence with apoprotein at 25.0 ± 0.1 °C. The binding energies of the semireduced and reduced complexes have been calculated using the thermodynamic cycles in Figure 9.

Unlike ordinary cofactors such as nicotinamide nucleotides, flavin cofactors tend to remain bound to their associated apoproteins throughout the catalytic cycle. To achieve this, flavins display high binding affinities for the apoproteins [typical values for the binding energy of the complexes between wild-type apoflavodoxins and oxidized FMN are about 12−13 kcal mol^{-1} (Ludwig & Luschinsky, 1992)]. Since the redox potentials of bound FMN in a flavoprotein only depend on the precise shape of the $\Delta G_{ox}-\Delta G_{sq}-\Delta G_{red}$ binding energy profile (Figure 10), profiles of identical shape, and thus leading to identical redox potentials, may provide tighter or weaker binding of the cofactor. It is characteristic of flavodoxin that the semiquinone is moderately stabilized with respect to the oxidized form and that the reduced form is strongly destabilized relative to both the oxidized and the semiquinone forms (Figure 10). When the amino acid at position 94 is a tyrosine, the energy profile is downshifted along the energy axis, and the value of ΔG_{red} is still low enough so as to preclude dissociation of reduced FMN. But if the tyrosine is replaced by phenylalanine or tryptophan, although the shape of the energy profile is not drastically changed, it is significantly upshifted toward a region of weaker binding energies where the binding of reduced FMN to apoflavodoxin becomes a little too weak. Data in Table 4 indicate that in these cases the binding energies are close to typical values of nicotinamide nucleotide−enzyme complexes: 5−7 kcal mol^{-1} (Fersht, 1984; also close to the value of ΔG_{ox} in the mutant Tyr94Leu that tends to lose FMN during purification; see Results), and dissociation of reduced FMN from the apoprotein may more easily occur. In this respect, one role that Tyr94 performs better than the other aromatic residues at that position is to strongly bind all FMN redox forms so as to ensure that FMN is not released from the apoprotein when it is reduced. We thus propose that this tyrosine may be conserved among flavodoxins because it provides a tighter FMN binding than phenylalanine or tryptophan. At present we do not know whether the reason for this tighter binding can be attributed to the structure of the tyrosine residue or rather to constraints imposed by the protein scaffold. We could also think of a different reason for the tyrosine to be so strongly conserved: tyrosine could be better than the other aromatic residues at helping the electron transfer reactions of apoflavodoxin-bound FMN. This possibility, however, remains to be tested. Whatever the case, it is clear that either of the other two aromatic residues is capable of inducing the asymmetrical V-shape of the binding energy profile that is responsible for the redox potentials, and therefore the conservation of tyrosine is unlikely to be related to a unique role in the modulation of the redox potentials.

A Stabilizing Interaction between Tyrosine 94 and Reduced FMN. Of all mutations tested, the one that more drastically changes the value of E_1 is that of Tyr94 to alanine. A similar effect has been reported for the *D. vulgaris* Tyr98Ala mutant flavodoxin. For this protein, Swenson and Krey (1994) suggested that the functional role of the equivalent Tyr98 is to substantially destabilize the flavin hydroquinone anion, but our data on *Anabaena* flavodoxin do not support this interpretation. In fact, we calculate from a thermodynamic cycle (Figure 9) that reduced FMN is bound by *Anabaena* wild-type apoflavodoxin more tightly than by the mutant where the tyrosine has been replaced by alanine (see Table 4 and Figure 10). The presence of a tyrosine (instead of alanine) at position 94 in *Anabaena* flavodoxin thus seems to stabilize the complex between the apoprotein and reduced FMN. Does this imply that the interaction between the tyrosine ring and the flavin is energetically favorable? Not necessarily. There are two issues, one general and one specific to our system, that need to be addressed before we can answer the above question. The general issue is that when a mutation is introduced in a protein the changes induced are not always local. Fortunately the NMR structure of the *D. vulgaris* mutant Tyr98Ala has been determined (Stockman et al., 1994), and it shows that this mutation yields a protein essentially identical to wild-type, except for the lack of the phenolic tyrosine ring. As for the *Anabaena* Tyr94Ala mutant the fluorescence and circular dichroism spectra of the apoprotein (Figures 5 and 6) are almost identical to those of the wild-type apoprotein, suggesting that the mutation does not alter the structure of the protein. But there is still another issue, specific to flavodoxin, that could complicate the interpretation of the binding energy data. When a small molecule binds to an "open" protein binding site, formation of the complex is mainly accompanied by the making of new bonds. If, however, the binding site is "closed", and it has to become more accessible to allow the binding of the ligand, then a number of internal protein bonds must be broken to allow complex formation. The latter is

the case for flavodoxin (at least for the flavodoxin from *Anabaena*). The X-ray structure of wild-type *Anabaena* apoflavodoxin (Genzor et al., 1996a) shows that Tyr94 interacts with Trp57, so closing the isoalloxazine binding site. In *Anabaena* apoflavodoxin, before the FMN can get in its place, the interaction between the two aromatic protein residues must be broken, and may certainly affect the affinity of the complex between apoflavodoxin and FMN. In the mutant Tyr94Ala, the aromatic interaction between the tyrosine and the tryptophan residue is already broken, and then (other things being equal) the binding of FMN (in any of the redox forms) would be stronger than in wild-type. Despite this effect, the complex between reduced FMN and the apoprotein with alanine at position 94 is weaker than the wild-type−FMN complex. We do not know at present the strength of the Tyr94/Trp57 pair in apoflavodoxin. The strength of a similar tyrosine−tyrosine pair present in the ribonuclease barnase was determined to be around 1 kcal mol^{-1} (Serrano et al., 1991). If this value is representative of the Tyr94/Trp57 interaction in apoflavodoxin, then the binding of FMN to Tyr94Ala apoflavodoxin, could be favored up to 1 kcal mol^{-1} in all redox states (relative to wild-type) because such interaction is not present in the apoprotein. The effect could, nevertheless, be smaller than that because, upon mutation, a protein tends to undergo a small reorganization in order to maximize its stability. It thus seems reasonable that the effect of structural differences in the apoproteins on the affinity of their complexes with FMN will be small. With this assumption, the differences in the binding energy profiles of the flavodoxins (Figure 10) should be attributed to differences in the interactions formed between the amino acids at position 94 and the isoalloxazine rather than to a different disruption of structure in the apoproteins on complex formation. The fact that ΔG_{red} in Tyr94Ala is less negative than in wild-type thus implies that the interaction between the tyrosine ring and the flavin is energetically favorable. If we assume that the effect of the Tyr/Trp interaction on the strength of the FMN−apoflavodoxin complexes is negligible, we calculate (from data in Table 4) that the energy of interaction between Tyr94 and reduced FMN in flavodoxin is −1.4 kcal mol^{-1}. If, however, we attribute to the Tyr/Trp interaction a weakening effect on the wild-type−FMN complex (of 1 kcal mol^{-1}), then the energy of interaction between the Tyr94 and reduced FMN in flavodoxin would be −2.4 kcal mol^{-1}. Whatever the case, Tyr94 stabilizes the reduced form of FMN.

Relative Destabilization of Reduced FMN by Tyrosine 94. If replacement of Tyr94 by alanine does not stabilize the reduced complex, why then does E_1 become much less negative in the alanine mutant? Since the value of E_1 depends on the difference in stability between the semireduced and reduced apoflavodoxin−FMN complexes, the available structural data on the different redox forms of flavodoxin (Watt et al., 1991; Ludwig et al., 1976) are of great interest. These data suggest that the shape of reduced and semireduced FMN is the same when bound to the protein and that the same FMN−protein bonds are present in the two redox forms. Comparison of the ΔG_{sq} in wild-type (also in Tyr94Trp and Tyr94Phe) with the ΔG_{sq} in Tyr94Ala indicates that Tyr94 (and the other aromatic residues at this position) strongly stabilizes the semireduced form of FMN. This is reasonable because Tyr94 makes extensive van der Waals contacts with the isoalloxazine ring, because on stacking onto the isoalloxazine there is a substantial burying of hydrophobic surface and because the off-set geometry of the stacking is energetically favorable. It is important to realize that all of these considerations also apply to the complex of apoflavodoxin and reduced FMN, and therefore they must also contribute to strongly stabilize the interaction between the tyrosine and reduced isoalloxazine. Why then is ΔG_{red} only moderately more negative in wild-type (and almost identical in Tyr94Trp and Tyr94Phe) than in Tyr94Ala where all these interactions are absent? The obvious answer is that there must be other effects that are counterbalancing the aforementioned stabilizing ones. Several possible causes of destabilization of reduced FMN in complex with apoflavodoxin have been proposed. One theory is based on the assumption that the most stable conformation of reduced FMN is nonplanar and that, since it is forced to be planar when interacting with some apoproteins (such as apoflavodoxin), it becomes destabilized with respect to the semireduced and oxidized forms that tend to be planar (Ludwig et al., 1976). This theory is supported by theoretical calculations (Hall et al., 1987; Vázquez et al., 1992; Zheng & Ornstein, 1996) but contested by NMR studies in water that indicated that reduced FMN is almost planar, this geometry being attributed to interaction with polar solvent molecules (Moonen et al., 1984). We note, however, that the protein interior might fail to exert a similar effect, and then the nonplanar conformation of bound reduced FMN could still be the more stable one. A second theory is based on the observed anionic character of apoflavodoxin-bound reduced FMN (Franken et al., 1984). This anion can be destabilized by the many negative charges near the FMN binding site that are found in flavodoxin as has been elegantly demonstrated by Zhou and Swenson (1995) in *D. vulgaris* flavodoxin. The protein-bound anionic hydroquinone could also be destabilized by being in an apolar environment where stabilizing interactions with water molecules are not possible. Swenson and Krey (1994) attribute to an increase in solvent exposure, on removal of the aromatic ring of tyrosine, the change in E_1 observed in *D. vulgaris* Tyr94Ala that is also evident in *Anabaena* Tyr98Ala. This is a reasonable explanation that is supported by structural data confirming a higher degree of FMN solvent exposure in the *D. vulgaris* mutant Tyr94Ala (Stockman et al., 1994). The increase in solvent exposure of FMN has also been detected by fluorescence in both the *D. vulgaris* and *Anabaena* tyrosine to alanine mutants. The possibility that a stressed conformation of reduced FMN could also be related to the change in E_1 observed in the alanine mutants cannot, however, be abandoned yet because in these mutants reduced FMN would be less constrained and upon reduction the flavin could relax to a more stable conformation without being kept planar by the tyrosine present in wild-type.

As for E_2, all mutants analyzed make it less negative than in the wild-type protein. Relative to alanine, Tyr94 makes E_2 slightly more negative because it stabilizes oxidized FMN a little more than the semiquinone. The two other aromatic residues make E_2 20 mV less negative than in the alanine mutant because they stabilize the semiquinone slightly more than oxidized FMN. Since E_2 in *Anabaena* flavodoxin is pH-dependent (Paulsen et al., 1990), any change of the relevant pK_a upon mutation would contribute to the observed differences in E_2. In *D. vulgaris* flavodoxin, the three aromatic residues make E_2 less negative relative to alanine

indicating that they preferentially stabilize the semiquinone relative to the oxidized form of FMN. The effect of the tyrosine on E_2 of *Anabaena* flavodoxin is thus different from its effect in *D. vulgaris*, but at present the physical reasons for this difference remain unknown.

The influence of tyrosine on the binding of FMN and its redox potentials can be summarized as follows. On the one hand, the tyrosine destabilizes the reduced complex relative to the semireduced and oxidized complexes, and this contributes to tailoring the binding energy profile that is responsible for the very low E_1 of flavodoxins. On the other hand, the tyrosine strongly interacts with all redox forms (probably by a combination of van der Waals interactions, offset aromatic−aromatic interactions, and the hydrophobic effect), thus shifting the entire energy profile of the complex toward a more stabilizing region, so opposing the release of reduced FMN. Since either of the two other aromatic residues seems to be able to tailor the binding energy profile as conveniently as tyrosine, the conservation of tyrosine may be related to its more efficient shifting of the binding energy profile that ensures a tighter binding of reduced FMN throughout the catalytic cycle.

Role of Tryptophan 57 in FMN Binding and in the Modulation of the Redox Potentials. Substitution of Trp57 by either tyrosine or phenylalanine yields flavodoxins with E_1 potentials very similar to that of the wild-type protein. This implies that the very negative E_1 potential in flavodoxins is not specifically related to the presence of a tryptophan at position 57 because any of the aromatic residues gives rise to similarly low potentials. Replacement of Trp57 by an alanine has a much less pronounced effect than the analogous replacement at position 94. An alanine at position 57 makes E_1 only 20 mV less negative than a tryptophan, which means that position 57 is less important than position 94 in setting E_1 at its characteristically low value. Mutation to alanine of the equivalent *D. vulgaris* flavodoxin Trp60 has been reported to exert a profound change in E_1 (Mayhew et al., 1995), in contrast to *Anabaena* flavodoxin. For *Anabaena* flavodoxin, the largest change in E_1 has been observed for the Trp57Leu mutant, but the change is only 30 mV. It thus seems that at position 57 the three aromatic residues are equivalent with respect to the value of E_1 exhibited by *Anabaena* flavodoxin and that replacing the naturally occurring tryptophan by smaller residues such as leucine or alanine does not drastically alter E_1.

The pertinent question is then the following: Why is a tryptophan preferred at position 57 rather than tyrosine or phenylalanine? For *Anabaena* flavodoxin, we can offer the same reason as in the case of position 94: tryptophan at position 57 shifts the energy profile downward (see Figure 10), making the binding of the hydroquinone tighter, so opposing the release of reduced FMN. We have observed that mutations at position 57 seem to affect E_2 more than E_1. Comparison of the E_2 values in wild-type and mutants at position 57 offers a second reason for preferring tryptophan at that position. Replacement of Trp57 by either tyrosine of phenylalanine makes E_2 some 60−70 mV less negative, which is a substantial departure from the wild-type value. The basis for this effect is that mutating the tryptophan to any of the other aromatic residues destabilizes the semireduced complex by around 1 kcal mol^{-1} while the oxidized complex is destabilized by around 2 kcal mol^{-1}. Tryptophan thus seems better suited than either tyrosine or phenylalanine to stabilize oxidized FMN relative to the semiquinone. Interestingly, the Trp57Phe and Trp57Tyr *Anabaena* mutants exhibit E_2 values very similar to those of wild-type *D. vulgaris* flavodoxin. It could be that, unlike *Anabaena* Trp57, *D. vulgaris* Trp60 does not stabilize oxidized FMN relative to semireduced FMN.

The V-shaped binding energy profile of flavodoxin, that determines its characteristic redox potentials, shows that the complex between apoflavodoxin and semireduced FMN is more stable than the complex with oxidized FMN. Does the tyrosine or the tryptophan in contact with the isoalloxazine ring play a role in establishing this difference? Supporting this possibility, a model system study by Draper and Ingraham (1970) showed preferential binding of tyrosine for semireduced FMN relative to oxidized FMN. We have, however, analyzed the effects of mutating the two aromatic residues flanking the FMN in *Anabaena* flavodoxin, and the difference in binding energy of the complexes formed between the apoprotein and these two redox forms of FMN has not disappeared in any of the mutants. It thus seems that none of the aromatic residues flanking the isoalloxazine ring is responsible for the higher stability of the semireduced complex relative to the oxidized one. The difference in stability must then be connected to interactions established by the FMN with other residues of apoflavodoxin. An obviously good candidate is the hydrogen bond that is formed between the FMN and the protein backbone when FMN becomes semireduced (Smith et al., 1977; Watt et al., 1991). The average semiquinone stabilization, relative to the oxidized complex in wild-type and the seven *Anabaena* mutants reported in this study, is 1.3 ±0.6 kcal mol^{-1}, which is consistent with the contribution of buried hydrogen bonds to protein stability as measured by site-directed mutagenesis (Fersht, 1987).

In summary, Trp57 seems to strengthen the interaction of apoflavodoxin with FMN in all its redox forms, thus helping to retain reduced FMN bound without altering much the redox potentials. In *Anabaena* flavodoxin, Trp57 destabilizes the semiquinone complex relative to the oxidized complex, but this might not be a general role exerted by equivalent tryptophans in other flavodoxins. Finally, we have noticed that reduction of mutants lacking Trp57 was generally slow. This, together with the fact that Trp57 directly interacts with the methyl groups of the isoalloxazine ring that might be the entry point of electrons into FMN [see Mayhew and Tollin (1992)], suggests that Trp57 might play a role in the kinetics of flavodoxin electron transfer reactions.

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