

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

Tomás Mayoral,¹ Milagros Medina,² Julia Sanz-Aparicio,¹ Carlos Gómez-Moreno,² and Juan A. Hermoso^{1*}

¹Grupo de Cristalografía Macromolecular y Biología Estructural, Instituto Química-Física Rocasolano, C.S.I.C. Serrano 119, Madrid, Spain

²Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, Zaragoza, Spain

ABSTRACT The three-dimensional crystal structure of the Glu301Ala site-directed mutant of ferredoxin-NADP⁺ reductase from *Anabaena* PCC 7119 has been determined at 1.8 Å 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.

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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.^{2,3} 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.^{1,5–13} 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.^{5,11,12,14–18} 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, sited-directed mutagenesis studies of the five conserved residues surrounding the isoalloxazine ring have been carried out on FNR from different species: (*Anabaena* numbering) Tyr79 (Tyr89 in pea and Tyr95 in spinach), Ser80 (Ser96 in spinach), Cys261 (Cys272 in spinach), Glu301 (Glu312 in spinach) and Tyr303 (Tyr308 in pea).^{5,11,12,14,18–20} 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.^{5,14} 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; Fld, 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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*Correspondence to: Dr. Juan A. Hermoso, Grupo de Cristalografía Macromolecular y Biología Estructural, Instituto Química-Física Rocasolano, C.S.I.C. Serrano 119, 28006-Madrid, Spain. E-mail: xjuan@iqfr.csic.es

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Glu312 could transfer protons to the isoalloxazine N-5 through Ser96 in the spinach enzyme.² This serine residue in *Anabaena* FNR is hydrogen bonded to the carboxylate of Glu301.⁴ However, the kinetic characterization and the three-dimensional structures reported by Aliverti et al.¹⁴ 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 Glu301 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 and a destabilized semiquinone,⁵ supporting the conclusions derived from the study of the mutants in the spinach enzyme. However, during characterization of Glu301Ala *Anabaena* FNR, no semiquinone formation was detected at all by photoreduction, in contrast with the data 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.⁵ 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,⁵ the two-electron midpoint potential estimated of the flavin cofactor in the spinach mutants appears to become more positive by ~10–30 mV.¹⁴ 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⁺,¹⁰ 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 TransformerTM Site-directed mutagenesis

TABLE I. Data Collection and Refinement Statistics

Data Collection		
Temperature (K)	293	100
Source	Rotating-anode	Synchrotron
Space group	P65	P65
Cell a, b, c (Å)	88.15, 88.15, 97.18	86.65, 86.65, 96.26
Resolution range (Å)	35.6–2.3	37.5–1.8
No. of unique refl.	18229	38021
Completeness of data (%)		
all data	95.9	98.6
outer shell	95.9	91.8
R _{sym} ^a	0.113	0.067
Refinement Statistics		
Sigma cutoff		0
Resolution range (Å)		7.0–1.8
No. of protein atoms		2334
No. of heterogen atoms		58
No. of solvent atoms		661
Rfactor ^b		0.19
Free Rfactor		0.24
RMS deviation		
bond lengths (Å)		0.008
bond angles (deg)		1.05
Ramachandran outliers		none

$$^a R_{\text{sym}} = \frac{\sum_{\text{hkl}} \sum_i |I_i - \langle I \rangle| / \sum_{\text{hkl}} \sum_i \langle I \rangle}{\sum_{\text{hkl}} \sum_i \langle I \rangle}$$

$$^b R\text{-factor} = \frac{\|F_o\| - \|F_c\|}{\|F_o\|}$$

kit from CLONTECH in combination with the synthetic oligonucleotides: 5'-GCTTAGTATGTTGCTACGTGCCAG-3', (base change is underlined) and 5'-AGTGCACCATCCGCGGTGTGA-3' as previously described.⁵ The expression of the mutant in *Escherichia coli* (*E. coli*) and its purification from IPTG induced cultures were also as described.^{5,21}

Crystallization 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 (BOG) 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.

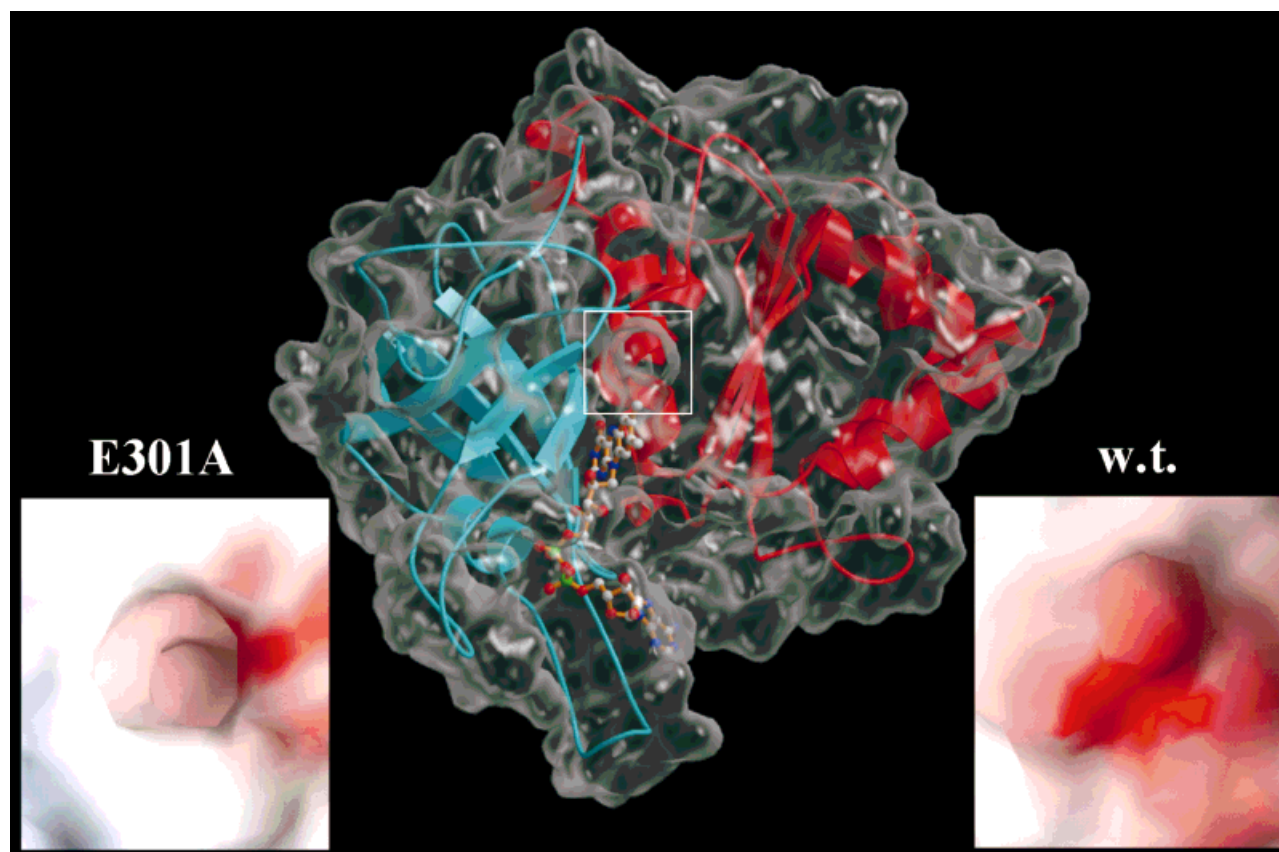


Fig. 1. GRASP³⁹ transparent molecular surface representation superimposed onto a MOLSCRIPT drawing⁴⁰ of the *Anabaena* Glu301Ala FNR mutant showing the secondary structure elements. The N-terminal FAD binding domain is on the left (cyan) and the C-terminal NADP⁺ binding domain is on the right (red). FAD is shown as balls and sticks. A new pocket is created in the mutant structure by the removal of Glu301 side chain (indicated by a square). The details of the molecular surface in this area, showing the

electrostatic surface potential, are presented for the mutant structure (bottom left) and for the wild-type enzyme (bottom right). Negative potential is shown in red. In the case of the Glu301Ala FNR this cavity, placed near the isoalloxazine ring, increases the solvent accessibility to the FAD group. The acidic character near the pocket is caused by the conformational change in the Glu139 side chain. Wild-type FNR shows a shallow cavity with a strong acidic character due to the presence of the Glu301 residue.

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 MOSFLM²² and SCALA²³ yielding 38021 unique reflections 98.6 % complete up to 1.8 Å resolution with $R_{\text{sym}} = 0.067$ ($R_{\text{sym}} = 0.072$ 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 AMoRe²⁴ on the basis of the 1.8 Å resolution native FNR model.⁴ FAD cofactor, SO₄²⁻ anion and water molecules were removed from the model, and all temperature factors were adjusted to a value of 15 Å.² An unambiguous single solution for the rotation and translation functions was obtained. This

solution was refined by the fast rigid body refinement program FITING.²⁵ Observed data up to 1.8 Å were included during the refinement, 7% of them being set aside for cross-validation analysis.²⁶ The model was subjected to alternate cycles of conjugate gradient refinement with the program X-PLOR²⁷ by using the Engh and Huber force field at all times. Electron density of Ala301 was clear in the 2F_o-F_c density map. At this point, manual model building was done where necessary using the software package O.²⁸ 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.²⁹ Of the 248 non-proline and non-glycine residues per molecule, 89.5% have conformations in the most favored regions, 10.5%

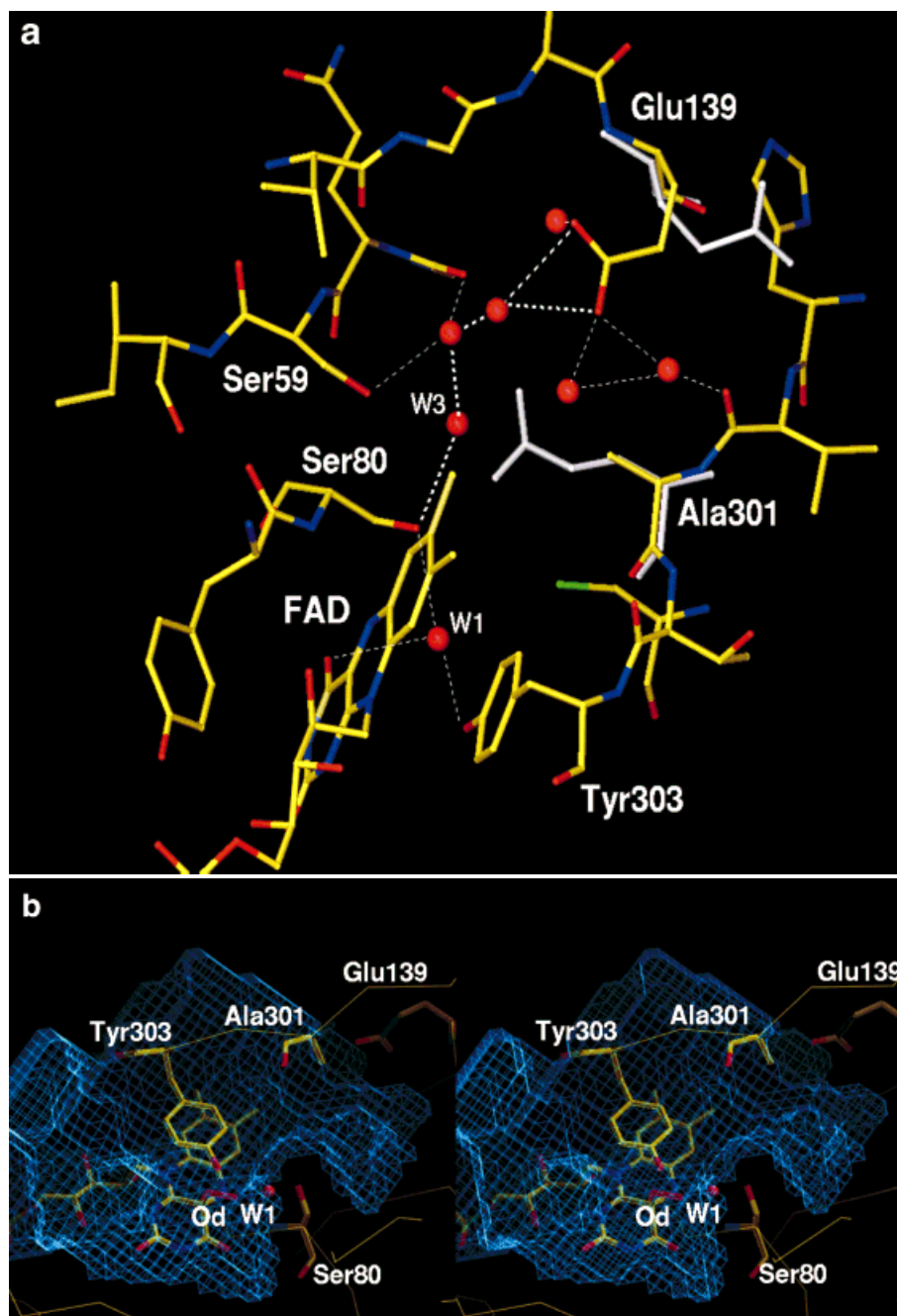


Fig. 2. A detailed view of the environment of the *Anabaena* Glu301Ala FNR active site. **a**: Hydrogen bonds are represented by broken lines. Significant structural differences with the wild-type enzyme are represented as white sticks (positions 139 and 301). The new conformation of Glu139 is stabilized by a network of water molecules linking the glutamate residue to Ser80. This hydrogen bonding pattern is exclusive to this mutant

structure. **b**: Stereo view of the cavity in the active site of the *Anabaena* Glu301Ala FNR structure calculated using the program VOIDOO⁴¹ and MAMA.⁴² In addition to the cavity in which FAD is packed, a new pocket is created. The proposed model of the flavin-C(4a)-hydroperoxide intermediate is presented with the distal oxygen in the most favorable position (see text). The figure was generated using the O graphics program.²⁸

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.^{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.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 the 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.^{5,33} 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 β -sheets, the bottom of which is capped by short α -helix and a long loop. The NADP⁺ binding domain consists of a core of five parallel β -strands surrounded by seven α -helices. 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 π - π 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 C α 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 Val300 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 C α atoms. The overall folding of both flavoproteins is very well conserved apart from one small extra α -helix in *Anabaena*, which is far from the active center, similar to what is observed in the WT enzymes. The FAD conforma-

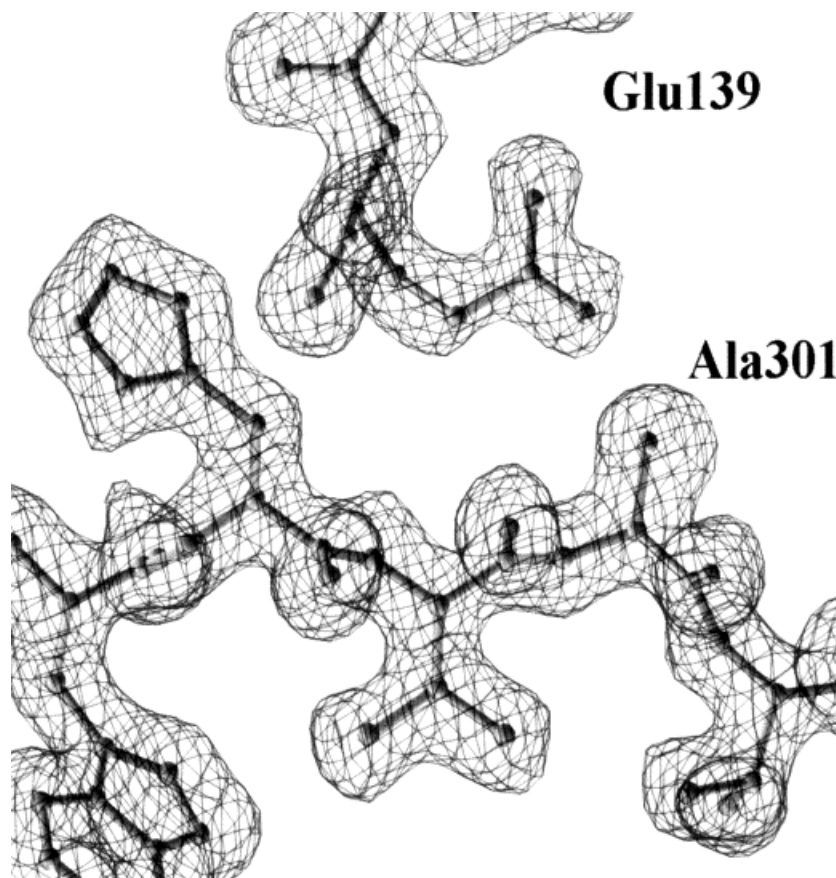


Fig. 3. $(2F_o - F_c)$ electron density map of the Glu301Ala mutant in the vicinity of the mutated residue. The map was contoured at 1σ . This figure was generated using the O graphics program.²⁸

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.¹⁴ 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.^{2,4,11,14,35} Figure 2 also shows the position of the relevant W1 water molecule, which is conserved in all crystal structures from *Anabaena* and spinach enzymes reported to date. This water molecule has been proposed to be a possible proton donor to the N5 position of the flavin ring, to which it is hydrogen bonded, in the oxidation process.⁴

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

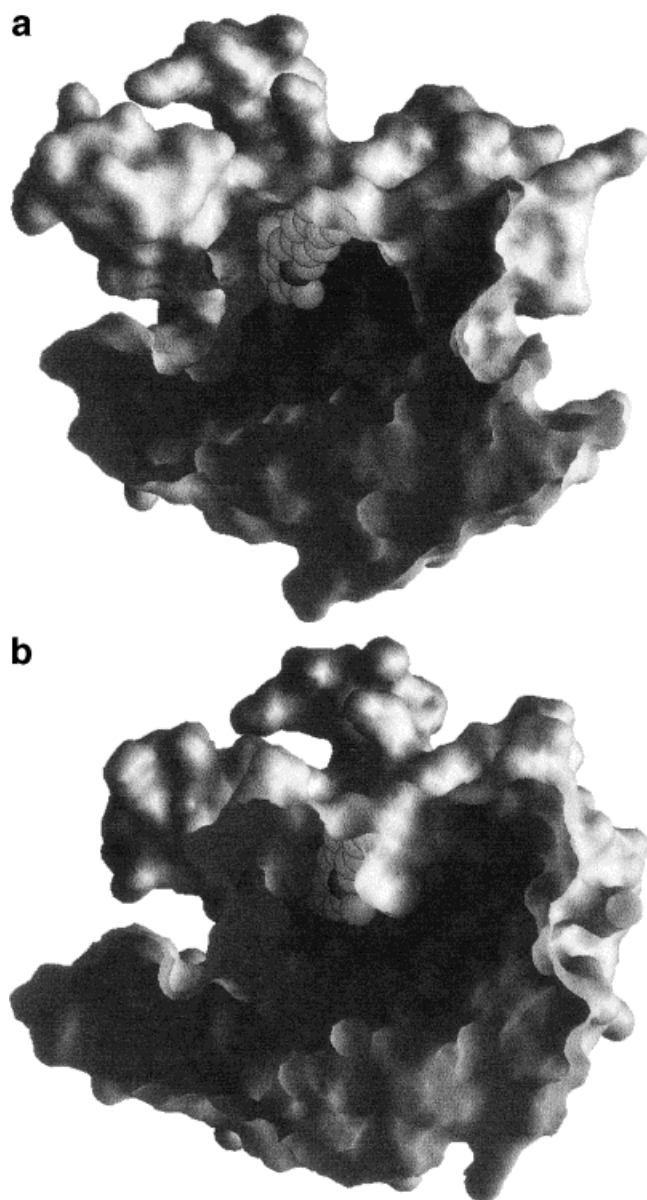


Fig. 4. A section of the molecular surface near the FAD group (represented as CPK and seen approximately in the same orientation) in (a) FNR wild-type enzyme and in (b) the Glu301Ala FNR. A new pocket is formed next to the FAD group increasing the solvent accessibility surface by 8 Å². The C4a atom of isoalloxazine ring is highlighted (see Fig. 5 for labeling). The figure was generated using GRASP.³⁹

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 O₂ 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 exists 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.³⁵ 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.

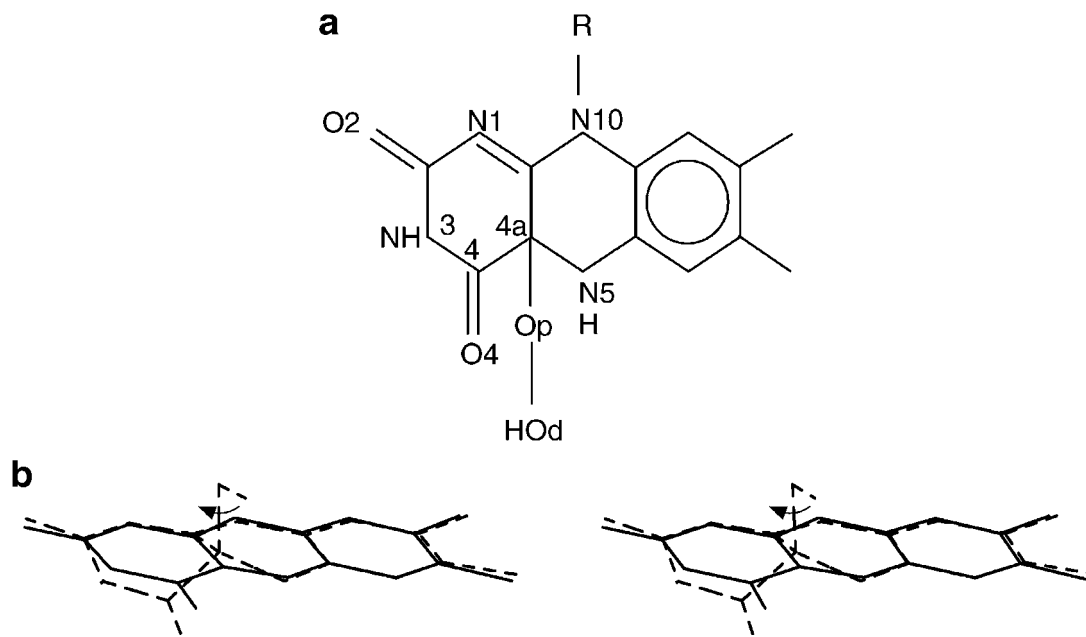


Fig. 5. The flavin-C(4a)-hydroperoxide model. **a:** Schematic diagram. **b:** Stereo view of the superposition of the flavin-C(4a)-hydroperoxide model (dashed lines) and the flavin ring of the Glu301Ala FNR structure (solid lines). The arrow indicates the rotation needed to generate the Od allowed region (see text).

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.⁵ 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 interac-

tions 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,¹⁰ 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.^{5,14} 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 monooxygenases 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 thermodynamically¹⁰), 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 peroxide 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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REFERENCES

1. Arakaki AK, Ceccarelli EA, Carrillo N. Plant-type ferredoxin-NADP⁺ reductases: a basal structural framework and a multiplicity of functions. *FASEB J* 1997;11:133–140.
2. Bruns CM, Karplus PA. Refined crystal structure of spinach ferredoxin reductase at 1.7 Å resolution: oxidised, reduced and 2'-phospho- 5'-AMP bound states. *J Mol Biol* 1995;247:125–145.
3. Correll CC, Ludwig ML, Bruns CM, Karplus PA. Structural prototypes from an extended family of flavoprotein reductases: comparison of phthalate dioxygenase reductase with ferredoxin reductase and ferredoxin. *Protein Sci* 1993;2:2112–2133.
4. Serre L, Vellieux FMD, Medina M, Gómez-Moreno C, Fontecilla-Camps JC, Frey M. X-ray structure of the ferredoxin-NADP⁺ reductase from the cyanobacterium *Anabaena* PCC 7119 at 1.8 Å resolution, and crystallographic studies of NADP⁺ binding at 2.25 Å resolution. *J Mol Biol* 1996;263:20–39.
5. Medina M, Martínez-Júlvez M, Hurley JK, Tollin G, Gómez-Moreno C. Involvement of Glutamic 301 in the catalytic mechanism of ferredoxin-NADP⁺ reductase from *Anabaena* PCC 7119. *Biochemistry* 1998;37:2715–2728.
6. Hurley JK, Fillat MF, Gómez-Moreno C, Tollin G. Electrostatic and hydrophobic interactions during complex formation and electron transfer in the ferredoxin-NADP⁺ reductase system from *Anabaena*. *J Am Chem Soc* 1996b;118:5526–5531.
7. Jelesarov I, De Pascalis AR, Koppenol WH, Hirasawa M, Knaff

- DB, Bosshard HR. Ferredoxin binding site on ferredoxin-NADP⁺ reductase. Differential chemical modification of free and ferredoxin-bound enzyme. *Eur J Biochem* 1993;216:57–66.
8. Medina M, Méndez E, Gómez-Moreno C. Lysine residues on ferredoxin-NADP⁺ reductase from *Anabaena* sp. PCC 7119 involved in substrate binding. *FEBS Lett* 1992a;298:25–28.
 9. Medina M, Méndez E, Gómez-Moreno C. Identification of arginyl residues involved in the binding of ferredoxin-NADP⁺ reductase from *Anabaena* sp. PCC 7119 to its substrates. *Arch Biochem Biophys* 1992b;299:281–286.
 10. Sancho J, Gómez-Moreno C. Interaction of ferredoxin-NADP⁺ reductase from *Anabaena* with its substrates. *Arch Biochem Biophys* 1991;288:231–238.
 11. Aliverti A, Bruns CM, Padini VE, Karplus PA, Vanoni MA, Curti B, Zanetti G. Involvement of Serine 96 in the catalytic Mechanism of ferredoxin-NADP⁺ reductase: structure-function relationship as studied by site-directed mutagenesis and X-ray crystallography. *Biochemistry* 1995a;34:8371–8379.
 12. Aliverti A, Piubelli L, Zanetti G, Lübbertstedt, Hermmann RG, Curti B. The role of cysteine residues of spinach ferredoxin-NADP⁺ reductase as assessed by site-directed mutagenesis. *Biochemistry* 1993;32:6374–6380.
 13. Batie CJ, Kamin H. Ferredoxin-NADP⁺ oxidoreductase. Equilibria in binary and ternary complexes with NADP⁺ and ferredoxin. *J Biol Chem* 1984;259:11976–11985.
 14. Aliverti A, Deng Z, Ravasi D, Piubelli L, Karplus PA, Zanetti G. Probing the function of the invariant glutamyl residue 312 in spinach ferredoxin-NADP⁺ reductase. *J Biol Chem* 1998;273:34008–34015.
 15. Schmitz S, Martínez-Júlvez M, Gómez-Moreno C, Böhme H. Interaction of positively changed amino acid residues of recombinant cyanobacteria ferredoxin-NADP⁺ reductase with ferredoxin probed by site directed mutagenesis. *Biochim Biophys Acta* 1998;1363:85–93.
 16. Martínez-Júlvez M, Hermoso J, Hurley JK, Mayoral T, Sanz-Aparicio J, Tollin G, Gómez-Moreno C, Medina M. Role of Arg100 and Arg264 from *Anabaena* PCC 7119 ferredoxin-NADP⁺ reductase for optimal NADP⁺ binding and electron transfer. *Biochemistry* 1998a;37:17680–17691.
 17. Martínez-Júlvez M, Medina M, Hurley JK, et al. Lys 75 of *Anabaena* ferredoxin-NADP⁺ reductase is a critical residue for binding ferredoxin and flavodoxin during electron transfer. *Biochemistry* 1998b;37:13604–13613.
 18. Orellano EG, Calcaterra NB, Carrillo N, Ceccarelli EA. Probing the role of the carboxyl-terminal region of ferredoxin-NADP⁺ reductase by site-directed mutagenesis and deletion analysis. *J Biol Chem* 1993;268:19267–19273.
 19. Ottado J, Calcaterra NB, Arakaki AK, Orellano EG, Carrillo N, Ceccarelli EA. On the role of aromatic amino acids interacting with FAD in plant-type ferredoxin-NADP⁺ reductase. In: Stevenson KJ, Massey V, Williams CH Jr, eds. *Flavins and flavoproteins*. Calgary: University of Calgary Press; 1996. p 501–504.
 20. Aliverti A, Padini VE, Sternieri FA, Corrado ME, Karplus PA, Zanetti G. In: Mathis P, ed. *Photosynthesis: from light to biosphere*. Dordrecht: Kluwer Academic Publisher; 1995b. p 653–656.
 21. Gómez-Moreno C, Martínez-Júlvez M, Fillat MF, Hurley JK, Tollin G. Molecular recognition in electron transfer proteins. In: Mathis P, ed. *Photosynthesis: from light to biosphere*. Dordrecht: Kluwer Academic Publisher; 1995. p 627–632.
 22. Leslie AGW. In *Proceedings of the CCP4 Study Weekend*. Helliwell JR, Machin PA, Papiz MZ, eds. SERC Daresbury Laboratory, Warrington, UK; 1987. p 39–50.
 23. Collaborative Computational Project Number 4. The CCP4 suite: programs for protein crystallography. *Acta Cryst* 1994;D50:760–763.
 24. Navaza J. AMoRe: an automated package for molecular replacement. *Acta Cryst* 1994;A50:157–163.
 25. Castellano E, Oliva G, Navaza J. Fast rigid-body refinement for molecular-replacement techniques. *J Appl Crystallog* 1992;25:281–284.
 26. Brünger AT. The Free R value: a novel statistical quantity for assessing the accuracy of crystal structures. *Nature* 1992;355:472–474.
 27. Brünger AT. X-PLOR: A system for X-ray Crystallography and NMR. New Haven, CT: Yale University Press; 1993.
 28. Jones TA, Zou JY, Cowan, SW, Kjeldgaard M. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Cryst* 1991;A 47:110–119, 1991.
 29. Laskowski RA, MacArthur MW, Moss DS, Thornton JM. PROCHECK: a program to check the stereochemical quality of protein structures. *J Appl Cryst* 1993;26:283–291.
 30. Schreuder HA, Hol WGJ, Drent J. Molecular modelling reveals the possible importance of a carbonyl oxygen binding pocket for the catalytic mechanism of p-hydroxybenzoate hydroxylase. *J Biol Chem* 1988;263:3131–3136.
 31. Schreuder HA, Hol WGJ, Drent J. Analysis of the active site of the flavoprotein p-hydroxybenzoate hydroxylase and some ideas with respect to its reaction mechanism. *Biochemistry* 1990;29:3101–3108.
 32. Bolognesi M, Ghisla S, Incoccia L. The crystal and molecular structure of two models of catalytic flavo(co)enzyme intermediates. *Acta Crystallogr* 1978;B34:821–828.
 33. Ghisla S, Massey V. Mechanisms of flavoprotein-catalyzed reactions. *Eur J Biochem* 1989;181:1–17.
 34. Rossman M, Moras D, Olsen K. Chemical and biological evolution of a nucleotide-binding protein. *Nature* 1974;250:194–199.
 35. Karplus PA, Daniels MJ, Herriot JR. Atomic structure of ferredoxin-NADP⁺ reductase: prototype for a structurally novel flavoenzyme family. *Science* 1991;251:60–66.
 36. Massey V. Activation of molecular oxygen by flavins and flavoproteins. *J Biol Chem* 1994; 269:22459–22462.
 37. Bes MT, De Lacey AL, Peleato ML, Fernández VM, Gómez-Moreno C. The covalent linkage of a viologen to a flavoprotein reductase transforms it into an oxidase. *Eur J Biochem* 1995;233:593–599.
 38. Mallet TC, Claiborne A. Oxygen reactivity of an NADH oxidase C42S mutant: evidence for a C(4a)-peroxyflavin intermediate and a rate-limiting conformational change. *Biochemistry* 1998;37: 8790–8802.
 39. Nicholls A, Bharadwaj R, Honig, B. GRASP: graphical representations and analysis of surface properties. *Biophys J* 1993;64:166.
 40. Kraulis PJ. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J Appl Cryst* 1991;24:946–950.
 41. Kleywegt GJ, Jones TA. Detection, delineation, measurement and display. *Acta Cryst* 1994;D50:178–185.
 42. Kleywegt GJ, Jones TA. Masks made easy. *ESF/CCP4 Newsletter* 1993;28:56–59.