Expression of ferredoxin–NADP⁺ reductase in heterocysts from *Anabaena* sp.

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The expression of ferredoxin–NADP⁺ reductase (FNR) from *Anabaena* sp. PCC 7119 in heterocysts and vegetative cells has been quantified. Specific reductase activity in heterocysts was approximately 10 times higher than in vegetative cells, corresponding to the increased FNR protein content. This was confirmed by immunoquantification of the FNR protein from whole filaments of *Anabaena* sp. PCC 7120 grown in media with and without combined nitrogen. Transcription of the *petH* gene was markedly enhanced in the absence of combined nitrogen.

This suggests that the increased RNA level is mainly responsible for the up-regulation of FNR in heterocysts. As has been observed for *nif* genes, iron deficiency also increased transcription of *petH*. Characterization of the FNR purified from isolated heterocysts showed no detectable differences from the enzyme from vegetative cells. Although nitrogen stress was a key regulatory factor, localization of the *petH* gene in the genomic map of *Anabaena* PCC 7120 showed that this gene is not physically associated with the *nif* cluster.

INTRODUCTION

Ferredoxin–NADP⁺ reductase (FNR; EC 1.18.1.2) is a FAD-containing protein which catalyses the photoreduction of NADP⁺ during oxygenic photosynthesis. The enzyme is found in all cyanobacteria and photosynthetic eukaryotic cells. Other enzymes with a similar specificity but different physiological roles have been described in several non-photosynthetic plant tissues [1,2], in mammalian mitochondria and in several bacteria [3,4].

Cyanobacterial FNR has been well characterized [5,6]. It is a membrane-bound component of the thylakoids and transfers electrons from ferredoxin or flavodoxin to NADP⁺. Moreover, the *petH* gene coding for FNR has been cloned from several cyanobacterial strains and the deduced amino acid sequence of the gene product is much longer than expected from N-terminal analysis of the purified protein [7]. The sequence of this additional domain of FNR is similar to that of the CpcD phycocyanin-associated linker protein. It has been proposed that the reductase interacts with the thylakoidal membrane by docking its N-terminal domain at the peripheral rods of the phycobilisomes [6].

Under nitrogen-fixation conditions, some filamentous cyanobacteria develop specialized cells called heterocysts. These cells provide the anaerobic environment necessary for the activity of nitrogenase, which reduces di-nitrogen to ammonia. In heterocysts, photosystem I (PSI) is still active but NADP⁺ photo-reduction does not take place. Even though there is not yet a clear idea of the different pathways that feed electrons to nitrogenase in cyanobacteria, it has been proposed that FNR is involved in the main route that supplies reducing power to nitrogenase through the specific ferredoxin from heterocysts (FdxH) [8,9]. *In vitro*, FNR can reduce nitrogenase in a light-independent pathway via ferredoxin [10]. In order to establish the presence and the role of FNR in the heterocyst and to investigate whether this enzyme is identical to the reductase present in vegetative cells, we have purified and characterized the FNR from heterocysts. Furthermore, localization of the FNR

gene in the genomic map of *Anabaena* and studies on FNR expression under different environmental conditions have been performed.

MATERIALS AND METHODS

Growth conditions and heterocyst isolation

Anabaena sp. PCC 7119 used for heterocyst isolation and immunoquantification was grown in BG11 medium [11] at 22 °C under light intensities of 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Cells were cultured in the presence or absence of nitrogen in the medium depending on the experimental procedure. *Anabaena* sp. PCC 7120 was used for RNA isolation and grown according to [12]. Heterocyst isolation was performed using a method based on that of Fay [13]. Filaments cultured in diazotrophic conditions were collected in the exponential phase by centrifugation at 3500 *g* for 10 min and washed with 50 mM Tris/HCl, pH 7.5, 10 mM EDTA and 1 mM MgCl₂. The amount of chlorophyll was measured [14] and adjusted to 150 $\mu\text{g}\cdot\text{ml}^{-1}$ with the same buffer. Lysozyme (1 $\text{mg}\cdot\text{ml}^{-1}$) was added and the mixture was incubated at 37 °C for 1 h with agitation. The cells were centrifuged for 10 min at 3000 *g*. The supernatant was stored at –20 °C and designated the protein extract of vegetative cells. The pellet was resuspended in 50 mM Tris/HCl, pH 7.5, and 1 mM MgCl₂ buffer to a chlorophyll concentration of 200 $\mu\text{g}\cdot\text{ml}^{-1}$. The suspension was sonicated for 3 min in a Branson apparatus at 10% of ultrasonic power and disruption of the vegetative cells was checked by light microscopy. The resulting homogenate was centrifuged several times at 150 *g* for 2.5 min. The pellet consisted of heterocysts, which appeared intact under light microscopy, at a purity of 95%.

Pure heterocysts were resuspended in 90% (v/v) acetone at –20 °C and sonicated at middle power for 3 min. The resulting solution was incubated for 1 h at 4 °C with agitation and the acetone-dried powders were centrifuged at 5000 *g* for 30 min and dried at room temperature. The pellet was resuspended in 50 mM

Abbreviations used: FNR, ferredoxin–NADP⁺ reductase; PSI, photosystem I; PSII, photosystem II.

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Tris/HCl, pH 8, and centrifuged at 10000 *g* for 20 min. The resulting supernatant was designated the crude protein extract of heterocysts.

Protein purification

FNR was purified from *Anabaena* sp. PCC 7119 using HPLC (Hydropore-5-SAX column; Rainin). The crude extract was precipitated with pre-cooled acetone and the pellet resuspended in 20 mM Tris/HCl, pH 7.4, containing 1 μ M PMSF, 0.1 mM EDTA and 0.1 mM 2-mercaptoethanol. The suspension was dialysed against the same buffer and applied to the column. A linear gradient of 0–0.5 M NaCl in buffer A (20 mM Tris/HCl, pH 7.4, 2 mM EDTA) was developed over 50 min. The absorbance of the eluate was recorded at 280 nm and 458 nm and fractions were collected and assayed for diaphorase activity [5]. FNR eluted in one of the first peaks, corresponding to a concentration of 0.05 M NaCl, together with phycobiliproteins. After dialysis (buffer A), the resulting solution was applied to a Cibacron–Blue Sepharose column (1 cm $\times$ 10 cm) equilibrated with 50 mM Tris/HCl, pH 8, and eluted with a linear NaCl gradient (0–0.5 M in equilibration buffer, vol. 100 ml). The pooled active fractions were dialysed against buffer A and applied to a Hydropore-5-SAX column, with a linear gradient of 0–0.15 M NaCl over 30 min, to yield the pure enzyme. The pooled fractions had a spectral ratio $A_{280}:A_{458}$ of 9, while pure protein isolated from vegetative cells has a ratio of 8. Total protein was measured by the method of Lowry et al. [15]. Electrophoresis was performed using a Phast System (Pharmacia). The N-terminal sequence of the first 10 amino acids was determined in two samples of 2 nmol each by the micro-sequencing facility of the University of Münster (Germany).

Immunological assays and activity measurements

Antibodies were raised according to the method described by Lampreave and Piñeiro [16]. Purified proteins used as standards were isolated from whole filaments [5]. Rocket immunoelectrophoresis was performed using a method described by Bog-Hansen [17]. At least three different determinations were performed for each data point. FNR activities were measured using the dichlorophenolindophenol-dependent diaphorase assay as described in [5].

RNA isolation and Northern blot analysis

A portion (200 ml) of each culture of *Anabaena* sp. PCC 7120 (1 g of cells) was harvested by centrifugation at 5000 *g* for 10 min and RNA was isolated as described in [12]. RNA (40 μ g) was glyoxylated and separated in 1% agarose gels, using 10 mM Na_2HPO_4 , pH 7, as running buffer. The RNA was transferred to a Hybond nylon filter. Hybridization of the probes and the washing steps were performed at 65 °C as described in [18].

DNA probes for Northern hybridization

The *petH* probe was obtained by PCR amplification using homologous primers for this gene and chromosomal DNA from *Anabaena* PCC 7120 as target DNA. Labelling was performed by random priming using [α - ^{32}P]dATP (Amersham).

Determination of heterocyst differentiation

The percentage of heterocysts in the filaments of *Anabaena* PCC 7120 was calculated by counting the vegetative cells between two heterocysts using the light microscope.

Localization of the *petH* gene in the genomic map

Total genomic DNA from *Anabaena* PCC 7120 was digested with the infrequently cleaving restriction endonucleases *Pst*I and *Sa*I. The fragments were separated in an agarose gel using pulsed homogeneous orthogonal field gel electrophoresis as described in [19]. DNA from the gel was transferred to a Hybond filter and hybridized with a labelled *petH* probe at 42 °C. Labelling of the probe was performed using dioxigenin-dUTP with a random priming kit from Boehringer Mannheim.

RESULTS AND DISCUSSION

Heterocysts have a different pattern of protein expression from vegetative cells, which is a consequence of their metabolism being focused on nitrogen fixation. Among these differences, we were interested in the functions of the enzyme FNR. In vegetative cells, FNR catalyses the photoreduction of NADP^+ , accepting electrons from reduced ferredoxin (Pet F). However, in heterocysts it has been suggested that FNR can act as electron carrier in a light-independent reaction between NADPH and the specific heterocyst ferredoxin (FdxH), with electrons flowing in the reverse orientation compared with photosynthesis [8–10]. In heterocysts, FNR can also transfer electrons in the light from NADPH, generated mainly from the pentose phosphate pathway, to PSI via the cytochrome b_6/f complex. Reduced PSI completes the electron flow to FdxH, the terminal donor to nitrogenase [9].

Changes in the expression of FNR from *Anabaena* associated with nitrogen fixation were detected by immunodetection of this enzyme in vegetative cells and isolated heterocysts. Table 1 shows that the amount of FNR when related to total protein was more than 10-fold greater in heterocysts than in vegetative cells, and diaphorase activity increased around 7-fold. This would indicate the importance of the enzyme in the nitrogen fixation process and possibly as a component of the main electron transfer chain to nitrogenase. These data are in agreement with the results obtained from *in vitro* reconstitution in *Anabaena* of different electron transfer chains to nitrogenase [10], which shows the ability of FNR to mediate electron transport in some of those pathways.

Considering the presence of a FdxH, specialized in electron donation to nitrogenase, it was of interest to determine whether the FNR present in such large amounts in the heterocyst was the same molecular species as the FNR expressed in vegetative cells. The first evidence of the presence of a single form of FNR came from the results of the immunoassays (results not shown). Moreover, FNR purified from 30 g of isolated heterocysts had a UV–visible absorption spectrum identical to that of the enzyme isolated from vegetative cells. SDS/PAGE exhibited a sole 36 kDa species, again identical to the pure enzyme from

Table 1 Measurement of FNR in vegetative cells and heterocysts isolated from *Anabaena* PCC 7119 grown in different conditions

	Amount (ng of FNR/mg of protein)	Activity (i.u./mg of protein)
With nitrate (20 mM)		
Vegetative cells	436	0.31
Without combined nitrogen		
Vegetative cells	476	0.22
Heterocysts	6595	1.46

Figure 1 Northern blot analysis of FNR expression in *Anabaena* sp. PCC 7120

RNA was isolated from cultures grown as follows: lane 1, BG11 (—NO₃[−], +0.9 μM Fe); lane 2, BG11 (+5 mM NO₃[−], +0.9 μM Fe); lane 3, BG11 (—NO₃[−], +30 μM Fe); lane 4, BG11 (+5 mM NO₃[−], +30 μM Fe).

vegetative cells. Isoelectric focusing of FNR purified from heterocysts revealed the same set of five isoforms as the protein purified from vegetative cells, with some difference in the relative amount of the bands obtained (results not shown). These isoforms are not proteolytic products arising during purification and have been reported to be due to interconversion of isoelectric species of FNR [20]. Since isoelectric focusing detects very small differences between proteins, including differential ion binding, variation of the oxidation states of the surface and various conformational states [20], the occurrence of the same five bands in both proteins was considered to be indicative of similarity.

The N-terminal sequencing of the first 10 amino acids of FNR isolated from heterocysts gave a single sequence which was identical with the sequence obtained from N-terminal analysis of the protein from vegetative cells, although this 36 kDa form was truncated from a 49 kDa FNR [21]. Consequently, all our data indicate that the FNR purified from heterocysts was the same molecular species as the one from vegetative cells.

Regulation of the expression of FNR was studied using Northern blots loaded with equal amounts of RNA extracted from cells grown under different conditions. Figure 1 shows that the *petH* transcript was constitutively expressed as a single message of 1.5 kb (lane 4) when photosynthesis but not nitrogen fixation occurred. Transcription of *petH* was enhanced under nitrogen-fixing conditions (lane 3), compared with the transcription in cells grown with ammonium nitrate (lane 4). The size of the transcripts is the same, in accordance with the finding of a single protein species, although under nitrogen-fixing conditions more smearing appeared above and below the strong 1.5 kb signal. The influence of iron starvation on the expression of *petH* was also studied, since iron stress has been shown to induce the transcription of some *nif* genes [12]. When iron was limiting *petH* transcription was more abundant (lane 2) than under non-starved conditions (lane 4). Lane 1 shows the level of transcription in cells grown under simultaneous nitrogen-fixing and iron deficiency conditions. Transcription in these cells was also higher than the transcription level in cells grown under nitrogen-repleted conditions. Therefore enhanced transcription of *petH* was in-

Table 2 FNR immunoquantified from filaments of *Anabaena* sp. PCC 7120 grown in different nutritional conditions

+ Fe, 30 μM; — Fe, 0.9 μM; + N, 5 mM ammonium nitrate; — N, no combined nitrogen present.

	Complete filaments (ng of FNR/mg of protein)	Heterocyst frequency (%) [*]
+ Fe/ + N	150	0
+ Fe/ — N	400	7
— Fe/ + N	235	2.5
— Fe/ — N	447	6.7

^{*} Taken from [12].

duced by iron deficiency. Lack of iron could lead to nitrogen starvation, since many enzymes involved in nitrogen assimilation, such as glutamate synthase, nitrate reductase and nitrite reductase, are iron–sulphur proteins. To investigate whether the observed transcripts were translated into functional protein, FNR was immunoquantified in aliquots of cells also used for RNA isolation. Table 2 shows that the amount of FNR generally correlated with the amount of transcript. Extracts from cells grown without combined nitrogen contained significantly more FNR than those grown under nitrogen-replete conditions.

Localization of *petH* in the genomic map of *Anabaena* [19] showed that the gene was present in the same 430 kb D fragment of *Pst*I digested DNA as the genes coding for the PSI structural subunits *PsaE1* and *PsaD1*, the photosystem II (PSII) subunit, *PsbD* and ferredoxin I. Although genes for PSI do not appear to be clustered, it is noticeable that the genes coding for PSI D and E subunits, ferredoxin and FNR, whose interaction is required for efficient photosynthetic electron transport, are located in the same DNA fragment restricted to 7% of the whole genome. We can conclude from this work that the expression of FNR is regulated in a different way in heterocysts and vegetative cells respectively. This regulation takes place mainly at the transcriptional level. Transcription is enhanced under nitrogen-fixing conditions and, to a lesser extent, under iron starvation. Our results also show that even though expression of FNR is very much enhanced under nitrogen-fixing conditions the *petH* gene is not associated with the major *nifHDK* cluster.

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