Structural Basis of the Catalytic Role of Glu301 in Anabaena PCC 7119 Ferredoxin-NADP⁺ Reductase Revealed by X-Ray Crystallography

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ABSTRACT The three-dimensional crystal structure of the Glu301Ala site-directed mutant of ferredoxin-NADP⁺ reductase from Anabaena PCC 7119 has been determined at 1.8Å resolution by x-ray diffraction. The overall folding of the Glu301Ala FNR mutant shows no significant differences with respect to that of the wild-type enzyme. However, interesting conformational changes are detected in the side chain of another glutamate residue, Glu139, which now points towards the FAD cofactor in the active center cavity. The new conformation of the Glu139 side chain is stabilized by a network of five hydrogen bonds to several water molecules, which seem to hold the carboxylate side chain in a rather fixed position. This interacting network connects the Glu139 side chain to the Ser80 side chain through a series of three water molecules. These observations are discussed in terms of the reactivity of Glu301Ala ferredoxin-NADP⁺ reductase towards its substrates, and the role of Glu301 in the catalysis is re-examined. Moreover, a structural explanation of the different reoxidation properties of this mutant is given on the basis of the reported structure by modeling the hypothetical flavin C(4a)-hydroperoxide intermediate. The model shows that the distal oxygen of the peroxide anion could be in an appropriate situation to act as the proton donor in the reoxidation process. Proteins 2000;38:60–69. © 2000 Wiley-Liss, Inc.

Key words: Glu301Ala FNR; catalysis; flavoprotein reoxidation; X-ray structure

INTRODUCTION Ferredoxin-NADP⁺ reductase (FNR, 1.18.1.2) catalyses NADPH production during photosynthesis in higher plants as well as in cyanobacteria. During this process, FNR accepts one electron from each of two molecules of the one-electron carrier ferredoxin (Fd) and uses them to reduce NADP⁺ to NADPH via hydride (H⁻) transfer from the N-5 atom of the FAD cofactor of the enzyme.¹ The three-dimensional structure of this enzyme has been shown to be the prototype of a family of flavoproteins having a large variety of biological functions involving transduction between nicotinamide dinucleotides (two-electron carriers) and one-electron carriers.²³ The three-dimensional structures of oxidized and reduced native spinach FNR and that of a complex with 2’-phospho-5’-AMP have been reported.² Moreover, the three-dimensional structure of Anabaena FNR and that of a complex with NADP⁺ have also been determined.⁴ Electron-transfer reactions and complex formation between FNRs from different sources and their physiological partners have been extensively studied.¹⁵–¹³ Recently, a large amount of structural, kinetic, and electrochemical data that demonstrate the crucial role that certain amino acid residues in FNR play in the reaction with its substrates have been assessed by site-directed mutagenesis.⁵¹¹,¹²,¹⁴–¹⁸ Some of the studied mutations mainly affected the ability of the enzyme to form efficient complexes with its substrates, and indicated that the mutated residues were not directly involved in the electron transfer process itself.¹⁵–¹⁷ Moreover, site-directed mutagenesis studies of the five conserved residues surrounding the isoalloxazine ring have been carried out on FNR from different species: (Anabaena numbering) Tyr79 (Tyr95 in pea and Tyr95 in spinach), Ser80 (Ser96 in spinach), Cys261 (Cys272 in spinach), Glu301 (Glu312 in spinach) and Tyr303 (Tyr308 in pea).⁵¹¹,¹²,¹⁴,¹⁸–²⁰ However, these studies appear to indicate that most of these residues, if involved in the enzyme function, are involved in the proper binding of the nicotinamide ring of NADP⁺ or in the stabilization of the transition state rather than playing a direct chemical role in catalysis.

Recently, the Glu301 position, which is conserved in most of the members of the FNR family, has been studied in detail in the Anabaena and (Glu312) in the spinach enzymes.⁵¹⁴ The 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, in the same manner as

Abbreviations: FNR, ferredoxin-NADP⁺ reductase; Fd, ferredoxin; Fd, flavodoxin; 5-dRF, 5-deazariboflavin; IPTG, isopropyl-β-D-thiogalactoside; PEG, polyethylene glycol; βOG, β-octylglucoside; Op, proximal oxygen; Od, distal oxygen

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Glu312 could transfer protons to the isoalloxazine N-5 through Ser96 in the spinach enzyme. However, the kinetic characterization and the three-dimensional structures reported by Aliverti et al. of several mutants at position Glu312 in the spinach enzyme (Glu312 to Asp, Gln, Leu, and Ala) indicate that Glu312 does not play a role as the proton donor, but appears to be required for proper orientation of the nicotinamide ring in the active site, and tuning of the redox potentials of the flavin semiquinone to enhance efficient electron transfer. The results reported for the characterization of the enzyme produced by replacement of the corresponding side chain of Glu312 in *Anabaena* FNR by Ala did not lead to the same conclusion (only one mutant was prepared), but are in general consistent with those reported for the spinach enzyme, because the mutant presented an impaired electron transfer efficiency with Fd prepared, but are in general consistent with those reported for the equivalent spinach mutant, and other interesting properties of the mutant were also revealed. Thus, it was shown that Glu301Ala FNR was able to form a complex with 5-dRf, which had not been detected previously for the wild-type, native, or any of the other mutated FNR forms assayed thus far. Furthermore, when reduced, this mutant was able to react with molecular oxygen via a mechanism different than that reported for any other FNR form. Moreover, although redox potential values have not been directly measured for any of the mutants, the reported data seemed to indicate that the negative side chain of Glu301 contributes to the redox potentials in both species, but affects the potentials in opposite direction. While a decrease of about 20 mV is proposed for the Glu301Ala *Anabaena* FNR with respect to WT for the oxidized/semiquinone couple, the two-electron midpoint potential estimated of the flavin cofactor in the spinach mutants appears to become more positive by –10–30 mV. Nevertheless, it is important to keep in mind that both estimations have been done in the presence of different FNR substrates. Finally, considering that difference spectroscopy has shown that *Anabaena* and spinach FNRs differ with respect to their interaction with the nicotinamide ring of the NADP+, the resolution of the three-dimensional structure of the Glu301Ala *Anabaena* FNR mutant could offer additional important information. In the present article, the three-dimensional structure of the *Anabaena* Glu301Ala FNR mutant is presented at 1.8 Å resolution in order to add structural information for the interpretation of the behavior observed for this mutant.

**MATERIALS AND METHODS**

**Biological Material**

The Glu301Ala FNR mutant was prepared by oligonucleotide-directed mutation of the FNR gene from *Anabaena* PCC 7119 FNR using the Transformer™ Site-directed mutagenesis kit from CLONTECH in combination with the synthetic oligonucleotides: 5′-GCTTAGTATGTGCTACGTCAGCAG-3′, (base change is underlined) and 5′-AGTGGACTTTACCGCTGTTAGC-3′ as previously described. The expression of the mutant in *Escherichia coli* (*E. coli*) and its purification from IPTG induced cultures were also as described.

**Crystallography and Data Collection**

Crystals of Glu301Ala FNR mutant were grown by the hanging drop method. The 5 µl droplets consisted of 2 µl of a 25.9 mg protein/ml solution buffered with 10 mM Tris-HCl (pH 8), 1 µl of unbuffered β-octylglucoside (βOG) at 5% (w/v) and 2 µl of reservoir solution containing 17% (w/v) polyethylene glycol (PEG) 6000, 20 mM ammonium sulphate, and 0.1 M Mes-NaOH (pH 5.0). The droplet was equilibrated against 1 ml reservoir solution at 20°C. Under these conditions crystals grew within 1 to 7 days in the presence of phase separation caused by the detergent up to a maximum size of (0.8 × 0.4 × 0.4 mm). These crystals were mounted in glass capillaries and screened on a Mar Research (Germany) IP area detector for intensity, resolution, and mosaicity. With the conventional rotating-anode generator, crystals diffracted to a maximum resolution of 2.3 Å. Cryo-protectant additives were tested in order to find suitable conditions to use cryo-techniques. Best results were obtained after soaking crystals in a solution containing 70% of mother liquor and 30% glycerol, for 1 min. In order to improve the resolution achieved with conventional X-ray source, synchrotron radiation was used.

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**TABLE I. Data Collection and Refinement Statistics**

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\[R_{sym} = \frac{\sum_{hkl} I_{hkl} - \left| I_{hkl} \right|}{\sum_{hkl} I_{hkl}}.\]

\[R_{free} = \frac{\sum_{hkl} I_{hkl}}{\sum_{hkl} I_{hkl}}.\]
A single crystal was mounted in a fiber loop and frozen at 100 K in a nitrogen stream. X-ray diffraction data were measured with a CCD detector using the ESRF Grenoble synchrotron radiation source at D2AM beamline. A total of 355 images were collected at 0.5° oscillation angles, with 2 sec exposure time per image. Maximum resolution observed in these conditions was 1.6 Å. Crystals were found to belong to the P65 hexagonal space group. They present one FNR molecule in the asymmetric unit and 60% solvent content with 3.0 Å³/Da. Refined cell dimensions were a = b = 86.65 Å, c = 96.26 Å. Data were processed and reduced with MOSFLM and SCALA yielding 38021 unique reflections 98.6 % complete up to 1.8 Å resolution with Rsym = 0.067 (Rsym = 0.072 for all data in the last shell, 1.9–1.8 Å).

Structure Solution and Refinement

The structure of the Glu301Ala FNR mutant was solved by molecular replacement using the program AMoRe on the basis of the 1.8 Å resolution native FNR model. FAD cofactor, SO₄²⁻ anion and water molecules were removed from the model, and all temperature factors were adjusted to a value of 15 Å³. An unambiguous single solution for the rotation and translation functions was obtained. This solution was refined by the fast rigid body refinement program FITING. Observed data up to 1.8 Å were included during the refinement, 7% of them being set aside for cross-validation analysis. The model was subjected to alternate cycles of conjugate gradient refinement with the program X-PLOR by using the Engh and Huber force field at all times. Electron density of Ala301 was clear in the 2Fo-Fc density map. At this point, manual model building was done where necessary using the software package O. Finally, water molecules were added. The resulting model was again subjected to further cycles of positional and B-factor refinement, which yielded final crystallographic R and R free factors of 0.19 and 0.24, respectively, for reflections between 7 and 1.8 Å resolution (Table I).

The final model comprises residues 9–303 (8 first residues were not observed in the electron density map), one FAD moiety, one SO₄²⁻ molecule, and 661 solvent molecules. The model exhibits good stereochemistry, with r.m.s.d. from ideality of 0.008 Å for bond lengths, 1.05° for bond angles, and 1.59° for improper torsion angles. A Ramachandran plot was calculated by using the program PROCHECK. Of the 248 non-proline and non-glycine residues per molecule, 89.5% have conformations in the most favored regions, 10.5%
being in the additional allowed regions. The atomic coordinates of the Glu301Ala FNR mutant have been deposited in the Protein Data Bank (code 1b2r).

**Model Building of Flavin C(4a)-Hydroperoxide**

Model building of the flavin intermediate in the FNR Glu301Ala mutant was based on the work by Schreuder et al.\textsuperscript{30,31} with the p-hydroxybenzoate hydroxylase. Following the same strategy, a model of flavin C(4a)-hydroperoxide intermediate was built aided by the crystal structure of a 4a,5-epoxyethano-3-methyl-4a,5-dihydrolumiflavin determined by Bolognesi et al.\textsuperscript{32} in which bond distances and angles of the peroxide moiety were fitted, taking into account the geometry reported in the literature. All the
adjustments were done manually using the O graphics program.28

RESULTS
Effects of the Glu301Ala Mutation in Anabaena FNR

As previously reported,5 replacement of Glu301 by Ala in Anabaena FNR did not produce gross structural rearrangements in the flavin environment as shown by the very small differences observed in the absorbance, CD, and fluorescence spectra of the mutated protein when compared with those of the WT enzyme. Moreover, no major changes were detected in the dissociation constants of the mutant to various FNR substrates with respect to those reported for the WT. However, electron transfer reactions between FNR and its substrates were clearly diminished, especially in those cases where an intermediate FNR semiquinone had to be produced.5 It is also important to point out that accumulation of the semiquinone form of this mutant upon photoreduction has not been detected either in the absence of NADP+ or in its presence. This result contrasts with that shown for the corresponding Glu312Ala spinach FNR mutant, where 8% of semiquinone is accumulated upon photoreduction.14

In addition, when reduced Glu301Ala Anabaena FNR mutant reacted with molecular oxygen it followed a different reoxidation mechanism than all the FNR forms reported thus far. Upon reaction with molecular oxygen reduced WT FNR reacts relatively fast to yield the neutral flavoprotein semiquinone and O2. Moreover, the reactivity of the formed semiquinone with molecular oxygen is several orders of magnitude smaller.5,31 However, upon examining the products formed by the reaction of reduced Glu301Ala FNR with molecular oxygen, neither semiquinone nor O2 formation were detected. Instead, substantial amounts of H2O2 were produced. Finally, Glu301Ala FNR is also able to form a complex with 5-dRF, which has not been detected thus far in any other FNR form.5

Overall Three-Dimensional Structure of the Glu301Ala FNR

The crystal structure of the FNR Glu301Ala mutant has been determined by X-ray diffraction. The first eight residues in the sequence were not included in the model, due to the poor definition of the electron density map in this region. The mutation site is on the surface of the FNR molecule, near the Tyr303 C-terminal residue and the isoalloxazine ring of the FAD cofactor (Fig. 1). As can be seen in the figure, the flavoenzyme folds into two well-defined domains: the FAD binding domain (residues 1 to 137) and the NADP+ binding domain (residues 139 to 303). The FAD binding domain is made up of a scaffold of six antiparallel strands arranged in two perpendicular b-sheets, the bottom of which is capped by short a-helices and a long loop. The NADP+ binding domain consists of a core of five parallel b-strands surrounded by seven a-helices. This corresponds to a variant of the typical dinucleotide binding fold.34,35 The FAD cofactor is bound to the protein through hydrogen bonds, van der Waals contacts and 3-3 stacking interactions. The isoalloxazine ring system, which constitutes the reactive part of the FAD, stacks on the aromatic rings of two tyrosine residues (Tyr79 and Tyr303). The ribityl moiety is also tightly bound to the protein through water molecules. On the contrary, the ribose and adenine moieties are less fixed to the polypeptidic chain.

Comparison of Anabaena Glu301Ala FNR Structure With That of Wild-Type Anabaena FNR

The overall folding of the Glu301Ala FNR mutant shows no significant differences with respect to the native structure, as shown by the r.m.s.d. of Cs backbones (0.3 Å) after superposition of both models. The most prominent differences are encountered in a loop comprising residues Tyr104 to Val113, but these differences are not significant due to the poor quality of the electron density map in the region from residue 106 to residue 111. This region has been described as highly flexible in the wild-type enzyme.4 Figure 2 displays a detailed view of the mutation site. The FAD cofactor environment is absolutely conserved with respect to that of the wild-type enzyme, except for two aspects. First, the Glu301-Ser80 hydrogen bond is missing due to the absence of the Glu301 side chain. Moreover, an important change in orientation is observed in another glutamate residue, Glu139, which is now pointing to the active site cavity towards the FAD cofactor. The new conformation is unambiguously defined in the electron density map (Fig. 3), which is of very good quality in this region, showing that in the Anabaena Glu301Ala FNR mutant, the oxygen atoms of the Glu139 side chain are involved in a network of five bifurcated hydrogen bonds to different water molecules. This situation is clearly different than that presented in the native enzyme, where the side chain of Glu139 is stabilized by a bifurcated hydrogen bond between one of its carboxylic oxygen atoms to Val130 NH and a water molecule.

Comparison of Anabaena Glu301Ala FNR Structure With Those of the Corresponding Spinach FNR Mutants

As previously mentioned, the position corresponding to Glu301 in the Anabaena FNR has also been studied in the spinach enzyme (Glu312).14 Several mutations have been performed at this position and their three-dimensional structures have also been determined.14 The structures of the Glu312Ala, Glu312Leu, Glu312Gln spinach FNR mutants did not show global perturbation of the protein structures. The structure of the Glu312Ala spinach FNR mutant shows an r.m.s.d. of only 1.3 Å with regard to that of the Glu301Ala Anabaena FNR after superimposing 285 Cα atoms. The overall folding of both flavoproteins is very well conserved apart from one small extra a-helix in Anabaena, which is far from the active center, similar to what is observed in the WT enzymes. The FAD confor-
tion and the main features of its environment are almost invariant in the mutants of both species. However, in contrast to what is observed for the *Anabaena* Glu301Ala FNR mutant, replacement of Glu312 by Ala does not cause a change in the conformation of Glu154 (corresponding to Glu139 in *Anabaena* FNR) in the spinach enzyme. The side chain of this glutamate keeps the same orientation and hydrogen bonding pattern as those seen in the spinach and *Anabaena* wild-type enzymes. Furthermore, replacement of Glu312 in spinach FNR by either Leu or Gln also revealed no changes in Glu154 conformation. On the other hand, this different conformation of Glu139 cannot be attributed to crystallization conditions because all the FNR proteins were crystallized at a similar pH. Therefore, the change in orientation observed in the side chain of Glu139 in *Anabaena* FNR upon replacement of Glu301 by Ala appears to be a unique feature of this mutant.

**The Role of Water Molecules in Glu301Ala FNR**

The new conformation of the Glu139 side chain is stabilized by a network of five hydrogen bonds to several water molecules, which seem to hold the carboxylate side chain in a rather fixed position. This hydrogen bonding pattern is depicted in Figure 2a. Further links of these water molecules to the peptide chain are also observed, and some of them are also hydrogen bonded to additional solvent molecules. It is interesting to note that this interacting network is connecting the Glu139 side chain to the Ser80 side chain through a string of three water molecules. Apparently, in the Glu301Ala FNR mutant, the shorter side chain of Ala301 allows a stronger interaction between W3 and the reactive Ser80 than in the case of the wild-type enzyme. It is also worth noting that no water molecule replaces the carboxylate Glu301 side chain. Finally, it is interesting to note that, apart from W3, all other water molecules involved in this network are exclusive to this mutant structure and have not been seen in any other FNR structures reported thus far.

**Solvent Accessibility**

The new water molecules present in the Glu301Ala FNR are localized in a pocket (Figure 2b) created by the removal of the Glu301 side chain. Apparently, this pocket enlarges the active center cavity in which the FAD is packed (a
perspective of this pocket is better seen in the center of the molecular envelope shown in Figure 1). The pocket is located over the α-helix, which is next to the N-terminal domain, and is also near the isoalloxazine ring of the FAD group. Therefore, the solvent accessibility of the isoalloxazine ring is significantly affected as can be observed in Figure 4, which represents a section of the molecular envelope of both the wild-type and the Glu301Ala mutant, in a view perpendicular to that of Figure 1. The calculated solvent accessibility surface of the FNR-FAD complex in the mutated enzyme is 8 Å² larger in this area than that corresponding to the wild-type enzyme (CCP4, 1994). This fact suggests that this feature might be related to the different behavior observed in the reaction of reduced Glu301Ala FNR with molecular oxygen with regard to the other FNR species analyzed thus far for this reaction.\(^5\)

**Flavin C(4a)-Hydroperoxide Model**

For most flavins and reduced flavoproteins it has been proposed that activation of dioxygen to form hydroperoxides occurs through formation of a covalent adduct at position 4a, which seems to be unfavorable for FNR (and for most of the electron transferases), due to the fact that it presents insufficient room to accommodate the hydroperoxide product, and the access to \(O_2\) is severely limited.\(^36\) However, in the Glu301Ala FNR enough room is made available in the flavin environment to accommodate such an intermediate species (Fig. 2b). Because it is known that the flavin C(4a)-hydroperoxide intermediates show lifetimes too short to be investigated by conventional X-ray crystallography, a model has been constructed of a C(4a)-hydroperoxide Glu301Ala FNR intermediate as reported in Materials and Methods. In this model, the flavin moiety of the C(4a)-hydroperoxide intermediate fits in very well with the isoalloxazine ring of FAD as determined for the free Glu301Ala FNR structure, except for N3, C4, O4, and C4a atoms that deviate significantly from the completely planar ring, which is present in the oxidized mutant structure (see Fig. 5). In the C(4a)-hydroperoxide adduct bond angles and lengths are more or less fixed, but torsional freedom exits about single bonds. Schreuder et al.\(^30,31\) assessed all possible positions of the distal oxygen (Od) by rotating around the C4a-proximal oxygen (Op) bond of the modeled flavin-C(4a)-hydroperoxide analogue within the active site of the \(p\)-hydroxybenzoate hydroxylase. These authors obtained mechanistic implications from the different possible positions of the Od relative to the protein. In the case of Glu301Ala FNR, superimposition of the flavin C(4a)-hydroperoxide model onto the flavin ring and rotation of Od around C4a-Op bond, reveals that the distal oxygen can only be found in a narrow region of the space, without involving important changes in the protein structure. This region corresponds to the position reported by Bolognesi et al.\(^32\) plus 30°, as shown in Figure 5. That is, Od is trans with respect to C10a and is in between C4 and N5. In this position, Od would be located 1.8 Å from the Ser80 and 2.0 Å from the Tyr303 hydroxyl group. However, these distances may be easily accommodated by small conformational changes in both residues, and, in fact, a movement of the C-terminal tyrosine has been proposed as a prerequisite for hydride and proton transfer in FNRs.\(^36\) Besides, Od is 1.6 Å away from the position of a conserved bound solvent molecule, W1 in Figure 2b. Therefore, although there is not an absolutely convincing experimental evidence that a flavin C(4a)-hydroperoxide intermediate is involved in the reoxidation process of Glu301Ala FNR, this model indicates that from the structural point of view this intermediate can be formed in the mutant, but not in the WT FNR.
DISCUSSION

The description of the three-dimensional structure of the Glu301Ala Anabaena FNR presented here shows some interesting unique features. The overall topology of the enzyme does not change with regard to that of the wild-type. Replacement of Glu301 by Ala does not produce a significant change in the overall charge distribution of the enzyme that could affect electrostatic interactions with its substrates, which is consistent with the fact that no major alterations have been reported for the dissociation constants for the different substrates of the enzyme. However, significant differences have been detected in the microenvironment of the Glu301 side chain and the flavin ring. As can be seen in Figures 1 and 2, these differences are mainly (i) the creation of a new pocket in the mutant structure and (ii) the conformational change observed in the side chain of Glu139, which compensates for the decrease of the acidic character of Glu301 side chain in the Glu301Ala FNR, producing only a slight modification of the electrostatic surface potential around the mutated position. The displacement of the side chain of Glu139 and the network of water molecules that is connecting it to the flavin ring through Ser80 side chain are prominent. Thus, the network of water molecules connecting Ser80 and Glu139 in the case of the Anabaena Glu301Ala FNR, may indicate that in this enzyme, the side chain of Glu139 might be performing some of the functions carried out by Glu301 in the wild-type enzyme. Interestingly, neither the network of water molecules nor a displacement of Glu154 can be observed in the structure of any of the spinach Glu312 FNR mutants, indicating that different interactions take place in the microenvironment of the flavin ring in the Glu301Ala Anabaena FNR and Glu312Ala spinach FNR. Differences in the interactions in the immediate environment of the flavin ring have already been reported between FNRs from different species. Thus, the study of the binding of NADP+ by difference spectroscopy in the Anabaena and spinach FNRs indicated that the flavin rings of these FNRs interact in different ways with the nicotinamide rings of the substrates, in that the flavin environment of spinach FNR is modified to a larger extent than that of the Anabaena enzyme. It is worth noting that difference spectroscopic studies also confirm that both Glu301Ala Anabaena FNR and Glu312Ala spinach FNR behave as their corresponding WT enzymes with regard to NADP+ binding. Thus, taking into account that changes in redox potentials for both mutants appear to take place in opposite directions and that while the Anabaena mutant is not able to stabilize the intermediate semiquinone form at all while the spinach mutant does, we propose that different interactions should be expected in the environment of the flavin rings of these mutants. We cannot overlook the fact that both mutated enzymes follow different mechanisms during the electron transfer reactions in which they can be involved. Consequently, the nature of the structural features found in the Glu301Ala Anabaena FNR mutant led us to the conclusion that we cannot rule out the role of Glu139 as a substitute of Glu301 in the transfer of protons from the external medium to the N5 atom of the isoalloxazine via Ser80. Relative to this, it is interesting to note that the conserved W1 water molecule, which has been proposed to substitute for Glu312 in the
spinach mutant, is placed farther away from the N5 flavin ring in the Glu301Ala *Anabaena* FNR than in the Glu312Ala spinach FNR (3.56 Å in *Anabaena* versus 3.28 Å in spinach). Thus, in the spinach case, the W1 water molecule could more easily play a role as proton donor. Moreover, it is worth mentioning that a special reactivity of the side chain of Glu139 has already been shown by chemical modification.37

Glu301Ala *Anabaena* FNR also showed altered FAD reoxidation properties, supporting the idea that the flavin ring exposure is an important factor in the mechanism of flavoprotein reoxidation. Reoxidation of free reduced flavins has been shown to proceed via formation of C(4a)-hydroperoxide intermediates. It is also generally accepted, although it lacks experimental support, that this must be a common route for reoxidation of a large number of flavoproteins, and that the ultimate products are dictated by the particular interactions between the flavin and the protein in each case.36 Although it is proposed that reoxidation of flavoproteins proceeds via the C(4a)-hydroperoxide intermediate, for many years such an intermediate has only been detected in the reaction of monoxygenases with O2. Nevertheless, Mallett and Claiborne38 have recently obtained evidence for a C(4a)-peroxyflavin in a NADH oxidase Cys42Ser mutant, a member of the oxidases group. However, in the group of the electron transferases, to which FNR belongs, it has been shown that the regions of the flavin ring that are candidates for formation of a covalent bond with O2 have insufficient room to accommodate the hydroperoxide product, and the access to O2 is severely limited, suggesting that in these cases the reaction will likely proceed without the formation of a flavin C(4a)-hydroperoxide.33

Thus, it is known that reduced FNR produces the neutral semiquinone form of FNR, (which is generally the one that electron transferases stabilize thermodynamically39), and O2- upon reaction with O2. Moreover, in FNR, as in most of the electron transferases, it is only the dimethylbenzene ring of the flavin that is freely accessible to solvent.4 However, upon examining the products formed by the reaction of reduced Glu301Ala FNR with O2, it was found that substantial amounts of H2O2 were obtained instead of O2 and stabilization of the FNR neutral semiquinone state was not detected. The three-dimensional structure for the Glu301Ala *Anabaena* FNR reported here clearly shows that replacement of Glu301 by Ala increases the degree of exposure of the dimethylbenzene flavin ring to solvent and thus may facilitate the access of oxygen, thereby allowing the production of H2O2 to predominate over O2- production. Moreover, taking into account the pocket produced in the environment of the isoalloxazine ring of FAD by replacement of Glu301 by Ala in FNR, a hypothetical C(4a)-hydroperoxide adduct has been modeled for the mutant (Figs. 2b and 5), which shows that enough room is available in the mutated FNR to accommodate the C(4a)-hydroperoxide. Only one region was found that was capable of accommodating the C(4a)-hydroperoxide intermediate without causing large changes in the protein fold. In this region, Od could be hydrogen bonded to the Ser80 and Tyr303 hydroxyl groups. Thus, in this situation, Ser80 could serve as a proton donor for the peroxy intermediate anion during the reoxidation process of the flavoprotein. Interestingly, the conserved W1 water molecule could be related to the position of the molecular oxygen for the oxidation process, as it is very close to the Od atom in the proposed model. On the other hand, this larger exposure of the flavin ring to the solvent may also account for the complex formation with dRf detected in this mutant.

**CONCLUSIONS**

The three-dimensional structure of Glu301Ala *Anabaena* FNR indicates that the side chain of Glu139, a residue situated 9.6 Å away from the N5 of the flavin ring, might be influencing some of the properties of the flavin and may assume catalytic functions of Glu301 to some extent. Important differences are found in the structure of the microenvironments of the flavin ring in this mutant and the corresponding one reported for the spinach enzyme. These differences may account for the different behaviors described for these mutants (i.e., essentially no detection of the semiquinone form by photoreduction in contrast to the 8% semiquinone form that accumulates in the spinach enzyme). Therefore, this indicates that small, but perhaps noticeable changes might be expected in the catalytic mechanisms of the FNRs from these two species. Finally, it has been shown that Glu301Ala FNR has enough room available to accommodate the hypothetical C(4a)-hydroperoxide intermediate during reoxidation. This could explain why this mutant follows a different reoxidation mechanism than the other flavoproteins of the electron transferase group.

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Three-dimensional structure of Glu301ala ANABAENA FNR


