REDUCTANT SUPPLY IN ISOLATED HETEROCYSTS OF *ANABAENA* SP. STRAIN CA

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Metabolically active heterocysts isolated from wild type *Anabaena* sp. strain CA under hydrogen incubation showed high endogenous acetylene-reducing activity with or without nickel supplementation. The acetylene reducing activity of Ar-incubated heterocysts was significantly enhanced by fructose, erythrose, and reduced glutathione. The possible pathways related to reductant supply to heterocysts are discussed.

Under aerobic nitrogen-fixing conditions some filamentous cyanobacteria differentiate 5 to 10% of their vegetative cells into heterocysts. In isolated heterocysts, high rates of acetylene reduction (nitrogen fixation) occur in the presence of light and H₂ (9, 10, 16, 23). Nitrogen fixation requires both ATP and a low potential electron donor (12). Although it is clear that H₂ provides electrons for acetylene reduction, the pathway of electron donation is still an open question (4, 6, 7, 13, 14, 18, 20). Sturzl and Böger (24) have shown for whole cells of *Anabaena variabilis* that, under diazotrophic conditions, reducing equivalents resulting from carbohydrate breakdown are preferentially supplied to photosystem I. It has been reported that in isolated heterocysts, electrons for nitrogen fixation essentially come from the dark reactions of glycolysis (21). However, there is some controversy about the sources of reductant for nitrogen fixation by cyanobacteria in vivo. The high endogenous nitrogenase activity reported in isolated heterocysts of *Anabaena* sp. strain CA (9) throws some doubt on the function/operation of the conventional pathways of reductant supply and their sources (21, 24). Here we report the possibility of existence of a novel mechanism for the generation and supply of reductant to nitrogenase in isolated heterocysts of *Anabaena* sp. strain CA.

MATERIALS AND METHODS

The organism used for these studies was *Anabaena* sp. strain CA (ATCC

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33047), a filamentous, heterocystous, marine cyanobacterium. Cultures were routinely grown in Borosil culture tubes containing 20 ml of ASP-2 medium (26) with no combined nitrogen and NaCl content of 5 g/l. 100 nM or 10 mM of filter-sterilized $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ or NH_4NO_3 respectively was added when required. The cultures were grown in 1% CO_2 -in-air at $39 \pm 0.1^\circ\text{C}$ and illuminated with six fluorescent lamps (F24/T12/D/HO) on either side of the bath ($300 \mu\text{E}/\text{m}^2/\text{s}$). Heterocyst isolation and other growth conditions were similar to those described earlier (10). For the isolation of heterocysts with high endogenous activity, cultures were grown to about 0.1 mg dry wt/ml in 1% CO_2 -in-air and then switched to 1% CO_2 -in-99% Ar for 12 h. In labelling experiments, 75 μCi of $\text{NaH}^{14}\text{CO}_3$ (specific activity, 1 $\mu\text{Ci}/10 \mu\text{g}$) in 3 ml was added into 17 ml of culture suspension. For sparging 1% CO_2 -in-air was used. Acetylene reduction and dry weights were determined as described previously (23).

Reagent or better grade biochemicals were purchased from Sigma Chemical Co., St. Louis, Missouri. Gold-labelled glucose and fructose were purchased from Aldrich Chem. Co., Milwaukee, Wisconsin.

RESULTS AND DISCUSSION

The kinetics of acetylene reduction by isolated heterocysts of *Anabaena* sp. strain CA under 90% H_2 + 10% C_2H_2 and 90% Ar + 10% C_2H_2 have been reported earlier (23). Here we compare the kinetics of acetylene reduction with erythrose and fructose. The acetylene-reducing activity of heterocysts isolated from cultures grown in 1% CO_2 -in-air was 2 and 1 $\mu\text{mol C}_2\text{H}_4/\text{mg dry wt/h}$ under 90% H_2 + 10% C_2H_2 and 90% Ar + 10% C_2H_2 , respectively. Nickel supplementation (100 nM $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$) in the presence of hydrogen increased acetylene reduction by isolated heterocysts twofold, but had no effect on heterocysts under Ar (Fig. 1). Of the various carbon compounds surveyed for their ability to support acetylene reduction in isolated heterocysts under argon, only fructose and erythrose supported the activity. Fructose (50 mM) had no effect on acetylene reduction by the isolated heterocysts, but at a higher level (350 mM) it increased the endogenous activity from about 1 μmol to almost 1.9 $\mu\text{mol C}_2\text{H}_4/\text{mg dry wt/h}$. This activity was almost equal to the activity reached under 90% H_2 . Erythrose (4 mM) stimulated the acetylene-reducing activity of the heterocysts from 1 μmol to 3.8 $\mu\text{mol C}_2\text{H}_4/\text{mg dry wt/h}$. Nickel supplementation had no effect on either fructose or erythrose-supported endogenous activity of isolated heterocysts (Fig. 1). Sorbose, mannitol, mannose, galactose, glucose, or glyceraldehyde-3-phosphate produced no significant effect on endogenous acetylene reduction by isolated heterocysts (data not shown).

The facts that the fructose concentration (350 mM) appears to be too high for any biochemical reaction and that fructose uptake is not a limiting factor (5) argues against the requirement for such a high concentration (350 mM) for enhancement of acetylene-reducing activity. At 250 mM, fructose did not affect oxygen uptake,

Fig. 1. Time course of acetylene reduction by isolated heterocysts from strain CA with or without added nickel in the growth medium and under an atmosphere of either 90% Ar + 10% C₂H₂ or 90% H₂ + 10% C₂H₂. NiCl₂·6H₂O was added to 100 nM upon culture transfer, and the cells were allowed to grow for at least five generations in the presence of nickel in 1% CO₂ in air atmosphere. 90% H₂ + 10% C₂H₂ with nickel supplementation (○); 90% Ar + 10% C₂H₂ with or without nickel + 4 mM erythrose (●); 90% H₂ + 10% C₂H₂ without nickel (△); 90% Ar + 10% C₂H₂ with or without nickel + 350 mM fructose (▲); 90% Ar + 10% C₂H₂ (□). Light intensity throughout the experiment was 300 μE/m²/s.

but it decreased oxygen production by about 30% in whole filaments. At present we are unable to explain the requirement of a higher concentration (350 mM) of fructose for enhancement of acetylene reduction by the isolated heterocysts. In contrast to the earlier observations (14, 18), the heterocysts isolated from *Anabaena* sp. strain CA do not show any inhibition of acetylene reduction under hydrogen by erythrose.

Erythrose- and fructose-supported endogenous acetylene-reducing activities, like H₂-supported activity, were sensitive to 2,5-dibromo-3-methyl-6-isopropyl-*p*-phenylenediamine (TMPD, 60 μM) (10). This suggests that electrons from the former pass through the cyt b₆/f-complex and PS I for ferredoxin reduction (21). The oxidative pentose phosphate pathway, which has erythrose-4-phosphate as an intermediate, is a major pathway in heterocysts for producing NADPH (11). Conversion of erythrose to triose before being metabolized via glycolytic enzymes, as believed to be present in heterocysts (1), appears unlikely in our case, since

neither dihydroxyacetone phosphate nor 3-phosphoglyceraldehyde enhanced endogenous activity (data not shown). Utilization of fructose and erythrose, independent of oxygen (during the argon gas phase), points to the existence of some pathway other than the conventional oxidative pentose phosphate pathway for the supply of reductant and electrons to nitrogenase (1, 11, 14, 15, 22). At present the possible presence of the bifidium pathway (17) in heterocysts of *Anabaena* sp. strain CA cannot be ruled out. However, the fate of acetate produced by this pathway remains to be explained.

Figure 2 shows the acetylene reduction rates of heterocysts isolated from cultures supplemented or not with nickel chloride and sparged with 1% CO₂-in-99% Ar for 12 h. Hydrogen-supported nitrogenase activity was significantly enhanced from 2.0 to 4.2 $\mu\text{mol C}_2\text{H}_4$ mg dry wt/h under argon sparging. Prein-

Fig. 2. Time course of acetylene reduction by isolated heterocysts of 1% CO₂ in 99% Ar-sparged cultures. The cultures were sparged for 12 h and rates were measured under the following conditions: 90% H₂ + 10% C₂H₂ with or without nickel (○); 90% Ar + 10% C₂H₂ + 0.3 mM GSH (●); 90% Ar + 10% C₂H₂ with or without nickel (△); 1.5 mM (□) and 0.15 mM GSH under 90% Ar + 10% C₂H₂ (■). Cultures were grown for at least five generations with 100 nM of NiCl₂·6H₂O.

cubation under 1% CO₂-in-99% Ar also increased the endogenous activity of isolated heterocysts from 1.0 μ mol to 2.0 μ mol C₂H₄ mg dry wt/h (Fig. 2). The increase in activity was not associated with the presence or absence of nickel. In fact nickel had no effect, even in a hydrogen atmosphere, on heterocysts isolated from cultures sparged in 1% CO₂-in-99% Ar for 12 h. Failure of nickel to increase acetylene reduction in the heterocysts isolated from culture sparged with 1% CO₂-in-99% Ar suggests that nickel supplementation may be conducive to the maximum obtainable activity from cultures grown under 1% CO₂-in-air.

The endogenous activity of isolated heterocysts was increased by the addition of different concentrations of reduced glutathione (GSH). The highest activity (3.8 μ mol C₂H₄ mg dry wt/h) was obtained with 0.3 mM of GSH (Fig. 2). Glutathione reductase activity has been demonstrated in isolated heterocysts of *Nostoc muscorum* (8). A protein from *Anabaena* (25) with thioredoxin activity, when reduced by dithiothreitol, inactivated glucose-6-phosphate dehydrogenase (G6PDH). Surprisingly, inactivated G6PDH was not reactivated oxidatively by oxidized thioredoxin or O₂, but could be reactivated by oxidized glutathione or H₂O₂ (25). These data indicate that if glucose-6-phosphate is active in supplying reductant in the heterocysts then there must be a mechanism to maintain it in an oxidized form. However, the heterocyst is generally viewed as an anaerobic to microaerobic system implying that oxidative activation may be restricted. The H₂O₂ activation of glucose-6-phosphate dehydrogenase may point to a photoreduction of O₂ in heterocysts forming H₂O₂ as in photosystem I (25).

As a whole, the above results shed some light on the nature of the reductant supply in heterocysts. A continuous transport of small molecule(s) from vegetative cells to heterocysts appears unlikely. Heterocysts can be readily labelled by ¹⁴CO₂ (added as NaH¹⁴CO₃) when incubated under 1% CO₂-in-99% Ar for 12 h. The incorporated radioactivity was not lost after repeated washings, which indicates the insoluble nature of the labelled product(s). Insignificant evolution of ¹⁴CO₂ (0.04 μ mol/mg dry wt) in 6 h and 6% loss of fixed/transferred radioactive material strongly precludes the functioning of the oxidative pentose phosphate pathway. Furthermore, the loss of only 20% of the dry weight of the heterocysts after 6 h (Table 1) under argon gas phase appears to support our view. To get 12 to 14 μ mol of C₂H₄/mg dry wt in 5 h under 90% Ar + 10% C₂H₂ with reduced glutathione from endogenously active heterocysts (Fig. 2), we need to utilize about half of the amount of glucose or in other words half of the dry weight of heterocysts. But the carbohydrate deposition in heterocysts is only 43% (3), the dry weight loss after 6 h incubation under 90% Ar + 10% C₂H₂ is only 20%, and microscopically heterocysts after 6 h incubation under argon look almost the same as at 0 h.

In light, photosystem I can reduce ferredoxin and generate ATP for reduction of N₂ to ammonia (2). There is now considerable evidence that H₂ (6, 7), NADH (7), and NADPH (21) can feed electrons into the photosynthetic electron transport chain of heterocysts. We agree on this point, but continuous supply of electrons is needed for the functioning of photosystem I. To get all these electrons from

Table 1. Accumulation of ^{14}C -labelled radioactive compounds, evolution of CO_2 , loss of dry weight and acetylene reduction by heterocysts isolated from *Anabaena* sp. strain CA sparged with 1% CO_2 in 99% Ar for 12 h. All experiments, except isolation, were performed under 90% Ar, 10% C_2H_2 . Isolation was performed under 100% Ar.

Sample	dpm/mg dry wt	$\mu\text{mol CO}_2$ produced mg dry weight h^{-6}	$\mu\text{mol C}_2\text{H}_2$ /mg dry weight h^{-6}	$\mu\text{g dry wt loss/mg}$ dry weight h^{-6}
Heterocysts (0 h)	16,052,333 (100%)	—	—	—
Heterocysts (6 h)	15,001,241 (94%)	0.04	8.6	203 (20%)

electron donors, the heterocysts must utilize their deposited/transferred carbon skeletons. In all carbon assimilation pathways, CO_2 must be evolved and heterocysts must lose a significant proportion of dry weight as carbon. The oxyhydrogen reaction seems improbable in isolated heterocysts under argon gas phase unless they split H_2O .

From the above experimental data we conclude that heterocysts cannot work under argon gas phase without splitting H_2O or utilizing some other novel mechanism for ATP and reductant supply to nitrogenase. However, it seems that no one has seen net O_2 evolution in isolated heterocysts, which would imply the presence of a functional photosystem II. The oxyhydrogen reaction or possibly something like the violaxanthin cycle (19) can take good care of the oxygen produced by providing extra ATP for nitrogenase (oxyhydrogen reaction) or reversible binding and releasing of any oxygen formed in the light in a tightly controlled manner (violaxanthin cycle).

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