Physiological Factors Determining Hydrogenase Activity in Nitrogen-Fixing Heterocystous Cyanobacteria

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ABSTRACT

Four species of nitrogen-fixing heterocystous cyanobacteria were compared with respect to induction of hydrogenase activity. Two of the strains contained phycoerythrin and built up high levels of carbohydrate storage material when grown in batch culture under nitrogen-fixing conditions and continuous illumination. These strains did not exhibit hydrogenase activity. Lack of activity in the phycoerythrin-containing species was determined by cell-free assays measuring both hydrogen-evolving and hydrogen-uptake activities. Apparently, expression of hydrogenase is negatively correlated with the carbohydrate pool present and concurrent respiration. Furthermore, there is an apparent relationship between the presence of phycoerythrin, carbohydrate accumulation, and the absence of hydrogenase activity.

Many nitrogen-fixing cyanobacteria contain an uptake hydrogenase, which consumes the hydrogen which is produced by nitrogenase during reduction of dinitrogen. Hydrogenase activity varies during the course of cultivation and is influenced by external factors such as light intensity (18) or availability of nickel (1, 6). Comparing hydrogenase activity in four strains of heterocystous cyanobacteria (three Anabaena strains and a Hapalosiphon species), we found that two of these did not exhibit any hydrogenase activity throughout the entire cultivation period. Since this was an unexpected finding, physiological parameters were studied, common to species without active hydrogenase and which differed from those containing hydrogenase activity. Comparative physiological investigations on factors involved in regulation of hydrogenase in heterocystous cyanobacteria are needed to broaden our knowledge on hydrogen metabolism, which still is poorly understood, although some details have been reported during the last decade (7, 9).

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MATERIALS AND METHODS

Anabaena CH1 and CH2 are isolates from Taiwan (4). Anabaena variabilis (A1 CC 29413) was originally obtained from Dr. C. P. Wolk, East Lansing, MI, and Hapalosiphon sp. (SAUG B-4679) from the Sammlung von Algenkulturen der Universität Göttingen, FRG. They were grown as described previously (5) with continuous illumination under nitrogen-fixing conditions in a mineral medium according to Arnon et al. (2). Cultures were started with a density equivalent to 1 μg Chl/mL (for Anabaena CH1 2 μg Chl/mL were used). After 24 and 48 h, a density of about 12 and 18 μg Chl/mL was attained for Anabaena CH1 and 15 to 22 μg Chl/mL for Anabaena CH2. Glycogen was determined according to Ernst et al. (8) and Chl measured after methanolic extraction of cells according to Mackinney (11).

Nitrogenase activity of intact filaments was determined as acetylene reduction (for details see Ref. 1). After harvest and immediate disruption of filaments with a French-pressure cell under a hydrogen atmosphere, hydrogenase activity was assayed in freshly prepared, cell-free homogenates either by gas chromatography as hydrogen evolution in the presence of reduced methylviologen (1), or spectrophotometrically as hydrogen uptake with triethylene blue as electron acceptor (15). All experiments were performed with at least three culture sets, and the respective activity assays repeated three to four times. Mean values are given throughout; for nitrogenase activity deviation from the mean was ±5%, for hydrogenase activity ±10%.

RESULTS AND DISCUSSION

Four strains of heterocystous cyanobacteria were studied, and we found that Anabaena CH2 and Hapalosiphon sp. did not exhibit uptake hydrogenase activity, whereas in Anabaena CH1 and Anabaena variabilis varying rates of enzyme activity were measured during the 72-h cultivation period (Table I). Aerobic or anaerobic homogenization gave the same results. In a further series of experiments, the Anabaena strains CH1 and CH2 were compared with respect to several physiological parameters, which may influence the regulation of hydrogenase activity. Nitrogenase activity in both strains showed a similar time course during the cultivation period, although it was consistently lower in Anabaena CH2. In contrast, the amount of carbohydrate storage material present in the cells was very high in Anabaena CH2, while in Anabaena CH1 a
Homogenates of Hapalosiphon and Anabaena CH1, Anabaena CH2, A. variabilis and Hapalosiphon sp. were incubated aerobically with decreasing levels of oxygen, followed by measurement of hydrogenase activity. Hydrogen uptake was measured with methylene blue as electron acceptor. Samples containing 30 μg Chl l$^{-1}$ x mL$^{-1}$ were suspended in phosphate buffer (20 mM, pH 7.2) and sparged with either argon or nitrogen. After addition of methylene blue (20 μM), the absorbance decrease at 670 nm was measured spectrophotometrically.

### Table I. Hydrogenase Activity Determined as Hydrogen Uptake in Homogenates of Anabaena CH1, Anabaena CH2, A. variabilis and Hapalosiphon sp.

<table>
<thead>
<tr>
<th>Cultivation time (h)</th>
<th>Hydrogenase Activity (μmol H$_2$ consumed x mg Chl$^{-1}$ x h$^{-1}$)</th>
<th>Anabaena CH1</th>
<th>Anabaena CH2</th>
<th>A. variabilis</th>
<th>Hapalosiphon</th>
</tr>
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<tr>
<td>24</td>
<td></td>
<td>3.6</td>
<td>0</td>
<td>2.7</td>
<td>0</td>
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<tr>
<td>48</td>
<td></td>
<td>2.8</td>
<td>0</td>
<td>3.5</td>
<td>0</td>
</tr>
<tr>
<td>72</td>
<td></td>
<td>1.9</td>
<td>0</td>
<td>3.9</td>
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</tr>
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</table>

somewhat lower maximum value for glycogen content was obtained during the first cultivation day. Thereafter, the amount of carbohydrate storage material decreased rapidly in Anabaena CH1 (Fig. 1). Respiration of Anabaena CH2 was found to be higher than that of Anabaena CH1 (40 and 70 μmol O$_2$ x mg Chl$^{-1}$ x h$^{-1}$, respectively, in 24 h old cultures).

Similar results were obtained comparing A. variabilis and Hapalosiphon sp.; the latter species also exhibited lower nitrogenase activity and a larger amount of carbohydrate storage material (data not shown). Apparently, the carbohydrate level present in nitrogen-fixing cyanobacteria is negatively correlated with uptake hydrogenase activity. A similar phenomenon has been observed in nonheterocystous cyanobacteria that fix nitrogen only under microaerobic conditions. In these strains as well, exhaustion of combined nitrogen, a prerequisite of nitrogenase induction, led to accumulation of carbohydrate storage material and no uptake hydrogenase was induced (10, 17). Presumably, the large amounts of glycogen present under nitrogen-fixing conditions supply nitrogenase with sufficient reductant. As reported previously (3), only minimum amounts of hydrogen are produced during N$_2$-fixation when the Mo-Fe protein is not limited by reductant supply, because under these conditions N$_2$ is the substrate which is preferentially reduced by nitrogenase. Upon limitation of reductant (i.e. low levels of carbohydrate present) more protons than nitrogen molecules will be reduced and enhanced hydrogen formation can be measured (3). Formation of hydrogen has been postulated to be a signal for the induction of hydrogenase (12). If true, this would explain the correlation between low levels of carbohydrate storage material and induction of hydrogenase.

To test this hypothesis, the glycogen pool was decreased in Anabaena CH2 by prolonged incubation under dark aerobic conditions (Table II). Indeed, hydrogenase could be detected with decreasing levels of glycogen. Nitrogenase activity, however, was absent after this incubation, most probably due to oxygen inactivation. Apparently, hydrogenase induction occurred during the first 24 h when nitrogenase was still active. These findings are indicative of Anabaena CH2 not being a mutant lacking hydrogenase. It is rather our culture conditions which prevent expression of hydrogenase activity in this species. Future studies will have to analyze the time course of hydrogenase induction and nitrogenase inactivation in more detail.

In a further series of experiments, hydrogen-evolving and hydrogen-consuming activities in cell-free systems were compared. As shown in Figure 2, the assay measuring hydrogen evolution with reduced methylviologen as electron donor gave similar results as the assay measuring hydrogen uptake with methylene blue as electron acceptor (Table II). No hydrogen-evolving activity could be detected in Anabaena CH2 and Hapalosiphon sp. These data confirm our previous observation that the hydrogen-evolution assay is a reliable indicator of uptake hydrogenase activity (1). A substantial fraction of membrane-bound nickel-dependent uptake hydrogenase, located exclusively within heterocysts (13, 14), becomes solubilized by the French-press treatment and can be assayed by hydrogen evolution. According to our experience the amount of a distinct soluble reversible hydrogenase is very low in heterocystous cyanobacteria. It should be mentioned that both assays for measurement of cell-free hydrogenase activity do not reflect the actual hydrogenase activity in vivo. These assays are, rather, a measure for the amount of hydrogenase present in the cells. Reliable determination of in vivo hydrogenase activity is impossible because nitrogenase-catalyzed hydrogen production superimposes hydrogen uptake activities which, in addition to hydrogenase, may even be catalyzed by nitrogenase itself (5, 16).

Since both Anabaena CH2 and Hapalosiphon sp. are phycocerythrin-containing strains we further considered that there might be a correlation between, on the one hand, the occurrence of phycocerythrin and the presence of a large glycogen pool and, on the other hand, the absence of hydrogenase in
heterocystous cyanobacteria. The correlation between these parameters is not likely to be coincidental, because phycoerythrin enables cyanobacteria to effectively use a broader share of the visible light spectrum than cyanobacteria lacking this pigment. Accordingly, our measurements showed that (under limiting light intensity) Anabaena CH2 and Hapalosiphon sp. exhibited higher rates of photosynthesis than Anabaena CH1 and A. variabilis (data not shown). Consequently, more carbohydrates will be produced by the phycoerythrin-containing species. Comparisons of additional cyanobacterial strains are required to determine whether this correlation may be generalized.

The absence of uptake hydrogenase activity has been unequivocally demonstrated in Anabaena CH2 and Hapalosiphon sp.; however, no net hydrogen evolution could be observed during dinitrogen fixation. Since H₂-formation is reported to be strictly correlated with N₂-reduction (3), hydrogen produced might be taken up by nitrogenase itself as suggested previously (5, 16), although our efforts to demonstrate H₂-uptake by cell-free nitrogenase preparations from Anabaena have so far been unsuccessful.

![Figure 2. Cell-free hydrogenase activity in Anabaena CH1 (○), A. variabilis (□), Anabaena CH2 (▲), and Hapalosiphon sp. (△). Hydrogenase activity was determined in homogenates prepared at the intervals indicated by the circles. Homogenates were incubated in glass vessels under an argon atmosphere (1) in the presence of 5 mM Na₂S₂O₄ and 2 mM methylviologen (1,1-dimethyl-4,4-bipyridinium dichloride).](image)

### Table II. Induction of Hydrogenase Activity in Anabaena CH after Long-term Incubation in the Dark

<table>
<thead>
<tr>
<th>Dark Incubation Time (h)</th>
<th>Glycogen Content (mg × mg Chl⁻¹)</th>
<th>Hydrogenase Activity, Cell-free (μmol H₂ evolved × mg Chl⁻¹ × h⁻¹)</th>
<th>Nitrogenase Activity, Cell-free (μmol C₂H₄ × mg Chl⁻¹ × h⁻¹)</th>
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</thead>
<tbody>
<tr>
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<td>1.12</td>
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### LITERATURE CITED
