Lys75 of *Anabaena* Ferredoxin−$NADP^+$ Reductase Is a Critical Residue for Binding Ferredoxin and Flavodoxin during Electron Transfer†

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ABSTRACT: Previous studies, and the three-dimensional structure of *Anabaena* PCC 7119 ferredoxin−$NADP^+$ reductase (FNR), indicate that the positive charge of Lys75 might be directly involved in the interaction between FNR and its protein partners, ferredoxin (Fd) and flavodoxin (Fld). To assess this possibility, this residue has been replaced by another positively charged residue, Arg, by two uncharged residues, Gln and Ser, and by a negatively charged residue, Glu. UV−vis absorption, fluorescence, and CD spectroscopies of these FNR mutants (Lys75Arg, Lys75Gln, Lys75Ser, and Lys75Glu) indicate that all the mutated proteins folded properly and that significant protein structural rearrangements did not occur. Steady-state kinetic parameters for these FNR mutants, utilizing the diaphorase activity with DCPIP, indicate that Lys75 is not a critical residue for complex formation and electron transfer (ET) between FNR and $NADP^+$ or NADPH. However, steady-state kinetic activities requiring complex formation and ET between FNR and Fd or Fld were appreciably affected when the positive charge at position of Lys75 was removed, and the ET reaction was not even measurable if a negatively charged residue was placed at this position. These kinetic parameters also suggest that it is complex formation that is affected by mutation. Consistent with this, when dissociation constants (K_d) for FNR_{ox}−Fd_{ox} (differential spectroscopy) and FNR_{ox}−Fd_{rd} (laser flash photolysis) were measured, it was found that neutralization of the positive charge at position 75 increased the K_d values by 50−100-fold, and that no complex formation could be detected upon introduction of a negative charge at this position. Fast transient kinetic studies also corroborated the fact that removal of the positive charge at position 75 of FNR appreciably affects the complex formation process with its protein partners but indicates that ET is still achieved in all the reactions. This study thus clearly establishes the requirement of a positive charge at position Lys75 for complex formation during ET between FNR and its physiological protein partners. The results also suggest that the interaction of this residue with its protein partners is not structurally specific, since Lys75 can still be efficiently substituted by an arginine, but is definitely charge specific.

Most of the energy used by living organisms in their biological functions originates from electron transfer (ET)[1] reactions between proteins. These reactions can only take place if proper binding of the proteins involved occurs. To obtain a better understanding of the structural basis for these reactions, we have used ferredoxin−$NADP^+$ reductase (FNR) from the cyanobacterium *Anabaena* PCC 7119 (FNR, 1.18.1.12) and its physiological reaction partners, ferredoxin (Fd) and flavodoxin (Fld), as a model system. FNR is a flavoenzyme which acts during photosynthesis, catalyzing ET from the one-electron carrier Fd to the two-electron acceptor $NADP^+$ (*1*), to produce NADPH, according to reaction 1.

$$2Fd_{rd} + NADP^+ + H^+ \rightarrow 2Fd_{ox} + NADPH \quad (1)$$

The three-dimensional structures of oxidized and reduced spinach FNR and that of a complex with 2′-phospho-5′-AMP have been reported (*2*). Moreover, the three-dimensional structure of this enzyme is the prototype of a large family of flavin-dependent oxidoreductases that function as transducers between nicotinamide dinucleotides (two-electron carriers) and one-electron carriers (*2*−*4*).

FNR consists of a single 303-amino acid chain (*5*) and a single molecule of FAD which interacts noncovalently with the apoprotein. When this organism is grown under low-

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[1] Abbreviations: FNR, *Anabaena* PCC 7119 ferredoxin−$NADP^+$ reductase; FNR_{ox}, FNR in the oxidized state; FNR_{rd}, FNR in the reduced state; FNR_{sq}, FNR in the semiquinone state; Fd, ferredoxin; Fd_{ox}, Fd in the oxidized state; Fd_{rd}, Fd in the reduced state; Fld, flavodoxin; Fld_{ox}, Fld in the oxidized state; Fld_{sq}, Fld in the semiquinone state; Fld_{rd}, Fld in the reduced state; IPTG, isopropyl β-D-thiogalactoside; dRf, 5-deazariboflavin; $dRfH^{\bullet}$, semiquinone form of dRf; DCPIP, 2,6-dichlorophenolindophenol; ET, electron transfer; PDR, phthalate dioxygenase reductase; wt, wild type; *I*, ionic strength.

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iron conditions, Fld is synthesized instead of Fd, and replaces it in the ET reaction from PSI to FNR (*6*). The ET reactions between FNR and its physiological partners ($NADP^+$, Fd, and Fld) have been extensively studied (*7*−*15*). The three-dimensional structure of *Anabaena* FNR has recently been determined (*16*). It resembles that of the spinach enzyme and consists of two distinct domains. The FAD binding domain contains a scaffold of six antiparallel strands arranged in two perpendicular β-sheets, the bottom of which is capped by a short α-helix and a long loop. The $NADP^+$ binding domain consists of a core of five parallel β-strands surrounded by seven α-helices. The edge of the dimethylbenzyl ring of FAD, which is the only part of the flavin isoalloxazine moiety exposed to the solvent, is putatively involved in the intermolecular ET with Fd or Fld. FNR and Fd (or Fld) form an electrostatically stabilized 1:1 complex, which has been extensively studied (*15*, *17*, *18*), although other studies have shown that both electrostatic interactions and hydrophobic forces must be important in the FNR−Fd ET interaction (*11*, *19*). It is also accepted that FNR contributes basic residues, while Fd contributes acidic residues, to the electrostatic stabilization of the complex (*18*). The site of Fd binding to FNR has been studied by a variety of techniques such as chemical modification (*12*, *13*, *21*), chemical cross-linking (*22*), site-directed mutagenesis (*23*, *24*), and the three-dimensional structural analysis of both proteins (*16*, *25*). All of these studies provide evidence that Fd must bind to FNR in a concave site in the FAD binding domain.

Attempts to crystallize the FNR−Fd complex are in progress**,** and FNR−Fd model complexes based upon the known crystal structures of both proteins and various biochemical data have been proposed (*25*, *26*). Use of the three-dimensional structure of PDR, a member of the FNR family which possesses a Fd domain bound to the FNR-like domain, has not led to the production of a satisfactory model for the FNR−Fd complex (*4*). However, there is a large volume of information concerning the binding of Fd to FNR. In the case of the *Anabaena* proteins, a dissociation constant (K_d) of 4 μM (*14*) has been determined. It is also proposed that Fd must bind within the large cleft which is situated between the two domains of FNR and that the Fd iron−sulfur center must be situated close to the methyl groups of the flavin ring, which is the only part of the FAD exposed to the solvent (*16*, *25*). A careful study of the FNR surface around the expected ET site reveals the presence of some hydrophobic residues that also could be involved in the binding between FNR and Fd (*3*). Nevertheless, these cannot be the only forces involved in complex formation, and several patches of positively charged residues have been implicated (*26*). Among these, there is one situated at the *si* face of the flavin ring, between residues Lys69 and Tyr79 of *Anabaena* PCC 7119 FNR, which includes four positively charged residues conserved among all the FNR sequences analyzed (Table 1). In the spinach enzyme, Lys85 and Lys88 (equivalent to Lys69 and Lys72 in *Anabaena*, respectively) have been implicated in complex formation with Fd (*22*). Also, site-directed mutagenesis studies of Lys72 in the *Anabaena* and the equivalent**,** Lys88**,** in the spinach enzymes have also pointed out the importance of such residues in both species (*23*, *24*). Thus far, no active mutants that properly bind FAD have been obtained at Arg77, a residue that is completely conserved in FNRs isolated from various sources and in other proteins belonging to the same family (Table 1), which would allow a study of its involvement in the interaction of FNR with Fd (*24*). Recently, we have also observed that replacement of Lys75 of FNR produced an enzyme with altered properties (*24*). The amine group of Lys75 is situated 12 Å from the exposed methyl groups of the flavin, so, in principle, it is not expected to be directly involved in the catalytic process. Nevertheless, it is conserved in all the FNR sequences analyzed thus far (Table 1), but not in other members of the family, indicating that although its function is preserved well in all of the FNRs, it is not a critical residue in the rest of the family members. In this study, further protein engineering on Lys75 has been carried out to investigate its role in complex formation and ET to Fd or Fld. Removal of this positive charge might be expected to produce significant changes in complex stabilization between FNR and its protein partners. We have replaced Lys75 by Arg, Gln, Ser, and Glu, thereby altering the side chain charge, polarity, and hydrogen-bonding capabilities. These mutants were studied by steady-state and transient kinetic measurements as well as by UV−vis absorption, fluorescence, and CD spectroscopies to rationalize their behavior.

Table 1: Conservation of the Lys69−Thr84 Loop of *Anabaena* PCC 7119 in the FNR Family[a]

FNRs	69	84	Ref
Anabaena PCC 7119	K N G K P E K L R L Y S I A S T		(*5*)
Spirulina. sp	N * * * * H * * * * * * * * * *		(*44*)
Spinach	* * * * * H * * * * * * * * * S		(*2*)
M. crystalinum	* * * * * H * * * * * * * * * S		(*45*)
P. sativum	* * * * * H * * * * * * * * * S		(*46*)
C. paradoxa	* D * * * H * * * * * * * * * *		(*47*)
Synechococcus 7002	* * * * * H * * * * * * * * * *		(*48*)
Other reductases of the FNR family			
Rat liver cytochrome P450 reductase	A L * N # L Q A * Y * * * * * -		(*49*)
Salmonella typhimurium sulfite reductase	A L V * # L T P * * * * * * * -		(*50*)
Human erythrocytes cytochrome b_5 reductase	- - # G N L V V * P * T P I * S		(*51*)
Pseudomonas cepacia phthalate dioxygenase reductase	- - - - - G S R * T * * L C N D		(*52*)

[a] The final alignments were obtained by visual inspection. Asterisks indicate exact matches of the sequences to that of *Anabaena* PCC 7119 FNR. Hyphens are packing characters introduced to align the sequences.

MATERIALS AND METHODS

Oligonucleotide-Directed Mutagenesis. FNR mutants at position 75 were produced using as a template a construct of the *petH* gene which had been previously cloned into the expression vector pTrc99a (*5*). The MORPH mutagenesis kit (5 Prime 3 Prime, Boulder, CO) was used to prepare the Lys75Gln, Lys75Arg, and Lys75Ser mutants. The mutagenic primers used were 5′ GTA GAG TCT CAA "ACG" TTC CGG CTT GC 3′ for Lys75Arg, 5′ GTA GAG TCT CAA "CTG" TTC CGG CTT GC 3′ for Lys75Gln, and 5′ GTA GAG TCT CAA "GGA" TTC CGG CTT GC 3′ for Lys75Ser. The Lys75Glu substitution was obtained using the Transformer site-directed mutagenesis kit from CLONTECH in combination with the synthetic oligonucleotide 5′ GAG TCT CAA TT"C" TTC CGG CTT GCC 3′ for Lys75Glu and the trans oligo *Nde*I−*Sac*II 5′ AGT GCA CCA

TCC GCG GTG TGA 3′. Mutations were verified by DNA sequence analysis. The constructs containing the mutated FNR gene were used to transform the *Escherichia coli* PC 0225 strain (*27*).

Purification of Proteins. FNR mutants were purified from IPTG-induced cultures as previously described (*14*, *27*). Recombinant Fd and Fld from *Anabaena* were prepared as described (*7*, *28*). UV−visible spectra and SDS−PAGE were used as purity criteria.

Spectral Analysis. UV−visible spectral analyses were carried out on either a CARY-15 (OLIS-modified), a Hewlett-Packard 8452 diode array, or a Kontron Uvikon 942 spectrophotometer. Circular dichroism (CD) spectra were obtained using either a Jasco 710 or an Aviv model 62A DS spectropolarimeter at room temperature in a 1 cm path length cuvette. The spectra were recorded on solutions having protein concentrations ranging from 55 to 77 μM in the visible region (300−620 nm), of 4 μM for the aromatic region, and of 0.7 μM for the far-UV region. Samples were prepared in 20 mM Tris-HCl (pH 7.3) for the visible region and in 1 mM Tris-HCl (pH 8.0) for the aromatic and far-UV regions. Protein and flavin fluorescence were monitored using a Kontron SFM 25 spectrofluorometer interfaced with a personal computer. Solutions for fluorescence measurements contained 4 μM protein in 50 mM Tris-HCl (pH 8.0).

Binding Constants. K_d values and binding energies of the complexes between oxidized Fd and the different oxidized FNR mutants were obtained at room temperature by difference absorption spectroscopy as previously described (*15*). Fd was titrated into a sample containing 10−17 μM FNR, except for Lys75Arg in which case the mutated FNR was titrated into 30 μM Fd. In addition to protein, solutions also contained 1 mM EDTA in 4 mM potassium phosphate buffer (pH 7.0). The ionic strength (I) was adjusted to 100 mM by adding 5 M NaCl (these conditions simulated those used in the laser flash photolysis experiments; see below). In those cases in which saturation was not obtained due to very weak binding, a value of K_d was estimated by calculation from the difference spectra, assuming that the extinction coefficient for complex formation with Fd of these mutants was the same as those obtained experimentally for the wt Fd−wt FNR and wt Fd−Lys75Arg FNR complexes ($\Delta\epsilon_{460nm} = 2000 \pm 300$ M^{-1} cm^{-1}).

Enzymatic Assays. The diaphorase activity using DCPIP as an electron acceptor, and the Fd or Fld FNR-dependent NADPH−cytochrome *c* reductase activity using Fd or Fld as a mediator, were measured as described previously (*14*, *29*, *30*). All measurements were taken at 25 °C in 50 mM Tris-HCl (pH 8.0), unless otherwise stated.

Laser Flash Photolysis Measurements. The pulsed laser photolysis apparatus used to obtain transient ET kinetics has been described previously (*20*, *31*, *32*), except that a Tektronix TDS410A digitizing oscilloscope was used in the current system. The photochemical reaction which initiates ET has also been described (*33*−*35*). Laser flash-induced kinetic measurements were taken at room temperature. In addition to protein, samples also contained 1 mM EDTA and 95−100 μM dRf in 4 mM potassium phosphate buffer (pH 7.0). The ionic strength of the solution was adjusted to 100 mM using 5 M NaCl. Samples were made anaerobic as described previously (*14*). Binding constants for the transient Fd_{rd}−FNR_{ox} complexes were determined by fitting the laser flash photolysis data (cf. Figure 2A) to the exact solution of the differential equation describing the minimal (two-step) mechanism (*36*, *37*).

Estimation of Reduction Potentials. Reduction potentials for the oxidized−semiquinone couples ($E_1°$) of the different FNR mutants were estimated by comparison of the extent of reoxidation of Fd_{rd} by FNR_{ox} observed at 507 nm in the laser flash photolysis measurements to that produced with wt FNR.

Stopped-Flow Measurements. ET processes between FNR and Fd, or Fld, were studied by stopped-flow methods using an Applied Photophysics SX17.MV spectrophotometer interfaced with an Acorn 5000 computer and the SX.17MV software of Applied Photophysics under anaerobic conditions as previously described (*14*) in 50 mM Tris-HCl (pH 8.0) at 13 °C. These conditions, as well as protein concentrations, were chosen to slow the very fast reactions observed for the wt protein, thereby making them detectable by this technique. Two wavelengths appropriate for following the reactions were chosen for each reaction since our system only allows the measurement of a single wavelength at a time. The ratio of proteins was chosen such that the FNR protein partner concentration was at least twice that of FNR and that the total protein concentration was low enough that the detector was operating in the linear range. The protocol for anaerobic stopped-flow sample production (*14*) does not allow an exact control of protein concentration, making it impossible to prepare all of the different mutant samples at the same concentration. Concentrations were recorded at the end of the experiment. However, we attempted to keep concentrations within certain limits to enable the observed rate constants to be used for comparative purposes.

RESULTS

Expression and Purification of the Lys75 FNR Mutants. The level of expression in *E. coli* of all the mutants prepared at position 75 of *Anabaena* PCC 7119 FNR was judged to be similar to that of the recombinant wt enzyme. All mutants were purified by following the same protocol that was used for the wt FNR, and about 5 mg of protein per liter of culture was obtained in homogeneous form, as shown by SDS−PAGE.

Spectral Properties of the Lys75 Mutants. No major differences were detected in the UV−visible, FAD, and protein fluorescence and circular dichroism spectra of the different FNR Lys75 mutants relative to those of the wt FNR (not shown). These data indicate that no major structural perturbations have taken place upon replacement of Lys75 in *Anabaena* PCC 7119 FNR by Arg, Gln, Ser, or Glu.

Steady-State Kinetics. The steady-state catalytic behaviors of the different FNR mutants at Lys75 were determined for two reactions catalyzed by FNR. All the kinetic parameters were obtained by fitting the data to the equation for a ping-pong mechanism. The values calculated for the FNR mutants are reported in Table 2 and compared with those obtained for the wt enzyme (*14*). No major effects were detected in any of the kinetic parameters for the diaphorase activity. K_m values for NADPH were in the same range as those reported for wt FNR. When the k_{cat} values were analyzed, the largest effect was found for Lys75Glu FNR, for which the k_{cat} value is 59% of that of wt FNR. Nevertheless, the catalytic efficiency of this mutant is still 79% of that of the wt enzyme.

Table 2: Steady-State Kinetic Parameters of Wild-Type FNR and Lys75 FNR Mutants

Diaphorase Activity with DCPIP			
FNR	k_{cat} (s^{-1})	K_m^{NADPH} (μM)	k_{cat}/K_m^{NADPH} (μM^{-1} s^{-1})
wild-type[a]	81.5 ± 3.0	6.02 ± 0.54	13.5 ± 0.5
Lys75Arg	71.7 ± 7.6	4.9 ± 1.1	14.6 ± 1.4
Lys75Gln	48.3 ± 1.7	3.0 ± 0.4	16.1 ± 1.4
Lys75Ser	65.0 ± 2.8	3.56 ± 0.51	18.3 ± 1.6
Lys75Glu	48.3 ± 1.0	4.57 ± 0.36	10.6 ± 0.6

NADPH-Dependent Cytochrome *c* Reductase Activity						
	with ferredoxin as a mediator			with flavodoxin as a mediator		
FNR	k_{cat} (s^{-1})	K_m^{Fd} (μM)	k_{cat}/K_m^{Fd} (μM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m^{Fld} (μM)	k_{cat}/K_m^{Fld} (μM^{-1} s^{-1})
wild-type[a]	200 ± 10	11 ± 2	18.2 ± 1.0	23.3 ± 1.6	33 ± 5	0.70 ± 0.05
Lys75Arg	270 ± 20	94 ± 9	2.9 ± 0.1	23.3 ± 2.2	15 ± 3	1.6 ± 0.2
Lys75Gln	190 ± 33	>500	<0.38	27 ± 6	632 ± 144	0.042 ± 0.001
Lys75Ser	200 ± 35	>500	<0.4	50 ± 7	800 ± 170	0.062 ± 0.004
Lys75Glu	–	–	–	–	–	–

[a] Data from ref *14*.

The NADPH-dependent cytochrome *c* reductase activity of the different FNR forms was studied using either Fd or Fld as a mediator for this activity. Replacement of Lys75 by Glu produced an enzyme that did not show any activity with Fd or with Fld. Moreover, the Lys75Gln and the Lys75Ser FNR mutants also showed low catalytic efficiencies compared to the wt protein (Table 2). However, these two mutants had k_{cat} values in the same range as the wt protein, while their K_m values were much larger than that for wt FNR. These results indicate that replacement of Lys75 by either Gln or Ser affects the complex formation process and not the ET process. Replacement of Lys75 by Arg produced a FNR with kinetic parameters, with either Fd or with Fld, similar to those for the wt enzyme. This indicates that this enzyme is capable of forming complexes with Fd and Fld and transferring electrons efficiently from NADPH to its protein partners. It is noteworthy that the kinetic parameters for the different FNR variants at position 75, obtained using either Fd or Fld as a mediator, are parallel although with different magnitudes.

Interaction of Lys75 FNR_{ox} Mutants with Fd_{ox}. To determine whether the mutations affected the Fd binding site of the reductase, the binding of the different FNR_{ox} mutant forms to Fd_{ox} was evaluated using difference absorption spectroscopy (*15*). The K_d values and the binding energies obtained for $FNR_{ox}-Fd_{ox}$ complex formation for wt and mutant FNRs are reported in Table 3. While Lys75Arg FNR appears to behave like the wt enzyme, no accurate K_d values could be obtained for the other three mutants, suggesting a very weak binding. K_d values were estimated for Lys75Gln and Lys75Ser (as described in Materials and Methods) and were 115 and 60 times larger than those measured for the wt FNR, respectively. No binding could be measured for the Lys75Glu $FNR_{ox}-Fd_{ox}$ interaction over the accessible concentration range.

Reduction of Lys75 FNR Mutants Studied by Laser Flash Photolysis. Upon laser flash photolysis of a solution containing dRf and EDTA, absorbance transients are produced corresponding to the rapidly formed dRf triplet state, and the dRf semiquinone radical ($dRfH^{\bullet}$) resulting from the abstraction of a hydrogen atom from EDTA by the dRf triplet. The $dRfH^{\bullet}$ radical decays by disproportionation and, in competition with this mode of decay, will reduce FNR_{ox} (or Fd_{ox}) if this is present in the reaction mixture (*19*, *33*−*35*, *38*). The reduction of all FNR mutants at Lys75 by laser-generated $dRfH^{\bullet}$ was monitored by the absorbance increase at 600 nm, corresponding to FNR_{sq} formation. Transients were fitted well by monoexponential curves, and the obtained rate constants were within a factor of 2 of that of the wt FNR protein (Table 4), indicating that the FAD of all the mutants is accessible and redox active. Reduction of Fd_{ox} by $dRfH^{\bullet}$ can be followed at 507 nm (a wavelength which is an isosbestic point for the transition between FNR_{ox} and FNR_{sq} and principally monitors absorbance changes due to $dRfH^{\bullet}$ and Fd_{rd}; see Figure 4 from ref *39* for details), where a rapid rise in absorbance is obtained due to the production of $dRfH^{\bullet}$, followed by an exponential decrease in light absorption to below the preflash baseline as Fd_{ox} is reduced by $dRfH^{\bullet}$. If both FNR_{ox} and Fd_{ox} are present in the reaction mixture, an initial decrease in absorbance is observed at 507 nm due to the reduction of Fd_{ox} by $dRfH^{\bullet}$ and a subsequent rise in absorbance at 507 nm (data not shown; cf. refs *7* and *40* for representative kinetic traces at 507 nm) due to the reoxidation of Fd_{rd} by FNR_{ox}. This rise in absorbance is accompanied by an absorbance increase at 600 nm (a wavelength that principally monitors absorbance changes due to FNR_{sq}) due to the formation of FNR_{sq} upon reduction of FNR_{ox} by Fd_{rd}. As expected, the absorbance increases at these two wavelengths follow the same kinetics. Figure 1

Table 3: Constants for Binding of Oxidized Wild-Type Fd to Oxidized Lys75 FNR Mutants and to Wild-Type FNR[a]

FNR	K_d (μM)	$\Delta G°$ (kcal/mol^{-1})
wild-type	3.3 ± 0.6[b]	−7.4 ± 0.1
Lys75Arg	4.8 ± 0.5	−7.2 ± 0.1
Lys75Gln	380 ± 20[c]	−4.6 ± 0.1
Lys75Ser	200 ± 60[c]	−5.0 ± 0.2
Lys75Glu	nd[d]	nd

[a] Solutions also contained 1 mM EDTA in 4 mM potassium phosphate buffer (pH 7.0). The ionic strength was adjusted to 100 mM using 5 M NaCl. [b] Taken from ref *31*. [c] Binding was too weak to observe saturation in the experimentally accessible protein concentration range. Therefore, K_d was determined from difference spectra, assuming that the difference extintion coefficient for the complex was the same as that $\Delta\epsilon$ calculated for the complexes of Lys75Arg and wild-type FNR with wild-type Fd ($\Delta\epsilon = 2000 \pm 300$ M^{-1} cm^{-1}). [d] The spectral perturbation due to binding was too weak to be measured for this mutant.

Table 4: Kinetic Parameters for the Reduction of the Different Lys75 FNR Mutants Studied by Laser Flash Photolysis[a]

FNR	reduction by dRfH• $k \times 10^{-8}$ (M^{-1} s^{-1})	reduction by ferredoxin K_d (μM)[b]	$\Delta G°$ (kcal/mol^{-1})	k_{ET} (s^{-1})[b]	$k \times 10^{-9}$ (M^{-1} s^{-1})[c]
wild-type[e]	2.1 ± 0.1[d]	1.7 ± 0.1[e]	-7.80 ± 0.04	5500 ± 400[e]	1.4 ± 0.1[f]
Lys75Arg	2.3 ± 0.1	3.0 ± 0.4[e]	-7.50 ± 0.08	4900 ± 600[e]	0.75 ± 0.10[f]
Lys75Gln	1.6 ± 0.1	–	–	–	0.038 ± 0.011[g]
Lys75Ser	1.6 ± 0.1	–	–	–	0.056 ± 0.002[g]
Lys75Glu	1.4 ± 0.1	–	–	–	0.00082 ± 0.00003[g]

[a] Deaerated solutions also contained 100 μM 5-deazariboflavin and 1 mM EDTA in 4 mM potassium phosphate buffer (pH 7.0). The ionic strength was adjusted to 100 mM using 5 M NaCl. [b] These values were evaluated from Figure 2 as described in the text. [c] These second-order rate constants were evaluated from Figure 2 as described in the text. [d] Taken from ref *31*. [e] Corrected for the concentration of the preformed complex. [f] These values were obtained, for the sake of comparison, from the initial slopes of the curves in Figure 2A. [g] Not corrected for the concentration of the preformed complex. The K_d values given in Table 3 are estimated values (see text), and given their magnitudes, any correction would be negligible.

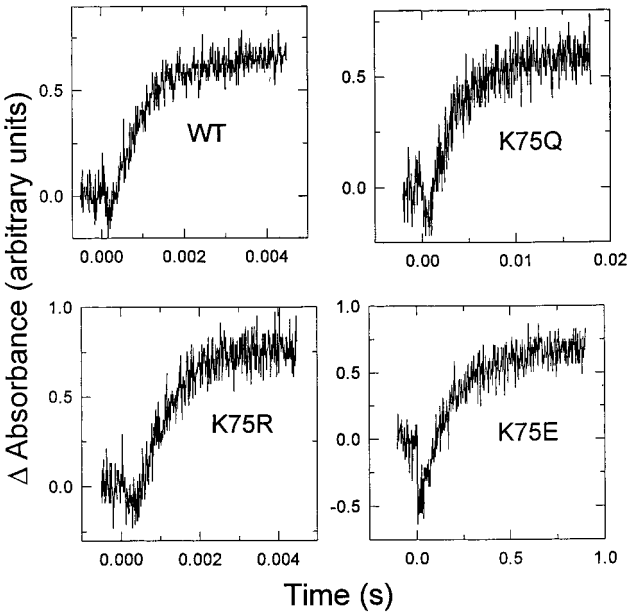

FIGURE 1: Transient kinetic traces observed upon laser flash photolysis of solutions containing 30 μM wt Fd and 5.2 μM wt FNR, 5.1 μM Lys75Arg, 5.5 μM Lys75Gln, and 4.8 μM Lys75Glu, respectively. The monitoring wavelength was 600 nm. Solutions also contained 1 mM EDTA and 95−100 μM dRf in 4 mM potassium phosphate buffer (pH 7.0). The ionic strength was adjusted to 100 mM using aliquots of 5 M NaCl. Lys75Ser (data not shown) was very similar to Lys75Gln.

shows kinetic traces observed at 600 nm which demonstrate the formation of the one-electron reduced (neutral semiquinone) forms of wt, Lys75Arg, Lys75Gln, and Lys75Glu FNR due to laser-induced reduction by Fd_{rd}. It is apparent from the time scales of Figure 1 (reaction conditions and concentrations used were such that these curves are directly comparable to one another) that Lys75Arg is very similar in its reactivity to wt FNR whereas Lys75Gln (as well as Lys75Ser; data not shown) is somewhat affected and Lys75Glu is severely impaired in its ability to accept electrons from Fd_{rd}.

Pseudo-first-order plots for the dependence of k_{obs} on FNR concentration for the reduction of wt FNR and the different FNR mutants at position 75 by Fd_{rd} at $I = 100$ mM are shown in Figure 2. Saturation was observed for wt and Lys75Arg FNR forms (Figure 2A), whereas linear concentration dependencies were observed for the Gln, Ser, and Glu mutants (Figure 2B). As we have shown previously (*11*), Fd_{rd} does not react with the $FNR_{ox}-Fd_{ox}$ complex. Therefore, using the K_d values for the complexes obtained for the wt and Lys75Arg mutants (Table 4), the concentration of unbound FNR was calculated and the kinetic data were replotted in Figure 2 (upper panel). The saturation behavior observed for these two mutants allowed determination of k_{ET}, a limiting first-order rate constant that includes contributions from structural rearrangements, changes in hydration of the protein, changes in solvent structure, and other factors leading to the attainment of an optimal orientation for interprotein ET, and K_d for the transient $Fd_{rd}-FNR_{ox}$ complex (*36*, *37*).

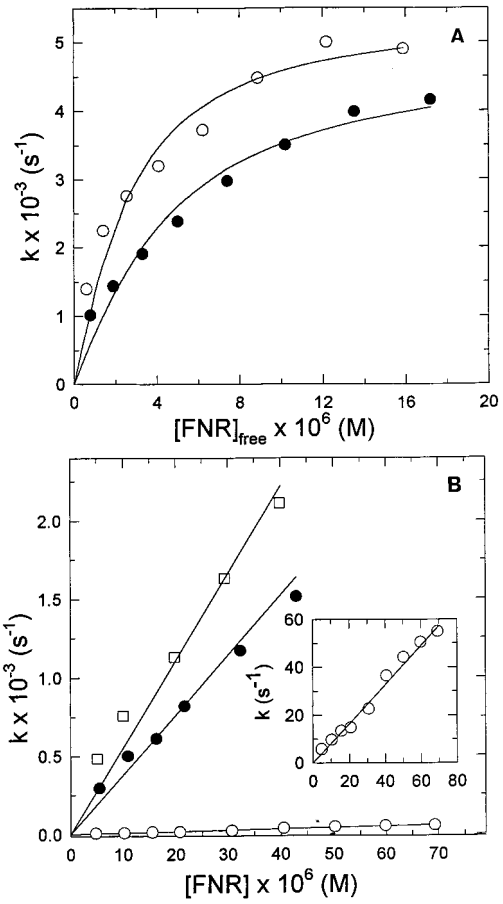

FIGURE 2: Dependence of pseudo-first-order rate constants for the reduction of (A) wt (○) and Lys75Arg (●) FNRs and (B) Lys75Gln (●), Lys75Ser (□), and Lys75Glu (○) FNRs by reduced Fd at an ionic strength of 100 mM. FNR was titrated into solutions containing 30 μM wt Fd, except for the case of wt FNR, where FNR was titrated into 9.8 μM wt Fd. Solution conditions were as described in the legend of Figure 1. The monitoring wavelength was 600 nm.

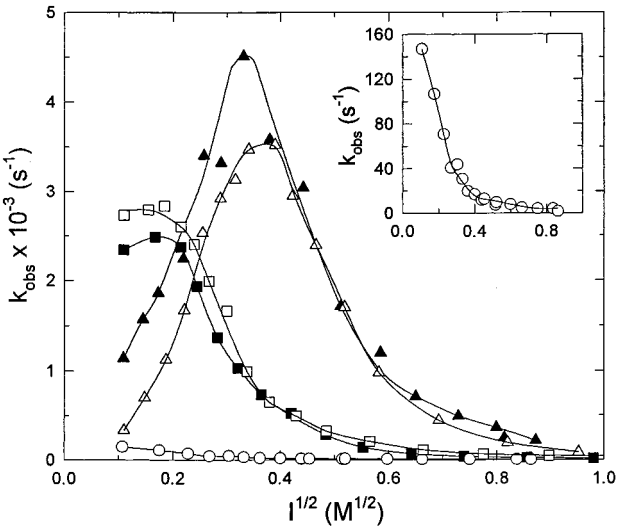

FIGURE 3: Ionic strength dependence of pseudo-first-order rate constants for the reduction of wt FNR (▲) and the Lys75Arg (△), Lys75Gln (■), Lys75Ser (□), and Lys75Glu (○) FNR mutants by reduced Fd. For the Lys75Glu reaction, the solution contained each protein at 30 μM. For the other reactions, solutions contained 40 μM wt Fd and 30 μM FNR. The ionic strength was adjusted using aliquots of 5 M NaCl. Other conditions were as described in the legend of Figure 1.

These kinetically derived constants are given in Table 4, where it is shown that although the k_{ET} values from Fd_{rd} to wt and to Lys75Arg FNR_{ox} are the same within experimental error, the K_d value for the intermediate $Fd_{rd}-FNR_{ox}$ complex involving Lys75Arg is almost twice that of the complex with wt FNR. Correction for unbound mutant FNR_{ox} was not used for the other FNR mutants, since binding constants for the oxidized protein−protein complexes either could not be measured (Lys75Glu) or were large enough that correction would be negligible (Lys75Gln and Lys75Ser). Also presented in Table 4 are the observed second-order rate constants for the ET processes with the different FNR forms determined from the plots in Figure 2B for the Gln, Ser, and Glu mutants, and estimated from the initial slopes of the plots in Figure 2A for wt and Lys75Arg FNR forms. These values confirm that Lys75Gln and Lys75Ser are kinetically quite similar to each other, and that Lys75Glu is severely impaired in its ET interaction with Fd.

Figure 3 shows the ionic strength dependencies of k_{obs} for wt and the Lys75 FNR mutants. As is evident from the data, Lys75Arg behaved very much like wt FNR at high ionic strengths, but is somewhat inhibited in the low-ionic strength region (approximately 20%). The value at which the maximal k_{obs} value is obtained is about the same ($I = 110-140$ mM) for the two proteins, although the observed rate constants are significantly different. Thus, whereas the effect of the Lys to Arg substitution is small, it is not inconsequential. Lys75Ser and Lys75Gln react quite similarly to each other over the whole ionic strength range, and reach their maximal value of k_{obs} at an ionic strength of approximately 30 mM, a value significantly lower than that observed for the Lys75Arg and wt FNR forms. In sharp contrast, Lys75Glu is highly impaired in its ET interaction with wt Fd, and if it has an optimal ionic strength at all, its value is shifted well below the accessible range. We have previously (*11*) associated the appearance of an optimum in the ionic strength curves with the electrostatic stabilization at low ionic strengths of a nonoptimal orientation for ET of the two proteins within the intermediate complex. It is thus quite apparent from these data that the positive charge of Lys75 plays an important role in this orientation process.

Reduction Potentials. The reduction potentials of the oxidized−semiquinone couple of the Lys75 mutant FNR forms have been estimated from the flash photolysis data (see Materials and Methods). The Lys75Arg, Lys75Gln, and Lys75Ser FNR mutants showed approximately equivalent extents of reoxidation of Fd_{rd} as wt FNR at similar concentrations, whereas approximately twice as much of the Lys75Glu mutant protein was required to obtain comparable extents of Fd_{rd} reoxidation. This implies that the one-electron reduction potentials of the Lys75Arg, -Gln, and -Ser FNR mutants are approximately equal to that of wt FNR, whereas the potential of Lys75Glu was decreased by approximately 20 mV (to −351 mV vs NHE) relative to that of wt FNR (−331 mV) (*40*). Note that this small shift still makes the reduction potential for Lys75Glu more positive than that measured for wt Fd (−384 mV vs NHE). It should also be noted that in a previous study (*40*), it was shown that the one-electron reduction potential of FNR in several complexes with wt and mutant Fds was shifted positively (making ET more thermodynamically favorable) by significant amounts (40−62 mV). On the basis of these considerations, it is unlikely that altered reduction potentials are responsible for the observed decreases in reactivity.

Rapid Reaction Stopped-Flow Studies of the FNR Mutants at Lys75. Stopped-flow kinetic studies were carried out for the different mutants prepared at Lys75 to further study the behaviors observed above. This technique allows us to study the time course of association and ET between FNR, in the oxidized and reduced states, and its two ET protein partners, Fd and Fld (*14*). Reactions between FNR and Fd were followed at 507 nm, although kinetic traces at 600 nm were also obtained to determine which reactions were taking place. The value of 507 nm is an isosbestic point for FNR_{ox} and FNR_{sq} (*39*), and although it is not an isosbestic point for the FNR_{sq} and FNR_{rd} transition, the absorbance change due to this FNR transition is very small compared to that due to the redox state change of ferredoxin. These facts make 507 nm an appropriate wavelength for following reduction or reoxidation of Fd in the presence of FNR. Previous stopped-flow studies of the ET process between Fd_{rd} and FNR_{ox} have shown that the ET from Fd_{rd} and to wt FNR_{ox} (k_{obs} of at least 1000 s^{-1}), producing FNR_{sq} and Fd_{ox}, must take place within the instrumental dead time, and that the rate constant observed by stopped-flow methods (250 s^{-1}) must correspond to the oxidation of another Fd_{rd} molecule and reduction of FNR_{sq} to FNR_{rd} (*14*, *41*). When this reaction for the different FNR mutants is studied at Lys75, varied behaviors were observed (Figure 4A), as shown by the observed rate constants reported in Table 5. A rate constant of 280 s^{-1} was obtained for the reduction of Lys75Arg FNR_{ox} when it reacted with Fd_{rd}. This behavior is very similar to that reported for wt FNR, and thus, the observed rate constant must correspond to the reduction of FNR_{sq} to FNR_{rd} by a second Fd_{rd} molecule. As shown by the laser flash photolysis data (Table 4), the expected k_{obs} for ET between Fd_{rd} and Lys75Arg FNR_{ox} to produce FNR_{sq} and Fd_{ox} should be in the same range as that for the reaction with wt FNR and much faster than the value that can be observed using

Table 5: Fast Kinetic Parameters for Electron Transfer Reactions of Wild-Type and Lys75 Mutant FNR Forms Studied by Stopped-Flow Methods[a]

	k_{obs} (s^{-1}) for the mixing of FNR_{ox} with		k_{obs} (s^{-1}) for the mixing of FNR_{rd} with	
FNR	Fd_{rd}[c]	Fld_{rd}[d]	Fd_{ox}[c]	Fld_{ox}[d]
wild-type[b]	nd[e]	nd[e]	nd[e]	2.5 ± 1.0
	250 ± 30			1.0 ± 1.0
Lys75Arg	nd[b]	>350	>350	9.4 ± 0.6
	280 ± 50	50 ± 1		1.46 ± 0.18
Lys75Gln	50 ± 12	26 ± 4	60 ± 15	1.0 ± 0.3
	1.7 ± 0.3	6.4 ± 0.4		0.45 ± 0.05
Lys75Ser	296 ± 32	25 ± 4	67 ± 15	1.3 ± 0.3
	50.2 ± 0.4	4.6 ± 1.8		0.43 ± 0.06
Lys75Glu	2.7 ± 0.4	0.52 ± 0.06	<0.0011	0.11 ± 0.02
	0.50 ± 0.05	0.08 ± 0.02		0.018 ± 0.001

[a] The samples were mixed in the stopped-flow instrument at the indicated concentrations (Figures 4 and 5) and redox states. $T = 13$ °C. All samples were prepared in 50 mM Tris-HCl (pH 8.0). [b] Data from ref *14*. [c] Reaction followed at 507 nm. [d] Reaction followed at 600 nm. [e] Reaction occurred within the dead time of the instrument.

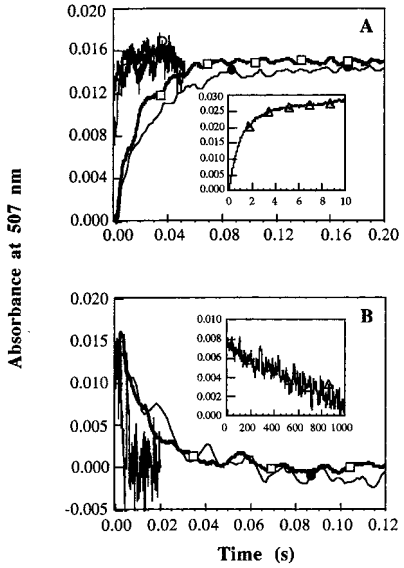

FIGURE 4: Time course of anaerobic reactions of FNR forms with Fd. Reactions were carried out in 50 mM Tris-HCl (pH 8.0) at 13 °C and followed at 507 nm. Final concentrations are given. (A) wt FNR_{ox} (8.4 μM, —) was reacted with 26 μM Fd_{rd}, 7.5 μM Lys75Arg FNR_{ox} (○) with 25 μM Fd_{rd}, 10.3 μM Lys75Gln FNR_{ox} (●) with 13.3 μM Fd_{rd}, 9.9 μM Lys75Ser FNR_{ox} (□) with 21.3 μM Fd_{rd}, and (inset) 9.8 μM Lys75Glu FNR_{ox} (△) with 23 μM Fd_{rd}. (B) Lys75Arg FNR_{rd} (9 μM, ○) was reacted with 22 μM Fd_{ox}, 8.8 μM Lys75Gln FNR_{rd} (●) with 25 μM Fd_{ox}, 10 μM Lys75Ser FNR_{rd} (□) with 25.8 μM Fd_{ox}, and (inset) 8.7 μM Lys75Glu FNR_{rd} (△) with 24.5 μM Fd_{ox}.

stopped-flow methods. Nevertheless, as shown in Table 5, the process of ET from Fd_{rd} to FNR_{ox}, as studied by stopped-flow methods, showed two different phases when the Lys75Gln, Lys75Ser, and Lys75Glu FNR_{ox} forms were analyzed. The two phases corresponded to two markedly different observed rate constants. For these three FNR mutants, the first rate constant must correspond to the production of the semiquinone state of the FNR, while the second process should correspond to the full reduction of this FNR_{sq} by a second Fd_{rd} molecule. For these mutants, the k_{obs} values for both processes were considerably slower than those obtained for wt and Lys75Arg FNR forms. Nevertheless, it is important to note that while Lys75Ser and Lys75Gln FNR were still able to accept electrons from Fd_{rd} with appreciable rate constants, replacement of Lys75 by a glutamic acid nearly abolished the enzyme's ET capability. However, despite the large differences observed in the rate constants and in the different protein concentrations used in each case, the amplitudes of the phases for all of the FNR forms are, within experimental error, in the same range, indicating that it is not the ET reaction itself that is affected by the mutations. All these data are consistent with the laser flash photolysis results in clearly indicating that the overall ET process between Fd_{rd} and FNR_{ox} to produce FNR_{sq} is greatly affected by removal of the positive charge at position 75.

Reduction of Fd by the different FNR_{rd} forms was also assayed. In all cases, the stopped-flow data were best fitted to a single-exponential process with amplitudes in the same range (Figure 4B and Table 5). This process, which was too fast for the wt FNR to be followed by stopped-flow spectrophotometry (*14*), showed three different ranges of observed rate constants for the different FNR mutants at Lys75. When reduction of Fd by Lys75Arg FNR_{rd} was studied, although nearly limited by the instrumental dead time, we were still able to detect a decay which corresponded to a rate constant of >350 s^{-1}, demonstrating that this process has been affected to a small degree which we are not able to quantitate. Lys75Gln and Lys75Ser FNR_{rd} showed rate constants for the reduction of Fd of only 60 and 67 s^{-1}, respectively, while there was almost no reaction when reduction of Fd by Lys75Glu FNR_{rd} was studied (Table 5).

ET reactions between FNR and Fld were analyzed for the different FNR forms by stopped-flow methods, and the results were compared with those reported for the wt enzyme (*14*). The reactions were followed mainly at 600 nm (to observe production of both Fld and FNR semiquinone forms), although kinetic traces at 460 nm were also obtained in some cases to determine which reactions were occurring. As previously described, the time course of wt FNR_{ox} reduction by reduced Fld cannot be followed by stopped-flow methods (*14*). Nevertheless, all the mutants prepared at Lys75 of *Anabaena* FNR showed reaction, and the traces for these reactions were analyzed and best fit by two-exponential processes with similar amplitudes. Furthermore, the total amplitudes of the reactions were similar for all the different FNR forms (Figure 5A). Reduction of Lys75Arg FNR_{ox} by Fld_{rd} showed one rate constant of >350 s^{-1} and a second rate constant of 50 s^{-1}, which is consistent with the simultaneous formation of both semiquinones, followed by the reduction of FNR_{sq} to the fully reduced state by a second

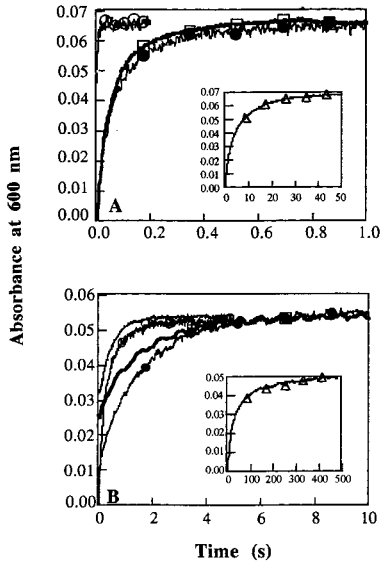

FIGURE 5: Time course of anaerobic reactions of FNR forms with Fld. Reactions were carried out in 50 mM Tris-HCl (pH 8.0) at 13 °C and followed at 600 nm. Final concentrations are given. (A) Lys75Arg FNR_{ox} (10.7 μM, ○) was reacted with 20.6 μM Fld_{rd}, 10 μM Lys75Gln FNR_{ox} (●) with 22.7 μM Fld_{rd}, 11.2 μM Lys75Ser FNR_{ox} (□) with 24.2 μM Fld_{rd}, and (inset) 10.7 μM Lys75Glu FNR_{ox} (△) with 22 μM Fld_{rd}. (B) Wt FNR_{rd} (9.6 μM, —) was reacted with 26 μM Fld_{ox}, 10.2 μM Lys75Arg FNR_{rd} (○) with 22.8 μM Fld_{ox}, 11.8 μM Lys75Gln FNR_{rd} (●) with 24.5 μM Fld_{ox}, 10.6 μM Lys75Ser FNR_{rd} (□) with 23.3 μM Fld_{ox}, and (inset) 9.7 μM Lys75Glu FNR_{rd} (△) with 26 μM Fld_{ox}.

molecule of Fld_{rd} (*14*). This result indicates that replacement of Lys75 by an Arg produced an enzyme with altered k_{obs} values for the reduction of FNR by reduced Fld. When the ET process from Fld_{rd} to Lys75Gln, Lys75Ser, and Lys75Glu FNR_{ox} mutants was studied, two different phases were also observed (Table 5 and Figure 5A). The observed rate constants for the reduction of Lys75Gln and Lys75Ser FNR_{ox} mutants by Fld_{rd} had similar magnitudes (26 and 25 s^{-1}, and 6.4 and 4.6 s^{-1}, respectively) and were considerably smaller than those reported for the reduction of the wt enzyme by Fld_{rd} (Table 5). Again, replacement of Lys75 of *Anabaena* FNR by a Glu produced an enzyme with a highly impaired ability to accept electrons from Fd_{rd}, yielding observed rate constants of 0.52 and 0.08 s^{-1} for the two processes.

The ET reaction from FNR_{rd} to Fld_{ox} was also investigated for the different FNR mutants at Lys75 (Figure 5B). As was observed for ET from wt FNR_{rd} to Fld_{ox}, two phases were also detected for all the Lys75 FNR mutants (Table 5). These phases correspond, as previously proposed, to the processes of ET from FNR_{rd} to Fld_{ox} to produce FNR_{sq} and Fld_{sq}, followed by reduction of a second molecule of Fld_{ox} to the semiquinone state by FNR_{sq} (*14*). However, although three different behaviors can again be observed for the different FNR mutants prepared at Lys75, the amplitudes of both phases are similar and all the FNR forms have similar total amplitudes for the process. Again, Lys75Arg FNR shows the maximal efficiency for this process (Figure 5B and Table 5), with observed rate constants slightly higher than those obtained for the wt FNR. The rate constants observed for the processes when Lys75Gln and Lys75Ser FNR_{rd} donate one electron to Fld_{ox} appear to be between 40 and 50% of those reported for the wt enzyme (Table 5), indicating that this process is still quite efficient for these mutants. Finally, replacement of Lys75 by Glu resulted in by far the largest decrease in the observed rate constants for reduction of Fld by FNR_{rd} (Table 5).

To determine if the reoxidation of the different FNR mutants at Lys75 followed a reoxidation process similar to that of the native enzyme (*14*), flavin reoxidation of the different FNR_{rd} mutants by molecular oxygen was followed by stopped-flow spectrophotometry at 460 and 600 nm. Reoxidation of the FAD can be followed at 460 nm. We observe the appearance of the neutral flavin semiquinone upon reaction of the fully reduced enzyme with O_2 while monitoring at 600 nm, and the subsequent slower reaction of the formed semiquinone with a second oxygen molecule (*42*). The traces obtained for these processes for the different Lys75 FNR mutants (not shown) showed profiles similar to those observed for wt enzyme at both wavelengths (*14*), indicating that the sequence followed in the reoxidation of these FNR_{rd} mutants is similar to that of the native enzyme, producing an intermediate semiquinone state.

DISCUSSION

In this study, we probe the importance of Lys75 in complex formation and in the ET processes between FNR and its protein partners. The positive charge provided by Lys75 has been replaced by neutral and positively and negatively charged residues as described above. Spectroscopic characterization of the different mutants produced at position 75 did not show significant differences in absorbance, circular dichroism, or fluorescence relative to those of the wt protein (*14*), indicating that gross protein structural rearrangements were not introduced by the mutation. This was expected, since Lys75 is located at the protein surface, and generally replacements of surface residues are less likely to alter the structure or stability of the protein. The analysis of the steady-state diaphorase kinetic parameters for the different Lys75 FNR mutants indicates that Lys75 is not a critical residue for complex formation and ET between FNR and $NADP^+$ or NADPH. However, when the steady-state kinetic activities were analyzed where complex formation and ET between FNR and Fd, or Fld, were required, a decrease was observed in the catalytic efficiency of the process. Nevertheless, not all the studied mutants were affected to the same extent (Table 2), and three groupings of reactivity were observed among these mutants. Similar reactivities were also observed when the binding of the different FNR mutants to Fd and their ET reactivities were measured.

Replacement of Lys75 by Arg, the most conservative replacement possible, produced an enzyme whose behavior most resembled that of the wt FNR. This enzyme was able to transfer electrons efficiently to and from Fd and Fld, as shown by the catalytic constants obtained in the NADPH-dependent cytochrome *c* reductase activities (Table 2), and by the laser flash photolysis (Table 4 and Figures 2 and 3) and stopped-flow (Figures 4 and 5) results, although the data also indicate that the reaction is somewhat inhibited in the low-ionic strength region and that some observed rate constants obtained by stopped-flow methods are slightly slower than that for the wt enzyme (Figures 4 and 5 and Table 5). The Lys75Arg FNR shows a catalytic efficiency in the NADPH-dependent cytochrome *c* reductase activity

slightly lower than that of the wt enzyme, due to a larger K_m. The K_d values obtained for the Fd_{ox}−Lys75Arg FNR_{ox} and for the Fd_{ox}−Lys75Arg FNR_{rd} complexes are slightly larger that those reported for the complexes with the wt FNR (Tables 3 and 4).

Lys75Gln and Lys75Ser reacted similarly, but were significantly impaired relative to the wt in ET with Fd and Fld**,** as seen in both transient and steady-state kinetic studies. Gln and Ser could still interact through hydrogen bond interactions with the Fd carboxyl expected to interact with Lys75. The high K_m values obtained in the NADPH-dependent cytochrome *c* reductase activity, using either Fd or Fld, suggest that complex formation and stability are affected (Table 2). This is confirmed by the K_d values obtained for the Fd_{ox}−FNR_{ox} complexes (Table 3) and by the linear concentration dependencies observed for these two FNR_{ox} mutants in the laser flash photolysis experiments (Figure 2B). Finally, binding studies and the steady-state and fast kinetic studies reported herein have shown that replacement of Lys75 by a Glu, a charge reversal mutation, produced an enzyme that, although retaining the ability to interact and transfer electrons with $NADP^+$ or NADPH and 5-deazariboflavin (Tables 2 and 4), was unable to interact efficiently in ET with Fd or Fld (Tables 4 and 5).

The binding data, the ionic strength dependence data, and the fast kinetic data reported herein all lead to the conclusion that the residue at position 75 in *Anabaena* FNR must bear a positive charge for maximal ET rates to be achieved with Fd and Fld, and also that altered complex stability is the major determinant of decreased reactivity within this series of mutants. Further, the facts that Lys75Arg, although it retains the positive charge at position 75, has maximal observed rate constant values which are 20% lower than that of wt FNR in the low-ionic strength region when it is studied by laser flash photolysis and has smaller k_{obs} values for the processes studied by stopped-flow methods are consistent with previous results (*9*−*11*, *20*) which indicate that there is a very precise surface complementarity at the protein−protein interface of the ET complex.

According to the amplitudes of the fast kinetic reactions reported here (Figures 1, 4, and 5), the reactions between the different FNR forms and its protein partners seem to occur to the same extent as with the wt enzyme, indicating that it is not the ET process itself that is affected by the mutation, but the stabilization of the complex formed between the two proteins (Tables 3 and 4). Therefore, it would be expected that one or more negatively charged residues on Fd or Fld would form a salt bridge with Lys75, which would provide much of the stabilization energy. The resolution of the three-dimensional structure of the complex would provide direct evidence about which amino acid residue on Fd interacts with Lys75 on FNR. Attempts have been made to restore the electron exchange ability of the complex involving the Lys75Glu FNR mutant by assaying it with Fd mutants in which a positive charge has replaced a negative residue on the surface of the protein. Thus far, these experiments have not yielded positive results. These results deserve an additional comment. Among the many site-directed mutants prepared for Fd (*7*, *8*, *40*), FNR (*24*), and Fld (*43*), several degrees of inhibition of the reaction in which they participated were observed. In those cases in which a drastic decrease in the rate of ET upon replacement of a charged residue was observed, it was not attributed to a lower stability of the complex formed, but to other factors, such as the inability to form a hydrogen bond. Mutants Glu94Lys, Phe65Ala, and Ser47Ala of Fd, which are severely impaired in ET with FNR (*7*, *40*), formed complexes with FNR that had stabilities similar to that of the complex involving the wt protein. These results are the first example in which a single mutation drastically alters the stability of the complex and, consequently, the rate of ET in the *Anabaena* Fd−FNR system. There is no doubt that much more information is required before a clear picture of the molecular basis for protein−protein recognition in ET reactions is obtained, but the results presented in this paper clearly contribute to the better understanding of this intricate problem.

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