Gene expression study of the flavodi-iron proteins from the cyanobacterium *Synechocystis* sp. PCC6803

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**Abstract**

The flavodi-iron proteins, also named FDPs, are an extensive family of enzymes able to reduce dioxygen to water and/or nitric oxide to nitrous oxide. These proteins are formed by a metallo-$\beta$-lactamase-like domain with a di-iron catalytic site fused to a flavodoxin-like module bearing an FMN. However, in cyanobacteria, which are oxygenic photosynthetic organisms widespread in Nature, FDPs have an extra NAD(P)H:flavin reductase-like domain as a C-terminal extension. Interestingly, cyanobacteria contain more than one gene encoding FDP-like proteins, with the genome of *Synechocystis* sp. PCC6803 containing four genes coding for putative FDPs. However, the function of those proteins remains unclear. In the present study, we have analysed the expression profile of these genes under oxidative and nitrosative stress conditions. The results indicate that one of the flavodi-iron genes, the so-called *flv1*, is induced in cells exposed to nitrosative stress. By conducting a broad analysis on the primary sequences of FDPs, we have identified that the FDPs of cyanobacteria and oxygenic photosynthetic eukaryotes may be divided into multiple types (1–12), according to the amino acid residues of the di-iron catalytic site.

**Introduction**

The FDPs (flavodi-iron proteins), previously named A-type flavoproteins, are a family of soluble and modular enzymes with a two-domain conserved core constituted by a metallo-$\beta$-lactamase-like domain, at the N-terminus, linked to a flavodoxin-like domain, at the C-terminus [1]. The metallo-$\beta$-lactamase module bears a di-iron catalytic centre, whereas the flavodoxin domain contains an FMN molecule [2–5]. However, these proteins may present extra modules, located at the C-terminal vicinity of the flavodi-iron core. According to their domain composition, FDPs are clustered into four distinct classes, from A to D. Class A is formed by the common core. FDPs of class B–D contain an extra C-terminal extension which is different for each class: a rubredoxin moiety for class B, a NAD(P)H:flavin oxidoreductase domain for class C and a combination of a rubredoxin domain fused to a NADH:rubredoxin oxidoreductase domain for class D [2–5].

The crystallographic studies on the FDPs reveal that these proteins are isolated as functional tetramers, dimers of dimers. Within each homodimer unit, the ‘head-to-tail’ arrangement of each monomer allows the electrons to flow from the flavodoxin domain of one of the monomers to the di-iron site of the other, as the di-iron site and the FMN are closely spaced in this arrangement [2,6].

An important feature of the FDPs is their ability to reduce dioxygen to water and/or nitric oxide to nitrous oxide [5,7–9]. However, until now, none of the studies conducted on FDPs allowed rationalization of the distinct affinity for each one of these substrates.

We have undertaken the study of class C FDPs from the cyanobacterium *Synechocystis* sp. PCC6803 (hereafter named *Synechocystis*). This organism contains four genes coding for putative FDPs (*flv1*, *flv2*, *flv3* and *flv4*) and the role of these proteins remains unknown.

Herein, we start by investigating the expression profile of the several FDPs from *Synechocystis* under oxidative and/or nitrosative stress conditions. Furthermore, a phylogenetic analysis of flavodi-iron proteins was also performed.

**Expression of *flv1* gene is induced by nitrosative stress**

Quantitative real-time RT (reverse transcription)–PCR experiments were utilized to access the transcription of the four *Synechocystis* flavodi-iron genes, *flv1–flv4*, in cells that, after reaching a $D_{730}$ of 1, were exposed to oxidative stress, generated by the addition of 3 mM H$_2$O$_2$ for 45 min. Under these conditions, the expression of all genes was approx. 1.5-fold (Figure 1A).

Previous studies have shown that the expression of the flavodi-iron genes is altered under several stress conditions, as follows. A very low induction of *flv1* (∼2-fold) has been reported for cells exposed to 3 mM H$_2$O$_2$, for 30 min [10]. Regarding *flv2*, this gene was induced under high light conditions (∼9-fold) and repressed (∼4-fold) in cells treated...
with H$_2$O$_2$ for 15 min. Nevertheless, for longer periods of time (up to 7 h) no alteration in the expression of flv2 was observed [10,11]. The flv4 gene expression was found to be increased in cells exposed to high light (∼6-fold) [11], to UV-B light (∼3-fold) [12], and to high light combined with Methyl Viologen (∼2-fold) [13], which was assumed to be due to oxidative stress. However, our results indicate that the changes reported for the expression of flv2 and flv4 in illuminated cells are not triggered by oxidative stress since we observed no alteration in their expression level in cells submitted to the oxidant H$_2$O$_2$.

The transcription of the *Synechocystis* flavodi-iron genes was also analysed under nitrosative stress conditions generated by GSNO (S-nitrosogluthatione). To this end, cells grown until reaching a $D_{730}$ of 1 were treated with 0.2 mM GSNO and samples were collected after 1 and 12 h for RNA extraction and subsequently analysed by real-time RT–PCR. The results showed that only flv1 gene transcription was always induced (Figures 1B and 1C). In addition, when *Synechocystis* was exposed to GSNO for longer times (12 h), the induction of flv3 and flv4 genes was also observed (Figure 1C). These results suggest that Flv1 is involved in the response to nitrosative stress, independent of the time of exposure.

**Flv1 has no-canonical residues at the di-iron catalytic site**

We also performed a comprehensive phylogenetic analysis of the primary structures of these proteins. To this end, a BLAST search was performed, which allowed us to retrieve nearly 500 putative sequences. Next, the flavodi-iron core of all the sequences was aligned with the program ClustalX [14], and a dendrogram was assembled [15]. Note that the signal peptides, present in some sequences, and the extra C-terminal domains were not considered in the aforementioned analysis.

The dendrogram obtained (Figure 2A) clearly highlights that the FDPs are widely distributed, both in prokaryotes and eukaryotes, as observed previously [16]. Furthermore, the scrutiny of the dendrogram also showed that the FDPs from cyanobacteria and oxygenic photosynthetic eukaryotes form a distinctive group from the sequences from Archaea, Bacteria and Protozoa, suggesting a common origin, in agreement with the hypothesis raised by Zhang et al. [17]. The analysis of the intact sequences from cyanobacteria and oxygenic photosynthetic eukaryotes revealed that all of them have the NAD(P)H:flavin reductase domain, being therefore class C FDPs. The sequences of
cyanobacteria and oxygenic photosynthetic eukaryotes were aligned with the sequences of prototypical FDPs, which were used as reference sequences. Two main clusters are obtained which is in agreement with previous analysis [16–18].

Our analysis, presented in Figure 2(B), revealed that 50% of the aligned sequences have conserved residues precisely matching the canonical ones known to be involved in iron coordination (H108-X-E110-X-D117-X-N112, Flv1 from Synechocystis sp. PCC6803 strain). Two main clusters are obtained which is in agreement with previous analysis [16–18].

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