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Regulation of Expression of Nitrate and Dinitrogen Assimilation by *Anabaena* Species

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Anabaena sp. strain 7120 appeared more responsive to nitrogen control than *A. cylindrica*. Growth in the presence of nitrate strongly repressed the differentiation of heterocysts and fixation of dinitrogen in *Anabaena* sp. strain 7120, but only weakly in *A. cylindrica*. Nitrate assimilation by ammonium-grown cultures was strongly repressed in *Anabaena* sp. strain 7120, but less so in *A. cylindrica*. The repressive effect of nitrate on dinitrogen assimilation in *Anabaena* sp. strain 7120, compared to *A. cylindrica*, did not correlate with a greater rate of nitrate transport, reduction to ammonium, assimilation into amino acids, or growth. Although both species grew at similar rates with dinitrogen, *A. cylindrica* grew faster with nitrate, incorporated more $^{13}NO_3^-$ into amino acids, and assimilated (transported) nitrate at the same rate as *Anabaena* sp. strain 7120. Full expression of nitrate assimilation in the two species occurred within 2.5 h (10 to 14% of their generation times) after transfer to nitrate medium. The induction and continued expression of nitrate assimilation was dependent on protein synthesis. The half-saturation constants for nitrate assimilation and for nitrate and ammonium repression of dinitrogen assimilation have ecological significance with respect to nitrogen-dependent growth and competitiveness of the two *Anabaena* species.

The studies of Fogg (7) demonstrating that ammonium represses heterocyst formation and dinitrogen fixation by *Anabaena* species have been confirmed by numerous studies on a variety of cyanobacteria (c.f. for reviews references 8, 10, and 13). The single reported exception comes from observation of a recently isolated marine *Anabaena* species in which nitrate but not ammonium prevents formation of heterocysts and fixation of dinitrogen (4). Nitrate has been reported to have variable and transitory inhibitory effects on heterocyst formation and dinitrogen fixation by other species (10, 31).

In a preliminary survey of freshwater cyanobacteria, including two strains of *A. cylindrica* (the Wolk and Fogg strains), *A. variabilis*, *A. doliolum*, *A. azollae*, *Nostoc muscorum*, and *Anabaenopsis circularis*, we too observed that growth in the presence of nitrate has a variable inhibitory effect on heterocyst differentiation; only in *Anabaena* sp. strain 7120 (formerly *Nostoc muscorum*) (10) was heterocyst differentiation consistently repressed by 5 mM nitrate.

Ammonium represses the synthesis of nitrate reductase by cyanobacteria (11, 12, 15, 19, 26). The effects of combined nitrogen on enzyme activities (including the nitrate and aerobic dinitrogen assimilatory systems) may reflect a form of nitrogen control similar to that observed in heterotrophic bacteria (18) and fungi (3). In cyanobacteria, exogenous ammonium per se does not appear to be the primary regulatory signal, at least for the synthesis of nitrogenase and differentiation of heterocysts (27). Rather, glutamine or a metabolic derivative of it has been implicated (10, 24, 30), as it also has been implicated in the control of nitrate metabolism in *Neurospora* species (22).

To gain further insight into the regulation of nitrogen metabolism in cyanobacteria, we compared nitrate assimilation by *Anabaena* sp. strain 7120 and *A. cylindrica* (Wolk strain). We chose *A. cylindrica* as the representative of cyanobacteria in which nitrate is a weak repressor because considerable information has already been accumulated on nitrogen metabolism in this species (16). In this report we compare these two species with respect to the effects of nitrate and ammonium on growth, nitrogenase activity, and heterocyst formation. We also compared the effects of various nitrogen sources on nitrate assimilation.

MATERIALS AND METHODS

Culture of cyanobacteria. Axenic cultures of *Anabaena* sp. strain 7120 (ATCC 27893) and *A. cylindrica* Lemm (ATCC 29414) were obtained from the American Type Culture Collection, Rockville, Md.

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Cyanobacteria were initially grown in 1- or 4-liter Erlenmeyer flasks fitted with sparging tubes and inlet and outlet ports and mixed by magnetic stirring, illuminated from the side with cool white fluorescence lamps at 7.5 W/m^2, and maintained as semicontinuous cultures by daily removal of cells and spent medium and addition of fresh medium. The basal medium was an eightfold dilution of that described by Allen and Arnon (2). For growth on nitrate or ammonium, the basal medium was supplemented with 5 to 20 mM NO_3^- (equimolar Na^+ and K^+ salts in all cases of supplementation or addition of NO_3^-) or 2.5 mM NH_4Cl plus, as buffer in all cases, 5 mM sodium *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), pH 7.2 to 7.5. In experiments to determine the immediate effect of high exogenous NO_3^- or NH_4^+ concentrations on nitrogenase activity, the experimental and control semicontinuous cultures were operated in a batch mode after addition of the nitrogen source. In all other cases, cells were harvested aseptically from the semicontinuous cultures, concentrated by centrifugation, and suspended in fresh medium to the cell concentration noted.

For growth experiments, the cyanobacteria were subcultured at room temperature in test tubes (2 by 15 cm) fitted with side arms and holding 15 ml of medium, with 8.5 W/m^2 of fluorescent illumination directed toward the sides of the tubes. The cultures were mixed by sparging with membrane-filtered (0.20-μm pore size; Millipore Corp., Bedford, Mass.) humidified gases at a flow rate of ca. 400 ml/min. In the case of N_2-supported growth, the cultures were sparged with air with or without 1% CO_2. In the cases of NO_3^- and NH_4^+-supported growth, cultures were sparged with air with or without 1% CO_2 or with argon-oxygen-carbon dioxide, 80:19:1 (vol/vol/vol), obtained by mixing cylinder gases in a rotameter (model 7471; Matheson, Newark, Calif.). Growth was monitored by changes in light scattering (measured at 750 nm in a Bausch & Lomb Spectronic 20) or chlorophyll *a* (Chl *a*) content. For Chl *a* determinations, 1.0-ml subsamples were harvested at 1,000 × *g* for 5 min, extracted with 90% (vol/vol) methanol, and quantitated at 663 nm, assuming a specific absorption coefficient of 78.74 (17).

To determine the long-term (up to 7 days of culture) effects of micromolar concentrations of NO_3^- and NH_4^+ on acetylene reduction and heterocyst formation, we suspended the harvested semicontinuous culture cells in basal medium and inoculated it to an initial concentration of approximately 5 × 10^4 cells/ml into 1 liter of basal medium containing TES-buffered nitrogen supplements in 2-liter Erlenmeyer flasks. The cultures were incubated at room temperature on an orbital shaker under 8.4 W/m^2 of fluorescent illumination. The rates of acetylene reduction and heterocyst frequencies were monitored at 8- to 48-h intervals by centrifuging 400 ml of culture and suspending the pellets in 5 ml of culture supernatant. Duplicate 2-ml subsamples were used to determine the rate of acetylene reduction, and the remainder was used for Chl *a* concentration and heterocyst frequency (400 to 700 total cells counted) determinations.

To avoid time delays and additional experimental manipulation before exposure to ^{13}N, the cyanobacteria in semicontinuous culture were first harvested by centrifugation at 500 × *g* for 5 min, washed twice with basal medium lacking combined nitrogen, suspended to 2.5 μg of Chl *a* per ml, and incubated under growth conditions for 2 to 3 h near the site of ^{13}N generation. The suspensions were then concentrated 10-fold by centrifugation and immediately exposed to $^{13}NO_3^-$. In some experiments, L-methionine-DL-sulfoximine (MSX; Sigma Chemical Co., St. Louis, Mo.) was added to the concentrated cell suspension (1.0 mM after dilution with $^{13}NO_3^-$).

Enzyme and protein assay. Nitrogenase activity was assayed by whole cell reduction of acetylene to ethylene; 2 ml of cyanobacterial suspension was incubated in 6.4-ml vials containing 5% (vol/vol) acetylene in air and at 30°C under tungsten illumination. Ethylene and acetylene were determined on a 0.1-ml subsample of the vial atmosphere on a Varian model 940 flame ionization detector gas chromatograph (Varian Associates, Palo Alto, Calif.) employing a column (⅛ in. by 6 ft. [ca. 3.2 mm by 1.8 m]) of Porapak R (Alltech Associates, Arlington Heights, Ill.).

Protein was assayed by the method of Lowry et al. (13), using bovine serum albumin as standard. Cells were lysed by cavitation for 150 s per ml of suspension (model W-225R sonicator, equipped with a microtip; Heat Systems Inc., Plainview, N.Y.), and protein was determined in the supernatant fraction after centrifugation at 1,000 × *g* for 5 min.

Nitrate uptake. Semicontinuous cultures grown with TES-buffered 1 to 5 mM NO_3^-, 0.5 to 1.0 mM NO_2^-, 1 mM NH_4^+, or N_2 were harvested aseptically, washed twice with basal medium lacking combined nitrogen, and suspended to a final concentration of approximately 5 × 10^7 cells/ml. One milliliter of concentrated suspension was added to 49 ml of basal medium supplemented with 5 to 250 μM NO_3^- in 125-ml flasks. The suspensions were incubated on an orbital shaker (100 rpm) at room temperature under fluorescent illumination. Subsamples (2.0 ml) were withdrawn at various times from 1 to 12 h and centrifuged at 1,000 × *g* for 5 min. The pellets were analyzed for Chl *a* content. Supernatant fractions (10 to 50 μl) were analyzed for nitrate and nitrite by separation on a high-performance liquid chromatography (HPLC) strong anion-exchange column (Partisil-10 SAX; Whatman Inc., Clifton, N.J.); nitrate and nitrite were eluted with 30 mM phosphate buffer, pH 3.0, and monitored at 210 nm, and the concentrations were determined from peak heights with a standard curve (28).

Labeling with $^{13}NO_3^-$. Details of the target system for the generation of ^{13}N at the Crocker Nuclear Laboratory on the University of California, Davis campus and the purification of $^{13}NO_3^-$ have been given previously (5, 16, 21). The $^{13}NO_3^-$ used in this study had a greater than 99.99% radiochemical purity and 1 to 4 mCi of ^{13}N per ml.

Labeling with $^{13}NO_3^-$ was done under air in 15-ml conical centrifuge tubes containing 0.25 ml of cyanobacterial suspension and 0.25 ml of purified $^{13}NO_3^-$ solution. The samples were incubated at room temperature on an orbital shaker (200 rpm) in front of a cool white fluorescent lamp (ca. 600 ft-c [ca. 7,056 lx]). The reactions were terminated after incubations of 5 to 900 s by the addition of 4 volumes of 100% methanol. The cells were extracted by mixing on a micromixer for 1 min, and the cellular debris was removed by centrifugation at 1,000 × *g* for 1 min. The methanolic extracts were processed either for amino acid separa-

tion by high-voltage electrophoresis and quantitation by integration of peaks in radioscans or for $^{13}NH_4^+$ determination by vacuum distillation and quantitation by scintillation spectroscopy as previously described (16).

RESULTS

Effects of inorganic nitrogen sources on growth, nitrogenase activity, and heterocyst formation. Both *Anabaena* sp. strain 7120 and *A. cylindrica* grew faster and produced a lower frequency of heterocysts when cultured with ammonium (in the presence or absence of dinitrogen) as compared with dinitrogen and nitrate (Table 1). However, the two species differed significantly with respect to their response to dinitrogen or nitrate as nitrogen sources. With nitrate, *A. cylindrica* grew almost as rapidly as it did with ammonium and slightly more rapidly than it did with dinitrogen, but it produced only about 30% fewer heterocysts. Repeated subculturing of this strain in 5 mM nitrate caused, at most, a 50% reduction in heterocyst frequency. In contrast, *Anabaena* sp. strain 7120 grew at about the same rate in 5 mM nitrate or with dinitrogen, but the frequency of heterocysts was reduced approximately 85% within 7 days of nitrate culture and was essentially 0 after two to three subcultures.

In both *Anabaena* species there was an immediate linear decline in nitrogenase activity upon the addition of either 5 mM nitrate or 2.5 mM ammonium to dinitrogen-grown cultures (Fig. 1). When ammonium was added to cultures of either species or nitrate to cultures of *Anabaena* sp. strain 7120, nitrogenase activity declined until little or no activity could be detected after 48 to 56 h. The time course of decline in nitrogenase activity was similar to that observed in other cyanobacterial strains (1, 27). Less than 24 h after the addition of nitrate to *A. cylindrica*, nitrogenase activity appeared to stabilize at 40 to 45% of the rate of dinitrogen-grown cultures and thereafter declined only slowly (Fig. 1).

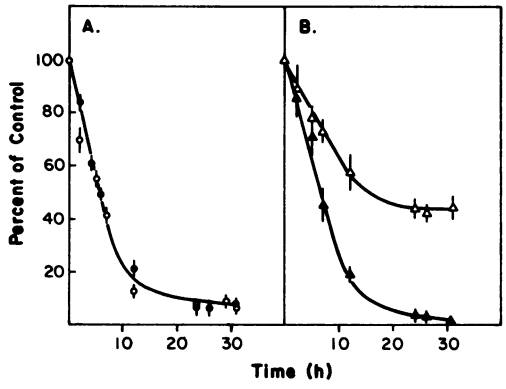

FIG. 1. Effect of added nitrate and ammonium to dinitrogen-growing cultures of *Anabaena* sp. strain 7120 (A) and *A. cylindrica* (B) on the activity of nitrogenase (acetylene reduction) as a function of time after the additions. Experiments were conducted on semicontinuous cultures converted to batch culture conditions at the time of addition of 5 mM nitrate or 2.5 mM ammonium. Open symbols refer to nitrate additions and closed symbols to ammonium additions. Each point is the mean ± standard error of the mean of three to five experiments. Control rates of acetylene reduction were 9.95 ± 0.7 and 20.20 ± 1.5 nmol per mg of protein per min for *Anabaena* sp. strain 7120 and *A. cylindrica*, respectively.

TABLE 1. Growth rates and heterocyst frequencies of *Anabaena* sp. strain 7120 and *A. cylindrica* cultured with dinitrogen, nitrate, and ammonium[a]

Species	Nitrogen source	Doubling time (h) ± SE	Heterocyst frequency (%) ± SE
Anabaena sp. strain 7102	N_2	21.5 ± 1.0	8.4 ± 0.3
	NO_3^-	21.1 ± 1.7	1.3 ± 0.2
	NH_4^+	18.8 ± 0.5	0
A. cylindrica	N_2	18.2 ± 1.0	6.3 ± 0.5
	NO_3^-	15.0 ± 1.6	4.3 ± 0.3
	NH_4^+	14.3 ± 0.3	0.1 ± 0.1

[a] Growth was measured by changes in light scattering at 750 nm or by Chl *a* content. In the experiments with combined nitrogen, the atmosphere was air with or without CO_2 or argon-oxygen-carbon dioxide, 80:19:1 (vol/vol/vol), and at NO_3^- and NH_4^+ concentrations of 5 and 2.5 mM, respectively. There were no significant differences between cultures grown with combined nitrogen under air or argon-oxygen-carbon dioxide. In the case of N_2, the atmosphere was air with or without 1% CO_2. Buffer in all cases was 5 mM TES, pH 7.5. Each value is the mean ± standard error of the mean of four to six separate experiments.

By using low-cell-density, large-volume culture conditions, we were able to extend observations on the effect of exogenous nitrate and ammonium concentrations on nitrogenase activity and heterocyst formation to micromolar levels. The results of these experiments (Fig. 2 and summarized in Table 2) showed that the half-saturation constants (K_i) for inhibition of acetylene reduction and heterocyst formation were in the very low micromolar range. The values were also reasonably similar for both species with respect to the specific nitrogen source and parameter analyzed. Nevertheless, there were distinct differences between nitrogen sources in terms of acetylene reduction, as well as inhibition of acetylene reduction compared with heterocyst formation. Ammonium inhibited expression of nitrogenase activity at three- to ninefold lower concentrations than did nitrate. The K_i values for acetylene reduction were also lower

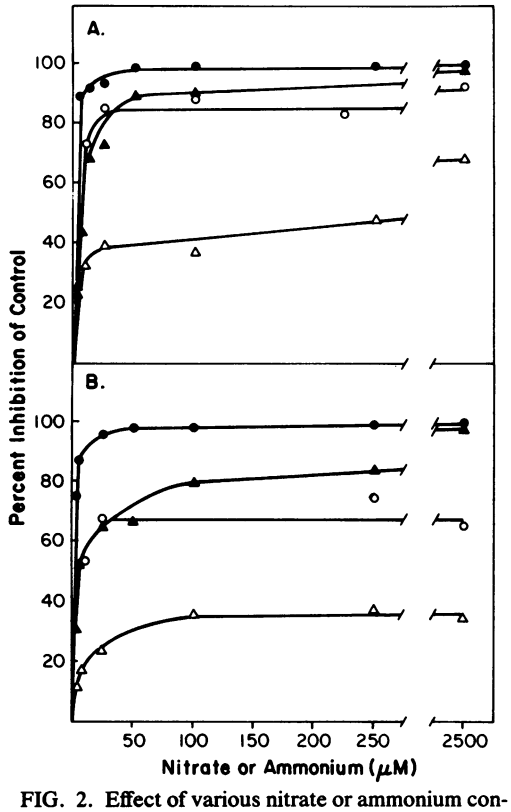

FIG. 2. Effect of various nitrate or ammonium concentrations on heterocyst formation and acetylene reduction by *Anabaena* sp. strain 7120 (A) and *A. cylindrica* (B). Open symbols refer to nitrate and closed symbols to ammonium: ○ and ●, acetylene reduction; △ and ▲, heterocyst frequency. The points represent the means of four to six experiments. Control rates of acetylene reduction were 0.21 ± 0.03 and 0.47 ± 0.06 (mean ± standard error of the mean of 15 experiments) μmol of C_2H_2 reduced per mg of Chl *a* per min for *Anabaena* sp. strain 7120 and *A. cylindrica*, respectively. Heterocyst frequencies in the absence of combined nitrogen were 7.8 ± 0.2 and 5.5 ± 0.1 (16 determinations) for *Anabaena* sp. strain 7120 and *A. cylindrica*, respectively. Mean Chl *a* concentration at the start of the experiments was 1.5 ng/ml, increasing to a maximum of 335 ng/ml within 6 to 7 days.

with either nitrogen source than they were for heterocyst formation.

The maximal inhibition of acetylene reduction and heterocyst formation was different in the two species (Fig. 1 and 2 and Tables 1 and 2). Exogenous ammonium inhibited the expression of both in the two species by greater than 97%. However, nitrate caused only 69 and 36% inhibition of acetylene reduction activity and heterocyst formation, respectively, in *A. cylindrica*. Maximal inhibition of acetylene reduction and heterocyst formation in *A. cylindrica* occurred between 25 and 100 μM and did not increase at higher nitrate concentrations. In contrast, higher nitrate concentrations increased inhibition of heterocyst formation in *Anabaena* sp. strain 7120. This increased inhibition in *Anabaena* sp. strain 7120 was not a direct function of time to dilute preexisting heterocysts since both species grew at their respective rates at all nitrate concentrations above 50 μM. Double reciprocal plots of nitrate concentration versus inhibition of heterocyst formation in *Anabaena* sp. strain 7120 showed a departure from linearity above 250 μM, which could imply a concentration dependence on nitrate transport or utilization.

Kinetics of nitrate assimilation. The effect of nitrate concentration on nitrate assimilation by *Anabaena* sp. strain 7120 and *A. cylindrica* was determined by HPLC analysis of nitrate disappearance from the growth medium (Fig. 3). Nitrate assimilation (including transport, reduction, and incorporation into organic metabolites) by both species showed saturation kinetics between 50 and 250 μM nitrate (Fig. 3). There were no significant changes in the rate of assimilation by either species at concentrations up to 2.5 mM (data not shown). Thus, the *Anabaena* species appear to have one nitrate assimilatory (transport) system, compared with two in *Klebsiella pneumoniae* (29). The calculated V_{max} values were 26.2 and 27.5 μmoles per mg of Chl *a* per h and the apparent K_s values were 29.7 and 26.2 μM for *Anabaena* sp. strain 7120 and *A. cylindrica*, respectively. The V_{max} values are approximately threefold greater than those previously calculated for a *Nostoc* species and an unrelated *Anabaena* species (6). The K_s values are similar to those reported for other cyanobacteria (33). Our initial experiments examining $^{13}NO_3^-$ accumulation in filtered cells resulted in curves with shapes similar to those in Fig. 3. However, the $^{13}NO_3^-$ experiments were difficult to quantitate due to variable counting efficiencies of the detectors available.

To determine whether differences in the reduction of nitrate to nitrite and to ammonium caused the differential effect of nitrate repression, we attempted to quantitate the intracellular concentrations and ratios of these ions by modifications of the transport assay of Thayer and Huffaker (29). Cells were exposed to $^{13}NO_3^-$ and then centrifuged at 15,000 × *g* through mixtures of silicone oils and into 1.0 M perchloric acid. The perchloric acid lysate was subjected to HPLC analysis for $^{13}NO_3^-$, $^{13}NO_2^-$, and $^{13}NH_4^+$ plus organic compounds. Due to extensive transfer of exogenous $^{13}NO_3^-$ through the silicone oils in the presence of *Anabaena* filaments, the results of these experiments were inconclusive. Mild cavitation of the *Anabaena* cultures to yield 10- to 15-cell filaments did not

TABLE 2. Kinetic constants of nitrate and ammonium repression of acetylene reduction and heterocyst formation in *Anabaena* sp. strain 7120 and *A. cylindrica*[a]

Species	Nitrogen source	Acetylene reduction			Heterocyst formation		
		K_i (μM)	Maximum % inhibition (6 day)	r^b	K_i	Maximum % inhibition (6 day)	r^b
Anabaena sp. strain 7120	NO_3^-	2.3	94.0	0.91	6.2	51.0	0.92
	NH_4^+	0.25	98.0	0.78	7.6	99.9	0.99
A. cylindrica	NO_3^-	2.6	69.0	0.83	9.5	36.0	0.99
	NH_4^+	0.74	99.0	0.92	3.2	97.0	0.97

[a] Kinetic parameters were calculated from the data in Fig. 2.
[b] The correlation coefficients (r) were determined from linear regression analysis.

reduce the amount of $^{13}NO_3^-$ contamination in the perchloric acid lysate.

Nevertheless, nitrite did not accumulate in media as it does in a "leaky" nitrite reductase mutant of a unicellular cyanobacterium (25). The lower limits of detection in the nonradioactive HPLC system were near 50 pmol of nitrite and 200 pmol of nitrate in a 50-μl injection volume (28). More than 50 pmol of nitrite did not accumulate in the growth medium of either *Anabaena* sp. strain 7120 or *A. cylindrica* at any external nitrate concentration after any incubation period. In parallel experiments with $^{13}NO_3^-$ plus 50 μM $^{14}NO_3^-$ as carrier, no $^{13}NO_2^-$ above that in the original ^{13}N solution (less than 0.01%) could be detected in the medium of either species after 120 s of incubation. Substantial amounts of ammonium also did not accumulate within the cells of either species. Less than 0.1% (lower limit of detection) of the no-carrier-added $^{13}NO_3^-$ (4 to 40 nM total nitrate) could be distilled as $^{13}NH_3$ from methanolic extracts after 900 s of incubation. However, when cultures were incubated in the additional presence of 1 mM MSX (an inhibitor of glutamine synthetase), 11.0 and 10.2% of the $^{13}NO_3^-$ distilled as $^{13}NH_3$ from extracts of *Anabaena* sp. strain 7120 and *A. cylindrica*, respectively.

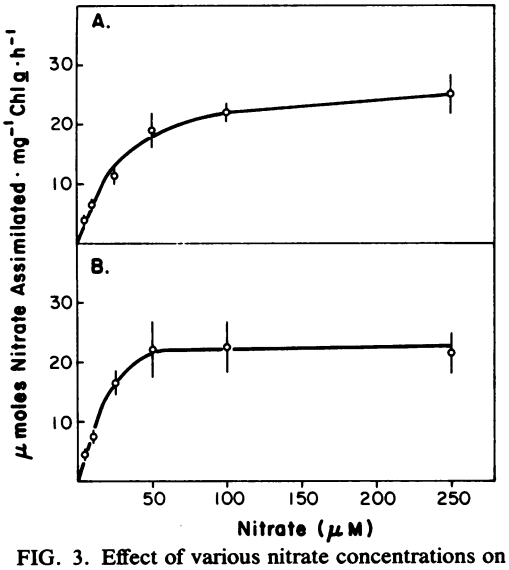

FIG. 3. Effect of various nitrate concentrations on the rates of nitrate assimilation by *Anabaena* sp. strain 7120 (A) and *A. cylindrica* (B). The data presented are means ± standard errors of the means of 8 to 14 experiments with cells of both species previously grown in the presence of 1 to 5 mM nitrate, 0.5 to 1 mM nitrite, 1 mM ammonium, or dinitrogen gas as the nitrogen source. After 2 to 4 h of incubation in the respective nitrate concentration, the rates of nitrate assimilation were determined by monitoring nitrate disappearance from growth medium, using HPLC as described in the text (see also Fig. 4).

Effect of nitrogen sources for growth on nitrate assimilation. The assimilation of nitrate by dinitrogen-fixing (11, 12, 20) and non-dinitrogen-fixing cyanobacteria (6, 15, 26) is reported to be lower in the presence of ammonium. We verified this observation in *Anabaena* sp. strain 7120 and *A. cylindrica* and also showed that the presence of ammonium did not completely repress nitrate assimilation in either species (Fig. 4 and Table 3). A low level of nitrate disappearance could be detected by HPLC analysis of ammonium-supplemented growth medium (Fig. 4), and significant amounts of $^{13}NO_3^-$ were incorporated into organic metabolites by ammonium-grown cells of both species (Table 3). The recovery from ammonium repression of nitrate assimilation occurred within 2.5 h after transfer to ammonium-minus, nitrate-plus growth conditions (Fig. 4). Full expression of nitrate assimilation after transfer from ammonium was dependent on new protein synthesis as shown by chloramphenicol treatment in *A. cylindrica* (Fig. 4). Treatment with chloramphenicol also reduced the rate of nitrate assimilation within 2 h of exposure in nitrate-grown cells (Fig. 4), implying a rapid turnover rate of an essential assimilatory pro-

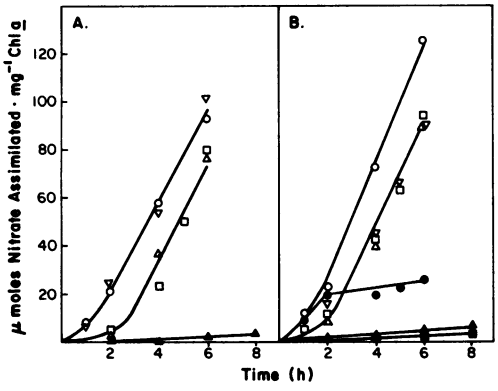

FIG. 4. Initial time course of the assimilation of 100 μM nitrate by suspensions of *Anabaena* sp. strain 7120 (A) and *A. cylindrica* (B) previously grown in the presence of 1 mM nitrate (○), 0.5 mM nitrite (▽), 1 mM ammonium (△), or nitrogen gas (□). Symbols: ▲, nitrate assimilation by suspensions incubated in the additional presence of 1 mM ammonium; ●, nitrate assimilation by nitrate-grown *A. cylindrica* in the presence of 50 μg of chloramphenicol per ml added at time zero; ■, nitrate assimilation by ammonium-grown cells of *A. cylindrica* in the presence of 50 μg of chloramphenicol per ml added at time zero. The data points reflect means of two to four individual experiments. The rates of nitrate assimilation were determined by monitoring disappearance of nitrate from the growth medium, using HPLC as described in the text.

tein. We have not attempted to identify the protein(s) required for continued expression by nitrate-grown cells or for induction in ammonium-grown cells.

Dinitrogen-grown cells showed a higher level of nitrate assimilation into organic metabolites than did ammonium-grown cells of both species (Table 3). The time course of full expression of nitrate assimilation by dinitrogen-grown cells when transferred to nitrate medium was similar to that of ammonium-grown cells (Fig. 4).

There were differences between *Anabaena* sp. strain 7120 and *A. cylindrica* in the effect of nitrogen sources for growth on nitrate assimilation. For example, growth on nitrite appeared to support full expression of nitrate assimilation in *Anabaena* sp. strain 7120 but not in *A. cylindrica* (Fig. 4). Compared to *A. cylindrica*, growth of *Anabaena* sp. strain 7120 in ammonium resulted in much greater repression of nitrate assimilation (Fig. 4 and Table 3). The differential effect of ammonium on nitrate assimilation was similar to the effect of nitrate on acetylene reduction and heterocyst formation (Fig. 2).

DISCUSSION

The two dinitrogen-fixing *Anabaena* species used in this study differed in their physiological responses to exogenous combined nitrogen. In general, *Anabaena* sp. strain 7120 was distinctly more responsive to nitrogen control (18) than was *A. cylindrica*. For example, growth in the presence of nitrate ultimately caused complete repression of heterocyst formation and dinitrogen fixation (the aerobic dinitrogen assimilatory system) in *Anabaena* sp. strain 7120, but only approximately 50 and 70% reductions in these activities, respectively, in *A. cylindrica* (Tables 1 and 2 and Fig. 1 and 2). We expected the repressive effect of nitrate on the dinitrogen assimilatory system of *Anabaena* sp. strain 7120, in comparison with *A. cylindrica*, to correlate with: (i) a greater rate of nitrate transport; (ii) more incorporation of $^{13}NO_3^-$ into amino

TABLE 3. Principal radioactive constituents observed after 900 s of assimilation of $^{13}NO_3^-$ by *Anabaena* sp. strain 7120 and *A. cylindrica* cultured with dinitrogen, nitrate, and ammonium

Species	Nitrogen source for growth	^{13}N found in compound (% ^{13}N added)[a]				
		Asp + Glu[b]	Gln	Cit + Ala[c]	Arg	Total amino acids
Anabaena sp. strain 7120	NH_4^+	0.01 ± 0.006	0.05 ± 0.03	0.001 ± 0.001		0.06 ± 0.04
	N_2	0.79 ± 0.35	0.29 ± 0.08	0.10 ± 0.06		1.19 ± 0.47
	NO_3^-	2.59 ± 0.51	0.35 ± 0.11	0.09 ± 0.04	0.08 ± 0.04	3.09 ± 0.63
A. cylindrica	NH_4^+	2.35 ± 1.48	0.50 ± 0.18	0.21 ± 0.13	0.016 ± 0.008	3.08 ± 1.73
	N_2	4.23 ± 0.46	0.51 ± 0.24	0.21 ± 0.07	0.06 ± 0.016	4.79 ± 0.48
	NO_3^-	3.25 ± 1.18	3.77 ± 0.65	1.31 ± 0.78	0.51 ± 0.28	8.88 ± 2.77

[a] Values are means ± standard errors of the mean of two to six experiments and were determined by integration and time correction of peaks of radioactivity from ^{13}N after electrophoresis at pH 9.2, in comparison with $^{13}NO_3^-$ radioactivity added as determined by scintillation spectroscopy. Asp, aspartate; Glu, glutamate; Gln, glutamine; Cit, citrulline; Ala, alanine; Arg, arginine.

[b] In *A. cylindrica*, the Asp + Glu peak was approximately 50% Asp in NO_3^--grown cultures and approximately 25% Asp in N_2- and NH_4^+-grown cultures. In *Anabaena* sp. strain 7120, the Asp + Glu peak was approximately 33% Asp in NO_3^--grown cultures and not observed in N_2- or NH_4^+-grown cultures.

[c] In all cases, the peak was primarily Cit with a variable small shoulder of Ala.

acids; and (iii) an increase in the growth rate relative to dinitrogen and comparable to that supported by ammonium. These correlations were not observed; in fact, they were generally reversed.

Based on the kinetics of preliminary short-term $^{13}NO_3^-$ accumulation experiments, we previously suggested that the rates of nitrate transport differed in the two species (1; J. C. Meeks, Third International Symposium on Photosynthetic Prokaryotes, Oxford, 1979, abstract D-15). However, with the data presented here, differences in rates of nitrate uptake or assimilation cannot explain the differential effect of nitrate on the aerobic dinitrogen assimilatory system. Nitrate disappeared from the growth medium of both species at the same rate (Fig. 3), although under identical culture conditions the rates of acetylene reduction and numbers of heterocysts formed differed markedly. When ammonium assimilation was inhibited by MSX, the two species accumulated nearly equal amounts of $^{13}NH_4^+$ from $^{13}NO_3^-$. Thus, their rates of reduction of nitrite to ammonium were also quite similar. The fact that detectable amounts of $^{13}NO_3^-$ derived $^{13}NH_4^+$ did not accumulate in the absence of MSX implies that ammonium was rapidly assimilated by both species. These observations suggest that dinitrogen fixation could have contributed to the growth of *A. cylindrica* in the presence of nitrate. The growth rate of *A. cylindrica* was the same in the presence of nitrate and the presence or absence of dinitrogen (Table 1). The rates of nitrate assimilation were measured under air; thus, it is possible that assimilation was greater when there was no contribution of fixed nitrogen by nitrogenase.

However, the relative amount of $^{13}NO_3^-$ incorporated into methanol-extractable amino acids differed; *Anabaena* sp. strain 7120 accumulated significantly less than *A. cylindrica*. These results imply that a significant fraction of nitrate-derived ammonium was converted into metabolites in *Anabaena* sp. strain 7120, including polypeptides that were not available for growth. In fact, nitrate did not stimulate growth of *Anabaena* sp. strain 7120 relative to dinitrogen (Table 1). Since the addition of 1% CO_2 and 5 mM fructose or glucose and higher light intensities did not increase the nitrate-dependent growth rate, availability of nitrogen was probably more rate limiting than carbon in *Anabaena* sp. strain 7120. In *A. cylindrica*, nitrate supported nearly as rapid a growth rate as ammonium. Assuming a nitrogen content of 7 to 10% and a Chl *a* content of 1% of the dry weight (32), the maximal rates of nitrate assimilation (Fig. 3) predict a generation time of 13 to 18 h in nitrate-supplemented medium; i.e., a generation time similar to that observed by *A. cylindrica* (Table 1). Thus, nitrate assimilation and growth appear to be closely coupled in *A. cylindrica*, but not in *Anabaena* sp. strain 7120.

The nitrate assimilatory systems of *Anabaena* sp. strain 7120 and *A. cylindrica* showed three general levels of expression: repressed (growth with ammonium), constitutive (growth with dinitrogen), and induced (growth with nitrate). There were differences between *Anabaena* sp. strain 7120 and *A. cylindrica* in their degree of repression by ammonium (Table 3). Nitrate assimilation by ammonium-grown cells of *A. cylindrica* was about 65% that of dinitrogen-grown cells. A similar small difference in nitrate reductase activity between dinitrogen and ammonium-grown *Nostoc* species and an unrelated *Anabaena* species was reported by Herrero et al. (12). In that study, growth in the presence of nitrate increased the rate of nitrate reductase activity about 3.8-fold relative to dinitrogen- and ammonium-grown cells. Nitrate-grown *A. cylindrica* showed similar increases of 1.9- to 2.8-fold relative to dinitrogen- and ammonium-grown cells, respectively (Table 3). However, ammonium-grown *Anabaena* sp. strain 7120 had only 5% of the rate of $^{13}NO_3^-$ incorporation by dinitrogen-grown cells, and there was an approximate 50-fold difference between nitrate- and ammonium-grown cells. In this respect, *Anabaena* sp. strain 7120 is similar to the unicellular non-dinitrogen- fixing cyanobacterium *Anacystis nidulans* (12). Nevertheless, both of the dinitrogen-fixing species used here and those used by Herrero et al. (12) differ from *A. nidulans* in that growth on nitrate gave higher rates of nitrate reductase activity and assimilation. Nitrate reductase activity in *A. nidulans* was fully expressed in the absence of both ammonium and nitrate (12).

Full expression of nitrate assimilation in *Anabaena* sp. strain 7120 and *A. cylindrica* was attained within 2 to 2.5 h after transfer from repressed or constitutive growth conditions to nitrate medium (Fig. 4). A comparable time span was plotted with nitrate uptake in ammonium-to-nitrate step-down experiments with *N. muscorum* (23). The time span for full expression of the nitrate assimilatory system in *Anabaena* sp. strain 7120 and *A. cylindrica* corresponds to less than 10 to 14% of their generation times. This is a relatively short time frame for complete synthesis and assembly of a complex assimilatory system. A comparable time span with respect to generation time was observed in *A. nidulans* for full expression of nitrate reductase activity during transitions from ammonium to nitrate or no combined nitrogen (12). In *A. cylindrica* (Fig. 4) and *A. nidulans* (12), induction of the activities did not occur in the presence of chloramphenicol

col, and the fully induced activity in *A. cylindrica* declined after 2 h of incubation. These data indicate de novo and continued synthesis of one or more essential proteins. However, the protein(s) responsible has not been identified. Thus, one cannot unequivocally state whether an inactive or less active form of nitrate reductase or of the nitrate assimilatory system was present in these cyanobacteria as has been suggested for eucaryotic cells (9).

The regulatory signal(s) for repressed or induced expression of the nitrate or aerobic dinitrogen assimilatory systems in cyanobacteria is unknown. The effect of MSX on reversal of short-term ammonium inhibition of nitrate uptake (6) and long-term ammonium repression of nitrate reductase activity (12) is identical to its effect on the aerobic dinitrogen assimilatory system (27). Thus, inhibition or repression of both nitrogen assimilatory systems in cyanobacteria appears to be dependent on incorporation of exogenous or nitrate-derived ammonium into glutamine. Whether a nitrogen regulatory compound is glutamine or a derivative of it remains speculative. The kinetics of $^{13}NO_3^-$ assimilation into amino acids by *Anabaena* sp. strain 7120 and *A. cylindrica* showed no significant variations in the rates or amounts of glutamine formed relative to other amino acids that indicate a direct role for glutamine in control of the aerobic dinitrogen assimilatory system (16).

The kinetics of nitrate and ammonium inhibition of the aerobic dinitrogen assimilatory system indicate that the effective concentrations are ecologically significant (K_i values of 0.25 to 9.5 μM; Table 2). Concentrations of 0.3 to 5 μM nitrate and 0.7 to 25 μM ammonium are commonly detected in mesotrophic to eutrophic alkaline freshwater lakes (14) where these organisms proliferate (8). The kinetics of nitrate assimilation also indicate that both species could complete for the low environmental concentrations of nitrate. The K_s values of 26 to 30 μM are within the same range as those determined for the non-dinitrogen-fixing dominant planktonic cyanobacterium, *Oscillatoria agardhii* (33), with which they would compete.

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