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A core of three amino acids at the carboxyl-terminal region of glutamine synthetase defines its regulation in cyanobacteria

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1 **Summary**

2 Glutamine synthetase type I (GS) is a key enzyme in nitrogen metabolism, and its activity is finely
3 controlled by cellular carbon/nitrogen balance. In cyanobacteria, a reversible process that involves
4 protein-protein interaction with two proteins, the inactivating factors IF7 and IF17, regulates GS.
5 Previously, we showed that three arginine residues of IFs are critical for binding and inhibition of GS. In
6 this work, taking advantage of the specificity of GS/IFs interaction in the model cyanobacteria
7 *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120, we have constructed different chimeric GSs
8 from these two cyanobacteria. Analysis of these proteins, together with a site-directed mutagenesis
9 approach, indicates that a core of three residues (E419, N456 and R459) is essential for the inactivation
10 process. The three residues belong to the last 56 amino acids of the C-terminus of *Synechocystis* GS. A
11 protein-protein docking modeling of *Synechocystis* GS in complex with IF7 supports the role of the
12 identified core for GS/IF interaction.

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1 Introduction

2 The glutamine synthetases (GSs, GS: E.C. 6.3.1.2) are a family of large, oligomeric enzymes that catalyze
3 the condensation of ammonium and glutamate to form glutamine, the main nitrogen source for protein
4 and nucleic acid synthesis. GS is present in both prokaryotic and eukaryotic organisms because it is
5 critical to nitrogen metabolism (Robertson & Tartar, 2006). The GS superfamily includes three distinct
6 classes, GS type I, II and III, each differing in molecular size and number of subunits in the holoenzyme
7 (Eisenberg *et al.*, 2000). GS type I, encoded by *glnA*, is a dodecameric enzyme composed of identical
8 subunits (*Mr*, ca. 50,000) and is found exclusively in bacteria and archaea (Brown *et al.*, 1994, Yamashita
9 *et al.*, 1990).

10 GS performs the enzymatic mechanism in two steps to yield glutamine and ADP. The first step of the
11 biosynthetic reaction is the formation of the activated intermediate γ -glutamyl phosphate. A metal ion
12 (magnesium or manganese) coordinates the phosphate oxygen atoms of ATP to allow phosphoryl transfer
13 to the carboxylate group of glutamate, yielding the intermediate. In the second step, ammonia attacks the
14 intermediate and releases the products, a free phosphate and glutamine (Eisenberg *et al.*, 2000, Gill *et al.*,
15 2002). Residues 323-330 of *Salmonella thyphimurium* form a loop, termed “the Glu327 flap”, that closes
16 the glutamate entrance to shield the intermediate from water hydrolysis. After the phosphoryl group is
17 transferred and the ammonia attacks the intermediate, the Glu327 flap opens the entrance to release
18 glutamine.

19 Countless studies have established that GS occupies a central position in the regulation of nitrogen
20 metabolism (Leigh & Dodsworth, 2007, Reitzer, 2003, Stadtman, 2001). In *E. coli*, GS is regulated by
21 several mechanisms, including (a) cumulative feedback inhibition by multiple end products of glutamine
22 metabolism, (b) regulated expression of *glnA*, and (c) reversible covalent modification of each subunit;
23 responding to carbon and nitrogen signals. Thus, in the presence of abundant carbon sources, nitrogen
24 deficiency results in a high level of GS activity. By contrast, when nitrogen is abundant, GS activity is
25 down regulated.

1 Two types of covalent modifications can regulate the catalytic activity of bacterial GS:
2 adenylylation/deadenylylation of a specific tyrosine residue (Ginsburg *et al.*, 1970) and oxidative
3 modification (Levine *et al.*, 1996, Liaw *et al.*, 1993). In *E. coli*, adenylylation of Tyr397 leads to
4 alteration of various catalytic properties, including the inactivation of the biosynthetic activity in the
5 presence of Mg²⁺ (Shapiro *et al.*, 1967, Wulff *et al.*, 1967). The degree of adenylylation depends on the
6 glutamine and 2-OG levels. For instance, higher glutamine level causes more monomers to be
7 adenylylated, thereby producing lower activity of glutamine synthetase.

8 In some archaea, GS is regulated by direct interaction with PII signaling proteins, specifically GlnK
9 (Ehlers *et al.*, 2005, Pedro-Roig *et al.*, 2013). In the case of *Bacillus subtilis*, GS itself has a regulatory
10 role by directly interacting with transcription factors (TnrA and GlnR) (Wray *et al.*, 2001). Although the
11 enzyme is mostly controlled by feedback inhibition, the TnrA protein also inhibits GS activity (Fedorova
12 *et al.*, 2013). In cyanobacteria we have previously shown that under high nitrogen conditions GS activity
13 in *Synechocystis* sp. PCC 6803 decreases when two small peptides that behave as inactivating factors are
14 present (IF7 and IF17, of 7 kDa and 17 kDa, encoded by *gifA* and *gifB* genes, respectively). The analysis
15 of mutant strains lacking one or both IFs revealed that each of these proteins contributes to GS regulation
16 by inactivation *in vivo*. A maximal level of inactivation of GS was observed when both proteins were
17 present (García-Domínguez *et al.*, 1999). In contrast, the filamentous cyanobacterium *Anabaena* sp. PCC
18 7120 possesses a single *gifA* gene that encodes an IF7-like protein, named IF7A (Galmozzi *et al.*, 2010).
19 The C-terminus of IF17 is 37% identical to IF7 and 34% identical to IF7A, whereas IF7 and IF7A are
20 53% identical.

21 We have previously shown that three arginine residues of IF7 (R8, R21 and R28) and their homologous
22 residues of IF17 (R90, R103 and R110) are essential for the interaction with GS (Saelices *et al.*, 2011a).
23 These residues, conserved in all ORFs homologous to IF7 and IF17, are located in the same positions in
24 IF7 and IF7A.

1 Expression of *gif* genes depends on nitrogen status (Galmozzi *et al.*, 2010, García-Domínguez *et al.*,
2 2000), which is perceived as changes in the intracellular pool of 2-oxoglutarate (Muro-Pastor *et al.*,
3 2001).

4 It is worth noting that there is a marked GS/IF specificity between *Synechocystis* and *Anabaena*, despite
5 the high similarity between their GSs. *Synechocystis* and *Anabaena* GS sequences are 77.7% identical.
6 While *Synechocystis* GS (SyGS) can be down regulated by IF7, IF17 and IF7A, *Anabaena* GS (AnGS) is
7 only inactivated by IF7A (Galmozzi *et al.*, 2010). Thus, we hypothesized that only a few residues should
8 be responsible for this specificity, and that those residues would likely be involved in IF recognition
9 and/or interaction.

10 Considering the different GS/IFs interactions of *Synechocystis* and *Anabaena*, we designed and analyzed
11 different chimeric proteins, as well as mutated variants of SyGS and AnGS. Our results indicate that IFs
12 down regulate GS through the C-terminal residues glutamate 419, asparagine 456 and arginine 459. A
13 computational model of the SyGS structure that predicts that the inactivating factor IF7 binds indeed to
14 this region further supports our studies.

15 **Results**

16 *Cyanobacterial GS inactivation takes place in the C-terminus.* *Anabaena* GS (AnGS) cannot be
17 inactivated by *Synechocystis* IFs; however, IF7A from *Anabaena* fully inactivates *Synechocystis* GS
18 (SyGS) (Galmozzi *et al.*, 2010). First, we constructed chimeric proteins in order to identify the region
19 responsible for this specificity. Three different fragments of *Synechocystis glnA*, encoding the N-terminus
20 of SyGS, were cloned and fused to various fragments of AnGS *glnA*, leading to three chimeric genes
21 (resulting proteins, Chi1, Chi2 and Chi3, are schematized in Fig. 1A). Purified chimeric proteins were
22 enzymatically active and characterized by gel electrophoresis (Fig. S1). They were analyzed for GS
23 inactivation with partners IF7, IF17 or IF7A (Fig. 1B). AnGS and SyGS were analyzed as controls. It is
24 noteworthy that Chi1, Chi2, and Chi3 chimeras behaved as AnGS. They are inactivated by IF7A, but not
25 by IF7 or IF17. Since Chi3 contains only a short sequence from AnGS (Fig. 1A), we inferred that the

1 region of 56 amino acid residues of the C-terminus corresponds to the region involved in the specificity
2 and hence critical for IF7 and IF17-mediated GS inactivation in *Synechocystis*. As a control, the reverse
3 version of Chi3, containing only the last 56 residues from SyGS (Chi4) was also cloned and analyzed
4 (Fig. 1A and 1B). As expected, Chi4 is inactivated by IF7, IF17 and IF7A, confirming that the C-terminal
5 56-residue segment from cyanobacterial GS is responsible for the IF specificity *in vitro*.

6 *In vivo* analysis of chimeric proteins was consistent with the *in vitro* results. The *Synechocystis glnA* gene
7 was replaced by each chimeric version by transformation, generating SChi1-SChi4 strains (Table 1 and
8 Fig. S1 C-D). The correct *glnA* expression in these strains was tested by Northern blot (Fig. S3) and their
9 GS specific activity was similar to that of the wild type *Synechocystis* strain (Fig. S1E). We studied GS
10 inactivation by ammonium addition in the strains containing chimeric versions, in comparison to wild
11 type (Syn6803), and $\Delta gifA \Delta gifB$ strains (García-Domínguez *et al.*, 1999) (Fig. 1C). Consistent with the *in*
12 *vitro* results, SChi1-SChi3 strains do not show GS inactivation (Fig. 1C). In addition we analyzed *in vivo*
13 accumulation of IF7 and IF17, that requires interaction with GS (Galmozzi *et al.*, 2007). IF7 and IF17 do
14 not accumulate in strains SChi1-SChi3, confirming lack of interaction (Fig 1D). However, the SChi4
15 strain shows GS regulation kinetics similar to that of the wild-type strain (Fig. 1C). In turn, IF7 and IF17
16 accumulate in SChi4 strain due to their interaction with GS (Fig. 1D). These results determine that the
17 last 56 residues of the GS drive the specificity of the enzymatic regulation in cyanobacteria.

18 *Two GS residues are involved in the specific GS/IF interaction in Synechocystis and Anabaena*. Taking
19 into account that the GS/IF interaction has an electrostatic nature (Mérida *et al.*, 1991a, Saelices *et al.*,
20 2011a), the observed specificity between *Synechocystis* and *Anabaena* GS inactivation could be due to
21 differential repulsion and/or attraction pattern. We analyzed the sequence alignment of the last 56 C-
22 terminal residues of SyGS and AnGS (Fig. 2A). We considered every residue that was differentially
23 charged between the two proteins for a mutational analysis (Fig. 2A, shaded residues). In SyGS we made
24 point substitutions of each of the identified residues to that in the corresponding position in AnGS. A
25 fixed amount of each purified GS variant was used in inactivation assays with 2 μ M of IF7, IF17 or IF7A.

1 As shown in Fig. 2B, two single mutations in SyGS that changed completely the inactivation outline,
2 were identified: the SyGS-N456K variant was unable to be inactivated by any inactivation factor (IF7,
3 IF17 or IF7A) and SyGS-R459Q displayed impaired inactivation by IF7 or IF17. We focused on these
4 two residues as the rest of the changes did not alter significantly the pattern of inactivation by the
5 different IFs. Two different experiments tested GS protein function. First, inactivation assays with
6 increasing amounts of IF7, IF17 or IF7A proteins were performed (Fig. 3A). Second, GS/IF interaction
7 was tested by protein-protein gel shift experiments (Fig. 3B). Surprisingly, the introduction of a lysine in
8 the position 456 of SyGS hindered inactivation by IF7A as well, although AnGS presents this lysine in
9 that position. Next, we constructed the double mutant SyGS-N456K/R459Q to check whether the
10 combination of both mutations allows the inactivation by IF7A. Interestingly, the SyGS-N456K/R459Q
11 mutant perfectly mimics the inactivation profile shown by AnGS, inactivation by IF7A but not by IF7 or
12 IF17 (Fig. 2B and Fig. 3A). In addition we constructed a reverse double mutant, AnGS-K457N/Q460R,
13 that contains *Synechocystis* GS residues at the positions homologous to N456 and R459. This mutant is
14 inactivated by IF7, IF17 and IF7A as SyGS (Fig. 3A). These results were consistent with protein-protein
15 gel shift assays, except for IF17, which showed interaction with both SyGS-N456K and SyGS-
16 N456K/R459Q mutants, although this interaction did not cause GS inactivation (Fig. 3B). To study if the
17 residues N456 and R459 of SyGS are also responsible for the specificity *in vivo*, *Synechocystis glnA* gene
18 was replaced by the SyGS-N456K, SyGS-R459Q or SyGS-N456K/R459Q expressing *glnA* variants by
19 transformation (Table 1 and Fig. S2). The *glnA* expression level in the resulting strains was tested by
20 Northern blot (Fig. S3). GS inactivation in these strains, compared with the $\Delta gifA \Delta gifB$ and wild type
21 (Syn6803) strains, supported the results obtained *in vitro*. Strains expressing SyGS-N456K, SyGS-R459Q
22 or SyGS-N456K/R459Q variants did not show inactivation by *Synechocystis* IFs (Fig. 4A). We analyzed
23 accumulation of IFs after ammonium addition, IF7 accumulated only in wild type (Syn6803). IF17
24 accumulation was barely detectable in SN456K and SR459Q strains and undetectable in SN456K/R459Q
25 strain (Fig. 4B). Taking together, these *in vitro* and *in vivo* results strongly indicate that the residues N456

1 and R459 in SyGS and their analogues K457 and Q460 in AnGS are critical for the specificity of the
2 *Synechocystis* and *Anabaena* GS/IF interaction and GS inactivation processes.

3 *Impaired GS-regulation leads to altered Gln/Glu balance.* We have previously shown that the addition of
4 ammonium to nitrate-grown *Synechocystis* cells provokes a quick and dramatic change in the intracellular
5 pools of Glu and Gln, that are completely restored to the original levels ~30 min after ammonium shift
6 (Mérida *et al.*, 1991b). This restoration to the steady-state levels is impaired in the $\Delta gifA\Delta gifB$ strain and
7 therefore it is the consequence of the GSI inactivation (Muro-Pastor *et al.*, 2001). We hypothesized that
8 mutant strains harboring GS variants not susceptible to inactivation by ammonium must also be impaired
9 in restoring amino acid homeostasis upon this strong change in nitrogen availability. As expected, similar
10 to what happens in a $\Delta gifA\Delta gifB$ strain (Muro-Pastor *et al.*, 2001) and in contrast to what happens in the
11 wild type strain (Syn6803), in the SN456K strain the Gln pool increased continuously after ammonium
12 was added (Fig. 4C).

13 *Inactivation of SyGS is driven by a core of three residues in vitro.* In order to address the question of
14 which residues of SyGS are involved in enzyme inactivation, we decided to examine the biochemical
15 environment of N456 and R459, using the crystal structure of SyGS (PDB ID 3NG0). It is noteworthy the
16 remarkable number of negatively-charged solvent-exposed residues close to N456 and R459. Since the
17 GS/IFs interaction is electrostatic (Mérida *et al.*, 1991a), N456 and R459, together with other charged,
18 solvent-exposed residues adjacent to them (Fig. 2A, squared residues) were substituted by the nonpolar
19 amino acid alanine or by an oppositely-charged residue; residue N456 was mutated to both charges.
20 These two types of substitutions would allow us to check both the contribution of a particular charged
21 residue (alanine substitutions) and, given the electrostatic nature of the GS/IF interaction, the putatively
22 more drastic effect of the introduction of an opposite charge (change of charge substitutions). The *in vitro*
23 GS inactivation analysis of single mutants showed that N456, R459, and a third amino acid residue E419
24 are key sites for interaction and inactivation of GS mediated by both IF7 and IF17 (Fig. 5B and Table 2).
25 Substitution of E419, N456 or R459 by alanine entailed partial or total loss of IF7 and/or IF17-mediated

1 inactivation. In order to determine whether an accumulative effect controls the GS-IF complex formation,
2 we designed and analyzed the triple alanine mutant SyGS-E419A/N456A/R459A that shows total loss of
3 IF7 and partial loss of IF17-mediated GS inactivation (Table 2). It is worth noting that some amino acid
4 substitutions differentially affect IF7 and IF17 function. SyGS-E423K and SyGS-E448K mutants were
5 mainly impaired in IF7-mediated inactivation while SyGS-D452K mutant is much more affected in IF17-
6 mediated inactivation (Table 2). However, the substitutions E419K, N456K, and R459E caused the total
7 loss of GS inactivation mediated either by IF7 or IF17 (Fig. 5B). For these three key residues (E419,
8 N456 and R459) we analyzed additional mutants harboring conservative changes. The replacements
9 E419D, N456Q or R459K (Table 2) caused a partial decay in GS inactivation. These data suggest that the
10 specific side chain identity is important for the regulation mechanism. These results were consistent with
11 protein-protein gel shift assays, except for IF17, which showed interaction with all mutants, although this
12 interaction did not always cause GS inactivation (Fig. 5C).

13 *GS inactivation is also coordinated by the three-residue core in vivo.* To corroborate whether the three-
14 residue core identified *in vitro* is also critical for GS regulation *in vivo*, the *Synechocystis glnA* gene was
15 replaced by mutated variants of these residues (Table 1 and Fig. S2). *glnA* expression was verified by
16 Northern blot (Fig. S3). Consistent with the *in vitro* data, GS inactivation after ammonium addition was
17 impaired in strains expressing SyGS variants with E419, N456 or R459 substitutions (Fig. 6A). Strains
18 with change-of-charge substitutions exhibited no GS inactivation while those with single alanine
19 substitutions showed partial GS inactivation. The strain with the triple alanine mutant displays little GS
20 inactivation (Fig. 6A). Accordingly, strains expressing GS variants able to bind IF7 or IF17 in gel shift
21 assays also showed IF7 or IF17 accumulation *in vivo*, respectively (Fig. 6B). Thus, SE419K, SN456K
22 and SR459E strains did not accumulate IF7 and slightly accumulated IF17. SE419A and SR459A strains
23 accumulate a significant amount of IF7 and IF17. The SN456A and SE419A/N456A/R459A strains
24 accumulated high amount of IF17 but no IF7. The results so far strongly indicate that IF/GS interaction

1 and inactivation require the coordination of the inactivating factors by the triangle formed by E419, N456
2 and R459 residues (Fig. 5A).

3 We wanted to investigate the reason way IF17 is able to interact, to some extent, with almost all the GS
4 variants, although in some cases such interaction does not cause inactivation. Previous results of our
5 group suggested that binding of IF17 to the GS is modulated not only by its C-terminal portion
6 (homologous to IF7 and essential for enzyme inactivation), but also by its N-terminus (82-residue-long
7 amino-terminal part not present in IF7) (Saelices *et al.*, 2011b). To analyse if IF17 N-terminal region, not
8 involved in GS inactivation, mediates its interaction with SyGS-N456K we have constructed a SN456K
9 strain expressing a previously characterized chimeric IF, containing the amino-terminal part of IF17 fused
10 to IF7 (IF17N/IF7) (Saelices *et al.*, 2011a), in a genetic background devoid of IF17. IF7 does not
11 accumulate in cells harbouring SyGS-N456 substitutions (Fig. 6B) because it does not interact with these
12 GS versions. Therefore we hypothesized that if the IF17N/IF7 protein accumulates in a SyGS-N456K
13 expressing mutant, it must be interacting with GS by its 82-residue-long IF17N region. The results
14 indicate, as expected, that the SN456K,IF17N/IF7 strain is not susceptible of GS inactivation but the
15 chimeric IF accumulates in the cells after ammonium addition and therefore, must interact with the
16 enzyme (Fig. 6C). This strongly suggests that the amino terminal part of IF17 is responsible for the
17 observed interaction of this factor even with GS versions not susceptible to inactivation.

18 *Modeled structure of the GS-IF7 complex supports mutational conclusions.* We achieved protein-protein
19 docking modeling using the SyGS structure (PDB ID 3NG0). Both IF7 and IF17 are intrinsically
20 disordered proteins (Muro-Pastor *et al.*, 2003, Saelices *et al.*, 2011b) and share three arginine residues
21 critical for their function (Saelices *et al.*, 2011a). We used the computer algorithm Phyre (Kelley &
22 Sternberg, 2009) to generate a hypothetical structural model of the segment IF7(1-38), which contains the
23 three critical arginine residues. We decided to use only a segment in order to increase the flexibility of the
24 molecule during docking. SyGS and modeled IF7 structures were used to generate a protein-protein
25 docking model of the complex, using the program ClusPro (Kozakov *et al.*, 2010). Among all the

1 outcomes, we selected the most energetically favorable model (Fig. 7). In this model, IF7 is allocated in a
2 polar pocket between each two GS rings, interacting with the three-residue (E419, N456 and R459)
3 triangle that we have identified in this work. It is worth noting that although the program found hundreds
4 of different possible complex structures, all of them presented the inactivating factor attached to the same
5 outer and polar pocket of the enzyme, with changes within IF7 orientation or packing. The stoichiometry
6 exhibited in this model is two monomers of GS per each inactivating factor, consistent with the
7 calculation made by CD (Saelices *et al.*, 2011b). Furthermore, the three critical arginines of IF7(1-38),
8 R8, R21 and R28, appear to interact with GS and stabilize the IF helix (Fig. 7B). R8, together with Q4, of
9 IF7(1-38) shows interactions with E419, N456 and R459 residues in GS, mainly by hydrogen bonds and
10 ionic interactions (Fig. 7C). R21 of IF7(1-38) seems to maintain the folding by an intra-molecular
11 hydrogen bonding interaction with the E32 residue. More interestingly, R28 of IF7(1-38) interacts with
12 E330 of GS forming a hydrogen bond (2.6 Å) (Fig. 7D). The computational model of the complex GS-
13 IF7(1-38) strongly supports the conclusions of the mutational analysis, that demonstrates that the pocket
14 formed by E419, N456 and R459 is essential for GS inactivation.

15 **Discussion**

16 We demonstrate here that GS inactivation by IFs occurs through the C-terminal region of the enzyme. In
17 addition, we propose an interaction SyGS/IFs mediated by the charged outer surface of the enzyme,
18 involving a three-residue core (E419, N456 and R459) and "the Glu327 flap".

19 According to our *in vitro* and *in vivo* mutational analysis, only two amino acid residues are involved in
20 the specificity between *Synechocystis* and *Anabaena* GS to the corresponding IFs, N456 and R459 from
21 SyGS and K457 and Q460 from AnGS. The replacement of both residues in SyGS by those present in
22 AnGS causes the loss of regulation by IF7 and IF17, and therefore mimics AnGS inactivation pattern.
23 Conversely, the replacement of K457 and Q460 in AnGS by the residues present in SyGS makes the
24 enzyme susceptible of inactivation by IF7, IF17 and IF7A like SyGS. Several filamentous cyanobacteria
25 genomes, including those of the *Anabaena* genus, show a proximal localization of the GS/IF coding genes

1 (Galmozzi *et al.*, 2010). This fact may be related to genome reorganization phenomena or co-evolutionary
2 processes that are responsible for the observed GS/IF interaction specificity. A comparative analysis of
3 the GS C-terminal sequence from different cyanobacteria clearly shows that both residues N456 and
4 R459 are not conserved, regardless of whether these cyanobacteria contain two, one or none IF gene. This
5 could also support possible coevolution of the genes encoding the GS/IFs system in different
6 cyanobacterial species.

7 The oceanic genus *Prochlorococcus* is interesting in this context because its GS C-terminus contains
8 similar or identical residues to SyGS in both positions (N456 and R459), but it lacks IF encoding
9 sequences. Probably the GS regulation mechanism would not be necessary in a relatively unchanged
10 environment as is the ocean (Garcia-Fernandez *et al.*, 2004) and therefore IF genes were lost in this
11 genus. In this regard, it would be interesting to check if the *Prochlorococcus* GS is susceptible to
12 inactivation by IF7 and/or IF17.

13 The total lack of GS inactivation in the strain harboring the SyGS-N456K variant is also supported by the
14 amount of Gln and Glu intracellular pools after ammonium addition (Fig. 4C). The SN456K strain has
15 similar behavior to that reported for the $\Delta gifA \Delta gifB$ strain, in which GS regulation is impaired because of
16 the absence of IFs (Muro-Pastor *et al.*, 2001). These observations demonstrate that the single substitution
17 N456K completely abolishes GS inactivation *in vivo* and confirm that this mechanism is responsible for
18 the maintenance of Gln and Glu cellular homeostasis during high nitrogen levels.

19 It is worth noting that substitution N456A leads to a partial loss of GS inactivation *in vivo* but has a
20 drastic effect on IF7/GS interaction both *in vitro* and *in vivo*. In fact no IF7 could be detected in cells of
21 the SN456A or the triple alanine substitution strains (Fig. 5B). Taking into account our previous data
22 demonstrating that target protection is required for IF7 accumulation *in vivo* (Galmozzi *et al.*, 2007,
23 Saelices *et al.*, 2011a), these results indicate that IF7 does not interact with the SyGS-N456A variant. On
24 the other hand, IF17 accumulation in SN456A or the triple alanine substitution strains is higher than in
25 the wild type strain. This observation suggests that both IF7 and IF17 somehow compete in their binding

1 to the GS. In addition, the results obtained with the IF17N/IF7 chimeric protein indicate that IF17 has an
2 additional anchorage site, most probably mediated by its N-terminus, which wouldn't take part in the
3 inactivation process.

4 Taxing into account the results of our mutational analysis and structural model of the complex, there are
5 three different processes that might explain the mechanism underlying GS regulation. First, a change in
6 the GS quaternary structure could lead to a dramatic alteration of the active sites to block enzymatic
7 activity. Second, key residues for substrates and/or products coordination are directly or indirectly
8 involved in the formation of interactions within the GS-IF complex. Finally, the positioning of IFs along
9 the outer surface of the enzyme could hinder the transit of molecules in/out of the active sites. The
10 GS/IF7 docking model suggests a possible mode of inactivation. The strong interaction between R28 of
11 IF7(1-38) and E330 of GS would give the enzyme a permanent closed or 'taut' state. This interaction
12 could hinder the entrance of the substrates or prevent the release of the products. This mechanism of GS
13 regulation is in some way similar to the one mediated by adenylylation/deadenylylation in enterobacteria,
14 which regulates intermediate formation by preventing closure of the E327 flap (Gill *et al.*, 2002).
15 Interestingly the residue adenylylated in enterobacteria, Y397 of *E. coli* GS, is also located in the C-
16 terminal region of the enzyme (Stadtman, 1991). Additionally, amino acid substitutions of Y397 that alter
17 the environment around it are sufficient to induce changes in GS activity (Luo *et al.*, 2005).

18 In *Bacillus subtilis*, mutational analysis has demonstrated that the interaction of TnrA with GS involves a
19 surface-exposed α -helix, next to the Tyr residue homologous to the adenylylated Y397 of enterobacteria
20 (Fisher *et al.*, 2002). This is the same region identified here to be involved in GS/IFs interaction.
21 Interestingly the TnrA/GS interaction also has an inhibitory effect on GS activity (Fedorova *et al.*, 2013).

22 In summary, in our model the GS/IFs interaction is electrostatic and the three critical arginines of IFs
23 decisively participate in the interactions with GS or IF stabilization. Thereby, IF7 interaction with the GS
24 outer surface seems to create a hydrogen bond between R28 of IF7(1-38) and E330 of GS, closing the
25 gate Glu330 to entrance and/or release of substrates and/or product. Finally, the residues Glu419, Asn456

and Arg459 of SyGS were found to be critical for enzyme regulation and play an important role in the interaction with IF7 through R8 and the helix wherein it is allocated.

Experimental Procedures

Strains and growth conditions. *Synechocystis* and *Anabaena* derivative strains were grown photoautotrophically at 30°C on BG11 medium (Rippka, 1988), using nitrate as nitrogen source, supplemented with 1 g l⁻¹ NaHCO₃ (BG11C) and bubbled with a continuous stream of 1% (v/v) CO₂ in air, under continuous illumination (50 μmol of photons m⁻² s⁻¹; white light). Ammonium treatment of cultures was performed by addition of 10 mM NH₄Cl, and the medium was buffered with 20 mM TES (pH 7.5). For plate cultures, BG11C liquid medium was solidified using 1% (w/v) agar. Strains used in this work are listed in Table 1

GS assay. GS activity was determined *in situ* by using the Mn²⁺-dependent γ-glutamyl-transferase assay in cells permeabilized with mixed alkyltrimethylammonium bromide (MTA) (Mérida *et al.*, 1991b). For the analysis of the *in vitro* GS/IFs interaction, binding reactions were performed in a final volume of 20 μl containing purified GS and increasing amounts of IF7, IF17 or IF7A, in Hepes-NaOH buffer, pH 7.0/50 mM KCl. Each sample was subjected to the same GS assay mentioned above for *in situ* samples, but without MTA addition. One unit of GS activity corresponds to the amount of enzyme that catalyzes the synthesis of 1 μmol min⁻¹ of γ-glutamylhydroxamate.

Plasmid and mutant constructions for purification. *Synechocystis glnA* was cloned into pBS-SK(+) plasmid (Stratagene) together with five histidine codons inserted after ATG start codon (Galmozzi *et al.*, 2007), generating pSyGS. By standard PCR with oligonucleotides An7F and An1R, *Anabaena glnA* was amplified bearing a His₅-tag after the ATG start codon. A *SalI-SalI* 1,441-bp fragment was cloned into the *SalI* restriction site of pBS-SK(+) plasmid, giving pAnGS. Using these two plasmids, pSyGS and pAnGS as templates, four chimeric genes were constructed using various fragments of *Synechocystis* and *Anabaena glnA*. For this purpose, we used a two-step PCR method that entails the synthesis of overlapping fragments (Higuchi *et al.*, 1988, Saelices *et al.*, 2011a). In addition, primers were designed to

1 produce site-specific mutations of *Synechocystis glnA* or *Anabaena glnA*. Mutagenesis was performed
2 using the same overlapping PCR method but incorporating the mutations into central overlapping primers
3 as previously described (Saelices *et al.*, 2011a). *BclI-XbaI* 796-bp fragment from *Synechocystis glnA* of
4 pSyGS was replaced by the different PCR fragments. *NheI-PacI* 162-bp fragment from *Anabaena glnA* of
5 pAnGS was replaced by the PCR fragment. All DNA constructs were confirmed by DNA sequencing.

6 *Protein purification.* *Synechocystis* and *Anabaena* GS and IFs expression and purification was carried out
7 as previously described (Galmozzi *et al.*, 2010, Saelices *et al.*, 2011a). Expression vectors used for
8 purification of the different GS or IF variants are listed in the supplemental Table S1. The proteins
9 purified in this work are listed in the supplemental Table S3.

10 *Protein-Protein Band Shift assay.* The binding reactions were carried out in a final volume of 20 μ l
11 containing 1.5 μ g (0.12 μ M) of purified GS and increasing quantities of IF7, IF17 or IF7A, in Hepes-
12 NaOH buffer (pH 7.0), 50 mM KCl. GS-IF complexes were allowed to form during 5 min at room
13 temperature. After the GS-IF complex formation, samples were subjected to 6% nondenaturing
14 polyacrylamide gels run at 25 $^{\circ}$ C in 25mM Tris-192 mM Glycine (pH 8.3), at 150 V for 2 h. Complexes
15 were visualized by gel staining with Coomassie blue.

16 *Generation of mutant strains of Synechocystis.* Targeting vector to obtain GS mutant strains, are listed in
17 supplemental Table S1. Previously generated pBS-SK(+) (Stratagene) containing *Synechocystis glnA*
18 locus (pMA1) (Mérida *et al.*, 1992) was used for mutant constructions. The *KpnI-DraI* fragment of the
19 *glnA* locus was cloned into pBS-SK(+) digested by *KpnI-SmaI*. After removing the *XbaI* site of the
20 multiple cloning site of the original plasmid pBS-SK(+), targeting vectors were generated by replacing
21 the 796-bp *BclI-XbaI* fragment, by the mutant variants obtained by site-directed mutagenesis as described
22 above. An $\text{Sm}^{\text{r}}\text{Sp}^{\text{r}}\text{C.S3}$ cassette (Prentki & Krisch, 1984) from pRL463 (pUC18/19 containing L.HEH1
23 and C.S3, nomenclature of Elhai & Wolk, (Elhai & Wolk, 1988) was cloned in the *XbaI* site of *glnA*
24 locus. In the case of Chi4 the strategy was different. The Chi4 chimeric gene used in the *in vitro* study
25 was joined to the upstream *Synechocystis glnA* region by PCR, using oligonucleotides SyChi4 5' HindIII,

1 SyChi4 5' R, SyChi4 and SyChi4R. This PCR-synthesized fragment was cloned into pBS-SK(+) digested
2 by HindIII-KpnI. A downstream *Synechocystis glnA* region was PCR-synthesized using oligonucleotides
3 SyChi4 3' KpnI and SyChi4 3'XhoI-KpnI. This fragment was cloned in the KpnI site of the above
4 described plasmid. Finally, an Sm^r Sp^r C.S3 cassette (Prentki & Krisch, 1984) from pRL463 (pUC18/19
5 containing L.HEH1 and C.S3, nomenclature of Elhai & Wolk, (Elhai & Wolk, 1988) was cloned in the
6 XhoI site incorporated previously in the SyChi4 3'XhoI-KpnI oligonucleotide. The resulting targeting
7 plasmids containing the mutant variants of *glnA* gene were used to transform the wild type *Synechocystis*
8 strain. For the generation of a *Synechocystis* strain expressing the SyGS-N456K and IF17N/IF7 proteins
9 (SN456K,IF17N/IF7), the targeting vectors pS-SyGS-N456K(2) and pCHV (Saelices *et al.*, 2011a) were
10 used to transform the $\Delta gifB$ *Synechocystis* strain (García-Domínguez *et al.*, 1999). All DNA constructs
11 were confirmed by DNA sequencing. Correct recombination was verified by PCR analysis (Fig. S1 and
12 S2), Oligonucleotides used for strains construction and verification are summarized in supplemental
13 Table S2.

14 *RNA isolation and Northern-blot analysis.* For Northern-blot analysis, total RNA was isolated and
15 extracted as previously described (García-Domínguez & Florencio, 1997). The concentration of total
16 RNA in each sample was quantified spectrophotometrically at 260 nm. RNA integrity was confirmed by
17 visualization of intact rRNA under UV light. Northern-blots were performed as previously described
18 (Saelices *et al.*, 2011a). PCR-synthesized fragments, encompassing the *glnA*, *gifA* or *gifB* genes were
19 used as probes. As a control the filters were reprobed with a 580-bp DNA fragment containing the
20 constitutively expressed RNase P RNA gene (*mpB*) from *Synechocystis* (Vioque, 1992). Hybridization
21 signals were quantified with a Cyclone Phosphor System (Packard).

22 *Western blot analysis.* Anti-IF7, anti-IF17 and Anti-TrxA antisera were obtained previously according to
23 standard immunization protocols (Galmozzi *et al.*, 2007, Marqués *et al.*, 1992, Navarro *et al.*, 2000). For
24 Western blot analysis, proteins were fractionated on 12-15% SDS-PAGE according to the method of
25 Laemmli (Laemmli, 1970) and immunoblotted with anti-IF7 (1:2,000), anti-IF17 (1:2,000) or anti-TrxA

1 (1:3,000). The ECL Plus immunoblotting system (GE Healthcare) was used to detect the different
2 antigens with anti-rabbit secondary antibodies.

3 *Preparation of crude extracts from Synechocystis cells.* For analysis of IF abundance in *Synechocystis*
4 cells grown under different conditions, crude extracts were prepared using glass beads as previously
5 described (Reyes *et al.*, 1995) in 50 mM Hepes-NaOH buffer (pH 7.0), 50 mM KCl. Equal volumes
6 (typically 10 μ l) of the processed samples were loaded on SDS-PAGE. Protein concentration in cell-free
7 extracts or purified protein preparations was determined by the method of Bradford, using ovalbumin as a
8 standard (Bradford, 1976).

9 *Amino acid determination.* Cells from 2 ml of culture were recovered by centrifugation, and cell lysates
10 were obtained by adding 0.45 ml of 0.2 N HCl, followed by vigorous shaking and incubation for 15 min
11 on ice. After centrifugation, supernatant was filtered through an Amicon Ultra-0.5ml, Ultracel-10K
12 centrifugal filter (Millipore) for deproteinization. The method used for the analysis of glutamate and
13 glutamine concentration in the deproteinized lysate involves a derivatization of amino acids with
14 phenylisothiocyanate (PITC) (Heinrikson & Meredith, 1984), which binds to primary or secondary
15 amines producing a derivative, phenylthiocarbamyl, that is detected by measuring the absorbance at 254
16 nm. Sixty microliters of sample were mixed with 60 μ L of the derivatizing solution
17 (ethanol:H₂O:triethanolamine:PITC, 7:1:1:1), incubated at room temperature for 30 min, and dried under
18 flowing N₂. The pellet was resuspended in 60 μ L of 4 mM sodium phosphate (pH 7.4) and 2%
19 acetonitrile and injected in a HPLC Elite LaChrom (Hitachi) system. The separation was performed using
20 a LichroCART 125-4 column. Amino acids were separated using a linear gradient from 70 mM sodium
21 acetate, 5% acetonitrile buffer (pH 6.55) to acetonitrile/water (50:50). Retention times for glutamate and
22 glutamine was 1.69 and 3.92 min, respectively.

23 *Secondary structure prediction and protein-protein docking analysis.* The 38 residue long primary
24 structure of the amino-terminus of the unfolded protein IF7 was used to generate a secondary structure

1 model by the application Phyre (Kelley & Sternberg, 2009). We selected a short segment in order to
 2 increase flexibility of IF7 in the docking. The (1-38) segment of IF7 includes the three critical arginines
 3 (Saelices *et al.*, 2011a). Protein-protein docking analysis was performed using the structure of
 4 *Synechocystis* GS (PDB ID 3NG0) and the computational model of IF7. The two structures were
 5 subjected to docking experiments using ClusPro (Kozakov *et al.*, 2010). The docking outputs were
 6 analyzed on energy provided by the application. Among all docking results, we selected the first and most
 7 energetically favorable model.

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6

Table 1: Cyanobacterial strains used in this study

<i>Synechocystis</i> strains	Description	Source or reference
<i>Synechocystis</i> sp. PCC 6803	wild type	
$\Delta gifA \Delta gifB$	$\Delta gifA::npt$, $\Delta gifB::cat$, Km ^R , Cm ^R	(García-Domínguez <i>et al.</i> , 1999)
SChi1	<i>glnASy</i> (726):: <i>glnAAn</i> (693):: <i>aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SChi2	<i>glnASy</i> (1104):: <i>glnAAn</i> (315):: <i>aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SChi3	<i>glnASy</i> (1251):: <i>glnAAn</i> (168):: <i>aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SChi4	<i>glnAAn</i> (1254):: <i>glnASy</i> (168):: <i>aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SE419A	<i>glnAE419A::aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SE419K	<i>glnAE419K::aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SE419A/N456A/R459A	<i>glnAE419A/N456A/R459A::aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SN456A	<i>glnAN456A::aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SN456D	<i>glnAN456D::aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SN456K	<i>glnAN456K::aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SR459A	<i>glnAR459A::aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SR459E	<i>glnAR459E::aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SR459Q	<i>glnAR459Q::aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SN456K/R459Q	<i>glnAN456K/R459Q::aadA</i> ⁺ , Sm ^R , Sp ^R	This study
SN456K,IF17N/IF7	<i>glnAN456K::npt</i> , <i>gifB</i> (1-246):: <i>gifA::aadA</i> ⁺ , Km ^R Sm ^R , Sp ^R	This study

Abbreviations: ^R denotes resistance to the indicated antibiotic: Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; and Sp, spectinomycin.

1 **Table 2: *In vitro* GS inactivation assay for the wild-type enzyme and different mutant versions.**

2

Protein name	GS Activity (%)		
	Ø	+ IF7	+ IF17
SyGS	100,0	6,9	5,8
SyGS-E419A	100,0	58,2	19,2
SyGS-E419K	100,0	86,2	90,9
SyGS-E419D	100,0	45,4	10,8
SyGS-E423A	100,0	7,0	4,8
SyGS-E423K	100,0	83,3	26,1
SyGS-E426A	100,0	8,8	6,1
SyGS-E426K	100,0	34,9	5,9
SyGS-E430A	100,0	14,3	9,7
SyGS-E430K	100,0	22,8	8,8
SyGS-D441A	100,0	9,2	4,9
SyGS-D441K	100,0	9,4	6,9
SyGS-E444A	100,0	8,2	8,3
SyGS-E444K	100,0	24,0	25,2
SyGS-E448A	100,0	14,6	10,0
SyGS-E448K	100,0	42,5	23,4
SyGS-D452A	100,0	8,0	27,0
SyGS-D452K	100,0	17,0	84,8
SyGS-N456A	100,0	98,8	14,6
SyGS-N456K	100,0	98,2	99,9
SyGS-N456D	100,0	95,7	93,0
SyGS-N456Q	100,0	85,6	35,0
SyGS-R459A	100,0	83,3	50,3
SyGS-R459E	100,0	100,0	100,0
SyGS-R459K	100,0	76,0	40,5
SyGS-N456A/R459A	100,0	93,4	36,8
SyGS-E419A/N456A/R459A	100,0	98,8	45,2

3

4 The same amount of each GS version (1,5 µg) was assayed alone (Ø) or after incubation (5 min) with 2

5 µM IF7 or IF17. 100% represent GS activity of each enzyme variant. The percentage of remaining

6 activity after incubation is showed in each case. The values represent arithmetic means from three

7 independent experiments.

1 **Figure Legends**

2 **Figure 1. Analysis of chimeric proteins between *Anabaena* GS (AnGS) and *Synechocystis* GS**
3 **(SyGS). A.** Scheme of the chimeric proteins constructed. Numbers into the boxes indicate the residues
4 corresponding to the GS of each organism. **B.** *In vitro* inactivation assay of the different chimeric
5 proteins. A fixed amount of each GS was incubated without (Ø) or with 2 µM of IF7, IF17 or IF7A. GS-
6 IF complexes were allowed to form during five minutes and GS transferase activity was determined. **C.**
7 Kinetics of the GS inactivation in *Synechocystis* strains expressing *glnA* chimeric genes. 10 mM NH₄Cl
8 was added to *Synechocystis* cells cultivated with nitrate as nitrogen source. Aliquots were withdrawn and
9 GS transferase activity was measured. The curves represent arithmetic means from three independent
10 experiments and their standard deviation values. **D.** Representative Western blot of IF7 and IF17 along
11 the GS inactivation for each strain. As a protein loading control, membranes were incubated also with
12 anti-TrxA. Thioredoxin A (TrxA) is constitutively expressed in *Synechocystis* cells.

13 **Figure 2. *In vitro* analysis of residues differentially charged between *Synechocystis* and *Anabaena***
14 **GS C-terminal region. A.** Sequence alignment of the last C-terminal 56 residues of SyGS and AnGS. ‘*’
15 indicates positions which have identical residues in the two sequences. Grey shadow represents difference
16 of charge. Charged and exposed residues spatially adjacent to N456 and R459 in the crystal structure of
17 SyGS are framed with a line. **B.** *In vitro* inactivation assays of the different GSs. Each GS protein (1.5
18 µg) was incubated without (Ø) or with 2 µM IF7, IF17 or IF7A. GS-IF complexes were allowed to form
19 during five minutes and GS transferase activity was determined.

20 **Figure 3. *In vitro* inactivation assays of SyGS, AnGS, and those mutants that are relevant for**
21 **specificity. A.** Each GS protein (1.5 µg) was incubated with increasing amounts of IF7 (black squares),
22 IF17 (white squares) or IF7A (black circles). GS-IF complexes were allowed to form during five minutes
23 and GS transferase activity was determined. **B.** GS variants (1.5 µg) were incubated with an excess of

IF7, IF17 or IF7A (11, 12 and 14 μ M, respectively). Then GS-IF complexes, together with GS alone ($\emptyset$), were separated in a 6% non-denaturing polyacrylamide gel and stained with Coomassie blue.

Con formato: Inglés (Estados Unidos)

Figure 4. Analysis of the GS inactivation in *Synechocystis* strains expressing SyGS-N456K, SyGS-R459Q and SyGS-N456K/R459Q variants. **A.** Kinetics of the GS inactivation in *Synechocystis* wild-type (Syn6803), $\Delta gifA\Delta gifB$ and strains expressing SyGS-N456K, SyGS-R459Q and SyGS-N456K/R459Q variants. 10 mM NH_4Cl was added to *Synechocystis* cells cultivated with nitrate as nitrogen source. Aliquots were withdrawn and GS transferase activity was measured. The curves represent arithmetic means from three independent experiments and their standard deviation values. **B.** Representative Western blot of IF7 and IF17 along the GS inactivation for each strain. **C.** Change in the intracellular Gln and Glu pools upon ammonium up-shift in wild-type (Syn6803) and SyGS-N456K expressing strains. Intracellular concentrations of Gln and Glu pools, relative to total protein, were determined before ($t = 0$) and after ammonium addition.

Figure 5. *In vitro* analysis of SyGS-E419, SyGS-N456 and SyGS-R459 variants. **A.** Structural overview of the C-terminal domain of SyGS (colored in pink). Charged and exposed residues together with N456 are labeled in the close view on the right. **B.** *In vitro* inactivation assays of GS variants. Each GS protein (1.5 μ g) was incubated with increasing amounts of IF7 (black squares) or IF17 (white squares). GS-IF complexes were allowed to form during five minutes and GS transferase activity was determined. **C.** GS variants (1.5 μ g) were incubated with IF7 or IF17 (11 and 12 μ M, respectively). Then GS-IF complexes, together with GS alone ($\emptyset$), were separated in a 6% non-denaturing polyacrylamide gel and stained with Coomassie blue.

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Figure 6. Analysis of the GS inactivation in *Synechocystis* strains expressing SyGS-E419, SyGS-N456 and SyGS-R459 variants. **A.** Kinetics of the GS inactivation in *Synechocystis* strains expressing different GS mutant variants. 10 mM NH_4Cl was added to *Synechocystis* cells cultivated with nitrate as nitrogen source. Aliquots were withdrawn and GS transferase activity was measured. The curves

1 represent arithmetic means from three independent experiments and their standard deviation values. **B.**
2 Representative Western blots of IF7 and IF17 along the GS inactivation for each strain. **C.** Analysis of the
3 GS inactivation in the SN456K,IF17N/IF7 strain. Kinetics of the GS inactivation after ammonium
4 addition in the SN456K,IF17N/IF7 and wild type strains. Representative Western blots of IF7, IF17 and
5 IF17N/IF7 along the GS inactivation for each strain.

6 **Figure 7. Protein-protein docking modeling of the complex GS-IF7.** **A.** Lateral view of the
7 electrostatic surface of GS, together with IF7, represented in light grey. The 38-residue-long IF7
8 structural model and the dodecamer from SyGS structure (PDB ID 3NG0) were used to generate a
9 protein-protein docking model of interaction. IF7 appears bound to the belt of the dodecamer, attached to
10 a charged pocket. A square marks the segment zoomed in **B.** Close-up view of the GS/IF7 interaction
11 region. Orange residues correspond to the three arginines critical for IF function (Saelices *et al.*, 2011a).
12 **C.** Close view of the binding between IF7-R8 and the three-residue core from GS. Interactions with less
13 than 3.2 Å of distance are marked with dotted lines. IF7-R8 and IF7-Q4 are coordinating the interaction
14 with E419, N456 and R459. **D.** Close view of the hydrogen bonding between IF7-R28 and SyGS-E330.

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