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Purification and Properties of Glutamine Synthetases from the Cyanobacteria *Synechocystis* sp. Strain PCC 6803 and *Calothrix* sp. Strain PCC 7601

ANGEL MÉRIDA,[1] LINE LEURENTOP,[2] PEDRO CANDAU,[1] AND FRANCISCO J. FLORENCIO[1]*

Departamento de Bioquímica Vegetal y Biología Molecular, Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-CSIC, Apartado 1113, 41080 Seville, Spain,[1] and Department of Biology, University of Antwerp, B-2610 Wilrijk, Antwerp, Belgium[2]

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Glutamine synthetases (GSs) from two cyanobacteria, one unicellular (*Synechocystis* sp. strain PCC 6803) and the other filamentous (*Calothrix* sp. strain PCC 7601 [*Fremyella diplosiphon*]), were purified to homogeneity. The biosynthetic activities of both enzymes were strongly inhibited by ADP, indicating that the energy charge of the cell might regulate the GS activity. Both cyanobacteria exhibited an ammonium-mediated repression of GS synthesis. In addition, the *Synechocystis* sp. showed an inactivation of GS promoted by ammonium that had not been demonstrated previously in cyanobacteria.

Ammonium assimilation takes place in cyanobacteria mainly by the sequential action of glutamine synthetase (GS) (L-glutamate:ammonia ligase [ADP-forming], EC 6.3.1.2) and glutamate synthase (L-glutamate:ferredoxin oxidoreductase [transaminating], EC 1.4.7.1) (11). GSs purified from a variety of procaryotic sources are quite similar in physicochemical parameters; for example, they have a molecular mass of about 600 kilodaltons and have a similar number of subunits and similar structures (dodecamers, with a single subunit type of about 50 kilodaltons). By contrast, their regulatory properties differ widely. The enzymes of *Escherichia coli* (22) and other gram-negative bacteria (20) are regulated at the activity level by a covalent modification of the enzyme through an adenylylation-deadenylylation system. Most gram-positive bacteria lack this system and exhibit a feedback regulation by products such as glutamine and other metabolites (3). The regulation of cyanobacterial GS activity is not clear, although amino acids (acting in a cumulative manner), divalent cations, and thiols have been proposed as modulators of the activity (6, 14, 19). There is no evidence of covalent modification of a GS from any cyanobacteria (4).

GSs from cyanobacteria of the genera *Anabaena* (13, 17), *Synechococcus* (5), and, more recently, *Phormidium* have been purified to homogeneity (2, 18). All of them were similar in size and subunit composition. We have purified GSs from the unicellular, facultative, heterotrophic cyanobacterium *Synechocystis* sp. strain PCC 6803 (section I) (15) and from the filamentous *Calothrix* sp. strain PCC 7601 (*Fremyella diplosiphon*) (section IV) (15) by a previously described method (5) that includes ion-exchange chromatography followed by affinity chromatography on 2′,5′-ADP-Sepharose. In both cases, this procedure provides a high yield of enzyme (about 40%) that is pure by electrophoretic criteria; however, GS activity is clearly more abundant in the *Calothrix* sp. than in the *Synechocystis* sp. (2.45 versus 0.056 U/mg of protein in the crude extracts).

We have carried out a structural study of *Calothrix* GS by using electron microscopy techniques and computerized image processing (9). This enzyme shows a typical procaryotic configuration, with a diameter of about 15 nm, and is formed by two superimposed hexagons, each composed of six subunits arranged radially with respect to the central hole but with a left-handed configuration. Rotational studies indicated that only rotation by 60° (or its multiples) gave a clear reinforcement of the intensities of the subunits (Fig. 1). In side view, the enzyme exhibited a quadrangular structure. This structure agrees with those described for other procaryotic GSs, such as those from *Salmonella typhimurium* (1) and *Anabaena* sp. strain PCC 7120 (13).

The physicochemical and kinetics parameters of both GSs were compared with those from two representative cyanobacteria, *Anabaena* sp. strain PCC 7120 (a dinitrogen fixer) and *Synechococcus* sp. strain PCC 6301 (an obligate photoautotrophic unicellular cyanobacterium and non-dinitrogen fixer) (Table 1). GS purified from the *Calothrix* sp. showed a high affinity for glutamate (0.35 mM) in the biosynthetic activity assay in comparison with other GSs, including the *Synechocystis* sp. enzyme (K_m, 1.2 mM). The K_m value for ammonia is very low (65 μM) but similar to those reported for other cyanobacterial GSs, such as those from the *Anabaena* sp. (12, 17). The high affinity for glutamate observed in the *Calothrix* sp., taken together with the K_m values for ammonia and ATP, indicates a GS with a high efficiency in ammonia assimilation, compared with other cyanobacterial GSs (2, 12, 18).

Antibodies raised against GS purified from *Synechococcus* sp. strain PCC 6301 (8) showed a strong cross-reaction with GSs from the *Synechocystis* sp. and the *Calothrix* sp. and also cross-reacted with other cyanobacterial GSs, such as those from *Anabaena* sp. strain ATCC 33047 (section IV) and *Fischerella muscicola* (section V) (data not shown). These results support the idea that GS is well conserved among the different cyanobacterial groups. These antibodies were used to immunotitrate both purified GSs. *Synechocystis* GS needed a smaller amount of antibodies than *Calothrix* GS to precipitate (Fig. 2). These results, taken together with those obtained by using immunodiffusion techniques (data not shown), confirm the view that GSs from unicellular cyanobacteria such as *Synechococcus* sp. strain PCC 6301 and *Synechocystis* sp. strain PCC 6803 are more similar to

* Corresponding author.

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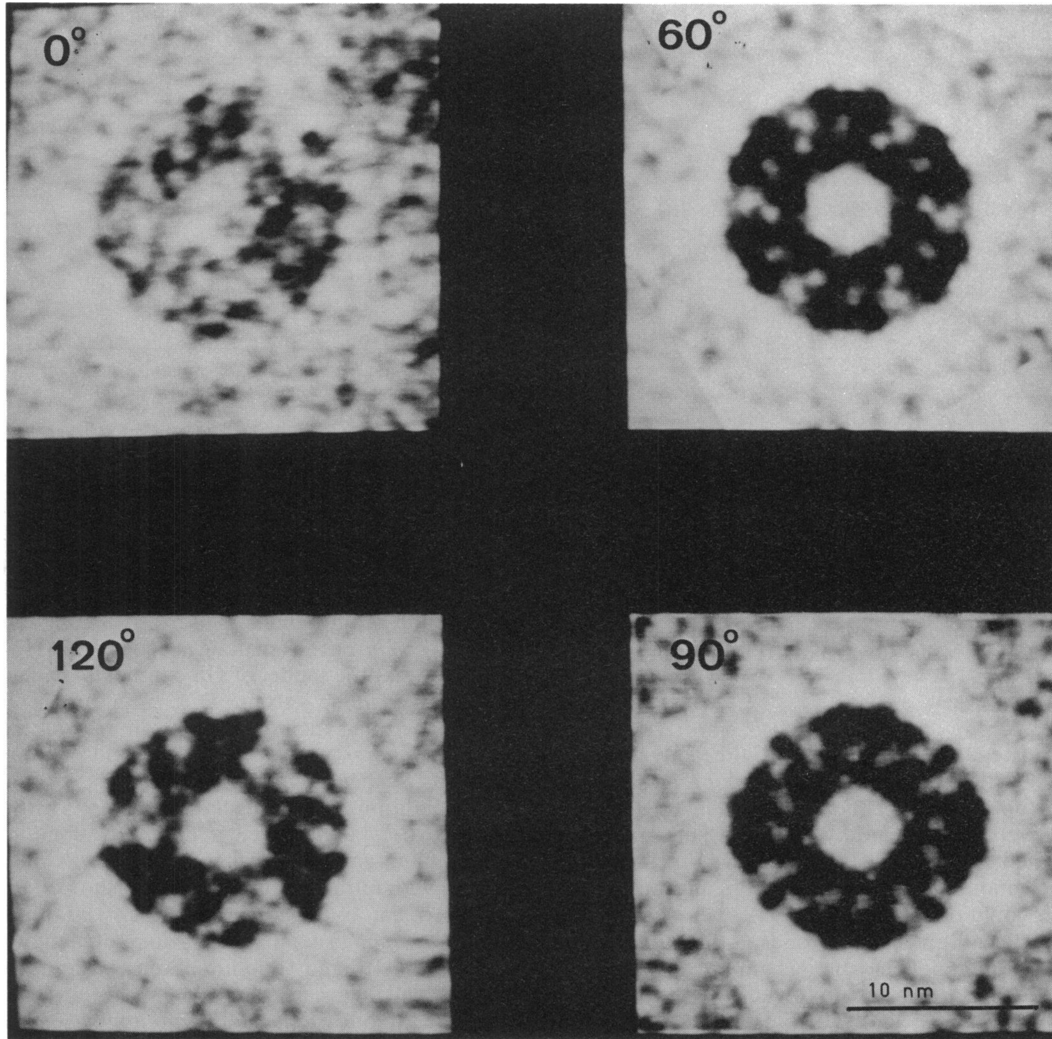

FIG. 1. Computerized image processing of a negatively stained molecule of GS from a *Calothrix* sp. The upper left panel shows the original electron micrograph of a single molecule. Rotation by 60° reinforced intensities with a periodicity of six. Magnification, ×1,700,000.

each other than the *Calothrix* sp. enzyme (a filamentous cyanobacterium) is to GSs of the unicellular species.

GSs from the *Synechocystis* sp. and *Calothrix* sp. were strictly dependent on divalent cations for their biosynthetic activity. In the *Synechocystis* sp., Mg^{2+} supported maximum activity at 25 mM (100%), followed by Co^{2+} (35%), Mn^{2+} (15%), and Ca^{2+} (1%) at the same concentrations. By contrast, *Calothrix* GS exhibited maximum activity with

TABLE 1. Physicochemical and kinetic parameters of GSs purified from *Synechocystis* sp. strain PCC 6803 and *Calothrix* sp. strain PCC 7601, compared with those of other cyanobacterial GSs

Species	Molecular mass (kDa)[a] of:		Optimal temp (°C)		Optimal pH		Apparent K_m					
							Transferase activity			Biosynthetic activity		
	Native enzyme	Subunit	Transferase assay	Biosynthetic assay	Transferase assay	Biosynthetic assay	Glutamine (mM)	ADP (nM)	Hydroxylamine (mM)	Glutamate (mM)	ATP (mM)	Ammonium (mM)
Synechocystis sp.[b]	624	52	34	35	6.5	7.2	14.3	220	14.5	1.2	0.55	0.17
Calothrix sp.[b]	588	49	40	37	6.5	7.0	10	62	12.5	0.35	0.30	0.065
Anabaena sp.[c]	610	50	—	—	—	7.6	—	—	—	2.1	0.32	<0.02
Synechococcus sp.[d]	580	47	35	38	6.4	7.1	—	—	—	5.0	0.7	0.02

[a] Subunit number for all species was 12, and for all species, there was only one type of monomer. kDa, Kilodaltons; —, not done.
[b] Data from this work.
[c] Data from reference 13.
[d] Data from reference 5.

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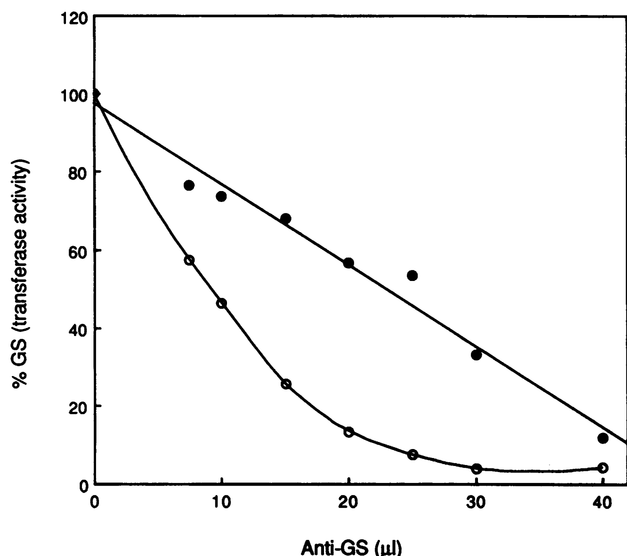

FIG. 2. Immunotitration of *Synechocystis* (○) and *Calothrix* (●) GSs. GSs from a *Synechocystis* sp. (30 µg) and a *Calothrix* sp. (25 µg) were titrated with antibodies (5 mg/ml) that were raised against pure GS from *Synechococcus* sp. strain PCC 6301.

Co^{2+} (164%) instead of Mg^{2+} (100%), with activities with Mn^{2+} and Ca^{2+} in the same range as those for *Synechocystis* sp. It is worth noting that all the kinetic values for biosynthetic activity were determined by measuring the amount of glutamine formed, as previously described (10), instead of measuring the amount of P_i released or using a coupling system, which could distort the results (2, 12).

For cyanobacteria, it has been proposed that intracellular levels of different metabolites, such as amino acids, nucleotides, and divalent cations, are involved in controlling GS activity in vivo (12, 16, 21, 23). The biosynthetic activity of the *Synechocystis* GS was inhibited by alanine and aspartic acid (52 and 41%, respectively, at a concentration of 1 mM). Aspartate is one of the amino acids that are found at higher intracellular concentrations in *Synechocystis* cells (data not shown), and its role might be important in the modulation of GS activity. The effects of alanine and aspartic acid in *Calothrix* GS were weaker (30 and 20% inhibition, respectively) than in the *Synechocystis* sp. It is also worth noting that, as in other GSs studied (19, 21), alanine, glycine, and serine strongly inhibited (about 90%) GS transferase activity; however, serine and glycine did not exhibit any effect on biosynthetic activity. These results revealed the limited relevance of the transferase reaction for in vivo studies of the regulation of GS. The strongest inhibition of the biosynthetic activity, in both enzymes, was obtained with ADP (90% at 1 mM) and AMP (80% inhibition at 1 mM). ADP is a competitive inhibitor with respect to ATP, with a K_i of 0.11 mM for GS biosynthetic activity from the *Synechocystis* sp. This activity is completely abolished with a 1:3 ADP/ATP ratio. Taking into account also that ADP is a product of GS activity, it can be suggested that the energy charge of the cell could play a key role in the regulation of GS activity.

Results obtained by using quantitative immunoelectrophoresis techniques (7) indicate unambiguously that GS synthesis in both cyanobacteria is regulated by the available nitrogen source. Both *Synechocystis* sp. and *Calothrix* sp. cells grown with ammonium as the nitrogen source (repressing conditions) had about half the GS protein that they did when the cells were grown in nitrate (derepressing conditions) (Table 2). With *Anabaena* sp. strain PCC 7120, it has been clearly demonstrated that regulation of GS synthesis is exerted by a differential transcription from different promoters of the *glnA* gene (structural gene for GS) (24). Our results suggest that the model of GS synthesis regulation described in *Anabaena* sp. strain PCC 7120 might be general in cyanobacteria, including both dinitrogen fixers, such as *Anabaena* spp. or *Calothrix* spp., and nondinitrogen fixers, such as *Synechocystis* spp.

TABLE 2. Levels of GS from *Synechocystis* and *Calothrix* cultures grown on different nitrogen sources[a]

Strain and nitrogen source	GS activity (U/mg of protein)	Amt of GS (µg of GS/mg of protein)
Synechocystis sp. strain PCC 6803		
Nitrate	0.554 (100)	3.90 (100)
Ammonium	0.107 (19)	2.00 (51)
Calothrix sp. strain PCC 7601		
Nitrate	2.9 (100)	38.8 (100)
Ammonium	1.13 (39)	16.4 (42)

[a] Cells of *Synechocystis* sp. and *Calothrix* sp. were grown on nitrate (10 mM) or ammonium (8 mM). GS transferase activity, total GS protein, and total protein in crude extracts were determined.

In addition to the regulation of GS synthesis, the *Synechocystis* sp. displayed a decrease of GS activity. With ammonium-grown cells, the activity was only 19% of that found with nitrate, but the cells still showed 51% of the total GS protein, a phenomenon which did not occur in the *Calothrix* sp. (Table 2). This loss of activity may not be attributable to allosteric inhibition by amino acids or nucleotides and suggests that GS in the *Synechocystis* sp. is modified to an inactive form. Preliminary studies with inactive GS obtained from ammonium-grown cells showed that GS is not reactivated by phosphodiesterase treatment, ruling out the existence of an adenylylation-deadenylylation system as has been demonstrated in enterobacteria (22). At present, we are studying the metabolic signals and molecular mechanisms which determine the loss of GS activity promoted by ammonium in this cyanobacterium.

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