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Control of Heterocyst and Nitrogenase Synthesis in Cyanobacteria

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The development of the heterocyst by filamentous nitrogen-fixing cyanobacteria provides an attractive model system for studying cellular differentiation. Heterocyst synthesis is repressed by the presence of exogenous combined nitrogen. In this report, it is shown that the tryptophan analog, D,L-7-azatryptophan (Aza-T), is capable of relieving the repressive effect of exogenous NH_4NO_3 on heterocyst and nitrogenase synthesis. In nitrogen-fixing cultures, the presence of 20 μM Aza-T increases the heterocyst frequency twofold. The glutamate analog, L-methionine-D,L-sulfoximine (MSX), has also been shown to cause a derepression in the synthesis of heterocysts and nitrogenase. However, unlike MSX, Aza-T does not appear to exert its effects by inhibiting the activity of glutamine synthetase. Therefore, glutamine synthetase may not be the sole key to the derepression of heterocyst and nitrogenase development in the cyanobacteria. It is hoped that a study of Aza-T action may lead to the elucidation of a novel control mechanism.

The control of cellular differentiation is one of the most basic and challenging problems in biology. Procaryotic model systems show much promise for investigating this process, and bacterial sporulation has been well-exploited for such studies (7). In recent years, attention has been focused on a novel and perhaps more attractive model: the filament of the nitrogen-fixing cyanobacteria (blue-green algae). These organisms contain some cells which are capable of differentiating into discrete forms called heterocysts in response to a deficiency of combined nitrogen in their environment (6). Under such conditions, the organism responds by fixing its own nitrogen, with the heterocyst apparently the major site of nitrogen fixation under aerobic conditions (5, 19). In addition to nitrogen fixation, the heterocyst shows several other physiological and biochemical characteristics which differ from adjacent vegetative cells (21, 22, 26, 27).

Kinetic studies on the synthesis of heterocysts and nitrogenase have shown that the two processes develop in a parallel fashion (9, 13), suggesting (but not proving) that there may be regulatory loci common to both. The glutamate analog, L-methionine-D,L-sulfoximine (MSX), a known potent inhibitor of glutamine synthetase (14), has been shown to relieve the repression exerted by exogenous ammonia on heterocyst formation and nitrogenase synthesis in *Anabaena cylindrica* (20). Furthermore, MSX-treated nitrogen-fixing cells excreted ammonia into the medium, without loss of nitrogenase activity, indicating that ammonia per se was not the repressor of nitrogenase and heterocyst synthesis (8, 20). Thus, on the basis of these results, it was postulated that the derepression of nitrogenase activity seen in the presence of MSX was due mainly to inhibition of glutamine synthetase, the first enzyme of the major ammonia assimilatory pathway in nitrogen-fixing cyanobacteria (4, 24). Furthermore, from such data, it was hypothesized that glutamine or a derivative of it is responsible for inhibiting the differentiation of vegetative cells into heterocysts (15, 23). In this paper we report observations concerning the effect of a tryptophan analog, D,L-7-azatryptophan (Aza-T), upon heterocyst and nitrogenase development in a filamentous cyanobacterium which confirm and substantially extend the findings of Mitchison and Wilcox with *Anabaena catenula* (11) and Bothe and Eisbrenner with *A. cylindrica* (1). Our data show that Aza-T simulates the effect of MSX by relieving the repression of nitrogenase synthesis and heterocyst differentiation by ammonia. However, in contrast to MSX, this compound does not inhibit the activity of glutamine synthetase, nor does it cause the cells to excrete ammonia or any other ninhydrin-positive material.

MATERIALS AND METHODS

Organism, media, and culture conditions. The organism used for these studies was *Anabaena* sp. CA, a filamentous cyanobacterium capable of rapid growth

on molecular nitrogen. This organism is ideally suited for studying heterocyst development because the process is relatively rapid; i.e., proheterocysts are observed 3 to 4 h after removing combined nitrogen. The growth conditions used in these studies have been described previously (17). Growth was measured turbidimetrically at 660 nm, and heterocysts were counted microscopically.

Reagents. The chemicals, MSX and Aza-T, were purchased from Sigma Chemical Co., St. Louis, Mo. These compounds were filter sterilized before addition to the media. Solutions of NH_4NO_3 were also filter sterilized before addition to the media to prevent the loss of ammonia upon autoclaving. All common compounds were of reagent grade quality.

Enzyme assays. Purified glutamine synthetase from *Anabaena* sp. CA was prepared by published methods (16). In vitro and in situ enzyme assays were performed as described previously (16). Isolated heterocysts for enzyme assays were prepared aerobically by the method of Thomas et al. (23).

Nitrogenase activity was measured by the acetylene reduction technique (18). Samples of 2 ml were removed from the growth tubes, and the cell suspension was incubated in 22-ml-capacity serum bottles under an atmosphere of argon:C_2H_2:CO_2 (86.5:12.5:1%, vol/vol). Saturating illumination was provided from a bank of 30-W incandescent reflector lamps placed 9 cm below the bottles. The bottles were shaken in a Warburg bath at 39°C. Portions of 0.5 ml from the gas phase were removed at 15-min intervals, and the amount of ethylene was determined by using a Tracor 550 gas chromatograph with a column packing of Porapak R. The method of Chaney and Marbach (2) following microdiffusion (3) was used to analyze culture filtrates for ammonia. The detection of ninhydrin-positive material was accomplished by using the procedures of Moore and Stein (12).

RESULTS AND DISCUSSION

The addition of 20 μM Aza-T to nitrogen-fixing cultures of *Anabaena* sp. CA increased the heterocyst frequency approximately twofold (Fig. 1a and b). The heterocyst pattern in these cultures was quite irregular (Fig. 1b), supporting previous observations obtained with *A. catenula* (11). However, we now report that 20 μM Aza-T allowed heterocyst differentiation to occur in *Anabaena* sp. CA in the presence of a level of NH_4NO_3 sufficient to repress heterocyst formation and nitrogenase synthesis in a control culture (Fig. 1c and d). Indeed, in contrast to the pattern seen with nitrogen-fixing cells, the heterocyst pattern of these cultures appeared to be regular and normal. Therefore, although Aza-T did relieve the repression of heterocyst formation in *Anabaena* sp. CA, NH_4NO_3 continued to influence development by maintaining a regular spacing of heterocysts.

Because the heterocyst is thought to be the major site at which nitrogen fixation takes place under aerobic conditions, we next examined the potential of Aza-T to derepress nitrogenase synthesis in NH_4NO_3-grown cultures (Fig. 2). Cells grown in the presence of 2.5 mM NH_4NO_3 and resuspended in medium containing 0.63 mM NH_4NO_3 grew logarithmically for about 9 h, whereupon the growth rate decreased to 28% of the initial rate (Fig. 2A). At this point, mature heterocysts were seen and acetylene reducing activity could be detected. In contrast, cells suspended in the same concentration of exogenous NH_4NO_3 and subsequently treated with 20 μM Aza-T showed several differences. Although growth was scarcely inhibited (17%) and occurred logarithmically for the same length of time as that of the control, proheterocyst differentiation could be seen by light microscopy within 3 to 3.5 h after Aza-T addition. Mature heterocysts became visible after 4 to 5 h, and nitrogenase activity was subsequently detected between 5.5 and 6.5 h after the addition of Aza-T. No pigmentation changes could be seen in these cultures throughout the time course of the experiment. The control culture during this period was still repressed both in terms of nitrogenase activity and heterocyst development. These results conflict with those of Bothe and Eisbrenner (1), who were unable to relieve ammonia repression of heterocyst and nitrogenase development by the addition of Aza-T to cultures of *A. cylindrica*. These differing results may be due to a heterogeneity among the cyanobacteria in their response to Aza-T. The time of induction was independent of Aza-T concentration over the range of 10 to 60 μM and appears to represent the minimum time needed to biosynthesize heterocyst and nitrogenase components. The effect exhibited by Aza-T is partial in that the heterocyst frequency obtained is about 50% of a normal nitrogen-fixing culture, while the nitrogenase activity obtained is 15 to 20% of the rate expected of a nitrogen-fixing culture. The rather low acetylene reducing activity in Aza-T-treated cultures is not due to direct inhibition of preformed enzyme because the addition of 20 μM Aza-T to a culture growing under nitrogen-fixing conditions does not result in an inhibition of nitrogenase activity over the same time period. Moreover, other workers have observed various degrees of derepression of nitrogenase activity by MSX which may depend in part upon the organism and experimental conditions used (8, 20, 25). It should also be stressed that the lack of complete derepression of nitrogenase activity and heterocyst differentiation mediated by Aza-T is not due to the formation of defective proteins because a secondary phase of induction takes place in the Aza-T-treated culture, concomitant with the initial induction of the control culture at the time

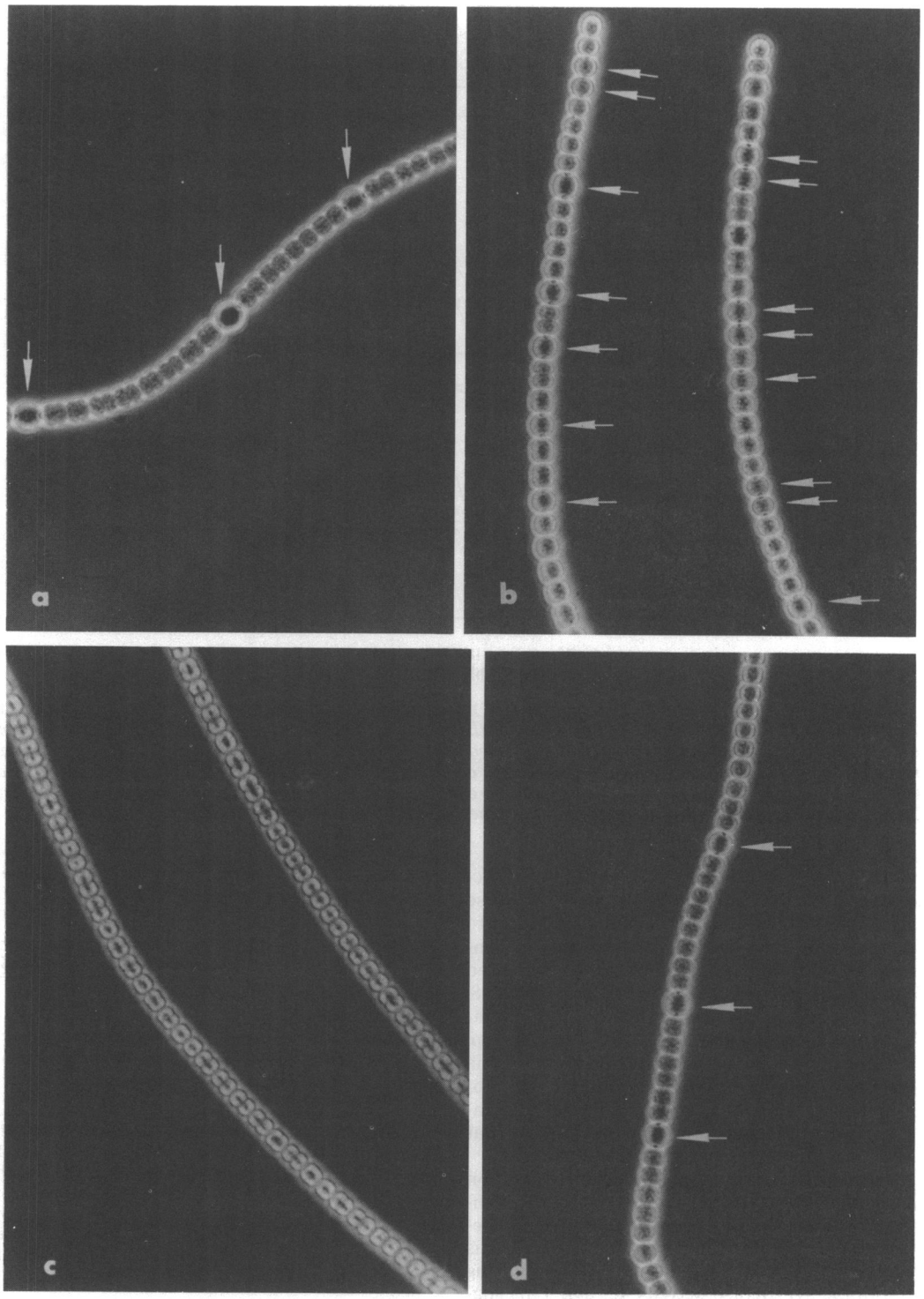

FIG. 1. *(a and b) Filaments of Anabaena sp. CA (magnification, ×200) grown at 39°C in modified ASP-2 medium minus exogenous fixed nitrogen. The cells were harvested, washed with sterile medium, and resuspended in fresh medium in the absence (a) and presence (b) of 20 μM Aza-T for 7 h under growth conditions. (c and d) Filaments of Anabaena sp. CA grown in modified ASP-2 medium plus 2.5 mM NH_4NO_3. The cells were washed and resuspended in fresh medium plus 0.63 mM NH_4NO_3 in the absence (c) and presence (d) of 20 μM Aza-T for 7 h under growth conditions. The arrows indicate those cells which are mature heterocysts.*

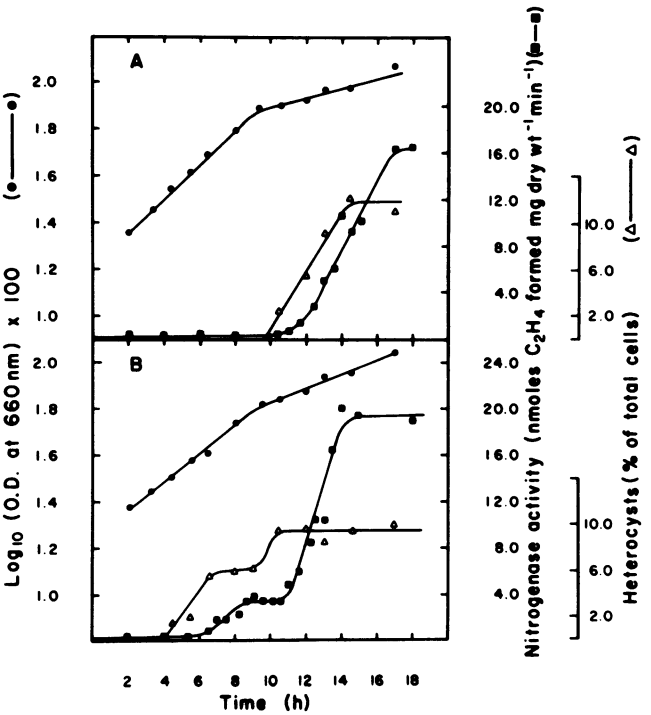

FIG. 2. *Time course of growth (●), induction of nitrogenase activity (■), and heterocyst formation (Δ) in Anabaena sp. CA in the absence (A) and presence (B) of Aza-T (20 μM final concentration). Cells were grown at 39°C, harvested in late exponential phase, washed once with sterile medium minus combined nitrogen, and resuspended in two 40-ml portions of fresh medium containing 0.63 mM NH_4NO_3. The initial cell density was 0.06 to 0.07 mg (dry weight) per ml. The cultures were placed back under growth conditions for 1 h before Aza-T (20 μM final concentration) was added to the experimental tube at time zero. O.D., optical density.*

when exogenous NH_4NO_3 is depleted. This secondary induction leads to values of nitrogenase activity and heterocyst frequency similar to those obtained by the control culture. In contrast to the data obtained with MSX-treated cultures of other nitrogen-fixing organisms (8, 20, 25), we have found that the addition of Aza-T to nitrogen-fixing cultures of *Anabaena* sp. CA does not lead to the excretion of ammonia or other ninhydrin-positive material (unpublished data). This suggests that the locus of Aza-T action is different from that of MSX, or that MSX may have more than one site of action.

To directly examine the effect of Aza-T on ammonia assimilation, we investigated its potential to inhibit the activity of glutamine synthetase (Table 1). As stated previously, MSX has been postulated to exert its effect by inhibiting glutamine synthetase activity (8, 20). This suggestion is supported by genetic evidence which implicates glutamine synthetase in the control of many aspects of nitrogen metabolism (10). The data in Table 1 show that Aza-T does not inhibit glutamine synthetase activity in whole cells of *Anabaena* sp. CA under nitrogen-fixing or NH_4NO_3 growth conditions. Furthermore, neither the purified enzyme nor the enzyme present in isolated heterocysts is inhibited by

TABLE 1. *Activity of glutamine synthetase in the presence of Aza-T or MSX*[a]

Enzyme source	% Activity remaining	
	1 mM Aza-T	1 mM MSX
Purified enzyme		
Transferase	108	22
Biosynthetic	108	0
N_2 whole cells	99	1.2
NH_4NO_3 whole cells	102	7.2
Heterocysts	97	27

[a] Whole cell assays contained 40 to 50 mU per assay. The specific activity of the purified enzyme was 16 μmol of P_i liberated per min per mg of enzyme for the biosynthetic assay and 120 μmol of γ-glutamylhydroxamate formed per min per mg of enzyme for the transferase assay. In all cases, enzyme was incubated in the presence of the inhibitor for 10 min before initiating the reaction.

Aza-T. Glutamine synthetase activity also does not decline in cultures sampled periodically after Aza-T addition. By contrast, MSX strongly inhibits the enzyme from all sources. The lack of complete inhibition by MSX in the transferase assay is due to the fact that under these conditions it is not an irreversible inhibitor, but merely a competitive inhibitor with respect to glutamine (14). Saturating levels of glutamine were used in these assays and, therefore, complete inhibition would not be expected. The differences in inhibition by MSX between the in vitro and in situ assays may reflect a difference in the environment of the enzyme.

Thus, the data are consistent with the idea that MSX is exerting its action by inhibiting glutamine synthetase. However, it appears that Aza-T may be causing derepression of heterocyst formation and nitrogenase synthesis by an independent mechanism. Alternatively, it is conceivable that both compounds may affect ammonia assimilation at some common locus beyond glutamine synthetase. We are currently examining the site of MSX and Aza-T action in more detail.

In summary, our results indicate that Aza-T is capable of relieving ammonia repression of heterocyst and nitrogenase formation. Furthermore, the data suggest that a novel control mechanism may be involved. It is possible that Aza-T may function primarily to initiate heterocyst synthesis because the degree of heterocyst formation is always greater than that of nitrogenase synthesis upon derepression with Aza-T. Certainly, at this point we are not able to discern whether the signal for heterocyst development and nitrogenase synthesis is the same. Further work on the molecular events surrounding the effects of Aza-T may lead to new insights into this and other questions surrounding heterocyst development. In any case, our results indicate that glutamine synthetase may not be the sole key to the derepression of heterocyst and nitrogenase development in cyanobacteria.

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