Amino Acid Residues in *Anabaena* Ferredoxin Crucial to Interaction with Ferredoxin–NADP$^+$ Reductase: Site-Directed Mutagenesis and Laser Flash Photolysis†

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ABSTRACT: Ferredoxin (Fd) functions in photosynthesis to transfer electrons from photosystem I to ferredoxin–NADP$^+$ reductase (FNR). We have made several site-directed mutants of *Anabaena* 7120 Fd and have used laser flash photolysis to investigate the effects of these mutations on the kinetics of reduction of oxidized Fd by deazariboflavin semiquinone (dRfH$^\bullet$) and the reduction of oxidized *Anabaena* FNR by reduced Fd. None of the mutations influenced the second-order rate constant for dRfH$^\bullet$ reduction by more than a factor of 2, suggesting that the ability of the [2Fe–2S] cluster to participate in electron transfer was not seriously affected. In contrast, a surface charge reversal mutation, E94K, resulted in a 20 000-fold decrease in the second-order rate constant for electron transfer from Fd to FNR, whereas a similar mutation at an adjacent site, E95K, produced little or no change in reaction rate constant compared to wild-type Fd. Such a dramatic difference between contiguous surface mutations suggests a very precise surface complementarity at the protein–protein interface. Mutations introduced at F65 (F65I and F65A) also decreased the rate constant for the Fd/FNR electron transfer reaction by more than 3 orders of magnitude. Spectroscopic and thermodynamic measurements with both the E94 and F65 mutants indicated that the kinetic differences cannot be ascribed to changes in gross conformation, redox potential, or FNR binding constant but rather reflect the protein–protein interactions that control electron transfer. Several mutations at other sites in the vicinity of E94 and F65 (R42, T48, D68, and D69) resulted in little or no perturbation of the Fd/FNR interaction. Kinetic experiments with the heterocyst Fd from *Anabaena* 7120, which functions in nitrogen fixation, are consistent with the above mutagenesis results and also indicate that Y98, which is also closely adjacent to E94 and F65, is not a critical residue for electron transfer to FNR. These results provide clear evidence for a high degree of localization and specificity in the interface region between the two proteins which is involved in the electron transfer process.

Ferredoxins (Fds)[1] comprise a class of low molecular weight (6–14 kDa) acidic electron transfer proteins found ubiquitously in nature. They function in photosynthetic electron transfer, nitrate reduction, and carbon and sulfur metabolism (Knaff & Hirasawa, 1991; Lovenberg, 1973–1977). These redox proteins contain one or more iron–sulfur [Fe–S] clusters as prosthetic groups, which can be of the [2Fe–2S], [4Fe–4S], or [3Fe–4S] type and have reduction potentials ranging from –600 to +200 mV (Cammack, 1984; Berg & Holm, 1982; Arnon & Buchanan, 1971). "Plant-type" ferredoxins contain a single [2Fe–2S] cluster in an 11-kDa peptide chain and are found in chloroplasts and cyanobacteria where they function as the terminal electron acceptor from photosystem I, transferring electrons to ferredoxin–NADP$^+$ reductase (FNR), which catalyzes the reduction of NADP$^+$ to NADPH. The ferredoxin from the cyanobacterium *Anabaena* strain 7120 is a member of this group and has a reduction potential of –430 mV [Salamon and Tollin (1992) and herein]. The three-dimensional structure of this Fd has recently been solved to 2.5-Å resolution (Rypniewski et al., 1991) and refined to 1.9 Å, making it a good candidate for the study of structure–function relationships in this class of proteins. Cyanobacteria which fix nitrogen (such as *Anabaena*) also have a ferredoxin isozyme which is the electron donor to nitrogenase and is located within specialized cells called heterocysts. In *Anabaena*, the heterocyst Fd is 51% identical to the vegetative form and the three-dimensional structure has also been determined (Jacobson et al., 1992, 1993). There are some interesting substitutions of otherwise conserved residues at positions R42, E95, and Y98 (see below).

Previous work from these and other laboratories has firmly established the role of electrostatics in protein–protein recognition in several redox systems [cf. Tollin and Hazzard (1991) and Roberts et al. (1991)]. Early studies (Foust et al., 1969) described the formation of complexes between various Fds and FNR and pointed to the importance of electrostatic interactions in the functioning of these proteins. Subsequent investigations (Batie & Kamin, 1984) confirmed these findings and postulated the existence of a cluster of positive charge on FNR at the Fd binding site. Indeed, recent chemical

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[1] Abbreviations: Fd, vegetative cell ferredoxin; HFd, heterocyst cell ferredoxin; FNR, ferredoxin–NADP$^+$ reductase; dRf, 5-deazariboflavin; dRfH$^\bullet$, 5-deazariboflavin semiquinone; wt, wild type; V_{ij}, electrostatic interaction energy; k_∞, reaction rate constant at infinite ionic strength; F65A, phenylalanine at position 65 replaced with alanine; other mutations are abbreviated in similar fashion.

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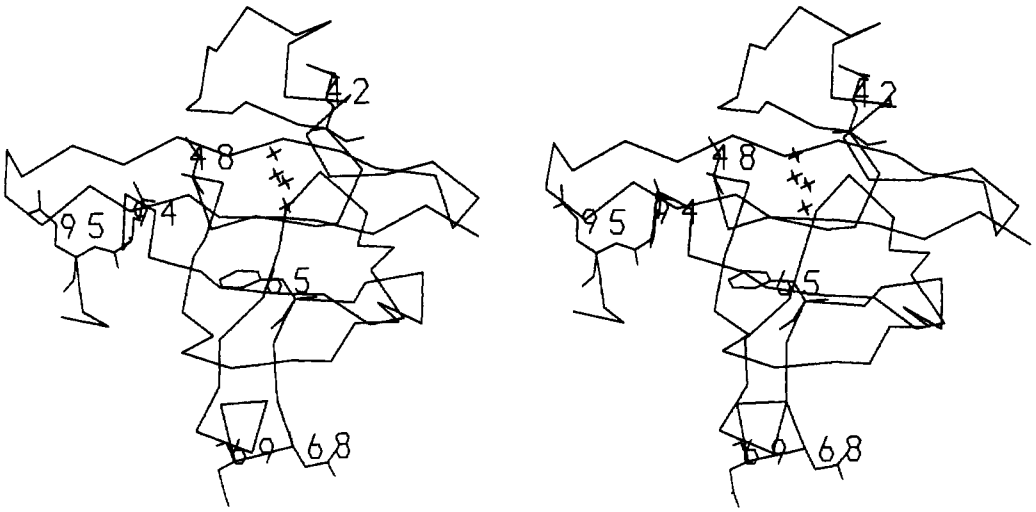

FIGURE 1: Stereoview of *Anabaena* 7120 vegetative Fd showing mutated residues. This was displayed on an Evans and Sutherland PS390 molecular graphics system using INSIGHT software (Biosym, Inc.). Coordinates were kindly provided by Dr. Hazel Holden, Department of Biochemistry, University of Wisconsin.

modification studies have identified an arginine residue (Sancho et al., 1990; Medina et al., 1992a) and two lysine residues (Medina et al., 1992b) in *Anabaena* FNR that are involved in binding Fd. In addition, two covalent cross-linking studies of the spinach proteins have implicated several acidic Fd residues within the regions 20–30, 65–70, and 92–94 (corresponding to regions 22–32, 67–72, and 94–96 in *Anabaena*) in binding to FNR (Vieira et al., 1986; Zanetti et al., 1988). Computer modeling based on the crystal structures of spinach FNR and *Spirulina* Fd also points to acidic residues in these segments of the Fd molecule (Karplus, 1991a,b).

Ionic strength effects on the kinetics of electron transfer from spinach Fd to spinach FNR studied by laser flash photolysis (Bhattacharyya et al., 1986; Walker et al., 1991) have indicated the strong influence of complementary electrostatic charges on complex formation and stabilization. In the case of the *Anabaena* proteins, the kinetic results (Walker et al., 1991) suggested that electrostatic forces are not a major contributor to complex stability, although they do influence the rate constant for intracomplex electron transfer.

Site-directed mutagenesis is a very powerful technique in which single or multiple amino acid changes can be introduced into a protein by altering the sequence of the coding DNA. The advantage this approach provides for studies of protein structure–function relationships is obvious and far-reaching (Baum, 1991). This methodology has been applied to homologous animal [2Fe–2S] Fds, which transfer electrons from NADPH–ferredoxin oxidoreductase to cytochromes P450. Site-directed mutations of D76 and D79 in the mitochondrial Fd (Coghlan & Vickery, 1991) to the neutral amino acids N and A led to large decreases in the binding affinity for both NADPH–ferredoxin oxidoreductase and cytochrome $P450_{scc}$. This identified these two acidic residues as being critical for recognition and indicated that the binding sites on mitochondrial Fd for its two redox partners overlapped significantly. In a further study (Coghlan & Vickery, 1992), these same two residues were changed to E, thus maintaining negative charge at these positions. These conservative changes also resulted in a dramatic decrease in binding affinity for both proteins and led to the conclusion that in the mitochondrial system, although overall electrostatic charge may be important in precollisional alignment of Fd with its redox partners, specific short-range interactions consisting of pairwise electrostatic interactions of fixed geometry are required for formation of the final, active complex.

Table I: Second-Order Rate Constants for Reduction of *Anabaena* Ferredoxins by Deazariboflavin Semiquinone at I = 12 mM

	$k \times 10^{-8}$ (M^{-1} s^{-1})		$k \times 10^{-8}$ (M^{-1} s^{-1})
WT	2.2 ± 0.2	F65A	1.2 ± 0.2
D68K/D69K	1.4 ± 0.2	F65I	1.1 ± 0.1
D68K	1.3 ± 0.2	R42A	1.4 ± 0.2
E94K/E95K	2.5 ± 0.4	R42H	1.3 ± 0.2
E95K	1.7 ± 0.1	T48A	1.3 ● 0.3
E94K	2.6 ± 0.4	HFd	1.3 ± 0.1

In the study reported here, we have used site-directed mutagenesis, coupled with laser flash photolysis measurements of electron transfer rate constants, to investigate the role of specific amino acid residues in *Anabaena* strain 7120 Fd in complex formation with and electron transfer to FNR obtained from a closely related organism (*Anabaena* strain 7119). The Fd mutants investigated are listed in Table I, and their locations relative to the iron–sulfur cluster are illustrated in Figure 1. The residues we have chosen to mutate are all highly conserved in cyanobacteria; all are also highly conserved in green algae and spinach except T48, for which S occupies the analogous position. As will be demonstrated below, several of these mutations have little or no effect on electron transfer to FNR. In sharp contrast, however, two amino acid residues (E94 and F65) are found to be crucial to this interaction, although mutations at these positions do not appreciably influence any other Fd properties that we have examined. The implications of these results are discussed.

MATERIALS AND METHODS

Vegetative cell wild-type (wt) and mutant ferredoxins from *Anabaena* strain 7120 were obtained at the University of Arizona using *Escherichia coli* strain JM109 which had been transformed with a plasmid (pAn662) containing the ferredoxin gene (Alam et al., 1986). This plasmid was a kind gift of Professor S. Curtis, North Carolina State University. Basically, the construct consisted of a *Hin*dIII fragment containing the gene which had been ligated into the plasmid vector pIBI25 (IBI, New Haven, CT). A 2-mL culture of the transformed bacteria, grown overnight at 37 °C with shaking in LB medium containing 150 μg of ampicillin/mL, was used to inoculate 1 L of the same medium containing 60 μM $FeSO_4$

(Böhme & Haselkorn, 1989). This in turn was used to inoculate 16 L of the same medium which was incubated overnight (16–17 h). Cells were collected by centrifugation for 20 min at 5000 rpm in a Sorvall RC-3 centrifuge. Typically, 60–70 g of wet pellet was obtained. The cells were lysed using a modification of a standard procedure (Sambrook et al., 1989). The pellet was resuspended in 300 mL of Tris-HCl buffer (100 mM, pH 8.0 containing 1 mM EDTA and 100 mM NaCl). Lysozyme (Sigma Chemical Co., St. Louis, MO, from chicken egg white) was added to a concentration of 0.8 μg/g of wet pellet. After approximately 20 min (when the suspension became viscous), DNase I (Calbiochem, San Diego, CA, from bovine pancreas) was added to a concentration of 20 μg/g of pellet. The suspension was stirred for 30 min until the viscosity decreased, sonicated (Branson Model W-220f) for 8 min in 2-min intervals, and centrifuged at 40 000 rpm in a Beckman Ti45 rotor for 90 min at 4 °C. The supernatant was applied to a 9-cm × 12-cm DEAE-cellulose column (DE52, Whatman, Maidstone, England) and washed with 250 mL of 20 mM Tris-HCl buffer, pH 7.3, containing 100 mM NaCl. The protein was then eluted with a linear NaCl gradient (200–500 mM in 20 mM Tris-HCl buffer, pH 7.3). Fractions containing Fd were pooled, concentrated (using an Amicon ultrafiltration device with a YM10 membrane), and applied to a Sephadex G50-f column (Pharmacia, Uppsala, Sweden). Fractions having an A_{422}/A_{276} ratio greater than 0.5 were pooled for use in kinetic and spectroscopic experiments. For some Fds, an additional step of ammonium sulfate (ultrapure, ICN Biomedicals, Cleveland, OH) fractionation was necessary to achieve the purity ratio given above. Yields of pure protein ranged from 3 to 12 μmol.

Site-directed mutants made at the University of Arizona (D68K, D68K/D69K, E94K, E95K, and E94K/E95K) used the unique site elimination (USE) method of Deng and Nickoloff (1992), which is available in kit form (transformer mutagenesis kit, Clontech, Palo Alto, CA). Oligonucleotide primers for mutagenesis ranged from 20 to 32 bases in length and were synthesized at the University of Arizona Division of Biotechnology Macromolecular Structures Facility. Base changes within the mutagenic oligonucleotides were made such that the resulting codons were those most frequently used by *E. coli* (Springer & Sligar, 1987). Molecular biology procedures (restriction digestion, ligation, transformation, DNA sequencing, etc.) followed standardized protocols (Sambrook et al., 1989; Ausubel et al., 1989). Transformed bacteria (*E. coli*, strain JM109) were plated on LB plates containing 75 μg/mL ampicillin and grown overnight at 37 °C. Single colonies were selected and grown overnight in 3 mL of LB containing 75 μg/mL ampicillin. DNA from these cultures was isolated and purified (Magic Minipreps DNA purification system, Promega, Madison, WI) and colonies containing the appropriate mutant proteins were identified by double-stranded DNA sequence analysis using the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, OH).

A full description of the subcloning, mutagenesis, protein overexpression, cluster reconstitution, and protein purification methods employed in making *Anabaena* 7120 variants at the University of Wisconsin (R42A, R42H, R42E, T48A, F65A, and F65I) will be presented elsewhere (H. Cheng, B. Xia, and J. L. Markley, manuscript in preparation). Briefly, the *petF* gene (Alam et al., 1988) from plasmid pAn662 (independent gift from Professor S. Curtis) was cloned into plasmid pET9a (Novogen, Madison, WI) containing the T7 promoter by using the *Nde*I and *Bam*HI restriction sites. The resulting construct pET9a/F was placed in *E. coli* host BL21(DE3)/pLysS. Protein expression was induced by adding isopropyl thiogalactoside (IPTG) into the growth culture when OD_{600} = 1.2 was reached. After the cells were harvested, they were lysed by a freeze–thaw cycle. The protein was reconstituted by a modification of the method of Coghlan and Vickery (1991).

Mutants were prepared at the University of Wisconsin by oligonucleotide-directed mutagenesis of recombinant M13mp18 DNA with the pET9a/F plasmid fragment inserted between the *Eco*RI and *Sal*I restriction sites. The method of single-strand DNA site-directed mutagenesis used was that described by Kunkel et al. (1987) and by Ausubel et al. (1987). The mutated DNAs were subcloned back into the pET9a vector. The resulting mutated recombinant plasmids were sequenced by a double-stranded dideoxy method (Lim & Pene, 1988) to confirm that mutagenesis was limited to the predicted sites.

FNR from *Anabaena* strain 7119 was isolated and purified as described previously (Pueyo & Gomez-Moreno, 1991). Recombinant Fd derived from heterocyst cells of *Anabaena* 7120 was prepared as described in Jacobson et al. (1992).

Absorption spectra in the visible and UV regions were obtained using an OLIS-modified Cary Model 15 spectrophotometer. Circular dichroism spectra were measured with an Aviv circular dichroism spectropolarimeter, Model 60 DS (Aviv Assoc., Lakewood, NJ). Ferredoxin concentrations were calculated using an extinction coefficient of 9700 M^{-1} cm^{-1} at 422 nm (Böhme & Haselkorn, 1989), and FNR concentrations were calculated using an extinction coefficient of 9400 M^{-1} cm^{-1} at 459 nm (Pueyo & Gomez-Moreno, 1991). Protein concentrations for CD spectra in the visible/near UV ranged from 82 to 97 μM and were diluted to 1.0 μM with water for spectra in the far UV.

Dissociation constants for complexes between the oxidized forms of FNR and wt and several mutant Fds were determined by a spectrophotometric method as previously described (Sancho & Gomez-Moreno, 1991). FNR was titrated into a 10 μM solution of Fd in 4 mM phosphate buffer, pH 7.0 (I = 12 mM, adjusted with NaCl). Difference spectra were obtained by using dual-compartment cuvettes, and absorbance changes due to complex formation were monitored at 460 nm. A double-reciprocal plot of the data ($1/\Delta A_{460}$ vs $1/[FNR]$) allowed calculation of a value of 5 ± 2 mM^{-1} cm^{-1} for the difference extinction coefficient at 460 nm for the wild-type Fd–FNR complex, which probably reflects contributions from both proteins. This is approximately 3 times larger than the value reported previously by Sancho and Gomez-Moreno (1991). The reason for this apparent discrepancy is unclear but may be due to the fact that we are using recombinant *Anabaena* 7120 Fd and *Anabaena* 7119 FNR in the present study, whereas the previous experiments were done with *Anabaena variabilis* proteins. Since the intercepts of double-reciprocal plots for those other Fds for which complex formation was measured varied by no more than 7%, this same extinction coefficient was used to calculate K_d values for all complexes.

Laser flash photolysis experiments utilized a nitrogen laser-pumped dye laser (BBQ 2A368 dye, 396-nm wavelength maximum, 0.1-mJ energy, pumped by a PRA Model LN100 nitrogen laser, 300-ps pulse duration; PRA, London, Ontario, Canada). The optical system used to monitor the reaction has been described previously (Przysiecki et al., 1985; Bhattacharyya et al., 1983). The photochemical reaction in which the 5-deazariboflavin (dRf) triplet initiates protein–protein electron transfer has also been described (Tollin & Hazzard, 1991). Briefly, laser-generated dRf triplet abstracts

a hydrogen atom from EDTA which is present in 10-fold excess. The resulting dRf semiquinone (dRfH•) then reduces oxidized protein in competition with its own disproportionation. All kinetic experiments were performed under pseudo-first-order conditions, in which protein is present in large excess over the dRfH• generated by the laser flash (<1 μM) [see Walker et al. (1991) for additional details]. Solutions of dRf (95–100 μM in 4 mM phosphate buffer, pH 7.0, containing 1 mM EDTA) were made anaerobic by bubbling for 1 h with H_2O-saturated argon. Microliter volumes of concentrated protein solutions were introduced into this solution in a 1-cm cuvette through a rubber septum, and Ar gas was blown over the sample surface to remove any added oxygen. Generally, data from 4–8 laser flashes were averaged. Ionic strength was adjusted by adding aliquots of 5 M NaCl. Kinetic traces were analyzed by using a computer fitting routine (Kinfit, OLIS Co., Bogart, GA). All data were fitted to a single exponential. The dRf was synthesized by a published procedure (Smit et al., 1986).

Reduction potentials were measured by cyclic voltammetry as described previously (Salamon & Tollin, 1992). This system utilized a gold electrode modified with a lipid bilayer consisting of a 1:1 mixture of egg phosphatidylcholine and dioctadecyldimethylammonium chloride. The protein concentration was 100 μM in a solution of 50 mM Tris-HCl buffer, pH 7.3, containing 30 mM $NaClO_4$. The potential was scanned at a rate of 20 mV/s, and reduction potentials were calculated with respect to the normal hydrogen electrode (NHE).

RESULTS AND DISCUSSION

Wild-Type Ferredoxin. Upon laser flash photolysis of dRf/EDTA solutions in the absence of other electron transfer agents, the dRfH• produced by the laser flash decays by disproportionation [cf. Walker et al. (1991)]. Figure 2A shows a typical transient decay measured at 505 nm (approximately the peak wavelength for dRfH•) under low ionic strength conditions (I = 12 mM). When oxidized Fd is present in such an experiment, dRfH• rapidly reduces Fd [cf. Walker et al. (1991)]. This reaction is conveniently monitored at 465 nm (Figure 2B), where oxidized Fd has an absorption maximum. The kinetic trace shows a rapid increase in absorbance due to dRfH• production, which subsequently decays in an exponential manner to below the preflash baseline, due to electron transfer to Fd resulting in a loss of absorption at this wavelength. By plotting pseudo-first-order rate constants for this decay as a function of Fd concentration (Figure 3), we obtain a second-order rate constant for the reaction of wt Fd with dRfH• of (2.2 ± 0.2) × 10^8 M^{-1} s^{-1} (Table I). This is similar to the value reported previously with nonrecombinant *Anabaena* 7119 Fd under similar conditions [(1.6 ± 0.2) × 10^8 M^{-1} s^{-1}; Walker et al., 1991].

With FNR present, reduced Fd is reoxidized by FNR as shown by the kinetic traces in Figure 2C,D. This is also consistent with the earlier results (Walker et al., 1991). The transient in Figure 2C was obtained at 507 nm, which is an isosbestic point for FNR reduction. The k_{obs} value determined from the exponential rise in absorbance at 600 nm (Figure 2D), which monitors the formation of the one-electron-reduced FAD moiety of FNR, is identical within experimental limits to that obtained from the absorbance increase at 507 nm, again as expected (Walker et al., 1991).

The variation of k_{obs} as a function of FNR concentration (plotted in Figure 4A from data obtained at 600 nm) yields a linear plot corresponding to a second-order rate constant for the reduction of FNR by reduced wt Fd of (1.2 ± 0.1) × 10^8 M^{-1} s^{-1}. In our earlier work with the *Anabaena* proteins (Walker et al., 1991), plots of k_{obs} vs FNR concentration showed saturation at all ionic strengths (10–310 mM), indicative of a (minimal) two-step mechanism consisting of

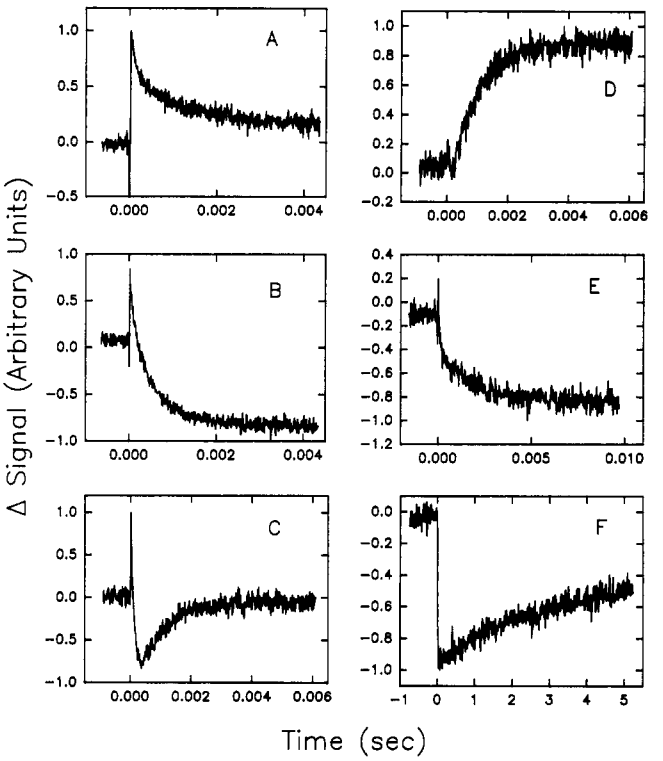

FIGURE 2: Transient decay curves of dRf/EDTA and dRf/EDTA solutions containing wt and F65A Fd plus or minus FNR at the monitoring wavelengths indicated. (A) 100 μM dRf, 505 nm, no Fd; (B) 7.4 μM wt Fd + 100 μM dRf, 465 nm, no FNR; (C) 40 μM wt Fd + 5 μM FNR + 100 μM dRf, 507 nm; (D) same as (C) at 600 nm; (E and F) 30 μM F65A mutant Fd + 30 μM FNR + 100 μM dRf, 507 nm. Solutions also contained 1 mM EDTA in 4 mM phosphate buffer, pH 7.0. Ionic strength was 12 mM. Note that the trace in panel F is on a 5-s time scale whereas the others are on millisecond time scales.

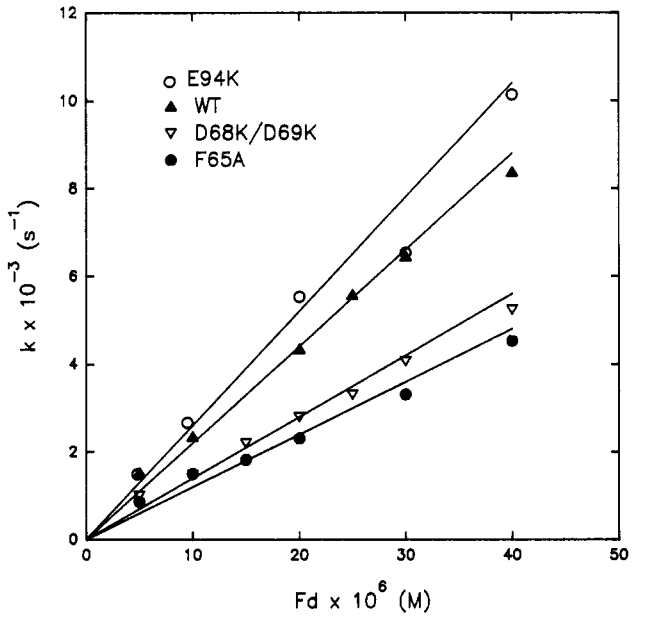

FIGURE 3: Second-order plots for the pseudo-first-order reduction of E94K, wt, D68K/D69K, and F65A Fds by dRf semiquinone. Decay kinetics were monitored at 465 nm. Solutions contained 1 mM EDTA and 95–100 μM dRf in 4 mM phosphate buffer, pH 7.0. Ionic strength was 12 mM.

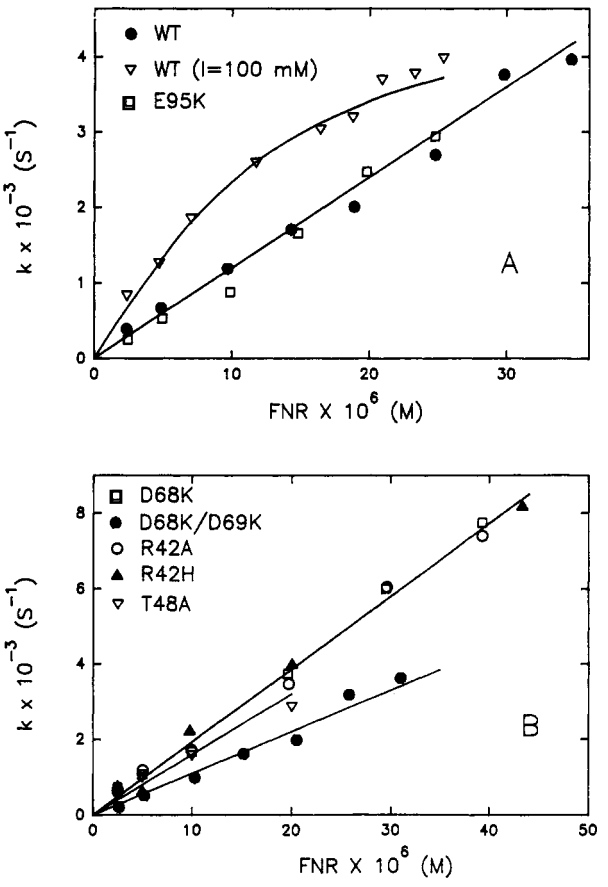

FIGURE 4: Pseudo-first-order rate constants for reduction of various concentrations of FNR by wt and mutant Fds. FNR was titrated into solutions containing (A) 30 μM wt Fd at 12 mM ionic strength, 40 μM wt Fd at 100 mM ionic strength, and 30 μM E95K Fd at 12 mM ionic strength and (B) 40 μM D68K Fd, 30 μM D68K/D69K Fd, 40 μM R42A Fd, 40 μM R42H Fd, and 40 μM T48A Fd. Decay kinetics were monitored at 600 nm. Solutions contained 1 mM EDTA and 100 μM dRf in 4 mM phosphate buffer, pH 7.0. Solutions in panel B had an ionic strength of 12 mM.

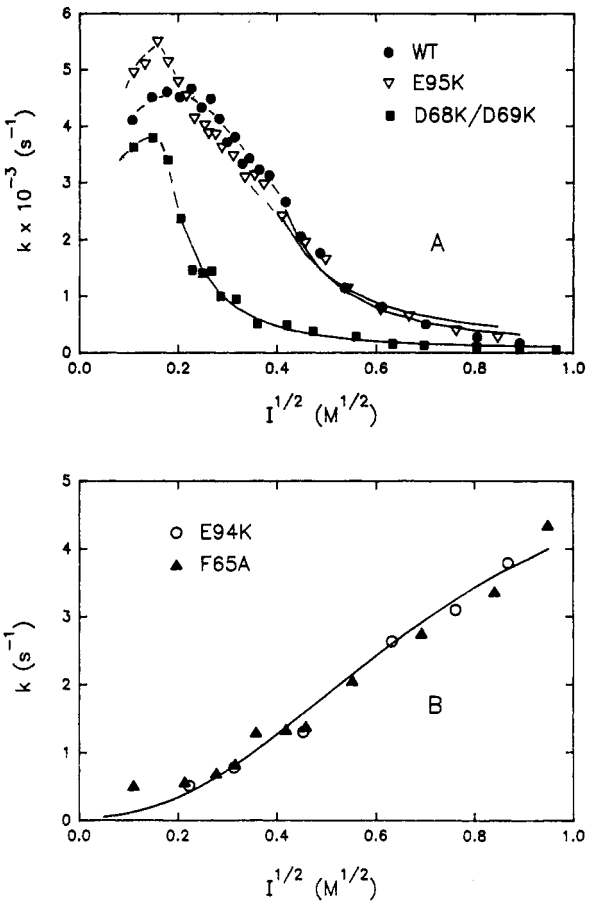

FIGURE 5: Ionic strength dependence of FNR reduction by wt and mutant Fds. Observed pseudo-first-order rate constants are plotted for reaction of FNR with (A) wt Fd, E95K Fd, and D68/D69K Fd and (B) E94K Fd and F65A Fd. Solutions contained 40 μM Fd and 40 μM FNR except for wt and E95K Fds, which were 30 μM in each protein. Other conditions were as in Figure 3. Solid curves are theoretical fits to an electrostatic model referenced in Table III.

a second-order reaction followed by a first-order reaction that becomes rate-limiting at high concentrations [cf. Strickland et al. (1975)]:

$$Fd_{red} + FNR_{ox} \overset{K_d}{\rightleftarrows} (Fd_{red}\text{–}FNR_{ox}) \overset{k_{et}}{\rightleftarrows} Fd_{ox} + FNR_{red}$$

The individual constants according to this mechanism can be obtained from a nonlinear least-squares computer fit of the hyperbolic data (Simondsen & Tollin, 1983; Simondsen et al., 1982). In the present study, we find that not only wt Fd but all of the mutant Fds that react rapidly with FNR (see below) show a linear dependence of k_{obs} on FNR concentration at I = 12 mM. This suggests a first-order rate constant much larger than the largest k_{obs} obtained in this experiment (i.e., >4000 s^{-1}). However, with wt Fd, increasing the ionic strength to 100 mM results in a hyperbolic concentration dependence (data shown in Figure 4A). From these latter data we obtain values of 6.7 μM for K_d and 5100 s^{-1} for k_{et}. These can be compared to values of 12 μM and 6500 s^{-1}, respectively, for these constants obtained previously with nonrecombinant Fd under similar conditions (Walker et al., 1991). Furthermore, in both sets of experiments, the apparent second-order rate constant for complex formation is larger at 100 mM ionic strength than at 12 mM. The reasons for the (relatively small) discrepancies between the present data and those of our earlier study are uncertain at present but may be related to one or more of the following: a recombinant Fd is used in the present work; the buffer conditions were not perfectly matched in the two sets of experiments (0.5 mM vs 1.0 mM EDTA), although this is probably not significant; and the earlier study used Fd and FNR from a slightly different strain of *Anabaena* (7119), although this also is probably not a significant factor based on sequence comparisons. It should be noted that the recombinant and nonrecombinant ferredoxins were identical by NMR criteria (H. Cheng and J. L. Markley, unpublished data). Despite the fact that further experiments will be necessary to clarify this, the data show that the recombinant wt Fd has redox properties that are reasonably close to those of the native *Anabaena* protein. We conclude that the two-step mechanism depicted above is operative in the present case and that the apparent second-order rate constant for the reduction of FNR by reduced Fd (which probably reflects both complex formation and electron transfer steps) can serve as a basis for assessing the effects of mutations on the interaction of Fd with FNR.

We have also examined the effect of ionic strength on the k_{obs} values for reduction of FNR by wt Fd (Figure 5A). The results demonstrate that at low ionic strengths the rate constant increases slightly with increasing ionic strength but that it decreases at higher ionic strengths, as expected for a reaction between oppositely charged species. The increase in k_{obs} with ionic strength in the low ionic strength region has been observed previously for the *Anabaena* 7119 proteins (Walker et al., 1991) and has also been seen in other protein–protein electron transfer systems including cytochrome *c*/cytochrome b_5 (Meyer et al., 1993) and cytochrome *c*/cytochrome *c* oxidase

Table II: Second-Order Rate Constants for Reduction of *Anabaena* FNR by *Anabaena* Ferredoxins at I = 12 mM

	$k \times 10^{-8}$ (M^{-1} s^{-1})		$k \times 10^{-8}$ (M^{-1} s^{-1})
WT	1.2 ± 0.1	F65A	≈0.00007
D68K/D69K	1.1 ± 0.1	F65I	≈0.0002
D68K	1.9 ± 0.2	R42A	2.0 ± 0.3
E94K/E95K	≈0.00007	R42H	2.0 ± 0.2
E95K	1.2 ± 0.1	T48A	1.6 ± 0.2
E94K	≈0.00005	HFd	1.8 ± 0.2

and cytochrome c/cytochrome c peroxidase (Hazzard et al., 1988, 1991). In these previous studies, this effect was attributed to a situation in which the most stable complex at low ionic strength is not optimal for electron transfer and some "loosening" of the complex is necessary to allow the two proteins to assume a mutual orientation that is more favorable to electron transfer. We presume that a similar explanation applies to the present system, although it is also possible that effects on the rate constant for complex formation are also contributing. In the earlier study with nonrecombinant *Anabaena* 7119 Fd (Walker et al., 1991), the results clearly demonstrated that k_{et} had an optimal value at intermediate ionic strengths.

The solid line through the higher ionic strength data in Figure 5A corresponds to a fit to the model of electrostatic interactions developed by Watkins (Watkins, 1986; Tollin et al., 1984), and the parameters derived from this treatment (electrostatic interaction energy, V_{ii}, and rate constant extrapolated to infinite ionic strength, k_∞) are listed in Table III. These values will be compared below to those obtained with some of the mutant Fds.

E94K, E95K, and E94K/E95K Mutants. As a means of assessing the importance of the negative charges at positions 94 and 95 in Fd, we made the double mutant E94K/E95K. Although this mutant Fd reacts with dRfH$^\bullet$ with essentially the same rate constant as does wt Fd (Table I), its reduction of FNR occurred with a second-order rate constant more than 20 000 times smaller than that of wt Fd (Table II; data not shown). The rate of this reaction was close to the lower limit of our instrumentation (due to diffusion of material into and out of the irradiated volume), and thus it was necessary to calculate an approximate second-order rate constant from a single experiment performed at a concentration of 30 μM in each protein. In order to determine whether both glutamate residues were responsible for this dramatic effect, we made the two single mutations E94K and E95K. Again, both mutants reacted with dRfH$^\bullet$ with rate constants similar to those of wt Fd (Table II). E95K reacted with FNR with a rate constant no different from that of wt (Figure 4 and Table II). However, E94K reacted with FNR just as slowly as the double mutant did (Table II). It is clear from these results that the mutation at E94 was the cause of the dramatic change in reaction rate constant with FNR.

In order to assess the possibility that mutagenesis at E94 caused a major structural perturbation in Fd, we carried out several characterizations. Thus, the UV–vis absorption spectrum of E94K is indistinguishable from that of wt Fd (Figure 6), and the UV–vis CD spectrum is also closely similar (Figure 7). Furthermore, the reduction potential of E94K is the same within experimental limits as that of wt Fd (Table III; Figure 8). These results, along with the similarity in behavior with respect to reduction by dRfH$^\bullet$, indicate no influence of the mutation on the properties of the [2Fe–2S] cluster or on the gross polypeptide backbone folding. Attempts are underway to determine the structure of this mutant by X-ray crystallography (H. Holden, personal communication).

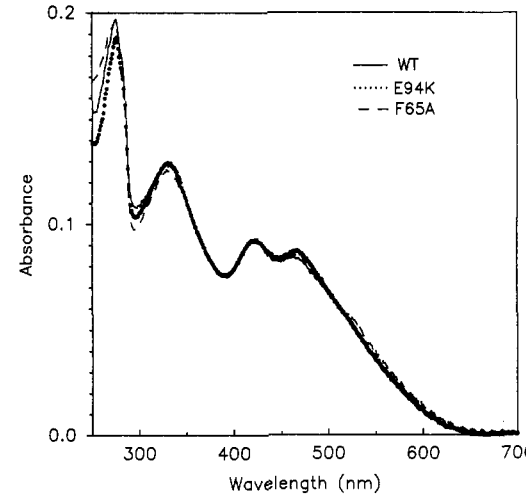

FIGURE 6: UV–vis absorption spectra of wt, E94K, and F65A Fds. Each protein was 9.5 μM in 20 mM Tris buffer, pH 7.3.

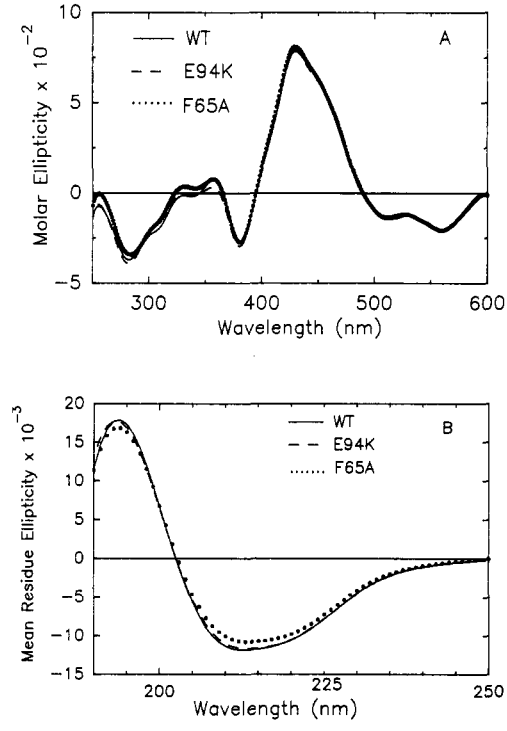

FIGURE 7: Circular dichroism spectra of wt, E94K, and F65A Fds. (A) Visible and near UV spectra. Protein concentrations were 82 μM for wt, 90 μM for E94K, and 97 μM for F65A Fds in 20 mM Tris-HCl buffer, pH 7.3. (B) Far UV spectra. Solutions described in (A) were diluted to 1 μM in water.

Inasmuch as the rate constants measured at low ionic strength for Fd reduction of FNR contain contributions from both complex formation and electron transfer (see above), we also determined a binding constant for the interaction between E94K and FNR (both in their oxidized forms) by spectrophotometric methods. Double-reciprocal plots of the binding data are shown in Figure 9 and the calculated binding constants are given in Table III. Although a significant decrease in complex stability was found (about 3-fold), this was clearly not large enough to account for the kinetic effect. We thus conclude that a substantial decrease in the rate constant of electron transfer within a transient complex with FNR is the major result of the mutation.

The observed rate constant for E95K reduction of FNR showed an ionic strength dependence similar to that of wt Fd (Figure 5A; Table III), although, as might be expected, the

Table III: Reduction Potentials, Electrostatic Parameters,[a] and FNR Dissociation Constants for WT and Some Mutant Ferredoxins

Fd	$E_{1/2}$[b] (mV)	K_d[c] (μM)	V_{ii} (kcal/mol)	$k_\infty \times 10^{-6}$ (M^{-1} s^{-1})
WT	−440 ± 15	9.4 ± 1.0	−11.6	5
E94K	−435 ± 15	26 ± 3	5.7	0.1
F65A	−445 ± 15	120 ± 10	5.7	0.1
D68K	nd	nd	−10.2	4
D68K/D69K	nd	nd	−7.4	2
E95K	nd	nd	−9.4	8
HFd	−445 ± 15	nd	−1.1	96

[a] Cf. Tollin et al. (1984) and Watkins (1986) for details of the methodology used for calculation of electrostatic parameters. An interaction radius of 6 Å was used. [b] Versus normal hydrogen electrode. [c] K_d values at I = 12 mM were calculated at each protein concentration in Figure 9 using the difference extinction coefficient given in Materials and Methods. The average value is presented in the table.

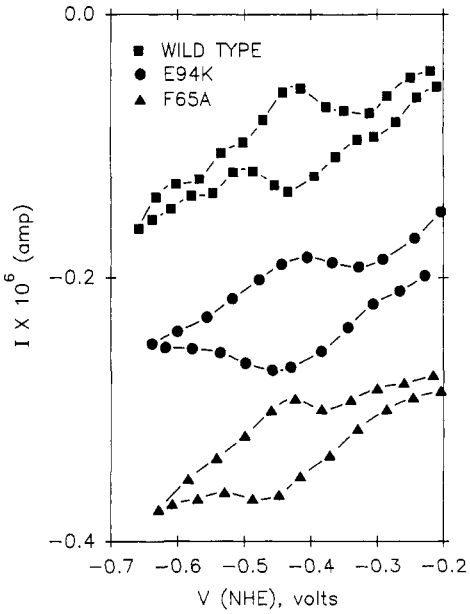

FIGURE 8: Cyclic voltammograms for wt, E94K, and F65A Fds. Curves are offset for clarity. Conditions were as described in Materials and Methods.

calculated electrostatic interaction energy (V_{ii}) is slightly smaller. In marked contrast, E94K showed a dependence that was dramatically different (Figure 5B). With the latter mutant, the rate constant increased in magnitude with increasing ionic strength up to 750 mM NaCl. A reasonable explanation for this result is that the orientation of the complex at all ionic strengths is so far from optimal that any "loosening" by salt addition allows the proteins to assume an orientation that is more favorable for electron transfer. Data over the entire ionic strength range could be fit by the Watkins equation. An electrostatic interaction energy for E94K was calculated whose magnitude is smaller than that of wt Fd and whose sign is positive; the k_∞ value is also much smaller than the wt value (Table III). Interestingly, the latter rate constant values are considerably closer to one another than are the second-order rate constants at I = 12 mM, consistent with the above explanation in terms of mutual intracomplex orientation.

The difference observed here between E94K and E95K is quite remarkable, considering how close these two adjacent residues are in the Fd structure (Figure 1). This demonstrates a high level of specificity in the Fd/FNR interaction. We will return to this point below. Furthermore, although these experimental results agree with the chemical modification studies (Zanetti et al., 1988; Vieira et al., 1986), they are in

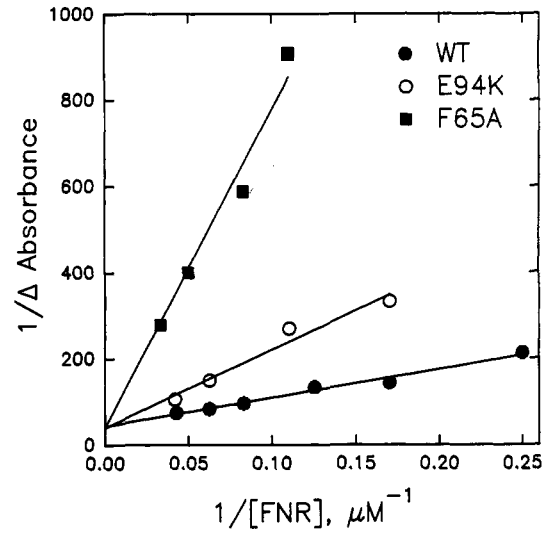

FIGURE 9: Double-reciprocal plot of absorbance change at 460 nm due to complex formation between oxidized *Anabaena* Fd and oxidized *Anabaena* FNR. FNR was titrated into a 10 μM solution of Fd as described in Materials and Methods. Ionic strength was 12 mM. Solid lines correspond to least-squares fits to the data points.

apparent disagreement with the model proposed by Karplus et al. (1991) based on computer graphics docking of *Spirulina* Fd with spinach FNR in which E95 was suggested as a key residue. [However, in another report Karplus (1991) suggested that E94 also was an important residue.] Whether or not this represents a species specificity remains to be determined. In this regard, Walker et al. (1991) found significant differences between the spinach and *Anabaena* systems with regard to the Fd/FNR electron transfer reaction.

F65A and F65I Mutants. F65A Fd reacts with dRfH$^\bullet$ with a rate constant (Figure 3; Table I) that is not too different from that of wt Fd, but like E94K, reduced F65A reacts with FNR with an approximate second-order rate constant more than 4 orders of magnitude smaller than wt Fd (Table II). The kinetic traces shown in Figure 2E,F demonstrate the slowness of this reaction relative to that of wt Fd. As was the case with E94K, the reduction potential is the same as that of wt Fd (Table III), and no major structural changes were indicated by the UV–vis (Figure 6) and CD spectra (Figure 7). The effect of ionic strength is also very similar to that obtained with E94K (Figure 5B; Table III), which is quite remarkable since the F65A mutation involves no change in the protein charge. This suggests the occurrence of a high degree of surface complementarity at the complex interface. It is interesting that the complex formed between oxidized F65A and oxidized FNR is even weaker than that for the E94K mutant (Table III). This may suggest an important contribution from hydrophobic interactions in complex stabilization [cf. Walker et al. (1991)]. However, again the change in binding constant is too small to completely account for the kinetic results, implying that intracomplex electron transfer is also being significantly altered.

In order to investigate whether or not a bulky aliphatic residue could effectively replace F65, we also made the F65I mutant. However, the electron transfer properties of this mutant were not significantly different from those of F65A (Tables I and II). These results open the possibility that the aromatic nature of the side chain at position 65 is an important component of the electron transfer process. This is currently being further investigated.

D68K and D68K/D69K Mutants. The acidic residues D68 and D69 are in a region implicated by cross-linking studies

in binding (Vieira et al., 1986) and are on the same side of the Fd molecule as E94 and F65. We therefore made the charge reversal mutants D68K and D68K/D69K. Both of these mutants reacted with dRfH$^{\bullet}$ and FNR with rate constants similar to those of wt Fd (Figures 3 and 4; Tables I and II). Furthermore, in its reaction with FNR, the double mutant D68K/D69K showed an ionic strength dependence of k_{obs} (Figure 5A) which has a shape similar to that of wt Fd, although it appears to be appreciably less reactive at higher ionic strengths than is wt Fd and has an optimum at I = 20 mM rather than I = 40 mM. Consistent with its decreased negative electrostatic charge, the V_{ii} value derived from the Watkins model is significantly smaller than that obtained with wt Fd, and the rate constant extrapolated to infinite ionic strength is also significantly smaller (Table III). Again, as expected on the basis of net charge, the D68K mutant yields a V_{ii} value only slightly smaller than that of wt Fd (Table III). We conclude from these data that although D68 and D69 are clearly not critical residues, this region of the Fd molecule does seem to have a significant, albeit small, interaction with FNR.

R42H, R42A, and R42E Mutants. R42 is a highly conserved residue (present in 36 of 38 sequenced plant-type Fds; Matsubara & Hase, 1983) and has been shown to be H-bonded through its amide nitrogen to one of the inorganic sulfurs of the [2Fe–2S] cluster (Skjeldal et al., 1991). Interestingly, one of the Fds in which R42 is not conserved is the heterocyst Fd from *Anabaena* 7120 (see below), where a histidine residue occupies position 42 (Böhme & Haselkorn, 1988). This latter Fd participates in electron transfer to nitrogenase during N_2 fixation but is not involved in photosynthetic electron transfer (Böhme & Schrautemeier, 1987). In the present experiments, both R42H and R42A reacted with dRfH$^{\bullet}$ and FNR with rate constants comparable to those of wt Fd (Figure 4; Tables I and II), and both mutant proteins showed ionic strength dependencies for the FNR reaction similar to that of wt Fd (data not shown). These mutant proteins also have normal UV–visible spectra (not shown). In preliminary experiments, we found that the R42E charge reversal mutant displays reactivity similar to that of wt Fd with regard to both dRfH$^{\bullet}$ reduction and electron transfer to FNR. Thus, it is quite clear that R42 is not critically involved in the electron transfer reactions of Fd, nor is a basic residue required at this position.

T48A Mutant. T48 is in the same region of the Fd molecule as the critical residues E94 and F65 and thus might be a good candidate for interaction with FNR. However, reduced T48A reacted with FNR just as well as wt Fd (Figure 4; Table II), and the oxidized protein had comparable reactivity with dRfH$^{\bullet}$ (Table I). Ionic strength effects on its interaction with FNR (data not shown) are similar to those of the wt Fd and the R mutants discussed above. Again, this mutant has normal UV–visible absorption spectra (not shown). Thus, T48, like D68, D69, and R42, is not a critical residue in the interaction of *Anabaena* Fd and FNR.

Heterocyst Fd. As stated above, HFd functions *in vivo* in N_2 fixation and not in photosynthetic electron transfer. However, steady-state experiments have demonstrated that HFd is as active as vegetative Fd in $NADP^+$ photoreduction with an *in vitro* system employing heterocyst thylakoids, cytochrome *c*553, and vegetative cell FNR (Schrautemeier & Böhme, 1985). Consistent with this, we find that HFd reacts with FNR with a rate constant comparable to that of wt vegetative Fd (Table II). It also has comparable reactivity with dRfH$^{\bullet}$ (Table I) and a similar redox potential (Table III). Thus, the properties of this "natural mutant" of Fd are consistent with our results with the genetically engineered R42 mutants. Also consistent with the mutagenesis experiments is the fact that both F65 and E94 are present in the heterocyst protein, whereas E95 is replaced by proline and T48 by serine.

A Watkins analysis of the k_{obs} vs ionic strength data for the reaction of the heterocyst Fd with FNR (not shown) yields V_{ii} = –1.1 kcal/mol and $k_\infty = 96 \times 10^6$ (Table III). The smaller electrostatic interaction energy is consistent with a smaller net negative charge for the heterocyst Fd (–9 vs –14 for the vegetative Fd), whereas the larger value for k_∞ suggests the possibility of a more favorable steric interaction when the electrostatic factors are no longer operative. Interestingly, the terminal amino acid at position 98 in HFd is alanine, whereas tyrosine occupies this position in wt vegetative Fd. This residue is located close to E94 and F65 in the vegetative protein. Experiments are presently underway to determine whether or not Y98 exerts a steric influence during electron transfer. At the very least, the present results demonstrate that Y98 is not a critical residue for the Fd/FNR interaction.

CONCLUSIONS

The present results demonstrate that a highly localized region of the ferredoxin surface contains amino acid side chains that are critical components of the electron transfer interaction between *Anabaena* Fd and FNR. Thus, the charge reversal mutation at E94 resulted in a dramatic decrease (approximately 20 000-fold) in the second-order rate constant for interprotein electron transfer, whereas a similar mutation at an adjacent site (E95) yielded approximately wild-type behavior. A rate constant difference of this magnitude could not be accounted for by the small increase in the K_d value for complex formation and was also not due to an altered reduction potential or to gross structural perturbations resulting from the mutation. Similarly, mutations at F65 led to analogous decreases in protein–protein electron transfer rate constants which also could not be attributed to the abovementioned effects. Alterations at four other residues (D68, D69, T48, and R42) located in the general vicinity of F65 and E94 also do not appreciably perturb the Fd/FNR interaction. These results provide clear evidence for a high degree of localization and specificity in the interface region between the two proteins which is involved in the electron transfer process. A similar high level of structural specificity has also been observed in the yeast cytochrome *c*/cytochrome *c* peroxidase system (Mauro et al., 1988) and in the mitochondrial Fd/NADPH–Fd oxidoreductase/cytochrome $P450_{scc}$ system (Coghlan & Vickery, 1992).

The precise roles of E94 and F65 in mediating electron transfer between the two proteins cannot be ascertained from the present experiments. Other mutations will be required in order to delineate this, e.g., E94Q, E94D, F65Y, and F65W. Such experiments are presently underway. Furthermore, it will be instructive to determine if other residues close to E94 and F65 (e.g., S47, D67, and Y98) are also important in the protein–protein interaction, and experiments along these lines are in progress. Finally, it is important to ascertain whether or not the same Fd residues that critically modulate Fd/FNR electron transfer also play analogous roles in electron transfer from photosystem I to Fd, and experiments designed to test this are being carried out.

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