Probing the Function of the Invariant Glutamyl Residue 312 in Spinach Ferredoxin-NADP\(^+\) Reductase*

(Received for publication, July 30, 1998, and in revised form, September 15, 1998)

Alessandro Aliverti, Zhan Deng, Daniela Ravasi, Luciano Piubelli, P. Andrew Karplus, and Giuliana Zanetti

From the §Dipartimento di Fisiologia e Biochimica Generali, Università degli Studi di Milano, Via Celoria 26, I-20133 Milano, Italy and the ¶Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853

Ferredoxin-NADP\(^+\) reductase, the prototype of a large family of structurally related flavoenzymes, pairs single electrons carried by ferredoxin I and transfers them as a hydride to NADP\(^+\). Four mutants of the enzyme, in which Glu-312 was replaced with Asp, Gln, Leu, and Ala to probe the role of the residue charge, size, and polarity in the enzyme activity, have been heterologously expressed, purified, and characterized through steady-state, rapid kinetic studies, ligand-binding experiments, and three-dimensional structure determination by x-ray crystallography. The E312L mutant was the only one that was almost inactive (~1%), whereas unexpectedly the E312A reductase was 10–100% active with the various acceptors tested. Rapid kinetic absorption spectroscopy analyses demonstrated that flavin reduction by NADPH was impaired in the mutants. Furthermore, NADP(H) binding was partially perturbed. These functional and structural studies lead us to conclude that Glu-312 does not fulfill the role of proton donor during catalysis, but it is required for proper binding of the nicotinamide ring of NADP(H). In addition, its charge modulates the two one-electron redox potentials of the flavin to stabilize the semiquinone form.

Ferredoxin-NADP\(^+\) reductase (FNR)\(^1\) from plants and cyanobacteria fulfills the role of electrical switch between one- and two-electron transfer processes during NADP\(^+\) photoreduction in the photosynthetic electron transport chain (1, 2). FNR became the structural prototype of a large family of structurally related flavoenzymes since the resolution of its three-dimensional structure, which highlighted a novel flavin binding fold (3). Indeed, FNR-like modules are building blocks for constructing both simple and complex flavoproteins with the most varied biological functions not only in prokaryotes and plants but also in animals (4, 5). Generally, the members of the family are highly specific either for NAD\(^+\) or NADP\(^+\), whereas they are more permissive with respect to the electron acceptor.

The non-physiological reactions catalyzed in vitro by FNR can be divided into two half-reactions corresponding to transfer of a hydride between NADPH and FAD, and transfer of single electrons between reduced FAD and electron carriers (A), such as Fd (cytochrome c), ferricyanide, and presumably INT, according to Scheme 1.

\[
\begin{align*}
FNR_{\text{ox}} + \text{NADPH} & \rightarrow FNR_{\text{red}} + \text{NADP}^+ \quad \text{(reductive half-reaction)} \\
FNR_{\text{red}} + n\text{A}_{\text{ox}} & \rightarrow FNR_{\text{ox}} + n\text{A}_{\text{red}} \quad \text{(oxidative half-reaction)}
\end{align*}
\]

**SCHEME 1**

The stoichiometric coefficient \(n\) can be 1 or 2, depending on the type of electron acceptor. The reductive half-reaction of FNR and other FNR family members occurs in discrete steps, which involve two Michaelis complexes (MC) and two charge-transfer complexes (CT) (6–10), according to Scheme 2.

\[
\begin{align*}
\text{FNR}_{\text{ox}} + \text{NADPH} & \rightleftharpoons \text{FNR}_{\text{red}} + \text{NADP}^+ \quad \text{MC1} \\
\text{FNR}_{\text{red}} + n\text{A}_{\text{ox}} & \rightleftharpoons \text{FNR}_{\text{ox}} + n\text{A}_{\text{red}} \quad \text{CT} \quad \text{MC2}
\end{align*}
\]

**SCHEME 2**

In order to clarify the mechanism of action of hydride transfer mediated by the enzyme through flavin-nicotinamide rings interactions, for which there was no support from crystal structures (3, 11, 12), our group and others have carried out site-directed mutagenesis of the five conserved residues surrounding the isooxalazine ring in the active center of FNR: Tyr-95, Ser-96, Cys-272, Glu-312, and Tyr-314 (spinach numbering). It was shown by changing Cys-272 to Ser that this residue, conserved in all the members of the family, is required for productive interaction of the nicotinamide ring of NADP\(^+\) with the flavin to facilitate hydride transfer between C-4 of nicotinamide and N-5 of the isooxalazine (13). Replacement of Ser-96 with Gly or Val clearly interfered with the proper binding of the nicotinamide ring and with the stabilization of the transition state during hydride transfer between NADPH and FAD (8). Substitution of the e for Tyr-95, which interacts with the si face of the isooxalazine and makes a H-bond to the ribityl 4′-OH group, did not alter significantly the physico-chemical properties of the enzyme (14).\(^2\) Mutation of the C-terminal Tyr-314, whose side chain covers the re face of the isooxalazine, was done for

---

\(^*\) This work was supported by grants from Ministero dell’Università e della Ricerca Scientifica e Tecnologica, from Consiglio Nazionale delle Ricerche, Italy, and from the National Science Foundation (NSF-MCB-9630474). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors codes 1BX1 (Gln), 1FRQ (Ala), and 1BX0 (Leu) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

¶To whom correspondence should be addressed. Tel.: 39-02-70644505; Fax: 39-02-2382451; E-mail: gzanetti@miucca.cs.unimi.it.

\(^1\) The abbreviations used are: FNR, ferredoxin-NADP\(^+\) oxidoreductase (EC 1.18.1.2); FNR\(_{\text{ox}}\), oxidized FNR; FNR\(_{\text{red}}\), reduced FNR; FADH\(^+\), neutral FAD semiquinone; Fd, ferredoxin I; MC, Michaelis complex; CT, charge-transfer complex; INT, 2-(p-iodomethyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride; 2′-P-ADP-ribose, 2′-monophospho-5′-diphosphoribose; 3′-AcPDP\(^+\), 3-acetylpyridine adenine dinucleotide phosphate; 3′-AmPDP\(^+\), 3-aminoimidazolone adenine dinucleotide phosphate; thio-NADP\(^+\), thionicotinamide adenine dinucleotide phosphate; 5-deazariboflavin, 5-carba-5-deazariboflavin.

pea FNR, where it could be replaced by Trp or Phe with only a 50% decrease in $k_{\text{cat}}$, indicating that it probably did not have a direct chemical role in catalysis (15).

In this paper we study the role of Glu-312, a residue that hydrogen-bonds to Ser-96 and has been hypothesized to play a role as proton donor during the one-electron reduction of FNR by ferredoxin (11). This residue is conserved in all the members of the FNR family, except cytochrome $b_{5}$ reductase and nitrate reductase (3). Here we replaced Glu-312 with Asp (conserving charge), Gln (conserving shape and polarity), Leu (conserving space but removing polarity), and Ala (removing the side chain and allowing replacement by water). Complete kinetic characterization of the four mutants and the three-dimensional structures of three of them lead to the conclusion that Glu-312 does not play a key chemical role as the proton donor, but rather is required for nicotinamide proper positioning in the active site. Preliminary results have been presented elsewhere (16).

**EXPERIMENTAL PROCEDURES**

Horse heart cytochrome c, INT, NADP(H), and NADP$^{+}$ analogs (2'-P-ADP-ribose, thio-NADP$^{+}$, 3-AcPADP$^{+}$, and 3-AmPADP$^{+}$) were obtained from Sigma. Factor X$_{s}$ was from Pierce. Restriction endonucleases and T4 DNA ligase were purchased from either Life Technologies, Inc. or Boehringer Mannheim. All other chemicals were of analytical grade.

**Oligonucleotide-directed Mutagenesis—** Site-directed mutagenesis of the FNR gene construct was carried out according to the phosphorothioate-based method (17), using the sculptor in vitro mutagenesis system (Amersham Pharmacia Biotech). The single-stranded template was obtained by reclaiming the wild-type FNR gene in M13mp9 as described (18). The sequence of the four synthetic oligonucleotides used for mutagenesis is reported in Table I. In addition to the base changes in the GAA triplet encoding Glu-312, designed to introduce the desired amino acid replacements at this position, the mutagenic primers carried a second, silent mutation in the following triplet (GTC → GTT), designed to disrupt a restriction AccI site, in order to facilitate subsequent screening steps. The presence of the desired mutation and the lack of second-site mutations were confirmed by sequence analysis of the recombinant genes. DNA sequencing was performed according to the chain termination method (19) using single-stranded M13mp9 templates and the T7 sequencing kit by Amersham Pharmacia Biotech.

**Expression and Purification of the Mutant FNRs—** The fragments carrying the mutated FNR genes were recloned in the expression vector pMAL-c (New England Biolabs), as described previously for other FNR mutants (8). Wild-type and mutant enzymes were purified according to the published E. coli (host strain RRLM15) essentially as described elsewhere (8), with the only difference that FPLC on SP-Sepharose (Amersham Pharmacia Biotech) was used as the last step instead of chromatography on phosphocellulose.

**Electrophoresis and Protein Sequence Analysis—** Nondenaturing polyacrylamide gel electrophoresis was performed on 12% polyacrylamide gel slabs. The C-terminal sequence of the FNR-E312Q mutant was determined using a Hewlett-Packard G1009A C-terminal protein sequencing system.

**Spectral Analyses—** Absorption spectra were recorded with the Hewlett-Packard 8452 or 8453 diode array spectrophotometers. The extinction coefficient of the protein-bound flavin was determined by resolving the FAD from the apoprotein and spectrophotometrically quantifying the released FAD, according to the procedure described in (8). Protein and flavin fluorescence were monitored on a Jasco FP-777 spectrophotofluorometer.

**FNR Photoreduction—** FNR reduction was achieved by one-electron donation from the 5-deazariboflavin semiquinone produced by light irradiation, EDTA serving as the final electron source (20). Photoreduction was performed both in the absence and in presence of NADP$^{+}$.

Reactions were carried out at 15°C in an anaerobic cuvette on 15–18 μM FNR samples in 10 mM HEPES, pH 7.0, in the presence of 15 mM EDTA and 1–1.2 μM 5-deazariboflavin. When present, NADP$^{+}$ was at a concentration of 17 μM. Reaction solutions were made anaerobic by successive evacuation and flushing with O$_{2}$-free N$_{2}$. Each mutant was subjected to successive short times of illumination to finally reach full reduction of enzyme flavin. Absorption spectra were recorded before and after successive periods of irradiation with a 150-watt light source. The spectrum obtained at each time may be a mixture of the three redox states of the enzyme FAD (oxidized, semiquinone, and hydroquinone).

**Kinetic Measurements—** Steady-state kinetics were performed as described previously (13). Rapid-reaction kinetics were studied with a Hi-Tech SF-61 stopped-flow spectrophotometer interfaced with a Macintosh computer using the KISS program (Kinetic Instruments, Inc.). Hydride transfer between NADPH and FNR was studied by mixing 100 μM NADPH with 30–40 μM oxidized FNRs in 50 mM HEPES, pH 7.0, at different temperatures, as reported previously (8, 13).

**Determination of the Dissociation Constant of the Complexes of the Mutant FNRs with Fd, NADP$^{+}$, and Thio-NADP$^{+}$—** The $K_{d}$ values of the complexes of the wild-type and the mutant enzymes with oxidized Fd were determined by titrating the reductase protein fluorescence with Fd in 20 mM HEPES, pH 7.0 (at 15°C), essentially as described in Ref. 8. To measure the $K_{d}$ of the complexes of the mutant FNRs with NADP$^{+}$ and thio-NADP$^{+}$, the proteins were spectrophotometrically titrated with the ligands. FNR samples were diluted in 10 mM Tris-HCl, pH 7.7 (at 15°C), to a final concentration of about 20 μM. Absorption spectra were recorded before and after successive additions of nucleotide. Difference spectra were computed subtracting from each spectrum the spectrum recorded in the absence of ligand, corrected to account for dilution. Data at several different wavelengths were fitted as described in Ref. 8.

**Determination of the Difference Spectra of the Complexes between Mutant FNRs and NADP$^{+}$ Analogs—** FNR samples were diluted in 10 mM Tris-HCl, pH 7.7 (at 15°C), to a final concentration of about 20 μM. Two successive additions of ligand (NADP$^{+}$, 2'-P-ADP-ribose, thio-NADP$^{+}$, 3-AmPADP$^{+}$, and 3-AcPADP$^{+}$) were then made to ensure saturation conditions. Difference spectra were computed as above.

**X-ray Crystallographic Methods—** Crystals of FNR mutants E312A, E312L, and E312Q grew under conditions similar to those for wild-type FNR (3, 11). These crystals were isomorphous, belonging to space group P2$_{1}$2$_{1}$2$_{1}$ (99.9° for E312Q, 99.7° for E312A, and 99.5° for E312L). The data were merged using SCALePACK (Z. Otwinowski, University of Texas, Dallas, TX), and crystallographic refinement was carried out using X-PLOR version 3.1 (22). The wild-type FNR structure (Ref. 11; Protein Data Bank (PDB) entry number 1fnb) was employed as starting model, with residue 312 changed from Glu to Asp. Two successive cycles of data collection and refinement yielded $R_{	ext{F}}$ values of 0.19–0.18 for E312A, 0.25 for E312L, and 0.16 for E312Q. Under similar conditions, the E312D mutant failed to produce single crystals. X-ray diffraction data were collected on a San Diego Multiview System area detector (21) with a Rigaku RU-200 rotating anode (Cu-Kα) as described (11). The data were merged using SCALEPACK (Z. Otwinowski, University of Texas, Dallas, TX), and crystallographic refinement was carried out using X-PLOR version 3.1 (22). The wild-type FNR structure (Ref. 11; Protein Data Bank (PDB) entry number 1fnb) was employed as starting model, with residue 312 changed from Glu to the appropriate residue, and residue 269 changed from Val to Phe (22, 11). Initial difference electron density maps also indicated that some residues surrounding Phe-269 (such as Leu-262) move slightly away from Phe-269, as was seen in the S96V mutant structure (Ref. 8; PDB entry number 1fnm). These models were then refined against their corresponding diffraction data between 10 Å and the highest resolution limit, using conventional positional refinement and then restrained B-factor refinement, followed by manual adjustment guided by $F_{o} - F_{c}$ maps and $F_{c} - F_{o}$ maps. Several rounds of refinement yielded the final structure. The data collection statistics, crystallographic refinement statistics and the final model quality are shown in Table II. Since the wild-type structure was refined using TNT version 5.1 (11), a control experiment was performed by refining this model against the same data using X-PLOR. This gave a structure that was almost identical to the TNT-refined model, indicating a consistency between TNT and X-PLOR.

## Table I

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Synthetic primer$^a$</th>
<th>Codon change</th>
</tr>
</thead>
<tbody>
<tr>
<td>E312D</td>
<td>GAATGAACCTTTGATGTATCTAATACCTTTAAGCTCGATTGCCTTTCTT</td>
<td></td>
</tr>
<tr>
<td>E312Q</td>
<td>GAACTTTGATGTATCTAATACCTTTGAACTCGATTGCCTTTCTT</td>
<td></td>
</tr>
<tr>
<td>E312A</td>
<td>GAACTTTGATGTATCTAATACCTTTGAACTCGATTGCCTTTCTT</td>
<td></td>
</tr>
<tr>
<td>E312L</td>
<td>GAACTTTGATGTATCTAATACCTTTGAACTCGATTGCCTTTCTT</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Base changes are underlined.
Role of Glu-312 in FNR Catalytic Mechanism

RESULTS

Expression and Purification of the Mutant FNRs—The four mutant enzymes were all expressed at wild-type level in E. coli and purified to homogeneity following the same procedure originally set up for the recombinant enzyme (see “Experimental Procedures”). The presence of the desired mutations was verified by DNA sequencing and further confirmed by checking the mobility of the mutated enzymes in nondenaturing gel electrophoresis. Indeed, only the FNR-E312D, as expected, showed the same mobility of the wild-type enzyme, whereas the other three mutants, having each lost a negative charge, were slightly retarded. The correct sequence of the E312Q mutant was confirmed by protein sequencing from the C terminus (-Q-V-Y).

Spectral Properties—All mutants exhibited the typical absorption spectrum of the wild-type enzyme. Extinction coefficients at 458 nm were determined to be 9.65, 9.62, and 9.8 mm\(^{-1}\) cm\(^{-1}\), for E312D, E312Q, E312A, and E312L mutants, respectively. The flavin fluorescence was quenched in the mutants as in the wild-type enzyme. Small but significant differences were observed in the visible absorbance spectra of the oxidized forms of the mutant FNRs with respect to the wild-type enzyme. These changes are qualitatively similar to those elicited by replacement of Ser-96 with Val or Gly (8), but of lower intensity. The E312L mutant is unique in that it has the 380-nm band increased but not red-shifted (16).

Crystallographic Structures of FNR Mutants E312A, E312L, and E312Q—Crystallization was attempted with all four E312 mutants, but only three crystallized (FNR-E312A, FNR-E312L, FNR-E312Q). Structures of the Ala, Leu, or Gln mutants reveal no global perturbation of the protein structures has occurred. Instead, only subtle changes are observed around the active sites (Fig. 1). Generally, the extent of these shifts is smaller than those observed in S96V mutant structure (8). Compared with the wild-type structure, the isoxaloxazine ring of FAD in FNR-E312A shifts slightly toward residue 312, with its C-7 atom moving about 0.5 Å. The hydroxyl group of Ser-96, which ordinarily hydrogen bonds to Glu-312 in the wild-type structure, moves about 0.4 Å away from FAD. In FNR-E312L, unlike FNR-E312A, the dimethylbenzene ring of FAD moves away from residue 312, with the C-7 atom shifting about 0.3 Å. The hydroxyl group of Ser-96 moves about 0.3 Å away from the isoxaloxazine ring. Water 407 also shifts 0.3 Å in the same direction, so that the distance between the O\(_y\) atom of Ser-96 and water 407 remains unchanged. Also, the side chain and main-chain atoms of Leu-312 move slightly away from FAD, compared with the wild-type structure. In FNR-E312Q, the shift of the isoxaloxazine ring is very small (<0.2 Å). The side chain atoms of Gln-312 swing away from Ser-96 by as much as 0.7 Å. We are unable to distinguish between the O41 and N2 atoms of Gln-312, as either assignment could satisfy the hydrogen bonding environment. The hydroxyl group of Ser-96 shifts 0.33 Å toward Gln-312, and it is within the hydrogen bonding distance to N2 (or O41) of Gln-312 (2.9 Å). In addition, the distance between the FAD-N-5 atom and Ser-96-O\(_y\) in all three mutants increases by ~0.2 Å compared with the wild-type structure.

Steady-state Kinetics—Steady-state kinetics of the mutants were performed for the diaphorase reaction using either K\(_3\)Fe(CN)\(_6\) or INT as the electron acceptor, because the reductive half-reaction is rate-limiting in the ferricyanide reaction, whereas the oxidative one is limiting with INT (Scheme 1). The kinetic parameters (Table III) reveal substantial differences using the two electron acceptors. In the case of ferricyanide, all the mutants showed a reduced \(k_{\text{cat}}\) and a \(K_m\) for NADPH almost unchanged with respect to that of the wild-type enzyme, except for the Ala mutant. Unexpectedly, this mutant was the most active, exhibiting about 60% of the wild-type \(k_{\text{cat}}\), whereas the FNR-E312D and the FNR-E312Q show a 4-fold decrease and the FNR-E312L mutant a 20-fold decrease. The \(K_m\) for ferricyanide of the Ala mutant was found also greatly decreased (3 versus 100 μM), and the \(k_{\text{cat}}/K_m\) is 20-fold higher than the wild-type one. Using INT as the second substrate, all the mutants, except the FNR-E312L, exhibited a substantial degree of activity with \(k_{\text{cat}}/K_m\) values ranging from 50 to 110% of that of the wild-type FNR. The \(K_m\) values for INT were always decreased and those for NADPH instead were increased, again except in the case of the Ala mutant (a 5-fold decrease). Thus, FNR-E312A is remarkable in that it shows a catalytic efficiency for each acceptor >5-fold higher than that of wild-type FNR.

Rapid Kinetic Studies of the Reductive Half-reaction of the Mutant FNRs—Analysis of the spectral changes during enzyme reduction by NADPH was performed by stopped-flow spectrophotometry in anaerobiosis (Fig. 2). For wild-type FNR, time traces at any wavelength were best fitted by two exponential phases (Fig. 2A): the first phase corresponding to the formation of a charge-transfer complex between enzyme-bound oxidized FAD and NADPH (CT1), the slower phase corresponding to the formation, by hydride transfer, of a charge-transfer complex between reduced FAD and NADPH (CT2) (Refs. 8 and 13; Scheme 2). Both charge-transfer complexes were present at the end of the reaction, since an equilibrium was established under these conditions. Only FNR-E312A (Fig. 2C) showed a pattern similar to that obtained with wild-type FNR, with the exception that the fast phase was completed in the dead time. Thus, only one phase was observable, corresponding to the second phase seen with the wild-type enzyme. Anaerobic reduction by NADPH of the other three mutants can be described as a single-exponential process with no rapid loss of absorbance at 460 nm (Fig. 2, B and C). For all mutants except FNR-E312L, the increase in the long-wavelength band was coincidental with flavin reduction (as monitored at 460 nm), and is thus likely due to the charge-transfer complex CT2 involving reduced FAD. By analysis of the extent of FAD reduction in the various FNRs, it appears that the flavin redox potential is increased for the mutants in the order E312D > E312Q > E312L. The calculated monophasic rates of flavin reduction for the various FNRs obtained in stopped-flow experiments at 25 °C are very similar to the \(k_{\text{cat}}\) values obtained in

### Table II

<table>
<thead>
<tr>
<th>FNR</th>
<th>Resolution limit</th>
<th>Measured reflections</th>
<th>Unique reflections</th>
<th>Completeness(^a)</th>
<th>(R_{\text{sym}}) b</th>
<th>(R_p)</th>
<th>Root-mean-square deviation from ideality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Å</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bond lengths</td>
</tr>
<tr>
<td>E312A</td>
<td>1.95</td>
<td>56,640</td>
<td>25,030</td>
<td>97.8 (96.4)</td>
<td>10.5 (28.7)</td>
<td>19.0</td>
<td>0.016</td>
</tr>
<tr>
<td>E312L</td>
<td>1.90</td>
<td>115,494</td>
<td>27,690</td>
<td>99.7 (97.8)</td>
<td>11.0 (37.5)</td>
<td>19.0</td>
<td>0.012</td>
</tr>
<tr>
<td>E312Q</td>
<td>1.90</td>
<td>74,220</td>
<td>27,319</td>
<td>98.8 (93.1)</td>
<td>8.6 (29.1)</td>
<td>18.3</td>
<td>0.012</td>
</tr>
</tbody>
</table>

\(^{a}\) Numbers in parentheses are completeness or \(R_{\text{sym}}\) values for the highest resolution bins.

\(^{b}\) \(R_{\text{sym}} = \frac{\sum_{i=1}^{n} \sum_{j=1}^{n} |I_i - I_j|/\sum_{i=1}^{n} I_i}{\sum_{i=1}^{n} \sum_{j=1}^{n} I_i}\).

\(^{c}\) \(R = \frac{\sum_{i=1}^{n} |F_o| - |F_c|}{\sum_{i=1}^{n} |F_o|}\).
the ferricyanide diaphorase activity (Table III), implying that the reductive half-reaction (Scheme 1) is impaired by the mutation. The temperature dependence of the observed reduction rates shows that the Ala mutant reduction rate is less sensitive to temperature than those of the other FNRs (data not shown).

Interaction with NADP+ and Analogs—The binding of NADP+ and its analogs to FNR can be monitored by changes in the visible absorption spectrum (7). Such studies not only provide affinities, but according to the hypothesis of Aliverti et al. (8, 13), the size of a positive difference peak at 505 nm gives information about the extent to which the nicotinamide ring is correctly positioned in the active center of FNR by replacing the side chain of Tyr-314. The compounds investigated here were NADP+, 2′-P-ADP-ribose, a substrate analog devoid of the nicotinamide ring, thio-NADP+, 3-AcPADP+, and 3-AmPADP+. The behavior of wild-type FNR is presented in Fig. 3A. 3-AcPADP+ and 3-AmPADP+ yielded difference spectra similar to that of 2′-P-ADP-ribose, indicating no significant pyridine-ring positioning in the active center. This NADP+ binding produced a difference spectrum that qualitatively resembles that induced by NADP+, but unexpectedly, it was much more intense. All four mutants titrated with NADP+, thio-NADP+, and 2′-P-ADP-ribose. They displayed essentially the wild-type difference spectrum with 2′-P-ADP-ribose (Fig. 3, dashed lines), although the trough at 505 nm is more intense for some mutants. NADP+ binding produced a difference spectrum (solid lines) similar to that induced by 2′-P-ADP-ribose in the case of FNR-E312Q and FNR-E312L (Fig. 3C), whereas the spectral changes induced in the FNR-E312A (Fig. 3B) were intermediate between the two types of difference spectra obtained with the wild-type enzyme. This different behavior of the mutants is further highlighted by analysis of the spectral effects induced by thio-NADP+ binding to the enzyme (dotted lines). Clearly, the FNR-E312A with thio-NADP+ (Fig. 3B) shows a difference spectrum similar to that exhibited by wild-type FNR with this ligand, whereas in the case of the Gln and Leu mutants (Fig. 3C) the spectrum is again similar to those induced by the other two ligands. FNR-E312D yielded difference spectra with NADP+ and thio-NADP+ similar to those of wild-type FNR, but of lower intensity (30% and 60%, respectively).

Although the Glu-312 mutants are spectrally quite distinct from wild-type FNR in their interaction with NADP+, the ligand affinities are not substantially altered. Kd values for NADP+ were in the range of 8–10 μM for all the enzymes except for the Gln mutant, which had Kd = 3.5 μM. The Kd value for thio-NADP+ binding was determined only for the wild-type enzyme and was found to be 5 μM, slightly lower than that for the real substrate.

Photoreduction of the Mutant FNRs—A stepwise anaerobic photoreduction with the 5-deazariboflavin/EDTA system (20) in the presence and absence of an equimolar amount of NADP+ has been used to assess shifts in the redox potential of the flavin (Fig. 4), and to monitor the maximal amount of FAD semiquinone (FADH*) and charge-transfer complexes (CT1 + CT2) that can be stabilized by each mutant (Table IV). The titration curves done in the presence of NADP+ (Fig. 4) reveal that for wild-type FNR the NADP+ is preferentially reduced, as is consistent, with the physiological direction of electron flow. For all mutants studied here, the enzyme-bound FAD potential becomes more positive relative to the NADP+ redox potentials.

Surprisingly, FNR-E312L has only a small apparent shift in redox potential and the other three mutants have similar larger shifts (Fig. 4).

With regard to semiquinone formation, in the absence of NADP+, only FNR-E312D presents as much FADH* as the...
wild-type enzyme; in the three other mutants, the maximal amount of FADH* during photoreduction is decreased by 70–80%. The only notable effect of NADP1 presence was to drop by 30% the maximal amount of FADH* in FNR-E312D (Table IV). All mutants stabilized the blue, neutral semiquinone as does wild-type FNR. The total presence of charge transfer complexes, CT1 and CT2, was quantified by the absorbance increases at 750 nm during the titration. The presence of measurable amounts of such complexes was verified in all the mutants except for FNR-E312L, confirming the stopped-flow data (see above). For these mutants, the maximal value of absorbance at 750 nm was 50% of that seen for wild-type FNR (Table IV).

Interaction with Ferredoxin I—Fluorescence quenching titrations revealed no significant differences in the affinity of the FNR-E312 mutants for oxidized Fd (data not shown). The functional competency of this interaction was assessed by measuring the cytochrome c reductase activity of FNR, which involves electron transfer from NADPH to Fd and subsequently to cytochrome c. The basal activity in the absence of ferredoxin, which is less than 1% with the wild-type enzyme, was found enhanced in the case of the Ala and Gln mutants, depending on the batch of cytochrome c used. Addition of superoxide dismutase and catalase decreased these rates substantially (16).

In Table V, the activity of wild type and two mutants with two different batches of cytochrome c show that, with the batch of cytochrome c not treated with trichloroacetic acid, the usual pattern of Fd-dependent electron transfer to cytochrome c is obtained and the mutants showed 11–16% of the wild-type activity with ferredoxin, apart from the FNR-E312L, which was only 1% active. Instead, with the other cytochrome c batch, the Ala mutant showed a terrific capacity to directly transfer electrons to the heme. A reason could be that clipped forms of cytochrome c have more access to the isoalloxazine ring when Glu-312 has been changed to Ala or Gln, because of the loss of a negative charge. The case of the Leu mutant may be different because it has very low activity with any acceptor, due to a major impairment of the reductive half-reaction.

DISCUSSION

The main purpose of our research concerns the elucidation of the mechanism of hydride transfer between flavin and pyridine...
Fig. 4. Photoreduction of the wild-type and mutant FNRs in the presence of an equimolar amount of NADP\(^+\): relationship between NADP\(^+\) and FAD reduction. ○, wild-type FNR; △, FNR-E312L; □, FNR-E312Q; ■, FNR-E312A; ●, FNR-E312D. Absorbance at 340 nm, which is practically an isosbestic point for the various reduction forms of FNR (24), is used to monitor NADPH formation. At each time of irradiation, the \(\Delta A_{340}\) as a fraction of the maximal \(\Delta A_{340}\) was plotted against the ratio of fully reduced enzyme FAD to the sum of fully reduced and oxidized enzyme flavin. The data were corrected for the presence of the FAD semiquinone.

![Absorption spectra of the fully reduced forms of wild-type and mutant FNRs.](image)

**Fig. 5. Absorption spectra of the fully reduced forms of wild-type and mutant FNRs.** Full reduction of the FNRs was achieved by photoreduction in the presence of 5-deazariboflavin and EDTA, at pH 7.0. For the sake of clarity, only the spectra of FNR-E312A (solid line) and wild type (dashed line) are reported. The spectra of FNR-E312L and FNR-E312Q look like that of the Ala mutant, whereas the spectrum of FNR-E312D is similar to that of the wild-type enzyme.

### Table IV

<table>
<thead>
<tr>
<th>FNR</th>
<th>NADP(^+)</th>
<th>FNR(_{red})</th>
<th>CT(_1) + CT(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>−</td>
<td>26</td>
<td>−</td>
</tr>
<tr>
<td>E312D</td>
<td>+</td>
<td>27</td>
<td>100</td>
</tr>
<tr>
<td>E312Q</td>
<td>+</td>
<td>24</td>
<td>−</td>
</tr>
<tr>
<td>E312A</td>
<td>+</td>
<td>17</td>
<td>73</td>
</tr>
<tr>
<td>E312L</td>
<td>−</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>8</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>9</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>

\(\text{Maximal amount of FAD semiquinone accumulated during enzyme photoreduction expressed as a percentage of total FAD present.}\)

\(\text{Maximal amount of total charge-transfer species accumulated during enzyme photoreduction expressed as a percentage of the amount formed in the case of wild-type FNR.}\)

The hypothesis that Glu-312 is acting as the proton donor/acceptor to FAD during FNR catalysis implies that the oxidative half-reaction would be affected (see Scheme 1), and because of the large pK\(_a\) difference between a Glu side chain and water, one would expect that the replacement of the Glu side chain with water would still result in a loss in activity of many orders of magnitude if the proton transfer step were limiting. We studied the diaphorase activity of the mutants both with ferricyanide and INT. In the case of the wild-type enzyme, the rate-limiting step of the former reaction is the reductive half-reaction, whereas the rate-limiting step of the latter is the oxidative one (8, 13). The values of \(k_{cat}\) of the mutants with ferricyanide match well the pre-steady-state rates of FAD reduction by NADPH. This indicates that the half-reaction of flavin reduction is responsible for the activity loss of the mutants. The INT reductase maximal activities of the mutants are less affected, further confirming that the oxidative half-reaction is not impaired by the mutation.

Several lines of evidence point to a perturbation of the interaction between NADPH and flavin in the FNR active center due to Glu-312 replacement. We observed a correlation between residual activities of the mutants and charge-transfer complex formation both in rapid reaction studies and photoreduction experiments. The FNR-E312L, which is the most impaired mutant, showed no evidence of charge transfer complex formation. This was also true for the highly impaired mutant FNR-S96V (8). Where there is left a measurable degree of activity (>10% of wild-type FNR activity), as in the case of the other mutants (C272S (13), S96G (8), and E312D/Q/A), the formation of CT1 or CT2 can be observed during photoreduction and enzyme reduction with NADPH by stopped-flow measurements. Interestingly, the replacement of Glu-312 with Asp, which is generally considered to be a conservative one, was more deleterious to the enzyme function than elimination of most of the residue side chain (Glu to Ala change). This suggests that hydrogen bonding properties, which can be mimicked by water, are more important than simple electrostatic effects, which would be conserved in the Asp mutant. The most impaired mutant was the FNR-E312L, which cannot hydrogen bond and does not leave room for water to replace the side chain. Further evidence for impaired nicotinamide binding in these mutants comes from the spectral titrations with NADP\(^+\) and analogs. Although all analogs bind to the mutants with near wild-type affinities, it must be recalled that structural...
studies have shown that the affinity of these ligands is dominated by the 2'-P-AMP portion of the dinucleotide, and does not give information about how well the nicotinamide portion interacts with the active-site pocket (3). However, Aliverti et al. (8, 13) have shown that the difference spectral changes can be used to monitor the level of proper nicotinamide positioning in the active-site pocket. A new discovery in this study is that theo-NADP⁺ yields much more intense spectral changes than NADP⁺, possibly due to a higher active-site occupancy of the thionicotinamide moiety. Thus, this NADP⁺ analog can be used as a more sensitive probe of nicotinamide positioning. Its use helped to discriminate more easily between the mutants with low and high activity, giving support that indeed the difference spectrum induced by NADP⁺ binding in the case of spinach FNR is indicative of the nicotinamide positioning in the active center. However, in the case of Asp mutant, the conservation of the side chain negative charge is important for the positioning of the nicotinamide ring but is not sufficient for maintaining activity. The hypothesis that Glu-312 may be required for proper binding of the nicotinamide ring and that most probably a two-step binding mechanism is involved, as reported for NAD(H) binding to alcohol dehydrogenase (26), can be envisaged. It remains to be shown by x-ray crystallography of the FNR-NADP complex whether this residue interacts with the nicotinamide carboxamide group, as has been seen in other pyridine-nucleotide-dependent enzymes (27–29).

In addition to this major effect on NADP(H)-flavin interaction, we observed several minor changes in the properties of the mutants, indicating a complex role for the Glu-312 side chain. It appears that the FAD semiquinone is destabilized in all the mutants except for the FNR-E312D form (Table IV), suggesting that the negative charge of the side chain plays a role. Another indicator of the importance of charge on flavin properties are the spectra of the hydroquinone forms of the enzymes, which reveal that the pKₐ of N-1H of hydroquinone is decreased in the neutral mutants. This electrostatic influence of Glu-312 (or Asp-312), which makes it less favorable for N-1H of the reduced isoalloxazine to become ionized, provides a mechanism by which the redox potentials may be affected. In a somewhat surprising result, the photoreduction experiments in the presence of equimolar NADP⁺ show that the two-electron midpoint potential of the flavin cofactor has become more positive (by ~10–30 mV) in all but the Leu mutant. The apparent Eₐ values that can be calculated from these experiments seem to be valid on two grounds. The E_a value of ~339 mV determined for the wild-type enzyme is in good agreement with that measured by potentiometry (~342 mV) (25), and the extent of flavin reduction of the enzyme mutants as seen in the stopped-flow experiments is increased in the same order. That the Leu mutant has a redox potential most like wild-type FNR is puzzling, but it should be recalled that these studies are measuring the redox potential in the presence of NADPH and it is known the NADPH binds differently to each of the mutants.

In terms of the interactions with Fd, the mutations to Ala and Asp do not affect the strength of binding but the electron transfer rates are diminished by about a factor of 10 (Table V).

It has been proposed that electron flow from or to Fd is through the FAD dimethylbenzene ring, which is exposed to solvent (3), and which is adjacent to the side chain of Glu-312. The equivalent activities of the Ala and Asp mutants indicates that the impairment is not just electrostatic in nature, but may involve a more specific effect of the Glu-312 side chain.

Recently, Medina et al. (30) have reported on the properties of *Anabaena* FNR having the corresponding Glu mutated to Ala (E301A). Insofar as similar experiments were done, their results match ours well, as that mutant folded properly, had unimpinged affinity for Fd, and had a destabilized semiquinone. Additional rapid kinetic experiments they carried out showed there was a 40-fold decrease in the rate of electron transfer from ferredoxin. They concluded that the active-site Glu residue was "a critical residue for electron transfer" but could not be very specific about its role. In our study, the additional Asp, Leu, and Gln mutants have allowed us to make more specific conclusions on the importance of charge, size, and polarity of this residue.

Based on these results, we derive the following three conclusions about the role of Glu-312 in FNR. 1) Glu-312 does not play an important role as a proton donor/acceptor, but it is involved in the proper binding of NADPH. The high activity of the E312A indicates that water is a reasonable substitute for the Glu side chain in the interactions with NADPH. 2) The charge of Glu-312 contributes to tuning the redox potentials of the flavin semiquinone to enhance efficient electron transfer. 3) The Glu-312 side chain has a little effect on Fd affinity or on electron transfer to dye molecules, but has a definite effect on the electron transfer between FNR and Fd.

**Acknowledgments**—We are grateful to Dr. Maria A. Vanoni for helping in the stopped-flow measurements. We thank Dr. Hubert Kaudewitz, Hewlett-Packard GmbH, Waldbronn, Germany, for performing the C-terminal protein sequencing of FNR-E312Q.

**REFERENCES**

Role of Glu-312 in FNR Catalytic Mechanism

Canada

Probing the Function of the Invariant Glutamyl Residue 312 in Spinach Ferredoxin-NADP + Reductase
Alessandro Aliverti, Zhan Deng, Daniela Ravasi, Luciano Piubelli, P. Andrew Karplus and Giuliana Zanetti

doi: 10.1074/jbc.273.51.34008

Access the most updated version of this article at http://www.jbc.org/content/273/51/34008

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 28 references, 9 of which can be accessed free at http://www.jbc.org/content/273/51/34008.full.html#ref-list-1