Ferredoxin-NADP⁺ reductase (FNR) catalyses the production of NADPH in photosynthetic organisms, where its FAD cofactor takes two electrons from two reduced ferredoxin (Fd) molecules in two sequential steps, and transfers them to NADP⁺ in a single hydride transfer (HT) step. Despite the good knowledge of this catalytic machinery, additional roles can still be envisaged for already reported key residues, and new features are added to residues not previously identified as having a particular role in the mechanism. Here, we analyse for the first time the role of Ser59 in *Anabaena* FNR, a residue suggested by recent theoretical simulations as putatively involved in competent binding of the coenzyme in the active site by cooperating with Ser80. We show that Ser59 indirectly modulates the geometry of the active site, the interaction with substrates and the electronic properties of the isalloxazine ring, and in consequence the electron transfer (ET) and HT processes. Additionally, we revise the role of Tyr79 and Ser80, previously investigated in homologous enzymes from plants. Our results probe that the active site of FNR is tuned by a H-bond network that involves the active site residues not previously identified, additional roles can still be envisaged for already reported key residues, and new features are added to residues not previously identified as having a particular role in the mechanism. Here, we analyse for the first time the role of Ser59 in *Anabaena* FNR, a residue suggested by recent theoretical simulations as putatively involved in competent binding of the coenzyme in the active site by cooperating with Ser80. We show that Ser59 indirectly modulates the geometry of the active site, the interaction with substrates and the electronic properties of the isalloxazine ring, and in consequence the electron transfer (ET) and HT processes. Additionally, we revise the role of Tyr79 and Ser80, previously investigated in homologous enzymes from plants. Our results probe that the active site of FNR is tuned by a H-bond network that involves the side-chains of these residues and that results to critical optimal substrate binding, exchange of electrons and, particularly, competent disposition of the C4n (hydride acceptor/donor) of the nicotinamide moiety of the coenzyme during the reversible HT event.

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isoalloxazine and nicotinamide rings, thus contributing to the optimal geometry among the N5i, the C4n and the hydrogen that have to be transferred between them [5,12,14–18]. As a second highly conserved aromatic side-chain, Tyr79, stacks at the isoalloxazine side-face with its hydroxyl H-bonding the 4′-ribityl hydroxyl of FAD, which is also connected through a complex H-bond network assisted by water molecules to the C2 of the isoalloxazine and to the side-chain Arg100 [8,19–22]. Other key highly conserved residues at the active site are the neighbours of Tyr303 at the re-face: Ser80, Cys261 and Glu301 [3,8,11,15,23–26]. They contribute to the fine modulation of the FAD midpoint reduction potential, the affinity for Fd, the architecture of the catalytically competent complex, and/or the ET and HT rates [5,7,10,11,26–28]. Despite the fact that structural changes detected upon spinach FNR reduction are minor, they implicate a slight approach of the hydroxyl of Ser96 (Ser80 in AnFNR) to N5i that loses its H-bond with Glu312 (Glu301 in AnFNR) leading to the displacement of Tyr314 (Tyr303 in AnFNR) away from the flavin ring (decreasing the π–π stacking with the reduced isoalloxazine), as well as the displacement of two highly conserved water molecules, W406 and W571, (W404 and W457 in AnFNR) located near the flavin ring.

**Fig. 1.** Key residues at the AnFNR active site. (A) Surface representation of the active site environment at the equilibrium of a MD simulation of a theoretical catalytically competent WT FNR, NADP+ complex [15]. NADP+, FAD, and selected key side-chains are shown in sticks with C in blue, orange and wheat, respectively. Selected water molecules at the active site are also shown as balls and sticks. (B) Sequence alignment of different members of the FNR superfamily (ClustalW2). Position of residues equivalent to those mutated in this work is shown in bold.
2. Materials and methods

2.1. Biological material

pET28a-AnFNR plasmids containing the S59A, S80A or Y79F mutations were constructed using Mutagenex® and used to produce and purify the corresponding proteins from Escherichia coli cultures as previously reported [30]. Samples were prepared in 50 mM Tris/HCl, pH 8.0. S80A FNR was further purified using a HiPrep 26/60 Sephacryl™ S-200 HR column (GE Healthcare). FNR_{h}q\_aq variants were obtained by anaerobic photoreduction of the samples in the presence of 2 μM 5-deazariboflavin (dRF) and 3 mM EDTA in 50 mM Tris/HCl, pH 8.0, by irradiation from a 250 W light source [15]. Deuterated FNR_{h}q\_aq (D-FNR_{h}q\_aq) variants were produced by photoreduction with EDTA and dRF of the corresponding FNR_{ox\_aq} previously dialysed in 50 mM Tris/DCl, pH 8.0. In D_{2}O. NADP\_d (4K-form, with the deuterium in the A face of the nicotinamide) was produced and purified as described [14,32]. Anabaena Fd (AnFd) was produced as previously described [12].

2.2. Spectroscopic assays

UV/vis spectra were recorded in a Cary-100 spectrophotometer. The molar absorption coefficient for each FNR variant was spectrophotometrically determined by thermal denaturation of the protein for 10 min at 90 °C, followed by centrifugation and separation of the precipitated apoprotein, and spectroscopic quantification of the FAD released to the supernatant [33]. Interaction parameters with NADPH, NAD\_d and Fd were determined by difference absorption spectroscopy at 25 °C in 50 mM Tris/HCl, pH 8.0, as previously described [7,34]. Titration curves were carried out by adding aliquots of 1 mM NADH or Fd, and 50 mM NAD\_d to 20–80 μM FNR solutions. Errors in the determination of K_d and ΔC were ±10% and ±5%, respectively.

2.3. Determination of midpoint reduction potentials of the FNR variants

Midpoint reduction potentials for the ox/hq couple (E_{ox/hq}, two-electron reduction process) of WT, S59A, Y79F, and S80A FNRs were determined at 25 °C by potentiometric titration under anaerobic conditions using a gold electrode and a calomel electrode as reference (E_{ref} = +244.4 mV at 25 °C). Due to the low degree of FNR semiquinone stabilisation it was not possible to measure the potential for the two-electron steps. Typically, the solution contained 20 μM FNR, 50 mM Tris/HCl buffer, pH 8.0, 3 mM EDTA and 2 μM dRF. 0.02% n-dodecyl-β-D-maltoside was also added to S80A FNR to increase its stability. MethylvioIogen (E_{m} = −446 mV) and benzylviologen (E_{m} = −359 mV) were additionally used as mediators. Solutions were made anaerobic over a 2–4 h period. Stepwise reduction of the protein was achieved by photoreduction using the equipment previously described [35]. The system was considered equilibrated when the potential (E), measured with a Fluke true-RMS multimeter, remained stable for at least 10 min. The UV/vis absorbance spectrum was then recorded and used to determine [FNR_{ox} and [FNR_{hq}] at the equilibrium after each reduction step. E_{ox/q\_aq} was calculated by linear regression analysis according to the Nernst equation. The values of each one-electron single step E_{ox/q\_aq} and E_{sq/hq} were derived from Eqs. (1) and (2) using the experimentally determined E_{ox/q\_aq} and the molar faction of the maximum percentage of SQ stabilised.

\[
E_{ox/q\_aq} - E_{sq/hq} = 0.11 \times \log \left( \frac{2[Q]}{[S]} \right) + \frac{2E_{ox/sq} + E_{sq/hq}}{2}.
\]

Error in the determined E_{ox/q\_aq} E_{ox/q\_aq} and E_{sq/hq} was estimated to be ±5 mV.

2.4. Steady-state kinetics measurements

The diaphorase activity of FNR was determined in a double beam Cary-100 spectrophotometer using either 2,6-dichlorophenolindophenol (DCPIP) (ΔE_{602 nm} 21 μM\_\text{cm}^{-1}\_\text{cm}^{-1}) or K_3Fe(CN)_{6} (ΔE_{620 nm} 1.05 μM\_\text{cm}^{-1}\_\text{cm}^{-1}) as two- or one-electron acceptors, respectively. The final reaction mixture contained 4 mM FNR, 0.1 mM DCPIP or 1.5 mM K_3Fe(CN)_{6}, and NADPH in the range 0–200 μM, while the reference cuvette contained 0.06 mM DCPIP when using this acceptor. Higher concentrations of FNR (1 μM) and/or nucleotide (0–5 mM) were required for the analysis of the reactions with NADH. The NADPH-dependent cytochrome c reductase activity was determined using AnFd, and horse heart cytochrome c (Cytc) as final electron acceptor. Reaction mixtures contained 4 mM FNR, 200 μM NADPH, 0.75 mg/ml Cytc and 0–15 μM AnFd. All measurements were carried out in 50 mM Tris/HCl, pH 8.0, at 25 °C. K_m and k_cat values were obtained by fitting the dependence of the observed initial rates on coenzyme concentration to the Michaelis–Menten equation. Estimated errors in K_m and k_cat were ±20% and ±10%, respectively.

2.5. Laser-flash induced kinetics

Laser-flash experiments were performed anaerobically at 25 °C in a 1 cm path-length cuvette using EDTA as electron donor and dRF as photosensitizer as previously described [7,36]. The standard reaction mixture contained, in a final volume of 1.5 mL, 4 mM sodium phosphate, 200...
transient charge transfer complex (CTC) formation and HT process-243
ings and competitive inhibition (inhibition constant $K_i$), respec-276
sponding to the redox couple (Table S3). The concentration of NADP+/H-265
$K_{\text{i}}$), respectively.

whereas data for the S80A FNR:NADP+ complex were collected on the-318

2.5. Crystal growth, data collection and structure refinement

2.7. Crystal growth, data collection and structure refinement

2.6. Stopped-flow pre-steady-state kinetic measurements

2.5. Crystal growth, data collection and structure refinement

2.4. Fluorescence kinetics

2.3. Reduced ferredoxin-NADP+ reductase

2.2. Protein chemistry

2.1. Materials and methods

1. Introduction

1.1. Background

1.1. Background

1.1. Background

1.1. Background

1.1. Background
assess final structures. S59A, Y79F and S80A FNRs diffracted up to 1.92, 2.0 and 1.9 Å, respectively, and belonged to the P6$_3$ space group. Their V$_{\text{m}}$ were 2.85, 2.86 and 2.68 Å$^3$/Da with one FNR molecule in their asymmetric units and 56.8, 57.0 and 53.7% solvent contents, respectively. Each model comprised residues 9-303 (S59A and Y79F FNRs) or 10-303 (S80A FNR), one FAD molecule, one SO$_4^{2-}$ ion and water molecules. The S80A FNR:NAD$^+$ complex was solved at 2.3 Å and crystals belonged to the I4 tetragonal space group. The model included residues 9-303, one FAD, one NAD$^+$ and waters. Data for collection and refinement processes can be found in Table S1. Coordinates and structure factors were deposited in the Protein Data Bank with accession codes 3ZBT for S59A FNR, 4BPR for Y79F FNR, 32BU for S80A FNR and 3ZC3 for S80A FNR:NAD$^+$.

3. Results

3.1. Interaction with partners, and oxido-reduction properties of the FNR variants

Purification of S59A, Y79F and S80A AnfNR variants produced protein yields and spectral properties (including UV-vis spectral shape, maxima position and $\Delta \varepsilon_{274 \text{ nm}}/\varepsilon_{458 \text{ nm}}$ ratio) similar to the WT, indicating that mutations prevented neither the assembly of FAD nor the protein folding. Nevertheless, S80A UV-vis spectral maxima slightly shifted to longer wavelengths (to 276, 397 and 466 nm), and its extinction coefficient in the flavin band-II was larger than that of WT (Fig. 2A). This suggests that Ser80 directly influences the electronic environment of the FAD isoalloxazine. Titration of the FNR ox variants with NAD$^+$ induced the typical difference spectra of cyano bacterial FNRs (Fig. 2B) indicative of coenzyme binding, lacking the positive band at 509 nm detected in enzymes from plants and related with direct stacking between the NNN and the isoalloxazine [5,30,34,47]. When Ala substituted for Ser80 changes in the position and intensities of spectral features were observed with respect to WT FNR. This again suggests modification of the isoalloxazine environment (Fig. 2B) [34,48]. Saturation of the difference spectra upon increasing NAD$^+$ concentration allowed determination of $K_\text{d}^{\text{NAD}^+}$ and $\Delta \varepsilon$ (see Supplementary material, Figure S1). The affinity of S59A and Y79F FNR$_\text{ox}$ for NAD$^+$ was within a factor of two of that of WT, but decreased up to 4-fold for the S80A variant (Table 1). Altogether these observations suggest that removal of the Ser80 side-chain modifies the nicotinamide disposition into the active site. Titration of the different FNR variants with NAD$^+$ did not induce the appearance of difference spectra. This indicated that the presence of NAD$^+$ does not have any effect in the environment of the isoalloxazine ring and suggested that binding of the coenzyme is not produced, similar to that described for the WT [30,34].

Difference spectra obtained upon titration of S59A and Y79F FNR$_\text{ox}$ with Fdox produced perturbations in the visible region very similar to those reported for the WT FNR$_\text{ox}$ [7], while the S80A variant showed a slight displacement of the maxima to shorter wavelengths (not shown). All the variants showed interaction parameters in the same range as for WT FNR$_\text{ox}$ with Fdox, the only exception was S59A, whose affinity for Fdox increased by 3-fold (Table 1).

Photoreduction of S59A and Y79F FNRs took place following similar spectral evolution and maximal percentage of semiquinone stabilisation (ranging 16–19%) as for the WT (Fig. 2C), but reduction of the S80A variant occurred with very little semiquinone stabilisation (<4%). Due to the low degree of FNR$_\text{sq}$ stabilisation it was not possible to independently measure the potential for the two one-electron steps; therefore, midpoint reduction potentials for the two electron processes ($E_{\text{ox/sq}}$) were determined for all of them. $E_{\text{ox/sq}}$ for the mutants yielded values only slightly less negative (8–12 mV) than for WT AnfNR (Fig. 2D, Table 2). This was clearly the consequence of less negative $E_{\text{sq/br}}$ values for the S59A and Y79F variants. The very little semiquinone stabilisation of S80A FNR also prevented estimation of $E_{\text{sq/br}}$ and $E_{\text{sq/hq}}$.
Table 1

Interaction parameters for complex formation of AnFNRox variants with NADP⁺ and AnFdox as determined by difference spectroscopy in 50 mM Tris/HCl, pH 8.0, at 25 °C.

<table>
<thead>
<tr>
<th>FNR variant</th>
<th>(K_d) (µM)</th>
<th>(\Delta A_{416-350}) (mM⁻¹ cm⁻¹)</th>
<th>(K_{off}) (µM)</th>
<th>(\Delta A_{416-400}) (mM⁻¹ cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4.0 1.15 6.7 1.95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S59A</td>
<td>8.5 1.28 6.7 2.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y79F</td>
<td>13.8 1.79 6.4 3.22</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2. Steady-state efficiency of the FNR variants

Kinetic parameters for the diaphorase activity of Y79F AnFNR reflected a very similar behaviour to the WT (Table 3). Replacement of Ser59 with Ala significantly increased the enzyme turnover \(k_{cat}\) with both one- and two-electron acceptors, but the effect in the catalytic efficiency only resulted relevant when using the two-electron acceptor, due to the increase in \(K_{cat}\) as the electron acceptor.

On the contrary, when Ser60 was substituted by Ala the catalytic efficiency decreased by 2- to 4-fold, due to a decrease in the enzyme turnover. None of the mutations improved the AnFNR ability to catalyse the FdIP diaphorase activity using NADH as electron donor. This activity was not detected at all in the S80A variant, making it even more specific towards the phosphorylated coenzyme.

3.3. Pre-steady-state kinetic analysis of the reduction of FNRox by Fdred

3.4. Transient kinetics of the hydride transfer reactions between FNR and the coenzyme. Kinetic isotopic effect (KIE) and dependence of the temperature
Steady-state kinetic parameters of the different AnFNR variants for the diaphorase (with either DCPiP or ferricyanide as electron acceptors) and cytochrome c reductase activities in 50 mM Tris/HCl, pH 8.0 at 25 °C.

<table>
<thead>
<tr>
<th>FNR variant</th>
<th>NADPH</th>
<th>DCPiP diaphorase</th>
<th>Fe(CN)₆³⁻ diaphorase</th>
<th>Cytochrome c reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kₐₙₐᵣ (µM)</td>
<td>kₑₜ (µM)</td>
<td>ΔEₐ (kcal/moL)</td>
<td>kₑₜ/ΔEₐ (µM⁻¹ s⁻¹)</td>
</tr>
<tr>
<td>WT</td>
<td>60</td>
<td>81.5</td>
<td>13.6</td>
<td>0.16</td>
</tr>
<tr>
<td>S59A</td>
<td>5.3</td>
<td>146.0</td>
<td>27.5</td>
<td>0.56</td>
</tr>
<tr>
<td>Y79F</td>
<td>3.8</td>
<td>73.0</td>
<td>19.2</td>
<td>0.21</td>
</tr>
<tr>
<td>S80A</td>
<td>4.6</td>
<td>30.4</td>
<td>6.6</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* Activity was not detected.

For this variant with respect to WT (Table 5), Replacements at Tyr79 and Ser80 produced more evident effects (Fig. 4, Table 5). A decrease in the amplitude of the spectral band for CTC-2 was observed upon reduction of Y79F FNRox by NADPH, while HT was slightly hampered in both directions (Fig. 4B and E, Table 5). More drastic effects were observed when Ala substituted for Ser80. Reduction of S80A FNRox by NADPH occurred without spectral CTC-2 stabilisation (Fig. 4C), and no spectral features of CTC at all were detected for the reverse reaction (Fig. 4F). Additionally, both reactions showed lower than 5% of the WT efficiency in HT (Table 5).

Due to the reversibility of the process, the production of the apparent decrease in the experimentally measured rate constants upon increasing coenzyme concentration and to the k₄ and k⁻¹ rates being in close agreement to the instrumental detection limit for some variants, equivolumetric concentrations of enzyme and coenzyme, as well as the use of the single-wavelength detector, were selected to further investigate this mechanism by analysing KIEs on the HT processes. The main observable difference between HT and DT processes was the considerable decrease in k₄ and k⁻¹ values with respect to the corresponding k₄ and k⁻¹ ones for all the FNR variants (Fig. 5A and B, Table 6), therefore, inducing moderate to important KIEs. For each particular variant the KIE was slightly larger for the reduction of FNRox by the coenzyme than for the reverse reaction. KIEs for the S59A and Y79F variants were in the same range as for WT, but processes with S80A FNRox showed considerably larger KIEs (up to 6-fold for the FNR reductive process).

S59A, Y79F and S80A FNRox showed overall crystal structures similar to that of WT (r.m.s.d. = 0.26 on Cα atoms aligned for all of them). Mutations did not lead to significant modification of the FAD isooxalazine environment and were constricted to the interactions involving the modified side-chains (Fig. 6A). The larger differences resulted in the loop 105–111, flexible in all AnFNR structures so far described and suggested to accommodate the adenosine moiety of the FAD [21,22,53]. Y79F FNR showed the highest B factors for the residues contained in this loop; from 103 to 114 the B factor is higher than 40 Å² (being its overall B factor of 21.54 Å²). This might be due to the fact that the cofactor is not engaged through its ribityl motif with this residue and no water molecule is mimicking the removed hydroxyl group. Ala substitution for Ser59 provoked removal of two defined water molecules that interact with the OG atom of Ser59 and with the OEt atom of Glu301, respectively, in WT FNR. Besides this, the FAD environment, especially the H-bond network involving Ser80 and Glu301, is not much affected by the mutation. Changes in the S80A FNR crystal structure are restricted to the H-bonds involving the removed hydroxyl group. Ala80 cannot form the side-chain of Glu301, being only in contact with N5i through a H-bond with its main chain N. Again, no water molecule mimics the H-bond network established by the Ser80 side-chain. The highly conserved water molecule, proposed to act as a proton donor to the N5i of FAD, is found in the S80A FNR structure, as in that of WT, interacting with the hydroxyl of Tyr303, but it is at closer distance with the O4 atom of FAD (2.9 Å) than in the WT structure (3.17 Å).

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Fig. 3. Laser-flash induced transient kinetic analysis for the reduction of the AnFNRox variants by AnFd: (A) Dependence of $k_{\text{obs}}$ on FNR concentration for the reduction by Fd of WT (○) FNRox at $I = 20$ mM, and of WT (●), Y79F (■) and S59A (♦) FNRox at $I = 120$ mM, and of S59A (●) FNRox at $I = 40$ mM. Reaction mixtures contained 40 μM Fd. (B) Dependence of $k_{\text{obs}}$ on the square root of $I$ for the reduction of WT (●), Y79F (■) and S59A (♦) FNRox by Fd. Reaction mixtures contained 40 μM Fd and 30 μM FNRox.

Fig. 4. Spectral evolution along the HT processes between AnFNR and the coenzyme followed by stopped-flow. Reduction by NADPH of (A) S59A FNRox (spectra recorded at 1.28, 3.84, 6.4, 8.96 and 49.92 ms after mixing), (B) Y79F FNRox (spectra recorded at 1.28, 3.84, 8.96, 24.32 and 49.92 ms), and (C) S80A FNRox (spectra recorded at 3.84, 29.4, 80.6, 157.4 and 997.1 ms). Reoxidation by NADP$^+$ of photoreduced (D) S59A FNRox (spectra recorded at 1.28, 3.84, 6.4, 8.96 and 49.92 ms), (E) Y79F FNRox (spectra recorded at 1.28, 3.84, 8.96, 19.2 and 49.92 ms) and (F) S80A FNRox (spectra recorded at 3.84, 29.4, 80.6, 157.4 and 997.1 ms). In all cases the thick line is the spectrum of the oxidised (for A, B and C) or reduced (D, E and F) protein before mixing. Insets show the time evolution of the absorption at 458 nm. Reactions were carried out with FNR at ~25 μM and coenzyme ~100 μM final concentrations in 50 mM Tris/HCl pH 8.0 at 6 °C.
Here we show that replacement of Ser59 with Ala improved the catalytic efficiency of the diaphorase activity of AnFNR (particularly the $k_{cat}$) with respect to the WT, as consequence of increasing the $k_{cat}/K_{m}$.

**4. Discussion**

(Fig. 6A). Another common characteristic in these structures, and in contrast with the WT, is the orientation of Arg264 towards the C-terminal. Its guanidinium H-bonding the C-terminal carboxylate of Tyr303 (Fig. 6A). This interaction was already predicted by MD simulations [15] and does not appear related with the introduced mutations, further suggesting a contribution for Arg264 in the displacement of Tyr303 to trigger the entrance of the nicotinamide into the catalytic site.

Finally, in the S80A FNR:NADP$^+$ complex, the N atom of Ala80 H-bonds N5i and O4i. In this structure, NADP$^+$ binds in a similar unproductive conformation to that previously reported for WT [22], although the isalloxazine-Tyr303 rings stacking distance gets slightly larger (around 0.4 Å) and the nicotinamide lies slightly closer to Tyr303, bridging its N7n atom and the OH of Tyr303.

**Table 5**

<table>
<thead>
<tr>
<th>FNR variant</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}$/K</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>300</td>
<td>285</td>
<td></td>
</tr>
<tr>
<td>S59A</td>
<td>390</td>
<td>253</td>
<td></td>
</tr>
<tr>
<td>Y79F</td>
<td>122.6</td>
<td>186</td>
<td></td>
</tr>
<tr>
<td>S80A</td>
<td>13.2</td>
<td>10.1</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Data from [14].

Fig. 5. Temperature dependence of the HT rates and KIEs. Arrhenius plots ($\ln k_{obs} = \ln A - E_a / RT$) of the kinetic data for the reactions of (A) FNRox with NADPH (closed symbols) or NADP$^+$ (open symbols) and of (B) FNRox or D-FNRox (open symbols) with NADP$^+$ (closed symbols). Temperature dependence of the KIEs for (C) FNRox reduction and (D) FNRox reoxidation by the coenzyme. Data correspond to WT (■), S59A (♦), Y79F (○), and S80A (▲) AnFNRs.

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well as the stabilisation of CTCs during HT, being major changes reduced to a decrease in $k_{H\text{I}}$ and $k_{H\text{II}}$ within 2-fold regarding the WT (Tables 1, 3, 5 and 6). Regarding Fd, the mutation increases the affinity of the Fd$_{\text{ox}}$, FNR$_{\text{mut}}$ complex and the ET rate between them (Table 4), while the reverse ET appears considerably hindered (Table 3). These effects with Fd might relate with the slightly less negative midpoint reduction potential of the variant, particularly $E_{\text{m}}(\text{FAD}_{\text{mut}})$ that will favour its reduction regarding the WT (Table 2). Therefore, altogether the observed effects suggest that the H-bond between the hydroxyl group of Tyr79 and the ribityl portion of the cofactor, despite not being critical for ET and HT, indirectly contributes to the reactivity in productive complexes. These data agree with previous mutations in the Lys75-Leu78 peptide, where side-chains, despite not being in direct contact with the isoalloxazine ring, have been shown to slightly displace its midpoint reduction potentials to less negative values [57,58]. Thus, Tyr79 can be included among the side-chains tuning the flavin midpoint potential by creating a defined environment that modulates the FAD conformation.

The last residue here analysed, Ser80, is a key one in the active site of plastidic type FNRs where together with Cys261 and Glu301, it constitutes a highly conserved catalytic triad (Fig. 1A) [22,27,28]. Its main-chain directly H-bonds the isoalloxazine N5i atom as well as the O4i atom via a conserved water molecule, while its hydroxyl interacts with another conserved water molecule that H-bonds Glu301, Tyr303, and N5i (Fig. 6A) [21,59]. Mutations at the equivalent position in spinach leaves FNR, Ser96, [11] impaired catalysis for the S96V and S96G variants with respect to the WT, while it was not possible to produce the S96A variant. We have succeeded in producing the S80A mutant in AnFNR. This replacement slightly modifies the electronic environment of the FAD isoalloxazine ring regarding the WT, and has a deleterious effect in its semiquinone stabilisation (Fig. 2), suggesting that this later process is finely controlled by the H-bond network involving Ser80, Glu301, Tyr303 and N5i. This observation is also consistent with the mutant lacking the ability to accept a single electron from $F_{\text{dox}}$ in the Fd-mediated laser-flash induced ET experiments as well as to donate a single electron to $F_{\text{dox}}$ in the Cyt reductase assay, given the formation of the FNR$_{\text{mut}}$ intermediate is necessary for both reactions. Noticeably, the S80A mutant shows a similar decrease in efficiency, regarding de WT, in the diaphorase assay when using either one-electron or two-electron acceptors. This suggests that the nature of the small, non-specific and non-physiological collisional $K_{\text{Fe(CN)}_6}$ one-electron acceptor allows it to extract a single electron from S80A FNR$_{\text{mut}}$ through the reduce amount of semiquinone that this mutant stabilizes (Table 2). On the contrary, processes with Fd include complex formation and dissociation rate limiting steps that modify FNR midpoint reduction potentials [13]. The results here present suggest that in the case of S80A FNR interaction with Fd further decreases the low stability of its semiquinone. Regarding the coenzyme the mutation produced minor effects in its affinity. Despite the fact that its $E_{\text{m}}$ slightly less negative than in WT (Table 2), would apparently favour HT events from the coenzyme, we observed important deleterious effects in turnover, catalytic efficiency, ET rate constants, and the stabilisation of CTCs during the HT event for both the forward and reverse HT reactions (Fig. 4, 6A, Tables 3 and 5). All these data are in agreement with those reported for the Ser96 mutants in spinach FNR [11], further indicating that this Ser is critical to generate the architecture of the catalytically competent complex upon coenzyme binding in both the cyano- and plastidic enzymes. Since the structures obtained for mutants at this Ser (both in AnFNR (Ser80) and the spinach enzyme (S96)) only show changes in the H-bond network involving this position (Fig. 6A), this network must be critical for the efficiency of the HT process. In agreement with this conclusion previous MD simulations suggested that the Ser80 side-chain might contribute to fix the position of the amide of NADPH and, as consequence, the position of the nicotinamide ring in the active site cavity [15]. The contribution of the Ser80 side-chain to the optimal architecture of the catalytically competent complex between FNR and the coenzyme is here further supported by the analysis of the active site dynamics during the HT event (Fig. 5 and Table 6). HT and DT reactions for WT FNR, in both the forward and reverse directions, have been explained applying a tunnelling model in which both environmental reorganisation (passive dynamics) and vibrational enhancement (active dynamics) contribute to the reaction [14,16]. However, KIE analyses for the processes of the S80A FNR mutant best fitted to the two extreme cases in which the tunnelling reaction is completely dominated by either environmental reorganisation or vibrational enhancement [60]. Thus, reduction of S80A FNR$_{\text{mut}}$ by NADPH/D was consistent with a full tunnelling model in which vibrational fluctuations of the active site (gating) are able to compress the hydrogen donor–acceptor distance during the HT event, making tunnelling more probable (specially for the light isotope) as long as the temperature increases. On the contrary, parameters measured for the oxidation of S80A FNR$_{\text{mut}}$ by NADP$^+$ suggest no gating contribution to the HT event, being the initial environmental thermal reorganisation of the whole active site the only dynamics contribution controlling the process. Therefore, modifications on the H-bond network caused by substitution of Ser80 with alanine entail important perturbations on the flexibility of the active site FNR along the HT reaction coordinate. In the case of the reaction of S80A FNR$_{\text{mut}}$ with NADPH, the organisation of the close environment of the donor (C4n) and acceptor (N5i) atoms allows them to undergo...
important vibrational fluctuations to attain the optimal distance and orientation for efficient HT. On the contrary, for the reverse reaction, once the NADP$^{+}$ nicotinamide reaches the active site of S80A FNRhq the reduced isoalloxazine and oxidised nicotinamide rings remain "frozen", without any further flexibility of the active site contributing to improve its relative distance and orientation to reach the hydrogen tunnelling ready conformation. Therefore, the S80A mutation highly compromises the active site environment fluctuations required in FNR to increase the HT probability, producing important thermodynamic and kinetic consequences in the process that are reflected in the enzyme efficiency, mechanism and reversibility of the process, all of them particular facts of plant type FNRs. Altogether these data confirm the importance of Ser80 in both the ET and HT processes, adding information about its roles. Thus, this Ser side-chain modulates the midpoint reduction potential of the flavin ring and contributes to the stabilisation of its semiquinone state, a key factor for efficient ET exchange between FNR and Fd. Additionally, it also contributes to stabilise the nicotinamide ring in the optimal

![Fig. 6. Influence of the mutations in the active site geometry of AnFNR.](image)

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geometric conformation regarding the isalloxazine ring for an efficient HT event through a tunnelling process following similar dynamics for both the photosynthetic and non-photosynthetic reactions, thus ensuring the efficiency and reversibility of the process.

In conclusion, as new information becomes available additional roles might be envisaged for residues in the active site of FNR. Among these residues are Tyr79 and Ser80, previously analysed in FNRs from higher plants [8,11,26]. Additionally, other residues not considered previously, such as Ser59, might indirectly contribute to the efficiency of the HT with the coenzyme by modulating the architecture of the reactive complexes [15]. Here, we present proofs for the implication of H-bond connections between the flavin and the polypeptide chain, direct or via water molecules, in the FNR catalytic efficiency, showing that the isalloxazine environment strongly influences FADαH stabilization and ET from Fd. Moreover, an additional key role is predicted for Ser80 during the HT step providing optimal active site geometry, including not only the final disposition between the reacting NSH and C4n atoms but also the active site motions required to achieve it.

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Appendix A. Supplementary data

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References
