

## Involvement of Glutamic Acid 301 in the Catalytic Mechanism of Ferredoxin-NADP<sup>+</sup> Reductase from *Anabaena* PCC 7119<sup>†</sup>

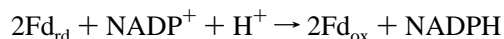
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**ABSTRACT:** The crystal structure of *Anabaena* PCC 7119 ferredoxin-NADP<sup>+</sup> reductase (FNR) suggests that the carboxylate group of Glu301 may be directly involved in the catalytic process of electron and proton transfer between the isoalloxazine moiety of FAD and FNR substrates (NADPH, ferredoxin, and flavodoxin). To assess this possibility, the carboxylate of Glu301 was removed by mutating the residue to an alanine. Various spectroscopic techniques (UV–vis absorption, fluorescence, and CD) indicate that the mutant protein folded properly and that significant protein structural rearrangements did not occur. Additionally, complex formation of the mutant FNR with its substrates was almost unaltered. Nevertheless, no semiquinone formation was seen during photoreduction of Glu301Ala FNR. Furthermore, steady-state activities in which FNR semiquinone formation was required during the electron-transfer processes to ferredoxin were appreciably affected by the mutation. Fast transient kinetic studies corroborated that removal of the carboxylate at position 301 decreases the rate constant approximately 40-fold for the electron transfer process with ferredoxin without appreciably affecting complex formation, and thus interferes with the stabilization of the transition state during electron-transfer between the FAD and the iron–sulfur cluster. Moreover, the mutation also altered the nonspecific reaction of FNR with 5′-deazariboflavin semiquinone, the electron-transfer reactions with flavodoxin, and the reoxidation properties of the enzyme. These results clearly establish Glu301 as a critical residue for electron transfer in FNR.

Ferredoxin-NADP<sup>+</sup> reductase (FNR,<sup>1</sup> EC 1.18.1.12) catalyzes the reduction of NADP<sup>+</sup> to NADPH during photosynthesis in all higher plants as well as in vegetative cells of the cyanobacterium *Anabaena* PCC 7119. This flavoprotein contains a single polypeptide chain and a noncovalently bound flavin adenine dinucleotide cofactor (FAD) as the only redox center. FNR accepts one electron from each of two molecules of the one-electron carrier ferredoxin (Fd) and uses them to convert NADP<sup>+</sup> into NADPH via hydride (H<sup>-</sup>) transfer from the N-5 atom of the flavin adenine dinucleotide (FAD), according to the reaction (1)



This protein 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). The three-dimensional structures of oxidized and reduced native spinach FNR and of the complex with 2′-phospho-5′-AMP have been reported (2).

*Anabaena* PCC 7119 FNR consists of a single polypeptide chain of 303 amino acid residues (5). When *Anabaena* is grown in a low-iron medium, flavodoxin replaces ferredoxin as electron donor to FNR (6). The electron-transfer reactions between *Anabaena* FNR and its physiological partners have been extensively studied (7–17). Recently, the three-dimensional structure of *Anabaena* PCC 7119 FNR and a model for the complex with NADP<sup>+</sup> were determined (18). The FNR molecule consists of two domains. The FAD binding domain is made up of a scaffold of six antiparallel strands arranged in two perpendicular  $\beta$ -sheets, the bottom of which is capped by a short  $\alpha$ -helix and a long loop. The NADP<sup>+</sup> binding domain consists of a core of five parallel  $\beta$ -strands surrounded by seven  $\alpha$ -helices. This corresponds to a variant of the typical dinucleotide binding fold (2, 19). The FAD cofactor is bound to the protein through hydrogen

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<sup>1</sup> Abbreviations: PSI, photosystem I; FNR, ferredoxin-NADP<sup>+</sup> reductase; FNR<sub>ox</sub>, FNR in the oxidized state; FNR<sub>rd</sub>, FNR in the reduced state; FNR<sub>sq</sub>, FNR in the semiquinone state; Fd, ferredoxin; Fd<sub>ox</sub>, Fd in the oxidized state; Fd<sub>rd</sub>, Fd in the reduced state; Fld, flavodoxin; Fld<sub>ox</sub>, Fld in the oxidized state; Fld<sub>rd</sub>, Fld in the reduced state; Fld<sub>sq</sub>, Fld in the semiquinone state; INT, 2-(4-indophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; IPTG, isopropyl  $\beta$ -D-thiogalactoside; dRf, 5-deazariboflavin; dRfH<sup>•</sup>, semiquinone form of dRf; DCPIP, 2,6-dichlorophenolindophenol; TN, turnover number.

Table 1: Conservation of the Glu301 Position of *Anabaena* PCC 7119 FNR in the FNR Family<sup>a</sup>

NADPH-dependent reductases		ref
FNR		
	295	303
<i>Anabaena</i> PCC 7119	A G R W H V E T Y	[5]
<i>Spirulina</i> sp.	K H * * * * * *	[45]
<i>Synechococcus</i>	E H * * * * * V *	[46]
spinach	* E Q * N * * * V *	[2]
pea	* E Q * N * * * V *	[47]
ice plant	* E Q * N * * * V *	[48]
rat liver cytochrome P450 reductase	K G * Y S L D V W S	[49]
<i>Salmonella typhimurium</i> sulfite reductase	E R * Y Q R D V Y	[50]
NADH-dependent reductases		
human erythrocytes cytochrome <i>b</i> <sub>5</sub> reductase	H P T E R C F V F	[51]
<i>Pseudomonas cepacia</i> phthalate dioxygenase reductase	S * T V * F * S F	[52]
<i>Arabidopsis thaliana</i> nitrate reductase	D M A N S F V V F	[53]

<sup>a</sup> The final alignments were obtained by visual inspection. An asterisk indicates exact matches of the sequences to that of *Anabaena* PCC 7119 FNR.

bonds, van der Waals contacts, and stacking interactions. The isoalloxazine ring system, which constitutes the reactive part of the FAD, interacts with the aromatic rings of two tyrosine residues (Tyr79 and Tyr303). The edge of the dimethylbenzyl ring, which is putatively involved in intermolecular electron transfer with ferredoxin or flavodoxin, is the only part of the flavin isoalloxazine moiety exposed to the solvent. It is noteworthy that N-5 of the flavin ring, which plays a crucial role in hydride transfer, only makes one weak hydrogen bond with Ser80 (Ser96 in the spinach enzyme). Mutations of Ser96 in spinach have confirmed that this residue is essential in stabilizing productive interactions between the isoalloxazine ring of FAD and the nicotinamide ring of NADP(H) (20). Ser80 in *Anabaena* is hydrogen bonded to the carboxylate of Glu301 (18). Because this glutamate residue is exposed to solvent, it appears to be a good candidate for transferring protons from the external medium to the buried N-5 atom of the isoalloxazine through Ser80, in the same manner as Glu312 appears to transfer protons to the isoalloxazine N-5 through Ser96 in the spinach enzyme (4). Moreover, most of the amino acid sequences in the FNR family (except nitrate reductase and cytochrome *b*<sub>5</sub> reductase) have a conserved acidic residue corresponding to Glu301 of *Anabaena* FNR (cf. Table 1). Recently, Zanetti et al. (21) changed Glu312 of spinach FNR to leucine, glutamine, aspartate, and alanine. The biochemical characterization of all these mutants and the structural studies on the spinach Glu312Leu FNR mutant led them to propose that the modifications are confined to the microenvironment of the mutated residue and that Glu312 must be required to protect the point of entry of electrons from the protein substrate.

According to the general catalytic mechanism proposed for flavin-dependent enzymes, the hydride transfer from reduced FAD to NADP<sup>+</sup> must involve the previous reduction of the flavin by addition of a proton and an electron to each of the nitrogen atoms at positions 1 and 5 of the flavin ring.

It is expected that the alteration of acidic residues in the environment of the flavin isoalloxazine ring, which are potential candidates for participating as proton donors, could produce significant changes in the reaction. Moreover, the one-electron transfer that takes place between the FNR electron-transfer protein partners (ferredoxin or flavodoxin) and the FAD cofactor also requires the exchange of a single proton that is, again, added to nitrogen 5 of the flavin. In this case, an intermediate neutral semiquinone species is formed. In the present study, protein engineering on Glu301 has been carried out in order to investigate its role in the catalytic process. Alterations of this residue might be expected to produce significant changes in those reactions where the flavin ring exchanges protons with the medium. We have replaced Glu301 by alanine, thereby removing the side-chain carboxyl group and altering its polarity and hydrogen-bonding capabilities as well as increasing the exposure of the flavin ring to the solvent. In the characterization of this mutant, we have used steady-state and transient kinetic measurements as well as different spectroscopies to rationalize its behavior.

## MATERIALS AND METHODS

**Oligonucleotide-Directed Mutagenesis.** The Glu301Ala mutant was prepared by site-directed mutagenesis using as template, a construct of the *petH* gene (22) which has been previously cloned into the expression plasmid pTrc99a (23). Glu301Ala substitution was carried out using the Transformer Site-Directed Mutagenesis Kit from Clontech in combination with the synthetic oligonucleotide 5'-GCTTAGTATGTTGC-TACGTGCCAG-3', (base change is underlined) in combination with the synthetic oligonucleotide, 5'-AGTGCACCATC-CGCGGTGTGA-3', which transforms an *NdeI* site into an *SacII* site, as selection primer. The presence of the desired mutation and the lack of second-site mutations were confirmed by sequence analysis of the entire mutated gene. The pTrc99a vectors containing the mutated FNR gene (i.e., Glu301Ala FNR) were transformed into *Escherichia coli* PC strain 0225. Prior to the large-scale purification of mutated FNR, overexpression of the mutant was tested by SDS-PAGE of a cell lysate formed by the sonication of cells harvested from IPTG-induced 10 mL cultures which had been resuspended in 0.2 mL of 50 mM Tris/HCl, pH 8.0.

**Purification of the Glu301Ala FNR Mutant.** Wild-type FNR and Glu301Ala FNR were purified from the corresponding IPTG-induced *E. coli* cultures as previously described (23). A final purification and concentration step was introduced by loading the protein onto a Mono-Q 10/10 column (Pharmacia) in an FPLC system (Pharmacia), using Tris/HCl pH 8. The protein was eluted with a linear gradient of 0 to 0.5 M NaCl. UV-visible absorption spectroscopy and SDS-PAGE electrophoresis results were used as purity criteria.

**Spectral Analysis.** Ultraviolet/visible spectral analyses were carried out either on a Hewlett-Packard diode array 8452 spectrophotometer or on a Kontron Uvikon 860 spectrophotometer. The molar absorption coefficient of the 459 nm peak due to protein-bound flavin was determined by removing the FAD from the apoprotein by heating for 10 min at 85 °C and spectrophotometrically quantitating the released FAD. Protein samples were denatured by incubation

of 40  $\mu\text{M}$  enzyme solutions in 50 mM Tris/HCl, pH 8.0, at 80 °C for 20 min. Circular dichroism was carried out on a Jasco 710 spectropolarimeter at room temperature in a 1 cm path length cuvette. The protein concentrations were 0.7  $\mu\text{M}$  for the far-UV and 4  $\mu\text{M}$  for the aromatic and visible regions of the spectrum. Samples were prepared in 1 mM Tris buffer, pH 8.0. Protein and flavin fluorescence were monitored using a KONTRON SFM 25 spectrofluorometer interfaced with a PC computer. Solutions used for fluorescence spectra contained 4  $\mu\text{M}$  protein in 50 mM Tris/HCl, pH 8.0.

Dissociation constants, binding energies and extinction coefficients of the complexes between oxidized FNR species and NADP<sup>+</sup>, oxidized ferredoxin, or oxidized flavodoxin were obtained as previously described (8). These experiments were performed on solutions containing approximately 30  $\mu\text{M}$  protein in 50 mM Tris/HCl buffer, pH 8.0 at 25 °C, to which aliquots of concentrated NADP<sup>+</sup>, oxidized ferredoxin, or oxidized flavodoxin (1–5  $\mu\text{L}$  additions) were added stepwise. Experimental data were fit to the theoretical equation for 1:1 stoichiometry by means of nonlinear regression using the program KaleidaGraph 2.1 from Abelbeck Software on a Macintosh computer.

*Enzymatic Assays.* Steady-state kinetic studies were carried out at 25 °C. The diaphorase (EC 1.8.1.4) activity, either with DCPIP or with ferricyanide as electron acceptors, and the FNR-dependent NADPH-cytochrome *c*, reductase activity were assayed as described (24, 25). The latter was determined using either ferredoxin, or flavodoxin. The standard reaction mixtures for the cytochrome *c* reductase activity contained, in a final volume of 1 mL, 50 mM buffer, 2.5 nM FNR, 0.75 mM cytochrome *c* and varied concentrations of NADPH, ferredoxin or flavodoxin in order to calculate the corresponding  $K_m$  values. All the measurements were carried out in 50 mM Tris/HCl, pH 8.0, unless otherwise stated. Photoreduction of NADP<sup>+</sup> was studied as described by Shin (25), except that *Anabaena* PCC 7119 thylakoidal membranes were used. These membranes were isolated from a 15 L culture of *Anabaena* PCC 7119. After centrifugation, the pellet was resuspended in 100 mL of buffer A (0.5 M sorbitol, 10 mM MgCl<sub>2</sub>, 10 mM HEPES, 5 mM phosphate, 12.5 mM EDTA, pH 7.5) and incubated with lysozyme (5 mg/mL) for 1 h at 37 °C in the dark. After centrifugation for 90 s at 2000 rpm in a Beckman JA-20 rotor, the supernatant was centrifuged at 20 000 rpm in the same rotor for 20 min. The pellet was washed with buffer B (10 mM HEPES, 10 mM MgCl<sub>2</sub> in 5 mM phosphate, pH 7.5) and a second cycle of centrifugations was performed. The new pellet was incubated in buffer B with 1 M NaNO<sub>3</sub> for 1 h at room temperature with gentle shaking. After a final centrifugation at 2000 rpm in a Beckman JA-20 rotor for 20 min, the pellet was washed, and the thylakoidal membranes were resuspended in buffer B and stored at –70 °C in the dark. Total chlorophyll was determined as described by Mackinney (26).

The oxidase activity of the wild-type and mutated enzymes were measured by the decrease in the absorbance at 340 nm using 0.1 mM NADPH and 0.25  $\mu\text{M}$  of enzyme samples in 50 mM Tris/HCl, pH 8.0. Production of hydrogen peroxide was determined by incubation of 1.4  $\mu\text{M}$  wild-type or Glu301Ala FNR samples with 600  $\mu\text{M}$  NADPH at 20 °C. The production of hydrogen peroxide by each FNR form

was determined at different time intervals by the peroxidase assay with 3,3',5,5'-tetramethylbenzidine and diocylsulfosuccinate (27).

*FNR Photoreduction.* Photoreduction of protein-bound flavin was performed at 4 °C in an anaerobic cuvette containing 15–25  $\mu\text{M}$  FNR in 50 mM Tris/HCl, pH 8.0, also containing 20 mM EDTA and 2  $\mu\text{M}$  5-deazariboflavin. Reaction solutions were made anaerobic by successive evacuation and flushing with O<sub>2</sub>-free Ar. Absorption spectra were recorded after successive periods of irradiation with a 150 W light source.

*Laser Flash Photolysis Measurements.* Laser flash photolysis experiments [cf. ref 12] were performed anaerobically at room temperature (25 °C) in the presence of 100  $\mu\text{M}$  dRf. Photoexcitation of dRf was accomplished with a Photochemical Research Associates model LN100 nitrogen laser-pumped dye laser (0.1 mJ energy, 300 ps fwhm pulse duration) which pumped a BBQ dye solution (PRA 2A386; emission wavelength, 395 nm). The optical system used to monitor the reaction has been previously described (28). The Nicolet 1170 signal averager has been replaced by a Tektronix TDS410A digitizing oscilloscope in the current laser system. The laser-generated dRf triplet abstracts a hydrogen atom from EDTA which is present in large excess and produces the dRf semiquinone (dRfH•) which, in competition with its own disproportionation, reduces oxidized protein (29). All kinetic experiments were performed under pseudo-first-order conditions, in which protein is present in large excess over the dRfH• generated by the laser flash. Under these conditions dRfH• reacts almost exclusively with Fd<sub>ox</sub> when both Fd and FNR are present simultaneously (7). This allows one to monitor electron transfer from Fd<sub>rd</sub> to FNR<sub>ox</sub>. Solutions of dRf (100  $\mu\text{M}$  in 5 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA) were made anaerobic by bubbling for 1 h with H<sub>2</sub>O-saturated Ar. Microliter volumes of concentrated protein solutions were introduced into this solution in a 1 cm cuvette through a rubber septum, and Ar gas was blown over the sample surface to remove any added oxygen. Generally, data from four to eight flashes were averaged. Ionic strength was adjusted by adding aliquots of 5 M NaCl. Kinetic traces were analyzed using a computer fitting routine (KINFIT, OLIS Co., Bogart, GA). The apparent complex dissociation constant and the limiting first-order rate constant for intracomplex electron transfer were evaluated from these data by a nonlinear least-squares computer-fitting procedure (30).

*Stopped-Flow Kinetic Measurements.* Rapid-reaction kinetics were performed anaerobically at 13 °C with an Applied Photophysics SX17.MV stopped-flow spectrophotometer interfaced with an Acorn 5000 computer using the SX.17MV software of Applied Photophysics. Unless otherwise stated, all samples were made anaerobic before introduction into the stopped-flow syringes. All the reactions were carried out in 50 mM Tris/HCl, pH 8.0, at the appropriate wavelengths, as indicated in the corresponding table and figure legends. The observed rate constants ( $k_{\text{obs}}$ ) were determined by fitting the data to mono- or biexponential processes. Absorption spectra were recorded after each measurement in order to calculate the precise concentration of each sample.

Samples were made anaerobic (in specially designed tonometers which fit the stopped-flow apparatus) by successive evacuation and flushing with O<sub>2</sub>-free Ar. Reduced

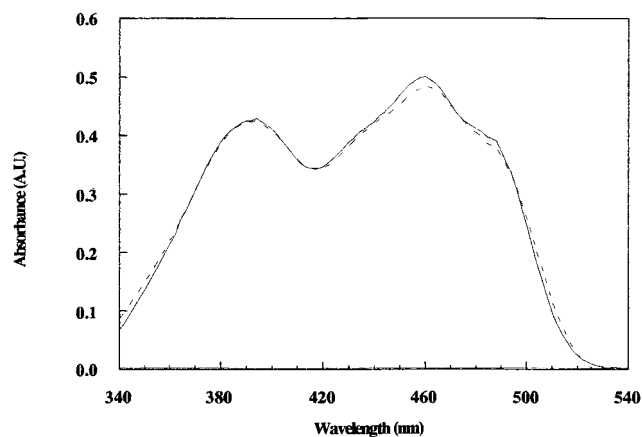


FIGURE 1: Absorption spectra of wild-type (—) and Glu301Ala (---) FNRs in the visible region. The spectra were recorded in 50 mM Tris/HCl, pH 8.0 at room temperature.

samples of ferredoxin, flavodoxin, and FNR for stopped-flow experiments were prepared by photoreduction as described above. The measurement of the rate of reoxidation of reduced FNR by molecular oxygen was carried out with a solution of air-saturated 50 mM Tris/HCl, pH 8.0.

## RESULTS

**Expression and Purification of the Glu301Ala FNR Mutant.** The level of expression in *E. coli* of the Glu301Ala mutant form of FNR was judged to be similar to that of the recombinant wild-type enzyme. The mutant was purified by following the same protocol as for the wild-type enzyme and was obtained in homogeneous form, as shown by SDS-PAGE electrophoresis, and in amounts suitable to perform the demanding characterization studies described herein.

**Spectral Properties.** The visible absorbance spectra of the oxidized form of the wild-type and Glu301Ala FNRs are shown in Figure 1. The alteration of the side chain of the amino acid residue at position 301 results in small, although significant, changes in the absorption properties of the flavin prosthetic group, perhaps resulting from alterations in the solvent exposure of the isoalloxazine ring. The absorbance maxima of both transitions I and II of the flavin (450 and 380 nm regions) [cf. 31] do not shift in the mutant relative to the wild-type FNR. Although no changes in absorptivity were detected in transition II, the extinction coefficient of transition I (at 459 nm) decreased slightly when Glu301 was replaced by alanine. The extinction coefficient for this transition in the mutant was calculated to be  $9.15 \text{ mM}^{-1} \text{ cm}^{-1}$ , whereas the value for the wild-type protein has been reported as  $9.4 \text{ mM}^{-1} \text{ cm}^{-1}$  (32). The FAD and the protein fluorescence of the mutated protein (not shown) were essentially the same as in the wild-type enzyme.

The circular dichroism spectrum of Glu301Ala FNR is compared with that of the wild-type enzyme in Figure 2. There are no shifts of the peak positions, and only small changes are observed in the relative molar ellipticities of the different bands, which correlates with the changes found in the absorption spectrum. Again, these small spectral perturbations observed by the replacement of Glu301 by Ala are likely due to subtle changes in the microenvironment of the isoalloxazine ring, which probably will be more exposed to the solvent in the mutated protein.

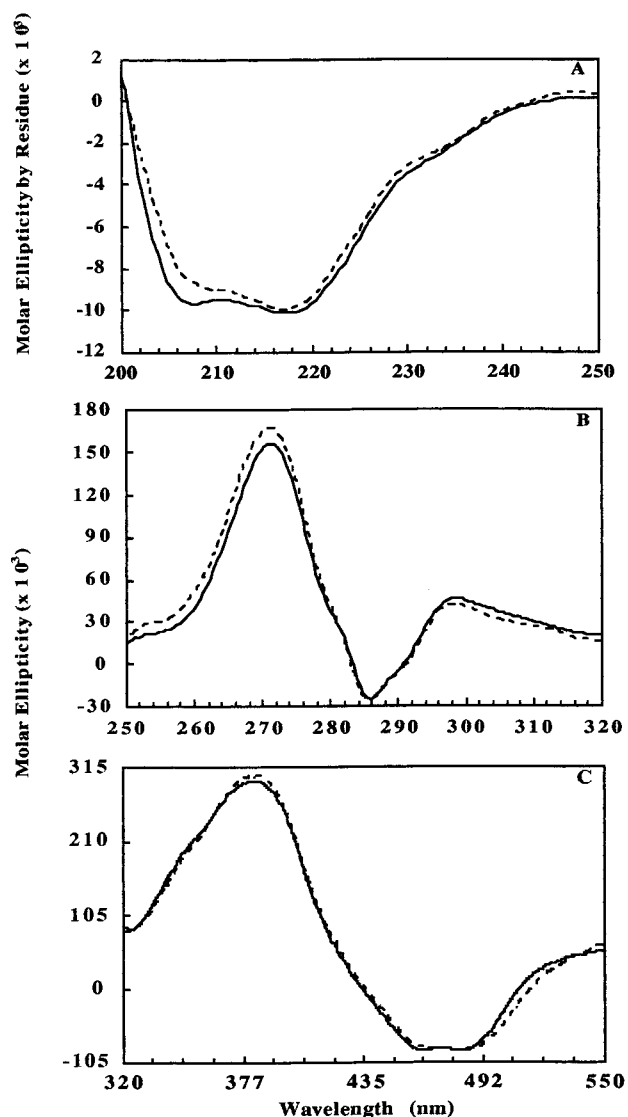


FIGURE 2: Circular dichroism spectra of wild-type (—) and Glu301Ala (---) FNR forms. Molar ellipticity by residue (A) in the far-ultraviolet, (B) near-ultraviolet, and (C) visible regions of the spectrum. All the spectra were recorded in 1 mM Tris/HCl, pH 8.0, at room temperature.

**Photoreduction of FNR Forms.** The FAD prosthetic group of the mutated Glu301Ala FNR was photoreduced with 5-deazariboflavin/EDTA; the rate of reduction was comparable to that of the wild-type enzyme. Although no significant changes were observed in the spectrum of the fully reduced Glu301Ala FNR form from that of the wild-type FNR, no absorbance changes attributable to a semiquinone transient species were observed in the case of the mutant. In contrast, wild-type FNR has been shown to accumulate about 15% of the total flavin as neutral blue semiquinone during the photoreduction process at pH 8.0 (33). This corresponds to a significant destabilization of the semiquinone by the mutation. It is not clear at present whether this is a kinetic or a thermodynamic effect; this will be discussed further below.

**Steady-State Kinetics.** Steady-state catalytic properties of wild-type and Glu301Ala FNRs were determined for various reactions catalyzed by FNR. The values of the kinetic parameters for the diaphorase reaction, using either DCPIP (a two-electron acceptor) or ferricyanide (a one-electron

Table 2: Steady-State Kinetic Parameters of Wild-Type and E301A Mutant FNRs

FNR	diaphorase activity							
	with DCPIP			with Fe(CN) <sub>6</sub> <sup>3-</sup>				
	$k_{cat}$ (s <sup>-1</sup> )	$K_m^{NADPH}$ ( $\mu$ M)	$k_{cat}/K_m^{NADPH}$ ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$K_m^{NADPH}$ ( $\mu$ M)	$k_{cat}/K_m^{NADPH}$ ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )	$K_m^{Fe(CN)_6^{3-}}$ ( $\mu$ M)	$k_{cat}/K_m^{Fe(CN)_6^{3-}}$ ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )
wild-type	81.5 ± 3.0	6.0 ± 0.6	13.5 ± 0.5	225 ± 3	23.0 ± 1.2	9.82 ± 0.15	172 ± 14	1.30 ± 0.02
E301A	75.0 ± 1.5	3.7 ± 0.2	20.2 ± 0.4	133 ± 5	32.0 ± 4.0	4.00 ± 0.15	50 ± 15	2.6 ± 0.1

FNR	NADPH-dependent cytochrome <i>c</i> reductase activity <sup>a</sup>							
	with ferredoxin as mediator				with flavodoxin as mediator			
	$k_{cat}$ (s <sup>-1</sup> )	$K_m^{NADPH}$ ( $\mu$ M)	$k_{cat}/K_m^{NADPH}$ ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )	$K_m^{Fd}$ ( $\mu$ M)	$k_{cat}/K_m^{Fd}$ ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$K_m^{Fld}$ ( $\mu$ M)	$k_{cat}/K_m^{Fld}$ ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )
wild-type	200 ± 10	6.0 ± 0.5	33.3 ± 1.7	11.0 ± 2.0	18.2 ± 1.0	23.3 ± 1.6	33 ± 5	0.70 ± 0.05
E301A	40.0 ± 1.6	8.3 ± 1.0	4.9 ± 0.2	2.4 ± 0.2	16.4 ± 0.7	11.1 ± 0.3	11.9 ± 1	0.92 ± 0.02

FNR	NADP <sup>+</sup> photoreduction with photosynthetic particles				electron-transfer kinetics between NADPH and O <sub>2</sub> using FNR as catalyst	
	with ferredoxin as mediator		with flavodoxin as mediator		TN (s <sup>-1</sup> )	
	TN (s <sup>-1</sup> )	%	TN (s <sup>-1</sup> )	%	TN (s <sup>-1</sup> )	
wild-type	2.45 ± 0.20	100	2.41 ± 0.20	100	0.06 ± 0.01	
E301A	0.96 ± 0.10	40	2.41 ± 0.15	100	0.23 ± 0.05	

<sup>a</sup> When calculating the  $K_m$  for one substrate of FNR the other one is present at saturating concentration.

acceptor), and for the NADPH-dependent cytochrome *c* reductase activity were obtained by fitting the data to the equation for a ping-pong mechanism. The values calculated for the Glu301Ala FNR are reported in Table 2 and compared with those obtained for the wild-type enzyme under the same conditions. The major effect of the mutation on the diaphorase activity using DCPIP as acceptor was on the  $K_m$  for NADPH, whereas  $k_{cat}$  decreased by only a minor extent. These results indicate that the catalytic efficiency of the mutant was about 149% that of the wild-type FNR in the reduction of DCPIP by NADPH. In contrast, when the diaphorase activity was assayed using ferricyanide as electron acceptor,  $k_{cat}$  for the Glu301Ala FNR was observed to decrease, with a minor increase in the  $K_m$  for NADPH. Thus, the mutant had 40% of the catalytic efficiency for NADPH as did wild-type FNR. When the Michaelis constant was determined for Fe(CN)<sub>6</sub><sup>3-</sup> in the diaphorase activity, we observed a decrease in the  $K_m$ . These data indicate that the mutant has a higher catalytic efficiency for this substrate.

The NADPH-dependent cytochrome *c* reductase activity of the Glu301Ala mutant was also studied and compared with that of the wild-type FNR. Both ferredoxin and flavodoxin were assayed as mediators for this activity. Replacement of Glu301 by alanine influenced mainly the  $k_{cat}$  value when ferredoxin was used as electron acceptor from FNR, producing a 5-fold decrease (Table 2). In this case, the  $K_m$  values for both substrates, NADPH and ferredoxin, were affected by the mutation, and the catalytic efficiency for NADPH in this reaction is only 14% that of the wild-type. A value of 16% of the activity of the wild-type spinach FNR has also been reported for this species when the Glu312Ala mutant was assayed in this reaction (21). It is noteworthy that, although the changes observed in the  $K_m$  for NADPH in the mutated FNR were minimal, they changed in the opposite direction from that found in the diaphorase activity with DCPIP, where a slight decrease of the  $K_m$  was observed, and in the same direction as in the diaphorase activity with ferricyanide, where a small increase in  $K_m$  was obtained.

In each of the activities noted above, the reaction is initiated by the reduction of FNR by the two-electron donor NADPH. In the NADPH-dependent cytochrome *c* reductase assay, however, ferredoxin is included in the reaction mixture. The ability of *Anabaena* FNR to form a ternary complex with ferredoxin and NADP<sup>+</sup> is well-known (8), and therefore it is possible that the presence of ferredoxin bound to the mutant affects the NADPH binding or electron transfer in a way different from that of the wild-type enzyme. On the other hand, ferredoxin is a one-electron acceptor protein and during the electron-transfer reaction between FNR<sub>rd</sub> and Fd<sub>ox</sub>, it is expected that an intermediate FNR<sub>sq</sub> species is produced. As noted above, the Glu301Ala FNR semiquinone is destabilized, making this one-electron process less favorable than with the wild-type enzyme and supporting the hypothesis that the electron transfer between FNR and ferredoxin has become the rate-limiting step in the catalytic mechanism involving the mutant FNR. This possibility is also supported by the fact that, whereas very minor changes were observed in the  $K_m$  for NADPH in all the activities assayed, the catalytic constants were appreciably affected in those processes where an intermediate semiquinone FNR species is expected to be produced after the transfer of a single electron (cf. Table 2).

The  $K_m$  for ferredoxin in the NADPH-dependent cytochrome *c* reductase activity of the mutant FNR decreased by a factor similar to the decrease of the  $k_{cat}$ , thereby retaining a value for the catalytic efficiency that is similar to that of the wild-type FNR (Table 2). When flavodoxin was used as mediator in this assay, the catalytic efficiency of the reaction for the wild-type FNR was only 3.8% of that of the reaction with ferredoxin. At this time is not clear if such behavior is due to the formation of nonspecific complexes between flavodoxin and cytochrome *c*, or to the fact that in this sequence of reactions, flavodoxin is forced to function between the fully oxidized and the semiquinone states, whereas normally it cycles between the hydroquinone and the semiquinone states. When Glu301Ala FNR was assayed with flavodoxin as mediator, a similar decrease of both the

$k_{\text{cat}}$  and the  $K_m$  values for flavodoxin were detected, keeping the catalytic efficiency at the same level.

The pH dependence of these reactions was also investigated (not shown), since it has been proposed that in the native enzyme, Glu301 is an excellent position to H-bond to the amide group of nicotinamide (4). When the pH was decreased below 7.0, the diaphorase activity of the mutant was affected to a greater extent than was the wild-type. In the NADPH-dependent cytochrome *c* reductase activity with ferredoxin as mediator, the maximum specific activity was found at a lower pH value for the wild-type protein (optimum pH, 7.0) than for the mutant (optimum pH, 8.5). This might indicate that protonation of Glu301 facilitates the electron-transfer reaction in the wild-type enzyme.

Photoreduction of NADP<sup>+</sup> by photosynthetic particles was also studied, thereby assaying the electron-transport chain in the physiological direction (Table 2). This reaction is the closest steady-state analogue to what occurs in vivo. The mutation of Glu301 produced an approximately 2.5-fold decrease in the specific activity when ferredoxin was used as the mediator (a 3.4-fold decrease was also reported for the spinach Glu312Ala mutant in such reaction (21)), whereas no changes were observed relative to the wild-type enzyme when the mutant protein was assayed with flavodoxin. It is also important to note that in this assay, flavodoxin substitutes for ferredoxin with the same efficiency as has been proposed to occur in vivo (6).

We have also observed that Glu301Ala reacted with NADPH in the absence of any exogenous electron acceptor and under aerobic conditions 3.5 times faster than the wild-type protein (Table 2). Since both proteins hardly showed any consumption of NADPH under anaerobic conditions, we concluded that Glu301Ala FNR reacted with oxygen faster than the wild-type protein. An oxidase activity has already been reported for a modified *Anabaena* FNR to which a viologen has been covalently linked (34). The possibility of superoxide radical formation from the interaction of the reduced mutated enzyme with the oxygen present in solution was checked with superoxide dismutase. While superoxide radical formation was detected for the wild-type protein, it was not detected for the Glu301Ala FNR. Since oxygen consumption was clearly evident (Table 2) and superoxide was not produced, other products of the reaction of the reduced flavin with oxygen were considered for the mutant. It was found that the incubation of NADPH with Glu301Ala FNR under aerobic conditions produced significant amounts of H<sub>2</sub>O<sub>2</sub>, which was not produced when the wild-type FNR was incubated under the same conditions (Figure 3). A lag time for the formation of H<sub>2</sub>O<sub>2</sub> was observed in the first minutes. We cannot explain the lag at this time but point out that it could be an artifact in the peroxidase assay at low H<sub>2</sub>O<sub>2</sub> concentrations.

**Interaction of FNR Forms with its Substrates.** To further investigate the effect of the mutation on the enzyme catalytic mechanism, the binding of substrates (NADP<sup>+</sup>, ferredoxin, and flavodoxin) to the mutant FNR was evaluated. Previous studies on *Anabaena* and spinach FNRs revealed that spectral perturbations appear, in the visible region, as a consequence of the formation of complexes of FNR with several electron-transfer proteins, such as ferredoxin, flavodoxin, or rubredoxin (8, 35), and with NADP<sup>+</sup> (8, 36). Difference absorption spectroscopy can then be used to measure the

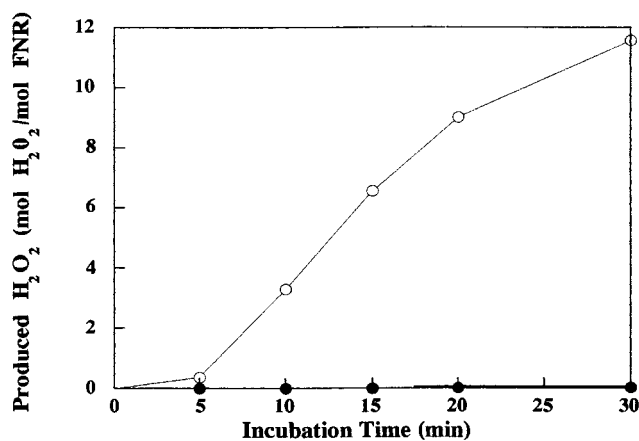


FIGURE 3: Hydrogen peroxide production by wild-type (●) and Glu301Ala (○) FNR forms when incubated with NADPH in an aerobic atmosphere at 20 °C; 1.4 μM wild-type and Glu301Ala FNR forms were incubated with 600 μM NADPH, and the hydrogen peroxide production was determined at different time intervals by the peroxidase assay.

binding constants for these complexes because they have a well-defined stoichiometry.

The interaction of the Glu301Ala FNR<sub>ox</sub> mutant with Fd<sub>ox</sub> was studied in order to evaluate whether structural changes introduced by the mutation could affect the Fd-binding site of the reductase. Comparison of the interaction of mutated FNR with both ferredoxin and flavodoxin was also of interest because both electron-transport proteins are natural substrates for the wild-type enzyme (6). The spectral changes produced when Glu301Ala FNR<sub>ox</sub> was titrated with Fd<sub>ox</sub> or Fld<sub>ox</sub> were similar to those found for wild-type FNR. Moreover, no major changes were observed in the  $K_d$  values, or in the binding energies (Table 3) for both substrates, indicating similar binding properties. Nevertheless, it is noteworthy that slightly higher extinction coefficients were found for the complexes formed with Glu301Ala FNR (Table 3). The significance of this is unclear.

When NADP<sup>+</sup> binds to wild-type FNR, the visible spectrum of the bound flavin undergoes a perturbation, yielding the difference spectrum shown in Figure 4. The same figure also shows the difference spectrum obtained by titrating the Glu301Ala FNR with NADP<sup>+</sup>. The spectral perturbations caused by the two FNRs upon NADP<sup>+</sup> binding were very similar, and only minor displacements of the minima and maxima were detected. Dissociation constants and binding energies were also similar (Table 3). The above results indicate that the NADP<sup>+</sup>, Fd, and Fld binding sites in FNR were not appreciably influenced by the replacement of Glu301 by alanine.

**Reduction of Glu301Ala FNR Studied by Laser-Flash Photolysis.** The reduction of Glu301Ala FNR by laser-generated dRfH<sup>•</sup> was monitored by the absorbance decrease at 460 nm and the increase at 600 nm, corresponding to FAD reduction and semiquinone formation, respectively. Transients were well fit by monoexponential curves, but the  $k_{\text{obs}}$  values showed a hyperbolic dependence on the concentration of FNR (Figure 5A). This latter behavior of the mutant was different from that observed for the wild-type FNR, where  $k_{\text{obs}}$  values were linearly dependent on the concentration of enzyme (inset Figure 5A), giving a second-order rate constant of  $(2.3 \pm 0.1) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$  at an ionic strength of 12 mM.

Table 3: Dissociation Constants and Free Energy for Complex Formation of Oxidized Wild-Type and E301A *Anabaena* PCC 7119 FNR Forms with NADP<sup>+</sup>, Ferredoxin, and Flavodoxin<sup>a</sup>

FNR form	$K_d^{\text{NADP}^+}$ ( $\mu\text{M}$ )	$\Delta\epsilon_{(480-394)}^{\text{NADP}^+}$ ( $\text{mM}^{-1}\text{cm}^{-1}$ )	$\Delta G^{\text{NADP}^+}$ ( $\text{kcal mol}^{-1}$ )	$K_d^{\text{Fd}}$ ( $\mu\text{M}$ )	$\Delta\epsilon_{(462)}^{\text{Fd}}$ ( $\text{mM}^{-1}\text{cm}^{-1}$ )	$\Delta G^{\text{Fd}}$ ( $\text{kcal mol}^{-1}$ )	$K_d^{\text{Fld}}$ ( $\mu\text{M}$ )	$\Delta\epsilon_{(465)}^{\text{Fld}}$ ( $\text{mM}^{-1}\text{cm}^{-1}$ )	$\Delta G^{\text{Fld}}$ ( $\text{kcal mol}^{-1}$ )
wild-type	$5.7 \pm 0.3$	$1.20 \pm 0.01$	$-7.10 \pm 0.05$	$4.0 \pm 1$	$2.00 \pm 0.08$	$-7.3 \pm 0.2$	$3 \pm 1$	$1.4 \pm 0.1$	$-7.4 \pm 0.3$
E301A	$3.5 \pm 0.5$	$1.00 \pm 0.01$	$-7.4 \pm 0.1$	$8.0 \pm 2$	$2.80 \pm 0.15$	$-6.9 \pm 0.2$	$4 \pm 2$	$3.0 \pm 0.1$	$-7.4 \pm 0.3$

<sup>a</sup> All the difference spectra were recorded in 50 mM Tris/HCl, pH 8.0, at 25 °C. All the proteins were in the oxidized state.

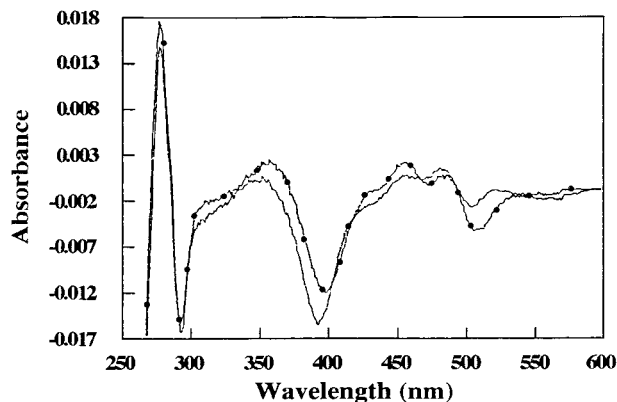


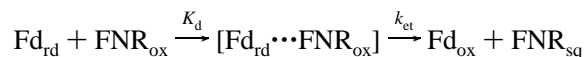
FIGURE 4: Difference absorbance spectra elicited by binding of FNR forms to NADP<sup>+</sup>. Binding of NADP<sup>+</sup> to oxidized wild-type (—) and Glu301Ala (●) FNRs. The experiment was performed at 25 °C with 39  $\mu\text{M}$  enzyme in 50 mM Tris/HCl, pH 8.0. Aliquots of a concentrated NADP<sup>+</sup> solution (4.1 mM) were added stepwise (1–5  $\mu\text{L}$  additions).

This result suggests that the FAD environment was modified by the mutation in a manner which allowed Glu301Ala FNR to form a complex with the dRfH<sup>\*</sup> during the electron-transfer process. Indeed, complex formation between 5-dRf<sub>ox</sub> and Glu301Ala FNR could be detected by differential spectroscopy (data not shown). From the laser flash kinetic data, values of  $1.7 \pm 0.1 \mu\text{M}$  and  $1800 \pm 100 \text{ s}^{-1}$  were calculated for the dissociation constant and the electron-transfer constants, respectively, based on a mechanism involving complex formation followed by electron transfer (see ref 30, and see below).

The kinetics of laser flash-induced reduction of *Anabaena* Fd by dRfH<sup>\*</sup> in the presence of wild-type *Anabaena* FNR was described previously (15, 17). The effect of ionic strength on the  $k_{\text{obs}}$  value for the reaction between reduced ferredoxin and Glu301Ala FNR is shown in Figure 5B and compared with that obtained for the wild-type enzyme (the latter data are from ref 17). It is clear that the electron-transfer process between Fd<sub>rd</sub> and FNR<sub>ox</sub> was greatly inhibited by the replacement of Glu301 by Ala in *Anabaena* FNR. It is also clear that the reaction with Glu301Ala FNR did not show the dramatic biphasic ionic strength dependence observed for the wild-type FNR. The sharp decrease in rate constant below 100 mM ionic strength has been attributed to the influence of electrostatic interactions, which cause the two proteins to form a less productive complex at low ionic strengths (15, 17). The absence of this effect in the mutant protein suggests that interactions involving the negative charge at Glu301 are a significant component of this behavior. Similar effects have been previously noted upon mutagenesis of acidic residues in Fd (Glu67, Glu68, Glu69; [ cf. ref 16]). This deserves further study.

The dependence of  $k_{\text{obs}}$  on FNR concentration for the reduction of Glu301Ala by Fd<sub>rd</sub> at  $I = 12$  and 100 mM are

shown in the main panel of Figure 5C. The hyperbolic behavior of these curves allowed the data to be fit using the analytical solution (30) of the differential equations describing the following two-step mechanism:



From this procedure,  $K_d$  for the formation of the intermediate complex can be calculated, as well as the value for the limiting rate constant,  $k_{\text{et}}$ . These values were  $15.2 \pm 1.0 \mu\text{M}$  (uncorrected  $K_d$  value; see below) and  $95 \pm 6 \text{ s}^{-1}$  at  $I = 12 \text{ mM}$  and  $11.1 \pm 0.6 \mu\text{M}$  (uncorrected  $K_d$  value; see below) and  $65 \pm 4 \text{ s}^{-1}$  at  $I = 100 \text{ mM}$ , respectively. The corresponding values for reduction of wild-type FNR by Fd at  $I = 100 \text{ mM}$  were previously reported as  $9.3 \pm 0.7 \mu\text{M}$  and  $6200 \pm 400 \text{ s}^{-1}$ , respectively (17).

As previously shown (17), the data obtained by this procedure must be corrected for the concentration of preformed  $[\text{Fd}_{\text{ox}} \cdots \text{FNR}_{\text{ox}}]$  complex, which prevents reduction of Fd<sub>ox</sub> by dRfH<sup>\*</sup>. Given the magnitude of the binding constant for this complex, a significant fraction of FNR will be present as this nonproductive species, especially at low ionic strength. Using difference spectroscopy, we have directly measured the dissociation constant for complex between Fd<sub>ox</sub> and Glu301Ala FNR<sub>ox</sub> at  $I = 12 \text{ mM}$ , obtaining a value of  $4.6 \pm 0.5 \mu\text{M}$ . After correction of the Glu301Ala FNR<sub>ox</sub> kinetic data with this value of  $K_d$ , analysis of the resulting  $k_{\text{obs}}$  vs FNR concentration curve at  $I = 12 \text{ mM}$  yielded values of  $2.6 \pm 0.4 \mu\text{M}$  and  $83 \pm 5 \text{ s}^{-1}$  for  $K_d$  and  $k_{\text{et}}$ , respectively. The corresponding values for wild-type FNR (17) were  $2.2 \pm 0.3 \mu\text{M}$  and  $3600 \pm 400 \text{ s}^{-1}$ , respectively. Thus, the free energies for complex formation between reduced ferredoxin and wild-type and Glu301Ala FNR forms are  $-7.68$  and  $-7.58 \text{ kcal/mol}$ , respectively, practically indistinguishable.

On the basis of a comparison of the extent of reoxidation of laser flash reduced Fd by wild-type and mutant FNRs, we estimate that the reduction potential for the oxidized/semiquinone couple ( $E_1^\circ$ ) of FNR is approximately 20 mV more negative in the Glu301Ala mutant than in the wild-type enzyme (this is based upon the observation that it requires approximately 2 times as much mutant FNR than wild-type to completely reoxidize Fd<sub>rd</sub>). This could contribute to the destabilization of the semiquinone form in the mutant noted above.

From these data we can conclude that the principal effect of mutating Glu301 of *Anabaena* PCC 7119 FNR to alanine is on the rate constant of the electron-transfer process between reduced Fd and FNR. It is important to note that the effect on electron transfer observed in the transient kinetic experiments is much larger than that seen in the steady-state assay of NADP<sup>+</sup> photoreduction (Table 2). This is presumably a consequence of the fact that the rate-determining step

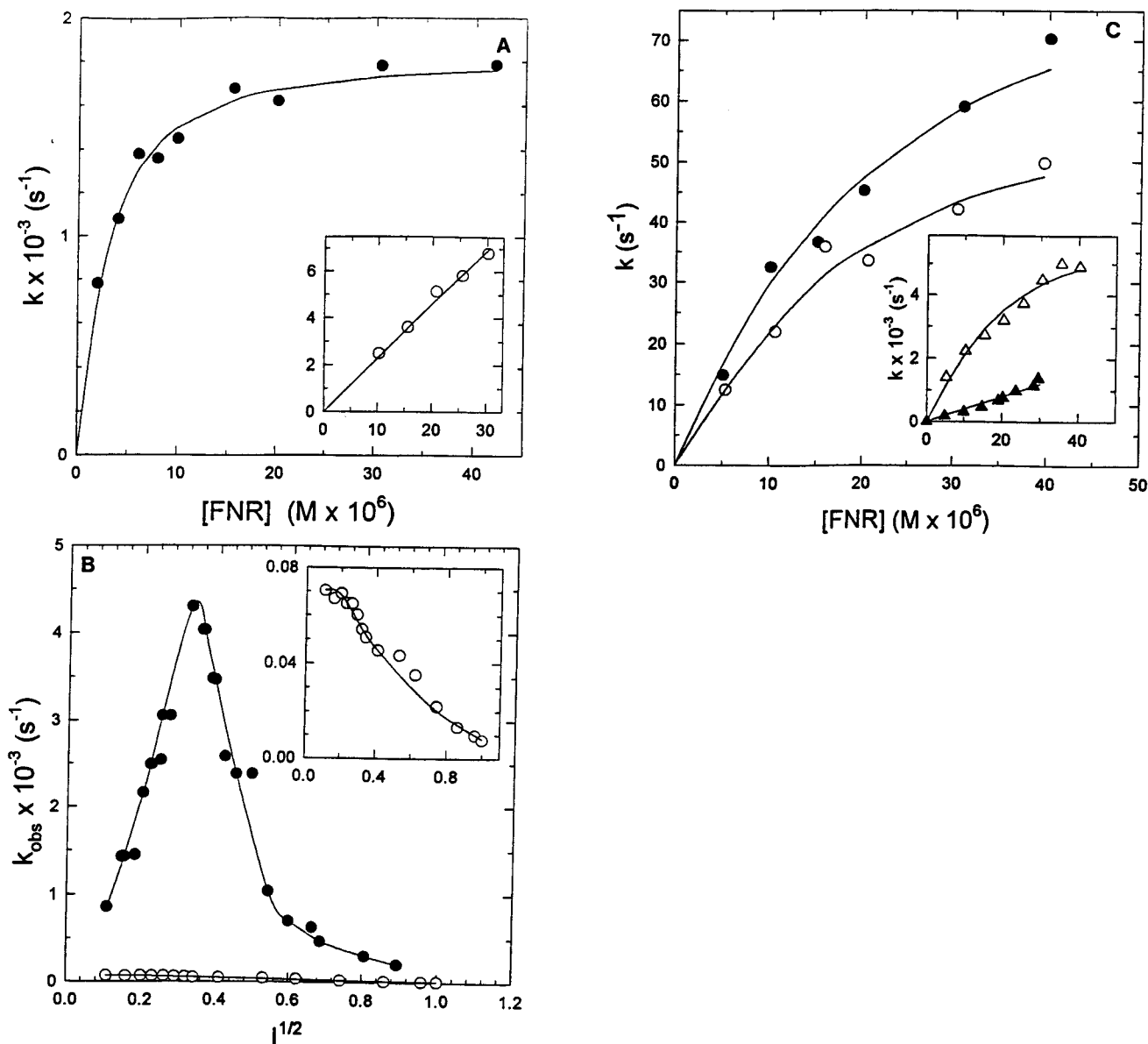


FIGURE 5: Laser-flash photolysis studies. (A) Second-order plots for the laser flash-induced reduction of Glu301Ala FNR by dRf semiquinone. Kinetics were monitored at 600 nm. The solution was buffered at pH 7.0 using 4 mM potassium phosphate buffer containing 1 mM EDTA and 100  $\mu$ M dRf. Ionic strength was 12 mM. The inset shows the corresponding plot for the wild-type enzyme. (B) Dependence of pseudo-first-order rate constants on ionic strength for the reduction of wild-type ( $\bullet$ ) and Glu301Ala ( $\circ$ ) FNR forms by  $\text{Fd}_{\text{rd}}$ . The ionic strength was adjusted with NaCl. The reaction mixtures contained 40  $\mu$ M Fd and 31  $\mu$ M FNR for the wild-type experiment, and 39  $\mu$ M Fd and 40  $\mu$ M FNR for the Glu301Ala experiment. Other conditions as in A. The inset shows the Glu301Ala data in an enlarged scale. (C) Pseudo-first-order rate constants for the reduction of Glu301Ala FNR by wild-type Fd at  $I = 12$  mM ( $\bullet$ ) and  $I = 100$  mM ( $\circ$ ). FNR was titrated into solutions containing 40  $\mu$ M Fd. The wild-type FNR data at  $I = 12$  mM ( $\blacktriangle$ ) and  $I = 100$  mM ( $\triangle$ ) shown in the inset were taken from Hurley et al. (17); 30  $\mu$ M Fd was present in each case. Other conditions as in A and B. All of the experiments were carried out at room temperature.

in the more complex steady-state system is something other than electron transfer from  $\text{Fd}_{\text{rd}}$  to  $\text{FNR}_{\text{ox}}$  (note that the turnover numbers in the  $\text{NADP}^+$  photoreduction assay are in the range of 1–3  $\text{s}^{-1}$ , whereas the electron-transfer rate constants are much larger than this).

**Rapid Reaction Stopped-Flow Studies of the Glu301Ala FNR.** To better understand the different behaviors observed for the Glu301Ala FNR in the various steady-state catalytic activities assayed, stopped-flow kinetic studies were conducted to investigate the time course of association and electron transfer between FNR and its different substrates. The kinetics of reduction of the *Anabaena* FNR forms by NADPH and the reoxidation of reduced enzyme by  $\text{NADP}^+$ , were determined by following the flavin spectral changes at

460 nm under anaerobic conditions. Unless otherwise stated, reactant concentrations (approximately 40  $\mu$ M NADPH or  $\text{NADP}^+$  and 7  $\mu$ M FNR, after mixing) were chosen to ensure pseudo-first-order conditions. The  $K_{\text{d}}$  value for the spinach FNR–NADPH complex, which has been reported to be 1  $\mu$ M (37), was used to ensure such conditions. Wild-type *Anabaena* FNR reacted very rapidly with NADPH (Figure 6A). The observed decrease of absorption at 460 nm was best fit by two exponentials: a fast phase with a rate constant greater than 600  $\text{s}^{-1}$  (this being the limit, i.e., dead time, of our instrument) and a second phase having a rate constant of at least 140  $\text{s}^{-1}$  (because most of these reactions were taking place within the dead time of the instrument, only the final traces could be analyzed) (Table 4). Similar



Table 4: Fast Kinetic Parameters for Electron Transfer Reactions of Wild Type and E301A FNRs Studied by Stopped-flow<sup>a</sup>

FNR form	$k_{\text{obs}}$ (s <sup>-1</sup> ) for the mixing of FNR <sub>ox</sub> with			$k_{\text{obs}}$ (s <sup>-1</sup> ) for the mixing of FNR <sub>rd</sub> with			
	NADPH <sup>c</sup>	Fd <sub>rd</sub> <sup>d</sup>	Fld <sub>rd</sub> <sup>e</sup>	NADP <sup>+</sup> <sup>c</sup>	Fd <sub>ox</sub> <sup>d</sup>	Fld <sub>ox</sub> <sup>e</sup>	O <sub>2</sub>
wild-type	> 600 <sup>b</sup> > 140 <sup>f</sup> ± 20	nd <sup>b</sup> 250 ± 30	nd <sup>b</sup>	> 600 <sup>b</sup>	nd <sup>b</sup>	2.5 ± 1.0 1.0 ± 1.0	5.0 <sup>e</sup> ± 1.0 0.76 <sup>e</sup> ± 0.08
E301A	> 600 <sup>b</sup> 130 ± 30	35 ± 10 10 ± 3	> 300 <sup>b</sup> 30 ± 10	130 ± 20	19 ± 6	0.4 ± 0.2 0.02 ± 0.01	1.09 <sup>e</sup> ± 0.30

<sup>a</sup> The samples were mixed in the stopped-flow spectrometer at the indicated concentrations (final concentration given) and redox states;  $T = 13$  °C. All samples were prepared in 50 mM Tris/HCl, pH 8.0. <sup>b</sup> Reaction occurred within the instrument dead time. <sup>c</sup> Reactions followed at 460 nm. <sup>d</sup> Reactions followed at 507 nm. <sup>e</sup> Reactions followed at 600 nm. <sup>f</sup> Most of the reaction took place within the instrument dead time.

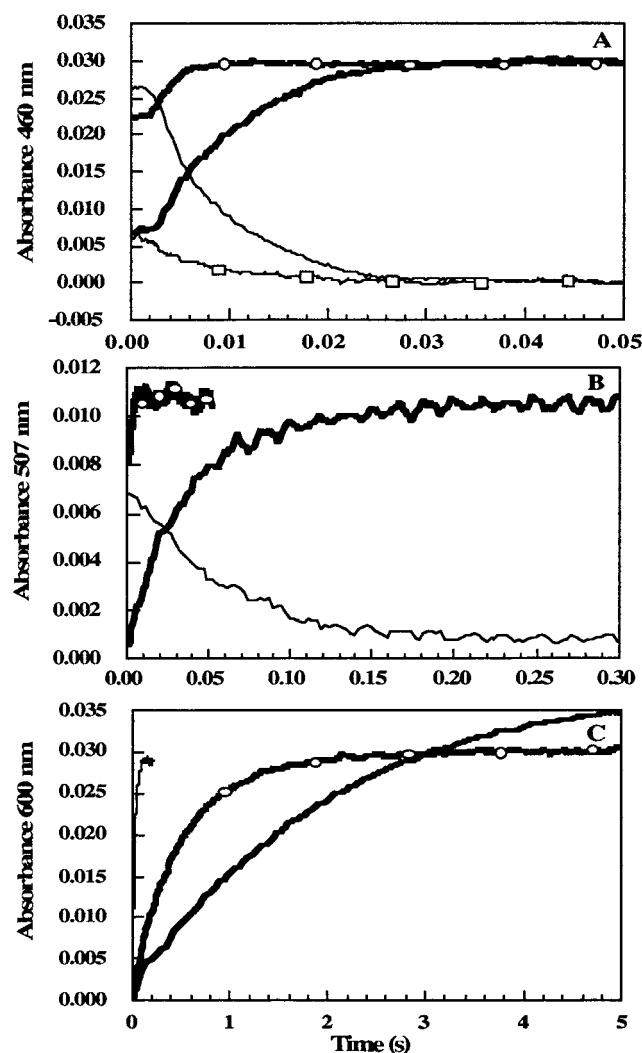


FIGURE 6: Time course of anaerobic reactions of FNR forms with the different FNR substrates. Reactions were carried out in 50 mM Tris/HCl, pH 8.0 at 13 °C. Final concentrations are given. (A) 6.5  $\mu\text{M}$  Glu301Ala FNR<sub>ox</sub> (—) was reacted with 44  $\mu\text{M}$  NADPH, 5  $\mu\text{M}$  wild-type FNR<sub>ox</sub> (---) with 44  $\mu\text{M}$  NADPH, 7.5  $\mu\text{M}$  wild-type FNR<sub>rd</sub> (—○—) with 44  $\mu\text{M}$  NADP<sup>+</sup> and 7.5  $\mu\text{M}$  Glu301Ala FNR<sub>rd</sub> (bold line) with 40  $\mu\text{M}$  NADP<sup>+</sup>. (B) 16.5  $\mu\text{M}$  Glu301Ala FNR<sub>ox</sub> (bold line) was reacted with 33  $\mu\text{M}$  Fd<sub>rd</sub>, 9.5  $\mu\text{M}$  wild-type FNR<sub>ox</sub> (—○—) with 28  $\mu\text{M}$  Fd<sub>rd</sub> and 14  $\mu\text{M}$  Glu301Ala FNR<sub>rd</sub> (—) with 25  $\mu\text{M}$  Fd<sub>rd</sub>. (C) 10.5  $\mu\text{M}$  Glu301Ala FNR<sub>ox</sub> (—) was reacted with 35  $\mu\text{M}$  Fld<sub>rd</sub>, 14  $\mu\text{M}$  Glu301Ala FNR<sub>rd</sub> (bold line) with 27.5  $\mu\text{M}$  Fld<sub>ox</sub> and 11.5  $\mu\text{M}$  wild-type FNR<sub>rd</sub> (—○—) with 27.5  $\mu\text{M}$  Fld<sub>ox</sub>.

behavior has already been reported for spinach FNR (38), concluding that the FNR rapidly formed an intermediate species, which had been reported to be a charge-transfer complex, [FNR<sub>ox</sub>-NADPH]. In the second phase, reduction of flavin occurred by hydride transfer from NADPH to FAD,

resulting in an equilibrium mixture of both complexes, [FNR<sub>ox</sub>-NADPH] and [FNR<sub>rd</sub>-NADP<sup>+</sup>]. A rate constant value for flavin reduction of 140 s<sup>-1</sup> could be consistent with those published for the spinach enzyme (38–40), particularly when one considers the differences in protein source, ionic strength, pH, and temperature. Reaction of Glu301Ala FNR with NADPH showed a similar behavior. A fast phase corresponding to charge-transfer complex formation ( $k > 600$  s<sup>-1</sup>) was followed by a slower phase having a rate constant of 130 s<sup>-1</sup>, which is again ascribed to flavin reduction (Figure 6A). This reaction was also studied at several lower NADPH concentrations. A nonlinear dependence of the slower  $k_{\text{obs}}$  was observed (data not shown).

The reaction of FNR<sub>rd</sub> with NADP<sup>+</sup> was also studied (Figure 6A). An increase in absorbance at 460 nm due to FNR reoxidation and an increase at 340 nm (approximately an isosbestic point for FNR) due to NADPH formation were obtained. The observed rates were similar at both wavelengths and were well fit by a single exponential with rate constants greater than 550 s<sup>-1</sup> (instrument limited) at all NADP<sup>+</sup> concentrations assayed. This reaction must be the electron-transfer process within the complex, i.e., [FNR<sub>rd</sub>-NADP<sup>+</sup>] → [FNR<sub>ox</sub>-NADPH]. It has been reported that when spinach FNR<sub>rd</sub> and NADP<sup>+</sup> were mixed, a new species was rapidly formed, probably [FNR<sub>rd</sub>-NADP<sup>+</sup>]. This species decayed rapidly with an increase in absorbance at 340 and 456 nm, indicating electron transfer from flavin to NADP<sup>+</sup> (40). The rate constant for this reaction reported by Batie and Kamin (40) was 500 s<sup>-1</sup>, which is consistent with the present study, despite differences in proteins and experimental conditions.

When this reaction was assayed with Glu301Ala FNR (Figure 6A), the rate constant for electron transfer within the complex, i.e., [Glu301Ala FNR<sub>rd</sub>-NADP<sup>+</sup>] → [Glu301Ala FNR<sub>ox</sub>-NADPH], was observed to decrease to a value of 130 s<sup>-1</sup>. As expected, the rate constant values obtained from transients collected at 340 nm were identical, within experimental error, to those calculated at 460 nm. It is clear from these results that the observed rate constant obtained with the mutated protein is at least 4 times smaller than that obtained with the wild-type FNR. Since, as for the wild-type FNR, the decays fit a first-order process and the absorbance changes corresponded with reoxidation of a large amount of the protein, we assumed that formation of the transient species [Glu301Ala FNR<sub>rd</sub>-NADP<sup>+</sup>] is as fast as for the wild-type protein and occurs within the dead time of the instrument. Moreover, electron transfer need not result solely in [FNR<sub>ox</sub>-NADPH], since an equilibrium mixture with [FNR<sub>rd</sub>-NADP<sup>+</sup>] would give similar kinetics (38).

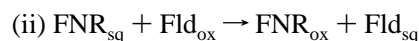
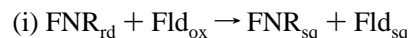
The rate of electron transfer between FNR, in the oxidized and reduced states, and its electron-transfer partners, ferredoxin and flavodoxin, was also studied by stopped-flow spectrophotometry. Electron-transfer reactions between FNR and Fd were followed at 507 nm. This wavelength is an isosbestic point for FNR<sub>ox</sub> and FNR<sub>sq</sub> and is near an isosbestic point for FNR<sub>sq</sub> and FNR<sub>rd</sub>. Thus, the reduction or reoxidation of ferredoxin can be followed at this wavelength. Solutions containing 56  $\mu\text{M}$  Fd<sub>rd</sub> (photoreduced by 5-dRf) and 19  $\mu\text{M}$  wild-type FNR<sub>ox</sub> were mixed in the stopped-flow spectrometer, and the absorbance changes followed at 507 nm. The observed kinetic profile was fit to a single-exponential process (Figure 6B) and gave a value of 250  $\text{s}^{-1}$ . However, according to the data obtained by laser-flash photolysis (Figure 5B), the expected  $k_{\text{obs}}$  for electron transfer between Fd<sub>rd</sub> and wild type FNR<sub>ox</sub> producing FNR<sub>sq</sub> and Fd<sub>ox</sub>, should be at least 1000  $\text{s}^{-1}$  under our stopped-flow experimental conditions. These data indicate that the process we were following by stopped-flow was not reduction of FNR<sub>ox</sub> by Fd<sub>rd</sub>. Complex formation and electron transfer from Fd<sub>rd</sub> to FNR<sub>ox</sub> must have occurred within the dead time of the instrument, and all of the FNR<sub>ox</sub> was converted into FNR<sub>sq</sub> very rapidly. Thus, the process we were observing in this experiment should be the oxidation of Fd<sub>rd</sub> and reduction of FNR<sub>sq</sub> to FNR<sub>rd</sub>. Moreover, similar behavior has been reported for the spinach FNR (40), where it has also been shown that reduction of FNR semiquinone by Fd is relatively slow ( $k_{\text{obs}} = 37\text{--}80 \text{ s}^{-1}$ ). When Glu301Ala FNR<sub>ox</sub> was mixed with photoreduced Fd, the increase in absorbance observed at 507 nm was best fit to a two-exponential process, with rate constants of 35 and 10  $\text{s}^{-1}$ , both exhibiting the same amplitude (Figure 6B). Because, as has been shown by laser flash photolysis, replacement of Glu301 by Ala produced a decrease of the electron-transfer rate for the process Fd<sub>rd</sub> + FNR<sub>ox</sub>  $\rightarrow$  FNR<sub>sq</sub> + Fd<sub>ox</sub> from 3600 to 83  $\text{s}^{-1}$ , we propose that this process is now being observed as the fast phase for the mutant in the stopped-flow experiment ( $k_{\text{obs}} = 35 \text{ s}^{-1}$ ), which should then be followed by the reduction of the FNR<sub>sq</sub> by reduced Fd with an observed rate constant of 10  $\text{s}^{-1}$ .

Reduction of ferredoxin by wild-type FNR<sub>rd</sub> was also assayed, but the reaction, producing Fd<sub>rd</sub> and FNR<sub>sq</sub>, was too fast to be followed by stopped-flow spectrophotometry. Nevertheless, when reduction of ferredoxin by Glu301Ala FNR<sub>rd</sub> was studied, a rate constant of only 19  $\text{s}^{-1}$  was obtained for the process (Table 4, Figure 6B), demonstrating that this reaction was also affected by the introduction of the mutation. All these data are consistent with the laser flash photolysis results in clearly indicating that the electron-transfer reaction between Fd and FNR to produce the FNR<sub>sq</sub> is greatly affected by the replacement of Glu301 by an alanine residue.

Electron-transfer reactions between FNR and flavodoxin were followed mainly at 600 nm, although kinetic traces at 460 nm were also obtained in order to elucidate which reactions were taking place. A wavelength of 600 nm was appropriate for the study of both reduction of FNR by Fld<sub>rd</sub> and reduction of Fld by FNR<sub>rd</sub>, since the final expected products of the reactions should be the semiquinone forms of both flavoproteins. As expected, the time course of wild-type FNR<sub>ox</sub> reduction by reduced flavodoxin was too fast to be followed by stopped-flow. However, when the reduction of Glu301Ala FNR by Fld<sub>rd</sub> was studied, an observed kinetic

trace was obtained which was best fit by a two-exponential process: one having a rate constant larger than 300  $\text{s}^{-1}$ , and the other having a rate constant of 30  $\text{s}^{-1}$ , both with similar amplitudes. The initial process is consistent with the formation of both semiquinones, by reduction of the mutated FNR<sub>ox</sub> to the semiquinone state by Fld<sub>rd</sub>, followed by reduction of FNR<sub>sq</sub> to the fully reduced state by the Fld<sub>rd</sub> which is still present in the mixture. This proposed mechanism for the mutant is consistent with the absorbance changes obtained at 460 nm.

Upon studying the reaction between wild-type FNR<sub>rd</sub> and Fld<sub>ox</sub> to yield FNR<sub>sq</sub> plus Fld<sub>sq</sub>, an unexpected very slow process was detected (Table 4, Figure 6C) that showed increasing absorbance at 600 nm. Experiments at increasing flavodoxin concentrations clearly showed that the observed increase in absorbance at 600 nm corresponded to two phases. At low flavodoxin concentrations (27.5  $\mu\text{M}$ ) the values obtained for the observed rate constants of both processes were very similar ( $k_{\text{obs}} = 1$  and 2.5  $\text{s}^{-1}$ ), and they could easily be mistaken for a single phase. Nevertheless, higher flavodoxin concentrations (200  $\mu\text{M}$ ) clearly showed the presence of two phases ( $k_{\text{obs}} = 10$  and 30  $\text{s}^{-1}$ ). These two processes could be ascribed to the following sequential processes, although we cannot unequivocally assign the experimentally determined rate constants to them:



Thus far, there have been no data reported for the electron-transfer rate from FNR<sub>rd</sub> to Fld<sub>ox</sub>. Nevertheless, our steady-state studies of the NADPH-dependent cytochrome *c* reductase activity with flavodoxin clearly indicate that oxidized FNR is not able to accept one electron from reduced FNR as efficiently as ferredoxin, since the rate of electron transfer from FNR<sub>rd</sub> to flavodoxin is much smaller than the corresponding rate with ferredoxin. However, the second process, reduction of flavodoxin by the semiquinone of FNR, has been studied by laser flash photolysis and shown to have a very fast electron-transfer rate constant ( $>7000 \text{ s}^{-1}$ ) (41). Nevertheless, in the reaction we are studying here, this fast process must occur following reaction (i), since FNR<sub>sq</sub> produced in reaction (i) is needed by reaction (ii). The mutant Glu301Ala FNR, when assayed like the wild-type protein described above under the same conditions, also showed two phases, but electron-transfer rates were markedly different (Table 4, Figure 6C). In this case, reduction of flavodoxin to the semiquinone state by the reduced mutant FNR was six times slower than for wild-type FNR<sub>rd</sub> (Table 4, Figure 6C). Again, these results indicate that electron transfer between Fld and FNR to produce the FNR<sub>sq</sub> is affected by replacement of Glu301 by an alanine residue.

As noted above, steady-state studies suggest that the reoxidation of Glu301Ala FNR<sub>rd</sub> by oxygen takes place by a process that differs from that involving the wild-type enzyme. To directly verify this hypothesis, the time course of flavin reoxidation by molecular oxygen was followed by stopped-flow spectrophotometry at 460 and 600 nm. It is known that electron transferases, such as flavodoxin and FNR, stabilize the neutral flavin semiquinone and produce substantial amounts of  $\text{O}_2^-$  on reaction of the fully reduced

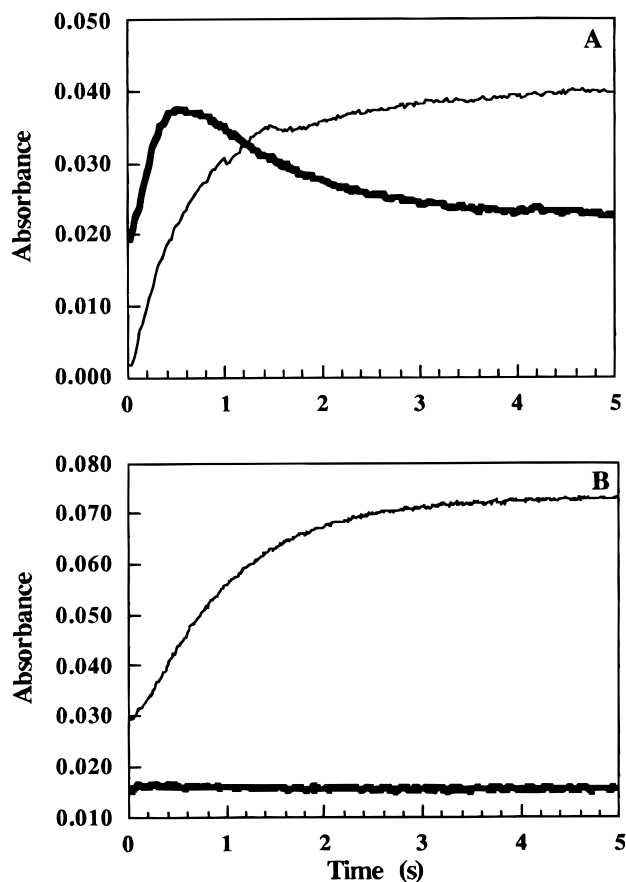


FIGURE 7: Time course of anaerobic reactions of FNR forms with molecular oxygen. (A) Reaction of 8.5  $\mu\text{M}$  wild-type FNR<sub>rd</sub> and (B) of 8.2  $\mu\text{M}$  Glu301Ala FNR<sub>rd</sub> with an air-saturated 50 mM Tris/HCl, pH 8.0, solution followed at (—) 460 and (bold line) 600 nm. Other conditions as in Figure 6.

protein with O<sub>2</sub>, inasmuch as the reactivity of the semiquinone with O<sub>2</sub> is several orders of magnitude smaller (42, 43). When 20  $\mu\text{M}$  wild-type FNR<sub>rd</sub> was reacted with aerobic 50 mM Tris/HCl, pH 8, reoxidation of the protein was observed at 460 nm as an increase in absorbance (Figure 7A). The process was best fit to two phases. When the reaction was followed at 600 nm, two sequential processes were observed. First, an increase in absorbance occurred that was ascribed to semiquinone formation, after which a decrease in absorbance occurred that must correspond to reoxidation of the semiquinone FNR by its reaction with O<sub>2</sub> (Figure 7A). The observed rate constants for such processes were 5 and 0.76 s<sup>-1</sup> (Table 4). When studying the reactivity of Glu301Ala FNR<sub>rd</sub> with molecular O<sub>2</sub>, we observed that the process at 460 nm was best fit to a single phase with an observed rate constant of 1 s<sup>-1</sup>, while no changes in absorbance were detected at 600 nm (Figure 7B). These results indicate that, upon reaction with O<sub>2</sub> Glu301Ala, FNR does not stabilize the semiquinone state, and thus this mutant does not behave as an electron transferase in its reaction with molecular O<sub>2</sub>. This result is consistent with the photo-reduction experiments noted above.

## DISCUSSION

Kinetic analysis of the reaction catalyzed by FNR is rather difficult due to the fact that the enzyme reactions can be assayed in two directions and can occur with a number of

different substrates. When NADPH is the reduced substrate, the electron acceptor can be either a nonphysiological compound (ferricyanide, DCPIP, INT), in which case the diaphorase activity is assayed, or ferredoxin, in what is called the NADPH-cytochrome *c* reductase reaction. The flow of electrons can also be assayed in the opposite direction, that is, using reduced ferredoxin as the electron donor and NADP<sup>+</sup> as the acceptor. This is the physiological reaction that uses electrons produced by photosynthesis, and it can be reproduced in vitro by incubation of the enzyme with ferredoxin and NADP<sup>+</sup> in the presence of illuminated photosynthetic membranes. The ferricyanide diaphorase reaction, the NADPH-dependent cytochrome *c* reductase activity, and the photoreduction of NADP<sup>+</sup> all involve the splitting of a two-electron reaction into two one-electron reactions or vice versa. In those reactions where NADPH is used as the reduced substrate, the enzyme receives two electrons to become fully reduced, and then two molecules of the one-electron acceptor (in the case of ferricyanide diaphorase activity and NADPH-dependent cytochrome *c* reductase activity) have to interact with it. This means that two substrate-approaching reactions (and release of the product) have to take place in every catalytic cycle. Any one of these processes can be rate-limiting under a specific set of conditions. Furthermore, for the NADP<sup>+</sup> reduction process, two molecules of reduced ferredoxin must interact with FNR and transfer two electrons before the hydride transfer to the NADP<sup>+</sup> molecule takes place. The mechanism is even more complicated if flavodoxin replaces ferredoxin as the substrate for FNR, as this protein might function between the fully oxidized and the semiquinone state, or between the semiquinone and the reduced state, depending on the assay. Thus, the analysis of the kinetic properties of FNR reactions must be approached using techniques which will provide information on specific steps in the reaction sequence. This requires the use of transient kinetic methodology, such as stopped-flow or laser flash photolysis.

In the present paper we wished to probe the involvement of the Glu301 residue in the catalytic mechanism of *Anabaena* PCC 7119 FNR. We report, for the first time, anaerobic stopped-flow measurement of the electron-transfer reaction between flavodoxin and FNR in both directions for both wild-type and mutant proteins and thereby add to the body of information on electron transfer between FNR and its protein partners. These are reactions that occur at rates too fast to be measured with this technique when the wild-type proteins are involved but might be in an accessible time regime for stopped-flow when the reactions are studied using mutated proteins. In this context, we have shown that the replacement of Glu301 by an alanine residue in *Anabaena* PCC 7119 FNR results in a dramatic decrease, in the catalytic activities of the enzyme. Absorbance, circular dichroism and fluorescence spectra of the mutated flavoprotein showed only small differences compared to wild-type FNR; thus, these spectroscopic probes, indicative of the flavin environment, show that gross protein structural rearrangements, which might explain the observed loss of activity, were not introduced by the mutation, nor were gross structural alterations reported for the three-dimensional structure of the spinach FNR when the equivalent position to Glu301 of *Anabaena* FNR, Glu312, was replaced by a leucine (21). Furthermore, affinities for the oxidized substrates (i.e., Fd,

Fld, and NADP<sup>+</sup>) (Table 3) were only slightly affected when Glu301 of *Anabaena* FNR was replaced by alanine, and thus alterations in these parameters also cannot account for the decrease observed in the catalytic activities of the mutant. This has been directly confirmed in the case of ferredoxin by laser flash photolysis methods. Thus, whereas the dissociation constant for the complex between Fd<sub>rd</sub> and FNR<sub>ox</sub> of the mutant was increased by a factor of only 1.1 relative to that of the wild-type enzyme,  $k_{et}$  was 45-fold smaller for the mutant. Direct measurements thereby confirm that replacement of Glu301 by alanine affects the electron-transfer process.

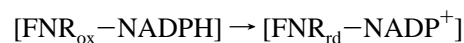
It is important to point out that the different activities of *Anabaena* FNR studied here were not affected to the same extent by the replacement of Glu301 by alanine (Table 2). Thus, the kinetic parameters for the DCPIP-dependent diaphorase activity were almost unaffected by the mutation. Moreover, when reduction of FNR to the fully reduced state by NADPH was followed by stopped-flow, no differences were detected for the mutant relative to the wild-type protein. However, when the diaphorase activity was assayed using a one-electron acceptor, ferricyanide, the catalytic constant was affected, and the enzyme showed just half the catalytic efficiency for NADPH. The value of  $k_{cat}$  for the mutant was also smaller in the NADPH-dependent cytochrome *c* reductase activity. This latter result indicates that the mutation affects the overall process of this reaction, in which FNR is first reduced by NADPH (in a two-electron-transfer process) and then transfers electrons, one at a time, to ferredoxin. The observed  $k_{cat}$  for this reaction is a first-order rate constant that includes the properties and reactions of the enzyme–substrate, enzyme–intermediate, and enzyme–product complexes. The  $K_m$  values for this activity using ferredoxin and flavodoxin were decreased by similar extents. Thus, the results obtained for the kinetic parameters of the Glu301Ala FNR indicate that the mutation produced enzyme-bound species which must transfer electrons to the substrates following different mechanisms from those followed by the wild-type FNR. It is worth noting that in the mechanisms of the wild-type FNR NADPH-dependent cytochrome *c* reductase and ferricyanide-diaphorase activities, the presence of an intermediate FNR semiquinone is required, and that such a semiquinone state seems to be highly unstable in the mutated protein. This could be the cause of the observed effects.

Another important result concerns the reaction between FNR<sub>rd</sub> and Fld<sub>ox</sub>. The stopped-flow measurements indicate that a single electron is transferred, with formation of both semiquinones, when the two species are mixed. Nevertheless, this reaction takes place at considerably lower rates than the corresponding reaction when Fd is the electron-acceptor protein. This fact could explain the much lower rate (1 order of magnitude difference, Table 2) determined for the Fld-dependent NADPH-cytochrome *c* reductase activity. However, it raises the question, why does flavodoxin behave so differently in this reaction as compared to Fd when they behave similarly in other reactions? This question is even more pertinent when one considers that the redox potential differences between the two reacting proteins would be much more favorable when Fld<sub>ox</sub> ( $E_m$  (Fld<sub>ox</sub>/Fld<sub>sq</sub>) = –261 mV) is the FNR electron acceptor than when Fd (–440 mV) is the reactant. While there is not a convincing explanation

for this fact, it emphasizes the importance of structural aspects of proteins participating in electron-transfer processes over thermodynamic factors. Furthermore, this indicates that FNR is not well suited for transferring a single electron to Fld<sub>ox</sub>, whereas it is to Fd<sub>ox</sub>. This result is even more relevant if we compare the rate constant for this reaction, FNR<sub>rd</sub> to Fld<sub>ox</sub> (2.5 s<sup>–1</sup>), with that reported by laser flash photolysis for the formation of Fld<sub>sq</sub> via intracomplex electron transfer from FNR<sub>sq</sub>. This latter rate constant has been reported as >7000 s<sup>–1</sup> (41).

The photoreduction of NADP<sup>+</sup> with photosynthetic particles was also influenced by the Glu301Ala FNR mutation. Here we note that this *in vitro* reaction is the closest to the physiological reaction and that an intermediate semiquinone state for FNR is also expected to be formed during this process. Contrary to what was seen for the NADPH-dependent cytochrome *c* reductase activity, flavodoxin and ferredoxin both photoreduce NADP<sup>+</sup> with the same efficiency, as expected, since *in vivo* this flavoprotein can replace the iron–sulfur protein. FNR functions with either protein because in the photoreduction of NADP<sup>+</sup>, and in the *in vivo* process, the Fld<sub>rd</sub>/Fld<sub>sq</sub> redox pair has an  $E_m$  of –435 mV (33), which is in the  $E_m$  range of ferredoxin (–440 mV). In the cytochrome *c* reductase reaction on the other hand, flavodoxin functions using the Fld<sub>ox</sub>/Fld<sub>sq</sub> redox pair, which has a redox potential value (–261 mV) very different from ferredoxin. When flavodoxin was used as mediator in the photoreduction of NADP<sup>+</sup>, no loss of activity was detected for the mutant, and, in the NADPH-dependent cytochrome *c* reductase activity, the decrease of the  $k_{cat}$  was not as dramatic as it was with ferredoxin. Here it is important to note that, in the electron-transfer reaction between flavodoxin and FNR, it is expected that the electrons are transferred one at a time, since in both proteins only the methyl groups of the dimethylbenzene ring are exposed to the solvent, so electron transfer must go through these groups. Moreover, the semiquinone form of flavodoxin is not expected to be able to reduce FNR. However, this behavior could be changed by the mutation, and flavodoxin could transfer two electrons at a time to Glu301Ala FNR, thereby avoiding the intermediate semiquinone species which, as has been demonstrated, seems to be destabilized in the mutant.

Stopped-flow measurements have allowed us to study fast electron-transfer processes between the redox pair FNR<sub>rd</sub>/FNR<sub>ox</sub> and its electron-transfer partners (Fd<sub>rd</sub>/Fd<sub>ox</sub>, Fld<sub>rd</sub>/Fld<sub>sq</sub>, Fld<sub>ox</sub>/Fld<sub>sq</sub>, and NADPH/NADP<sup>+</sup>) for the wild-type FNR and the Glu301Ala mutant. Most of the reactions involving wild-type FNR were too fast to be studied by this technique. Only a second component for the reduction of FNR by NADPH, having a rate constant of at least 140 s<sup>–1</sup>, could be measured with precision. This is ascribed to the two-electron reduction of flavin occurring by hydride transfer from NADPH to FAD (37, 39).



An additional process that could be measured for the wild-type FNR was the reduction of flavodoxin by FNR<sub>rd</sub>. This process could be observed due to the fact that it takes place much more slowly than the other reactions. The rate constant for this reaction was just 2.5 s<sup>–1</sup>.

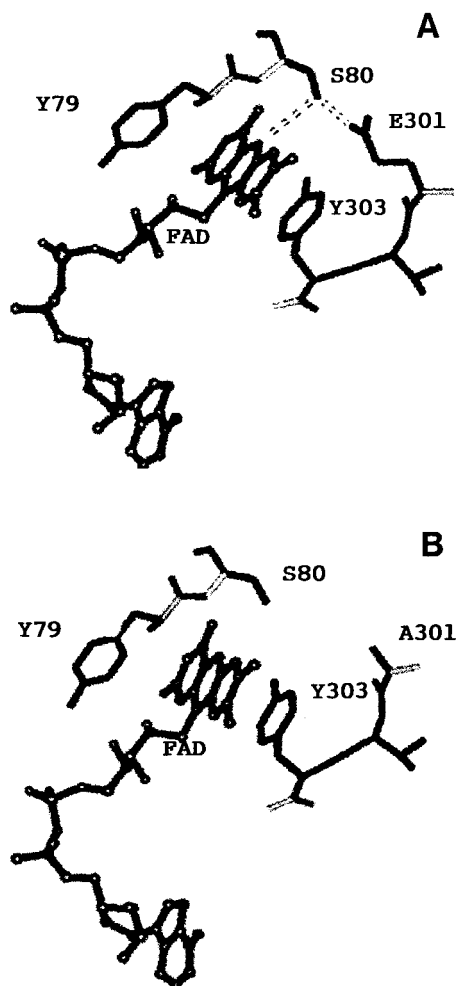


FIGURE 8: (A) View of the FAD and the residues at the catalytic site as seen in the three-dimensional crystal structure of *Anabaena* PCC 7119 FNR. (B) Computer simulation of the replacement of Glu301 by alanine in the three-dimensional structure of *Anabaena* FNR.

Replacement of Glu301 by Ala resulted in significant decreases in the observed electron-transfer rate constants between FNR species and its substrates (Table 4). Our initial hypothesis was that Glu301, which is exposed to solvent, was a good candidate to transfer protons from the external medium to the buried N-5 atom of the isoalloxazine through Ser80 (Figure 8A). Although this was not directly confirmed in this study, it is clear that the side-chain carboxylate group of Glu301 is very important for electron transfer in *Anabaena* FNR. Glu301 appears to stabilize the intermediate FAD semiquinone, and this property may be crucial in controlling the kinetics of electron transfer.

During characterization of Glu301Ala FNR other interesting properties of the mutant were revealed. Thus, it was shown that it was able to form a complex with 5-dRf, which has not been detected previously for the wild-type, native, or any other of the mutated FNRs thus far assayed. Laser flash photolysis has allowed the determination of the dissociation constant for such a complex as well as the  $k_{et}$ . The second interesting property of this mutant was that when reduced, it was able to react with oxygen via a mechanism different than that of the native and wild-type FNRs. Based on the rapidity of reaction with O<sub>2</sub> and the nature of the products formed, FNR belongs to the electron transferase

family. Reduced FNR reacts with one-electron acceptors, or donors, and produces the semiquinone form of FNR and O<sub>2</sub><sup>-</sup> upon reaction with O<sub>2</sub> (43). In general, electron transferases thermodynamically stabilize the neutral flavin semiquinone. Also, in these proteins, it is generally only the dimethylbenzene ring of the flavin that is freely accessible to solvent. In this context, it is noteworthy that modification of the 8-CH<sub>3</sub> position greatly modulates the redox potential of flavins (44). In electron transferases, it has been shown that the regions of the flavin ring which are candidates for formation of a covalent bond with O<sub>2</sub><sup>-</sup> have insufficient room to accommodate the hydroperoxide product, and the access to O<sub>2</sub> is severely limited (42). Thus, in these cases the reaction will likely proceed without the formation of a flavin 4 $\alpha$ -hydroperoxide. Upon examining the products formed by the reaction of reduced Glu301Ala FNR with O<sub>2</sub>, we found that substantial amounts of H<sub>2</sub>O<sub>2</sub> were obtained instead of O<sub>2</sub><sup>-</sup>. Moreover, stabilization of the FNR semiquinone state was not detected during the reoxidation process. For most flavins and flavoproteins, it has been proposed that activation of dioxygen to form hydroperoxides occurs through formation of a covalent adduct at position 4 $\alpha$ , which seems to be unfavorable for electron transferases. Replacement of Glu301 by Ala in *Anabaena* FNR increases the degree of exposure of the dimethylbenzene flavin ring to solvent (Figure 8B) and thus may facilitate the access of oxygen, thereby making the production of H<sub>2</sub>O<sub>2</sub> predominate over O<sub>2</sub><sup>-</sup> production.

## CONCLUSION

The aforementioned studies reveal important conceptual information about the reaction mechanism of FNR and suggest that the physicochemical environment of the flavin in the active site might play an important role in catalysis. Studies on the mutant characterized in this study establish the important function of the side-chain carboxyl group of Glu301 in *Anabaena* FNR. Removal of this acidic residue destabilizes the intermediate semiquinone state of the FAD cofactor and thus confirms that semiquinone formation is important for effective electron transfer between FNR and its substrates. The mutant also showed altered FAD reoxidation properties, supporting the idea that flavin ring exposure is an important factor in the mechanism of flavoprotein reoxidation.

## ACKNOWLEDGMENT

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## REFERENCES

- Serrano, A., Rivas, J., and Losada, M. (1984) *FEBS Lett.* 170, 8–88.
- Karplus, P.A., Daniels, M. J., and Herriott, J. R. (1991) *Science* 251, 60–66.
- Correll, C. C., Ludwig, M. L., Bruns, C. M., and Karplus, P. A. (1993) *Protein Sci.* 2, 2112–2133.
- Bruns, C. M., and Karplus P. A. (1995) *J. Mol. Biol.* 247, 125–145.
- Fillat, M. F., Bakker, H. A. C., and Weisbeek, P. J. (1990) *Nucleic Acids Res.* 18, 7161.
- Fillat, M. F., Sandman, G., and Gómez-Moreno, C. (1988) *Arch. Microbiol.* 150, 160–164.

7. Walker, M. C., Pueyo, J. J., Navarro J. A., Gómez-Moreno, C., and Tollin, G. (1991) *Arch. Biochem. Biophys.* 287, 351–358.
8. Sancho, J., and Gómez-Moreno, C. (1991) *Arch. Biochem. Biophys.* 288, 231–233.
9. Medina, M., Mendez, E., and Gómez-Moreno, C. (1992) *FEBS Lett.* 298, 25–28.
10. Medina, M., Mendez, E., and Gómez-Moreno, C. (1992) *Arch. Biochem. Biophys.* 299, 281–286.
11. Medina, M., Gómez-Moreno, C., and Tollin, G. (1992) *Eur. J. Biochem.* 210, 577–583.
12. Hurley, J. K., Salamon, Z., Meyer, T. E., Fitch, J. C., Cusanovich, M. A., Markley, J. L., Cheng, H., Xia, B., Chae, Y. K., Medina, M., Gómez-Moreno, C., and Tollin, G. (1993) *Biochemistry* 32, 9346–9354.
13. Hurley, J. K., Cheng, H., Xia, B., Markley, J. L., Medina, M., Gómez-Moreno, C., and Tollin, G. (1993) *J. Am. Chem. Soc.* 115, 11698–11701.
14. Hurley, J. K., Medina, M., Gómez-Moreno, C., and Tollin, G. (1994) *Arch. Biochem. Biophys.* 312, 480–486.
15. Hurley, J. K., Fillat, M. F., Gómez-Moreno, C., and Tollin, G. (1995) *Biochimie* 77, 539–548.
16. Hurley, J. K., Schmeits, J. L., Genzor, C., Gómez-Moreno, C., and Tollin, G. (1996) *Arch. Biochem. Biophys.* 333, 243–250.
17. Hurley, J. K., Fillat, M. F., Gómez-Moreno, C., and Tollin, G. (1996) *J. Chem. Soc.* 118, 5526–5531.
18. Serre, L., Vellieux, F. M. D., Medina, M., Gómez-Moreno, C., Fontecilla-Camps, J. C., and Frey, M. (1996) *J. Mol. Biol.* 263, 20–39.
19. Rossman, M., Moras, D., and Olsen, K. (1974) *Nature* 250, 194–199.
20. Aliverti, A., Bruns, C. M., Pandini, V., Karplus, P. A., Vanoni, M., Curti, B., and Zanetti, G. (1995) *Biochemistry* 34, 8371–8379.
21. Zanetti, G., Aliverti, A., Ravasi, D., Curti, B., Deng, Z., and Karplus, P. A. (1996) in *Flavins and Flavoproteins* (Stevenson, K. J., Massey, V., and Williams, C. H. Jr., Eds.) pp 509–512, University of Calgary Press, Calgary, Alberta, Canada.
22. Fillat, M. F., Flores, E., and Gómez-Moreno, C. (1993) *Plant. Mol. Biol.* 22, 725–729.
23. Gómez-Moreno, C., Martínez-Júlvez, M., Fillat, M. F., Hurley, J. K., and Tollin, G. (1995) in *Photosynthesis: from Light to Biosphere* (Mathis, P., Ed.) Vol. 2, pp 627–632, Kluwer Academic Publishers, The Netherlands.
24. Avron, M., and Jagendorf, A. T. (1956) *Arch. Biochem. Biophys.* 65, 475–490.
25. Shin, M. (1971) *Methods Enzymol.* 23, 440–442.
26. Mackinney, G. (1941) *J. Biol. Chem.* 140, 315–322.
27. Gershoni, J. M., and Palade, G. E. (1983) *Anal. Biochem.* 131, 1–15.
28. Przysiecki, C. T., Bhattacharyya, A. K., Tollin, G., and Cusanovich, M. A. (1985) *J. Biol. Chem.* 260, 1452–1458.
29. Tollin, G., Hurley, J. K., Hazzard, J. T., and Meyer, T. E. (1993) *Biophys. Chem.* 48, 259–279.
30. Simonsen, R. P., and Tollin, G. (1983) *Biochemistry* 22, 3008–3016.
31. Müller, F. (1991) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) Vol. 1, pp 1–71, CRC Press, Boca Raton, FL.
32. Pueyo, J. J., and Gómez-Moreno, C. (1991) *Prep. Biochem.* 21, 191–204.
33. Pueyo, J. J., Gómez-Moreno, C., and Mayhew, S. G. (1991) *Eur. J. Biochem.* 202, 1065–1071.
34. Bes, M. T., De Lacey, A. L., Peleato, M. L., Fernandez, V. M., and Gómez-Moreno, C. (1995) *Eur. J. Biochem.* 233, 593–599.
35. Foust, G. P., Mayhew, S. G., and Massey, V. (1969) *J. Biol. Chem.* 244, 964–970.
36. Bookjans, G., and Böger, P. (1979) *Arch. Biochem. Biophys.* 194, 387–393.
37. Batie, C. J., and Kamin, H. (1986) *J. Biol. Chem.* 261, 11214–11223.
38. Massey, W., Matthews, R. G., Foust, G. P., Howell, L. G., Williams, C. H., Jr., Zanetti, G., and Ronchi, S. (1970) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., Ed.) pp 393–411, Springer-Verlag, Berlin.
39. Aliverti, A., Piubeli, L., Zanetti, G., Lübberstedt, T., Hermann, R. G., and Curti, B. (1993) *Biochemistry* 32, 6374–6380.
40. Batie, C., and Kamin, H. (1984) *J. Biol. Chem.* 259, 8832–8839.
41. Walker, M. C., Pueyo, J. J., Gómez-Moreno, C., and Tollin, G. (1990) *Arch. Biochem. Biophys.* 281, 76–83.
42. Ghisla, S., and Massey, V. (1989) *Eur. J. Biochem.* 181, 1–17.
43. Massey, V. (1994) *J. Biol. Chem.* 269, 22459–22462.
44. Williamson, G., and Edmondson, D. E. (1985) *Biochemistry* 24, 7790–7797.
45. Yao, Y., Tamura, T., Wada, K., Matsubara, H., and Kodo, K. (1984) *J. Biochem.* 95, 1513–1516.
46. Schluchter, W. M., and Bryant, D. A. (1992) *Biochemistry* 31, 3092–3102.
47. Newman, B. J., and Gray, J. C. (1988) *Plant Mol. Biol.* 10, 511–520.
48. Michalowski, C. B., Schmitt, J. M., and Bohnert, H. J. (1989) *Plant Physiol.* 89, 817–822.
49. Haniu, M., McManus, M. E., Birkett, D. J., Lee, T. D., and Shively, J. E. (1989) *Biochemistry* 28, 8639–8645.
50. Ostrowski, J., Barber, M. J., Tueger, D. C., Miller, B. E., Siegel, L. M., and Kredich, N. M. (1989) *J. Biochem. (Tokyo)* 96, 579–582.
51. Yubisui, T., Miyata, T., Iwanaga, S., Tamura, M., Yoshida, S., Takeshita, M., and Nakajima, H. (1984) *J. Biochem.* 96, 579–582.
52. Correll, C. C., Batie, C. J., Ballow, D. P., and Ludwig, M. L. (1992) *Science* 258, 1604–1610.
53. Hyde, C. E., and Campbell, W. H. (1990) *Biochem. Biophys. Res. Commun.* 168, 1285–1291.

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