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Nitrogen Starvation Mediated by DL-7-Azatryptophan in the Cyanobacterium *Anabaena* sp. Strain CA

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The addition of DL-7-azatryptophan (AZAT), a tryptophan analog, to continuous cultures of *Anabaena* sp. strain CA grown with 10 mM nitrate as the nitrogen source resulted in the differentiation of heterocysts. Analysis of the intracellular amino acid pools of *Anabaena* sp. strain CA after the addition of AZAT showed a marked decline in the intracellular glutamate pool and a slight increase in the levels of glutamine. The in vitro activity of glutamate synthase, the second enzyme involved in primary ammonia assimilation in *Anabaena* spp., was partially inhibited by the presence of AZAT at concentrations which are effective in triggering heterocyst formation (15% inhibition at 10 μM AZAT and up to 85% inhibition at 1.0 mM AZAT). Azaserine, a glutamine analog and potent glutamate synthase inhibitor, had no effect on the triggering of heterocyst development from undifferentiated batch and continuous cultures of *Anabaena* sp. strain CA. However, the presence of 1.0 μM azaserine significantly decreased the intracellular glutamate pool and increased the glutamine pool. The addition of AZAT also caused a decrease in the C-phycocyanin content of *Anabaena* sp. strain CA as a result of its proteolytic degradation. AZAT also had an inhibitory effect on the nitrogenase activity of *Anabaena* sp. strain CA. All these results suggest that AZAT causes a general nitrogen starvation of *Anabaena* sp. strain CA filaments, triggering heterocyst synthesis.

The heterocyst is a morphologically and physiologically distinct cell which develops from vegetative cells of certain filamentous cyanobacteria (30). The heterocyst is formed in response to nitrogen starvation and is the major site of nitrogen fixation in filamentous cyanobacteria under aerobic conditions (1, 10, 24). The heterocyst has attracted much attention and intense study because it provides an excellent model system for the study of procaryotic cell differentiation.

Various studies on the regulation of heterocyst development in a filamentous cyanobacterium *Anabaena* sp. have searched for molecules that permit or provoke differentiation in normally repressing media. These studies used a wide variety of amino acid analogs, among them L-methionine-DL-sulfoximine (MSX), a glutamine analog, and DL-7-azatryptophan (AZAT), a tryptophan analog. These compounds have been reported to derepress and initiate the development of heterocysts from undifferentiated filaments (2, 4, 17, 20, 22, 26, 28). Mitchison and Wilcox (17) tested a wide range of amino acids and analogs for their effect on heterocyst formation. Of these compounds, only AZAT had any effect, including both a reduction in the number of cells between heterocysts (interval width) and the formation of adjacent (multiple) heterocysts in *Anabaena catenula*. Bothe and Eisbrenner (2) confirmed the effect of AZAT on heterocyst pattern in *Anabaena cylindrica*. Heterocyst frequency began to increase within 24 h of AZAT addition, the extent of the increase being dependent on the concentration of analog. Recent work indicated that AZAT can relieve the inhibitory effect of nitrate on heterocyst formation. Stacey et al. (22) have shown that the addition of AZAT (20 μM) to ammonium nitrate-grown cultures of *Anabaena* sp. strain CA devoid of heterocysts resulted in the derepression of heterocyst and nitrogenase synthesis. Later, Rogerson (20) also showed that AZAT can partially induce heterocyst formation but not nitrogenase activity, even under microaerophilic conditions, in *Anabaena variabilis*.

An explanation for the mode of action of MSX on the triggering of heterocyst formation has been provided by the studies of Stewart and Rowell (26). MSX directly or indirectly inhibits the activity of glutamine sythetase (GS), the first enzyme invovled in the primary ammonia assimilation of *Anabaena* spp. (15), and lowers the levels of intracellular glutamine. Thus, on the basis of these results, it has been suggested that the most likely candidate for a regulatory molecule is glutamine or some derivative of it (26, 29, 31). However, unlike MSX, AZAT did not inhibit the activity of GS (22). Therefore, glutamine or its metabolic byproducts may not be the sole key to the derepression of heterocyst development in *Anabaena* spp., and it remains an open question how AZAT exerts its effect on heterocyst development in *Anabaena* sp. strain CA.

In this study, continuous chemostat cultures grown with various nitrogen sources were used to systematically determine the effect of AZAT on the derepression of heterocyst development in *Anabaena* sp. strain CA.

MATERIALS AND METHODS

Organism. The organism used for these studies is a filamentous, heterocystous, marine cyanobacterium, *Anabaena* sp. strain CA (ATCC 33047), which has been described previously (23).

Batch culture conditions. Batch cultures of *Anabaena* sp. strain CA were routinely grown in Pyrex test tubes (2.5 by 15.0 cm) containing 20 ml of ASP-2 medium at 39°C, as described previously (23). Growth was monitored at 660 nm in a Spectronic 20 spectrophotometer, and heterocysts were examined microscopically. A mature heterocyst was defined as a cell with visible polar bodies observed with the light

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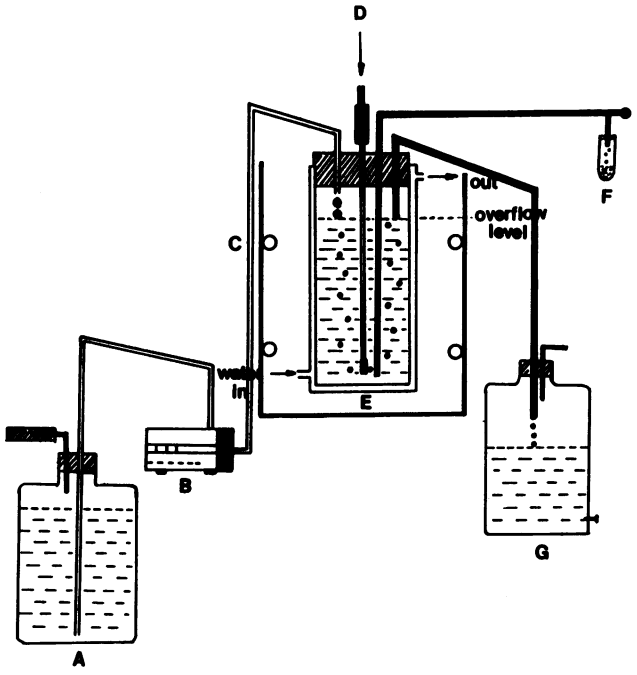

FIG. 1. Steady-state culture apparatus used for the continuous cultivation of *Anabaena* sp. strain CA. (A) Sterile medium reservoir containing ASP-2 medium; (B) LKB 2132 MicroPerpex peristaltic pump; (C) light source (four General Electric F36T12/D/HO fluorescent lamps on each side of the apparatus); (D) gas inlet (filter-sterilized air plus 1.0% CO_2); (E) 500-ml continuous-culture apparatus; (F) sample collector; (G) effluent receiver.

microscope at ×430 magnification. Heterocyst frequency was expressed as a percentage of the total cells; more than 500 cells were counted for this determination.

Continuous-culture conditions. Continuous cultures of *Anabaena* sp. strain CA (culture volume, 350 ml) were grown in 500-ml culture vessels. The inoculated continuous culture was allowed to grow in the batch mode for 24 h and reached a cell density of 25% transmittance (T) (264 μg [dry weight] per ml) before being put on stream for continuous-flow operation. A peristaltic pump was used to control the flow rate and maintain the preset culture density. Samples were withdrawn aseptically and monitored periodically for cell density and purity. The continuous culture was allowed to equilibate for at least 7 days, and after steady-state conditions were verified the experiments began. A schematic representation of the continuous culture apparatus is shown (Fig. 1).

Extraction of intracellular amino acid pools. Intracellular amino acid pools of intact filaments were extracted by the method of Dharmawardene and Stewart (8) after the cells were washed twice with fresh ASP-2 medium.

Determination of amino acid pools. Amino acid analyses were done with a Beckman amino acid analyzer (model 121 MB; Beckman Instruments Inc., Palo Alto, Calif.). A three-buffer single column (2.8 by 300 mm) of AA-10 resin (Beckman 121 MB application notes, 121 MB-TB-017) was used for the analysis of physiological fluids. The amino acids were detected by ninhydrin reagent and identified by comparing their elution times with those of known standards (physiological calibration mixture, Sigma Chemical Co. A 9906) and quantified with reference to the internal standard, β-(2-thienyl)-DL-alanine, by a Spectra-Physics computing integrator (model 4100; Spectra-Physics Inc., Mountain View, Calif.) as the data acquisition system. The results reported here are based on several single pilot experiments, and the values are the means of two separate determinations.

Measurement of C-phycocyanin content. The levels of C-phycocyanin were determined on the residues remaining after extraction of the cultures with 70% (vol/vol) ethanol as described previously (12). The concentration of C-phycocyanin was then calculated from the specific absorption coefficient at 620 nm: $[E]^{1\%} = 73.0$ (5, 6).

Preparation of cell extracts. *Anabaena* sp. strain CA cells were suspended in Tris chloride buffer (100 mM, pH 7.8), containing 5 mM EDTA and 10 mM 2-mercaptoethanol and disrupted by two passages through a French pressure cell at 12,000 lb/in^2. The cell debris was removed by centrifugation at 24,000 × *g* for 30 min at 4°C. The supernatant was used for enzyme assay after passage through a Sephadex G-25 column (1.5 by 45.0 cm) or after dialysis against the same buffer system overnight.

GOGAT assay. Glutamate synthase (GOGAT; EC 1.4.7.1) activity was determined by measuring the formation of L-[^{14}C]glutamate from 2-keto-[1-^{14}C]glutarate and unlabeled glutamine in the presence of the aminotransferase inhibitor aminooxyacetate under anaerobic conditions as described by Rai et al. (19) with a slight modification. The reaction mixture, in a total volume of 1.1 ml, contained 100 mM Tris chloride buffer (pH 7.8), crude enzyme extract (0.5 to 1.0 mg of protein), 2.5 mM 2-keto-glutarate containing 1.0 μCi of 2-keto-[1-^{14}C]glutarate per ml, 5.0 mM glutamine, 5.0 mM aminooxyacetate, 1.0 mM methyl viologen, and 12.5 mM sodium dithionite. The reaction was initiated by the addition of sodium dithionite, and the assay was kept anaerobic by flushing with dinitrogen gas. After incubation at 39°C for 15 min, the reaction was terminated by the addition of 1.0 ml of 95% ice-cold ethanol. The reaction mixture was then subjected to ascending chromatography on Polygram silica gel plates (20 by 20 cm; Brinkmann Instruments Co., Westbury, N.Y.), with *n*-butanol–acetic acid–water (4:1:1 by volume) as the solvent system. The glutamate spots, which cochromatographed with a known standard, were cut out, and the radioactivity was counted in a toluene-based scintillation cocktail with a Beckman liquid scintillation counter (model LS-100C; Beckman Instruments, Inc.). GOGAT activity was linear with respect to time of incubation and proportional to the amount of cell extract used.

Preparation of L-[*methyl*-^{14}C]methionine-labeled C-phycocyanin. *Anabaena* sp. strain CA was grown in 100 ml of ASP-2-nitrate medium for 24 h and then labeled with L-[*methyl*-^{14}C]methionine (0.20 μCi/ml) in 0.1 mM carrier methionine for another 24 h. During this time period, more than 80% of the L-[*methyl*-^{14}C]methionine was incorporated into protein. The cells were harvested by centrifugation, washed, and suspended in 5 ml of Tris chloride buffer (100 mM, pH 7.8). The cell suspension was then passed twice through a French pressure cell at 12,000 lb/in^2. The cell debris was removed by centrifugation at 18,000 × *g* for 15 min. L-[*methyl*-^{14}C]methionine-labeled C-phycocyanin was partially purified by passing the supernatant through DEAE-Sephacyl and Sephadex G-75 gel filtration columns. The purified ^{14}C-labeled C-phycocyanin had an A_{620}/A_{280} ratio of greater than 3.6 and contained 5.0 × 10^5 cpm of protein per ml at 245 μg/ml; this material was used as the substrate for the protease assay.

Determination of protease activity during AZAT treatment. Continuous cultures of *Anabaena* sp. strain CA were treated with 10.0 μM AZAT. After 0, 1, 2, 3, 4, 6, and 8 h, 10-ml

samples were taken and the cells were collected by centrifugation. Cell extracts were prepared by the method described above. Each protease assay solution contained 100 μCi of ^{14}C-labeled C-phycocyanin solution, 100 μl of crude extract, and 50 μl of Tris chloride buffer (100 mM, pH 7.8). The assay solutions were incubated in a shaking water bath at 39°C for 30 min. The reaction was terminated by adding 250 μl of 10% trichloroacetic acid (TCA), and the precipitate was removed by centrifugation. The radioactivity of the supernatant containing degradation products of ^{14}C-C-phycocyanin was counted in a liquid scintillation counter. Protease activity was expresed as counts of ^{14}C released per milligram of protein per hour.

Measurement of nitrogenase activity. Nitrogenase activity of whole cells of *Anabaena* sp. strain CA was determined by the acetylene reduction technique described previously (25). The specific activity of nitrogenase was expressed as nanomoles of ethylene produced per milligram of protein per minute.

Protein determination. Whole cell protein was calculated from standard curves relating cell protein and culture turbidity (3). An A_{660} of 1.0 in a Spectronic 20 spectrophotometer with a 2.5-cm light path is equal to 220 μg of protein per ml of culture. The protein content of cell extracts of *Anabaena* sp. strain CA was determined by the Coomassie blue-binding method (7), with bovine serum albumin as the standard.

Chemicals. AZAT and all other biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo., except azaserine (AZAS), which was the product of Calibiochem-Behring Corp., La Jolla, Calif. AZAT and AZAS were filter sterilized before addition of the media. 2-*keto*-[^{14}C]glutaric acid, sodium salt (specific activity, 54 mCi/mmol), was purchased from Amersham Corp., Arlington Heights, Ill. L-[*methyl*-^{14}C]methionine (specific activity, 41 mCi/mmol) was purchased from ICN Pharmaceuticals, Inc., Irvine, Calif. All common chemicals were of reagent grade.

RESULTS

Effect of amino acids and tryptophan precursors on AZAT-mediated heterocyst differentiation. Whatever the molecular basis for the ability of AZAT to trigger heterocyst development under conditions in which heterocyst synthesis is normally repressed (3, 22), AZAT must first be transported into the cells. It was asumed that this tryptophan analog was transported via the general amino acid transport system. The simultaneous addition of neutral and aromatic amino acids, precursors of tryptophan, and tryptophan itself with AZAT to batch cultures of *Anabaena* sp. strain CA can prolong or reverse the effect of AZAT in triggering heterocyst development (AZAT effective time was 22 h with tryptophan, 20 h with serine, and 10 h with glutamine, versus 5 h with AZAT alone). Acidic amino acids had no effect in reversing the derepression of heterocyst formation (AZAT effective time was 5 h with aspartic acid and glutamic acid).

AZAT-mediated heterocyst differentiation with different nitrogen sources. The ability of AZAT to trigger heterocyst differentiation of *Anabaena* sp. strain CA cultured with different nitrogen sources (10 mM sodium nitrate, 10 mM ammonium chloride, or dinitrogen) was examined (Table 1). The addition of AZAT to cultures of *Anabaena* sp. strain CA initiated the development of heterocysts and increased the heterocyst frequency under all conditions of nitrogen nutrition. Although multiple heterocysts were often observed, complete derepression of heterocyst formation was never obtained in filaments of *Anabaena* sp. strain CA.

TABLE 1. Effect of 10 μM AZAT on heterocyst differentiation in *Anabaena* sp. strain CA[a]

Nitrogen source	Heterocyst frequency (% of total cells)[b]	
	0 h	6 h
Sodium nitrate		
Control	0	0
AZAT	0	8
Ammonium chloride		
Control	6	6
AZAT	6	10
Dinitrogen		
Control	10	10
AZAT	10	14

[a] *Anabaena* sp. strain CA was grown in the continuous culture conditions described in Materials and Methods.

[b] Heterocyst frequency is expressed as the percentage of heterocysts among total cells (vegetative cells plus heterocysts). For each determination, at least 500 cells were counted.

Intracellular amino acid pools after AZAT or AZAS addition. Continuous cultures of *Anabaena* sp. strain CA were kept at the desired growth stage for at least 7 days to maintain the cells in a stable physiological state. At this time, AZAT or AZAS was added, and the effect on the intracellular amino acid pools of continuous cultures of nitrate-grown *Anabaena* sp. strain CA was noted. Of the amino acids analyzed, the intracellular levels of glutamate and glutamine were most affected. The addition of 10 μM AZAT to continuous cultures of *Anabaena* sp. strain CA caused a significant decrease in glutamate levels and a slight transient increase in the glutamine levels (Fig. 2A). This change in intracellular glutamate and glutamine levels corresponded to the derepression of heterocyst formation noted previously (4, 22). It was also noted that the addition of AZAT caused a marked increase in the pool of a nonprotein amino acid, citrulline (Fig. 2B). AZAS, a glutamine analog and potent GOGAT inhibitor, had no efect on the triggering of heterocyst formation in undifferentiated continuous cultures of *Anabaena* sp. strain CA, similar to the results in previous studies with batch cultures (4). However, the addition of AZAS (1 μM) to the continuous culture resulted in a significant increase in the glutamine pool, followed by a precipitous decrease; the glutamate pool gradually decreased with time (Fig. 3). AZAS also caused a general decrease in the total intracellular amino acid pools of other major intracellular amino acids, such as alanine, glycine, and serine (results not shown). Thus, the effect of AZAS was found to be similar to its effect on *A. cylindrica*, in which the conversion of glutamine to glutamate was reduced (21).

Effect of AZAT on GOGAT activity. Extracts prepared from cells grown in continuous cultures were used to measure GOGAT activity. The levels of AZAT normally used in the continuous culture studies (10 μM) resulted in only 15% inhibition of GOGAT activity in the in vitro assay. When the concentration of AZAT was increased beyond 100 μM, more than 75% of the GOGAT activity was inhibited, and 1 mM AZAT caused 88% inhibition (Fig. 4). GOGAT activity also declined in extracts taken from cells sampled periodically after the addition of 10 μM AZAT to the continuous culture of nitrate-grown *Anabaena* sp. strain CA (results not shown).

Degradation of C-phycocyanin and increase in protease activity specific for C-phycocyanin. The C-phycocyanin content of *Anabaena* sp. strain CA was determined by a

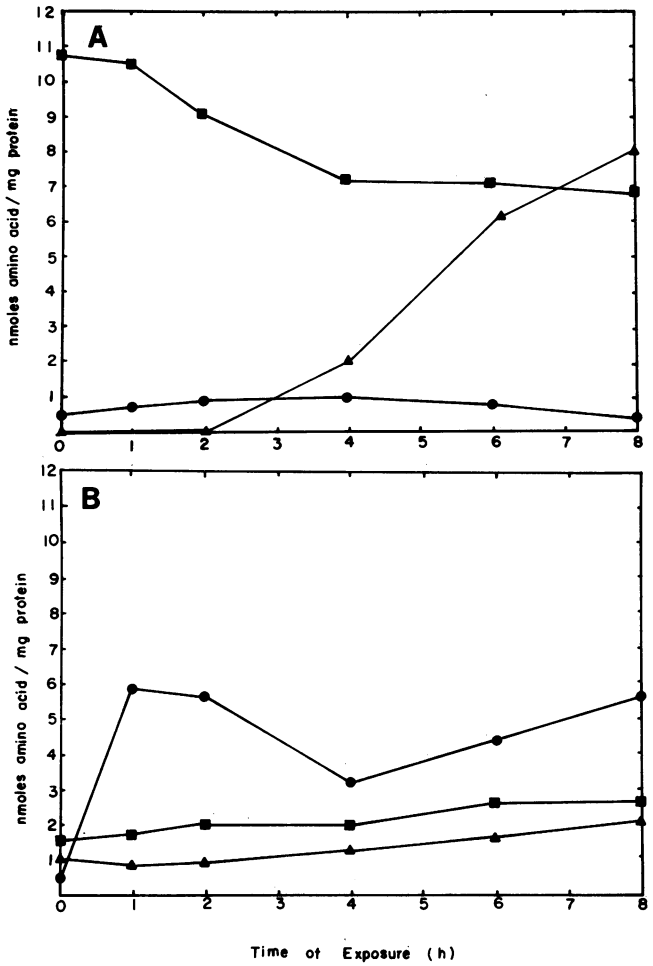

FIG. 2. Effect of AZAT (10 μM) added at time zero on the intracellular amino acid pools of a nitrate-grown continuous culture of *Anabaena* sp. strain CA. (A) Intracellular pools of glutamate (■) and glutamine (●) and heterocyst frequency (▲) and (B) intracellular pools of aspartic acid (■), citrulline (●), and ornithine (▲).

spectrophotometric assay. Previous experiments indicated that purified *Anabaena* sp. strain CA C-phycocyanin exhibited a typical absorption spectrum, with a maximal absorbance at 620 nm. The addition of AZAT to the culture of *Anabaena* sp. strain CA caused degradation of the storage protein C-phycocyanin whether the organism was grown with nitrate, ammonium, or dinitrogen as the nitrogen source. Nitrate-grown cultures of *Anabaena* sp. strain CA were more responsive to the addition of AZAT than dinitrogen-fixing cultures (Fig. 5). A marked increase in C-phycocyanin-degradative activity in vitro correlated with the decline of C-phycocyanin absorbance in vivo (Fig. 6).

Effect of AZAT on nitrogenase activity. Nitrogenase activity was determined after the addition of AZAT to continuous cultures of dinitrogen-fixing *Anabaena* sp. strain CA. The effect of AZAT on acetylene reduction itself was also determined. AZAT inhibited acetylene reduction activity in both cases (Fig. 7 and 8). A dinitrogen-fixing culture of *Anabaena* sp. strain CA lost about 70% of its acetylene reduction activity after 6 h of incubation with AZAT (Fig. 8). These results were consistent with the suggestion that AZAT does not function through an effect on the initiation of nitrogenase biosynthesis in *Anabaena* spp. (20), even though

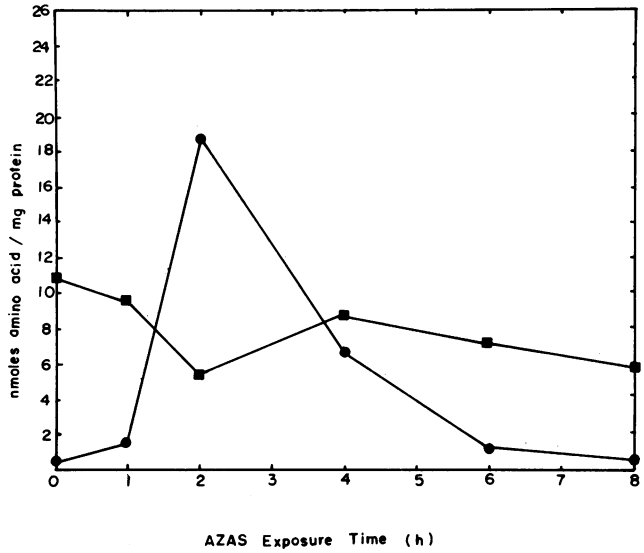

FIG. 3. Effect of AZAS (1 μM) added at time zero on the intracellular amino acid pools of nitrate-grown culture of *Anabaena* sp. strain CA. Symbols: ■, glutamate; ●, glutamine.

a previous report (4) showed low nitrogenase activity (10 to 15% of the activity of a dinitrogen-fixing culture) after the addition of AZAT to an ammonium nitrate-grown culture of *Anabaena* sp. strain CA.

DISCUSSION

AZAT, a tryptophan analog, has been shown to induce both a reduction in the number of vegetative cells between heterocysts and the formation of multiple heterocysts in undifferentiated cultures of the filamentous nitrogen-fixing cyanobacteria *A. catenula* (17), *A. cylindrica* (2), *Anabaena* sp. strain CA (4, 22), and *Anabaena* variabilis (20). The site of action and the mechanism by which AZAT provokes heterocyst development are completely unknown. It was previously postulated that AZAT might inhibit enzymes of tryptophan biosynthesis, leading to general nitrogen starvation and subsequent induction in the synthesis of heterocysts

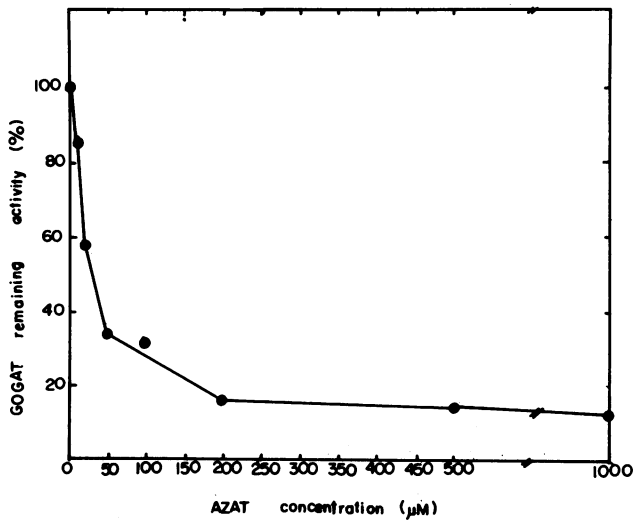

FIG. 4. Effect of AZAT on in vitro GOGAT activity in *Anabaena* sp. strain CA; 100% GOGAT activity was 26.7 nmol of glutamate formed per min per mg of protein.

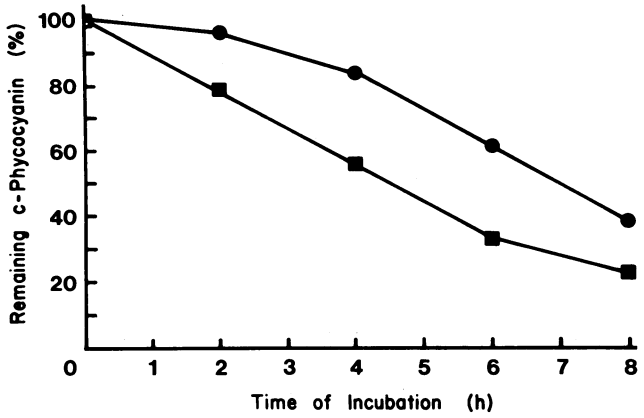

FIG. 5. Effect of AZAT on the C-phycocyanin content of continuous cultures of *Anabaena* sp. strain CA. AZAT (10 μM) was added at time zero. C-phycocyanin content was measured over time by the spectrophotometric method (12). The levels of C-phycocyanin are expressed as a percentage of the C-phycocyanin content at time zero; 100% C-phycocyanin content was 230.1 μg/ml of protein in dinitrogen-fixing cultures and 320.4 μg/mg of protein in nitrate-grown cultures of *Anabaena* sp. CA. Nitrogen source: 10 mM nitrate (■) or dinitrogen (●).

(4). Alternatively, AZAT might replace an amino acid in a protein so that its regulatory function in heterocyst formation is lost, similar to penicillinase synthesis in *Staphylococcus* spp. (11). In this study, we report that AZAT inhibits primary ammonia assimilation, which leads to symptoms characteristic of general nitrogen starvation in nitrate-grown cultures of *Anabaena* sp. strain CA. The evidence was provided by (i) an analysis of the intracellular amino acid pools, (ii) the determination of GOGAT activity, the second enzyme involved in primary ammonia assimilation in *Anabaena* spp., (iii) measurement of the content of the storage protein C-phycocyanin, and (iv) assay of the levels of a C-phycocyanin-specific protease induced after the addition of AZAT to continuous cultures of *Anabaena* sp. strain CA.

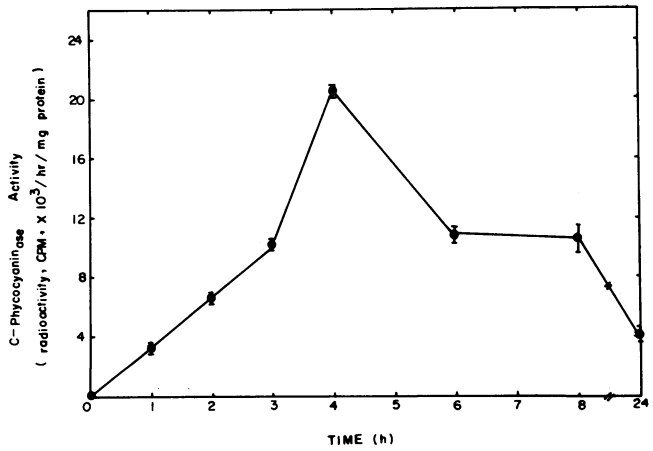

FIG. 6. Effect of AZAT on the induction of C-phycocyanin-specific protease. Protease activity was measured as described in Materials and Methods and assayed by the release of trichloroacetic acid-soluble radioactivity from purified L-[methyl-^{14}C]-methionine labelled C-phycocyanin. Activity is expressed as radioactivity (10^3 cpm) released per milligram of protein per hour.

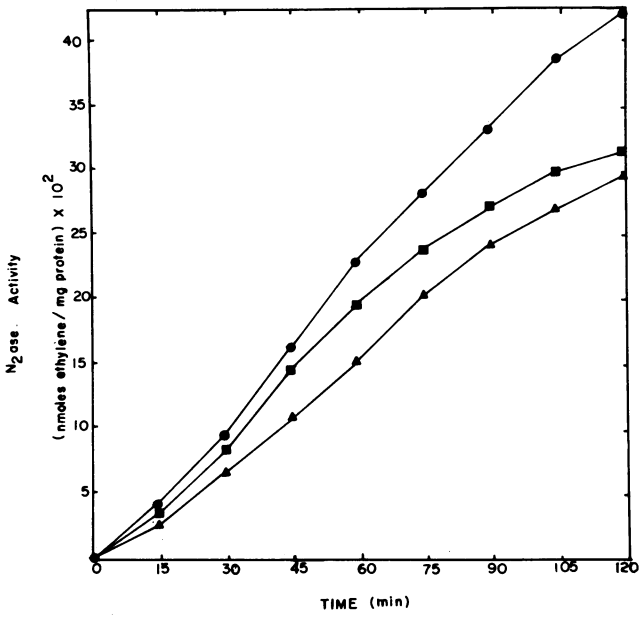

FIG. 7. Effect of AZAT on acetylene reduction. Various concentrations of AZAT were added to the acetylene reduction assay vials. Acetylene reduction was measured as described previously (3). Acetylene reduction activity is expressed as nanomoles of ethylene produced per milligram of protein. Symbols: ●, control, no AZAT; ■, 10 μM AZAT; ▲, 100 μM AZAT.

Quantitation of the intracellular amino acid pools of nitrate-grown continuous cultures of *Anabaena* sp. strain CA showed that glutamate was present at much higher concentrations than alanine, serine, glycine, aspartate, cystine, and ornithine. A very low level of glutamine was detected in these pools (only about 2.5% of the total intracellular amino acids). The addition of AZAT to nitrate-grown cultures of *Anabaena* sp. strain CA caused a change in the pool pattern of the intracellular amino acids. In the presence of AZAT, intracellular pools of glutamate decreased, followed by a slight transient increase in the levels of glutamine. The levels

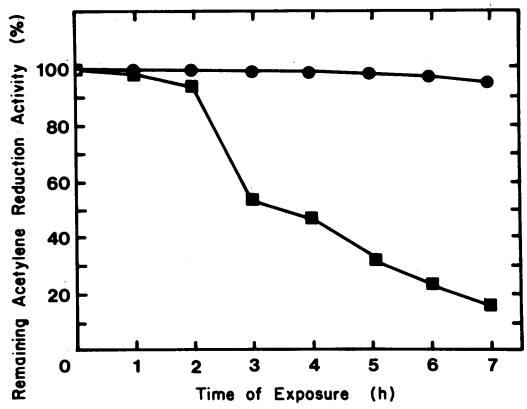

FIG. 8. Effect of AZAT on the nitrogenase activity of *Anabaena* sp. strain CA after incubation with cells grown on dinitrogen. The specific activity of nitogenase was determined after incubation with AZAT by removing samples at the indicated times and measuring acetylene reduction activity. Symbols: ●, control, no AZAT; ■, 10 μM AZAT. The 100% value for nitrogenase activity was 43.3 nmol of ethylene per mg of protein per min.

of alanine, glycine, and serine showed little change. A significant increase in the levels of intracellular citrulline and slight increases in aspartate and ornithine were noted. It is known that AZAT, unlike MSX, does not exert its effect by direct inhibition of the activity of GS in whole cells of *Anabaena* sp. CA under dinitrogen-fixing or ammonium nitrate growth conditions. Moreover, neither the purified GS nor the enzyme present in the isolated heterocysts was inhibited by AZAT. GS activity also does not decline in cultures sampled periodically after the addition of AZAT (22). However, determination of in vitro GOGAT activity in extracts of *Anabaena* sp. strain CA indicated that AZAT at the applicable concentration (10 μM) can inhibit about 15% of GOGAT activity; up to 88% of its activity was inhibited by 1.0 mM AZAT. It is possible that more severe inhibition of GOGAT activity by low concentrations of AZAT may occur in vivo due to the accumulation of this compound inside the cell. AZAS, a potent inhibitor of GOGAT and other amidotransferases (14, 16, 21, 27), did not induce the formation of heterocysts in nitrate-grown cultures of *Anabaena* sp. strain CA. The addition of AZAS (1.0 μM) to the nitrate-grown cultures did cause a transient accumulation of high levels of intracellular glutamine and a gradual decrease in the glutamate pool (Fig. 3). A significant change in the pools of alanine, serine, and glycine was also caused by AZAS (results not shown). The differences in the change of the pool patterns of amino acids caused by AZAT or AZAS and the sensitivity of GOGAT to AZAT or AZAS may explain why AZAS failed to initiate the formation of heterocysts in *Anabaena* sp. strain CA. However, tryptophan metabolism, as suggested previously (4), also appears to be implicated in heterocyst development in *Anabaena* sp. strain CA.

In addition to its inhibitory effect on the activity of GOGAT, AZAT also induced the synthesis of a C-phycocyanin-specific proteolytic activity, leading to a decrease in the C-phycocyanin content of nitrate-grown cultures of *Anabaena* sp. strain CA. Since the earliest-characterized enzymatic response to nitrogen starvation in *Anabaena* spp. is the induction of a specific protease (9, 13, 18, 31), the increase in proteolytic activity assayed in vitro was correlated with the loss of C-phycocyanin absorbance in vivo in nitrate-grown cultures treated with AZAT. These results also indicated that AZAT can cause nitrogen starvation-like symptoms in *Anabaena* sp. strain CA.

The inhibition of acetylene-reducing activity of dinitrogen-fixing cultures of *Anabaena* sp. strian CA by treatment of cultures with AZAT and by the addition of AZAT to the assay system suggests that AZAT does not function by affecting nitrogenase biosynthesis. Although a previous report showed low nitrogenase activity (10 to 15% of the activity of a dinitrogen-fixing culture) after the addition of AZAT to ammonium nitrate-grown cultures of *Anabaena* sp. strain CA (4),this low nitrogenase activity might merely be a consequence of the development of heterocysts.

In conclusion, it is difficult to attribute a specific site for the role of AZAT in mediating the observed effects. AZAT does appear to inhibit general nitrogen metabolism, and when vegetative cells encounter nitrogen starvation, all the cellular and metabolic activities must adjust. One adjustment appears to be the synthesis of heterocysts so that nitrogen fixation may be used for nitrogen nutrition under conditions of nitrogen starvation. It is also known that AZAT can be efficiently incorporated into and replace tryptophan in proteins. AZAT not only inhibits the biosynthesis of tryptophan but also competes with tryptophan in the charging of $tRNA^{Trp}$ and the incorporation of tryptophan into proteins. The AZAT-containing proteins might be seen as error proteins by the cells (4). Since the fundamental role of intracellular protein degradation is to protect the cells against the accumulation of abnormal or harmful proteins, an increase in the protein turnover rate by the induction of proteolytic enzymes to preferentially degrade the error proteins may occur. Whatever the precise mechanism by which AZAT exerts its effect on heterocyst derepression in *Anabaena* sp. strain CA, the fact that heterocyst differentiation occurs even when there is a slight increase in the intracellular glutamine pool suggests that this amino acid may not be a diffusible inhibitor which blocks heterocyst development in this organism.

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