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A hydrogen bond network in the active site of *Anabaena* ferredoxin-NADP$^+$ reductase modulates its catalytic efficiency

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ABSTRACT

Ferredoxin-nicotinamide–adenine dinucleotide phosphate (NADP$^+$) reductase (FNR) catalyses the production of reduced nicotinamide–adenine dinucleotide phosphate (NADPH) in photosynthetic organisms, where its flavin adenine dinucleotide (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 isoalloxazine 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.

1. Introduction

In the photosynthetic electron transfer (ET) chain of plants, algae and cyanobacteria, the isoalloxazine ring of the flavin adenine dinucleotide (FAD) cofactor of ferredoxin-NADP$^+$ reductase (FNR) gets reduced to its hydroquinone state by sequentially accepting two electrons from two ferredoxin (Fd) molecules. Subsequently, it transfers a hydride from the N5 atom (N5Hi) of the isoalloxazine of its FAD cofactor to the nicotinamide C4 atom of nicotine adenine diculeotide (NADP$^+$) (C4n) to provide the cell with reduction power in the form of NADPH [1–3]. The overall ET process from Fd to NADP$^+$ is reversible, with transitory ternary complexes, Fd:FNR:NADP$^+$, formed during catalysis [4]. Structural, mutational and theoretical studies revealed residues on the protein surface and in the isoalloxazine environment involved in the interaction and ET with the protein partner, contributing to the optimal architecture of the active site for proton and electron transfer, as well as playing key roles in the catalytic binding of the nicotinamide moiety of the coenzyme (NMN) during the hydride transfer (HT) event [5–13]. Among them, a particular role is proposed for the C-terminal Tyr (Tyr303 in *Anabaena* FNR (*An*FNR), numbering used herein) (Fig. 1A). This residue stacks at the *re*-face of the isoalloxazine ring of FAD, modulates its midpoint reduction potential and reduces

Abbreviations: NADP$^+$, NADPH, nicotinamide–adenine dinucleotide phosphate in its oxidised and reduced forms; FAD, flavin adenine dinucleotide; FNR, FNR_{ox}, FNR_{hq}, FNR_{sq}, ferredoxin-NADP$^+$ reductase and FNR in the fully oxidised, anionic hydroquinone (fully reduced) and neutral semiquinone (one-electron reduced) states, respectively; Fd, Fd_{rd}, ferredoxin and in its reduced state; 2′-P, 2′-phosphate group of NADP$^+$/H; dRf, 5-deazariboflavin; ET, electron transfer; HT, hydride transfer; DT, deuteride transfer; WT, wild-type; CTC, charge-transfer complex; CTC-1, FNR_{ox}-NADPH CTC; CTC-2, FNR_{hq}-NADP$^+$ CTC; NMN, nicotinamide nucleotide moiety of NADP$^+$/H; 2′-P-AMP, 2′-P-AMP moiety of NADP$^+$/H; PP_i, pyrophosphate; N5Hi, N5i, N5 hydride donor/acceptor of the FADH$^-$/FAD isoalloxazine ring of FNR; C4n, C4Hn, C4 hydride acceptor/donor of the NADP$^+$/H nicotinamide ring; $k_{A\rightarrow B}$, $k_{B\rightarrow C}$, apparent/observed rate constants obtained by global analysis of spectral kinetic data; k_{obsHT}, $k_{obsHT\text{-}1}$, k_{obsDT}, $k_{obsDT\text{-}1}$, observed conversion HT and DT rate constants for the forward and reverse reactions; k_{HT}, $k_{HT\text{-}1}$, hydride transfer first-order rate constants for the forward and reverse reactions, respectively; k_{DT}, $k_{DT\text{-}1}$, deuteride transfer first-order rate constants for the forward and reverse reactions, respectively; K_d^{NADPH}, $K_d^{NADP^+}$, dissociation constants for the intermediate complexes in the reduction and reoxidation of FNR, respectively; KIE, kinetic isotopic effect; A_H, A_D, Arrhenius preexponential factors for hydrogen and deuteride, respectively; E_{aH}, E_{aD}, activation energies for hydride and deuteride transfer, respectively; k_{et}, first-order electron transfer rate; k_2, second-order rate constant for bimolecular electron transfer; *I*, ionic strength

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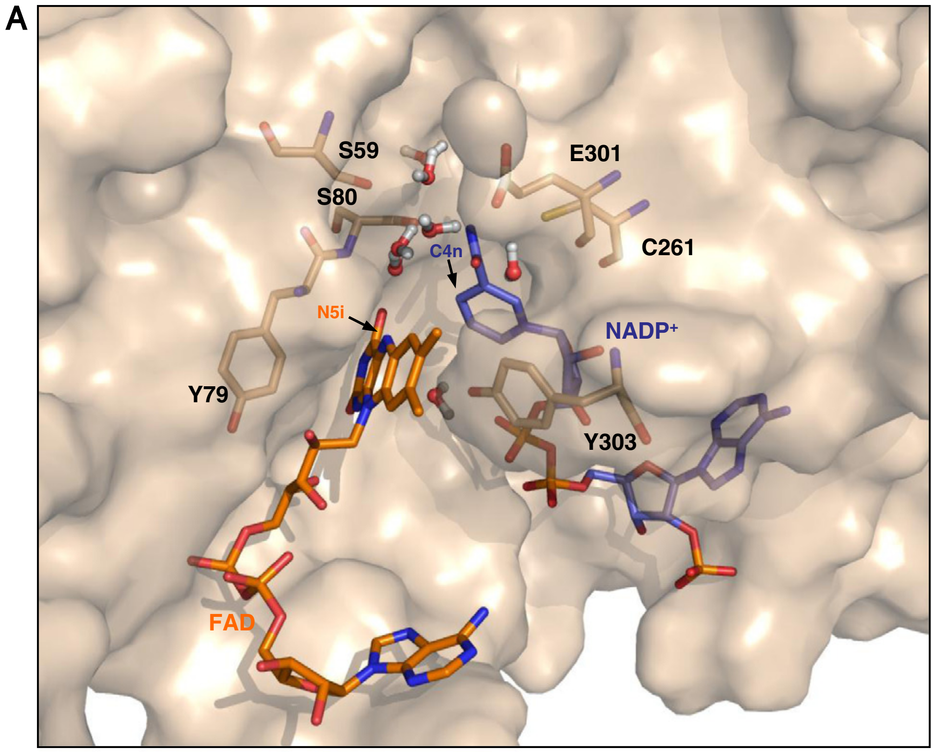
A



B

	60 70 80
Anabaena PCC7119 FNR	IEGQ**S**IGIIPPGVD--KNGK-----------------PEKLRL**YS**IASTR
Spirulina sp. FNR	LEGQ**S**IGIIPPGTD--NNGK-----------------PHKLRL**YS**IASTR
Synechococcus sp. FNR	LEGQ**S**IGIIPPGED--KNGK-----------------PHKLRL**YS**IASTR
Synechocystis sp. FNR	LEGQ**S**IGIIPPGED--DKGK-----------------PHKLRL**YS**IASTR
Arabidopsis thaliana leaf FNR	REGQ**S**IGVIPEGID--KNGK-----------------PHKLRL**YS**IASSA
Nicotiana tabacum leaf FNR	REGQ**S**IGVIADGVD--ANGK-----------------PHKLRL**YS**TASSA
Oryza sativa leaf FNR	REGQ**S**IGVIADGVD--KNGK-----------------PHKLRL**YS**IASSA
Pisum sativum leaf FNR	REGQ**S**IGIVPDGID--KNGK-----------------PHKLRL**YS**IASSA
Spinacea oleracea FNR	REGQ**S**VGVIPDGED--KNGK-----------------PHKLRL**YS**IASSA
Arabidopsis thaliana root FNR	WEGQ**S**YGVIPPGENPKKPGA-----------------PHNVRL**YS**IASTR
Nicotiana tabacum root FNR	WEGQ**S**YGVIPPGENPKKPGN-----------------PHNVRL**YL**IASTR
Oryza sativa root FNR	WEGQ**S**YGIIPPGENPKKPGA-----------------PHNVRL**YS**IASTR
Pisum sativum root FNR	WEGQ**S**YGVIPPGENPKKPGS-----------------PHNVRL**YS**IASTR
Leptospira interrogans FNR	VIGQ**S**GGVIPPGEDPEKKAKGLA------------DVGYTVRL**YS**IASPS
Azotobacter vinelandii FPR	ENGQ**F**VMIGLEVD-----------------------GRPLMRA**YS**IASPN
Escherichia coli FPR	TAGQ**F**TKLGLEID-----------------------GERVQRA**YS**YVNSP
Rhodobacter capsulatus FPR	RSGE**F**VMIGLLDDN----------------------GKPIMRA**YS**IASPA
Zea mays NR	PIGK**H**IFVCASIE-----------------------GKLCMRA**YT**PTSMV
Rattus norvegicus cb5R	PIGQ**H**IYLSTRID-----------------------GNLVIRP**YT**PVSSD
Sus scrofa cb5R	PVGQ**H**IYLSARID-----------------------GNLVIRP**YT**PVSSD
Bos taurus cb5R	PVGK**H**VYLSARID-----------------------GSLVIRP**YT**PVTSD
Plasmodium falciparum FNR	LEGH**T**CGIIPYYNELDNNPNNQINKDHNIINTTNHTNHNNIAL**SH**IKKQR

Fig. 1. Key residues at the *An*FNR active site. (A) Surface representation of the active site environment at the equilibrium of a MD simulation of a theoretical catalytically competent WT FNR_{hq}:$NADP^+$ 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.

the probability of a too strong stacking interaction between the isoalloxazine and nicotinamide rings, thus contributing to the optimal geometry among the N5i, the C4n and the hydrogen that has to be transferred between them [5,12,14–18]. A second highly conserved aromatic side-chain, Tyr79, stacks at the isoalloxazine *si*-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 *An*FNR) to N5i that loses its H-bond with Glu312 (Glu301 in *An*FNR) leading to the displacement of Tyr314 (Tyr303 in *An*FNR) 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 *An*FNR) located near the ribityl of FAD [29]. Theoretical calculations for the HT process