A novel mechanism of glutamine synthetase inactivation by ammonium in the cyanobacterium *Synechocystis sp.* PCC 6803. Involvement of an inactivating protein

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Abstract The glutamine synthetase of the cyanobacterium *Synechocystis sp.* PCC 6803 can be inactivated in vivo by ammonium addition by a new mechanism that involves the binding to the enzyme of an inactivating factor. This binding provokes a different mobility of the inactive enzyme with respect to the active form in non-denaturing PAGE, but not in SDS-PAGE. This modification of glutamine synthetase is for the first time visualized by Western blot analysis of the active and inactive forms. Cross-linking experiments using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) demonstrate the existence of two main complexes of 56 kDa and 67 kDa between the inactivating factor and the glutamine synthetase subunit (53 kDa) in the inactive but not in the active form of glutamine synthetase.

Key words: Glutamine synthetase; *Synechocystis* 6803; Cyanobacteria; Ammonium assimilation; Enzyme inactivation

1. Introduction

Glutamine synthetase (GS) (EC 6.3.1.2) is a key enzyme in the nitrogen assimilation process in most microorganisms. This central role of GS requires the synthesis and the activity of this enzyme to be finely regulated in response to the available nitrogen source [1,2]. GS from *Escherichia coli* and other Gram-negative bacteria is modulated at the activity level by a mechanism of adenylylation/deadenylylation of the enzyme, being active the deadenylylated form [3].

In cyanobacteria, ammonium assimilation takes place mainly by the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway [4]. In the unicellular cyanobacterium *Synechocystis sp.* PCC 6803 we have previously reported a short-term ammonium-promoted GS inactivation, in a process that comprises the non-covalent binding of a phosphorylated factor, whose nature was then unknown [5,6]. The inactive GS could be reactivated in vitro by different treatments that include alkaline phosphatase, increasing pH or high ionic strength [6]. In this study we show for the first time, by using Western blot analysis and cross-linking techniques, that the ammonium-promoted inactivation of *Synechocystis* 6803 GS implicates, probably, the non-covalent binding of a 14 kDa inactivating protein.

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Abbreviations: GS, glutamine synthetase; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; PAGE, polyacrylamide gel electrophoresis; SDS sodium dodecyl sulfate.

2. Experimental

2.1. Strains and growth conditions

Synechocystis sp. strain PCC 6803 was grown photoautotrophically at 35°C on BG11 medium [7] with continuous fluorescent illumination (25 $W \cdot m^{-2}$; white light). The cultures were bubbled with 1.5% (v/v) CO_2 in air. When ammonium was used as nitrogen source, nitrate was replaced by 10 mM NH_4Cl and the medium was buffered with 20 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES) buffer. Ammonium-promoted GS inactivation was carried out by addition of 2 mM NH4Cl to a nitrate grown culture.

2.2. Cell-free extract

Cells were harvested by centrifugation at 3,000 × *g* for 10 min, washed with fresh medium and resuspended in 50 mM HEPES-NaOH buffer, pH 7. Cells were disrupted by sonication (20 kHz, 75 W) for 30 s (in 15 s periods). The homogenated was centrifuged at 40,000 × *g* for 15 min and the resulting supernatant constituted the cell-free extract.

2.3. Polyacrylamide gel electrophoresis and Western blot analysis

Samples of crude extract were run in polyacrylamide SDS gel, using 6% (w/v) to lO% acrylamide gradient gel, according to Laemmli [8]. Marker proteins were Prestained SDS-PAGE Standards (low and high range) from Bio-Rad Laboratories. Non-denaturing gel electrophoresis was carried out in 6.25% (w/v) acrylamide gels and run at 4°C. Identification of the glutamine synthetase activity in non-denaturing gel electrophoresis was carried out by the Mn^{2+}-dependent γ-glutamyl-transferase assay [9], modified as follows: after non-denaturing PAGE, the gel was incubated at 30°C in a reaction mixture containing, in a final volume of 10 ml, 600 μmol of HEPES–NaOH buffer (pH 7.0), 400 μmol of L-glutamine, 40 μmol of $MnCl_2$, 600 μmol of hydroxylamine, 10 μmol of ADP and 200 μmol of sodium arsenate. After 20 min the reaction was stopped by transferring the gel to $FeCl_3$ in acid solution that stains the bands where γ-glutamylhydroxamate was formed by the GS activity [9].

Western blot analysis of crude extract samples subjected to denaturing or native electrophoresis were carried out as previously described [10]. Purified polyclonal monospecific antibodies obtained against *Synechococcus sp.* PCC 6301 glutamine synthetase were used [11].

2.4. Cross-linking treatment

Cross-linking reactions were performed at 25°C in 50 mM HEPES–NaOH buffer, pH 7.0, by addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to a final concentration of 4 mM. Final volume of cross-linking reaction was 200 μl with about 2 mg of total protein. Aliquots of 20 μl were taken at various intervals and mixed with an equal volume of dissociation buffer (1% SDS, 1% 2-mercaptoethanol, 30 μM sucrose and 0.4 M sodium phosphate, pH 4.0). Alternatively samples were taken for GS activity determination.

2.5. Other methods

GS biosynthetic activity was measured in vitro from the rate of glutamine formation as described in [6]. Glutamine was determined by high performance liquid chromatography (HPLC) as described [12]. Changes of the sample buffer were carried out by fast filtration through a Sephadex G-25 gel using the method described by Penefsky [13]. Protein in cell-free extracts was determined by the method of Bradford, using ovalbumin as a standard [14]. Alkaline phosphatase used for GS reactivation treatment was of molecular biology grade (Boehinger,

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Mannheim, Germany), free of phosphodiesterase activity. GS reactivation by in vitro phosphatase treatment was as described [6].

3. Results

3.1. Change in electrophoretic mobility of inactive native GS

As previously reported, ammonium addition to nitrate-grown *Synechocystis* cells promotes a dramatic decrease in GS activity [5]. Crude extracts from either nitrate-grown cells or nitrate-grown cells treated with ammonium (2 mM) for one hour, were subjected to non-denaturing gel electrophoresis and analyzed by Western blotting. Only one GS band was recognized by the antibodies in extracts from nitrategrown cells, by contrast several bands with a lower migration velocity were recognized in GS-inactivated crude extract (Fig. 1A). Only the band common to both extracts exhibited GS transferase activity in an assay in non-denaturing gel (Fig. 1B). In vitro treatment with alkaline phosphatase or a pH increase of the GS-inactivated extracts, previously demonstrated to reactivate the inactive enzyme [6], provoked the disappearance of the bands with lower migration (Fig. 1, lanes 3–7). This change in electrophoretic mobility was only observed in Western blots of non-denaturing-PAGE but not in Western blots of SDS-PAGE (Fig. 2).

The lower mobility of the native inactivated GS in comparison with the active GS, taking together with the high molecular mass (about 630 kDa) [11] of the dodecameric native enzyme suggests that the inactivating factor is a polypeptide rather than a metabolite. The fact that inactive GS appears with different electrophoretic mobilities indicates that a variable amount of the same inactivating protein could be bound to GS, in a similar way as occurs with the different grade of adenylylation of *E. coli* and in other bacterial GS [2].

3.2. Cross linked complex characterization

Treatment of GS-inactivated extracts with the water-soluble carbodiimide EDC resulted in the formation of a covalent complex between GS subunits and the inactivating factor as was evidenced by Western-blot of SDS-PAGE (Fig. 3). In fact, anti-GS recognized three main bands: the GS single subunit (53 kDa), and two cross-linked complexes between one GS subunit and the inactivating factor, one of about 56 kDa and other of 67 kDa. Western blot of electrophoresed EDC treated extracts from nitrate grown cells showed the band corresponding to the single subunit but not the 56 and 67 kDa bands. Other upper bands were also formed by the EDC-treatment that correspond to two GS subunits with or without the inactivating factor, in a range from 104 to 120 kDa (Fig. 3).

Crude extracts treated for 1 hour with EDC retained the same GS activity as the non-treated extracts, indicating that, in our condition, the catalytic site of GS was not affected by the EDC modification (Table 1). As it has been mentioned above, ammonium-inactivated GS can be reactivated in vitro by increasing the pH of the extract. However, very low reactivation was observed in EDC-treated extracts from ammonium-treated cells (Table 1). By contrast, complete reactivation of GS was observed when extracts from ammonium-treated cells were subjected to the same pH increase (Table 1), suggesting that EDC treatment maintains the inactivating factor bound to GS. Alkaline phosphatase was also unable to reactivate cross-linked inactive GS. Pretreatment of inactive GS extract with alkaline phosphatase prevented the formation of EDC cross-linked complex, suggesting that dephosphorylation of the inactivating protein was enough to release it from GS (not shown).

4. Discussion

In cyanobacteria, ammonium represses the nitrate assimilation system which includes nitrate transport and its further reduction to ammonium [15]. Furthermore, ammonium also reduces glutamine synthetase synthesis [11,16] and, in certain species such as *Synechocystis* 6803, is able to inactivate the GS

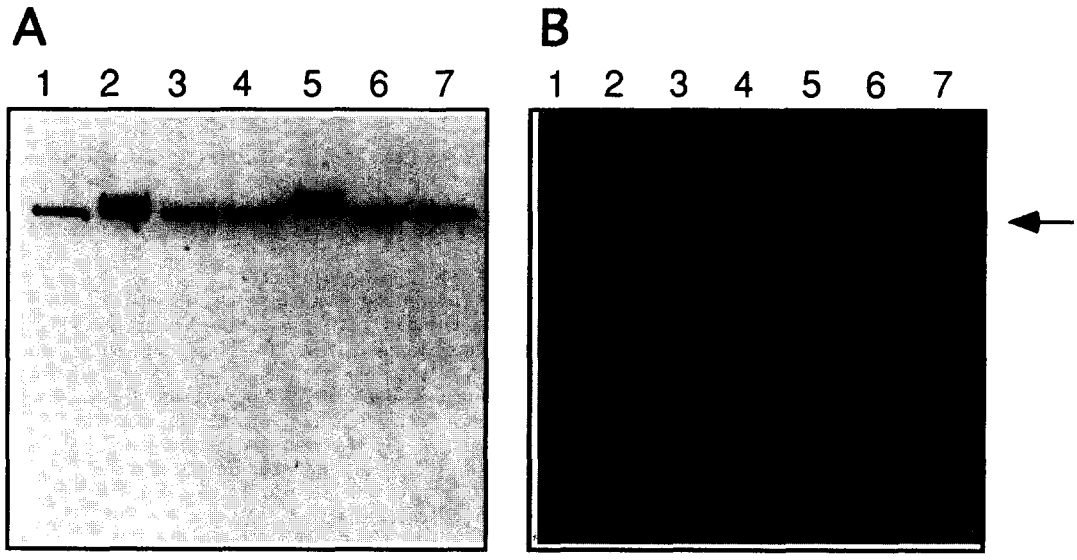

Fig. 1. Identification by Western blot analysis and activity staining of active GS and ammonium-inactivated GS. (A) GS-active extract and GS-inactive extract were subject to non-denaturating gel electrophoresis and Western blot analysis. (B) Identification of GS-transferase activity in non-denaturating gel electrophoresis. Lane 1 = crude extract from nitrate grown cells; lane 2 = crude extract from ammonium treated cells; lane 3 = crude extract from ammonium-treated cells after GS reactivation by changing the pH from 7–9; lane 4 = crude extract from nitrate grown cells after changing the pH from 7 to 9; lane 5 = crude extract from ammonium treated cells after filtration at pH 7; lane 6 = crude extract from nitrate grown cells after treatment with alkaline phosphatase; lane 7 = crude extract from ammonium-treated cells after treatment with alkaline phosphatase.

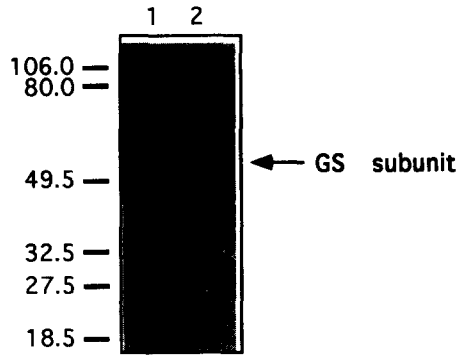

Fig. 2. SDS-PAGE Western blot of glutamine synthetase SDS-PAGE Western blot of crude extracts from nitrate grown cells (lane 1) and ammonium treated cells (lane 2). 20 μg of total protein were loaded per lane.

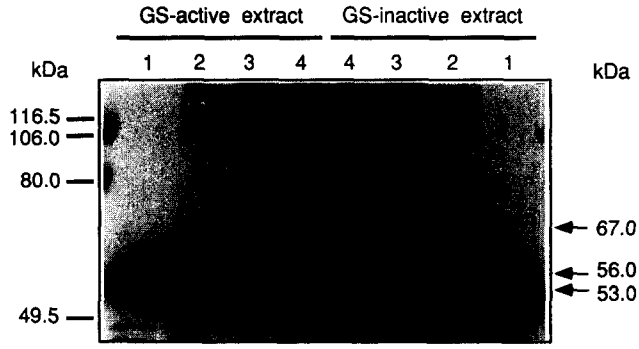

Fig. 3. SDS-PAGE Western blot of EDC cross-linked extract GS-active and GS-inactive extracts were treated with EDC as described in section 2 and aliquots were taken at the indicated times. The standard markers are indicated on the left. The arrows indicates the deduced molecular weight of the signaled bands.

by a process that requires the metabolization of ammonium by the GS-GOGAT pathway [5]. The lower GS activity in ammonium with respect to nitrate is related to the low amount of ammonium available by the cells using nitrate as nitrogen source. In addition high levels of glutamine are toxic for *Synechocystis* cells so a way to maintain the intracellular concentration of glutamine is by decreasing GS activity [5]. We have previously reported that GS inactivation is mediated by a phosphorylated factor, which binds to GS in a non-covalent manner [6]. In fact, the binding of one or more molecules of the inactivating factor to the *Synechocystis* 6803 GS promotes a change in the electrophoretic mobility of the enzyme only when electrophoresis is carried out in non-denaturing condition, supporting the view that the modification is not covalent. In order to confirm that the inactivating-factor is a protein we have carried out cross-linking experiments. Two main cross-linked complexes were produced with the GS subunit, one of about 56 kDa and another of about 67 kDa (Fig. 3), suggesting the existence of two inactivating factor forms, one of 3 kDa and another one of 14 kDa. However the changes observed in electrophoretic mobility of inactive GS in nondenaturing PAGE (Fig. 1) were higher than the expected mobility for the binding of a 3 kDa protein per GS subunit, taking into account that the molecular mass of native GS is about 630 kDa. The 56 kDa band could represent a different cross-linked product between the 14 kDa protein and the GS subunit that gave a different electrophoretic mobility, as occur in one of the subunit of Ca^{2+}-ATPase of sarcoplasmic reticulum due to an intramolecular cross-linking in this subunit [17].

This inactivation system clearly differs from the well-established one in enterobacteria, where an adenylyl group is transferred to a specific tyrosine residue of each GS subunit promoting the GS inactivation [3]. In order to establish the cascade system that promotes ammonium-mediated GS modification, several lines of evidences are now available. Thus, ammonium addition causes a primary signal by producing a transitory change of the intracellular concentration of amino acids, especially those related to the GS-GOGAT pathway, increasing glutamine and decreasing glutamate and 2-oxoglutarate levels [5]. Recently the existence in the unicellular cyanobacterium *Synechococcus* 7942 of a protein of 12.4 kDa similar to the PII protein from *E. coli* has been demonstrated [18]. In enterobacteria, the PII protein is modified depending on the intracellular glutamine/2-oxoglutarate ratio by the action of the uridylyltransferase/uridylyl-removing enzyme, being PII deuridylylated in a nitrogen rich medium and uridylylated in a nitrogen poor medium. Unmodified PII stimulates the adenylylation of GS, and modified PII stimulates the deadenylylation reaction [2,3]. Although it has not been demonstrated that the cyanobacterial PII protein is uridylylated, it has been shown that the protein is phosphorylated, depending on the nitrogen nutritional conditions. Thus, in cells grown on nitrate but not in ammonium grown cells ^{32}P is incorporated into PII [18]. This pattern of modification seems to discard that PII protein binds directly to GS since the GS inactivating protein seems to be phosphorylated in the presence of ammonium, since alkaline phosphatase reactivated the enzyme (Fig. 2, lane 7). In addition in the cyanobacterium *Anacystis nidulans*, the existence of a membrane protein kinase and a cytosolic phosphatase activities that are sensitive to ammonium [19] has been described. Both enzymes, together with the PII protein could be involved in the signal transducing cascade that regulates GS activity in *Synechocystis*.

In conclusion, a new mechanism for GS activity regulation seems to be present in cyanobacteria which is different from that exhibited by enterobacteria and from the inhibition of GS by metabolites described in Gram-positive bacteria [20]: evidently, the system chosen by *Synechocystis* to regulate GS activity requires a constitutive expression of the inactivating protein in order to be available when the nitrogen source changes;

Table 1
Effect of EDC on in vitro GS reactivation by pH increase

	GS activity (mU/mg)	
	Reactivation treatment	
Extract	none	pH increase
GS active	29.5 + 2	31.9 + 3
GS active plus EDC	28.6 + 4	27.3 + 3
GS inactive	4.3 + 1	26.5 + 3
GS inactive plus EDC	3.8 + 1	8.6 + 2

GS biosinthetic activity was measured by glutamine formation. One enzymatic unit was the μmol of glutamine formed by min. Crude extracts were treated with EDC for 1 h at 25°C as described in section 2. Reactivation of GS was carried out by pH change from 7.0 to 9.0; ± indicates standard error.

this conclusion is supported by the fact that ammonium inactivation is a short-time process [5]. We are currently trying to isolate the inactivating protein in order to address further studies about the phosphorylation reaction and amino acid residues implicated in the binding to glutamine synthetase subunit.

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