Synthesis of Nitrogenase and Heterocysts by Anabaena sp. CA in the Presence of High Levels of Ammonia

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Anabaena sp. CA fails to synthesize heterocysts and nitrogenase when grown with KNO₃ as the nitrogen source. By contrast, both heterocysts and proheterocysts are synthesized in NH₄Cl-containing media to a level nearly commensurate with cells grown in the absence of combined nitrogen. The growth rate of the organism in NH₄Cl-containing media was similar to that obtained with KNO₃ as the nitrogen source and was independent of the presence of N₂ in the atmosphere. Thus, our results indicate that the organism assimilated nitrate and ammonium nitrogen equally well to meet the nitrogen requirements for growth. Moreover, in contrast to previous studies with other cyanobacteria, the repressor signal for heterocyst differentiation in Anabaena sp. CA is not derived from the metabolism of ammonia but appears to be involved with nitrate metabolism. Nitrogenase activity was partially expressed in NH₄Cl-grown cultures. Increasing the level of nitrogenase activity to a value representative of a N₂-grown culture required both the inhibition of ammonia assimilation and de novo protein synthesis. An increase in the number of mature heterocysts was not required. The fact that high levels of exogenous ammonia only partially repress the synthesis of proteins required for the maximum expression of nitrogenase activity in Anabaena sp. CA has important implications.

The heterocystous cyanobacteria (blue-green algae) are a large group of phototrophic microorganisms capable of assimilating atmospheric nitrogen under aerobic conditions, using sunlight as an energy source. These distinct properties have led investigators to study members of this group as model systems for converting solar energy into biomass (16) and hydrogen gas (2, 10) and for improving the nitrogen budget of certain agricultural systems (16).

Biological nitrogen fixation is an energetically expensive process. It has been calculated that, in vivo, 20 to 30 mol of ATP is required per mol of N₂ fixed into ammonia (1, 3, 11). Thus when the reaction product, ammonia, is present in the environment, synthesis of nitrogenase is normally repressed. In recent years several laboratories have been engaged in elucidating the mechanism(s) by which synthesis of nitrogenase is controlled in various heterotrophic bacteria (13, 17, 20, 25, 26). From such studies it is hoped that strains of nitrogen-fixing organisms can be developed which will derepress the synthesis of nitrogenase even in the presence of combined nitrogen. Successful development of such strains with agriculturally important organisms will be of deep practical significance.

In this communication we report on the novel properties of a recently isolated rapidly growing halotolerant heterocystous cyanobacterium (22). This natural isolate partially derepresses the synthesis of nitrogenase while growing in the presence of high levels of ammonia.

MATERIALS AND METHODS

Organism. Anabaena sp. CA (ATCC 33047) is a recently isolated heterocystous cyanobacterium (22). The organism is maintained on slants of modified medium ASP-2 (27) at 35°C.

Culture conditions. The organism was routinely grown in Pyrex test tubes (25 by 150 mm) containing 20 ml of ASP-2 medium at 40°C. The cultures were illuminated by two F36T12/D/HO fluorescent lamps placed 10 cm from the growth tube positions on either side of the growth bath. The cultures were bubbled at 8 to 9 ml/min with 1% CO₂ in air. The system has been described in detail previously (14) and enables one to obtain maximum growth rates up to a density of 0.6 mg (dry weight) of cells per ml of culture.

The nitrogen sources for growth were added to the medium to the required final concentration from stock solutions which had been neutralized and filter sterilized through 22.5-mm-diameter Nucleopore filters (0.45-μm pore size). Growth was routinely followed turbidimetrically at 660 nm in a Spectronic 20 colorimeter. A conversion factor for absorbance against dry
weight was obtained such that an absorbance of 1.0 at 660 nm was equivalent to 0.43 mg (dry weight) per ml. Growth in the absence of atmospheric $N_2$ was conducted in modified growth tubes. These consisted of tubes fitted with white rubber stoppers through which two glass tubes (5-mm diameter) were inserted; one tube extended to the bottom of the growth tube. Rubber tubing was fitted onto the two glass tubes extending from the top of each rubber stopper such that the internal atmosphere of the vessel could be sealed off from the laboratory atmosphere during the time intervals when the cultures were not being gassed. Cultures grown in this manner were continuously bubbled with a stream of argon-oxygen-$CO_2$ (78: 21:1, vol/vol/vol; Big 3, Houston, Tex.) through the longer tube; the short tube acted as a vent. Cultures of Anabaena sp. CA, placed in this system in medium ASP-2 containing no combined nitrogen source, did not grow. Thus, the system successfully eliminated molecular nitrogen from the growth tubes. Samples of culture were removed from the growth vessel with a gas-tight syringe and bore tubing. This unit was large enough to extend to the bottom of the culture tube.

Whole cell protein measurements. Samples for protein determinations were handled as follows. Samples (5 to 10 ml) of each culture were harvested by centrifugation (4°C) at 11,500 × $g$ for 10 min. The pellets were washed once with 5 ml of deionized, distilled water and dried overnight under a gentle air stream. The pellets were then suspended in 2.5 ml of 0.1 N NaOH containing 2% $Na_2$CO$_3$ (wt/vol), and the protein was solubilized at 100°C for 15 to 20 min. Appropriate subsamples were taken, and protein was determined by the method of Lowry et al. (12). Crystalline bovine serum albumin was used as a standard.

Nitrogenase activity. Assays were determined with whole cells by the acetylene reduction technique (R. Schollhorn and R. H. Burris, Fed. Proc. 25:710, 1967) under the conditions described previously (21). One milligram of activity is the amount of whole-cell nitrogenase activity required to reduce 1 nmol of $C_2H_2$ in 1 min.

RESULTS

When Anabaena sp. CA was cultured in the presence of various inorganic nitrogen sources, distinct effects were seen on the numbers of proheterocysts and mature heterocysts and the level of expression of nitrogenase activity in exponential-phase cells (Table 1). Both $NH_4NO_3$ and KNO$_3$ completely repressed heterocyst development and nitrogenase activity. Attention is drawn, however, to cells grown in medium containing $NH_4Cl$. Such cells, although maintaining a generation time similar to that of KNO$_3$-grown cells, synthesized about 65% of the total complement of mature and proheterocysts as compared to cells growing with molecular nitrogen as the sole nitrogen source. Moreover, 25 to 30% of the level of nitrogenase present in N$_2$-grown cells was also expressed in cultures grown with

<table>
<thead>
<tr>
<th>Nitrogen source for growth</th>
<th>Generation time (h)*</th>
<th>Nitrogenase activity*</th>
<th>Mature heterocysts*</th>
<th>Proheterocysts*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N_2$</td>
<td>4.8</td>
<td>30.0—37.0</td>
<td>8—9</td>
<td>2—3</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>4.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$NH_4Cl$</td>
<td>4.3</td>
<td>8.5—10.0</td>
<td>5—6</td>
<td>2</td>
</tr>
<tr>
<td>$NH_4NO_3$</td>
<td>3.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* All sources of inorganic nitrogen were present at 10 mM.
* Generation times were obtained from the exponential phase of the growth curve.
* Expressed in nanomoles of $C_2H_2$ produced per milligram (dry weight) per minute.
* Mature heterocysts are expressed as a percentage of the total cells and are defined as those containing distinct polar bodies as seen with a light microscope at a magnification of ×400.
* Proheterocysts are those cells which, although obviously differentiated, have no clear polar bodies. All samples were taken from cultures in midexponential phase of growth.

$NH_4Cl$.

The ability of nitrate, and the inability of ammonia, to repress heterocyst differentiation in a cyanobacterium have not been observed previously. Furthermore, the growth rates of Anabaena sp. CA were similar within the concentration range of 2.5 to 10.0 mM $NH_4Cl$ and independent of the presence of molecular nitrogen in the atmosphere (Table 2). The numbers of mature and proheterocysts were also independent of the ammonia concentration. However, the expression of nitrogenase activity was more finely controlled; the specific activity was somewhat higher in cultures grown with concentrations of 5 mM $NH_4Cl$ and less, and also higher in cultures grown in the atmosphere devoid of molecular nitrogen. The latter observation is rather similar to results obtained with Clostridium pasteurianum (4). Further experiments have shown that the initial growth rate of Anabaena sp. CA at low concentrations of ammonia (as low as 0.1 mM) does not change, and that exponential cells from the low-ammonia-grown cultures of Table 2 have the same amount of protein (0.4 mg) per mg (dry weight) of cells as that found in cells grown at high levels of ammonia. Moreover, when the growth rate was diminished at optimum levels of ammonia and nitrate (either the potassium or sodium salt) by culturing the organism at a low light intensity, the same pattern of heterocyst and nitrogenase synthesis was obtained, i.e., repression of synthesis in nitrate medium and continued synthesis of nitrogenase and heterocysts in ammonia medium. Thus, a decrease in the metabolic rate...
as a result of energy deprivation has no effect on the control mechanism for heterocyst and nitrogenase synthesis. These data indicate that the expression of heterocysts and nitrogenase activity in NH₄Cl-grown *Anabaena* sp. CA is not due to an inherent inability of this organism to accumulate exogenous NH₄⁺ at a rate sufficient to meet its bulk nitrogen requirements for growth. The results of these experiments also support our previous observations that the control of heterocyst development in this organism is distinct from the primary ammonia assimilatory system (21). It should be noted that ammonia repressed the synthesis of heterocysts and nitrogenase in *Anabaena* strain 1F in experiments performed in parallel with strain CA (J. F. Grillo, C. Van Baalen, and F. R. Tabita, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, K132, p. 167).

The partial expression of nitrogenase activity and heterocyst development in *Anabaena* sp. CA in the presence of high ammonia concentration was studied further. The results in Fig. 1a show that the total units of nitrogenase activity increased during exponential growth of *Anabaena* sp. CA on N₂ such that the specific activity of nitrogenase remained relatively constant at a level of 27 to 37 nmol of C₂H₄ produced per mg (dry weight) per min. Also, the percentage of mature heterocysts (8%) and proheterocysts (2 to 3%) remained constant (Fig. 1b). However, when 10 mM KNO₃ was added to such a culture, both the specific activity of nitrogenase (Fig. 2a) and the numbers of mature and proheterocysts decreased (Fig. 2b) as growth proceeded (Fig. 2a). Nitrate had no effect on the culture for 2 h. This is shown by the continued increase in the total units of nitrogenase and the constancy of the specific activity of the enzyme. However, after 2 h there was no further development of proheterocysts, and after 6 h there was no further increase in either the total number of mature heterocysts in the culture or the total units of nitrogenase. Moreover, between 10 and 13 h, the total units of nitrogenase in the culture began to decrease, indicative of turnover of the preexisting enzyme. These data show that nitrate metabolism is involved in the repression of

![Graph](http://jb.asm.org/)
Fig. 2. (a) Effect of the addition of KNO₃ (10 mM final concentration) at zero time on the nitrogenase activity and growth of a culture of Anabaena sp. CA previously growing on N₂; (b) effect of KNO₃ on the numbers of mature and proheterocysts. See Fig. 1 for symbols.

Fig. 3. (a) Effect of the addition of NH₄Cl (10 mM final concentration) at zero time on the nitrogenase activity and growth of a culture of Anabaena sp. CA previously growing on N₂; (b) effect of NH₄Cl on the numbers of mature and proheterocysts. See Fig. 1 for symbols.

both the development of heterocysts and further synthesis of new nitrogenase. In contrast, when 10 mM NH₄Cl was added to a culture growing on N₂ some differences were seen. Ammonia immediately inhibited any further increase in the total units of nitrogenase in the culture. This resulted in a decrease in the specific activity of the enzyme as growth proceeded (Fig. 3a). However, in contrast to the nitrate-treated culture, the specific activity began to stabilize after approximately 10 to 12 h at 30% of the initial level. This was accompanied by an increase in the total units of enzyme in the culture. In addition, the percentage of mature and proheterocysts was only reduced by 25 and 33%, respectively, even though the total cell mass increased sixfold during this time period. Figures 4a and b show that even after prolonged subculturing (at least 20 generations in medium containing 10 mM NH₄Cl) Anabaena sp. CA coordinately synthe-
sized new heterocysts and nitrogenase as growth proceeded, such that the units of enzyme activity per unit mass of cells remained constant. Moreover, since the addition of chloramphenicol (5 µg/ml, final concentration) caused a rapid loss of nitrogenase activity (Fig. 4a), nitrogenase activity is undoubtedly maintained by the continuous synthesis of new protein.

If ammonia assimilation was inhibited in these cultures either by blocking the major ammonia assimilatory enzyme, glutamine synthetase, with L-methionine DL-sulfoximine (Fig. 4c and d) or by removing ammonia from the medium (Fig. 4e and f), then the specific activity of nitrogenase increased. However, in neither case was the increase in activity immediate. This increase could be abolished by chloramphenicol (Fig. 4c and e), showing that de novo protein synthesis is required. Finally, the kinetics of increase in nitrogenase activity were identical in both cases between 0 and 7 h. However, the specific activity of the L-methionine DL-sulfoximine-treated culture increased threefold between 0 and 6 h without being accompanied by any significant increase in the total number of mature heterocysts in the culture (Fig. 4d). In contrast, the culture resuspended in medium minus ammonia continued to grow (data not shown) and synthesized mature and proheterocysts (Fig. 4f) at a rate similar to the control (Fig. 4a). However, the total units of nitrogenase activity in this culture were also threefold greater than in the control culture after 6 h.

DISCUSSION

Several important findings have emerged from these studies with Anabaena sp. CA. The ability of this organism to grow at reproducible growth rates to high cell densities in the presence of up to 20 mM NH₄Cl has not been seen previously in the cyanobacteria. This property has enabled us to look at the development of heterocysts and nitrogenase activity in the presence of the elevated levels of ammonia commonly used to repress nitrogenase biosynthesis in other bacteria (4, 18, 19).

The ability of Anabaena sp. CA to completely repress heterocysts and nitrogenase synthesis when growing with nitrate as the nitrogen source, and its ability to differentiate a large number of heterocysts while growing on NH₄Cl, are also novel observations. All heterocystous cyanobacteria heretofore studied extensively have been shown to completely repress the synthesis of heterocysts and nitrogenase in medium containing 2 to 3 mM NH₄Cl (6). Moreover, our observations of rapid growth of the organism in NH₄Cl-containing medium in an atmosphere devoid of molecular nitrogen shows unequivocally that it has the ability to assimilate ammonia nitrogen as efficiently as nitrate nitrogen. These results thus support our previous observations that the trigger for heterocyst development is distinct from the primary ammonia assimilatory mechanism (21). Further studies in this area are currently under way.

The ability to express nitrogenase activity in the presence of excess ammonia has been observed previously in several different N₂-fixing microorganisms under circumstances in which the primary ammonia assimilatory mechanism had been either chemically (9, 23, 28) or genetically (19, 25, 26) modified. Rarely had this phenomenon been observed and attributed to other causes (8, 21). The data presented here show that ammonia per se does not cause immediate inactivation of preexisting nitrogenase in Anabaena sp. CA, as observed in several species of photosynthetic bacteria (7, 15, 29), and they prove unequivocally that ammonia metabolism does not completely repress synthesis of the nif gene products in this organism. Moreover, although there is partial repression of some protein(s) required for maximum expression of nitrogenase activity, synthesis of these can occur in Anabaena sp. CA grown for many generations on ammonia. Full derepression of their synthesis can occur without necessarily being accompanied by development of new heterocysts. Further work is required to study the components of the NH₄Cl-induced heterocysts in more detail.

Finally, it has often been proposed that it would be potentially useful for agricultural purposes to "engineer" strains of nitrogen-fixing microorganisms that were derepressed for nitrogenase in the presence of ammonia (5, 8, 24). Certainly, our ability to isolate such a phototrophic organism from the natural environment suggests that this property may be more common than previously thought.

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