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REGULATION OF GLUTAMINE SYNTHETASE IN THE BLUE-GREEN ALGA *ANABAENA* L-31

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Summary

In N_2-grown cultures of *Anabaena* L-31, in which protein synthesis was prevented by chloramphenicol, presence of NH_4^+ caused a drastic decrease of glutamine synthetase (L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2) activity indicating NH_4^+-mediated inactivation or degradation of the enzyme. The half-life of glutamine synthetase was more than 24 h, whereas that of nitrogenase (reduced ferredoxin:dinitrogen oxidoreductase (ATP-hydrolysing), EC 1.18.2.1) was less than 4 h, suggesting that glutamine synthetase may not act as positive regulator of nitrogenase synthesis in *Anabaena*.

Glutamine synthetase purified to homogeneity was subject to cumulative inhibition by alanine, serine and glycine. The amino acids, however, exhibited partial antagonism in this behaviour. Glyoxylate, an intermediate in photorespiration, virtually prevented the amino acid inhibition. Kinetic studies revealed inhibition of the enzyme activity by high Mg^{2+} concentration under limiting glutamate level and by high glutamate in limiting Mg^{2+}. Maximum enzyme activity occurred when the ratio of glutamate to free Mg^{2+} was 0.5 to 1.0. The results demonstrate that the enzyme is subject to multiple regulation by various metabolites involved in nitrogen assimilation.

Introduction

The enzymological evidence favouring the glutamine synthetase/glutamate synthase pathway as the major route of ammonium metabolism in microbes [1] and plants [2] has been confirmed by the use of radioactive nitrogen (^{13}N)

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

as tracer [3—6]. Moreover, glutamine synthetase (L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2), which is the key enzyme in ammonium utilization also in animals [7], catalyses the first major step in the formation of a variety of nitrogenous constituents in various organisms [8]. In Gram-negative bacteria, a fine mechanism of regulation of glutamine synthetase by adenylylation cascade has been established [9]. The enzyme in *Klebsiella pneumoniae* has been shown to function as a positive regulator of nitrogenase (reduced ferredoxin:dinitrogen oxidoreductase (ATP-hydrolysing), EC 1.18.2.1) synthesis [10,11].

In nitrogen-fixing blue-green algae, glutamine synthetase does not seem to be regulated by adenylylation cascade [12—14]. Feedback inhibition by amino acids has been demonstrated [13—15]. Adenine nucleotides and inorganic phosphate were also found to inhibit enzyme activity [13—15]. Here, we examine in detail the effects of ammonium and amino acids on glutamine synthetase of the nitrogen-fixing blue-green alga *Anabaena* L-31, and demonstrate the role of the substrates of the enzyme and of glyoxylate in regulation of the enzyme activity. The relationship between glutamine synthetase and nitrogenase is also considered.

Materials and Methods

Materials

Anabaena L-31, a nitrogen-fixing blue-green alga [16] was the experimental organism. All biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Inorganic chemicals were bought from BDH laboratories, India. Electrophoresis material came from LKB Produckter AB, S161 25 Bromma-1, Sweden.

Methods

Media and growth. Anabaena L-31 was grown without combined nitrogen as described elsewhere [17]. NH_4^+ was added, when desired, as NH_4Cl buffered with equimolar Hepes (pH 7.0). Growth was determined by measuring chlorophyll *a* [18]. All the experiments were done using exponential phase (48—72 h old) cultures.

Preparation of cell-free extracts and purification of glutamine synthetase. Cell-free extracts of *Anabaena* L-31 were prepared as described elsewhere [17]. The proteins precipitated by 40% $(NH_4)_2SO_4$ were discarded and the supernatant was resolved by affinity chromatography on Blue Sepharose [17] to yield homogeneously purified glutamine synthetase. Our procedure was developed independently of that described by Tabita et al. [14] using the same active moiety for affinity chromatagraphy.

Assays. Glutamine synthetase activity was measured by biosynthetic and γ-glutamyl transferase assays [17] similar to those described by Shapiro and Stadtman [19]. Protein was determined by the method of Lowry et al. [20]. Nitrogenase activity was measured by acetylene reduction as described by David and Fay [21].

Acrylamide gel electrophoresis. Electrophoresis was done as described by Davis [22]. The gels were incubated in the transferase assay mixture and the

bands for enzyme activity were developed by dipping the gels in 10% $FeCl_3$.

Isoelectric focusing. The procedure described in the instruction manual for LKB Ampholene 8100 (Electrofocusing Equipment) was followed using 110 ml column with the cathode at the top. The enzyme was mixed in sucrose gradient containing equal mixture of 1% ampholine of pH 4—6 and pH 5—7. The electrofocusing was performed at 600 V and 4°C for 24 h and fractions of 2 ml were collected.

Results

Effect of NH_4^+

In N_2-grown cultures of *Anabaena* L-31 glutamine synthetase activity increased during the exponential phase (Fig. 1). The increase was arrested in the presence of chloramphenicol and the specific activity remained stable at the original level during the 24 h experimental period. Addition of NH_4^+ to the N_2-grown cultures also prevented the enhancement of glutamine synthetase activity during exponential growth. Remarkably, in the presence of NH_4^+ and chloramphenicol together, enzyme activity decreased sharply to 40% of the control after 8 h.

Experiments similar to that described above were done to investigate parallel effects, if any, of NH_4^+ on nitrogenase. The enzyme showed a half-life of about

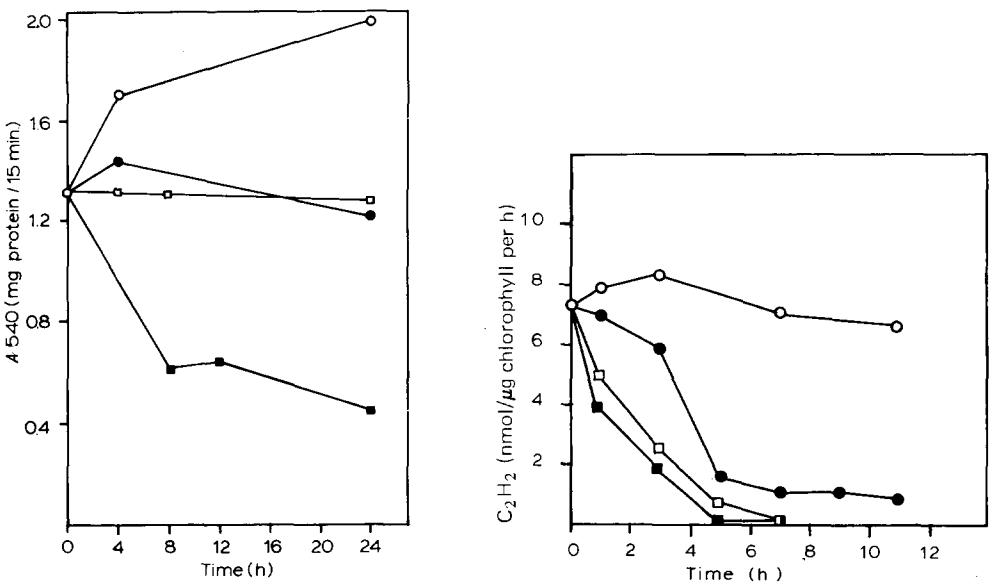

Fig. 1. Ammonium inactivation of glutamine synthetase from *Anabaena* L-31. To aliquots of 48-h-old N_2-grown cultures 5 mM NH_4Cl buffered with 5 mM Hepes (pH 7.0) (●); 200 μg per ml chloramphenicol (□); or 5 mM buffered NH_4CL + 200 μg per ml chloramphenicol (■) were added. Aliquots without addition (○) served as control. 70-ml portions were withdrawn at different intervals for the estimation of Mn^{2+}-dependent transferase activity in cell-free extracts.

Fig. 2. Ammonium inactivation of nitrogenase in *Anabaena* L-31. Details of the treatments were as described in Fig. 1. Nitrogenase activity was estimated as described in methods. ○, control; ●, NH_4^+; □, chloramphenicol; ■, NH_4^+ plus chloramphenicol.

2 h in the presence of chloramphenicol (Fig. 2). The activity sharply declined to 15% of the original level after 7 h when NH_4^+ was present. NH_4^+ and chloramphenicol together decreased nitrogenase activity more quickly.

Two types of glutamine synthetase have been reported in nitrogen-fixing bacteria [23] and in plants [24]. Because N_2-grown filaments of *Anabaena* have two different cell types, having differential activities of the enzyme [12,25], it is conceivable that two types of glutamine synthetase may exist in such algae. However, the crude enzyme preparations (proteins precipitated from cell-free extract by 40—80% $(NH_4)_2SO_4$) obtained after growth of N_2 and NH_4^+, when examined by polyacrylamide gel electrophoresis, showed transferase activity at a single band located at the same place towards the cathode. Enzyme preparation from whole filaments of N_2-grown culture formed a single band on isoelectric focusing, at a pH of 4.3 (data not included).

Inhibition by amino acids and its modification

Inhibition of glutamine synthetase from *Anabaena* by amino acids has been reported [13—15]. We have examined amino acid inhibition with a view to bring out possible differences, if any, in the enzyme purified to homogeneity from N_2-grown and NH_4^+-grown cultures of *Anabaena* L-31. Major inhibition of the enzyme from both situations was caused by alanine, serine and glycine (Table I). Glutamine synthetase from both the culture conditions was equally sensitive to amino acid inhibition, unlike the enzyme from *E. coli* where sensitivity increases with NH_4^+-induced adenylylation [26]. Transferase activity was more susceptible to inhibition than biosynthetic activity. These results are in agreement with those obtained by Stacey et al. [14]. The amino acids inhibited cumulatively but not additively. Such cumulative inhibition was observed even at low concentrations (2 mM) of amino acids. However, inhibition of enzyme activity by a mixture of amino acids was less than that expected, if the amino acids were inhibiting cumulatively and independently.

TABLE I

INHIBITION OF PURIFIED GLUTAMINE SYNTHETASE BY AMINO ACIDS

Amino acids were present at 5 mM concentration. Biosynthetic assay mixture contained 100 μmol Hepes (pH 7.6), 10 μmol L-glutamate, 10 μmol NH_4Cl, 20 μmol $MgCl_2$ and 5 μmol ATP in 0.9 ml. Figures in parentheses indicate the inhibition expected if the amino acids were inhibiting cumulatively and independently [27].

Inhibitor	Percent inhibition			
	Biosynthetic		Transferase	
	N_2-grown	NH_4^+-grown	N_2-grown	NH_4^+-grown
L-Ala	45	47	88	89
β-Ala	29	30	70	73
L-Ser	42	44	60	54
Gly	36	37	53	51
L-Ala + L-Ser	56 (68)	57 (70)	89 (95)	89 (95)
L-Ala + Gly	53 (65)	55 (67)	88 (94)	87 (95)
Gly + L-Ser	54 (63)	55 (65)	67 (81)	66 (78)
L-Ala + Gly + L-Ser	68 (80)	65 (80)	92 (98)	90 (98)

We interpret our results to mean that the presence of one amino acid results in partial antagonism to the inhibitory effect of the other [27].

10 mM of glycolate, glyoxylate, formate, malate, oxalate, pyruvate or α-ketoglutarate had no significant effect on transferase activity of glutamine synthetase. Of these organic acids, glyoxylate and pyruvate afforded protection for the enzyme against amino acid inhibition. Whereas protection by pyruvate was marginal, that by glyoxylate was remarkable. The more significant protection by glyoxylate was examined in detail using the purified enzyme. Inhibition of transferase activity by 2 mM alanine, serine and glycine (75, 42 and 36%, respectively) could be effectively prevented by glyoxylate. While 10 mM glyoxylate protected the enzyme from serine inhibition completely, the percentage inhibition by glycine and alanine decreased to 20 and 30%, respectively. To ascertain whether glyoxylate can protect completely against glycine and alanine also at higher concentrations, the inverse of the fraction protected was drawn against the reciprocal of glyoxylate concentration (Fig. 3). Plots for both amino acids intercept the y-axis below the value 1, indicating that glyoxylate can offer total protection against these amino acids [28]. Similar results were obtained for protection of Mg^{2+} dependent biosynthetic activity against inhibition by 5 mM of the three amino acids. Double-reciprocal plots of percentage inhibition of biosynthetic activity versus glyoxylate showed that it was competitive with glycine and serine. Against alanine, protection increased in a sigmoidal fashion. The Hill plot for glyoxylate protection against glycine had a slope of 1.1 and that for protection against alanine was biphasic with slopes of 0.8 and 1.9. The results suggest that protection against glycine and serine is due to direct competition of glyoxylate with these amino acids for

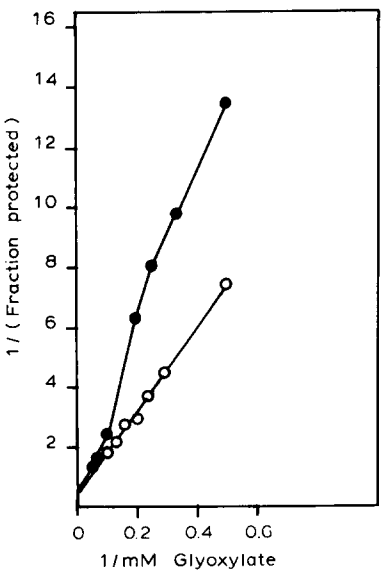

Fig. 3. Double-reciprocal plots for glyoxylate protection from amino acid inhibition of transferase activity of purified glutamine synthetase from N_2-grown *Anabaena* L-31. Fractional protection against inhibition by Gly (○); and Ala (●) was calculated from values for percentage inhibition by 2 mM of the amino acid in the absence or presence of 1 to 10 mM glyoxylate.

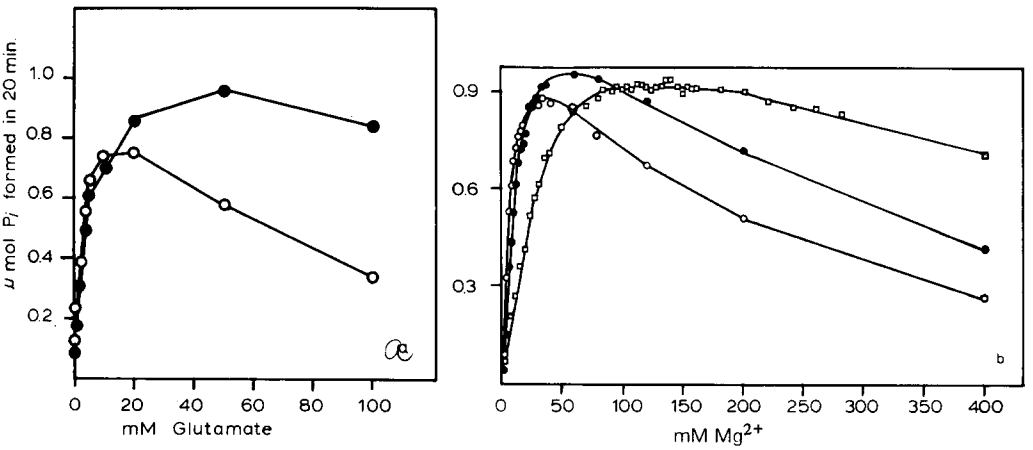

Fig. 4. a. Effect of Mg^{2+} concentration on substrate saturation curves for glutamate. Biosynthetic activity of purified glutamine synthetase from N_2-grown *Anabaena* L-31 was estimated as described in Methods except that amounts of glutamate varied. 15 mM, (○) and 70 mM (●) Mg^{2+}. b. Effect of glutamate levels on substrate saturation curves for Mg^{2+}. Biosynthetic activity of purified glutamine synthetase from N_2-grown *Anabaena* L-31 was estimated and the amounts of Mg^{2+} varied. Glutamate: ○, 10 mM; ●, 30 mM; and □, 100 mM.

binding sites on the enzyme. However, alanine inhibition appears to be prevented allosterically by glyoxylate.

Kinetic behaviour

In the Mg^{2+} dependent biosynthetic assay, the apparent K_m of glutamine synthetase for glutamate was 4 mM at high NH_4^+ (10 mM) and Mg^{2+} (70 mM) concentrations. However, the K_m for glutamate was dependent on Mg^{2+} concentration. For instance, by increasing Mg^{2+} concentration from 20 to 100 mM, the K_m for glutamate changed from 1.3 to 6.1. mM. High glutamate inhibited the enzyme but the inhibition could be averted by raising the level of Mg^{2+} (Fig. 4a). Similarly, Mg^{2+} at high concentration also inhibited the enzyme. The inhibitory effect of Mg^{2+} could be averted by raising the concentration of glutamate (Fig. 4b). Maximum enzyme activity was obtained when the ratio glutamate to free Mg^{2+} was between 0.5 to 1.0. Free Mg^{2+} was roughly estimated as the Mg^{2+} available in excess to the Mg-ATP complex assuming a 1 : 1 ratio of Mg^{2+} to ATP [29].

Saturation curves for NH_4^+ gave an apparent K_m of 0.2 mM NH_4^+ at 70 mM Mg^{2+} and 100 mM glutamate. At saturating glutamate and low Mg^{2+} some substrate inhibition by NH_4^+ was observed.

Discussion

Our results on the effect of addition of NH_4^+ and chloramphenicol to N_2-fixing cultures of *Anabaena* L-31, indicate inactivation or degradation of glutamine synthetase on confrontation with excess NH_4^+. The long half-life of the enzyme demonstrated by us substantiates the suggestion [14] that repression alone may not be the sole means of regulating its activity. Investigations

by various workers [12—14] have failed to reveal modulation of blue-green algal glutamine synthetase by adenylylation cascade, characteristic of other Gram-negative bacteria. In contrast to the results obtained with bacteria [30], provision of glutamate or glutamine with NH_4^+ also did not produce changes characteristic of adenylylation cascade (Tuli and Thomas, unpublished data). Further, the behaviour of the enzyme purified from N_2- and NH_4^+-grown cultures with respect to amino acid inhibition and the result from electrophoretic mobility and isoelectric focusing, discount the possibility of the presence of two types of enzyme in *Anabaena* L-31 unlike in other nitrogen-fixing organisms [23].

The rapid decline of nitrogenase when compared to the long half-life of glutamine synthetase in the presence of high NH_4^+ suggests that, in contrast to other N_2-fixing bacteria [10], glutamine synthetase may not be involved as a positive regulator of nitrogenase synthesis.

In vitro regulation of glutamine synthetase is controlled by serveral effector molecules. We have shown that amino acid inhibition can be relieved by glyoxylate. Photorespiration is prevalent in blue-green algae [31] and it is significant that glyoxylate, which is an intermediate in this process and is a precursor for the synthesis of these amino acids, is involved in regulating glutamine synthetase activity. The role of metal ions in regulating glutamine synthetase activity is well known [26]. For instance, that free Mg^{2+} is required in excess of ATP has been reported in bacteria [26] and blue-green algae [14]. But that free Mg^{2+} has an effect on the interaction of the blue-green algal enzyme with glutamate has not been shown earlier.

References

1 Nagatani, H., Shimizu, M. and Valentine, C.R. (1971) Arch. Microbiol. 79, 164—175
2 Miflin, B.J. and Lea, P.J. (1977) Annu. Rev. Plant Physiol. 28, 299—329
3 Thomas, J., Wolk, C.P., Shaffer, P.W., Austin, S.M. and Galonsky, A. (1975) Biochem. Biophys. Res. Commun. 67, 501—507
4 Wolk, C.P., Thomas, J., Shaffer, P.W., Austin, S.M. and Galonsky, A. (1976) J. Biol. Chem. 251, 5027—5034
5 Skokut, T.A., Wolk, C.P., Thomas, J., Meeks, J.C. Shaffer, P.W. and W.S. Chien (1978) Plant Physiol. 62, 299—304
6 Meeks, J.C., Wolk, C.P., Schilling, N., Shaffer, P.W., Avissar, Y. and Chien, W.S. (1978) Plant Physiol. 61, 980—983
7 Tate, S.S. and Meister, A. (1973) in the Enzymes of Glutamine Metabolism (Prusiner, S. and Stadtman, E.R., eds.), pp. 77—127, Academic Press, New York
8 Stadtman, E.R. (1973) in The Enzymes of Glutamine Metabolism (Prusiner, S. and Stadtman, E.R., eds.), pp. 1—6, Academic Press, New York
9 Ginsburg, A. and Stadtman, E.R. (1973) in The Enzymes of Glutamine Metabolism (Prusiner, S. and Stadtman, E.R., eds.), pp. 9—43, Academic Press, New York
10 Streicher, S.L., Shanmugam, K.T., Ausubel, F., Morandi, C. and Goldberg, R.B. (1974) J. Bacteriol. 120, 815—821
11 Tubb, R.S. (1974) Nature 251, 481—485
12 Dharmawardene, M.W.N., Haystead, A. and Stewart, W.D.P. (1973) Arch. Microbiol. 90, 281—295
13 Rowell, P., Enticott, S. and Stewart, W.D.P. (1977) New Phytol. 79, 41—54
14 Stacey, G., Baalen, C.V. and Tabita, F.R. (1979) Arch. Biochem. Biophys. 194, 457—467
15 Sawhney, S.K. and Nicholas, D.J.D. (1978) Biochim. Biophys. Acta 527, 485—496
16 Thomas, J. (1970) Nature 228, 181—183
17 Tuli, R., Jawali, N. and Thomas, J. (1979) Indian J. Exp. Biol. 17, 1239—1241
18 Mackinney, G. (1941) J. Biol. Chem. 140, 315—322
19 Shapiro, B.M. and Stadtman, E.R. (1970) Methods Enzymol. 17A, 910—922
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275

21 David, K.A.V. and Fay, P. (1977) Appl. Environ. Microbiol. 34, 640—646
22 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
23 Darrow, R.A. and Knotts, R.R. (1977) Biochem. Biophys. Res. Commun. 78, 554—559
24 Stasiewicz, S. and Dunham, V.L. (1979) Biochem. Biophys. Res. Commun. 87, 627—634
25 Thomas, J., Meeks, J.C., Wolk, C.P., Shaffer, P.W., Austin, S.M. and Chien, W.S. (1977) J. Bacteriol. 129, 1545—1555
26 Shapiro, B.M. and Stadtman, E.R. (1970) Annu. Rev. Microbiol. 24, 501—524
27 Woolfolk, C.A. and Stadtman, E.R. (1967) Arch. Biochem. Biophys. 118, 736—755
28 Wedler, F.C., Carfi, J. and Ashour, A.E. (1976) Biochemistry 15, 1749—1755
29 Perrin, D.D. and Sharma, V.S. (1966) Biochim. Biophys. Acta 127, 35—41
30 Wohlhueter, R.M., Schutt, H. and Holzer, H. (1973) in The Enzymes of Glutamine Metabolism (Prusiner, S. and Stadtman, E.R., eds.), pp. 45—64, Academic Press, New York
31 Lex, M., Silvester, W. and Stewart, W.D.P. (1972) Proc. R. Soc. Ser. B. 180, 87—102