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ScienceDirect
Archives of Biochemistry and Biophysics 467 (2007) 206–217
ABB
www.elsevier.com/locate/yabbi

Tuning of the FMN binding and oxido-reduction properties by neighboring side chains in *Anabaena* flavodoxin

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Received 9 May 2007, and in revised form 13 August 2007
Available online 29 August 2007

Abstract

Contribution of three regions (phosphate-binding, 50's and 90's loops) of *Anabaena* apoflavodoxin to FMN binding and reduction potential was studied. Thr12 and Glu16 did not influence FMN redox properties, but Thr12 played a role in FMN binding. Replacement of Trp57 with Glu, Lys or Arg moderately shifted $E_{ox/sq}$ and $E_{sq/hq}$ and altered the energetic of the FMN redox states binding profile. Our data indicate that the side chain of position 57 does not modulate $E_{ox/sq}$ by aromatic stacking or solvent exclusion, but rather by influencing the relative strength of the H-bond between the N(5) of the flavin and the Asn58-Ile59 bond. A correlation was observed between the isoalloxazine increase in solvent accessibility and less negative $E_{sq/hq}$. Moreover, $E_{sq/hq}$ became less negative as positively charged residues were added near to the isoalloxazine. Ile59 and Ile92 were simultaneously mutated to Ala or Glu. These mutations impaired FMN binding, while shifting $E_{sq/hq}$ to less negative values and $E_{ox/sq}$ to more negative. These effects are discussed on the bases of the X-ray structures of some of the Fld mutants, suggesting that in *Anabaena* Fld the structural control of both electron transfer steps is much more subtle than in other Flds.

Keywords: Flavodoxin; FMN reduction potential; FMN binding; X-ray structures

Flavodoxins (Flds) are small α/β flavoproteins involved in numerous electron transfer (ET)[1] reactions in prokaryotic organisms and certain algae [1,2]. Non-covalently bound FMN confers Fld with its ability to act as a low potential electron carrier. Complex formation causes $E_{ox/sq}$ and $E_{sq/hq}$ of FMN to be inverted and to become well-separated [3,4].

Three main regions of apoflavodoxin (ApoFld) are involved in FMN binding: the phosphate-binding (P-binding) loop, the 50's loop and the 90's loop [5,6]. Although the phosphate group of FMN is charged, electrostatic interactions do not seem to play a major role, and instead

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[1] *Abbreviations used:* ox, sq or hq, oxidized, semiquinone or hydroquinone forms of the flavin ring; ET, electron transfer; $E_{ox/sq}$, midpoint reduction potential for the ox/sq couple; $E_{sq/hq}$, midpoint reduction potential for the sq/hq couple; WT, wild-type; P-binding, phosphate-binding; Fld, flavodoxin; ApoFld, apoflavodoxin; Fld_{ox}, Fld in the oxidized state; Fld_{hq}, Fld in the hydroquinone state; Fld_{sq}, Fld in the semiquinone state; K_d, dissociation constant; FNR, ferredoxin-$NADP^+$ reductase; PSI, Photosystem I; ΔG_{ox}, ΔG_{sq}, ΔG_{hq}, free energy of binding for ApoFld:FMN_{ox}, ApoFld:FMN_{sq} and ApoFld:FMN_{hq} complexes.

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doi:10.1016/j.abb.2007.08.024

the P-binding loop contributes to stabilize phosphate through H-bonds provided by side chains of several Thr (or Ser) and main chain NH groups of the consensus sequence T/S-X-T/S-G-X-T/S-X (10-T-Q-T-G-K-T-E-16 in *Anabaena* Fld) [5–8]. Residues in the 50's and 90's loops make close contacts with the isoalloxazine ring and modulate its reduction properties [9–15]. Despite the high sequence similarity between Flds, the structure around the flavin and the specific interactions of the flavin with the ApoFld vary in these loops, tuning different reduction potentials. In most Flds the 90's loop provides a Tyr stacked against the *si*/outer face of FMN (Tyr94 in *Anabaena*). The residue from the 50's loop that stacks at the *re*/inner face is commonly a Trp, as in *Anabaena* (Trp57) [7,16–20], but non-aromatic residues have also been found; Leu in *Azotobacter vinelandii* [21] and *Klebsiella pneumoniae* Flds [22], Met in *Clostridium beijerinckii* Fld [3], His in Fld MioC from *Escherichia coli* [23] and Ala in *Helicobacter pylori* Fld [20]. Other side chains at the 50's loop have also been suggested to play a role in $E_{ox/sq}$ modulation [24]. While the H-bond network observed in *Anabaena* Fld is essentially conserved in Fld structures from different species [25], the 56–60 segment varies considerably [3,19,26]. When Flds from *C. beijerinckii* and *Desulfovibrio vulgaris* are reduced to the sq, a rearrangement of the 58–59 peptide bond occurs (*Anabaena* numbering), allowing a main chain carbonyl O (contributed by a Gly) to flip from an "O-down" conformation to an "O-up" conformation. In this "O-up" conformation, formation of a H-bond between this main chain carbonyl O and the N(5)H from the neutral sq occurs [27,28]. In *Anacystis nidulans* Fld (high sequence and structure conservation with *Anabaena* Fld and position 58 also contributed by an Asn) this flip involves the breaking of a weak H-bond present in the ox state between the FMN N(5) and the NH group of Val59, in favor of the stronger H-bond between the carbonyl group of Asn58 ("O-up" conformation) and FMN N(5)H in the sq state. In *Anabaena* Fld, the NH group of Ile59 is 3.7 Å from N(5) but the orientation presents a favorable geometry that suggests such an H-bond also stabilizes *Anabaena* Fld_{ox} [7]. This loop geometry and N(5) interaction appears common to long-chain Flds from *A. nidulans, E. coli* and *Anabaena*, but has not been found in the long-chain Fld from *A. vinelandii*, nor in the short-chain Flds from *D. vulgaris* and *C. beijerinckii* [21]. The fact that the sq states of *A. nidulans* and *Anabaena* Flds are less stable than those of Flds from other species has been correlated to the weaker H-bond that is formed between the N(5)H of the flavin and the backbone CO of Asn compared to the bond formed with the smaller Gly and to the presence in the oxidized state of the N(5)–HN59 H-bond that is absent in other Flds [3,26]. Such backbone rearrangements appear to provide a versatile device for modulating the reduction potential, and a similar role for the Asn58-Ile59 peptide to that found in *A. nidulans* Fld can be predicted in *Anabaena* Fld [24].

Mutational and structural studies can be used to investigate the contributions of an amino acid side chain to modulate the reduction potentials and the binding affinities of the different redox states of FMN. Replacement of Asn58 by Lys in *Anabaena* Fld decreased the $E_{ox/sq}$ value, effect related to an increase in the conformational energy of the peptide Asn58-Ile59 in the sq state [24]. Ile59 and Ile92 are the only two hydrophobic residues exposed to solvent and situated in the isoalloxazine ring environment where interaction of Fld with its ET partners is expected. Single mutational studies at these positions in *Anabaena* Fld indicated that the nature of these side chains tunes $E_{ox/sq}$ and that these residues appear to play a role in complex formation and ET between photosystem I (PSI) and Fld [29]. The role of *Anabaena* Fld Trp57 in FMN binding and reduction potential tuning has also been analyzed by replacing it with non-charged residues, concluding that it plays a role in FMN binding rather than in redox properties modulation, and that it is involved in setting an appropriate environment to modulate *in vivo* ET from PSI to FNR [9,30].

In the present manuscript we examine the effect on FMN binding and reduction potentials when residues in the FMN binding site, that are thought to be involved in the interaction of Fld with other proteins, are modified [30–32]. The highly conserved Thr12 (occurring only as Thr or Ser) and Glu16 (usually Glu or Arg) at the terminal end of the P-binding loop have been mutated to Lys. The effect of introducing charged side chains close to the isoalloxazine moiety of FMN has also been tested by replacing Trp57 with Glu, Lys and Arg. Finally, Ile59 or Ile92 have been simultaneously substituted by Ala and Glu, to investigate the effect of replacing the only surface hydrophobic residues close to the FMN. The side chains of all these residues are exposed to the solvent and are thought to be involved not only in binding the FMN and modulation of reduction potentials, but also in the interaction of Fld with PSI and FNR [6,31,32].

Materials and methods

Biological materials

Mutations were introduced into the pTrc99a-cloned structural gene encoding the WT Fld from *Anabaena* PCC7119 by using the QuikChange mutagenesis kit (Stratagene) in combination with the following synthetic oligonucleotides (modified bases shown in bold):

5′-CTACGGTACTCAAAA**GG**GGTAAAACTGAATCAGTAGCAG-3′ for Thr12Lys,

5′-CTCAAACTGGTAAAACT**AAG**TCAGTAGCAGAAATCATTCG-3′ for Glu16Lys,

5′-GGCTGTCCTACT**CG**C**A**ATATTGGCGAACTGCAAAGCG-3′ for Trp57Arg,

5′-GGCTGTCCTACT**GA**GAATATTGGCGAACTGCAAAGCG-3′ for Trp57Glu,

5′-GGCTGTCCTACT**AA**GAATATTGGCGAACTGCAAAGCG-3′ for Trp57Lys,

5′-GCTTTGCAGTTCGCC**TTC**ATTCCAAGTAGGACAGCC-3′ for Ile59Glu,

5′-GAAAATTATCTGCGTAACCT**TC**TTGGTCACCAGTCCC-3′ for Ile92Glu,