

The GS-GOGAT pathway is not operative in the heterocysts. Cloning and expression of *glsF* gene from the cyanobacterium *Anabaena* sp. PCC 7120

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Abstract The gene encoding the ferredoxin-dependent glutamate synthase (Fd-GOGAT), *glsF*, from the heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120, has been cloned and sequenced. Unlike other cyanobacteria, *Anabaena* 7120 contains only Fd-GOGAT, lacking NADH-GOGAT. The amount of *glsF* transcript and Fd-GOGAT activity were similar under all the nitrogen growth conditions tested. Enzyme activity, Western and Northern blot analyses indicated that Fd-GOGAT is absent in the heterocysts, while glutamine synthetase (GS) and NADP-isocitrate dehydrogenase (IDH) were present in these specialised cells. Our results clearly indicate that the GS-GOGAT pathway is not operative in the heterocysts, and hence glutamate must be imported from the adjacent vegetative cells, to sustain GS activity. Heterocysts probably export glutamine or another nitrogen rich compound like arginine to the vegetative cells. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: GS-GOGAT; Nitrogen assimilation; Heterocyst; Cyanobacterium

1. Introduction

In most photosynthetic organisms, ammonium assimilation takes place by the sequential action of glutamine synthetase (GS) and glutamate synthase (GOGAT). This pathway, known as the GS-GOGAT cycle, constitutes the link between nitrogen and carbon metabolisms [1,2]. Many studies have been devoted to the regulation of the GS-GOGAT pathway, since this cycle ultimately controls the nitrogen flux contributing to cell growth. The regulation of GS activity and the expression of the corresponding gene (*glnA*) have been extensively analysed in different cyanobacteria [3]. In the filamentous N₂-fixing cyanobacterium *Anabaena* sp. 7120, *glnA* gene expression and GS activity are low in cells growing under ammonium as nitrogen source and high under dinitrogen-fixing conditions [4,5].

In contrast, little is known about glutamate synthase (GOGAT) in cyanobacteria. The unicellular cyanobacterium *Synechococcus* shows Fd-GOGAT activity and the corresponding enzyme has been purified and characterised [6]. Two enzymes

showing Fd-GOGAT activity have been described in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, encoded by the *gltS* (actually known as *glsF*) and *gltB* genes [7]. However, a more detailed analysis indicates that *gltB* encodes for the large subunit of the NADH-GOGAT (Martín-Figueroa et al., unpublished) which also contains a small subunit encoded by the *gltD* gene. In addition, two GOGATs (Fd- and NADH-dependent) have recently been reported to exist in the unicellular cyanobacterium *Plectonema boryanum* [8]. The existence of glutamate synthase activity in heterocysts of N₂-fixing cyanobacteria is controversial. Therefore, the putative role of Fd-GOGAT in these organisms has been investigated mainly in the context of a possible complete GS-GOGAT pathway in the heterocysts. Thomas et al. [9] failed to detect Fd-GOGAT activity in heterocysts of *Anabaena cylindrica* (W), while Gupta and Carr [10] observed the formation of [¹⁴C]glutamate from [¹⁴C]glutamine in heterocyst extracts from the same cyanobacterium. Similar results have been attributed to a contaminant glutaminase activity by Rai et al. [11].

In this work we have cloned and analysed the expression in whole filaments and in isolated heterocyst, of the only gene coding for glutamate synthase in *Anabaena* sp. PCC 7120, *glsF*. Enzyme activity, Western and Northern blot studies clearly indicate the absence of Fd-GOGAT in the heterocyst, in contrast with the high levels of GS and NADP-isocitrate dehydrogenase activities. Our results point to the lack of a complete GS-GOGAT pathway in the heterocyst. The implications for the heterocyst nitrogen metabolism are discussed.

2. Materials and methods

2.1. Organisms and growth conditions

Escherichia coli strains: DH5 α (BRL), and MC1069 were used for plasmids and gene library constructions respectively. Cultures were grown in Luria broth [12] supplemented with ampicillin at a final concentration of 100 μ g/ml.

Anabaena sp. PCC 7120 was cultured at 30°C in BG11 medium [13] containing nitrate, ammonium or dinitrogen as nitrogen source, and bubbled with a continuous stream of 1% (v/v) CO₂ in air.

2.2. Isolation of heterocysts

For cell-free extracts, heterocysts were isolated from 5 l culture of *Anabaena* 7120, growing under N₂-fixing conditions. Cells were then harvested and resuspended in buffer A (50 mM potassium phosphate buffer, pH 7, 1 mM EDTA, 14 mM 2-mercaptoethanol and 1 mM PMSF), at a ratio of 1 ml buffer A/100 μ g of chlorophyll. The suspension was passed through a French press once at 20000 psi, and then centrifuged at 150 $\times$ g for 3 min. The pellet was washed with buffer A and centrifuged again at 150 $\times$ g for 3 min to further elim-

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inate cell debris. The pellet obtained was used as the heterocyst preparation.

For RNA isolation, a different method described in [14] was used. In both cases the enrichment in heterocysts of the preparations was followed by optic microscope analysis.

2.3. Nucleic acid manipulations

Total *Anabaena* DNA was isolated as described in [15]. All DNA manipulations and *E. coli* transformations were performed following standard procedures [12]. PCR amplifications were carried out as described in [12]. The two oligonucleotides used for PCR amplifications were the same as were used to clone the *Synechocystis* 6803 *glsF* and *gltB* genes [7]. Southern blot hybridisations were performed as described in [12]. Colony hybridisation was carried out as indicated [16]. A 1.2 kb *glsF* internal fragment obtained by PCR with the aforementioned oligonucleotides, was used as probe in Southern blot and colony hybridisations.

Total RNA was isolated as described [17]. Separation of RNA, transfer to nylon membranes (Hybond N+, Amersham) and hybridisation conditions were according to the instruction manuals from Amersham. Total RNA (15 µg) was loaded in each lane. A probe corresponding to nucleotides –140 to +59 (considering the first nucleotide of the translation start codon as +1) was used for the detection of *glsF* mRNA. Internal probes from *rbcL*, *nifH*, *glnA* and *icd* genes were used for detecting their respective mRNAs. The constitutively expressed *rnpB* gene from *Anabaena* 7120 [18] was used as a loading control. Probes were ³²P-labelled using [α-³²P]-dCTP by random primer, and quantifications of radioactive signals were performed using a Cyclone Phosphor System apparatus (Packard).

2.4. Cell-free extracts, enzyme assays and Western blot analysis

Cell-free extracts from whole filaments or isolated heterocysts were performed by grinding the cells in a mortar with liquid nitrogen, resuspension in buffer A and centrifugation at 12 000 × g for 15 min. Protein concentrations of the supernatants were determined by the method of Bradford [19], using ovalbumin as standard.

Ferredoxin-dependent glutamate synthase activity was determined by measuring glutamate formation as previously described [20], using ferredoxin from *Synechocystis* 6803 as electron donor [21]. One unit of enzyme activity corresponds to the formation of 1 µmol of glutamate per min.

Glutamine synthetase and NADP-isocitrate dehydrogenase activities were determined as described previously [22,23].

For Western blot analysis, cell-free extracts were subjected to SDS-PAGE on 6% (w/v) polyacrylamide for Fd-GOGAT detection and 12% (w/v) polyacrylamide for GS or IDH detection [24]. The Western blot procedure was carried out as described [23], using polyclonal antibodies raised against *Synechocystis* 6803 Fd-GOGAT [25], *Synechococcus* sp. PCC 6301 GS [26] and *Synechocystis* 6803 NADP-IDH [23] respectively. The Western blots were developed by ECL detection reagents (Amersham) according to manufacturers instructions.

3. Results

3.1. Fd-GOGAT activity in *Anabaena* 7120. Immunodetection of *GlsF* protein

The Fd-GOGAT activity from cell-free extracts of *Anabaena* 7120 cultured in either NO₃[−], NH₄⁺ or N₂ was assayed in order to determine if the enzyme activity was regulated by the nitrogen source. As shown in Table 1, Fd-GOGAT activity was almost similar under the different nitrogen conditions used. NADH-GOGAT activity was not detected under any conditions tested. As the data reported about the existence of this enzyme in the heterocyst are contradictory [27], we measured the Fd-GOGAT activity in crude extracts of isolated heterocysts. In contrast to the high levels of GS and IDH activities observed, there was no detectable Fd-GOGAT activity (Table 1). These results pointed to the lack of Fd-GOGAT in the heterocyst. To further investigate whether the Fd-GOGAT enzyme is present in an inactive form in these cells, heterocyst extracts were subjected to Western blot, using

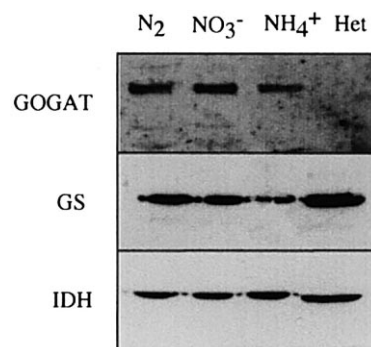


Fig. 1. Fd-GOGAT is not present in the heterocysts. Crude extracts from *Anabaena* 7120 vegetative cells grown on: dinitrogen (1), nitrate (2), ammonium (3) or from isolated heterocysts (4) were subjected to Western blot analysis using antibodies against Fd-GOGAT, GS and IDH. Total protein (15 µg) was loaded in each lane.

antibodies raised against *Synechocystis* 6803 Fd-GOGAT (*GlsF*) [7]. However no cross-reacting bands were observed in heterocyst preparations, while a band corresponding to *GlsF* was always observed in the lanes corresponding to the whole filaments (Fig. 1). Furthermore, the amount of *GlsF* in vegetative cells was similar under all the nitrogen conditions tested. In order to corroborate that this result was not due to a general degradation of proteins provoked by the heterocyst isolation, we carried out additional Western blots using antibodies raised against *Synechococcus* 6301 GS [26] and *Synechocystis* 6803 IDH [23]. As shown in Fig. 1, bands that cross-reacted with both GS and IDH antibodies were detected in vegetative cells as well as in heterocysts. These results clearly point out that Fd-GOGAT is not present in the heterocysts from *Anabaena* 7120, while the amounts of GS and IDH are higher than in vegetative cells.

3.2. Cloning and sequence analysis of *glsF* gene

To extend our analysis, we proceeded to clone the gene coding for the *Anabaena* 7120 Fd-GOGAT. For that purpose we carried out a PCR amplification, using the primers detailed in Section 2, obtaining a unique DNA fragment of 1.2 kb that was used as a probe in a Southern blot hybridisation with total *Anabaena* DNA, only one copy of the corresponding gene, *glsF* was found (data not shown). No hybridisation bands were obtained using as a probe an internal fragment from *Synechocystis* 6803 *gltB* gene coding for the large subunit of the NADH-GOGAT, (not shown). In order to clone

Table 1
Levels of Fd-GOGAT, GS and NADP-IDH activities in *Anabaena* 7120

Source	Enzyme activity (mU/mg protein)		
	Fd-GOGAT	GS	IDH
Vegetative cells			
N ₂	32.3	883	101.5
NO ₃ [−]	28.8	600	72.7
NH ₄ ⁺	25.3	339	60.1
Heterocysts	n.d.	1634	146.4

The activities were determined in cell-free extracts from vegetative cells grown with N₂, NO₃[−] or NH₄⁺ as nitrogen source and from isolated heterocysts. Assays were performed as described in Section 2. Data are averages of three independent experiments, and the standard error for all values was always less than 10%. n.d.: not detected.

Fig. 2. Analysis of GlsF deduced amino acid sequence. A: Identities of *Anabaena* 7120 GlsF and *Arabidopsis thaliana* Fd-GOGAT, *Zea mays* Fd-GOGAT, *P. boryanum* NADH-GOGAT and Fd-GOGAT, *Synechocystis* 6803 NADH-GOGAT and Fd-GOGAT, *Medicago sativa* NADH-GOGAT and *E. coli* NADPH-GOGAT using the PileUp program. The identity showed corresponds to the shortest sequence of each two glutamate synthases compared. B–D: Comparison of the conserved regions presented in all GOGATs: glutamine amidotransferase domain (B) the FMN binding site (C) and 3Fe–4S cluster (D). The putative maturation site is upperlined and the conserved Cys-33 is in boldface. The Asp and the Lys residues involved in the binding of the ribityl chain of FMN are in boldface. The three Cys of the iron–sulfur cluster are denoted by Δ . Asterisks indicated conserved amino acid residues in at least seven sequences. The glutamate synthase sequences were obtained from the EMBL/GenBank database.

(4647 bp) of *glsF* gene has been deposited in the EMBL/GenBank database with the accession number AJ249913. Recently, the complete genome of *Anabaena* 7120 has been sequenced, showing that *glsF* is the unique gene encoding for GOGAT. The *glsF* gene encodes a protein of 1549 amino acid residues. Comparative analysis of the deduced amino acid sequence (Fig. 2), shows a strong identity with the counterpart Fd-GOGATs from the cyanobacteria *P. boryanum* and *Synechocystis* 6803 (about 70%) and with the corresponding Fd-GOGATs from higher plants (about 60%). Lower identity was found with the large subunit of NAD(P)H-GOGAT from *E. coli* (about 45%). A more detailed analysis reveals the existence of functional domains highly conserved in all glutamate synthases (Fig. 2B to D) [28]. The glutamine amidotransferase domain contains a cysteine, Cys-33, which is absolutely conserved in all GOGAT sequences, and is involved in the release of the amide group of the glutamine during the enzyme catalysis (Fig. 2B). A second strongly conserved region corresponds to the FMN binding domain that comprises the critical residues Asp-1137 and Lys-1141 which probably interact with the ribityl side-chain of FMN (Fig. 2C). Finally, a third region responsible for the accommodation of the [3Fe-4S] cluster containing the three cysteines, Cys-1169, Cys-1174 and Cys-1179 which define the motif CX₅CX₄C (Fig. 2D) strictly conserved in all GOGATs known is also found.

3.3. *glsF* transcript accumulation in vegetative cells and in heterocysts of *Anabaena* 7120

To detect if *glsF* was expressed in the heterocyst, as well as to determine changes in *glsF* transcript levels when *Anabaena* 7120 is cultured under different nitrogen source, we carried out Northern blot experiments using total RNA isolated from whole filaments grown using NO₃[−], NH₄⁺ or N₂ or from heterocyst (Fig. 3). The probe used was a DNA fragment comprising the first 59 bp of the *glsF* coding region plus 140 bp of the promoter region. *glsF* transcript was almost constant in whole filaments under all conditions tested (Fig. 3), indicating that expression of *glsF* in vegetative cells from *Anabaena* 7120 is not regulated by the nitrogen availability. In contrast, *glsF* mRNA was virtually absent in heterocysts. The low level of *glsF* mRNA detected in this case is in concordance with the level of *rbcL* mRNA (Fig. 3), which is known to be expressed only in vegetative cells [29]. So, the trace amounts of *rbcL* mRNA as well as *glsF* mRNA observed in heterocysts can therefore be concluded as results of vegetative cell contamination. These results clearly demonstrated that the *glsF* gene is not transcribed in the heterocyst. Moreover, using a fragment of the *nifH* gene that is strongly expressed in the heterocyst [29] as a probe for Northern blot, showed a 46-fold higher hybridisation in the heterocyst than in vegetative cells grown using NH₄⁺ as nitrogen source, and 6-fold higher than

in whole filaments under N₂-fixing conditions, indicating the high enrichment in heterocyst RNA of the sample (Fig. 3).

In addition, probes of *glnA* and *icd* genes were used to confirm if their expressions were higher in heterocyst than in vegetative cells. The hybridisations carried out with these probes showed, in both cases, approximately 1.6-fold higher levels in heterocyst than in whole filaments under N₂ conditions. 100% corresponds in each case to the mRNA level in whole filaments of cells grown in N₂ conditions.

These results, together with those obtained by Western blot and assays for enzymatic activity, clearly show that GOGAT is absent from heterocysts of *Anabaena* 7120.

4. Discussion

In cyanobacteria, the GS-GOGAT pathway is the only way to incorporate ammonium to carbon skeletons [1,2], and it controls the flux of nitrogen by regulating the first enzyme of the pathway, GS, following the criterion of the nitrogen

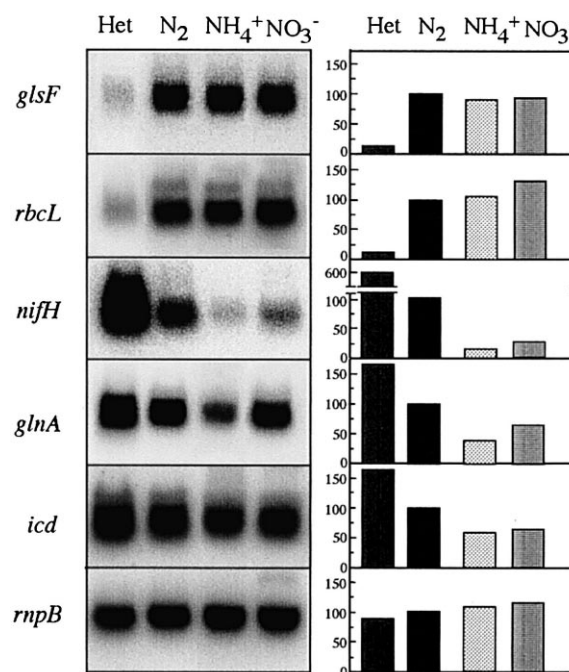


Fig. 3. Levels of *glsF*, *rbcL*, *nifH*, *glnA* and *icd* transcripts in *Anabaena* 7120 cells under different nitrogen conditions and in isolated heterocysts. Total RNA was isolated from heterocysts of *Anabaena* 7120 or from whole filament cells that used nitrate, ammonium or dinitrogen as nitrogen source. A total of 15 μ g of RNA was loaded per lane. The relative levels of mRNA are expressed as % of the level detected in whole filaments under N₂-fixing conditions. *rnpB* RNA levels were examined as a control for equal loading of the lanes.

source used for growth. Thus, in presence of ammonium, a reduced nitrogen form, GS activity is low and the expression of the corresponding gene, *glnA*, is also attenuated. However, when the nitrogen source is nitrate or dinitrogen, in nitrogen-fixing cyanobacteria, both GS activity and *glnA* gene expression are increased [3–5,30–32]. In cyanobacteria, the second enzyme of this pathway, GOGAT, mainly uses ferredoxin as reductant. In addition, some cyanobacteria like *P. boryanum* and *Synechocystis* 6803 have a second glutamate synthase that uses NADH as electron donor [8]. Here, we show that in the case of *Anabaena* 7120 only the *glsF* gene coding for the Fd-GOGAT is present, and the corresponding enzyme Fd-GOGAT is not regulated by the nitrogen source (Table 1 and Fig. 1). It is well established that GS is present in heterocysts and is capable of incorporating the ammonium synthesised by the nitrogenase complex into carbon skeletons, this notion raises the question whether also GOGAT is present in these specialised cells. This question remained controversial, since some authors reported evidence that Fd-GOGAT is present in the heterocysts [10,33], but others failed to detect it [9,11]. In all these studies this question has been explored only by determination of GOGAT activity. Here, for the first time, we use antibodies raised against Fd-GOGAT and Northern hybridisations to clearly conclude that Fd-GOGAT is absent in the heterocyst (Figs. 1 and 3). Since GS and NADP-IDH are clearly present in the heterocyst (Fig. 1) [27,34], our model for the nitrogen metabolism in these cells would be as follows: (i) Nitrogenase reduces dinitrogen to ammonia, which is incorporated to glutamate to produce glutamine by the action of GS. (ii) NADP-isocitrate dehydrogenase produces 2-oxoglutarate and NADPH. The last compound is used as electron donor to reduce ferredoxin, while 2-oxoglutarate is exported to the vegetative cells. (iii) Glutamate, which can not be produced in the heterocyst because of the lack of GOGAT, has to be imported from the adjacent vegetative cells. (iv) Glutamine, the product of ammonia assimilation, has to be exported or converted to arginine. Arginine together with aspartate could be used to synthesise cyanophycin, a polymer that serves as nitrogen reserve easily mobilised [27,34]. In this regard, the enzymes required for the arginine biosynthesis are present in the heterocysts [27]. This process also suggests as a reasonable hypothesis that 2-oxoglutarate could be exchanged by glutamate, between the heterocyst and the adjacent cells.

In summary, our work shows clearly that the heterocyst-forming cyanobacterium *Anabaena* 7120 contains only a Fd-GOGAT that is absent from the heterocysts, suggesting that the glutamate required for ammonia incorporation would be synthesised in the vegetative cells and then transported to the heterocysts.

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