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Biochemical Characterization of Glutamine Synthetase from the Diazotrophic Cyanobacterium, *Anabaena doliolum*

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Abstract. In cyanobacteria, the glutamine synthetase–L-glutamine-2-oxoglutarate aminotransferase (GS-GOGAT) pathway is the major ammonia-assimilating route. The GS of *Anabaena doliolum* was synthesized more under N_2-fixing conditions, followed by ammonium, nitrate, and nitrite as nitrogen sources. The activities of both the glutamine synthetase, Mg^{2+}-dependent biosynthetic and Mn^{2+}-dependent γ-glutamyl transferase were optimum at pH 7. The active site of the enzyme bears sulfhydryl (-SH) groups; this was confirmed with the -SH group inhibitors, para-chloromercuribenzoate (pCMB) and N-ethylmaleimide (NEM). The biosynthetic and γ-glutamyl transferase activities showed specificity for the divalent cations, Mg^{2+} and Mn^{2+}, respectively. The other divalent cations Co^{2+}, Cu^{2+}, and Ni^{2+} were poor substitutes. This enzyme also required these divalent cations to stabilize its structure and function under extreme conditions such as high and low temperatures and urea denaturation. The glutamate analog L-methionine-D,L-sulfoximine, inactivated the enzyme, whereas the GOGAT inhibitor, azaserine, had no effect on the enzyme activity. The GS enzyme required de novo protein synthesis.

Inorganic nitrogen is assimilated via the glutamine synthetase/glutamate synthase pathway under conditions of a limited supply of ammonia [2–6, 8, 9, 11, 13, 14, 18, 24, 26, 29]. NH_4^+ incorporation into glutamine is catalyzed by glutamine synthetase (GS) followed by the reductive reaction of glutamine with α-oxoglutarate to yield two molecules of glutamate, the reaction catalyzed by glutamate synthase (GOGAT) [2–6, 9, 11, 13, 14, 18, 24, 26, 29]. In cyanobacteria, this pathway has been shown to be the major ammonia-assimilating route [5, 8, 13, 18, 24, 26, 29].

Glutamine synthetase catalyzes a number of reactions [15, 17, 25] forming the basis of a number of different assays.

(1) Biosynthetic reaction:

$$\text{ATP} + \text{L-glutamate} + \text{NH}_3 \xrightarrow{Mg^{2+}/Mn^{2+}} \text{L-glutamine} + \text{ADP} + \text{Pi}$$

(2) Transferase reaction:

$$\text{Glutamine} + \text{NH}_2\text{OH} \xrightarrow[Mg^{2+}/Mn^{2+}]{\text{ADP, Pi, or As}} \gamma\text{-glutamyl hydroxamate} + \text{NH}_3$$

Biologically, the most significant reaction catalyzed by GS is the biosynthetic reaction involving the conversion of L-glutamate to L-glutamine, utilizing the available ammonia.

The work reported in this paper represents isolation and biochemical characterization of glutamine synthetase biosynthetic and γ-glutamyl transferase activities from the diazotrophic, filamentous cyanobacterium *Anabaena doliolum*.

Materials and Methods

Organism and culture conditions. Cells of *Anabaena doliolum* were grown in eight times diluted Allen and Arnon medium [1], without any nitrogen source and containing different nitrogen sources, viz., 0.1 mM ammonium chloride, 20 mM potassium nitrate, 1 mM sodium nitrite, and 2 mM glutamine; buffered with 50 mM 4-(hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)/NaOH to pH 7.0. The cultures were grown at 28°C under illumination of 10 W/m² provided by white fluorescent tubes. Cyanobacterial cells were collected in the exponential phase and used for the preparation of the enzyme extract [20].

Extraction and assay of glutamine synthetase enzyme. Two hundred milliliters of cyanobacterial cultures in the exponential phase were centrifuged and washed with 30 mM Tris buffer (pH 8.0) containing 10 mM $MgCl_2 \cdot 6H_2O$, 1 mM $MnCl_2 \cdot 4H_2O$ and resuspended in the same buffer [20]. The cells were broken by passage

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Table 1. Effect of nitrogen source on the activity of glutamine synthetase biosynthetic and γ-glutamyl transferase activity

Nitrogen source	Biosynthetic activity[a]	γ-Glutamyl transferase activity[a]
N_2-fixing	4.7	25.9
NH_4^+ grown	2.4	13.3
NO_3^- grown	1.4	7.7
NO_2^- grown	0.8	4.6
Glutamine grown	0.03	20.9

[a] An average of three independent readings was taken into consideration and the activity expressed as μmoles product formed/mg protein per min.

through a chilled French Pressure Cell twice at 20,000 lb/in^2 [20]. The homogenate was centrifuged at 6000 *g* for 20 min, and the supernatant thus obtained was used for the estimation of glutamine synthetase biosynthetic and γ-glutamyl transferase activities.

The Mn^{2+}-dependent γ-glutamyl transferase was estimated by determining the rate of γ-glutamylhydroxamate formed [21]. The Mg^{2+}-dependent biosynthetic activity of glutamine synthetase was determined from the rate of release of Pi [21].

Analytical methods. Protein contents in the extracts were measured with bovine serum albumin (BSA) as the standard protein [12].

Various inhibitors were first dissolved in the minimum possible volumes of ethanol diluted with distilled water or only in distilled water to obtain stock solutions. Stock solutions were filter-sterilized before being added to the cultures.

Chemicals. All chemicals at their analytical grades were obtained from Sigma Chemical Co. (USA), BDH, and SRL (India).

Results

Influence of different nitrogen sources on GS activity. γ-Glutamyl transferase activity was maximum in extracts obtained from N_2-fixing cultures, followed by glutamine, ammonium, nitrate, and nitrite (Table 1). The GS biosynthetic activity was nearly five to six times lower than the transferase activity with repressive response of nitrogen sources similar to the transferase activity.

Effect of pH on the GS γ-glutamyl transferase and biosynthetic activity. Enzyme activities were optimum at pH 7. The biosynthetic activity showed a gradual decline in activity in the alkaline range. The transferase activity, on the other hand, had a

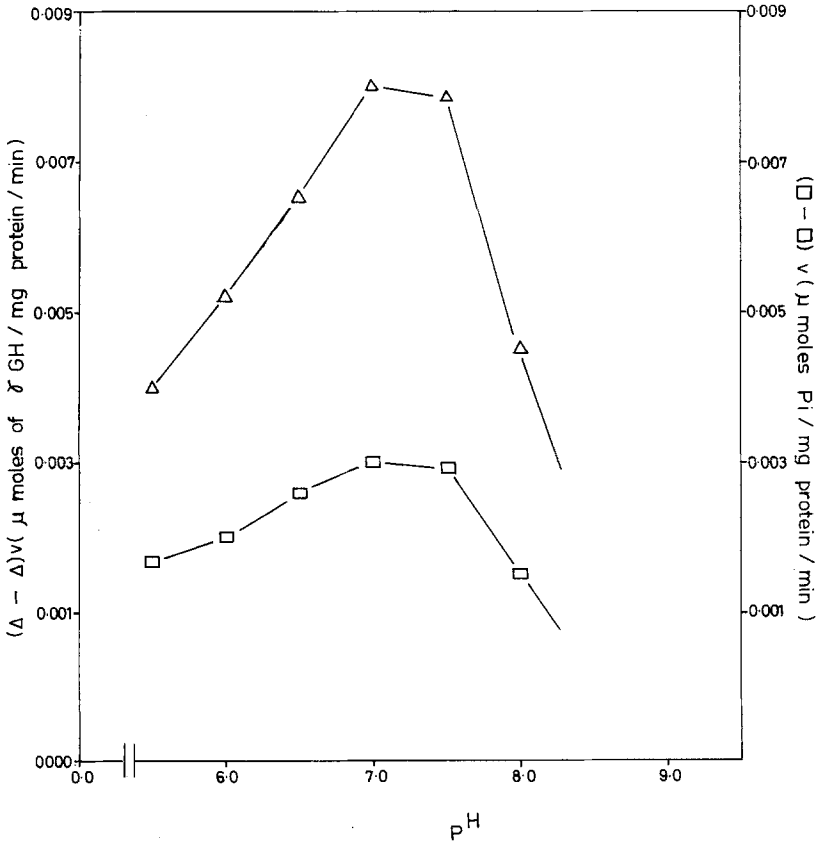

Fig. 1. Effect of pH on glutamine synthetase biosynthetic and transferase activities.

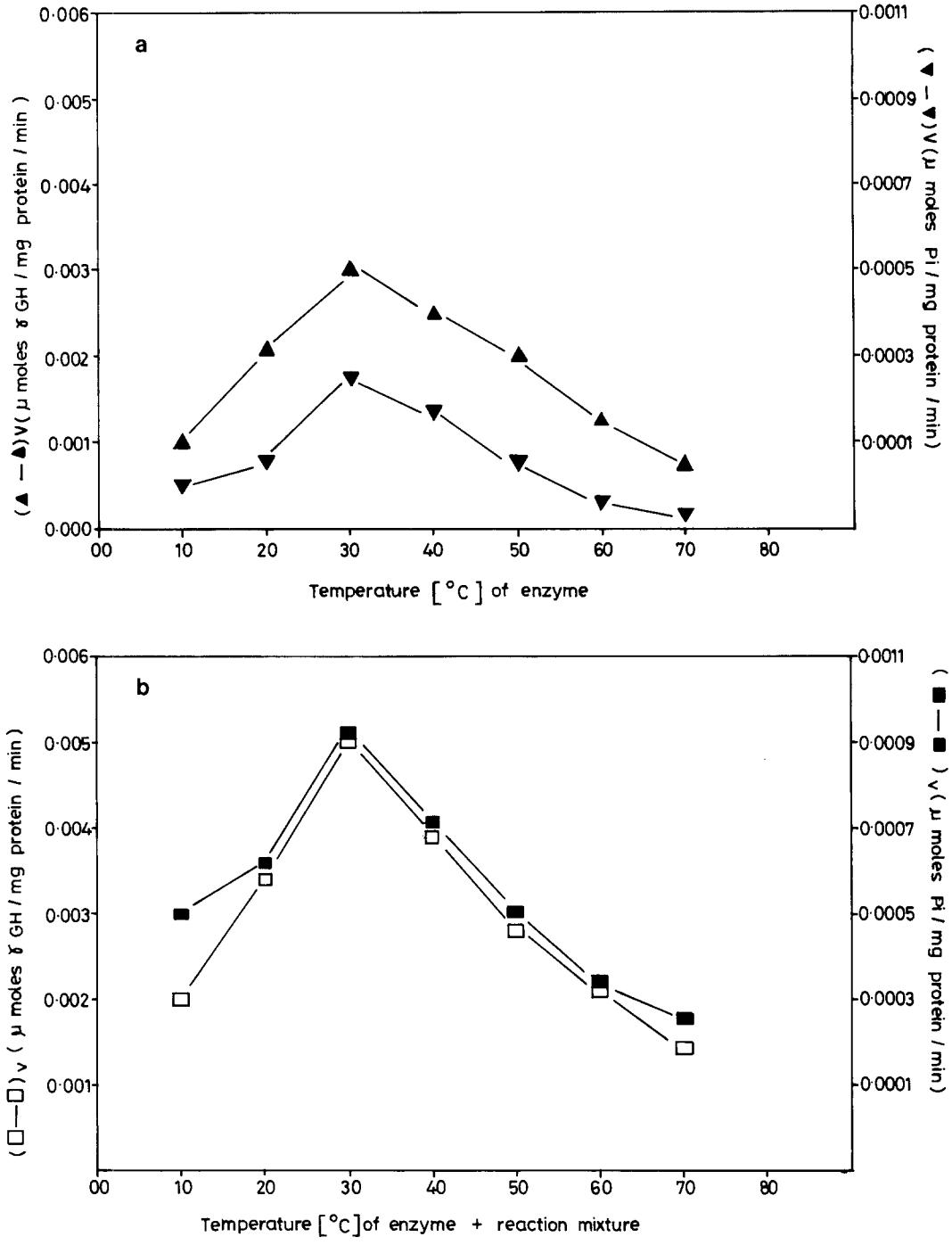

Fig. 2. (a) Rate of glutamine synthetase biosynthetic and transferase activities at varying temperatures of the enzyme. (b) Rate of glutamine synthetase biosynthetic and transferase activities at varying temperatures of the enzyme and reaction mixture.

much sharper drop on either side of the optimal pH (Fig. 1).

Effect of temperature on the enzyme activity. The enzyme activity was assayed over a wide range of temperature (10°–70°C). In one set, the enzyme was incubated at these temperatures for a suitable time period before being added to the reaction mixture, and the GS activity was assayed (Fig. 2a). In the second set, both the enzyme and the reaction mixture were incubated at different temperatures and the activity estimated (Fig. 2b). In both sets, the

Table 2. Effect of various divalent cations on glutamine synthetase biosynthetic and γ-glutamyl transferase activities

Metal ion	Biosynthetic activity[a] metal ion (mM)[b]	Transferase activity[a] metal ion (mM)[c]
Mg^{2+}	11.3	2.17
Mn^{2+}	1.1	25.9
Co^{2+}	0.013	2.19
Cu^{2+}	0.0	2.58
Ca^{2+}	0.0	0.0
Ni^{2+}	0.0	0.0

[a] An average of three independent readings was taken into consideration in terms of μM product formed/mg protein per min.
[b] 125 mM of other divalent cations was added in place of Mg^{2+} in the reaction mixture, and the enzyme activity was determined.
[c] 0.3 mM of other divalent cations was added in place of Mn^{2+} in the reaction mixture, and the enzyme activity was determined.

Table 3. Effect of various inhibitors on the activity of glutamine synthetase enzyme

Additions	% residual activity
Control	100
MSX (1 μM)	2.4 (97.6)[a]
MSX (2 μM)	0.8 (99.2)
MSX (4 μM)	0.1 (99.1)
Azaserine (4 μM)	98.9 (1.1)
Chloramphenicol (200 μg/ml)	2.1 (97.9)
pCMB (0.1 mM)	1.6 (98.4)
NEM (2.5 mM)	6.8 (93.2)

[a] Percent inhibition given in parentheses.

temperature required for optimal γ-glutamyl transferase and biosynthetic activity was 30°C.

Divalent cation specificity. The relative effectiveness of various divalent cations in catalyzing the biosynthetic and γ-glutamyl transferase reaction is shown in Table 2. Maximum transferase activity was recorded at 0.3 mM Mn^{2+}, whereas Mg^{2+}, Co^{2+}, and Cu^{2+} expressed low activity at the same concentration. Ca^{2+} and Ni^{2+} did not support the enzyme activity.

Biosynthetic activity was maximum with 125 mM Mg^{2+}, Mn^{2+} and Co^{2+} showed very little biosynthetic activity at the same concentration. Cu^{2+}, Ca^{2+}, and Ni^{2+} showed no activity (Table 2).

Effect of urea on GS activity. Urea with increasing concentration was very effective in denaturing the native enzyme, resulting in loss of transferase as well as biosynthetic activity (Fig. 3a and b). Both Mg^{2+} and Mn^{2+} were effective in partially reducing the denaturation caused by urea. Mg^{2+} gave 40% protection to the biosynthetic enzyme with 5 mM urea, while Mn^{2+} was much more effective and gave 60% protection with same urea concentration.

Effect of inhibitors on GS activity. Inhibition of GS activity by L-methionine-D,L-sulfoximine (MSX) was concentration dependent from 1 to 4 μM. Azaserine, an inhibitor of GOGAT, did not inhibit the glutamine synthetase activity. The enzyme activity was arrested by the protein synthesis inhibitor, chloramphenicol. The -SH group blocker, para-chloromercuribenzoate (pCMB),and the thiol reagent, N-ethylmaleimide, blocked the enzyme activity, indicating that -SH groups are present at the active site of the enzyme.

Discussion

The data presented here are on partial characterization of the biochemical parameters of glutamine synthetase from *Anabaena doliolum*. A variety of effectors in the cellular milieu have been proposed to have a regulatory role.

Glutamine synthetase (GS) and glutamate synthase are the key ammonia-assimilating enzymes in cyanobacteria [8, 18, 19, 26, 29]. The enzyme was synthesized more under N_2-fixing conditions, followed by ammonium, nitrate, and nitrite. Similar inhibitory response of GS synthesis to the different nitrogen sources was also observed in *Anabaena variabilis* [19], *Anabaena cylindrica* [18], and *Nostoc muscorum* [22].

The pH optimum of 7.0 for both Mg^{2+}-dependent biosynthetic and Mn^{2+}-dependent transferase activities resembled that for the enzyme from the cyanobacterium *Anacystis nidulans* [7] and from animal, plant, and bacterial sources [16, 28], but differed slightly from that in the cyanobacteria *A. cylindrica* [20] and *Anabaena* L-31 [27], where the pH optimum was around 7.8.

GS in *Anabaena doliolum* has been found to be much more stable in the presence of divalent cations, Mg^{2+} and Mn^{2+}. This was evident when the activities were compared in the presence and absence of divalent cations at high and low temperatures with urea treatment. It was suggested that under these denaturing conditions, the -SH groups on the enzyme become more exposed after the removal of the divalent cations, resulting in the conversion of the active enzyme to a relaxed state. The divalent cat-

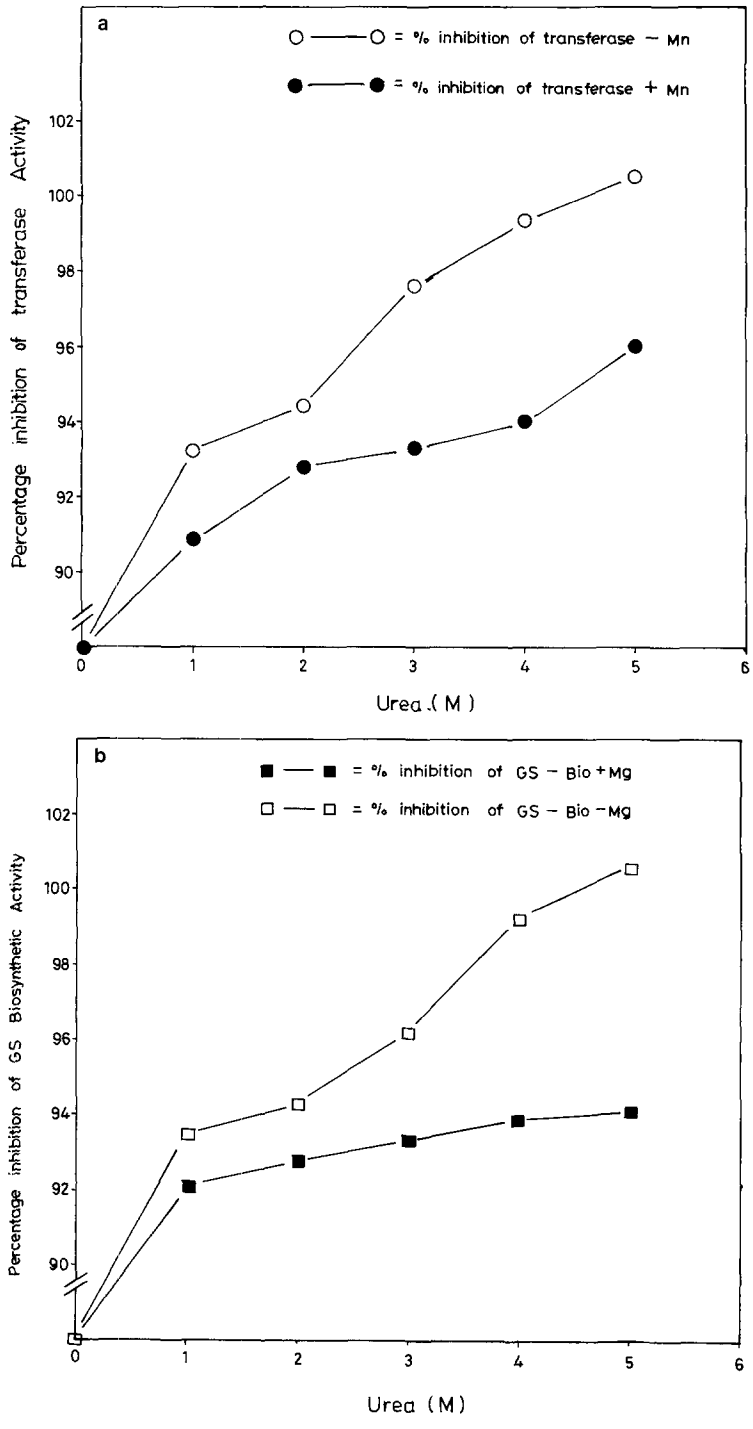

Fig. 3. (a) Percentage inhibition of the γ-glutamyl transferase activity by urea and the alleviation of the inhibition by manganese. (b) Percentage inhibition of the glutamine synthetase biosynthetic activity by urea and its alleviation by magnesium.

ions, Mg^{2+} and Mn^{2+}, decrease the denaturation owing to their capacity to induce aggregation of the enzyme molecules, causing less exposure of the vulnerable portions of the enzyme to the external influences by reducing the surface area [27, 28]. These observations are in agreement with the results for the GS from *E. coli* [10, 21], *A. cylindrica* [20], and *Anabaena* L-31 [27].

The important role of the -SH groups present at the active site of the enzyme is further confirmed by pCMB and NEM, which block the -SH groups and inhibit the enzyme activity. *Anabaena* L-31 GS also showed inhibition by pCMB [27].

Mg^{2+} and Mn^{2+} stabilize the enzyme structure and are specific for biosynthetic and γ-glutamyl transferase activities respectively [20, 23, 27]. This

was further confirmed with other divalent cations, Co^{2+}, Cu^{2+}, Ca^{2+}, and Ni^{2+}. These cations proved poor substitutes, possibly because they cannot regulate the enzyme structure and, hence, the catalytic function of the enzyme. A similar role of divalent cations has been observed in *Anabaena* CA [23], *A. cylindrica* [20], and *Anabaena* L-31 [27].

The analog of glutamate, MSX, competes with it for a common binding site on the catalytic part of the enzyme. Once firmly and irreversibly bound, it causes irreversible inactivation of the enzyme activity [8, 22, 24]. In contrast to MSX, which decreased the intracellular glutamine pool, azaserine, a specific glutamate synthase inhibitor, increased the glutamine pool and decreased the glutamate pool with no effect on GS activity [8, 18]. Lastly, the inhibition of GS activity with chloramphenicol suggested that the enzyme system was synthesized de novo on the ribosomes [18, 27].

Thus, studies on the regulation of glutamine synthetase in *A. doliolum*, a major inorganic nitrogen assimilatory enzyme, were crucial to be able to understand how this enzyme regulated the production of a diverse array of nitrogenous compounds essential for the maintenance and survival of living systems.

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