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High Endogenous Nitrogenase Activity in Isolated Heterocysts of *Anabaena* sp. Strain CA After Nitrogen Starvation

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Metabolically active heterocysts were isolated from a mutant of *Anabaena* sp. strain CA with fragile vegetative cells. Heterocysts isolated from cultures grown in 1% CO_2 in air reduced C_2H_2 at 57 and 10 nmol of C_2H_2 per mg (dry weight) per min under H_2 and Ar, respectively. However, if whole filaments were sparged with 1% CO_2 in 99% Ar for 12 h before heterocyst isolation, these heterocysts showed C_2H_2 reduction rates of 83 nmol of C_2H_4 per mg (dry weight) per min under either H_2 or Ar, or 40% the activity of whole filaments grown in 1% CO_2 in air. Heterocysts isolated from cultures sparged with 100% Ar or 1% CO_2 in 99% N_2 had the same C_2H_2 reduction pattern as heterocysts from cultures grown in 1% CO_2 in air, i.e., low activity under Ar and high activity under H_2. Labeling of whole filaments incubated with $NaH^{14}CO_3$ for 12 h under 1% CO_2 in air or 1% CO_2 in 99% Ar resulted in a twofold higher accumulation of ^{14}C-labeled compounds in vegetative cells and heterocysts of Ar-incubated cells. Our results suggest that during incubation under 1% CO_2 in 99% Ar, presumably a nitrogen starvation condition, continuing photosynthetic fixation of CO_2 leads to accumulation of material(s) in the heterocysts that supports a high, persistent endogenous rate of C_2H_2 reduction. This material appears to be, in part, glycogen.

In recent years, the heterocyst of filamentous nitrogen-fixing cyanobacteria has become an attractive model system for studying the process of dinitrogen fixation (8, 13, 22). These cells develop at fixed intervals along a filament deprived of combined nitrogen (10). During the process of differentiation, many biochemical changes occur that are distinct from vegetative cells; new proteins are synthesized while the synthesis of others is shut off (9). Heterocysts form a thick cell envelope and polar nodules at both ends which morphologically differentiate them from the adjacent vegetative cells. Much of the evidence suggests that these cells are the sole site of N_2 fixation, at least under aerobic conditions. They lack the capacity for O_2 evolution (photosystem II) (7, 17, 26). This specialization is thought to contribute to O_2 protection of nitrogenase inside the heterocysts (13, 22) and also requires a continuous transfer of reductant from vegetative cells to heterocysts. Such movement of labeled carbon from vegetative cells to heterocysts was shown in autoradiography experiments by Wolk (29).

Recovery of nitrogenase activity in the isolated heterocysts has been shown (5, 13a, 15, 16, 18, 23, 25, 27). However, in most of the preparations, the C_2H_2 reduction assay mixture contained constituents similar to those of the in vitro assay for nitrogenase, which questions the metabolic competence of such heterocysts (3, 6, 15, 16, 23, 25). In a few cases (11, 13a, 18) C_2H_2 reduction in the absence of added cofactors was stimulated by H_2 5 to 10 times over the rate in Ar alone. As yet, no added reductants other than H_2 have been reported to stimulate C_2H_2 reduction activity to such an extent.

Stimulation of nitrogenase activity in whole filaments of *Anabaena* spp. has been reported by preincubation in 1% CO_2 in Ar or only acetylene (4, 19, 20). In this paper, we report that heterocysts isolated from an N_2 fixing culture sparged with 1% CO_2 in 99% Ar showed high and similar activity under either Ar or H_2.

MATERIALS AND METHODS

Organism and culture conditions. *Anabaena* sp. strain CA-V, a vegetative cell wall fragile mutant originally isolated from *Anabaena* sp. strain CA (ATCC 33047), was routinely grown in Pyrex culture tubes (22 by 175 mm) in 20 ml of ASP-2 medium (21) with no added combined nitrogen at 39 ± 0.1°C. The

general growth conditions were similar to those described earlier (21).

Heterocyst isolation. Heterocysts were isolated as described previously (13a). The isolation mixture was composed of fresh ASP-2 medium modified by increasing the KCl concentration to 0.03 M and the NaCl concentration to 0.37 M and by the addition of 0.5 mM dithiothreitol and 1 mg of lysozyme per ml. Isolation of heterocysts from cultures grown in 1% CO_2 in air was performed strictly under an H_2 atmosphere, whereas Ar was solely used during isolation of heterocysts from cultures sparged in 1% CO_2 in 99% Ar. Heterocysts were used immediately after isolation.

Incubation conditions. Heterocyst-containing cultures (0.1 mg [dry weight]/ml), grown on 1.05% CO_2 in air were switched to 1.04% CO_2 in 98.96% Ar or 1.05% CO_2 in 98.95% N_2 and kept in continuous light. The bubbling rate was about 35 ml/min. In labeling experiments, 75 μCi of $NaH^{14}CO_3$ (specific activity, 1 μCi/10 μg) in 3 ml was added aseptically to 90-ml cultures (0.08 mg [dry weight]/ml) in a growth bath. After 1 h, sparging was started with 1% CO_2 in air or 1% CO_2 in 99% Ar.

Hydrolysis of cells and glucose assay. A 5-ml portion of whole filaments (0.12 mg [dry weight]/ml) or 2 ml of isolated heterocysts (ca 0.4 mg [dry weight]) were centrifuged at 6,000 × *g* for 5 min and then suspended in sterile distilled water. After centrifugation, the supernatant was decanted, and 1 ml of 2 N H_2SO_4 was added to the pellet. The tube was put into a steam bath and hydrolyzed for 6 h. The hydrolyzed sample was neutralized with saturated barium hydroxide. After the removal of precipitated $BaSO_4$ by centrifugation and filtration, the clear extract was used for glucose assays. Glucose content was estimated by the glucose oxidase assay method. Dry weight determinations were made as described elsewhere (2).

Acetylene reduction assay. C_2H_2 reduction was measured as described earlier (12). Ethylene was measured by injecting 0.2 ml of the gas phase into an Antek model 464-IPC gas chromatograph fitted with a 6-ft (ca. 183-cm) Chromosorb 104 (Johns-Manville Celite Division, Denver, Colo.) column at 50°C, with He at 40 ml/min as carrier gas.

Chemicals. Lysozyme was purchased from Calbiochem, La Jolla, Calif. L-Methionine-DL-sulfoximine (MSX) and glucose assay kit no. 510 were products of Sigma Chemical Co., St. Louis, Mo. $NaH^{14}CO_3$ was obtained from New England Nuclear Corp., Boston, Mass. All common compounds were of reagent grade.

RESULTS

Changes in *Anabaena* sp. strain CA-V cultures after sparging in 1% CO_2 in 99% Ar. When an actively N_2-fixing culture of *Anabaena* sp. strain CA-V (0.1 mg [dry weight]/ml) was transferred from 1% CO_2 in air to 1% CO_2 in 99% Ar, the color of the culture perceptibly changed from blue-green to yellow-green within 2 h. After 10 to 12 h of sparging, the vegetative cells lost almost all the phycobiliproteins, but heterocysts still retained a typical blue-green color, as evident from microscopic observation. The frequency of heterocysts varied from 11 to 14%. The dry weight of the culture increased to only 0.12 mg/ml, which reflects a lack of appreciable growth during the 12-h sparging with 1% CO_2 in 99% Ar.

Nitrogenase activity in isolated heterocysts. Earlier we reported (13a) that heterocysts isolated from cultures grown in 1% CO_2 in air showed acetylene reduction rates of 57 and 10 nmol of C_2H_4 per mg (dry weight) per min under H_2 and Ar, respectively. A variety of carbon sources, namely, glucose, fructose, erythrose, malate, formate, and succinate, added to the C_2H_2 reduction assay mixture under Ar, failed to stimulate C_2H_2 reduction (data not shown). It has been reported that preincubation under a 1% CO_2 in Ar gas phase led to a five- to sixfold increase of nitrogenase activity in whole filaments (4, 20). We therefore examined the activity of heterocysts isolated from cultures preincu-

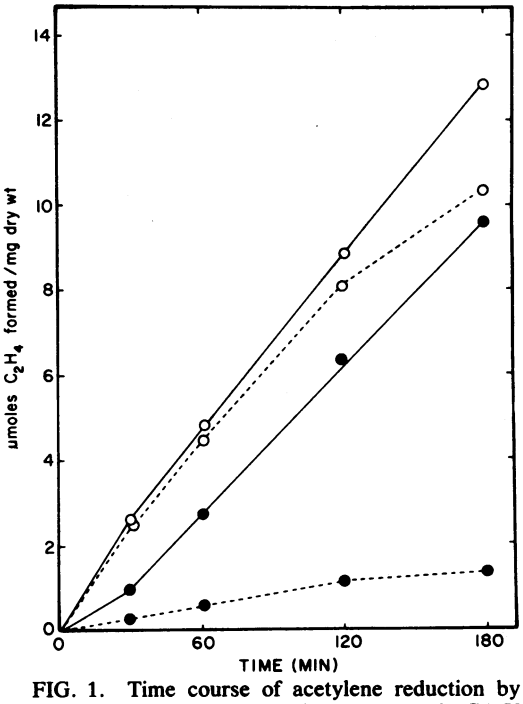

FIG. 1. Time course of acetylene reduction by heterocysts isolated from *Anabaena* sp. strain CA-V grown in 1% CO_2 in air (●) or incubated in a growth bath in the light under 1% CO_2 in 99% Ar (○) for 12 h. Rates of acetylene reduction were assayed under the following conditions: ●-----●, 90% Ar + 10% C_2H_2; ●——●, 90% H_2 + 10% C_2H_2; ○-----○, 90% Ar + 10% C_2H_2; ○——○, 90% H_2 + 10% C_2H_2. Each 7-ml vial contained 1 ml of reaction mixture plus 0.1 mg (dry weight) of heterocysts. Assay vials were incubated in a growth bath at 39°C, illuminated on each side with four F20T12D/HO fluorescent lamps placed 8 cm from the lamp centers to tubes (250 μE/s per m^2; model LI-185A radiometer; LI-Cor Inc., Lincoln, Nebr.). The vials were frequently shaken by hand.

bated under 1% CO_2 in 99% Ar. Figure 1 shows the acetylene reduction rate of heterocysts isolated from cultures sparged in 1% CO_2 in air and 1% CO_2 in 99% Ar for 12 h. It was evident that preincubation in 1% CO_2 in 99% Ar had two effects. First, the rate of C_2H_2 reduction was higher in heterocysts from Ar-treated cultures (83 nmol of C_2H_4 per mg [dry weight] per min); second and most important, the rate was the same under either Ar or H_2. The acetylene reduction rate of the heterocysts from Ar-treated cultures was 40% of that in intact filaments grown in 1% CO_2 in air. Although the effect of incubation in 1% CO_2 in 99% Ar was maximum at 12 h, an effect was seen as early as 3 h (Table 1).

Effect of incubation under other gases on C_2H_2 reduction activity. Sparging with 100% Ar resulted in an increased basal C_2H_2 reduction activity of isolated heterocysts, but there was still a marked difference in rates under Ar or H_2 (Table 1); after 14 h in Ar, activity diminished greatly. If cultures were sparged with 1% CO_2 in 99% N_2, the results were similar to those found with heterocysts isolated from 1% CO_2 in air (data not shown).

Effect of MSX. MSX is an irreversible inhibitor of glutamine synthetase in N_2-fixing cyanobacteria (13, 24). It derepresses nitrogenase activity even in the presence of ammonia. Stewart and Rowell (24) showed continued nitrogenase activity and excretion of ammonia in the medium after the addition of 1 μM MSX in *Anabaena cylindrica*. Because sparging in 1% CO_2 in 99% Ar also caused nitrogen starvation without inhibiting nitrogenase activity, it was desirable to use MSX as another means of bringing about nitrogen starvation. N_2-fixing cultures were treated with 20 μM MSX, and after 12 h, heterocysts were isolated. Unlike heterocysts isolated from cultures treated with 1% CO_2 in 99% Ar, these heterocysts behaved like heterocysts isolated from cultures grown in 1% CO_2 in air (Table 1).

$^{14}CO_2$ fixation in cultures incubated in 1% CO_2 in 99% Ar. Cultures were incubated with $NaH^{14}CO_3$ for 12 h under either 1% CO_2 in 99% Ar or 1% CO_2 in air; then the heterocysts were isolated. The whole filaments and the heterocysts isolated from cultures incubated in 1% CO_2 in 99% Ar contained twice as much radioactivity as the cultures grown in 1% CO_2 in air (Table 2). The radioactivity fixed in the heterocysts was not washed out, even after repeated washings. At least part of this increased $^{14}CO_2$ fixation could be accounted for as an accumulation of a nonsoluble glycogen-like reserve material (Table 3). These nitrogen-fixing cultures incubated under nitrogen starvation conditions (1% CO_2 in 99% Ar) behaved like other nitrogen-starved cultures of blue-green algae and increased their glycogen content (1, 28).

TABLE 1. Effect of pretreatments on acetylene reduction activity of isolated heterocysts

Treatment[a]	Time (h)	Activity[b] under the following gas phase:	
		90% H_2 + 10% C_2H_2	90% Ar + 10% C_2H_2
1% CO_2-99% Ar	0	2.8	0.5
	3	3.6	1.5
	6	3.2	2.1
	12	4.6	4.2
100% Ar	1	2.0	1.0
	2	3.1	1.3
	4	3.8	1.0
	14	0.6	0.1
MSX (20 μM)	12	2.6	0.8

[a] The procedure is described in the legend to Fig. 1.
[b] Ethylene formed (micromoles per milligram [dry weight] of heterocysts per hour).

DISCUSSION

Our results provide direct evidence that heterocysts with high and equal rates of C_2H_2 reduction under Ar or H_2 can be isolated after preincubation of whole filaments in 1% CO_2 in 99% Ar, wherein photosynthetic CO_2 fixation continued, but N_2 fixation did not occur. In earlier work with isolated heterocysts, maximal recovery of activity required an ATP-generating system (3, 6, 15, 16, 23). Peterson and Wolk (18) and more recently, Kumar et al. (13a) showed that although heterocysts did not need an added ATP-generating system, C_2H_2 reduction was greatly stimulated by the addition of H_2, presumably a source of reductant. Although we have no adequate explanation for the high endogenous rate of C_2H_2 reduction evidenced by heterocysts isolated from cultures incubated in 1% CO_2 in 99% Ar, several factors may be operating. Considering the possible roles of H_2 as reductant or in oxygen protection, one might

TABLE 2. Accumulation of ^{14}C-labeled radioactive compounds in whole filaments and heterocysts isolated from *Anabaena* sp. strain CA-V cultures grown in 1% CO_2 in air or sparged in 1% CO_2 in 99% Ar[a]

Sample	dpm/mg (dry wt)
Whole filaments (air)	137,720
Whole filaments (Ar)	348,237
Isolated heterocysts (air)	150,188
Isolated heterocysts (Ar)	386,097

[a] For details of $NaH^{14}CO_3$ labeling, see text.

TABLE 3. Glucose content in hydrolyzed whole filaments and isolated heterocysts

Sample	Glucose content (% dry wt)
Whole filaments[a]	21
Isolated heterocysts[b]	25
Whole filaments[c]	48
Isolated heterocysts[d]	56

[a] Cultures were grown in medium free of combined nitrogen at 39°C with continuous bubbling with 1% CO_2 in air. A 5-ml portion of the culture (0.12 mg [dry weight]/ml) was harvested and used for the glucose assay.

[b] Heterocysts were isolated from 80-ml cultures grown as described in footnote *a*.

[c] Cultures were grown in medium free of combined nitrogen at 39°C on 1% CO_2 in air to about 0.08 mg (dry weight)/ml and then sparged with 1% CO_2 in 99% Ar for 12 h. A 5-ml portion of the culture was removed for the glucose assay.

[d] Heterocysts were isolated from 80-ml cultures previously sparged with 1% CO_2 in 99% Ar for 12 h.

argue that the high rate of C_2H_2 reduction in Ar alone is somehow related to H_2 recycling. Nitrogen-fixing cyanobacteria do produce H_2 at higher rates when incubated under 1% CO_2 in 99% Ar (14), but this requires a continuous source of electrons and, for maximal activity in heterocysts isolated from cultures grown in 1% CO_2 in air, an H_2 level of 20% (13a). It is difficult to visualize either of these as an explanation for the enhanced activity under Ar alone of heterocysts isolated from cells incubated in 1% CO_2 in 99% Ar.

It is evident that heterocysts isolated from cultures sparged in 100% Ar had increased basal activity, but still there was a marked difference between the Ar and H_2 rates (Table 1). The increase in basal activity during the early part of the sparging (up to 4 h) might be due to increased synthesis of nitrogenase or reductant as the cells depleted residual CO_2 and N_2. It is also interesting to note that the increased nitrogenase activity after incubation under Ar is indeed reflected in the heterocysts, and there is no need to invoke the induction of vegetative cell nitrogenase under low O_2 (19). Heterocysts isolated from cells grown in 1% CO_2 in 99% N_2 behaved like heterocysts from cultures grown in 1% CO_2 in air, indicating that low O_2 alone will not induce heterocysts with high nitrogenase activity under Ar. MSX-treated heterocysts also behaved like those isolated from cultures grown in 1% CO_2 in air. MSX is expected to lead to nitrogen starvation, but the reduction of N_2 to NH_3 should continue (24), and perhaps this electron drain or some unknown aspect of NH_3 regulation prevents development of heterocysts with high activity under Ar alone. To check the involvement of the bulk fixation of CO_2, 10^{-5} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea was added before sparging the cultures in 1% CO_2 in 99% Ar, and after 12 h, heterocysts were isolated. These heterocysts were again comparable in activity to heterocysts isolated from cultures grown in 1% CO_2 in air: low C_2H_2 reduction under Ar and high C_2H_2 reduction under H_2. Together these data suggest that a lack of N_2 fixation and continued bulk photosynthetic CO_2 fixation are necessary for inducing heterocysts with high and persistent endogenous C_2H_2 reduction activity. It is reasonable to assume that these heterocysts accumulate a pool of reductant. Such an assumption is corroborated by data from the $^{14}CO_2$ labeling experiments and the increased glucose content of hydrolyzed whole filaments and heterocysts after sparging with 1% CO_2 in 99% Ar (Tables 2 and 3). For the present, it is simplest to view this accumulation as occurring via the demonstrated transfer from vegetative cells to heterocysts (29). It is likely that the increased heterocyst glycogen content formed as a result of nitrogen starvation in 1% CO_2 in 99% Ar helps support the high rate of C_2H_2 reduction under Ar alone. A partial loss of radioactivity during C_2H_2 reduction indicates heterocyst metabolism and indirectly supports such a conclusion. However, it should be noted that glucose alone cannot lead to the enhanced Ar rate because the glycogen content increased only twofold, whereas the C_2H_2 rate compared to that in heterocysts isolated from cultures grown in 1% CO_2 in air increased eightfold, equal to the H_2-supported rate. Thus, there must be some other compound(s) or mechanism involved in the development of the high endogenous activity in heterocysts during incubation of whole filaments under 1% CO_2 in 99% Ar.

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