Glutamine synthetase inactivation by protein–protein interaction

(nitrogen metabolism/cyanobacteria/*Synechocystis* 6803/enzyme regulation)

Mario García-Domínguez, José C. Reyes, and Francisco J. Florencio*

Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-Consejo Superior de Investigaciones Científicas, Américo Vespucio s/n, E-41092 Sevilla, Spain

ABSTRACT Glutamine synthetase (GS; EC 6.3.1.2) is the pivotal enzyme of nitrogen metabolism in prokaryotes. Control of bacterial GS activity by reversible adenylylation has provided one of the classical paradigms of signal transduction by cyclic cascades. By contrast, in the present work we show that cyanobacterial GS is controlled by a different mechanism that involves the interaction of two inhibitory polypeptides with the enzyme. Both inactivating factors (IFs), named IF7 and IF17, are required *in vivo* for complete GS inactivation. Inactive GS-IF7 and GS-IF17 complexes were reconstituted *in vitro* by using *Escherichia coli*-expressed purified proteins. Our data suggest that control of GS activity is exerted by regulating the levels of IF7 and IF17.

Glutamine synthetase (GS; EC 6.3.1.2) is the key enzyme of nitrogen metabolism in prokaryotes, and it is subjected to a sophisticated regulatory control that has been studied extensively (reviewed in refs. 1–3). Depending on the organism, the GS activity is regulated by at least one, and often by all, of the following three levels: feedback inhibition of the activity, reversible covalent modification of the enzyme, and transcriptional regulation of the structural gene. In most of the systems studied, control of GS activity responds to carbon and nitrogen signals. In the presence of abundant carbon sources, nitrogen deficiency results in a high level of GS activity. On the contrary, when nitrogen source is abundant, GS activity is down-regulated.

GS type I (referred to as GS), the most common type of GS in prokaryotes, is a dodecameric enzyme composed of 12 identical subunits (M_r, about 55,000) arranged in two superimposed hexagonal rings (4, 5). Regulation of enterobacterial GS by reversible adenylylation has provided one of the classical paradigms for signal transduction by cyclic cascades. Stadtman and colleagues discovered that GS from *Escherichia coli* exists in two interconvertible forms, an adenylylated form that is highly sensitive to feedback inhibition and a deadenylylated form that is relatively insensitive to feedback inhibition (reviewed in refs. 1, 6, and 7). Adenylylation involves the transfer of an adenylyl group from ATP to a Tyr residue on each of the 12 subunits of the enzyme. The adenylylation state of GS is controlled by a bicyclic cascade involving two bifunctional proteins, the adenylyltransferase and the uridylyltransferase, and the signal-transducing protein PII. From an evolutionary point of view, control of GS activity by adenylylation has been a very successful system because it is present in numerous eubacterial groups (2). In contrast, GSs from *Bacillus*, *Clostridium*, and cyanobacteria are not modified by adenylylation (8, 9). Although regulation of GS activity in *Bacillus* and *Clostridium* seems to be mostly at the level of feedback inhibition, an *in vivo* reversible inactivation of GS has been reported in the cyanobacterium *Synechocystis* sp. PCC 6803

(10–12). As for the adenylylation of enterobacterial GS, control of *Synechocystis* GS depends on the nitrogen–carbon balance of the cell. Thus, addition of ammonium to nitrate-growing *Synechocystis* cells results in a rapid and drastic decay of GS activity. The construction of a *Synechocystis* strain harboring a histidine-tagged modified version of GS has allowed us to purify the inactive enzyme. We describe in the present work the inactivating mechanism of the cyanobacterial GS that involves the direct interaction of two different inhibitory polypeptides with the enzyme.

MATERIALS AND METHODS

***Synechocystis* Growth Conditions.** *Synechocystis* sp. strain PCC 6803 and its derivatives were grown photoautotrophically at 30°C on BG11 medium (13) (18 mM nitrate as nitrogen source) under continuous illumination (50 W·m^{-2}; white light). The cultures were bubbled with 1% (vol/vol) CO_2 in air. BG11$_0$ medium was BG11 medium lacking nitrogen source. When ammonium was used as nitrogen source, BG11$_0$ medium was supplemented with 10 mM NH$_4$Cl and the medium was buffered with 20 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (Tes) buffer at pH 7.0. Kanamycin (50 or 200 μg/ml) and chloramphenicol (20 μg/ml) were added when required.

Purification of Inactive Histidine-Tagged GS. A *Synechocystis* strain harboring a histidine tag-modified GS was generated as follows. Five histidine codons were inserted after the *glnA* ATG start codon by standard PCR techniques. Then, a 1.3-kb kanamycin resistance (Km^R) cassette (C.K1) (14) was inserted into the *Sca*I site, 189 bp upstream of the *glnA* translation start codon. This plasmid (pHIT4) was used to transform *Synechocystis* as described previously (15). Total replacement of the wild-type (wt) *glnA* gene by the tagged version was verified by Southern blot (16) and PCR of purified genomic DNA from the Km^R *Synechocystis* clones. This strain was named *Synechocystis* HTGS1. Nitrate-grown *Synechocystis* HTGS1 cells or nitrate-grown cells treated for 2 h with 5 mM ammonium were harvested by centrifugation and resuspended in 50 mM Hepes·NaOH buffer, pH 7.0. Cells were disrupted by sonication (20 kHz, 75 W) for 2 min and centrifuged at 40,000 × *g* for 15 min. The resulting supernatant constituted the cell-free extract. His-GS was purified from the cell-free extracts by Ni-affinity chromatography by using His-Bind Resin matrix (Novagen) and following the manufacturer's instructions.

Amino-Terminal Determination of Inactivating Factors IF7 and IF17. Protein samples were subjected to SDS/PAGE (17) and transferred to a poly(vinylidene difluoride) membrane

This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: GS, glutamine synthetase; wt, wild type; IF, inactivating factor.
*To whom reprint requests should be addressed at: Instituto de Bioquímica Vegetal y Fotosíntesis, Centro de Investigaciones Científicas Isla de la Cartuja, C/. Americo Vespucio s/n, 41092 Sevilla, Spain. e-mail: floren@cica.es.

(Immobilon-P; Millipore). Membrane-bound protein was subjected to automatic Edman degradation by using an Applied Biosystems Procise Sequencer. Determined amino-terminal sequences were STQQQAR for IF7 and MQLSYR for IF17.

Insertional Mutagenesis of *gifA* and *gifB* Synechocystis Genes. Loci *ssl1911* and *sll1515* were amplified by PCR, using purified *Synechocystis* sp. PCC 6803 genomic DNA, and cloned into pGEM-T (Promega), generating the plasmids pSIF1 and pLIF1, respectively. Targeting vectors pSIF3 and pSIF4 were generated by replacing a 334-bp *Acc*I fragment, which contains the entire *gifA* gene, by C.K1 and C.C1 (14) (chloramphenicol resistance, Cm^R) cassettes, respectively. pLIF2 was generated by replacing a 286-bp, *Nhe*I-*Bst*EII fragment containing almost the entire *gifB* gene by a C.C1 (Cm^R) cassette. To generate Δ*gifA* or Δ*gifB* strains, wt *Synechocystis* cells were transformed with pSIF3 (Km^R) or pLIF2 (Cm^R), respectively. To generate Δ*gifAgifB* double mutants, *Synechocystis* Δ*gifA* cells were transformed with the *gifB* gene-targeting vector pLIF2, and Km^R/Cm^R colonies were selected. To generate Δ*gifA* and Δ*gifB* mutants in the *Synechocystis* strain harboring the modified His-GS, pSIF4 and pLIF2 were used to transform *Synechocystis* HTGS1 (Km^R) strain, and Km^R/Cm^R colonies were selected. Mutants were tested by Southern blot analysis.

GS Assay. GS biosynthetic activity and GS transferase activity were determined as described previously (11, 18). One unit of GS activity corresponds to the amount of enzyme that catalyzes the synthesis of 1 μmol of glutamine or γ-glutamyl-hydroxamate per min.

RNA Isolation and Northern Blot Analysis. Total RNA from *Synechocystis* was isolated as described previously (19). For Northern blots, 15 μg of total RNA was loaded per lane and electrophoresed in 1% agarose denaturing formaldehyde gels. Transfer to nylon membranes (Hybond N$^+$; Amersham), prehybridization, hybridization, and washes were in accordance with Amersham instruction manuals.

GS, IF7, and IF17 Expression and Purification. To express *Synechocystis* GS type I in *E. coli*, a *Sal*I fragment from pJCR3 (20) containing the entire *Synechocystis glnA* gene was cloned into pBluescript SK(+) in the same orientation as the *plac* promoter. GS was purified from *E. coli* by ammonium sulfate precipitation and affinity chromatography in ADP-Sepharose. Specific activity of the pure enzyme was 175 milliunits/mg. To express IF7 and IF17, PCR-synthesized fragments encompassing *gifA* and *gifB* genes were inserted into pET-3a to generate pSET2 and pLET2, respectively. Exponentially growing *E. coli* BL21 cells transformed with the indicated plasmids were treated with 0.5 mM of isopropyl β-D-thiogalactoside for 5 h. IF7 was purified from the soluble fraction by cation-exchange chromatography on CM-52 cellulose (Whatman). IF17 was found to accumulate as insoluble inclusion bodies. The insoluble inclusion bodies were isolated, washed extensively with 1% Triton X-100, and then solubilized in U buffer (7 M urea/50 mM Hepes, pH 7.0/50 mM KCl). Solubilized inclusion bodies were subjected to ion-exchange chromatography on DEAE-cellulose. The flow-through fraction contained 95% purified IF17. Denatured IF17 was renatured by a 10-fold dilution in 50 mM Hepes, pH 7.0/50 mM KCl buffer. Urea was eliminated further by dialysis.

Protein–Protein Band Shift Experiments. The binding reactions were carried out in a final volume of 20 μl containing 2.25 μg (0.18 μM) of purified GS and increasing quantities of IF7 or IF17 in Hepes·NaOH buffer, pH 7.0/50 mM KCl. GS-IF complexes were allowed to form during 5 min at room temperature and then separated in 6% nondenaturing polyacrylamide gels run at 4°C. Complexes were visualized by gel staining with Coomassie blue.

Cross-Linking Experiments. 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 to a final concentration of 4 mM. Reactions were stopped after

15 min by addition of SDS-containing Laemmli loading buffer (17). Proteins were separated by SDS/PAGE, and gels were stained with Coomassie blue.

RESULTS

Purification of Inactive *Synechocystis* 6803 GS. Efforts to purify inactive GS from *Synechocystis* by classical chromatographic procedures were unsuccessful, because the GS became active during the purification procedure. Therefore, we decided to use Ni-affinity chromatography as a fast and efficient method to purify inactive GS from *Synechocystis*. For this purpose a *Synechocystis* strain (HTGS1) was constructed by replacing the wt *glnA* gene (structural gene for GS) by a histidine-tagged modified version. Ammonium-mediated inactivation of the modified His-GS *in vivo* was indistinguishable from the inactivation of wt GS (data not shown). The active His-GS was purified from nitrate-grown *Synechocystis* HTGS1 cells by Ni-affinity chromatography. The inactive enzyme was purified by the same procedure from nitrate-grown *Synechocystis* HTGS1 cells that were incubated with ammonium for 2 h. Whereas purified His-GS from nitrate-grown cells showed a specific activity of 175 units/mg, His-GS purified from ammonium-treated cells showed a specific activity of 63.5 units/mg, indicating that about 65% of the enzyme remained inactive after elution from Ni-affinity chromatography. These data were confirmed by *in vitro* reactivation of the inactive enzyme by using reactivation treatments described previously (pH or ionic strength increase) (10). Both active and inactive purified His-GS were subjected to SDS/PAGE. Surprisingly, two polypeptides of about 7 and 17 kDa copurified with the inactive His-GS but not with the active enzyme (Fig. 1*A*). These polypeptides were named IF7 (inactivating factor of 7 kDa) and IF17 (inactivating factor of 17 kDa).

IF7 and IF17 Are Encoded by Two Different Genes. Sequencing of IF7 and IF17 amino termini (see *Materials and Methods*) and comparison with the *Synechocystis* 6803 genome database (21) revealed that these polypeptides correspond to the ORFs *ssl1911* and *sll1515,* respectively. IF7 and IF17 encoding genes were named *gifA* and *gifB*, respectively (for glutamine synthetase inactivating factor). Comparative analysis of the amino acid sequences demonstrated significant sequence similarity between IF7 and the carboxyl terminus of IF17 (Fig. 1*B*). Comparison of *gifA*- and *gifB*-deduced amino acid sequences with the database revealed the existence of a previously unidentified ORF from *Anabaena* sp. PCC 7120, a filamentous cyanobacterium, that shares homology with both factors (Fig. 1*B*). Interestingly, this ORF is placed downstream and in the opposite orientation with respect to the *Anabaena glnA* gene (22).

***gifA* and *gifB* Mutants Are Impaired in GS Inactivation.** To test whether IF7 and IF17 were involved in the inactivation of *Synechocystis* GS, we constructed single (Δ*gifA* and Δ*gifB*) and double (Δ*gifAgifB*) deletion mutants. Δ*gifA*, Δ*gifB*, and Δ*gifA-gifB* mutants grew normally by using nitrate as nitrogen source. Levels of GS biosynthetic activity were determined at different times after ammonium addition to nitrate-grown wt and mutant cells. As described previously (11), wt GS activity decreased dramatically, reaching about 20% of the initial level 40 min after ammonium addition. However, GS inactivation was impaired severely in both the Δ*gifA* and the Δ*gifB* mutants and completely absent in the Δ*gifAgifB* double-mutant (Fig. 2*A*). These results clearly demonstrate that IF7 and IF17 are involved in the *Synechocystis* GS-inactivating mechanism. Because IF7 and IF17 copurified with GS, the mechanism of inactivation could involve the physical interaction of both inactivating factors with the enzyme. To verify whether each factor is able to bind independently to GS *in vivo*, we constructed Δ*gifA* and Δ*gifB* mutants in the *Synechocystis* HTGS1 strain harboring the modified His-GS. Small quantities of IF7
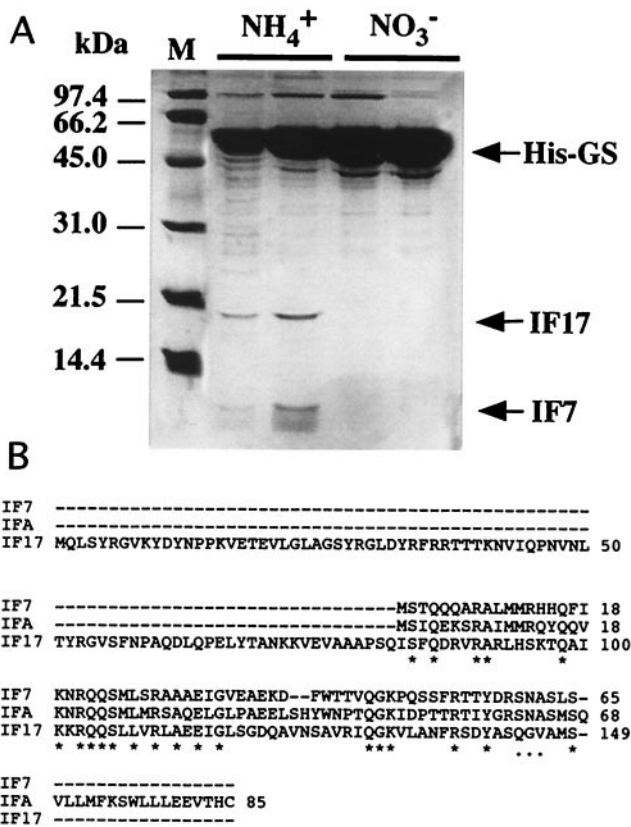
FIG. 1. (*A*) IF7 and IF17 copurify with inactive GS. His-GS was purified by Ni-affinity chromatography from nitrate-grown HTGS1 *Synechocystis* cells (NO$_3^-$) or from nitrate-grown HTGS1 *Synechocystis* cells treated with 5 mM ammonium chloride for 2 h (NH$_4^+$). Approximately 20 μg of protein was separated by SDS/PAGE and stained with Coomassie blue. (*B*) Alignment of the deduced amino acid sequence of *Synechocystis* IF7 (ORF *sll1911*), IF17 (ORF *ssl1515*), and *Anabaena* IF7 (previously unidentified ORF from GenBank accession no. X00147). Alignment was carried out by using the CLUSTALX program (29). Conserved residues are indicated by asterisks.

or IF17 copurified with His-GS from ammonium-treated HTGSI-Δ*gifB* or HTGSI-Δ*gifA* cells, respectively, correlating with the low level of GS inactivation observed in these strains. These results demonstrated that both inactivating factors were able to bind independently to the GS *in vivo* (Fig. 2*B*).

Expression of *gifA* and *gifB*. Northern blot experiments demonstrated that *gifA* and *gifB* mRNA levels were transiently induced by ammonium. Thus, *gifA* and *gifB* mRNA levels in
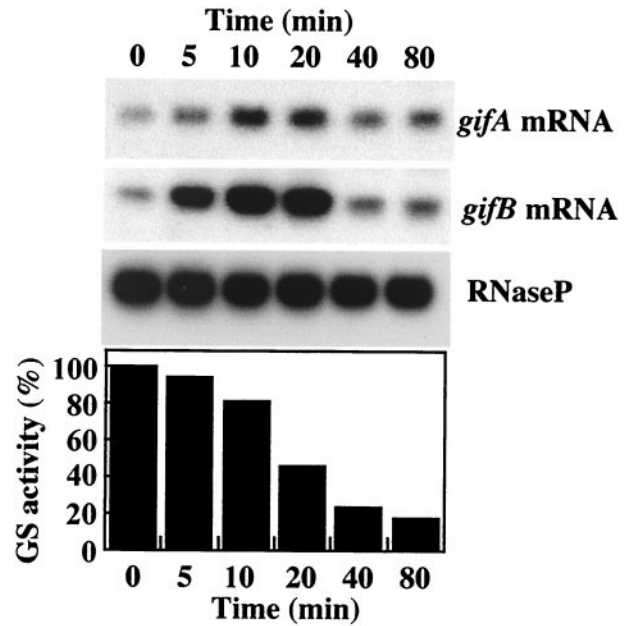

FIG. 3. Transient induction of *gifA* and *gifB* mRNA levels. Ammonium chloride (5 mM) was added to mid-log *Synechocystis* wt cells at *t* = 0. Samples were taken at the indicated times, and total RNA was isolated and analyzed by Northern blotting. PCR-synthesized fragments, encompassing the entire *gifA* or *gifB* genes, were used as probes. The filter was rehybridized with a probe for the constitutively expressed RNase P RNA gene as control (31). GS transferase activity of the same cultures, at the indicated times, is shown in the graph at the bottom. One hundred percent activity corresponds to 1.5 units/mg of protein.

nitrate-grown cells were low, increasing dramatically 5 min after ammonium addition and reaching the highest levels at *t* = 20 min. One hour later, mRNA quantity returned to levels only 2-fold higher that those present in nitrate (Fig. 3). Determination of GS activity levels in the same experiment showed a temporal delay between the maximal *gifA* and *gifB* mRNA accumulation and the maximal GS inactivation. Thus, *gifA* and *gifB* mRNA levels started to decay between 20 and 40 min after ammonium addition, when GS has not reached complete inactivation (Fig. 3). These experiments suggest that levels of *gifA* and *gifB* mRNA are finely controlled by a feedback mechanism and that GS inactivation may be the consequence of increasing the intracellular levels of IF7 and IF17.

***In Vitro* Reconstitution of the GS Inactivation.** To characterize further the GS-IF interaction *in vitro*, we purified *Synechocystis* GS, IF7, and IF17 expressed in *E. coli* (Fig. 4*A*).
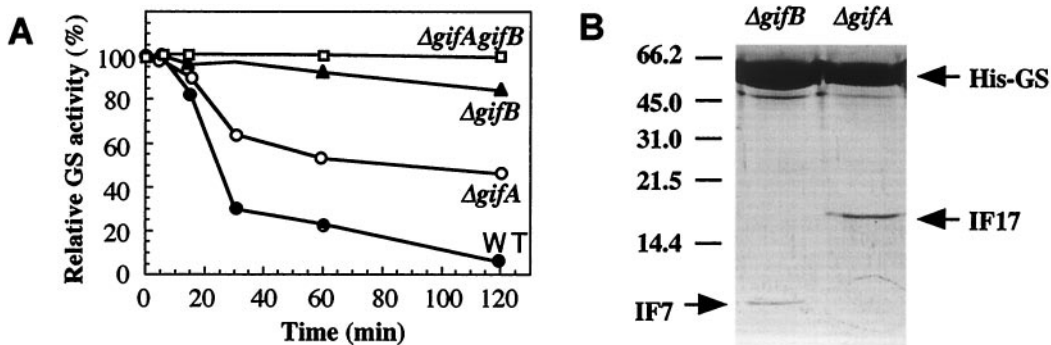

FIG. 2. (*A*) *In vivo* ammonium-dependent GS inactivation in *Synechocystis* wt and Δ*gifA*, Δ*gifB*, and Δ*gifA*Δ*gifB* mutants. wt, Δ*gifA*, Δ*gifB*, and Δ*gifA*Δ*gifB* *Synechocystis* cells were grown in BG11 medium by using nitrate as nitrogen source. Ammonium chloride (5 mM) was added at *t* = 0, and GS biosynthetic activity was determined, *in situ*, at the indicated times. (*B*) Δ*gifA* and Δ*gifB* *Synechocystis* HTGS1 cells were grown in BG11 medium and treated with 5 mM of ammonium chloride for 2 h. His-GS was purified by Ni-affinity chromatography and subjected to SDS/PAGE and Coomassie blue staining.
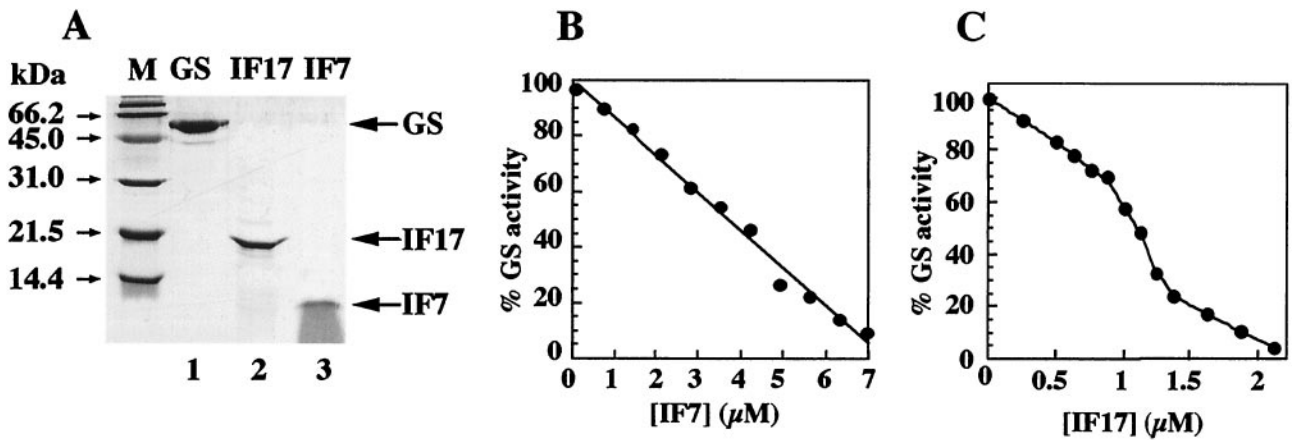
FIG. 4.   Reconstitution of *Synechocystis* GS inactivation *in vitro*. (*A*) SDS/PAGE (15%) of purified GS, IF17, and IF7 proteins. Lanes: 1, 2 μg of purified GS; 2, 1 μg of purified IF17; 3, 1 μg of purified IF7. *Synechocystis* GS (2.25 μg) was incubated with increasing quantities of IF7 (*B*) and IF17 (*C*) in a final volume of 20 μl. Inactive GS-IF complexes were allowed to form during 5 min, and GS transferase activity was determined. One hundred percent activity corresponds to 0.4 unit of GS.

Purified IF7 and IF17 inhibited GS activity *in vitro* (Fig. 4 *B* and *C*). Addition of both factors together in equimolecular concentrations did not increase the inhibitory effect (data not shown). These results demonstrate that either IF7 or IF17 is sufficient *per se*, without additional modifications, for GS inactivation. GS inactivation exhibited a sigmoidal dependence on IF17 concentration, suggesting that this factor binds cooperatively to the GS. However, a linear response was observed for IF7-dependent inactivation. GS activity could be recovered by increasing the pH of the sample (up to pH 9), a treatment that has been shown previously to reactivate inactive GS in extracts from ammonium-treated cells (10). GS-IF interaction was demonstrated and visualized by protein–protein band shift experiments. Increasing amounts of IF7 and IF17 retarded the GS protein band in nondenaturing gels (Fig. 5 *A* and *B*). The

presence of IF7 or IF17 in retarded complexes was demonstrated by excision of the slower-migrating band from the nondenaturing gels and migration on SDS/PAGE gels. GS and IF7 or IF17 were separated (data not shown). Purified GS from the cyanobacterium *Anabaena azollae* was not retarded by IF7 or IF17, indicating that both factors interact specifically with the *Synechocystis* GS. The mobility shift caused by IF7 was minor compared with the one caused by IF17. Whereas one major band was observed in GS-IF7 interaction experiments, up to six different GS-IF17 forms were visible in GS-IF17 band shift assays (Fig. 5*B*). Direct interaction was demonstrated further by cross-linking experiments. Thus, treatments of mixes containing GS and IF7 or IF17 with the water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide resulted in the formation of covalent complexes between
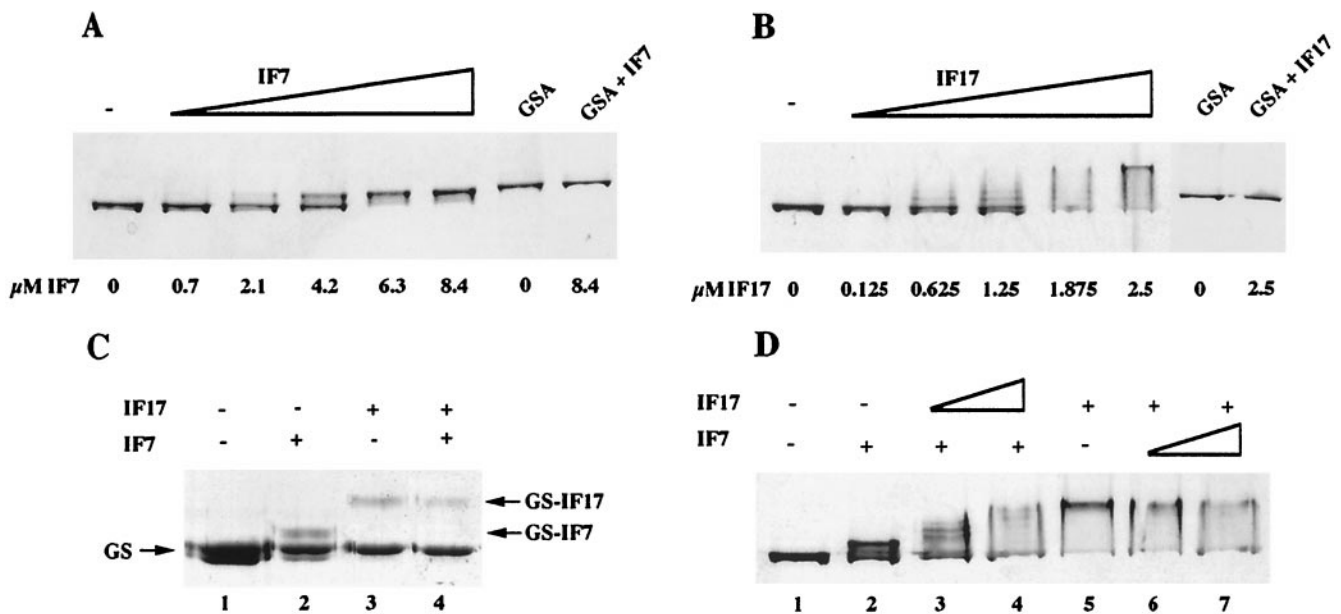

FIG. 5.   IF7 and IF17 interact *in vitro* with GS. Synechocystis GS (0.18 μM) was incubated with increasing quantities of IF7 (*A*) and IF17 (*B*) in a final volume of 20 μl. Inactive GS-IF complexes were allowed to form during 5 min and then separated in a 6% nondenaturing polyacrylamide gel and stained with Coomassie blue. Purified *Anabaena azollae* GS (GSA) was incubated with IF7 (*A*) or IF17 (*B*). (*C*) Purified GS (0.18 μM) was incubated in the absence of IF (lane 1), with 7 μM IF7 (lane 2), 2 μM IF17 (lane 3), or 7 μM IF7 and 2 μM IF17 (lane 4) in a final volume of 20 μl. Cross-linking reactions with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were carried out for 15 min. Cross-linking products were visualized by SDS/PAGE and Coomassie blue staining. (*D*) Purified GS (0.18 μM) was incubated with 7 μM of IF7 (lanes 2–4) or 2 μM IF17 (lanes 5–7) for 10 min. Then, increasing amounts of IF17 (1 and 2 μM) were added to samples 3 and 4, and increasing amounts of IF7 (3.5 μM and 7 μM) were added to samples 6 and 7. Competition binding was allowed for an additional 10-min period, and GS-IF complexes were visualized by 6% nondenaturing PAGE.

GS subunits and IF7 or IF17, which were resolved by SDS/PAGE (Fig. 5C). However, only IF17 was cross-linked to GS subunits when both IF7 and IF17 were added simultaneously to the enzyme (Fig. 5C). In addition, mobility-shift competition assays showed that GS-IF7 complexes were disrupted in the presence of IF17 but GS-IF17 complexes were not disrupted by IF7 (Fig. 5D). These results suggest that IF17 has a higher affinity for the GS than IF7. This conclusion also was supported by the inactivation kinetic data (Fig. 4 B and C).

DISCUSSION

Phylogenetic analysis has revealed that the structural gene for GS is one of the oldest functioning genes (23), and, therefore, GS could be one of the oldest functioning enzymes. Probably, this long evolutionary history together with its central role in metabolism have determined the existence of very sophisticated mechanisms to control GS activity. We describe a regulation mechanism of the cyanobacterial GS activity that is different from the classical adenylylation mechanism present in many prokaryotes. The *Synechocystis* system involves the direct interaction of two inhibitory peptides (IF7 and IF17) with the GS. The results presented here suggest a model in which the presence of ammonium, a nitrogen-rich source, triggers the transcriptional induction of *gifA* and *gifB* genes, increasing the synthesis of IF7 and IF17. These factors then are able to bind to the GS, provoking enzyme inactivation.

Two different aspects of the amino acid composition of IF7 and IF17 are remarkable. First, glutamine and arginine are the most abundant amino acids in both IF7 (13.7% Gln and 12.5% Arg) and IF17 (9.2% Gln and 12.2% Arg). Gln is the enzymatic product of GS. Glutamine intracellular pool increases about 60-fold after ammonium addition to nitrate-grown cyanobacteria (11), coinciding with the time when IF7 and IF17 are synthesized. Arginine is the amino acid with the highest nitrogen content, and, therefore, its synthesis is favored under rich nitrogen conditions. In addition, arginine and citrulline (an intermediate in arginine biosynthesis) are labeled shortly after assimilation of $^{13}NH_4$ in some cyanobacteria (24). That Gln and Arg, which constitute about 25% of the amino acids of IF7 and IF17, are abundant during the time that both factors are synthesized may enhance the rate of *gifA* and *gifB* mRNA translation. In addition, this particular amino acid composition of both factors may be considered a kind of product-feedback mechanism regulating the ammonium assimilation pathway. The second interesting point in the amino acid composition of IF7 and IF17 is the abundance of positively charged residues. The isoelectric points of IF7 and IF17 are 11.2 and 10.9, respectively. GS can be reactivated in crude extracts by increasing the pH or the ionic strength (10). These results also have been confirmed with purified GS and IFs (data not shown). These experiments indicate that GS-IF complex formation may be determined by electrostatic interactions. The positive charge of both IFs suggests that the GS-interaction site could be a negatively charged region of the enzyme.

Predicted secondary structure (using PHDSEC program from the PredictProtein Server, European Molecular Biology Laboratory) (25) of both IFs revealed the probable presence of an α-helix comprising amino acids 85–115 of the IF17 sequence and amino acids 3–45 of the IF7 sequence. The three IF sequences available show a high amino acid identity in this region (Fig. 1B), suggesting that it may be involved in the interaction with the GS. That both *Synechocystis* IFs show structural and sequence similarities also suggests that both factors interact with the same region of the GS. Which region this is and how binding of IF7 and IF17 leads to the inhibition of GS activity are points that remain to be elucidated. Mobility-shift experiments revealed one major band containing GS-IF7 complexes. In contrast, up to six different bands were visible in GS-IF17 interaction experiments, suggesting that

binding of at least six IF17 polypeptides is required for complete GS inactivation. Further experiments are required to determine the exact stoichiometry of the inactive GS-IF complexes *in vivo*.

IF7 and IF17 expressed in *E. coli* are able, without further modification, to bind and inactivate the GS. This result together with the rapid increase in *gifA* and *gifB* expression after ammonium addition suggest that binding of IFs to GS is determined only by the intracellular concentration of both factors. The effects of IF7 and IF17 *in vivo* seem to be cumulative, raising the question of why two inactivating factors are required. One possibility is that expression of IF7 and IF17 responds to different environmental cues in addition to the nitrogen source. Moreover, the different inactivation kinetics displayed by each factor points to a distinct role for each IF under specific conditions.

That a protein homologous to IF7 and IF17 is present in *Anabaena* sp. strain PCC 7120, a cyanobacterium phylogenetically distant from *Synechocystis,* suggests that a system of GS activity control similar to the one that we describe here is extended broadly in cyanobacteria. Ammonium-promoted down-regulation of GS from other cyanobacterial strains also has been observed (ref. 26 and our unpublished observations). However, it is unknown whether a similar system of GS inactivation operates in other prokaryotic groups.

One major difference between the system of GS modification by adenylylation and the cyanobacterial system is that whereas adenylylation provokes a high sensitivity to feedback inhibition, GS-IF complex formation seems to yield a completely inactive enzyme. Adenylylation constitutes an example of the regulatory cascades in which enzymes at one level modulate the activities of enzymes at the subsequent level. Such cascades provide distinct control potential, including signal amplification and increased controllability (27, 28). In addition, covalent modifications are faster response systems than those involving alterations in gene expression. From this point of view, the cyanobacterial mechanism could be considered more rudimentary than the adenylylation system. However, regulation by binding of inhibitory peptides is a solution used extensively to modulate critical enzymatic activities. For example, control of cell cycle progression in eukaryotes is regulated by a number of cyclin-dependent kinase inhibitors such as p15, p19, p21, p27, etc. (25). Furthermore, protease enzyme activities from eukaryotic and prokaryotic origins also are often controlled by inhibitory peptides (29, 30). Studies on the enzymology and the metabolic control of GS have contributed enormously to biochemical knowledge in the past. The molecular interactions involved in the binding of IF7 and IF17 to GS and the molecular bases of the inhibition of GS constitute an interesting model to understand biochemical regulatory processes based on protein–protein interaction.

1. Reitzer, L. J. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology*, eds. Neidhardt, F. C., Curtiss, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. & Umbarger, H. E. (Am. Soc. Microbiol., Washington, DC), 2nd Ed., pp. 391–407.
2. Merrick, M. J. & Edwards, R. A. (1995) *Microbiol. Rev.* **59,** 604–622.
3. Magasanik, B. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology*, eds. Neidhardt, F. C., Curtiss, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B.,

Reznikoff, W. S., Riley, M., Schaechter, M. & Umbarger, H. E. (Am. Soc. Microbiol., Washington, DC), 2nd Ed., pp. 1344–1356.

4. Yamashita, M. M., Almassy, R. J., Janson, C. A., Cascio, D. & Eisenberg, D. (1989) *J. Biol. Chem.* **264,** 17681–17690.
5. Almassy, R. J., Janson, C. A., Hamlin, R., Xuong, N.-H. & Eisenberg, D. (1986) *Nature (London)* **323,** 304–309.
6. Rhee, S. G., Chock, P. B. & Stadtman, E. R. (1989) *Advances in Enzymology*, ed. Meister, A. (Wiley Interscience, New York), Vol. 62, pp. 37–92.
7. Stadtman, E. R. (1990) *Methods Enzymol.* **182,** 793–809.
8. Deuel, T. F. & Prusiner, S. (1974) *J. Biol. Chem.* **249,** 257–264.
9. Fisher, R., Tuli, R. & Haselkorn, R. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3393–3397.
10. Mérida, A., Candau, P. & Florencio, F. J. (1991) *Biochem. Biophys. Res. Commun.* **181,** 780–786.
11. Mérida, A., Candau, P. & Florencio, F. J. (1991) *J. Bacteriol.* **173,** 4095–4100.
12. Reyes, J. C. & Florencio, F. J. (1995) *FEBS Lett.* **367,** 45–48.
13. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. (1979) *J. Gen. Microbiol.* **111,** 1–61.
14. Elhai, J. & Wolk, C. P. (1988) *Gene* **68,** 119–138.
15. Chauvat, F., De Vries, L., Van der Ende, A. & Van Arkel, G. A. (1988) *Mol. Gen. Genet.* **204,** 165–191.
16. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
17. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
18. Marqués, S., Florencio, F. J. & Candau, P. (1989) *Anal. Biochem.* **180,** 152–157.
19. García-Domínguez, M. & Florencio, F. J. (1997) *Plant. Mol. Biol.* **35,** 723–734.
20. Reyes, J. C. & Florencio, F. J. (1994) *J. Bacteriol.* **176,** 1260–1267.
21. Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., *et al.* (1996) *DNA Res.* **3,** 109–136.
22. Tumer, N. E., Robinson, S. J. & Haselkorn, R. (1983) *Nature (London)* **306,** 337–342.
23. Kumada, Y., Benson, D. R., Hillemann, D., Hosted, T. J., Rochefort, D. A., Thompson, C. J., Wohlleben, W. & Tateno, Y. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3009–3013.
24. Meeks, J. C., Wolk, C. P., Thomas, J., Lockau, W., Shaffer, P. W., Austin, S. M., Chien, W. S. & Galonsky, A. (1977) *J. Biol. Chem.* **252,** 7894–7900.
25. Rost, B. & Sander, C. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7558–7562.
26. Rowell, P., Sampaio, M. J. A. M., Ladha, J. K. & Stewart, W. D. P. (1979) *Arch. Microbiol.* **120,** 195–200.
27. Mura, U., Chock, P. B. & Stadtman, E. R. (1981) *J. Biol. Chem.* **256,** 13022–13029.
28. Mura, U. & Stadtman, E. R. (1981) *J. Biol. Chem.* **256,** 13014–13021.
29. Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680.
30. Laskowski, M., Jr., & Kato, I. (1980) *Annu. Rev. Biochem.* **49,** 593–626.
31. Vioque, A. (1992) *Nucleic Acids Res.* **20,** 6331–6337.