Vol. 96, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1980 Pages 975-983

REGULATION OF GLUTAMINE SYNTHETASE IN THE BLUE-GREEN ALGA *ANABAENA FLOS-AQUAE*

B.J. McMaster, M.S. Danton, T.A. Storch, and V.L. Dunham*

Department of Biology and Environmental Resources Center
State University College
Fredonia, New York 14063

Received August 20,1980

Summary: Glutamine synthetase (GS) was isolated from log phase cells and purified to a single protein as evidenced by gel electrophoresis. Protamine and ammonium sulfate precipitation and chromatography on DEAE-cellulose and Bio-Gel resulted in 380-fold purification. The enzyme was most sensitive to alanine (85% inhibition at 0.1 mM) but was also inhibited by glycine, arginine and serine. Combinations of inhibitory amino acids or nucleotides (AMP, ADP, ATP) exhibited cumulative inhibition. Cooperative inhibition was noted with CTP and any single nucleotide. Inhibition by CTP alone was uncompetitive with respect to glutamine. The enzyme was also regulated by the energy charge of the cell.

Glutamine synthetase (GS) [L-glutamate:ammonia ligase, ADP, EC 6.3.1.2] has been shown to function as a key enzyme in the assimilation of nitrogen in numerous organisms including bacteria (1,2), fungi (3), blue-green algae (4,5) and higher plants (6,7). The prokaryotic enzyme is regulated by repression, fluctuating levels of divalent cations *in vitro*, feedback inhibition, energy charge, and adenylylation (8). Although adenylylation has not been shown to regulate the algal enzyme, several of the above mechanisms may be active in blue-green algae. For example, the algal enzyme is regulated by feedback inhibition by amino acids and nucleotides (5,9). In addition, environmental factors such as iron and nitrate may regulate the levels of the algal enzyme (10). Regulation may be more complex in that multiple GS activities have been identified in bacteria (11,12) and higher plants (13,14).

This paper presents the results of an investigation of GS activity in the blue-green alga *Anabaena flos-aquae* as to the possible presence of

*Present address: Department of Plant Science, University College, P. O. Box 78, Cardiff CF1 1XL, United Kingdom.

0006-291X/80/180975-09$01.00/0

multiple activities, its regulation by various nitrogenous compounds, and a comparison of the enzyme's regulatory properties with those from other prokaryotic organisms.

Materials and Methods: Cultures of *Anabaena flos-aquae* (Lyng.) Bréb were obtained from Dr. R.C. Starr, Austin, Texas and maintained axenically under constant aeration and fluorescent light (2.9 W/m^2) in Bold's basal medium (15).

Crude homogenate--Six liters of log phase cells were harvested by centrifugation and suspended in 15 ml of buffer containing 200 mM imidazole-HCl (pH 7.5), 10 mM $MnCl_2$ and 2 mM 2-mercaptoethanol. Following sonication (at intervals for a total of 10 min), the sonicate was centrifuged at 14,500 x *g* for 15 min and the pellet discarded.

Purification--A 1% (w/v) solution of protamine sulfate was added to the supernatant to yield a concentration of 12% (v/v). Following stirring for 10 min and centrifugation at 22,000 x *g* for 20 min, the pellet was discarded. The supernatant was brought to 70% saturation with crystalline ammonium sulfate. Following centrifugation as above, the pellet was suspended in 2 ml of sonication buffer and dialyzed overnight against sonication buffer. Following centrifugation, the desalted preparation was applied to a DEAE-cellulose column and eluted with a linear gradient of KCl (0.1-1.0 M) in sonication buffer. Following concentration using membrane filtration, the enzyme was applied to a Bio-Gel A-0.5 column (2.3 x 113 cm) and eluted with sonication buffer containing 0.1 KCl. Purity of the preparation was monitored by disc gel electrophoresis (16). Approximate molecular weights were determined by gel filtration chromatography of the enzyme and commercially prepared proteins.

Assays--Enzyme activity was monitored by two assay procedures. The biosynthetic reaction

1) $ATP + L\text{-glutamate} + NH_3 \xrightarrow{Mg^{2+}} L\text{-glutamine} + ADP + Pi$

was performed by a procedure described by O'Neal and Joy (17). Pi released was monitored by the method of Chen *et al.* (18). The transfer reaction

2) $L\text{-glutamine} + NH_2OH \xrightarrow[AsO4,ADP]{Mn^{2+}} \gamma\text{-glutamyl hydroxamate} + NH_3$

employed a procedure described by Woolfolk *et al.* (19). GS activity was expressed in terms of μmoles γ-glutamyl hydroxamate formed in 10 min at 37° C (one unit). Protein was determined by the method of Lowry *et al.* (20) using bovine serum albumin as the standard.

Characterization--Various known inhibitors of GS were added to the assay separately and in combinations over an individual concentration range of 1-10 mM. Double reciprocal and Dixon plots were used to characterize the type of inhibition. Three methods were employed to determine if GS is regulated by adenylylation: a) a spectrophotometric assay (8), b) an assay of activity in the presence of Mg^{2+} or Mn^{2+} (2), and c) snake venom phosphodiesterase treatment of the enzyme (1,4).

Results: The isolation and purification procedures resulted in a 380-fold purified enzyme preparation (Table 1) which appeared as a single band of protein after gel electrophoresis. The one enzyme activity observed following chromatography on DEAE-cellulose and Bio-Gel had an approximate molecular weight of 430,000. Activity monitored by the transfer assay was 50-fold greater than the biosynthetic assay and was dependent on all assay

Table 1. Purification of Glutamine Synthetase from *Anabaena*

Purification Step	Volume (ml)	Protein (mg/ml)	Total Units*	Specific Activity**	Purification
Crude Homogenate	18.0	5.8	1100	11	1
Protamine Sulfate	17.6	2.5	1680	38	4
Ammonium Sulfate	4.3	8.1	1750	50	5
DEAE-cellulose	23.4	0.01	670	260	25
BioGel A-0.5	39.0	0.004	560	4000	380

*1 Enzyme unit = 1 μmole γ-glutamylhydroxamate/10 min at 37° C.
**Specific activity = total enzyme units/total protein.

components except ADP. In the absence of ADP, 27% of the complete assay activity remained.

The apparent K_m with respect to glutamine was determined to be 10 mM (Fig. 1). The enzyme was most sensitive to feedback inhibition by alanine. At a concentration of 0.1 mM, alanine inhibited GS by 85% (Table 2). The enzyme was also inhibited by glycine and serine at 1 mM concentrations and aspartate at 5 mM. No inhibition of GS occurred with arginine, asparagine, glutamine, glutamate, histidine, isoleucine, leucine, or threonine at concentrations as high as 10 mM. All nucleotides tested were inhibitory with ATP showing 90% inhibition at 10 mM. Other known inhibitors of GS (Table 2) were inhibitory to the algal enzyme at concentrations above 1 mM.

A comparison of the observed inhibition with the calculated cumulative or additive effect of combined inhibitors indicated that the algal GS was inhibited in a cumulative manner (Table 3). It is notable that when CTP was combined with any other single nucleotide, a cooperative inhibition was observed. The addition of phosphate groups to AMP resulted in increased inhibition of GS.

Further analysis of the type of inhibition by each inhibitor indicated a possible mechanism for CTP and alanine. Double reciprocal plots showed

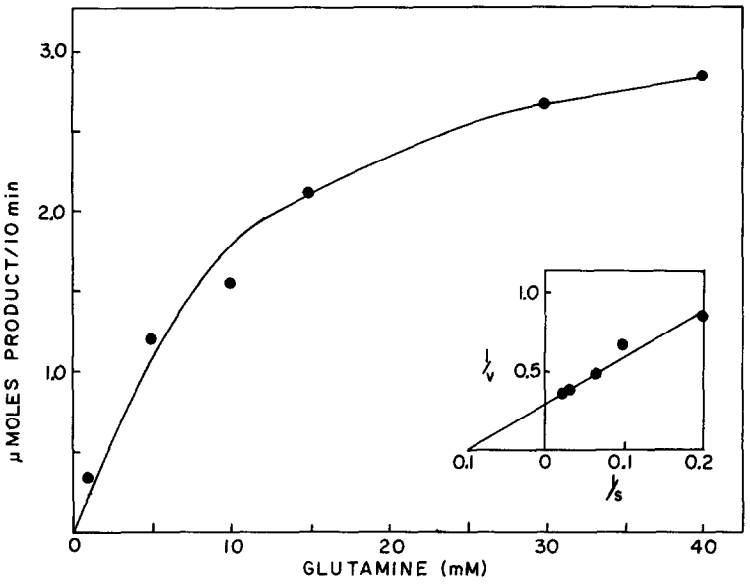

FIGURE 1. Effect of substrate concentration on the glutamine synthetase from *Anabaena*. Insert is a double reciprocal plot of the data. Product is γ-glutamylhydroxamate.

that GS is uncompetitively inhibited by CTP, with respect to glutamine, up to 10 mM CTP (Fig. 2). The most effective amino acid, alanine, exhibited mixed inhibition (Fig. 3).

Spectrophotometric analysis of dialyzed enzyme at 260 and 290 nm (260/290 = 0.71) indicated that the enzyme was not adenylylated (22). However, when Mg^{2+} was substituted in the transfer assay for Mn^{2+} (2), only 6% of the GS activity remained, suggesting the possibility of adenylylation. In addition, a slight release of phosphate was observed when 8 μg of GS were treated with 48 μg of snake venom phosphodiesterase (1,4) although GS activity was not altered.

Discussion: Although regulation of GS from *A. flos-aquae* by amino acids and nucleotides was generally similar to the enzyme isolated from other sources, GS was very sensitive to serine, glycine and, especially, alanine. For example, the enzyme was 2-fold more sensitive to alanine and serine when compared to the biosynthetic activity of the GS from *A. cylindrica* (5). Alanine, as analyzed by double reciprocal plots, resulted in a mixed

Table 2. Inhibitors of Glutamine Synthetase Activity from *Anabaena*

Compounds	% of Control Activity at 1 mM	5 mM	10 mM
amino acids			
L-alanine	16*	0.3	0.0
L-aspartate	100	75	76
glycine	59	21	18
L-serine	66	31	26
nucleotides			
AMP	89	88	72
ADP	97	88	78
ATP	84	59	12
CTP	91	76	57
other compounds			
L-methionine DL sulfoximine	89	84	67
carbamyl phosphate	92	76	74
glucosamine 6-phosphate	101	72	64
pyrophosphate	94	48	47

*Control activity = 1.68 μmole γ-glutamylhydroxamate formed in 10 min at 37° C. Glutamine concentration was 30 mM in all assays. Average of three experiments.

type of inhibition with respect to glutamine at alanine concentrations of 0.1-1.0 mM. Although internal concentrations of serine are low in blue-green algae (21), other inhibitory amino acids such as alanine and glycine are known to increase following exposure of cultures to increased nitrogen (22). Therefore, these amino acids may be important regulators of GS *in vivo*.

The increased inhibition of GS with increased phosphate groups on AMP indicated that the transfer reaction was extremely sensitive to ATP and

Table 3. Inhibitor Combination Studies of GS Activity from *Anabaena*

Inhibitor (1 mM each)	Observed	% Inhibition Cumulative	Additive
ala(85)+gly(33)*	83	90	118
ala(85)+asp(0)	87	85	85
ala(85)+ser(27)	86	89	112
gly(33)+asp(0)	31	33	33
gly(33)+ser(27)	47	51	60
asp(0)+ser(27)	30	27	27
ala(85)+gly(33)+asp(0)	88	88	118
gly(33)+asp(0)+ser(27)	43	50	60
ala(85)+asp(0)+ser(27)	84	89	112
ala(85)+gly(33)+ser(27)	89	90	145
ala(85)+gly(33)+ser(27)+asp(0) (2.5 mM each)	90	90	145
AMP(3)+ADP(10)	11	13	13
AMP(3)+ATP(26)	30	28	29
AMP(3)+CTP(8)	17	11	11
ADP(10)+ATP(26)	26	33	36
ADP(10)+CTP(8)	23	17	18
ATP(26)+CTP(8)	38	32	34
AMP(3)+ADP(10)+ATP(26)	25	33	39
ADP(10)+ATP(26)+CTP(8)	31	40	44
AMP(3)+ATP(26)+CTP(8)	46	38	37
AMP(3)+ADP(10)+CTP(8)	25	23	21
AMP(3)+ADP(10)+ATP(26)+CTP(8)	31	40	47

*Numbers in parentheses indicate percent inhibition of enzyme activity by the individual compound. Average of three experiments.

that the enzyme may be controlled by the energy charge of the cell. These data agree with other reports on the involvement of energy charge (5,23) and are notable since internal levels of these nucleotides are altered by environmental changes (24). In addition, any released phosphate would inhibit the enzyme as well (Table 2). Although most of these inhibitors regulated the enzyme in a cumulative manner as shown in other organisms (4,5,8), a synergistic type of inhibition with respect to CTP and any other single adenine nucleotide was observed in these studies. CTP may

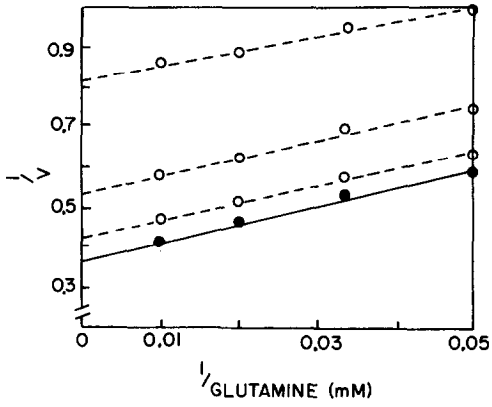

FIGURE 2. Inhibition of glutamine synthetase from *Anabaena* by CTP. Graph represents linear regression analysis of data. Control (o—o). Ascending lines (o---o) indicate CTP concentrations of 1, 5, and 10 mM, respectively.

bind, therefore, at a site which interacts with the binding site of adenine nucleotides. This may not be the glutamine binding site since CTP proved to be uncompetitive with respect to glutamine.

Preliminary evidence indicated that the enzyme may be regulated by covalent modification. Although ADP has not been shown to be covalently linked to the enzyme (5), the purified, dialyzed enzyme retained 27% of its activity in the absence of ADP. Although some phosphate was released

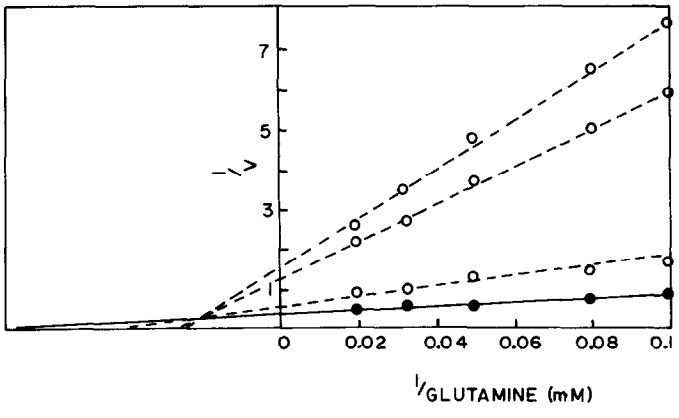

FIGURE 3. Effect of alanine on the glutamine synthetase from *Anabaena*. Graph represents linear regression analysis of data. Control (o—o). Ascending lines (o---o) indicate alanine concentrations of 0.1, 0.5, and 1 mM, respectively.

after treatment with phosphodiesterase, the release was not accompanied by a change in GS activity.

The data presented here indicate that the one form of GS from *A. flos-aquae* is regulated by the amino acids alanine, glycine, and serine and the nucleotides ATP and CTP. The regulation by the amino acids appears to be a cumulative inhibition. In addition, the energy charge of the cell appears to play a key role in the assimilation of ammonia.

Acknowledgement: We thank Mr. David Verstreate for his technical assistance and the Department of the Interior, Office of Water Research and Technology for financial support (BO-74 NY).

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