Role of Hydrophobic Interactions in the Flavodoxin Mediated Electron Transfer from Photosystem I to Ferredoxin-NADP$^+$ Reductase in *Anabaena* PCC 7119[†]

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ABSTRACT: Hydrophobic interactions play an active role in effective complex formation between ferredoxin-NADP$^+$ reductase (FNR) and ferredoxin (Fd) from *Anabaena*, where an aromatic amino acid residue on the Fd surface (F65) and three hydrophobic residues (L76, L78, and V136) on the reductase surface have been shown to be essential for the efficient electron transfer (ET) reaction between Fd and FNR (Martínez-Júlvez et al. (2001) *J. Biol. Chem. 276*, 27498−27510). Since in this system flavodoxin (Fld) can efficiently replace Fd in the overall ET process, we have further investigated if such hydrophobic interactions are also critical in complex stabilization and ET in the FNR/Fld association. Different ET behaviors with Fld are observed for some of the mutations made at L76, L78, and V136 of *Anabaena* FNR. Thus, the ET interaction with Fld is almost completely lost upon introduction of negatively charged side chains at these positions, while more conservative changes in the hydrophobic patch can influence the rates of ET to and from Fld by altering the binding constants and the midpoint redox potentials of the flavin group. Therefore, our results confirm that nonpolar residues in the region close to the FAD group in FNR participate in the establishment of interactions with Fld, which serve to orient the two flavin groups in a manner such that ET is favored. In an attempt to look for the counterpart region of the Fld surface, the effect produced by the replacement of the only two nonpolar residues on the Fld surface, I59 and I92, by a Lys has also been analyzed. The results obtained suggest that these two hydrophobic residues are not critical in the interaction and ET processes with FNR. The reactivity of these I92 and I59 Fld mutants toward the membrane-anchored photosystem I (PSI) complex was also analyzed by laser flash absorption spectroscopy. From these data, significant effects are evident, especially for the I92 position of Fld, both in the association constant for complex formation and in the electron-transfer rate constant in the PSI/Fld system.

Electron transfer (ET)[1] reactions between proteins only take place upon optimal orientation of their redox centers. To achieve such orientation, it is assumed that both electrostatic and hydrophobic interactions are involved (*1*, *2*). Much effort has been put into determining the structural requirements for complex orientation, reorganization, and ET between the proteins involved in the ET chain that transfers electrons from photosystem I (PSI) to ferredoxin-NADP$^+$ reductase (FNR), via a [2Fe-2S] plant-type ferredoxin (Fd), to reduce NADP$^+$ in plants, algae, and cyanobacteria (*3*−*13*). Biochemical and structural data support the idea that Fd binds in a concave cavity around the FAD of the reductase (*10*, *11*, *13*−*15*). These data also indicate that although multiple electrostatic interactions might play a critical role in the initial complex formation (*5*, *7*, *12*, *16*−*18*), probably by orienting the proteins during their mutual approach, in the particular case of the *Anabaena* system only two charged residues on FNR, K75 and E301, and one on Fd, E94, have been shown to play a crucial role in effective complex

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[1] ET, electron transfer; FNR, 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_{rd}, Fld in the reduced state; Fld_{sq}, Fld in the semiquinone state; IPTG, isopropyl-β-D-thiogalactoside; dRf, 5-deazariboflavin; PSI, photosystem I; Cytc, horse heart cytochrome *c*; $Cytc_{ox}$, cytochrome *c* in the oxidized state; $Cytc_{rd}$, cytochrome *c* in the reduced state; K_a, complex association constant; K_d, complex dissociation constant; k_{et}, electron-transfer first-order rate constant; k_{ap}, apparent observed rate constant; k_{obs}, pseudo-first-order observed rate constant; PMS, phenazine methosulfate; WT, wild type; $E_{ox/sq}$, redox potential for the oxidized-semiquinone couple; $E_{sq/rd}$, redox potential for the semiquinone-reduced couple.

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stabilization (*6*, *9*, *10*, *17*, *19*). Recently, it has also been shown that hydrophobic interactions are critical in the formation of the most optimal complex for ET between FNR and Fd (*4*, *5*, *13*). Thus, an aromatic amino acid on the Fd surface, F65, as well as a hydrophobic patch, formed by residues L76, L78, and V136, on the FNR surface and in the vicinity of the FAD isoalloxazine ring, are essential for an efficient ET reaction between FNR and Fd (*4*, *5*, *13*). These data are in agreement with the three-dimensional structures reported for the *Anabaena* (*10*) and *maize* (*11*) FNR/Fd complexes. In the case of the *Anabaena* crystalline complex, it is clearly shown that the interface is formed mainly by hydrophobic and van der Waals interactions that involve F65 and Y98 from Fd and L76, L78, and V136 from FNR, while a single salt bridge between E94 Fd and K75 FNR is also formed.

When certain algae and cyanobacteria are grown under iron-deficient conditions, they synthesize an FMN-dependent flavoprotein, flavodoxin (Fld), that replaces Fd in the one-electron transfer from PSI to FNR by oscillating between the semiquinone and the hydroquinone states (*20*, *21*). It is generally accepted that both ET proteins, Fd and Fld, interact with the same region of the reductase during complex formation, although it has been shown that each individual charged residue on the reductase does not participate to the same extent in the interaction with each of the ET protein carriers (*12*, *17*, *18*). Thus, although K75 in *Anabaena* FNR also seems to be critical for an optimal interaction between the reductase and Fld (*12*, *17*), thus far there is no candidate residue on the Fld surface with which it appears to interact. Moreover, although the role of several negatively charged residues on the *Anabaena* Fld surface has been analyzed by site-directed mutagenesis, namely E61, E67, D100, D126, D144, and E145 (*22*, *23*), the data reported thus far indicate that none of them appears to be critical for the complex formation and ET processes between Fld and its substrates. Finally, despite much effort, the three-dimensional structure of the FNR/Fld complex remains unsolved.

Since no information about hydrophobic interactions between FNR and Fld or Fld and PSI is available and taking into account the critical role they play in the FNR/Fd interaction (*10*, *13*), in the present study we have analyzed the role of hydrophobic interactions in the processes of binding and ET between Fld and FNR and between PSI and Fld. Thus, those mutants previously constructed in the hydrophobic region of FNR, at positions L76, L78, and V136 (*13*), have now been characterized in their interaction with Fld. Moreover, to look for possible counterparts of this region of FNR on the Fld surface, the role of the only two hydrophobic residues on the Fld surface around FMN, I59 and I92 (Figure 1), the putative region for interaction with the reductase, have also been analyzed by independently replacing both side chains by Lys. Such mutants have also been analyzed for their reactions with PSI, to further analyze the role of hydrophobic forces in the PSI/Fld interaction. Thus far, it has been shown that the PsaC, PsaE, and PsaD subunits in PSI are involved in the high affinity binding of Fd and Fld (*24*), although these subunits contribute differently to binding and complex stabilization. Thus, PsaD appears to be important for the electrostatic guidance of the acceptor proteins into the binding pocket, PsaE, especially its R39 residue, and plays an important role in stabilizing the final electron-transfer complex, whereas the key residue for the interaction seems to be K35 of PsaC (*25*). However, a specific Lys PsaD residue that has shown to be critical for the interaction with Fd (*26*) is dispensable for the flavodoxin-mediated $NADP^+$ reduction, suggesting that the interaction of PSI with Fd and Fld might take place through a nonidentical mechanism (*27*). Finally, the I59K and I92K mutants have also been analyzed in their reactions with cytochrome *c* (Cytc).

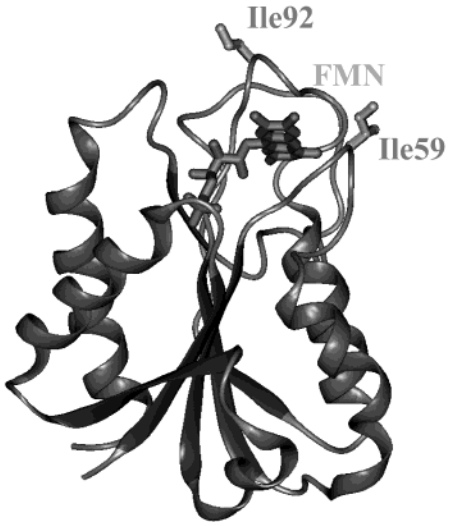

FIGURE 1: Ribbon representation of *Anabaena* WT Fld showing the mutated residues (I59 and I92) in stick representation. The FMN cofactor is also shown.

MATERIALS AND METHODS

Biological Material. Anabaena FNR mutants at L76, L78, and V136 were obtained by site-directed mutagenesis as previously described (*13*). I59K and I92K *Anabaena* Flds were produced using the Transformer Site-directed Mutagenesis Kit from CLONTECH in combination with the synthetic oligonucleotides: 5'-AAAATTATCTGCGTAACCTTTTTG-GT-3' for I92K, 5'-TTTGCAGTTCGCCTTTATTCCAAG-TAGG-3' for I59K, and the Fld gene cloned into the expression vector pTrc99a (*28*). Mutations were verified by DNA sequence analysis. The pTrc99a vectors containing the mutated Fld gene were transformed into *Escherichia coli* TG1. Recombinant WT FNR, WT Fld, and their mutants were purified from LB cultures of IPTG-induced *E. coli* (*13*, *28*). UV−vis spectra and SDS−PAGE were used as purity criteria. PSI particles from *Anabaena* sp. PCC 7119 were obtained as described (*29*, *30*). The P700 content in PSI samples was calculated from the photoinduced absorbance changes at 820 nm, $\epsilon = 6.5\ mM^{-1}\ cm^{-1}$ (*31*). Chlorophyll concentration was determined according to Arnon (*32*). The chlorophyll/P700 ratio of the PSI preparations was 140/1.

Spectral Analysis. UV−vis spectral analyses were carried out on a Kontron Uvikon 942 or on a HP 8452. Circular dichroism was carried out on a Jasco 710 spectropolarimeter at room temperature in a 1 cm path length cuvette in 1 mM Tris/HCl, pH 8.0, at 25 °C. The protein concentrations were 0.7 μM for the far-UV and 4 μM for the aromatic and visible regions of the spectrum. Protein fluorescence was monitored using an AMINCO-BOWMAN 2 spectrofluorometer from Spectronic Instruments. Solutions contained 5 μM protein in 50 mM Tris/HCl, pH 8.0. K_d values and binding energies

of the [FNR_{ox}/Fld_{ox}] complexes were obtained at room temperature in 50 mM Tris/HCl, pH 8.0, by difference absorption spectroscopy as previously described (*9*). Errors in the estimated K_d, $\Delta\epsilon$, and $\Delta G°$ values were ±15, ±15, and ±10%, respectively. The FNR-dependent NADPH-cytochrome *c* reductase activity was assayed using Fld as ET carrier from FNR to Cytc (Sigma) in 50 mM Tris/HCl, pH 8.0, as described (*9*). Errors in the estimated values of K_m and k_{cat} were ±15 and ±10%, respectively. Photoreduction of the Fld forms was studied under anaerobic conditions as reported (*9*). The stoichiometry of the reaction of Fld_{rd} with $Cytc_{ox}$ was determined by measuring the μmol of $Cytc_{rd}$ produced after mixing, under anaerobic steady-state conditions, of a solution of Fld_{rd} (10 μM, final concentration) with $Cytc_{ox}$ (30 μM, final concentration) in Tris/HCl 50 mM, pH 8.0, at room temperature.

Stopped-Flow Kinetic Measurements. Stopped-flow measurements were carried out under anaerobic conditions using an Applied Photophysics SX17.MV spectrophotometer as previously described (*9*). Reduced samples of FNR and Fld were prepared by photoreduction with dRf (*9*, *33*). Reactions between FNR and Fld were followed at 600 nm after mixing the proteins at a ~1:2.5 ratio, the final FNR concentration was ~10 μM. Reactions between Fld_{rd} and Cytc were followed at 550 nm, with a final concentration of ~10 μM Fld and the following Cytc concentrations: 10, 30, 90, and 150 μM. All reactions were carried out in 50 mM Tris/HCl, pH 8.0, at 13 °C. The apparent observed rate constants (k_{ap}) were calculated by fitting the data to mono- or biexponential processes. Errors in their estimated values were ±15%.

Laser-Flash Absorption Spectroscopy. ET processes between PSI and Fld were studied at 580 nm by laser-flash absorption spectroscopy following the procedure and using the laser flash system previously described (*33*). The standard reaction mixture contained, in a final volume of 1 mL, 20 mM Tricine/KOH, pH 7.5, 0.03% β-dodecyl maltoside, an amount of PSI-enriched particles equivalent to 35 μg of chlorophyll mL^{-1}, 0.1 μM PMS, 2 mM $MgCl_2$, 2 mM sodium ascorbate, and Fld at the indicated concentration. For most experiments, the estimated error in the observed rate constants was less than 20%, based on reproducibility and signal-to-noise ratios. Data collection and exponential analyses were as previously described (*34*). Kinetic analyses were carried out according to the two-step reaction mechanism previously proposed (*34*, *35*). Errors in the estimated values of complex association constants (K_a) and ET rate constants (k_{et}) were ±15 and ±5%, respectively.

Midpoint Redox Potential Measurements. The $E_{ox/sq}$ and $E_{sq/rd}$ potentials of I92K Fld were determined at 25.0 ± 0.5 °C in 10 mM potassium phosphate, pH 7.0, by anaerobic photoreduction in the presence of dRf as previously described (*36*). Alternative reduction by sodium dithionite was used for the determination of the $E_{ox/sq}$ potential of I59K Fld. The same conditions and mediators for I92K Fld were also used for the I59K Fld mutant. The $E_{sq/rd}$ potential of I59K Fld was derived according to the following equation: $E_{ox/sq} - E_{sq/rd} = 0.11 \log\{2[SQ]/(1 - [SQ])\}$. The error in the determinations was estimated in ±3 mV. Potentials are reported versus the standard hydrogen electrode.

Table 1: Steady-State Kinetic Parameters of WT and Mutated FNR Forms in the NADPH-Dependent Cytochrome *c* Reductase Activity Using Fld as Electron Carrier and Dissociation Constants, Extinction Coefficient Changes, and Free Energy for Complex Formation of WT and Mutated FNR_{ox} Forms with Fld_{ox}

FNR form	k_{cat} (s^{-1})	K_m^{Fld} (μM)	k_{cat}/K_m^{Fld} ($\mu M^{-1} s^{-1}$)	K_d (μM)	$\Delta\epsilon_{(465)}$ ($M^{-1} cm^{-1}$)	ΔG (kcal mol^{-1})
WT[a]	23.3	33	0.70	3.0	1.4	−7.4
L76A	35	37	2.4	12.5	3.4	−6.7
L76V	6.3[b]	n.d	n.d	102	5.7	−5.5
L76F	5.2[b]	n.d	n.d	7.8	0.60	−7.0
L76S	n.d[c]	n.d[c]	n.d[c]	6.7	0.93	−7.1
L78A	2.2[b]	n.d	n.d	43.9	1.5	−6.0
L78V	43	52	1.4	10.7	0.70	−6.8
L78F	3.1[b]	n.d	n.d	17.0	1.10	−6.5
L78S	n.d[c]	n.d[c]	n.d[c]	31.7	1.6	−6.2
L76F/L78F	n.d[c]	n.d[c]	n.d[c]	27.4	1.3	−6.3
V136A	8.5	121	<0.07	16.4	1.5	−6.5
V136L	14	142	<0.1	2.5	2.4	−7.7
V136S	n.d[c]	n.d[c]	n.d[c]	5. 3	0.75	−7.2

[a] Data from ref *9*. [b] Values for k_{cat} could only be estimated because of the very small extent of reaction observed that was Fld concentration independent. [c] No reaction was observed.

RESULTS

Role of the Anabaena FNR Hydrophobic Patch Formed by Residues L76, L78, and V136 in the Processes of Interaction and ET with Fld. Steady-State Kinetic Analysis of the FNR Mutants. The NADPH-dependent cytochrome *c* reductase activity of FNR was assayed for the different FNR mutants at positions L76, L78, and V136 using Fld as an electron carrier from FNR to Cytc (Table 1). Significant differences were observed for the kinetic parameters of some mutants relative to those of the WT. L76A and L78V FNRs exhibit slightly larger k_{cat} values, about 1.5-fold, whereas the rest of the FNR mutants show appreciable decreases in their k_{cat} values (Table 1). Thus, V136A and V136L had k_{cat} values that were decreased by 3- and 2-fold, respectively, while L76V, L76F, L78A, and L78F had estimated k_{cat} values up to 11-fold lower, and no measurable reaction at all was detected for L76D, L78D, L76D/L78D (data not included in Table 1), L76S, L78S, L76F/L78F, and V136S. K_m^{Fld} values for most of these FNR mutants could not be estimated because of the very low activity. Thus, K_m^{Fld} values could be only estimated for V136A and V136L FNRs, which exhibited a 4-fold increase in this value as compared with that of the WT and for L76A and L78V, which had slightly larger K_m^{Fld} values, suggesting a weaker interaction between these FNR mutants and Fld (Table 1). As a consequence of the K_m^{Fld} and k_{cat}^{Fld} values, L76A and L78V FNRs show catalytic efficiencies close to that of WT, while efficient processes were not observed for the other mutants.

Interaction of FNR_{ox} Forms with Fld_{ox}. To further evaluate the effect of the mutations introduced at positions 76, 78, and 136 of *Anabaena* FNR on its binding to Fld, difference absorption spectroscopy was used to determine the dissociation constants and binding energies of the complexes formed between the different FNR_{ox} forms and Fld_{ox} (*9*) (Table 1). The difference spectrum of the WT Fld_{ox}/FNR_{ox} complex, which has been proposed to arise from alteration of the FNR flavin environments upon Fld association, shows absorption maxima around 390 and 465 nm (*9*, *33*). Spectral perturbations similar to WT FNR (Figure 2A) were detected when analyzing the interaction between Fld and most of the FNR_{ox}

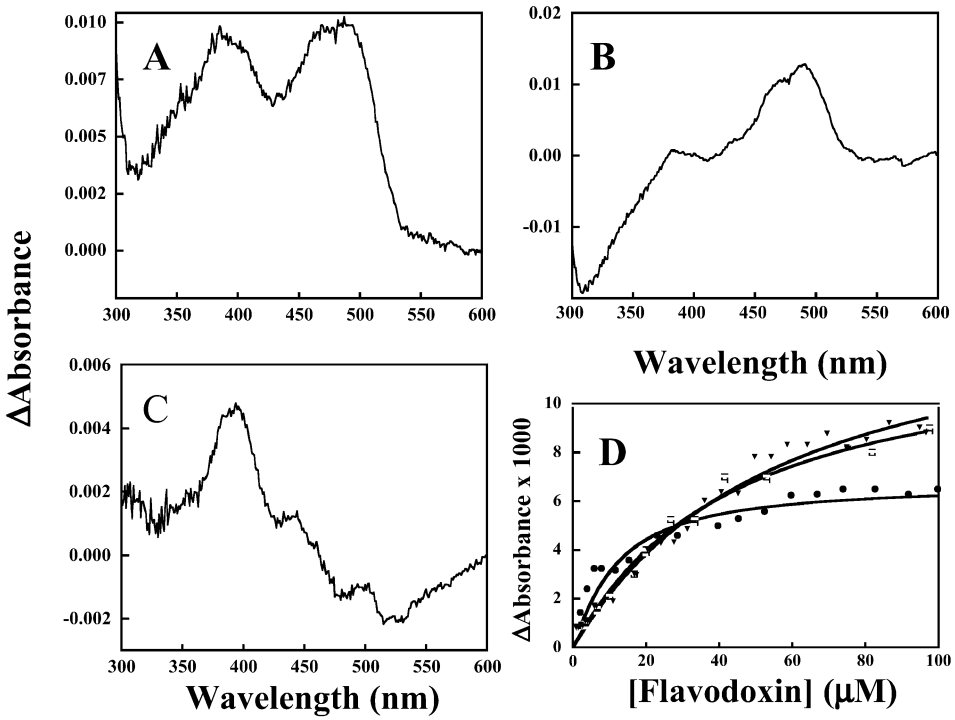

FIGURE 2: Spectroscopic characterization of the complexes formed between FNR_{ox} forms and WT Fld_{ox}. (A) Difference absorption spectrum obtained during the titration of WT FNR_{ox} (14 μM) with WT Fld_{ox} (70 μM). (B) Difference absorption spectrum obtained during the titration of L76F/L78F FNR_{ox} (15.3 μM) with WT Fld_{ox} (90 μM). (C) Difference absorption spectrum obtained during the titration of L78S FNR_{ox} (13.7 μM) with WT Fld_{ox} (60 μM). (D) Spectrophotometric titration (ΔAbs at 462 nm) of the following selected FNR forms with Fld_{ox}: (●) WT FNR, (□) L76F/L78F FNR, and (▼) L78S (for this mutant ΔAbs at 390 nm is presented).

forms assayed. Only two exceptions to this behavior were found. The L76F/L78F and L78S FNR mutants had characteristic difference spectra with Fld_{ox} showing modifications in the relative intensities of the bands around 390 and 460 nm relative to the spectra of WT and the other FNR mutants (Figure 2B,C). These effects might indicate a slightly different flavin environment in the complexes of these mutants with Fld. Moreover, those FNR mutants in which a negative charge was introduced at positions 76 and/or 78 showed no difference spectra at all (data not included in Table 1). These latter results confirm that the introduction of a negative charge at positions 76 or 78 completely eliminates the ability of the enzyme to form a stable complex with Fld. The difference spectra obtained at different Fld concentrations allowed the determination of the dissociation constants and binding energies for the corresponding complexes (Figure 2D, Table 1). Thus, while the L76A, L76F, L76S, L78V, L78F, V136A, V136L, and V136S FNR_{ox} mutants exhibited K_d values for complex formation with Fld similar to or slightly higher than those of the WT enzyme, considerably weaker complexes are apparently formed by L76V, L78A, L78S, and L76F/L78F, with K_d values that were increased up to 34-fold relative to WT (Table 1). In most of the cases, only slight changes of the $\Delta\epsilon$ values were observed, with the only exception of L76A and L76 that have shown larger changes for this value.

Stopped-Flow Kinetic Analysis of the Reaction between FNR Mutants and Fld. To further investigate the role of *Anabaena* FNR residues L76, L78, and V136 in association with an ET to Fld, anaerobic stopped-flow kinetic experiments were carried out. Reactions between the different redox states of FNR and Fld were analyzed at 600 nm, a wavelength at which formation of both protein semiquinone species can be followed. As previously discussed, reaction between WT FNR_{ox} and Fld_{rd} occurs within the dead time of the instrument (*9*). However, when each of the FNR_{ox} mutants was mixed with Fld_{rd}, an increase in absorbance at 600 nm, previously ascribed to formation of both semiquinones (*9*, *12*, *17*), was detected for all the FNR mutants (Figure 3A) showing that in most of the cases the observed processes were considerably slower than with WT FNR (Table 2). With the only exceptions of L78S and V136S FNR_{ox} reactions with Fld_{rd}, the rest of the kinetic traces were best fit to a two-exponential reaction, with the initial process consistent with the formation of the two semiquinones (reaction 1), followed by reduction of FNR_{sq} to the fully reduced state by Fld_{rd}, which is still in the reaction mixture (reaction 2) (*9*, *33*):

$$FNR_{ox} + Fld_{rd} \underset{}{\overset{K_d}{\rightleftharpoons}} [FNR_{ox}\cdots Fld_{rd}] \xrightarrow{k_{et}} FNR_{sq} + Fld_{sq} \qquad (1)$$

k_{ap1}

$$Fld_{rd} + FNR_{sq} \underset{}{\overset{K_d'}{\rightleftharpoons}} [FNR_{sq}\cdots Fld_{rd}] \xrightarrow{k_{et}'} Fld_{sq} + FNR_{rd} \qquad (2)$$

k_{ap2}

Among the analyzed FNR mutants fit to a two-exponential process, three groups of behaviors can be found based on their k_{ap} values. Thus, L76A, L76V, L78V, V136A, and V136L FNR forms are reduced by Fld_{rd} with k_{ap} values lower than that of WT but still with appreciable rate constants, indicating that the ET process from Fld_{rd} to FNR is still taking place with some efficiency for these FNR forms. However, replacement of L76 and L78 by Asp or the

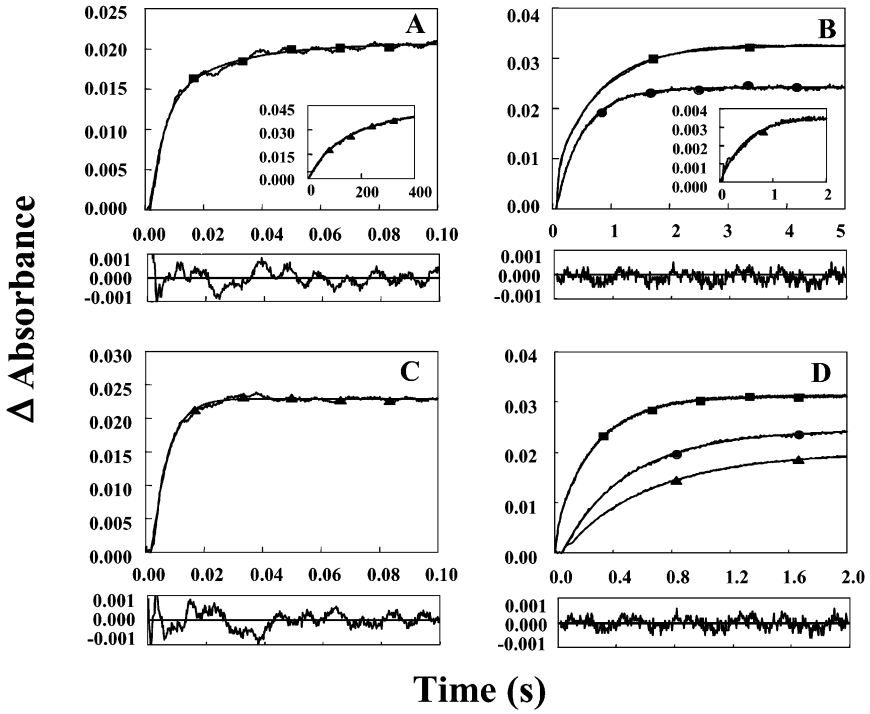

FIGURE 3: Time course of the anaerobic reactions of selected FNR forms with Fld forms as measured by stopped-flow. The monitoring wavelength was 600 nm. Final concentrations are given in parentheses. (A) Reaction of L76A FNR_{ox} (20 μM) (■) with WT Fld_{rd} (47 μM) (residuals for this fit are shown at the bottom) and of L76D/L78D FNR_{ox} (15 μM) (▲) with WT Fld_{rd} (44 μM) (inset). (B) Reactions of WT FNR_{rd} (20 μM) (●) with WT Fld_{ox} (50 μM) (residuals for this fit are shown at the bottom), L76A FNR_{rd} (14 μM) (■) with WT Fld_{ox} (47 μM) and L76F/L78F FNR_{rd} (18 μM) (▲) (inset) with WT Fld_{ox} (57 μM). (C) Reaction of I59K Fld_{rd} (21 μM) (▲) with WT FNR_{ox} (11 μM) (residuals for this fit are shown at the bottom). (D) Reactions of WT Fld_{ox} (50 μM) (●) with WT FNR_{rd} (20 μM) (residual for this fit is shown at the bottom), I59K Fld_{ox} (24 μM) (■) with WT FNR_{rd} (10 μM) and I92K Fld_{ox} (27 μM) (▲) with WT FNR_{rd} (9 μM).

Table 2: Fast Kinetic Parameters for the Reaction of WT and Mutated FNR Forms with Fld as Studied by Stopped-Flow[a]

	k_{ap} (s^{-1}) for the mixing of FNR_{ox} with Fld_{rd}		k_{ap} (s^{-1}) for the mixing of FNR_{rd} with Fld_{ox}	
FNR form	k_{ap1}	k_{ap2}	k_{ap1}	k_{ap2}
WT	n.d.[b]		2.5	1.0
L76D	6.1	1.6	0.6	0.12
L76A	>250	55	16	1.4
L76V	170	10	9	0.61
L76F	46	7.0	3.0	0.8
L76S	42	0.5	1.4	1.4
L78D	0.5	0.14	0.08	0.03
L78A	77	18	3.4	0.55
L78V	>400	140	3.0	2.1
L78F	28	3.6	2.8	0.5
L78S	24[c]		n.r.[d]	
L76D/L78D	0.02	0.005	n.r.[d]	
L76F/L78F	8.2	1.6	20	2.4
V136A	150 ± 20	17	1.3	1.1
V136L	>350	75.1	7.8	1.0
V136S	21.3[c]		n.r.[d]	

[a] Reaction followed at 600 nm. [b] Reaction occurred within the instrumental dead time. [c] These reactions were fit to a monoexponential equation. [d] No reaction was detected.

simultaneous replacement of L76 and L78 by Phe, and especially by Asp, produces FNR enzymes that are highly impaired in their abilities to accept electrons from Fld_{rd} (Figure 3A), while an intermediate behavior is observed for the rest of the FNR mutants. Finally, mutants whose kinetic behaviors were best fit to a monoexponential process also had considerably smaller k_{ap} values.

The reverse reaction has also been investigated. Previous stopped-flow studies on the reaction of WT FNR_{rd} with WT Fld_{ox} indicated that this reaction was a relatively slow process for which two phases were detected (*9*, *33*). These were assigned to the following processes:

$$Fld_{ox} + FNR_{rd} \underset{}{\overset{K_d}{\rightleftharpoons}} [FNR_{rd}\cdots Fld_{ox}] \xrightarrow{k_{et}} Fld_{sq} + FNR_{sq} \quad (3)$$

k_{ap1}

$$Fld_{ox} + FNR_{sq} \underset{}{\overset{K_d'}{\rightleftharpoons}} [FNR_{sq}\cdots Fld_{ox}] \xrightarrow{k_{et}'} Fld_{sq} + FNR_{ox} \quad (4)$$

k_{ap2}

When this reaction was studied using the different FNR mutants at L76, L78, and V136, a two-exponential process, with two phases of similar amplitude, was detected for most of the mutants (Figure 3B), as in the WT FNR reaction, although different kinetic behaviors were observed. Thus, while L76F, L78A, L78V, L78F, and V136A had k_{ap} values similar to those of WT, L76A, L76V, V136L, and particularly the double mutant L76F/L78F had increases in the k_{ap1} values of 6-, 3-, 2-, and 10-fold, respectively, with respect to the WT FNR reaction (Table 2, Figure 3B). Slower processes were observed for L76S and V136A FNRs and especially for the reaction of L76D and L78D $FNRs_{rd}$ with WT Fld_{ox}. Finally, no reaction at all was detected for the reaction of L78S, L76D/L78D, and V136S FNR_{rd} forms with WT Fld_{ox}. It should also be noted that this process occurred with similar amplitudes for all the different FNR forms, with only two exceptions: the reaction of the L76A mutant showed an increase in amplitude for this process and that of the L76F/78F FNR mutant, which had an amplitude that was 10 times smaller, suggesting that in this case the reaction occurred to a lesser extent.

Role of Hydrophobic Residues on the Anabaena Fld Surface in the Interaction and ET with FNR, Cytochrome c, and PSI. Spectral Properties of the Different Fld Mutants. Mutants of Fld at positions I59 and I92 were prepared using the procedure used for the WT protein. In all cases, the level of expression as well as the spectral properties (UV−vis, fluorescence, and CD spectra) were similar to those of the WT Fld (not shown). These data indicate that no major structural perturbations have been produced by the introduced mutations. Moreover, when photoreduced the mutants stabilize a maximal amount of semiquinone that was similar to that of the WT Fld (96−100%).

Redox Potentials of Fld Forms. The midpoint redox potentials calculated from the Nerst plots at pH 7.0 are $E_{ox/sq} = -223$ mV and $E_{sq/rd} = -440$ mV for I59K Fld and $E_{ox/sq} = -190$ mV and $E_{sq/rd} = -418$ mV for I92K Fld. Thus, whereas replacement of I59 by Lys in Fld does not produce major changes in the midpoint redox potential values relative to those of WT Fld ($E_{ox/sq} = -212$ mV and $E_{sq/rd} = -436$ mV) (*36*), introduction of a Lys residue at position I92 caused alterations in both $E_{ox/sq}$ (+30 mV) and $E_{sq/rd}$ (+18 mV) to more positive values.

Steady-State Kinetics of WT FNR Using the Different Fld Mutants as Protein Carriers. The FNR k_{cat} values obtained using the I59K and I92K Fld mutants as electron carriers to Cytc were 14.5 and 17.4 s^{-1}, respectively, values that are only slightly lower (up to 1.5-fold) than that observed with WT Fld, 23.3 s^{-1}. The FNR K_m value for the I59K Fld mutant ($K_m = 37.8$ μM) was similar to that for WT Fld ($K_m = 33$ μM), while the FNR K_m value for I92K Fld was increased by a factor of 2 ($K_m = 72.5$ μM). These data suggest that the affinity of the FNR/Fld complex is not modified by replacement of Fld I59 by a Lys, while introduction of a Lys at position I92 only slightly destabilizes the interaction. Therefore, when the catalytic efficiency (k_{cat}/K_m) of FNR was determined using the different Fld variants in the cytochrome *c* assay, it turned out that both Fld mutants had an FNR catalytic efficiency only slightly smaller than that of WT (up to 3-fold).

Interaction of Different Fld_{ox} Forms with FNR_{ox}. Similar spectral perturbations were detected by difference absorption spectroscopy for I59K and I92K Fld_{ox} mutants upon mixing with FNR_{ox} as were obtained using WT Fld (not shown), suggesting that the orientation of the two proteins in the complex is not appreciably altered by the mutations studied here. Accordingly, the K_d values obtained (8.3 μM and 7.1 μM for the complex of FNR with I59K and I92K Fld forms, respectively, vs the value of 3.0 μM for the complex with WT Fld) indicate that introduction of positive charges either at positions 59 or 92 of *Anabaena* Fld only produces a slight weakening of the FNR_{ox}/Fld_{ox} interaction. Moreover, the $\Delta\epsilon$ values obtained for the transition band at 460 nm are not too different from the value obtained for the WT Fld complex (1.4 mM^{-1} cm^{-1} for WT Fld vs 1.4 and 2.1 mM^{-1} cm^{-1} for I59K and I92K Flds, respectively), suggesting that I59 and I92 exert little influence on the orientation of the FNR_{ox}/Fld_{ox} complex.

Stopped-Flow Kinetic Analysis of the Reaction between WT FNR and I59K and I92K Fld Mutants. The reaction of WT FNR_{ox} with I92K Fld_{rd} occurs, as also shown for WT Fld, within the dead time of the instrument (Table 3). However, when this process was assayed using the I59K Fld_{rd} mutant, an increment of absorbance at 600 nm, which was best fit to a two-exponential process, was observed with a k_{ap1} value >300 s^{-1} and a k_{ap2} value of 160 s^{-1} (Figure 3C; Table 3), indicating that both reactions (1 and 2) are slowed by the introduction of a positive charge at position 59 of *Anabaena* Fld.

Table 3: Kinetic Parameters for the Reactions of WT FNR with WT and Mutated Flds as Studied by Stopped-Flow[a]

	k_{ap} (s^{-1}) for the mixing of Fld_{rd} with FNR_{ox}		k_{ap} (s^{-1}) for the mixing of Fld_{ox} with FNR_{rd}	
Fld form	k_{ap1}	k_{ap2}	k_{ap1}	k_{ap2}
WT[b]	n.d[c]		2.5	1.0
I59K	>300	160	27	3.5
I92K	n.d[c]		2.0	0.9

[a] Reaction followed at 600 nm. [b] Data from ref *9*. [c] Reaction occurred within the dead time of the instrument.

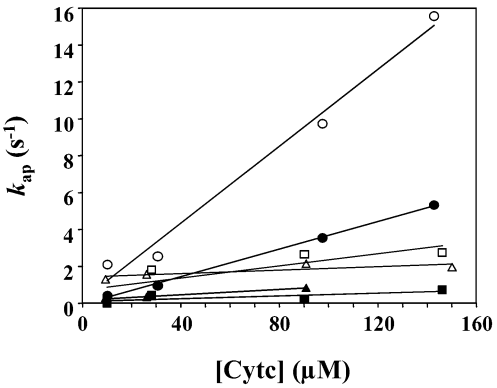

FIGURE 4: Apparent observed rate constants for the reduction of $Cytc_{ox}$ by different Fld_{rd} forms. $Cytc_{ox}$ concentration dependence of k_{ap1} (open symbols) and k_{ap2} (closed symbols) for WT Fld_{rd} (○, ●), I59K Fld_{rd} (□, ■), and I92K Fld_{rd} (▲, △). The monitoring wavelength was 550 nm, and the temperature was 13 °C. All samples were prepared in 50 mM Tris/HCl, pH 8.0.

When analyzing the reverse process (the reaction of FNR_{rd} with Fld_{ox}), again I92K Fld behaved similarly to the WT Fld (Table 3), while the process with I59K occurred considerably faster (Figure 3D), having 10- and 3.5-fold larger values, respectively, for k_{ap1} and k_{ap2} corresponding to reactions 3 and 4. Since in all the cases the amplitudes of the reactions were similar to those with WT Fld, it can be assumed that they occur to the same extent.

The reactivity of the L76D and L78D FNR mutants with the I59K and I92K Fld mutants has also been assayed in both directions. The obtained data (not shown) indicate that, as for WT Fld, there is no interaction that leads to an efficient electron-transfer process with any of the Fld mutants.

Stopped-Flow Kinetic Analysis of the Reaction between the Fld_{rd} Mutants and $Cytc_{ox}$. To elucidate if the changes observed in FNR k_{cat} values obtained by measuring the NADPH-cytochrome *c* reductase activity when using Fld mutants could be caused by the less efficient interaction and ET between Fld_{rd} and Cytc, fast kinetic analysis of the reaction between different Fld_{rd} forms and $Cytc_{ox}$ was carried out using stopped-flow methodology. When WT Fld_{rd} reacted with $Cytc_{ox}$ an increase in absorbance at 550 nm was observed, consistent with $Cytc_{ox}$ reduction, which was fit to a two-exponential process (not shown). The dependence of such k_{ap} values on Cytc concentration was linear (Figure 4), indicating that either there is no complex formation during

the ET process or the K_d value for the intermediate complexes is very large. Moreover, stoichiometric studies indicate that one Fld_{rd} μmol is reducing 1.8−2 μmol of $Cytc_{ox}$. Such observations might be consistent with an initial collisional one-electron transfer from Fld_{rd} to a $Cytc_{ox}$ molecule (reaction 5), followed by reduction of a second $Cytc_{ox}$ molecule by the Fld_{sq} generated in reaction 5 (reaction 6):

$$Cytc_{ox} + Fld_{rd} \xrightarrow[k_{ap1}]{} Cytc_{rd} + Fld_{sq} \quad (5)$$

$$Fld_{sq} + Cytc_{ox} \xrightarrow[k_{ap2}]{} Fld_{ox} + Cytc_{rd} \quad (6)$$

When analyzing this reaction for I59K and I92K Flds the same two-exponential behavior was observed (not shown), allowing the determination of both k_{ap} values, which again were linearly dependent on Cytc concentration (Figure 4). However, the k_{ap1} and k_{ap2} values obtained with the Fld mutants are consistently smaller than the corresponding values obtained for WT Fld (Figure 4), suggesting a weaker interaction and/or slower ET from Fld to Cytc upon replacement of either I59 or I92 by a Lys.

Kinetic Analysis of the Laser Flash Photolysis Reduction of Fld Forms by PSI. Reduction of *Anabaena* Fld_{ox} to the semiquinone state by PSI particles can be followed using laser-flash absorption spectroscopy. Previous studies have shown that although the physiological reaction is the reduction of the Fld_{sq} form to the fully reduced state, this can be a useful reaction model to analyze the interaction and ET parameters involved in the reduction of Fld by PSI (*22*, *33*, *37*, *38*). Reduction of I59K and I92K Flds by *Anabaena* PSI particles followed monoexponential kinetics, as has been reported for WT (*37*). The observed pseudo-first-order rate constants (k_{obs}) for the reduction of WT and I59K Fld reduction by PSI depend nonlinearly on the concentration of flavoprotein, showing a saturation profile (Figure 5). This suggests that a bimolecular transient [PSI/Fld] complex is formed prior to ET, according to the following minimal two-step reaction mechanism, as described previously in this and other PSI/Fld systems (*22*, *33*, *37*, *38*):

$$PSI_{rd} + Fld_{ox} \underset{}{\overset{K_a}{\rightleftharpoons}} [PSI_{rd}\cdots Fld_{ox}] \xrightarrow{k_{et}} PSI_{ox} + Fld_{sq} \quad (7)$$

in which the ET first-order rate constant (k_{et}) can experimentally be inferred from the limiting k_{obs} at infinite Fld concentration, while K_a is the equilibrium constant of the complex. Minimal values for K_a and k_{et}, which can be estimated from data similar to those in Figure 5 (*33*, *35*), are shown in Table 4 for WT and I59K Flds at pH 7.5. From these values, it is evident that the I59K mutation causes both a 50% decrease in the equilibrium constant for complex formation and a 4-fold increase in k_{et}.

The most dramatic changes in Fld reduction by PSI are observed with the I92K Fld mutant, in which a linear dependence of k_{obs} on Fld concentration is obtained (Figure 5). This denotes a collisional-type mechanism in which no formation of a stable transient complex is observed. From the linear plot in Figure 5, a second-order bimolecular rate constant can be estimated (Table 4). It is interesting to note that although the observed linear fit indicates a decrease in the K_a for complex formation, the k_{obs} values obtained at high Fld concentration are higher for the I92K mutant than for the WT Fld, thus also indicating that the ET process itself is more efficient in this mutant. This can be easily explained taking into account the above determined $E_{ox/sq}$ value for the I92K Fld mutant, which is 30 mV more positive than that reported for the WT Fld (*36*). Taking into account the electrostatic nature of the interaction between Fld and PSI in *Anabaena* (*37*), the experimental k_{obs} values were determined at varying $MgCl_2$ concentrations, to check the effect of the mutations on the electrostatic interactions with PSI. The obtained profiles (not shown) indicated that whereas WT and I92K proteins have similar ionic strength dependencies, the I59K mutant was more efficient in ET in the range of salt concentration used in this study.

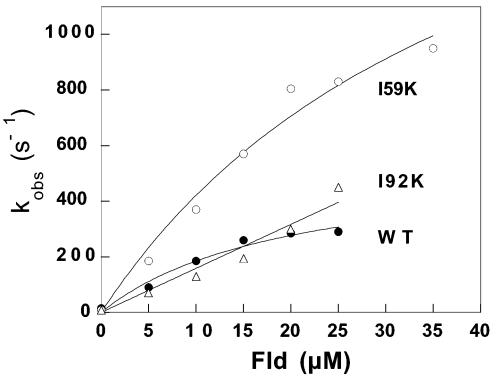

FIGURE 5: Dependence of the k_{obs} upon Fld concentration for reduction of WT and mutated Fld forms by *Anabaena* PSI. The solid curves for WT and I59K proteins correspond to the theoretical fitting according to the two-step formalism described by Meyer et al. (*35*).

Table 4: Kinetic Parameters for the Reduction of WT and Mutated Flds by *Anabaena* PSI as Studied by Laser Flash-Photolysis[a]

Fld form	K_a (M^{-1})	k_{et} (s^{-1})	k_2 ($M^{-1}\,s^{-1}$)
WT	6.0×10^5	500	
I59K	2.7×10^5	1900	
I92K			1.6×10^7

[a] The rate constants were estimated from kinetic traces followed at 580 nm.

DISCUSSION

The three-dimensional structure of FNR shows that the FAD redox center is partially exposed at the bottom of a concave cavity to which, as indicated by all available biochemical and structural data, either Fd or Fld must associate, donate one electron, and dissociate from during catalysis (*10*, *12*). In the case of the FNR/Fd system, it has been shown that both electrostatic and hydrophobic interactions play an important role in these processes of association and dissociation (*6*, *12*, *13*, *16*−*19*), as has also been reported in other systems (*38*, *39*). However, thus far neither data about the role of hydrophobic interactions in the FNR/Fld system nor about the three-dimensional structure of the [FNR/Fld] complex are available. In the present study, the site-directed mutants of *Anabaena* FNR made at the three hydrophobic amino acid residues, L76, L78, and V136, that were shown to play a critical role in the interaction with Fd (see Figure 1A from ref *13*) have been analyzed in the corresponding processes with Fld. Moreover, the role of two

hydrophobic residues on the Fld surface close to the FMN cofactor, I59 and I92 (Figure 1), has also been studied.

Our results clearly indicate that the introduction of a negatively charged residue at positions 76 and/or 78 of *Anabaena* FNR impairs the ET transfer processes that take place to and from Fld (Table 2). Binding constants between the oxidized forms of these mutants and Fld_{ox} could not be measured (Table 1), while no reaction at all could be detected in the steady-state kinetic assay with Fld as protein carrier, presumably because complex formation was too weak. Stopped-flow data also indicate an important impairment for the ET processes between these FNR mutants and Fld, while the observed amplitude changes suggest that the reactions between the protein partners seem to occur to the same extent as with the WT enzyme. All these data taken together indicate that it is not the ET transfer process per se that is affected by the mutation but the stabilization of the complex. As previously shown (*13*), placement of a negative charge at positions 76 or 78, and especially at both positions simultaneously, produces a change of polarity in the FAD environment that must have a marked destabilizing effect on the complex since it has been shown that R16, K72, and K75 provide a positively charged region on the FNR surface that is particularly important in the binding and orientation of both protein carriers, Fd and Fld, during ET (*12*). Moreover, since it has been shown that no major changes are produced in the midpoint redox potential upon the introduction of such negative charges at positions 76 and 78 (*13*), the effect of the mutations on the observed rates for electron exchange between FNR and Fld cannot be due to an effect on the free energy of the reaction. Therefore, as previously concluded for the FNR/Fd interaction, the drastic effect observed on the rates of reaction for these mutants with Fld is most likely a consequence of a decrease in the ability of the reductase to recognize Fld and to form an efficient complex because of the introduction of negative charges at positions 76 and 78 of the reductase.

The L76A, L76V, L78A, L78V, V136A, and V136L FNRs represent conservative substitutions, where the hydrophobic and nonpolar character of the L76, L78, and V136 side chains are maintained, with the main difference between them being the side chain volume that extends from the protein surface. Our results indicate that although all these mutants are able to form complexes and to transfer electrons to and from Fld, the reactions are slightly modified by the introduced mutations as indicated by their interaction and kinetic parameters with Fld (Tables 1 and 2). For example, L76V, L78A, and V136A FNR forms have rate constants (Tables 1 and 2) consistently smaller than those of the WT FNR and have a significant increment in their K_d value for [FNR/Fld] complex, whereas L76A, L78V, and V136L donate electrons more efficiently to Fld (Tables 1 and 2) than do L76V, L78A, and V136A. These data suggest that FNR establishes specific hydrophobic interactions with Fld. Also noticeable is the effect observed by replacement of L76 by Val, which produces an important impairment in the interaction and ET processes with Fld, whereas when this position is occupied by the less bulky residue Ala a behavior more similar to that of WT is observed. In addition, much larger $\Delta\epsilon_{(465)}$ values are observed upon complex formation of both of these mutants with Fld than for any other FNR form, suggesting a different environment of the FAD upon complex formation. Such observations indicate that it is not only the volume or the nature of the side-chain but also its three-dimensional conformation that influence the complex formation and the relative position of both proteins within the complex formed.

Replacement of L76, L78, or V136 in *Anabaena* FNR by Ser apparently only affects the [FNR/Fld] complex formation for the L78S mutant (Figure 2; Table 1). Thus, for this mutant the K_d value is 10-fold larger than that for the WT complex. However, much less efficient reactions with Fld are observed for all these FNR mutants than with the WT FNR (Tables 1 and 2). Since it has been reported that the introduction of such mutations produces a shift of the flavin midpoint redox potential to more positive values, +20 mV for L76S and V136 FNRs and +39 mV for L78S (*13*), the altered midpoint redox potential values can explain the less efficient processes where electrons must flow from FNR to Fld, especially if full reduction of the latter must be accomplished ($E_{ox/sq} = -212$ mV, $E_{sq/rd} = -436$ mV) (*36*) (Tables 1 and 2). However, faster processes would be expected in those reactions where electrons flow from Fld_{rd} to FNR, as reported for the reduction by Fd_{rd} (*13*), since the redox potential differences between the two reacting centers will be more favorable for the Ser mutants than for the WT enzyme**.** However, for this latter process all three Ser mutants appear highly impaired in their ability to accept electrons from Fld_{rd} (Table 2), suggesting that the main effect is on structural aspects of the FNR/Fld association that are altered by the introduced mutations leading to the formation of less productive interactions.

Finally, replacement of either or both of the L76 or L78 residues by the larger hydrophobic aromatic residue Phe produces enzymes that interact only slightly less effectively with Fld than the WT (Table 1). Moreover, the complex involving L76F/L78F results in a somewhat different electronic arrangement around the flavin ring as judged by the difference spectrum (Figure 2). ET reactions from Fld and these mutants are considerably less favorable than those of the WT (Table 2), especially for the double mutant. In these cases, the ET process occurred with smaller rate constants and also to a much smaller extent (Figure 3B). Therefore, our results indicate that subtle changes in the hydrophobic patch of FNR involving residues L76, L78, and V136 can influence the rates of ET to and from Fld by changing the binding constants, altering the midpoint redox potential of the FAD group, and changing the environment around the cofactor. We have previously reported similar effects when FNRs having mutations in this hydrophobic patch were assayed for their reactivity with Fd (*13*), which again supports the idea that FNR uses the same site for the interaction with Fd and Fld (*12*). However, careful inspection of the results shows that, in general, the introduction of some of the mutations on FNR produced more drastic effects in the processes involving Fld than those involving Fd, suggesting that each of these *Anabaena* FNR residues do not participate to the same extent in the processes of complex formation and ET between the two proteins.

Since our data suggest that this hydrophobic FNR patch is critical for efficient Fld association, it is expected that a nonpolar region on the Fld surface would interact with this region of FNR during complex formation and ET, as has been shown in the case of the FNR/Fd association (*5*, *10*). The three-dimensional structure of *Anabaena* sp. PCC 7119

Fld shows I59 and I92 to be the only two hydrophobic residues close to the isoalloxazine ring where interaction with its ET substrates is expected (Figure 1). Therefore, in the present study we have analyzed the effect of replacing these two Ile residues on the interaction of Fld with its two physiological redox partners, FNR and PSI, and also with its artificial ET acceptor, Cytc.

The kinetic parameters obtained for FNR, using the different Fld variants as protein carriers in the NADPH-dependent cytochrome *c* reductase assay, as well as the data derived from differential spectroscopic analysis of the FNR_{ox}/Fld_{ox} interaction, indicate that the introduced mutations do not produce major effects in the stability of the [FNR_{ox}/Fld_{ox}] complex and only slightly decrease the k_{cat} values of the overall process. However, stopped-flow analysis of the ET from Fld_{rd} to $Cytc_{ox}$ clearly indicates that each of the Fld mutants is less effective in ET to Cytc than WT Fld, suggesting that I59 and I92 play a role in the interaction or ET process between Fld and Cytc. Moreover, stopped-flow analysis suggests that replacement of I59 by Lys appears to only slightly alter the ET processes between Fld and FNR. Reactions involving ET from FNR_{rd} to Fld_{ox} have rate constants up to 10-fold higher than those obtained with WT Fld (Figure 3D, Table 3). However, when analyzing the reverse process, reduction of FNR by Fld variants, the ET reaction of I59K is slightly impaired relative to WT Fld (Figure 3C, Table 3). Since the ability to form a complex is apparently not affected and the E_2 value for this mutant is similar for that reported for the WT, structural aspects of the protein−protein interaction may account for the observed slight enhancement or impairment of the interaction. In summary, our data indicate that although I59 Fld slightly modulates the ET processes between FNR and Fld, neither this residue nor I92 appears to be critical for the interaction and ET processes. Therefore, so far no residue on the Fld surface that has been investigated has been shown to be critical for the interaction and ET processes between Fld and FNR (*22*, *23*). Such observations, together with the difficulties in obtaining a homogeneous crystal for the [FNR/Fld] complex, might suggest a lower specificity in the FNR/Fld interaction than that of the FNR/Fd one.

Interaction and ET between reduced PSI and the I59K and I92K Flds were analyzed by using laser flash photolysis. The obtained results indicate that the I59K mutation in Fld causes both a 50% decrease in the association constant and a 4-fold increase in k_{et}. In the case of the I92K Fld mutant, a collisional-type mechanism in which no formation of a stable transient complex is formed appears to be operative. Therefore, these data clearly indicate that I92 from Fld plays an important role in the interaction of the flavoprotein with PSI, and probably in the formation of the productive ET complex, whereas I59 plays a much modest role in those processes. Actually, the introduction of additional positive charges in Fld could induce repulsive interactions with positive groups in PSI subunits (K35 in PsaC and R39 in PsaE) previously proposed to be involved in PSI/Fld complex stabilization (*25*).

In summary, in the ET process from PSI to FNR via Fld, the I59, and especially, the I92 residues of *Anabaena* Fld appear to play a role in the association and ET processes from PSI to Fld, whereas they do not appear to be a determinant in the corresponding processes between Fld and FNR. Moreover, our results confirm that, although the initial interaction between FNR and Fld involves electrostatic forces between the two proteins, the arrangement of the complex formed by these electrostatic interactions is not the most efficient for ET, probably because of an improper orientation of the two redox centers (*5*, *12*, *16*−*18*). In this context, nonpolar residues in the region close to the flavin group in FNR participate in the establishment of hydrophobic interactions between the two interacting proteins, which serve to orient the two groups in a manner such that ET is favored. Thus, in the present study we have shown the importance of the hydrophobic surface comprised of residues L76, L78, and V136 in *Anabaena* FNR, in complex formation, and ET to Fld. However, the counterpart region on the Fld surface is thus far not known.

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