Involvement of Serine 96 in the Catalytic Mechanism of Ferredoxin-NADP$^+$ Reductase: Structure−Function Relationship As Studied by Site-Directed Mutagenesis and X-ray Crystallography[†,⊥]

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Received January 24, 1995; Revised Manuscript Received April 24, 1995[⊗]

ABSTRACT: The crystal structure of ferredoxin-NADP$^+$ reductase (FNR) suggests that Ser96 is directly involved in hydride transfer between the isoalloxazine moiety of FAD and the nicotinamide ring of NADP(H). To probe its role, Ser96 has been mutated to valine (S96V) and glycine (S96G). These mutations primarily affected the interaction of the nicotinamide ring with the flavin. Absorbance, fluorescence, and circular dichroism spectra and the crystal structure of FNR-S96V indicate that this mutant folds properly. FNR-S96V shows only 0.05% of wild-type activity, while the affinities for both ferredoxin and NADP$^+$ are virtually unchanged. However, spectral perturbations induced by NADP$^+$ binding to FNR-S96V strongly resemble those elicited by the binding of 2′-monophosphoadenosine−5′-diphosphoribose, a substrate analog lacking the nicotinamide ring, both to the mutant and wild-type enzymes. Rapid reaction studies on the valine mutant failed to detect charge-transfer intermediates during flavin reduction by NADPH. In addition, no semiquinone formation was seen during photoreduction of FNR-S96V. The three-dimensional structure of the valine mutant shows small, albeit definite, changes only in the isoalloxazine microenvironment. The glycine mutant of FNR displays behavior intermediate between that of wild-type enzyme and that of the valine mutant. It maintains *ca.* 2% of the wild-type activity as well as the ability to form the charge-transfer species between reduced FNR and NADP$^+$. In photoreduction experiments, the same degree of flavin semiquinone stabilization was observed with FNR-S96G and with the wild-type enzyme. NADP$^+$ binding to the glycine mutant was very similar to that observed in the case of the valine mutant. Thus, these mutations of Ser96 clearly interfere with the proper binding of the nicotinamide and with the stabilization of the transition state during hydride transfer between nicotinamide and FAD. Furthermore, both mutations seem to alter the redox properties of FAD, leading to either destabilization of semiquinone (FNR-S96V) or stabilization of the reduced flavin (FNR-S96G).

Ferredoxin-NADP$^+$ reductase (FNR,[1] EC 1.18.1.2) is the prototype of a large family of flavin-dependent oxidoreductases (more than 20 different enzymes) that function as transducers between nicotinamide dinucleotides (two-electron carriers) and one-electron carriers (Karplus et al., 1991; Correll et al., 1993; Karplus & Bruns, 1994). All of the members of the family display, as a basic module, the two-domain motif of FNR, to which other domains can be added. Thus, an understanding of the mechanism of electron transfer in FNR could be of more general interest, although the very important physiological function of the enzyme itself makes it worthy of attention. FNR is the flavoenzyme responsible for storage, as reducing equivalents in NADPH, of the light energy captured through the photosynthetic process in plants and cyanobacteria (Carrillo & Vallejos, 1987; Zanetti & Aliverti, 1991). The sequences of the enzyme from seven different sources are now available, and the three-dimensional structure of the spinach enzyme has been refined to 1.7 Å (Karplus & Bruns, 1994; Bruns & Karplus, 1995). A role for a few residues in the catalytic mechanism has been suggested in the past by chemical modification studies (Carrillo & Vallejos, 1987; Zanetti & Aliverti, 1991). More recently, the powerful tool of site-directed mutagenesis in

[†] This work was supported by grants from Consiglio Nazionale delle Ricerche target project "Biotechnologies and Bioinstrumentations", from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (60%), and from the National Science Foundation (MCB 9112699).

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[⊥] Coordinates have been deposited in the Brookhaven Protein Data Bank under the filename 1FRN.

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[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1995.

[1] Abbreviations: FNR, ferredoxin-NADP$^+$ oxidoreductase; FNR_{ox}, oxidized ferredoxin-NADP$^+$ oxidoreductase; FNR_{red}, reduced ferredoxin-NADP$^+$ oxidoreductase; Fd, ferredoxin; INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride; 2′-P-ADP−ribose, 2′-monophosphoadenosine−5′-diphosphoribose; 5-deazariboflavin, 5-carba-5-deazariboflavin.

0006-2960/95/0434-8371$09.00/0

conjunction with X-ray crystallography has allowed us to probe more directly the bearing of protein structure on enzymatic catalysis.

We have cloned and expressed in *Escherichia coli* the spinach FNR (Aliverti et al., 1990), and by site-directed mutagenesis we have succeeded in defining a role for several residues of the active center. Lys116 was found to be involved in the formation of the Michaelis complex with NADP(H). The FNR-K116Q mutant exhibited a 50-fold increase in K_m for NADPH and a 10-fold decrease in k_{cat}, with respect to the wild-type enzyme. This suggests that the modification of Lys116 might affect the orientation of the nicotinamide ring (Aliverti et al., 1991). The role of Cys272, a residue conserved in all of the members of the FNR family and positioned near the isoalloxazine ring of the flavin (Karplus et al., 1991), was probed by changing it to serine (Aliverti et al., 1993). It was shown that Cys272 is required for productive interaction of the nicotinamide ring of $NADP^+$ with the flavin (*i.e.*, correct orientation of the two rings) to facilitate hydride transfer between C_4 of the nicotinamide and N_5 of the isoalloxazine. Tyr314, the side chain of which covers the *re* face of the isoalloxazine (Karplus et al., 1991), has been shown in the pea enzyme by Orellano et al. (1993) to be important, but not essential for enzyme activity, since it can be replaced by tryptophan or phenylalanine with only a 50% decrease in k_{cat}. This residue is present as either Tyr, Phe, or Trp in most FNR family members (Karplus et al., 1991). As can be seen from the crystal structure of FNR (Karplus et al., 1991), Ser96 occupies a strategic position in the active center of FNR. This residue interacts through the amide nitrogen and the side chain with the N_5 position of the isoalloxazine. It is a conserved residue in all known FNR's, and occurs as serine or threonine in all of the FNR family members (Karplus et al., 1991; Taylor et al., 1993).

Alteration of Ser96 might be expected to produce significant changes in the rate of hydride transfer between NADP(H) and flavin (Karplus & Bruns, 1994). We therefore planned to replace Ser96 with glycine, alanine, or valine. These changes remove the side-chain hydroxyl group and its polarity and hydrogen-bonding capabilities, with minimal structural change. The larger valine substitution was chosen because the smaller side chains of glycine and alanine might permit a water molecule to fit into the resulting hole, which would provide structural and functional replacement for the missing hydroxyl group. Although we were unable to produce the alanine mutant (S96A), we present here the properties of the S96V and S96G mutants and the three-dimensional structure of the S96V mutant. We combine data from steady-state and rapid kinetics with information coming from the structural analysis of the FNR-S96V enzyme to rationalize the behavior of the mutants.

EXPERIMENTAL PROCEDURES

Horse heart cytochrome *c*, INT, NADP(H), and 2′-P-ADP-ribose were obtained from Sigma. Factor X_a was from Pierce. Restriction endonucleases and T4 DNA ligase were purchased either from Gibco BRL or Boehringer Mannheim. All other chemicals were of analytical grade.

Oligonucleotide-Directed Mutagenesis. Site-directed mutagenesis of the FNR gene construct was carried out using the phosphorothioate-based method (Sayers et al., 1988), with the "Sculptor in vitro mutagenesis system" by Amersham International. The single-stranded template was obtained by recloning the wild-type FNR gene in M13mp9 as described (Aliverti et al., 1991). The sequences of the three synthetic oligonucleotides used for mutagenesis are reported in Table 1. The presence of the desired mutation and the lack of second-site mutations were confirmed by sequence analysis of the entire mutagenized gene. DNA sequencing was performed according to the chain termination method (Sanger et al., 1977) using single-stranded M13mp9 templates and the T7 sequencing kit by Pharmacia Biotech.

Table 1: Nucleotide Sequences of Synthetic Oligonucleotides Used for Mutagenesis

mutation	synthetic primer[a]	codon change
S96V	TTGAGATTGTACGTTATCGCCAGCAG	TCG→GTT
S96G	GTTGAGATTGTACGGTATCGCCAGCAGTG	TCG→GGT
S96A	TGAGATTGTACGCGATCGCCAGC	TCG→GCG

[a] Base changes are underlined.

Expression and Purification of the Mutant FNR Forms. The fragments carrying the two mutated FNR genes (*i.e.*, FNR-S96G and FNR-S96V) were recloned in the expression vector pMAL-c (New England Biolabs), which directs the synthesis of fusion proteins where the protein of interest is linked to the C-terminus of the *E. coli* maltose-binding protein. The resulting expression plasmids, namely, pMBPFNR-S96G and pMBPFNR-S96V, were found to support much higher FNR expression than that obtained with previous constructions based on the pDS12 vector (Aliverti et al., 1994). Wild-type and mutant enzymes were purified from *E. coli* (host strain RRIΔM15) according to a combination of described procedures (Aliverti et al., 1990, 1991, 1994). After hydrophobic interaction chromatography, the FNR fusion proteins were further purified by FPLC on a NeoBar AQ4 column (quaternary ammonium-based exchanger, Dyno Particles A.S., Norway). Proteolytic cleavage was then carried out overnight at 12 °C with factor X_a at a mass ratio of 1:1000 with respect to the fusion protein. The final purification of the processed FNR forms was accomplished by phosphocellulose chromatography. A detailed description of the preceding procedure will be published elsewhere (A. Aliverti et al., manuscript in preparation).

Spectral Analyses. Absorption spectra were recorded with a Hewlett-Packard diode array 8452A spectrophotometer interfaced to a Hewlett-Packard 89500A ChemStation. Derivative spectra were computed by means of the Hewlett-Packard 89510A general scanning software. The extinction coefficient of the peak near 460 nm due to protein-bound flavin was determined by resolving the FAD from the apoprotein and spectrophotometrically quantitating the released FAD. Enzyme solutions (20−30 μM) in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA were used to obtain the spectra of the FNR forms. Protein samples were denatured by adding sodium dodecyl sulfate to 0.2% final concentration. FAD release from FNR was complete within a few minutes at 25 °C. Circular dichroism was performed on a Jasco J-500A CD spectrometer, courtesy of Dr. G. Vecchio (CNR, Milano). Proteins were diluted to about 50 μM in 50 mM Tris-HCl (pH 7.4). Spectra were recorded at 22 °C in 1 cm path cuvettes at a scan rate of 5 nm/min. Protein

and flavin fluorescence was monitored on a Jasco FP-777 spectrofluorometer.

FNR Photoreduction. Photoreduction of bound flavin was performed at 15 °C in an anaerobic cuvette containing 12−20 μM FNR samples in 10 mM HEPES (pH 7.0) in the presence of 15 mM EDTA and 1.2−1.5 μM 5-deazariboflavin. Reaction solutions were made anaerobic by successive evacuation and flushing with O_2-free N_2. Absorption spectra were recorded before and after successive periods of irradiation with a 150 W light source.

Steady-State Kinetic Measurements. Steady-state kinetics was performed as previously described (Aliverti et al., 1993).

Stopped-Flow Kinetic Measurements. Rapid-reaction kinetics was studied with a Hi-Tech SF-61 stopped-flow spectrophotometer interfaced with a MacIntosh computer using the KISS program (Kinetic Instruments, Inc). Hydride transfer between NADPH and oxidized FNR forms was studied at various NADPH concentrations and at different temperatures, as already reported (Aliverti et al., 1993).

Determination of the Dissociation Constant of the Enzyme/Ferredoxin Complexes. The K_d values of the complexes of the wild-type and the mutant enzymes with oxidized Fd were determined by titrating the reductase protein fluorescence with Fd in 20 mM HEPES (pH 7.0) at 15 °C, essentially under the same conditions as reported by Aliverti et al. (1994). Experimental data were fitted to the theoretical equation for a 1:1 binding stoichiometry, as described in Wang et al. (1992), by means of nonlinear regression using the program GraFit (Erithacus Software Ltd., UK).

Determination of the Dissociation Constant of the Enzyme/NADP$^+$ Complexes. To measure the K_d values of the complexes of the FNR forms with pyridine nucleotide substrate, the proteins were spectrophotometrically titrated with NADP$^+$. FNR samples were diluted in 10 mM Tris-HCl (pH 7.7) (at 15 °C) to a final concentration of 15−20 μM. Absorption spectra were recorded before and after successive additions of NADP$^+$. Difference spectra were computed by subtracting from each spectrum the spectrum recorded in the absence of ligand, corrected to account for dilution. The detection of the spectral perturbation elicited by NADP$^+$ binding was substantially improved in terms of sensitivity and selectivity by converting the difference spectra into their first derivatives. Data at several different wavelengths were fitted as described for Fd.

Determination of the Difference Spectra of the Enzyme/2′-P-ADP−Ribose Complexes. FNR samples were diluted in 20 mM Tris-HCl (pH 7.7) (at 15 °C) to a final concentration of about 20 μM. Three successive additions of 2′-P-ADP−ribose were then made (equimolar, 5-fold excess, and 50-fold excess over FNR) to ensure saturation conditions. Difference spectra were computed as before.

X-ray Crystallography. Crystals of the FNR-S96V grew under the same conditions as those used to solve the structure of natural spinach FNR (Karplus et al., 1991). Crystals of natural enzyme and FNR-S96V were isomorphous and belonged to space group $C2$, with unit cell parameters a = 90.7 Å, b = 57.7 Å, c = 68.1 Å, and β = 100°. Under identical crystallization conditions, protein of FNR mutants S96G, C272S, and C132S failed to yield diffraction-quality crystals. The FNR-S96V crystals were less well formed than those of natural FNR, but a few diffraction-quality crystals were grown. The largest of these measured 600 × 300 × 200 μm^3. Diffraction data to 2.0 Å resolution were collected on a San Diego Multiwire Systems multiwire area detector (Hamlin, 1985) at Cornell University. A similar FNR-S96V crystal was soaked for 6 h in artificial mother liquor supplemented with 50 mM NADP$^+$ prior to data collection, which lasted 24 h. The data were merged by using the computer program SCALEPACK (Z. Otwinowski, University of Texas at Dallas), and crystallographic refinement was performed by using the refinement program TNT version 5a (Tronrud et al., 1987). The structure of the protein purified from spinach leaves was used as a starting model, with residue 96 changed from serine to valine and residue 269 changed from valine to phenylalanine (residue 269 happens to be different in the cloned FNR gene; Jansen et al., 1988), using the crystallographic modeling program CHAIN (Sack, 1988). These modified residues were initially placed in conformations consistent with the difference Fourier electron density calculated between the crystal forms. The FNR-S96V mutant structure (without NADP$^+$) was refined for 20 cycles of conjugate gradient minimization to a final R-factor of 16.2% for all data between infinity and 2.0 Å resolution. The rms deviation from ideality was 0.015 Å for bond lengths and 2.3° for bond angles.

RESULTS

Expression and Purification of the FNR Mutants. Although we originally planned to produce three mutants of spinach FNR in which Ser96 would be replaced with glycine, alanine, or valine, we failed in obtaining positive clones from the Ser to Ala mutagenesis. FNR-S96G and FNR-S96V were expressed as fusion proteins at the C-terminus of the *E. coli* maltose-binding protein. Both mutant fusion proteins were synthesized in *E. coli* at a lower level than the wild-type fusion protein (*ca.* 2.5 and 5 mg per g of fresh weight cells, for the mutant and the wild-type FNR forms, respectively) and required an additional anion-exchange column compared to wild type to obtain mutant fusion proteins pure enough to allow efficient proteolytic splitting. After cleavage by factor X_a to restore the correct N-terminus and phosphocellulose chromatography, the two FNR mutants were obtained in homogeneous form in amounts suitable to carry out demanding characterization studies, such as stopped-flow kinetics and crystallization.

Spectral Properties. The near-ultraviolet and visible absorbance spectra of the oxidized form of the wild-type and mutant FNRs are shown in Figure 1A. Alteration of the side chain of the amino acid residue at position 96 results in small but significant changes in the absorbance properties of the flavin prosthetic group. In the mutants, the absorbance maxima of both transitions I and II of the flavin (in the 450 and 380 nm regions, respectively; Müller, 1991) were red shifted by 2−6 nm relative to wild type. Furthermore, the extinction coefficient of transition II (in the 380 nm region) increased markedly in going from Ser to Gly to Val enzyme forms. The second-derivative spectra (Figure 1B) more clearly show the positions of the maxima of the transition components, which correspond to the troughs in the second-derivative spectra (Butler, 1972). The changes in transition II are much more evident than those in transition I, both qualitatively and quantitatively. The circular dichroism spectrum of FNR-S96V in the visible wavelength region is compared with that of wild-type enzyme in Figure 1C. There is a red shift in the peak positions, which correlates with that found in the absorption spectrum (Figure 1A,B). The

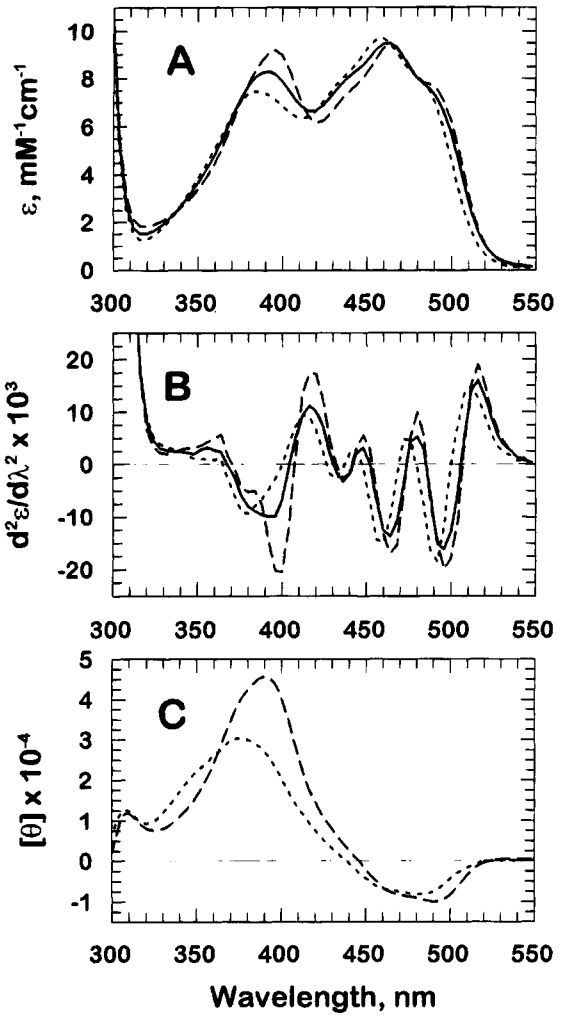

FIGURE 1: Absorbance and circular dichroism spectra of mutant and wild-type FNRs: (A) extinction coefficients in the near-ultraviolet and visible regions *vs* wavelength are reported for the three FNR forms; (B) second derivative of the spectra shown in panel A; (C) molar ellipticity in the near-ultraviolet and visible regions of the spectrum reported for S96V and wild-type FNR forms. Symbols: short dashes, wild-type FNR; solid line, FNR-S96G; long dashes, FNR-S96V.

Table 2: Specific Activities of the FNR Forms

enzyme form	acceptor		
	INT (units/FAD)	$K_3Fe(CN)_6$ (units/FAD)	cyt *c*[a] (units/FAD)
wild type	2800	20000	3900
S96V	4.5	6.2	5.9
S96G	174	280	234

[a] Ferredoxin-dependent cytochrome *c* reductase activity.

spectral perturbation in the mutant FNR is likely due to a change in the microenvironment of the isoalloxazine ring brought about by the replacement of Ser by Val. The protein fluorescence and the FAD fluorescence of the mutant flavoproteins were essentially the same as in wild-type enzyme.

Steady-State Kinetics. The two mutations at position 96 resulted in different degrees of impairment of the catalytic activity of FNR (Table 2). Wild-type FNR shows different activity values when assayed with different substrates, depending on the ability of the various acceptors to take electrons from the reduced enzyme, with ferricyanide being the most effective. As shown in Table 2, there is very little substrate-dependent rate for either mutant. This observation is a strong indication that it is the reductive half-reaction, *i.e.*, the reduction of FNR by NADPH, that is primarily affected in the mutant FNR forms.

Steady-state kinetics of the various FNR forms were determined for the diaphorase reaction using either INT or $K_3Fe(CN)_6$ as the electron acceptor. The values of the kinetic parameters, obtained by fitting the data to a ping-pong mechanism, are reported in Table 3. The major effect of mutation is on the k_{cat}'s, the K_m's for NADPH being increased only to a minor extent. FNR-S96G showed a k_{cat} value 36-fold lower than that of the wild-type enzyme, when measured with ferricyanide as electron acceptor. The activity of FNR is almost completely abolished by replacing Ser96 with valine; the k_{cat} for ferricyanide reduction of the FNR-S96V mutant is almost 2000-fold lower, and its catalytic efficiency (k_{cat}/K_m) is reduced by about 4 orders of magnitude compared with those of the wild-type FNR. Significantly, the k_{cat}'s of FNR-S96G and FNR-S96V measured with the two different acceptors tend to become equal, supporting the hypothesis that the reductive half-reaction has become the limiting step in the catalytic mechanism of the mutant FNR forms. In both the FNR mutant forms, the K_m's for the electron acceptors (INT or ferricyanide) were so diminished that their accurate measurement was difficult. This behavior had already been observed with other FNR mutants (Aliverti et al., 1991), and it is the expected effect of a mutation that greatly reduces the velocity of the reductive half-reaction in a ping-pong mechanism reaction.

Rapid-Reaction Studies. To directly verify this hypothesis, the time course of flavin reduction by NADPH was studied by stopped-flow spectrophotometry. Reactant concentrations (about 50 μM NADPH and 20 μM FNR forms, after mixing) were chosen to ensure pseudo-first-order conditions, with the K_d for NADPH being 1 μM as reported by Batie and Kamin (1986). The kinetics of reduction of wild-type FNR (Figure 2A), including the dependence on NADPH concentration, confirmed our previous reports (Aliverti et al., 1993). Time traces recorded at any wavelength in the range 340−750 nm were best fit by the same two exponential phases: a fast phase occurring at a rate of 820 s^{-1} and a slower phase occurring at a rate of 105 s^{-1}. During the fast phase an absorbance decrease was observed at wavelengths between 350 and 500 nm that accounted for about 30% of the total absorbance change. Conversely, the same phase resulted in an absorbance increase at wavelengths extending from about 530 nm to greater than 750 nm. During the second phase, an absorbance decrease occurred over the entire visible range. The final spectrum showed a considerable level of oxidized flavin and substantial absorbance in the 550−800 nm region (Figure 3). These data are consistent with a reaction mechanism in which the formation of the Michaelis complex between NADPH and oxidized FNR is extremely fast. In the first observable phase, the formation of a transient charge-transfer complex between NADPH and oxidized FNR occurs. In the second phase, this species is partially converted to the charge-transfer complex between reduced FNR and $NADP^+$ by hydride transfer from NADPH to FAD. An equilibrium is reached where most of the FNR is present as a mixture of both charge-transfer complex forms, with FNR_{ox}•NADPH prevailing (Figure 3).

Table 3: Kinetic Parameters of Mutant and Wild-Type FNRs for the Diaphorase Activity Assayed with Two Different Electron Acceptors

	acceptor					
	INT			$K_3Fe(CN)_6$		
enzyme form	k_{cat} (e^- eq s^{-1})[a]	K_m^{NADPH} (μM)	k_{cat}/K_m (e^- eq s^{-1} M^{-1})	k_{cat} (e^- eq s^{-1})[a]	K_m^{NADPH} (μM)	k_{cat}/K_m (e^- eq s^{-1} M^{-1})
wild type	220	48	4.58×10^6	500	35	1.43×10^7
S96V	0.41	131	3.10×10^3	0.26	106	2.47×10^3
S96G	10.4	86	1.21×10^5	13.7	144	9.51×10^4

[a] Electron equivalents per second.

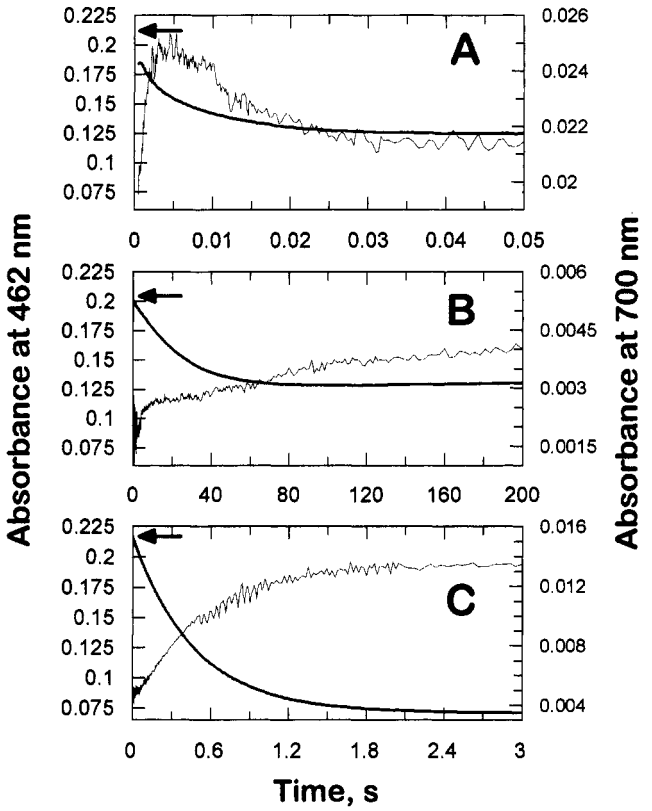

FIGURE 2: Time course of anaerobic reduction of FNR forms by NADPH: comparison of mutant forms with the wild-type enzyme. Linear plots of A_{462} (bold trace) and A_{700} (thin trace) *vs* time. The arrows indicate the initial absorbance at 462 nm of the samples. Reactions were carried out in 50 mM HEPES (pH 7.0) at 4 °C. (A) 41.4 μM wild-type FNR was reacted with 98.4 μM NADPH. (B) 40.0 μM FNR-S96V was reacted with 96.0 μM NADPH. (C) 40.8 μM FNR-S96G was reacted with 107 μM NADPH.

Unlike wild-type FNR, the reduction of the mutant FNR forms by NADPH can be described as a single-exponential process (Figure 2B,C). We observed no rapid loss of absorbance with either mutant such as what had been seen with wild-type FNR and ascribed to the formation of the FNR_{ox}•NADPH complex. The calculated monophasic rates of reduction in the mutants were 0.04 and 2.1 s^{-1} for FNR-S96V and FNR-S96G, respectively. With FNR-S96V, the decrease in absorbance at 462 nm is of the same magnitude as that observed with the wild-type enzyme, whereas a much lower A_{462} value was reached at the end of the reaction of FNR-S96G with NADPH (Figure 3). This suggests that the redox potential for FNR-S96G is higher than that for the wild-type or S96V FNR forms. As shown in Figure 2B, very little spectral change was observed at 700 nm during the reduction of FNR-S96V. This mutant reacted slowly enough with reduced NADPH to allow the fast recording of spectra (at a scan rate of 100 nm/s) during flavin reduction

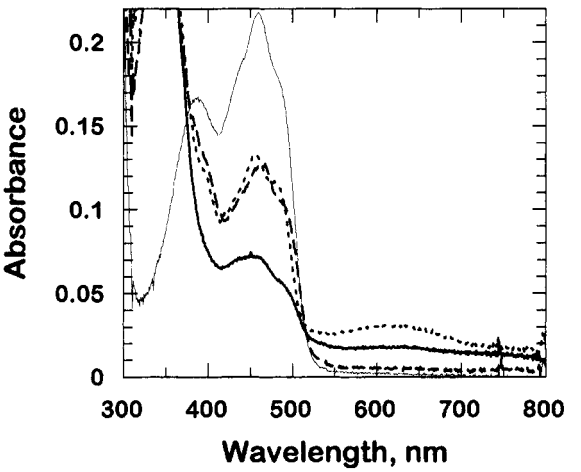

FIGURE 3: Spectra of the FNR forms after anaerobic reaction with NADPH. Spectra were recorded by the stopped-flow spectrophotometer *ca.* 60 s after mixing. Conditions were the same as in the legend of Figure 2. Symbols: short dashes, wild-type FNR; solid line, FNR-S96G; long dashes, FNR-S96V. The thin line represents the spectrum of the oxidized wild-type FNR.

(not shown), demonstrating the absence of any absorbance band attributable to charge-transfer species. Although no transient intermediates were seen before the hydride-transfer step during FNR-S96G reduction, a charge-transfer species accumulated as an end product (Figure 3). As shown in Figure 2C, the increase in absorbance at 700 nm occurred at the same rate as the absorbance decrease at 460 nm, suggesting that the long-wavelength-absorbing species appearing at the end of the reaction is the charge-transfer complex comprising $NADP^+$ and reduced enzyme. Indeed, in reactions with NADPH (as measured at 462 nm) the flavin became significantly more reduced than in the wild-type enzyme (Figure 3).

Kinetics of FNR reduction was studied at different temperatures in the range 4−25 °C. Both phases of wild-type FNR reduction increased with temperature, the faster phase soon becoming too fast to be measured as the temperature was raised. The temperature dependence of the reduction rate of mutant FNRs, as well as the second phase of wild-type enzyme reduction, was analyzed by the Eyring plot (Segel, 1975). Thermodynamic parameters of activation for the reaction were calculated, and the values are shown in Table 4. The activation enthalpy for the reduction of FNR-S96G is identical to that of the wild-type enzyme, the lower reactivity of the mutant being ascribed to a less favorable activation entropy. The opposite was observed in the case of FNR-S96V: a much higher activation enthalpy was obtained, whereas the activation entropy was even more favorable than that of the wild-type enzyme.

Photoreduction. The FAD prosthetic groups of both FNR-S96G and FNR-S96V were photoreduced by EDTA in the

Table 4: Thermodynamic Parameters of Activation of FAD Reduction by NADPH in Mutant and Wild-Type FNR Forms

enzyme form	$\Delta H^{\ddagger}$ (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal mol^{-1} K^{-1})	$\Delta G^{\ddagger}$ [a] (kcal mol^{-1})
wild type	9.8	−13.9	13.9
S96V	16.0	−7.5	18.2
S96G	9.8	−21.5	16.2

[a] Calculated for T = 298 K.

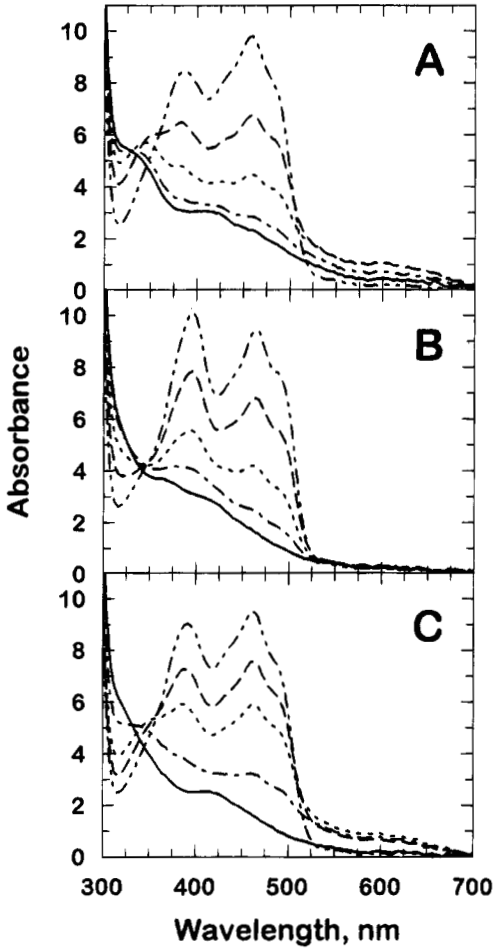

FIGURE 4: Photoreduction of FNR forms. Absorbance spectra of anaerobic FNR solutions recorded after successive periods of 60 s of illumination. Spectra were not corrected for the absorbance contribution of 5-deazariboflavin. Conditions were 10 mM HEPES (pH 7.0), 15 mM EDTA, and 1.2−1.5 μM 5-deazariboflavin. (A) 12 μM wild-type FNR; (B) 15 μM FNR-S96V; (C) 21 μM FNR-S96G. To facilitate comparison, spectra were normalized with respect to those expected for 1 mM enzyme solutions.

presence of 5-deazariboflavin at rates comparable to that observed in the case of the wild-type enzyme (Figure 4). No significant differences were observed between the spectra of the fully reduced FNRs. During photoreduction of wild-type FNR, a substantial amount of neutral blue semiquinone accumulated (Figure 4A), accounting for about 26% of the total flavin present, as calculated on the basis of an ϵ_{600} value of 4000 M^{-1} cm^{-1} (Batie & Kamin, 1984). Approximately the same amount of semiquinone (23%) was observed during the reduction of FNR-S96G (Figure 4C) as in the case of wild-type FNR photoreduction, whereas no absorbance changes attributable to a semiquinone transient species were observed in the case of FNR-S96V (Figure 4B).

Interaction with NADP$^+$. Since a productive interaction between NADPH and FNR seemed to be impaired by

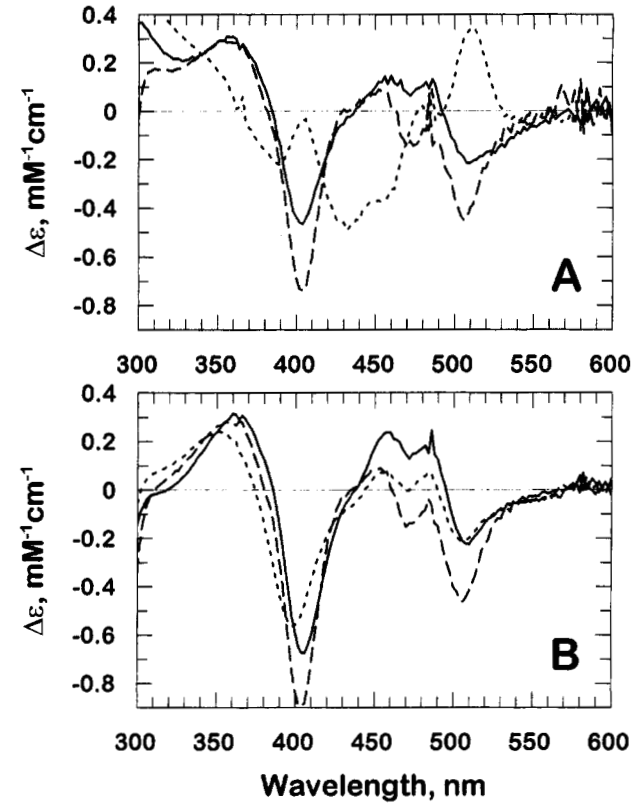

FIGURE 5: Difference absorbance spectra elicited by binding of NADP$^+$ and 2′-P-ADP−ribose to the FNR forms. Difference spectra obtained by subtracting from the spectra recorded at the titration end points those of the corresponding unliganded FNR form. To facilitate comparison, differences in the extinction coefficients are reported. Symbols: short dashes, wild-type FNR; solid line, FNR-S96G; long dashes, FNR-S96V. (A) Difference spectra of the NADP$^+$/FNR complexes recorded in 10 mM Tris-HCl (pH 7.7) at 15 °C. (B) Difference spectra of the 2′-P-ADP−ribose/FNR complexes recorded in 20 mM Tris-HCl (pH 7.7) at 15 °C.

mutations at position 96 of the amino acid sequence, it was of interest to study the interaction of the mutant enzymes with the oxidized pyridine nucleotide substrate. When NADP$^+$ binds to wild-type FNR, the visible spectrum of the bound flavin undergoes a perturbation, yielding the difference spectrum shown in Figure 5A. In the same figure, the difference spectra obtained by titrating the mutant FNR forms with NADP$^+$ are presented for comparison. The spectral perturbations elicited in the two mutant flavoproteins by NADP$^+$ binding are very similar to each other, but they are greatly different from those displayed by the wild-type FNR. Dissociation constants, obtained from the titration data by nonlinear curve fitting, were identical within the error of the analysis: 10 ± 2, 13 ± 3, and 13.8 ± 3 μM for FNR-S96V, FNR-S96G, and wild-type FNR, respectively. In Figure 5B, the difference spectra elicited in the various FNR forms by the addition of 2′-P-ADP−ribose, an NADP$^+$ analog lacking only the nicotinamide ring, are shown; all of the difference spectra are very similar in shape. Surprisingly, in the mutant FNRs the difference spectra produced by NADP$^+$ binding are superimposable with those obtained by 2′-P-ADP−ribose binding (Figure 5A,B). This suggests that the nicotinamide ring of NADP$^+$ does not interact properly with the flavin in these mutants, even though the interaction of the 2′-P-ADP−ribose moiety is virtually the same.

Interaction with Ferredoxin. The interaction of both mutant FNR forms with Fd was studied in order to rule out

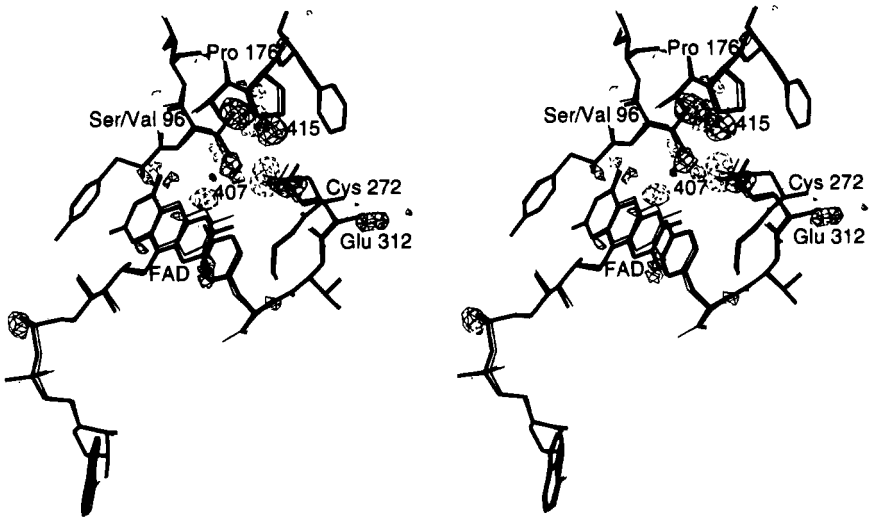

FIGURE 6: Structure of FNR-S96V. The thick bonds show the refined structure of the active site of FNR, with a valine residue at position 96. The thin bonds show the wild-type structure with serine at position 96. The cage contours show difference Fourier electron density (FNR-S96V *vs* wild-type FNR) using phases from the refined wild-type structure. The solid cages show positive difference Fourier density contoured at $+4.0\sigma$. The stippled cages are contoured at -4.0σ. The largest differences in atomic positions are observed for Glu312, water415, and the dimethylbenzene moiety of FAD. Smaller motions are observed for residues Tyr314, Pro176, and Gly173.

possible long-range conformational effects of mutations, which could affect the Fd-binding site of the reductase. The K_d values of the complexes between FNR forms and oxidized Fd were determined through fluorescence quenching titrations. The fluorescence quenching reached at saturating Fd concentrations was the same in the mutant proteins and in the wild-type one. The K_d values of the wild-type and mutant FNR forms were all near 15 nM in 20 mM HEPES (pH 7.0) at 15 °C.

Three-Dimensional Structure of the FNR-S96V Mutant. The replacement of residue Ser96 with valine caused several small changes in the positions of atoms in the active site of FNR (see Figure 6). To simplify the discussion, we will use verbs describing movement to discuss differences in atomic position between the wild-type structure with a serine at position 96 and the structure with valine at that position. Of course there is no actual movement, since distinct proteins are being compared. Most of these changes in atomic positions correspond to atoms moving away from residue 96 in the mutant structure. This general expansion of the active site allows accommodation of the increase in side-chain volume at position 96.

The main-chain atoms of residue 96 itself undergo some small conformational changes in response to the valine substitution. One carboxylate oxygen of glutamate 312, which ordinarily hydrogen bonds to Ser96, moves 1.1 Å away. Water415, which is closely associated with Glu312, moves 1.0 Å. The dimethylbenzene end of the isoalloxazine ring of FAD shifts by 0.6 Å away from residue 96. This shift in FAD is transmitted as far as the pyrophosphate linkage. The valine mutation is also associated with motions of Tyr314 and Pro176 about 0.3 Å away from the active site. An $NADP^+$ soak of the FNR-S96V crystals was also analyzed. As seen with crystals of FNR purified from spinach leaves (Bruns & Karplus, 1994), this soak resulted in ordered binding of only the adenosine moiety of $NADP^+$, not the nicotinamide moiety (data not shown). The Ser to Val mutation appeared to have no effect upon the binding of the adenosine moiety of $NADP^+$.

DISCUSSION

FNR-S96V Mutant. The replacement of Ser96 by a valine residue in spinach ferredoxin-$NADP^+$ reductase results in a dramatic decrease in the catalytic activity of the enzyme. Although absorbance and circular dichroism spectra of the mutant flavoprotein show small changes, the protein and FAD fluorescence quenching, indicative of the environment of the flavin, and the affinities for the substrates (*i.e.*, $NADP^+$ and ferredoxin) were all unchanged. Thus, a gross structural rearrangement of the protein conformation cannot be the cause of the loss in activity. This has been confirmed by the determination of the three-dimensional structure of the mutant FNR; the changes are quite small and involve only atoms near the active center of FNR. The active site is expanded slightly to make room for the larger side chain of valine. The slight shifts seen for the flavin ring and Tyr314 (the position of this latter ring could be a model for nicotinamide positioning in analogy to the situation in glutathione reductase; Pai et al., 1988) and other active site atoms are consistent with the spectral perturbations (absorption and CD), which indicate a definite, albeit small, modification of the isoalloxazine microenvironment.

The fact that the enzyme activity of the mutant FNR was practically independent of the electron acceptor used suggested that the rate-limiting step in catalysis is the reduction of the enzyme flavin by NADPH. Indeed, a rate of 0.04 for the mutant *vs* 105 s^{-1} for the wild-type FNR was measured in stopped-flow experiments of enzyme reduction by NADPH. Moreover, no evidence was found for the presence of charge-transfer species, either FNR_{ox}·NADPH or FNR_{red}·$NADP^+$, during FNR-S96V reduction by NADPH. $NADP^+$ titrations of FNR-S96V yielded a value for K_d of the complex that was very similar to that of the native one. The difference spectrum produced by the mutant FNR was very similar to those elicited by $NADP^+$ analogs binding to FNR from spinach (Batie & Kamin, 1986). Indeed, the same difference spectra were obtained with either $NADP^+$ or 2′-P-ADP−ribose in the case of the mutant enzyme. It has been shown that the nicotinamide ring does not make a net contribution to the binding energy of $NADP^+$ (Batie &

Kamin, 1986). Thus, the K_d value obtained for $NADP^+$ binding to the mutant FNR would be expected to be similar to that for wild type, although the correct positioning of the nicotinamide ring did not take place. That the Ser to Val mutation has no effect upon the binding of the adenosine moiety of $NADP^+$ has also been verified by the structure of the complex by X-ray crystallography. We conclude that, for the mutant, the nicotinamide moiety cannot be positioned as in the wild-type enzyme so as to give the characteristic spectral perturbations of the isoalloxazine ring. This is also consistent with the absence of any charge-transfer species during reduction of the enzyme by NADPH. Thus, the replacement of the OH of Ser96 with the side chain of valine disrupts the proper geometry of the active site, so that the isoalloxazine and nicotinamide rings cannot form a stable stacking interaction, which should favor hydride transfer between C_4 of NADP(H) and N_5(H) of the flavin. In this context, the movement of the side chain of Glu312 away from the native enzyme position could be of importance, since it has been suggested to be in an excellent position to H-bond to the amide group of nicotinamide (Bruns & Karplus, 1995).

FNR-S96G Mutant. The replacement of Ser96 with glycine yielded an enzyme that maintains *ca.* 2−5% of the wild-type activity, depending on the electron acceptor used. As observed with FNR-S96V, the rate-limiting step of the overall reaction for FNR-S96G clearly is the flavin reduction by NADPH: 2.1 *vs* 105 s^{-1} for the wild-type FNR. FNR-S96G occupies a position intermediate between the wild-type enzyme and FNR-S96V. Some properties of the glycine mutant are more similar to those of the wild-type enzyme than to those of the valine mutant: semiquinone stabilization, the enthalpy of activation in flavin reduction by NADPH, and formation of the charge-transfer species, FNR_{red}•$NADP^+$. In regard to the last point, it is possible that correct positioning of the two rings (flavin and nicotinamide) is elicited about 2% of the time in FNR-S96G and that formation of the transient FNR_{ox}•NADPH escapes detection in the stopped-flow study due to sensitivity problems as well as to kinetic reasons. Instead, a buildup of the charge-transfer FNR_{red}•$NADP^+$ is clearly seen in the course of the stopped-flow reaction, which is the result of the higher concentration of reduced flavin in the case of the glycine mutant. Apparently, reduction of the isoalloxazine ring stabilizes the correct interaction with the nicotinamide moiety of $NADP^+$. This does not happen in the valine mutant.

Other properties of FNR-S96G are similar to those of the valine mutant, *i.e.*, the red shift of the absorbance spectrum and the spectral changes induced by $NADP^+$ binding. Unfortunately, the glycine mutant did not crystallize, and thus, we do not have its three-dimensional structure. However, the two mutants show important differences: (a) the extent of flavin reduction by NADPH was much larger than in the FNR-S96V case; (b) the thermodynamic parameters of activation in the flavin reduction by NADPH were substantially different in the two mutants. For the S96G mutant an increase in $\Delta G^{\ddagger}$ is due to an increase in $\Delta S^{\ddagger}$ (more negative), while for the S96V mutant the $\Delta H^{\ddagger}$ was greatly enhanced. The higher degree of flavin reduction obtained in the case of FNR-S96G by the same NADPH concentration to flavin ratio used for the other two FNR forms points to a shift in the redox potential of FAD in the glycine mutant to a more positive value. This suggests that the mutant protein binds the reduced flavin more tightly than the wild type or less tightly the oxidized FAD. In the case of FNR-S96V, the E_m of FAD is apparently unchanged, but the semiquinone is highly destabilized. The results suggest that there may be a higher separation of the two one-electron potentials for flavin reduction in FNR-S96V than in wild-type FNR. In this context, it should be mentioned that the Thr94 residue of NADH-cytochrome b_5 reductase, which should be the equivalent of Ser96 of FNR in this enzyme of the FNR family, has been replaced with Ala, Ser, Cys, and Asn by Shirabe et al. (1994). The alanine mutant was inactive and was not purified. The Ser, Cys, and Asn mutant reductases were 100, 25, and 0.2% active, respectively. In all cases, the rate of flavin reduction was unchanged or even much increased (for the cysteine mutant) while the flavin semiquinone was destabilized in comparison to the wild-type NADH-cytochrome b_5 reductase.

Why does FNR-S96G have higher activity than the S96V enzyme? The interaction between the isoalloxazine ring of FAD and the nicotinamide ring of NADP(H) is disrupted more in the FNR-S96V mutant than in the FNR-S96G mutant. The lack of a side chain in the glycine mutant may permit a water molecule to occupy the position ordinarily held by the serine side chain. This hypothetical water molecule could perform a function similar to that of the side-chain hydroxyl group of serine and restore partial activity. This would not be possible with the valine mutant. Alternatively, the bulkier and apolar side chain of valine may directly interfere with the binding of the nicotinamide in a way that the glycine mutant does not. Nicotinamide binding might also be influenced indirectly through the effects of the Ser96 mutations on the positions of surrounding groups such as Glu312, water415, or FAD, as observed in the crystal structure of the FNR-S96V mutant (Figure 6). The positions of these groups probably are less affected in the glycine mutant than in the valine mutant, although in the absence of a crystal structure for FNR-S96G we cannot be certain. We have made a Glu312 to Leu mutant, and if its production is successful, its properties will help to clarify the role of this residue.

CONCLUSIONS

The mutants characterized in this study point out important functions of the side-chain hydroxyl group of Ser96 in FNR. Ser96 stabilizes the interaction between the isoalloxazine ring of FAD and the nicotinamide ring of NADP(H), and it influences the reduction properties of FAD. The interaction between flavin and nicotinamide is disturbed in both mutants studied, expecially the S96V mutant. Disruption of the interaction between flavin and nicotinamide can explain the decreased activity of the mutants. The transition state of hydride transfer is destabilized insofar as the flavin−nicotinamide interaction is part of that transition state. The two mutants both showed altered FAD reduction properties, but in different ways. FNR-S96V apparently has unaltered two-electron midpoint potential, but the semiquinone form is destabilized. FNR-S96G exhibits normal semiquinone formation, but the two-electron midpoint potential appears to be increased.

We currently are unable to present a detailed structural interpretation of the mechanism by which the FNR-S96G and FNR-S96V mutants exhibit their effects. This is in part

because the details of the interaction between the nicotinamide and isoalloxazine rings have escaped crystallographic analysis (Bruns & Karplus, 1995). Any such mechanism will have to account for not only the effect upon nicotinamide binding but also the effects upon the reduction properties of FAD.

ACKNOWLEDGMENT

The authors are grateful to Dr. P. Arosio and Dr. G. Vecchio for access to their molecular graphics instrumentation and CD spectrometer, respectively.

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BI9501601