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Role of critical charged residues in reduction potential modulation of ferredoxin-NADP$^+$ reductase

Differential stabilization of FAD redox forms

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Reduction potential determinations of K75E, E139K and E301A ferredoxin-NADP$^+$ reductases provide valuable information concerning the factors that contribute to tune the flavin reduction potential. Thus, while E139 is not involved in such modulation, the K75 side-chain tunes the flavin potential by creating a defined environment that modulates the FAD conformation. Finally, the E301 side-chain influences not only the flavin reduction potential, but also the electron transfer mechanism, as suggested from the values determined for the E301A mutant, where $E_{ox/rd}$ and $E_{sq/rd}$ shifted +41 and +102 mV, respectively, with regard to wild-type. Reduction potentials allowed estimation of binding energies differences of the FAD cofactor upon reduction.

Keywords: reduction potential; ferredoxin-NADP$^+$ reductase.

Ferredoxin-NADP$^+$ reductase (FNR, EC 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 to the nicotinamide ring of the pyridine nucleotide [1]. When, in cyanobacteria and certain algae, the organism is grown under iron-deficient conditions, flavodoxin (Fld) replaces Fd in this reaction [2]. In the proposed catalytic mechanism, upon reduction of FNR by the first Fd a transient FNR semiquinone is produced [1,3,4]. Three-dimensional structures of FNRs from different species, either in the oxidized or the reduced states, show that no significant conformational differences exist between oxidized and reduced FNR [5,6]. Crystal structures for complexes of the enzyme with NADP$^+$ [6,7] and, more recently, three-dimensional structures of the complex between FNR and Fd have also been reported [8,9]. The geometry of these FNR:NADP$^+$ and FNR:Fd complexes suggest nonsteric impediments to the proposed [NADP$^+$:FNR:Fd] ternary complex. Moreover, the structures reported for the FNR:Fd complexes [8,9] indicate that an FNR molecule interacts specifically with a single Fd molecule before each one-electron transfer process, also suggesting that disassembly of the FNR–Fd interaction takes place upon a redox linked conformational change in the Fd molecule once the electron has been transferred to FNR [8]. Although FNRs from different species have been thoroughly investigated [3,4,10–15], the mechanism of proton and electron transfer (ET) between FNR and its substrates is still not clear.

The molecular interface between Fd and FNR [8] consists of a core of hydrophobic residues from both molecules, whose role in complex stabilization and ET has been confirmed [12,16,17]. In addition, charged groups on both molecules are also critical [4,10–13,17]. Among these, in *Anabaena* PCC 7119 FNR, K75, residue which is conserved in all the FNR sequences analysed but not in other members of the FNR family, seems essential for stabilization of the intermediate FNR:Fd complex [10], evidence supported by the H-bond between K75 FNR and E94 Fd observed in the complex [8] (Fig. 1). Structural analysis also suggested that the E301 carboxylate might be involved in the catalysis by transferring protons from the external medium to the buried N5 atom of the isoalloxazine through S80 (Fig. 1) [4–6,15]. Replacement of E301 by Ala impaired the FNR ability to exchange electrons in those processes where a transient semiquinone FNR was expected [4]. Such behaviour was related to the very low stability of the E301A FNR semiquinone and, consequently, modification of the flavin reduction potential might be expected for this mutant. Although the E301A FNR overall folding was absolutely conserved with respect to the wild-type, the E301–S80 H-bond was absent, and a conformational change was observed in the E139 side-chain, which now points towards the FAD cofactor [18]. This E139 conformation was stabilized by a network of H-bonds to several new water molecules that connect E139 and S80 side-chains, suggesting that E139 might influence the FAD properties. Previous

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Abbreviations: FNR, ferredoxin-NADP$^+$ reductase; Fd, ferredoxin; Fld, flavodoxin; dRf, 5-deazariboflavin; $E_{ox/rd}$, $E_{ox/sq}$, $E_{sq/rd}$, oxidized-reduced, oxidized-semiquinone, semiquinone-reduced couples reduction potentials; ET, electron transfer.
Enzyme: ferredoxin-NADP$^+$ reductase (EC 1.18.1.2).

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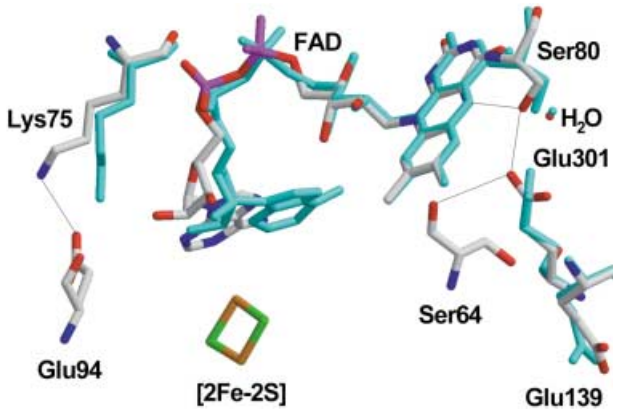

Fig. 1. Three-dimensional structural comparison, in *Anabaena* PCC 7119, of the conformations of the FAD and the FNR K75, S80, E139 and E301 side-chains. Free FNR (coloured in blue), FNR:Fd complex (coloured following CPK). E94, S64 and [2Fe-2S] of complexed Fd are also shown.

site-directed mutagenesis studies indicate that the E139 charge has a significant effect on the geometry of the interacting FNR-Fd surfaces but not on the ET process itself [13].

Versatility of protein-bound flavins arises from the interaction of its redox centre, the isoalloxazine ring, with the apoprotein, which determines its reduction potential within the protein environment [19–21]. For many years, no reduction potentials for FNR mutants were reported [4,15], and only recently have we been able to achieve its measurement [16]. The close proximity of K75, E139 and E301 to the flavin ring and the reported characterizations of K75E, E139K and E301A FNRs make it interesting to test the influence of these charged residues on FNR reduction potential.

MATERIALS AND METHODS

Protein production

K75E, E139K and E301A *Anabaena* PCC 7119 FNR mutants were obtained using as a template a construct of the *petH* gene which had been previously cloned into the expression vector pTrc99a, as previously described [4,10,13]. Mutants and wild-type FNR forms from *Anabaena* PCC 7119 were purified from the corresponding *E. coli* cultures by previously described methods [4]. UV-Visible absorption spectroscopy and SDS/PAGE electrophoresis were used as purity criteria.

FNR photoreduction

Photoreduction was carried out at 10 °C in an anaerobic cuvette containing 15–25 μM FNR, 1 mM EDTA and 2 μM dRf in 50 mM Tris/HCl, pH 8.0 [4,20]. Solutions were made anaerobic by successive evacuation and flushing with O_2-free Ar. Absorption spectra were recorded after successive periods of irradiation with a 150-W light source and were used to calculate the FNR_{ox}, FNR_{sq} and FNR_{rd} concentrations throughout reduction. The extinction coefficients used at 458 and 600 nm were, respectively, 9400 [22] and 200 $M^{-1}\cdot cm^{-1}$ [12] for FNR_{ox}; 3400 [3] and 5000 $M^{-1}\cdot cm^{-1}$ [22] for FNR_{sq}; and 900 [22] and 300 $M^{-1}\cdot cm^{-1}$ [12] for FNR_{rd}.

Spectroelectrochemistry for reduction potential measurements

Potentiometric titrations of FNRs were performed in a three-electrode electrochemical cell [23] using a gold working and a silver/silver chloride reference electrodes. Reducing equivalents were provided either electrochemically, with methyl-viologen as mediator, or photochemically. Both methods yielded the same results. Experimental solutions contained 12–20 μM protein, 1–3 μM indicator dyes, 10% (v/v) glycerol and 100 μM methyl-viologen (electrochemical reduction) or, 1 μM dRf and 1 mM EDTA (photoreduction), in 50 mM Tris/HCl buffer at pH 8.0. Indicator dyes included; lumiflavin 3-acetate (−223 mV), benzyl-viologen (−348 mV); and methyl-viologen (−443 mV). Solutions were made anaerobic over a 2-h period. After each reduction step, the cell was held at 10 °C. Once equilibration of the system was achieved, the UV-Visible spectrum was recorded (PerkinElmer 2S). Prior to redox species quantitation, turbidity and dye contributions were subtracted. Due to the low degree of FNR semiquinone stabilization it was not possible to measure the potential for the two one-electron steps. Values for $E_{ox/rd}$ of FNRs were determined according to the Nernst equation:

$$E = E_{ox/rd} + (0.056/n)\cdot\log([ox]/[red])$$

Each FNR displayed a two-electron redox behaviour based on the slopes of the Nernst plot, ≈30 mV. The reduction potentials are reported vs. the standard hydrogen electrode. The error in the E determinations was estimated in ± 3 mV.

RESULTS

Photoreduction

Photoreduction enabled the visible spectral properties of the different FNRs to be monitored throughout the reduction process, thereby allowing an accurate quantitation of the maximal amount of the total flavin semiquinone stabilized without spectral interference from the mediators. Thus, the concentrations of the different redox species at each reduction step were calculated by solving a mass balance equation and two Beer's law relationships (458 and 600 nm). Our data indicate that although wild-type, K75E and E139K FNRs accumulate a maximum of 22, 27 and 21%, respectively, of the total flavin as neutral semiquinone (Table 1), almost no absorbance changes attributable to a semiquinone were detected for E301A FNR [4].

Spectroelectrochemistry for reduction potential determination

Wild-type FNR. Figure 2A shows the spectra of the wild-type FNR species generated throughout potentiometric titration. The corresponding Nernst plot is consistent with a two-electron reduction (inset), as described previously for spinach and *Anabaena* FNRs [12,22,24], and with an $E_{WTox/rd}$ of −325 mV at pH 8.0. Despite the small differ-

Table 1. Midpoint reduction potentials and differences in binding energies of the oxidized, semireduced and reduced apoFNR:FAD complexes of wild-type and mutated FNR forms at pH 8.0.

FNR	$E_{ox/rd}$ (mV)	$E_{ox/sq}$ (mV)	$E_{sq/rd}$ (mV)	%SQ	$\Delta\Delta G_{sq\text{-}ox}$ (kcal·mol^{-1})	$\Delta\Delta G_{rd\text{-}sq}$ (kcal·mol^{-1})	$\Delta\Delta G_{rd\text{-}ox}$ (kcal·mol^{-1})
Wild-type	−325	−338	−312	22	−1.4	4.2	2.8
K75E	−305	–312	–298	27	−2.0	3.8	1.8
E139K	−326	−341	−311	21	−1.35	4.1	2.8
E301A	−284	−358[a]	−210	2	−1.0	1.8	0.9
FAD	−265[b]	−400[b]	−130[b]	0.2			

[a] Data from [4]. [b] Data for free FAD at pH 8.0 estimated from [29].

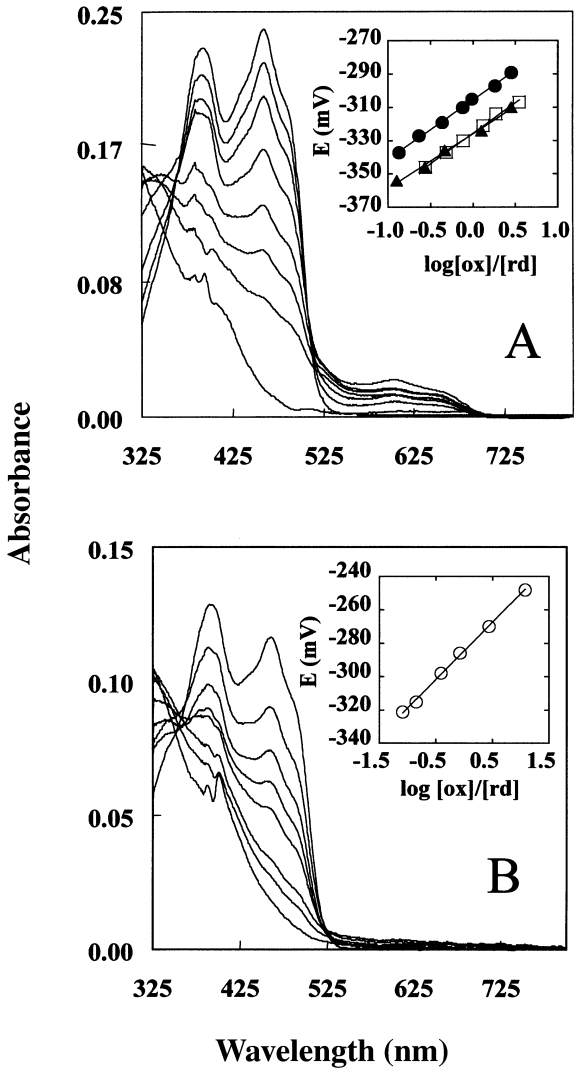

Fig. 2. Spectra obtained during potentiometric titration of (A) wild-type and (B) E301A FNRs. The insets show the corresponding Nernst plots: K75E (●), E139K (▲), wild-type (□) and E301A (○) FNRs.

ences observed among wild-type and native *Anabaena* FNRs, due to proteolytic cleavage of six residues at the N-terminus of the native enzyme purified from *Anabaena* cells, the value here obtained for wild-type FNR is in good agreement with those previously reported for native *Anabaena* FNR ($E_{FNRox/rd} = -376$ mV, pH 8.0 [22] and $E_{FNRox/rd} = -320$ mV, at pH 7.0 [26]), for the *Anabaena* wild-type FNR ($E_{WTox/rd} = -323$ mV, pH 7.5 [12]) and also with that described for the spinach FNR ($E_{WTox/rd} = -380$ mV [24], pH 8.0).

The reduction potentials of the one-electron reduction steps can be derived according to the equations

$$E_{ox/sq} - E_{sq/rd} = 0.11 \log\{2[SQ]/(1 - [SQ])\}\ [25] \quad (1)$$

$$(E_{ox/sq} + E_{sq/rd})/2 = E_{ox/rd} \quad (2)$$

once $E_{ox/rd}$ ($E_{WTox/rd} = -325$ mV) and the maximum concentration of semiquinone stabilized by the wild-type enzyme ([SQ] = 22.4% as determined by photoreduction experiments) are known. Thus, by simultaneously solving this Nernst derived two-equation system, the reduction potentials for the two individual ET processes for wild-type FNR have been calculated to be $E_{WTox/sq} = -338$ mV and $E_{WTsq/rd} = -312$ mV in Tris/HCl, pH 8.0 (Table 1).

K75E FNR. Spectra obtained through potentiometric titration of K75E FNR are nearly identical to those of wild-type. The corresponding Nernst plot yields an $E_{ox/rd}$ value 20 mV more positive than that of wild-type (Fig. 2A, inset). By considering the maximum of semiquinone stabilized, the values of $E_{K75Eox/sq} = -312$ mV and $E_{K75Esq/rd} = -298$ mV were calculated (Table 1). Therefore, a charge-reversal replacement of K75 is somehow affecting the flavin reduction potential.

E139K FNR. The spectra generated by E139K FNR during reduction exhibit properties indistinguishable from those of wild-type and, the corresponding Nernst plot yields a midpoint potential for the ox/rd couple almost identical to that of the wild-type. According to Eqns (1,2), the values of $E_{E139Kox/sq} = -341$ mV and $E_{E139Ksq/rd} = -311$ mV were calculated (Table 1). Thus, the $E_{ox/rd}$, $E_{ox/sq}$ and $E_{sq/rd}$ values obtained for E139K FNR are the same, within experimental error, as those of the wild-type.

E301A FNR. Potentiometric titration of E301A FNR shows that no detectable levels of the semiquinone intermediate state accumulated (Fig. 2B), which is consistent with the photoreduction analyses and with previous studies [4]. Moreover, the midpoint reduction potential calculated from the Nernst plot of E301A (inset) is 41 mV more positive than that of the wild-type (Table 1). Due to the lack of semiquinone stabilization it was not possible to perform the analysis above described to calculate the one-electron reduction potentials. However, based on the fact that

reoxidation of laser flash reduced Fd requires approximately twice as much E301A FNR than wild-type FNR, it was previously estimated that $E_{E301Aox/sq}$ should be 20 mV more negative than the corresponding wild-type value [4]. Therefore, using $E_{E301Aox/sq} = -358$ mV, the experimental value of $E_{E301Aox/rd}$ and Eqns (1,2), a +102 mV shift of the $E_{E301Asq/rd}$ ($E_{Glu301Ala\ sq/rd} = -210$ mV) and a maximal amount of only 2% of semiquinone are shown by E301A FNR (Table 1).

Binding affinities of apoFNR variants for the different redox states of FAD

Due to the irreversible denaturation of FNR upon FAD dissociation, we were not able to determine experimentally either the K_d or the binding energies for the apoFNR:FAD complexes in any redox state. However, as the reduction potentials of free and bound FAD are linked to the binding affinities of the FAD redox forms to apoFNR, differences between the binding energies for the interaction of the different FAD redox states can be calculated once the reduction potential values of complexed and free FAD are known [21]. Thus, according to:

$$\Delta G_{sq} = \Delta G_{ox} - F(E_{ox/sq} - E^{freeFAD}_{ox/sq}) \quad (3)$$

$$\Delta G_{rd} = \Delta G_{sq} - F(E_{sq/rd} - E^{freeFAD}_{sq/rd}) \quad (4)$$

differences between the free energies for the FAD:apoFNR complexes in the different redox states:

$$\Delta\Delta G_{sq\text{-}ox} = \Delta G_{sq} - \Delta G_{ox} = -F(E_{ox/sq} - E^{freeFAD}_{ox/sq}) \quad (5)$$

$$\Delta\Delta G_{rd\text{-}sq} = \Delta G_{rd} - \Delta G_{sq} = -F(E_{sq/rd} - E^{freeFAD}_{sq/rd}) \quad (6)$$

$$\Delta\Delta G_{rd\text{-}ox} = \Delta G_{rd} - \Delta G_{ox} = -F(E_{ox/sq} - E^{freeFAD}_{ox/sq} + E_{sq/rd} - E^{freeFAD}_{sq/rd}) \quad (7)$$

can be obtained (Table 1). As the three-dimensional structures of the oxidized and reduced forms of the spinach FNR do not show major structural differences in FAD conformation and binding to the protein [5], the shifts observed in the binding affinities of the three FAD redox forms to apoFNR cannot be a result of redox linked conformational changes in the flavin environment. For all the FNRs, complexes with semireduced FAD are considerably more stable than those of the oxidized forms, while reduced FAD complexes are less stable than the semiquinone or oxidized ones. Thus, in the wild-type protein, the FAD semiquinone is bound slightly more tightly (by 1.4 kcal·mol^{-1}) to the apoFNR than the oxidized form, while the reduced cofactor considerably destabilizes the complex compared with both the oxidized (2.8 kcal·mol^{-1}) and the semiquinone forms (4.2 kcal·mol^{-1}). E139K FNR, has identical reduction potential values as wild-type FNR (Table 1) and therefore an identical binding energy profile. Replacement of K75 by Glu produced an enzyme that upon reduction stabilized more the semireduced complex than the wild-type. Moreover, the reduced complex is destabilized relative to the oxidized and the semireduced ones, although, for both cases, the magnitude of the destabilization (1.8 and 3.8 kcal·mol^{-1}, respectively) is slightly smaller than that found for the wild-type FNR complexes. In the case of E301A, although the semiquinone and the reduced complexes are again more and less stable, respectively, than the oxidized, differences in the magnitude of the shifts are observed. Thus, in comparison with wild-type, the semiquinone complex is less stabilized with respect to the oxidized and, on the contrary, the reduced is much less destabilized relative to both the oxidized and the semireduced complexes.

DISCUSSION

Knowledge of the reduction potentials of the FNR mutants enables us to interpret their behaviours in thermodynamic terms and, consequently, the role of specific side-chains in the ET processes. Substitution of K75 by Glu produced an enzyme whose semiquinone appears to be stabilized to a slightly larger extent than that of the wild-type, and which had ox/rd, ox/sq and sq/rd FAD reduction potentials more positive by 20, 26 and 14 mV, respectively, suggesting that the K75 side-chain is somehow influencing the FAD reduction potential within the protein environment. FNR structure shows that K75 side-chain is not making any contact with the FAD-isoalloxazine [12.40 Å from K75-NH_2 to CH_3(8)] (Fig. 1). Moreover, K75 is not involved in any intraprotein interaction, but is situated at the entrance of a water cavity, at the bottom of which are the pyrophosphate and the ribose from the FAD. Therefore, K75 cannot be directly modulating the potential of the flavin ring by itself, but replacement of its positive charge by a negative one may produce a different organization of this solvent cavity. This might force the different regions of the FAD to adopt a slightly different conformation, which could produce the differences observed in flavin binding and reduction potentials. Such differences in relative conformation of the FAD moieties are, for instance, detected in the structure of the Fd:FNR complex, where a displacement of K75 side-chain from the water cavity to form a salt-bridge with Fd E94 side-chain is accompanied by a displacement of the pyrophosphate and the ribose of FAD towards the water cavity, which produces a less tight FAD L conformation (Fig. 1) [8]. Such complex formation has been shown to produce changes in the flavin reduction potentials [12]. We can conclude that K75 side-chain, which is conserved in all the FNR sequences analysed, apart from being a key residue in stabilizing complex formation with Fd prior to ET [10], modulates the protein/flavin interaction and contributes to a long distance modulation of the flavin reduction potential.

All the properties of E139K FNR analysed here were identical to those of the wild-type (Table 1). Therefore, the negative E139 side-chain does not influence the potential of the flavin within the protein environment, nor is involved in the stabilization of the FAD:apoFNR complex. This was expected, due to the long distance between the E139 side-chain and the FAD [10.87 Å from carboxylate to CH_3(7) of (FAD)] (Fig. 1) [6]. This is consistent with previous interpretations, which indicate that the large decrease in the ability to accept electrons from Fd_{rd} exhibited by this mutant, is not due to an alteration of its reduction potential, but more likely to a nonoptimal mutual orientation of the cofactors within the intermediate complex [13].

Replacement of E301 by Ala produced an enzyme that does not stabilize the semiquinone state at all and has a

reduction potential for the two-electron transfer process that is 41 mV more positive than the wild-type enzyme, i.e. $E_{Glu301Alaox/rd} = -284$ mV (Table 1). These two facts imply an important alteration of the reduction potentials for the two one-electron reduction processes, $E_{ox/sq}$ and $E_{sq/rd}$. Previous characterization of the reactivity of this E301A FNR mutant in complex formation and ET to its substrates had already indicated that the lack of its semiquinone stabilization was the cause for its highly impaired ET ability, as compared to the wild-type, in those processes in which a transient neutral semiquinone intermediate should be produced, i.e. with Fd and Fld [4]. Based on these transient kinetic results, which showed that it is required twice as much E301A mutant than wild-type FNR to completely reoxidize Fd_{rd}, a reduction potential value 20 mV more negative than the corresponding value for the wild-type was estimated for the ox/sq couple, i.e. $E_{Glu301Alaox/sq} = -358$ mV [4]. Taking into account both values, $E_{Glu301Alaox/rd} = -284$ and $E_{Glu301Alaox/sq} = -358$ mV, and according to Eqn (2), a large shift is expected for the reduction potential of the sq/rd couple to a much more positive value, $E_{Glu301Alasq/rd} = -210$ mV, which would set up a thermodynamic barrier for semiquinone stabilization. This is consistent with the experimental observations. E301A does not stabilize the semiquinone and its $E_{ox/rd}$ is 41 mV more positive than the wild-type one, which implies alteration of the one-electron potentials. This also indicates that, in E301A FNR, the H-bond network connecting E139 and N5 of the isoalloxazine does not substitute for E301 in modulating the flavin potential [18], and that it might only provide an alternative means of providing protons to the flavin ring to produce the hydroquinone form upon reduction of the enzyme when E301 is not present to provide them. Replacement of E301 by Ala also shifts the binding energy differences between the different FAD redox states compared with the wild-type. In this mutant the stabilization of the semiquinone complex relative to the oxidized is less pronounced, while the fully reduced state does not introduce such a large destabilization, relative to both the oxidized and the semireduced. Although in FNR it is accepted that reduction by the first electron is accompanied by the uptake of a proton, this is not the case for the second electron transfer [3,22], being the anionic hydroquinone formed. Therefore, it seems likely that an electrostatic repulsion between it and the neighbouring E301 results in destabilization of the hydroquinone FAD:apoFNR complex relative to those complexes involving the quinone and semiquinone. Such effect would not be produced in the case of the E301A mutant, and could account for the decreased destabilization of the complex observed upon reduction. The roles of E301 stabilizing the transient semiquinone, destabilizing the flavin hydroquinone complex and therefore influencing the FAD reduction potential, support the original hypothesis of its role in proton transfer from the solvent to the isoalloxazine N(5) position, via S80, to yield the neutral semiquinone [4,6]. Such a mechanism is also supported by the structures reported for FNR:Fd complexes [8,9]. Thus, in the *Anabaena* complex, the carboxylate group of E301 is not exposed to solvent but is H-bonded to the hydroxyl group of Fd S64, which is in turn exposed to solvent and could initiate the solvent proton transfer chain [8]. The maize complex shows structural changes around the FAD, where E312 (equivalent to E301 in *Anabaena* FNR) is displaced towards S96 (S80), bringing both side-chains into H-bonding distance [9]. In the *Anabaena* complex, changes are observed in the relative distances and organization between the atoms of the S80 and the E301 side-chains, and a torsion is introduced into the isoalloxazine of the FAD (Fig. 1). The structural perturbations in the environment and the conformation of the isoalloxazine are very likely related to the reduction potential shifts observed upon complexation [12,22,27,28]. In fact, complex formation between wild-type FNR and Fd not only shifts the ox/rd reduction potential of the flavin by +25 mV, but also inverts the two one-electron potentials, resulting in a stabilization of the semiquinone [12,28]. Therefore, an 'anchoring' role can be proposed for the side chain of E301, which is situated in the structure in such a way as to promote the crucial H-bonding network that stabilizes the flavin semiquinone. This effect is likely enhanced when, upon complexation with Fd, structural changes in the active site of the enzyme are induced.

These results also allow interpretation of the different behaviour of E301A FNR in accepting electrons from Fd or Fld [4]. In such a reaction, it is proposed that Fld cycles between the semiquinone and reduced states, as Fld_{sq} is not able to further reduce FNR. However, in the case of E301A FNR, the two-electron reduction of E301A FNR by Fld becomes thermodynamically favourable, avoiding the intermediate semiquinone, which has to be produced with the one-electron carrier Fd. Moreover, in the ET reaction between Fld and wild-type FNR it is expected that the electrons are transferred one at a time, as only the methyl groups of the FNR dimethylbenzene ring, proposed to be the entry point of electrons, are exposed to the solvent [20]. Replacement of E301 by Ala increases the degree of exposure of the dimethylbenzene flavin ring to solvent [18], which might also contribute to a different mechanism for the reduction of E301A FNR by Fld.

In conclusion, the determination of the reduction potential values for K75E, E139K and E301A FNR forms and their comparison with those of the wild-type provides additional information concerning factors that contribute to tune the reduction potential of the flavin within the protein environment. Thus, our results suggest that some side-chains may modulate the reduction potential value of the flavin ring by creating defined environments that modulate the conformation of the FAD, which in turn seems to have an effect on the flavin redox properties, as shown for the K75 side-chain. Moreover, it has also been shown that other residues located close to the flavin ring influence not only its reduction potential, but also the mechanism of ET for the enzyme.

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