Hydrogen Evolution Catalyzed by Hydrogenase in Cultures of Cyanobacteria

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Cultures of *Anabaena cylindrica*, grown on media containing 5 mM NH_4Cl (which represses heterocyst formation), evolved hydrogen after a period of dark incubation under an argon atmosphere. This hydrogen production was not due to nitrogenase activity, which was nearly undetectable, but was due to a hydrogenase. Cultures grown on media with tungsten substituted for molybdenum had a high frequency of heterocysts (15%) and inactive nitrogenase after nitrogen starvation. The hydrogenase activity of these cultures was three-fold greater than the activity of non-heterocystous cultures. The effects of oxygen inhibition on hydrogen evolution by heterocystous cultures suggest that two pools of hydrogenase activity exist – an oxygen sensitive hydrogen evolution in vegetative cells and a relatively oxygen-resistent hydrogen evolution in heterocysts. In either case, inhibition by oxygen was reversible. Light had an inhibitory effect on net hydrogen evolution. Hydrogen production *in vitro* was much higher than *in vivo*, indicating that *in vivo* hydrogenase activity is limited by endogenous reductant supply.

Introduction

Some species of green algae possess a hydrogenase that, after a period of dark anaerobic incubation, catalyzes the evolution of hydrogen both in a dark, fermentative reaction and in a light-dependent reaction. The following generalizations about this hydrogenase may be made [1, 2]: Hydrogen evolution commences only after the algae have been anaerobically adapted in the dark for 2–30 h. The period of most rapid H_2 production is only a few h long, and H_2 production ceases after about one day. Oxygen at a pO_2 above 0.002 atm is inhibitory. H_2 evolution is several-fold higher at low light intensities than in the dark; however, moderate to high light intensities inhibit the reaction because of increased photosynthetic O_2 production. Hydrogen evolution by N_2-fixing, filamentous cyanobacteria has been previously demonstrated [2]. Under the conditions used, most of the H_2 was evolved via nitrogenase as evidenced by the requirement for the absence of fixed N_2, insensitivity to carbon monoxide, and inhibition by N_2 or acetylene [3–6]. In reductant-depleted cultures, H_2 can support nitrogenase activity [7, 8]. Both isolated heterocysts [9] and intact filaments [10] are capable of consuming H_2 in an oxy-hydrogen reaction. This H_2 consumption activity has been ascribed to an uptake hydrogenase, a membrane-bound enzyme that is saturated at a relatively low pH_2 [7, 11]. In N_2-fixing cyanobacteria, this hydrogenase may act to increase the efficiency of N_2 fixation by recycling reductant lost in the N_2 fixation process [4].

Evolution of H_2 (via hydrogenase) has been demonstrated with crude cyanobacterial extracts [11–14] and has been implied from studies with whole cells [3]. Whereas H_2 consumption activity appears to be membrane bound and localized primarily in the heterocysts [9], the soluble H_2 evolution activity is thought to be equally concentrated in both heterocysts and vegetative cells [8, 11]. In addition, the activities for H_2 consumption and H_2 evolution have been differentially sedimented from a fragmented, isolated heterocyst preparation [11]. However, little is known about the physiology of H_2 evolution, in part because any possible H_2 evolution by this enzyme is usually masked by nitrogenase catalyzed H_2 evolution. *In vivo* H_2 evolution driven by dithionite-reduced methyl viologen has been recently demonstrated in both heterocystous and non-heterocystous cultures of *Anabaena cylindrica* [15] (methyl viologen inhibited nitrogenase mediated H_2 evolution). Since little is known about the role of this

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enzyme in the hydrogen metabolism of cyanobacteria, we have investigated the presence of this enzyme under various culture conditions and determined the conditions necessary for expression of its activity *in vivo* without added reductant.

Materials and Methods

Organisms and culture methods. *Anabaena cylindrica* (629), *Nostoc muscorum* (6719), *Phormidium luridum* (426), and *Chlorogloea fritschii* (6912) (all cultures were obtained from the Culture Collection of Algae at the University of Texas at Austin) were grown in 2-liter culture vessels on modified Allen and Arnon Media [16] to which 5 mM NH_4Cl was added. Cultures were sparged with 0.3% CO_2 in air: which, in conjunction with 5 mM $NaHCO_3$ in the media, maintained the pH between 7.8 and 8.2. Lighting and cell density measurements were as previously described [16]. All cell densities are given in gms dry wt/liter or mgs dry wt/ml. When the density of ammonium-grown cultures reached 0.525–0.700 g/l, NH_4Cl was depleted and additional NH_4Cl (to bring the media to 5 mM NH_4Cl) was added. *A. cylindrica* was also grown in 8-liter vessels with modified Allen and Arnon media from which molybdenum had been omitted and sufficient 1 M sodium tungstate had been added to give 8 mg W per 1. This concentration effectively inhibited utilization of trace amounts of molybdenum without inhibiting growth. NH_4Cl (1 mM) provided fixed N. When the ammonium was depleted, the cultures yellowed due to phycocyanin degradation induced by N starvation. These cultures were used when the heterocyst frequency had increased to 15%. The nitrogenase that was synthesized under these conditions was inactive and these cultures had very low acetylene reduction activities. Preparations of N_2-starved cultures with active nitrogenase and the microscopic determination of heterocyst frequencies were also done as previously described [16]. Cultures were concentrated by settling to a density of 3.5–10.5 g/l. The concentrated cells were then made anaerobic by sparging with argon: CO_2 (99.7:0.3) for 10 min before injection into assay flasks. Additions of liquids or gases to assay flasks were made prior to the addition of the culture sample.

H_2 production assays. All assays were conducted anaerobically (under argon) unless otherwise noted. Two ml portions of the concentrated cultures were injected into 6.4 ml serum-stoppered Fernbach flasks which had been previously flushed with argon. For dark assays, flasks were darkened with aluminum foil. Lighting, when required, was via a bank of 30-W tungsten spotlights which gave a light intensity of 6.0×10^4 ergs/cm^2 per s. The flasks were incubated on a thermostated (25 °C) shaker for various times. Reactions were terminated with the injection of 0.20 ml of 25% trichloroacetic acid. Gas samples were withdrawn from the assay flasks with a 100 µl gas-tight syringe and injected into a Varian model 3700 gas chromatograph equipped with a thermal conductivity detector and a 0.32×183 cm stainless steel column packed with molecular seive 5A. Argon was used as the carrier gas. H_2 volumes were obtained from peak heights by reference to a standard curve. The minimum volume of H_2 measurable in the 6.4 ml assay flasks was about 0.01 µl H_2 per flask.

Acetylene reduction assays were performed essentially as described [16], except that the flasks were incubated in the dark. This procedure indicates the upper limit of the possible contribution of nitrogenase to the observed H_2 evolution.

Cell-free extract preparation and assay. Cell-free extracts were prepared by injecting 20 ml of concentrated cells (previously incubated anaerobically) into an anaerobic, serum-stoppered sonicator cell. The suspension was disrupted at 4 °C for 6 min with a Heat Systems Ultrasonics model W200 sonicator set at medium power and pulsed with a 65% duty cycle. This procedure destroyed all vegetative cells and 80–90% of the heterocysts. 0.75 ml of extract was injected into serum-stoppered, argon-flushed 6.4 ml Fernbach flasks. 0.20 ml of buffered sodium dithionite (25 mM) was added. 50 µl of a 20 mM solution of methyl viologen were added to start the reaction. Assays were run for 20 min, terminated with the injection of 0.2 ml of 25% trichloracetic acid, and the H_2 produced was measured as in whole-cell assays (described above). Results are expressed as µl H_2 evolved per mg dry weight (of the original cell mass) per h, since the volume change during disruption was negligible. This allows direct comparison between *in vitro* and *in vivo* assays.

Results

Ammonia-grown (5 mM NH_4Cl) and N-starved (tungsten) cultures showed similar time courses for

the development of hydrogenase activity *in vivo*, with maximum rates of H_2 production being achieved within one to three h of the onset of dark anaerobiosis (Fig. 1). After reaching a maximum, rates of H_2 evolution were constant for at least seven h (*i.e.* H_2 production was linear with time). Rates of hydrogen production were not affected by incubation under gas phases of either nitrogen or 15% acetylene in argon. The requirement for a period of dark anaerobiosis for H_2 production by cyanobacteria is similar to that found for H_2 production by green algae [1, 2]. The maximum rates of H_2 production (per mg dry weight) obtained with N_2-starved (15% heterocysts), tungsten-poisoned cultures were three to four-fold higher than with ammonium-grown cultures (< 0.1% heterocysts). These results suggest that the heterocysts contain a proportionately larger amount of hydrogenase, or that the hydrogenase present in heterocysts is more active.

To rule out the possibility of indirect effects of tungsten or ammonium on hydrogenase activity, the *in vitro* and *in vivo* hydrogenase activity of different culture types was examined (Table I). Since added reductant and electron carrier are in excess in *in vitro* assays, rates of H_2 evolution are limited only by the amount of active enzyme present. As Table I shows, *in vitro* hydrogenase activity was correlated to some extent with heterocyst frequency. Cultures that had been grown on 5 mM NH_4Cl had practically no heterocysts (< 0.1%) and possessed a small but significant amount of *in vitro* hydrogenase activity. The air-grown culture (with 5% heterocysts) has an *in vitro* hydrogenase activity that was intermediate between the activity of ammonium-grown cultures and N_2-starved (15% heterocysts) cultures. *In vitro* hydrogenase activity was invariably found to be much higher than *in vivo* hydrogenase activity (Table I). H_2 evolution by cultures was only 3–7% of the activity obtainable *in vitro*, indicating that *in*

Table I. Comparison of *in vivo* and *in vitro* hydrogenase activity of *Anabaena cylindrica* [a].

Culture Media	*In vitro* H_2 evolution [μl/mg dry wt · h]	*In vivo* H_2 evolution [μl/mg dry wt · h]	Dark acetylene reduction [μl C_2H_2/mg · h]
Tungsten (-Mo)	1.18	0.057	0.0008 [b]
Air grown and nitrogen starved	1.79	0.096	0.0054 [b]
Air grown	0.98	0.038	0.0081
5 mM NH_4Cl	0.30	0.009	0.0007

[a] Cultures were grown, anaerobically adapted in the dark for 4 h and assayed as described in Materials and Methods. The results shown for tungsten and ammonium chloride cultures are the average of three experiments for each culture type, the data for the air-grown cultures were obtained from duplicate cultures from which a portion was anaerobically adapted for 4 h and then assayed for hydrogenase *in vivo* and *in vitro*. The remaining culture was N_2-starved, anaerobically adapted as above, and assayed for hydrogenase *in vivo* and *in vitro*.

[b] Light driven acetylene reduction activities of the tungsten culture was 0.8 μl C_2H_2/mg · h, while the normally air grown and then nitrogen starved culture had a light driven acetylene reduction activity of 42 μl C_2H_2/mg · h.

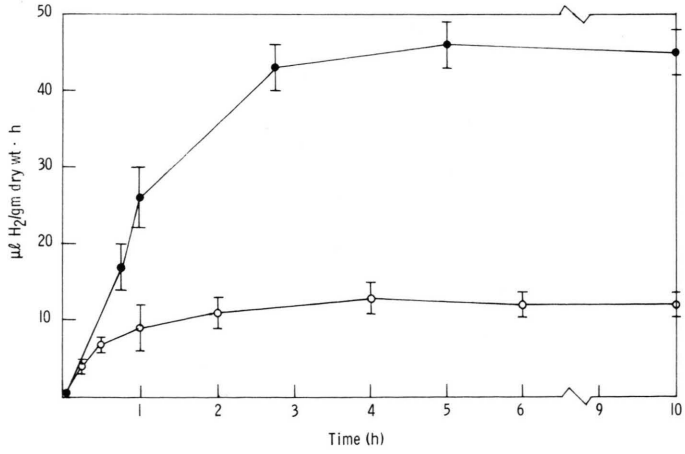

Fig. 1. Time course of dark activation of hydrogenase in *A. cylindrica*. ⌀, 5 mM NH_4Cl batch-grown culture (< 0.1% heterocysts). The results shown are the average of three cultures. ●, tungsten-grown culture (15% heterocysts). The results shown are the average of two cultures. Dark acetylene reduction rates for both cultures were about 1 μl C_2H_4/g dry wt-h. Cultures were concentrated by settling, anaerobically incubated (under an argon atmosphere) in the dark, and assayed for H_2 production at the indicated times.

vivo activity is severely limited by supply of reductant. Similar results were obtained with *Nostoc muscorum* (not shown). In experiments with ammonium (5 mM) grown cultures of *Phormidium luridum* and *Chlorogloea fritschii*, no detectable H_2 evolution, either *in vivo* or *in vitro*, was obtained. Thus, the ability to evolve H_2, either *in vivo*, or *in vitro*, is not universal among the cyanobacteria.

That the higher rates of H_2 production by N-starved, tungsten-poisoned cultures is due to disproportionately higher activity in heterocysts is also suggested by the inhibitory effects of various O_2 concentrations (Fig. 2). H_2 evolution by heterocyst-free cultures was almost completely abolished by 5% O_2. At 5% O_2, H_2 production by cultures with 15% heterocysts showed approximately the same decrease in absolute H_2 production. The remaining H_2 evolution activity (~ 2/3 of the original) was fairly O_2 resistant, with 50% inhibition at 15% O_2 in the gas phase. Heterocystous cyanobacteria also contain the extremely O_2 labile enzyme, nitrogenase, and indirect evidence has led to the hypothesis that nitrogenase is localized in the heterocysts and, therefore, is resistant to O_2 inactivation. The results shown in Figure 2 suggest that a large proportion of the hydrogenase activity in the N_2-starved cultures is contained in the heterocyst and, thus, protected, to some extent, from the inhibitory effects of O_2. However, O_2 inhibition was almost completely reversible *in vivo*, and, as previously shown, *in vitro*

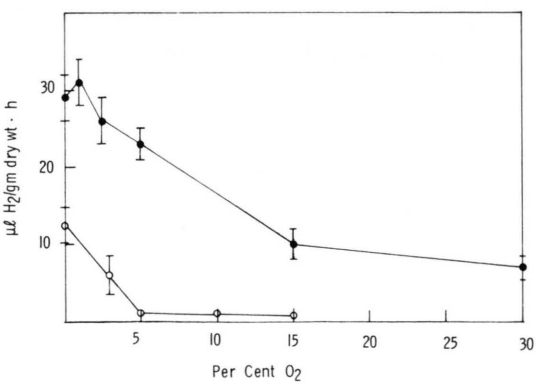

Fig. 2. O_2 inhibition of hydrogenase activity in *A. cylindrica*. Φ, NH_4Cl batch-grown culture (0.1% heterocysts). Average of two experiments. Dark acetylene reduction activity = 0.6 μl C_2H_4/g-h. ●, tungsten-grown culture (15% heterocysts). Average of two experiments. Dark acetylene reduction activity = 1 μl C_2H_4/g-h. Cultures were concentrated by settling, anaerobically adapted in the dark for 4 h, and then assayed for H_2 production for one h at the indicated O_2 concentrations.

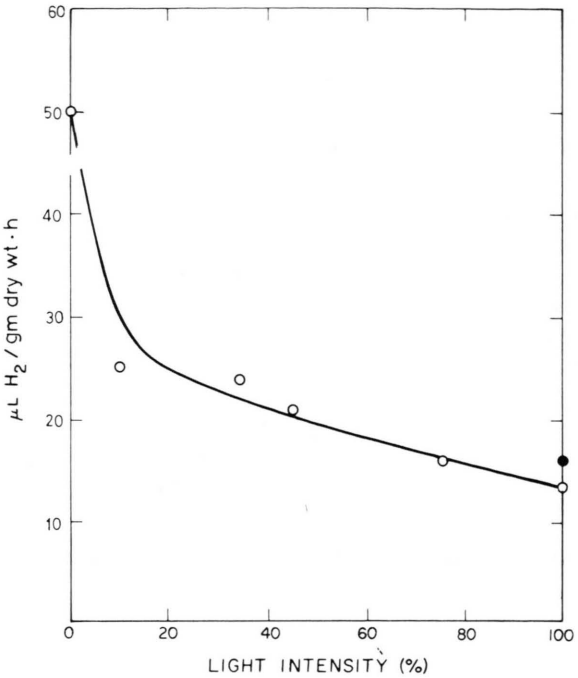

Fig. 3. Effects of varying light intensities on reversible hydrogenase activity in *A. cylindrica*. ○, tungsten-grown culture with dark acetylene reduction activity of 0.5 μl C_2H_4/g-h. One-hundred percent light intensity equaled 6×10^4 erg/cm$_2$ · s. ●, + 20 μM DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea]. The culture was anaerobically adapted in the dark for 4 h and then assayed for one h at the indicated light intensities.

[14]. Thus oxygen may act directly in a manner which is readily reversible upon removal of oxygen from the gas phase, or oxygen may act indirectly by competing for some reduced compound which drives hydrogen evolution.

Light also has an inhibitory effect (Fig. 3). At the lowest light intensity tested (6.0×10^3 ergs/cm^2 per s), H_2 evolution was already inhibited fifty percent over that of the dark control. Higher light intensities were increasingly inhibitory. In green algae, high light intensities were thought to inhibit H_2 evolution indirectly via photosynthetic O_2 evolution. However, unlike the case with green algae, where addition of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) alleviates inhibition by high light intensities [17], addition of 2×10^{-5} M DCMU had little effect on activity at the highest light intensity tested.

Broken cell preparations of *Anabaena cylindrica* can evolve H_2 from some electron donors such as glucose, glucose-6-phosphate, isocitrate, and di-

thionite when supplied with an artificial electron carrier [11, 12]. We found that glucose, succinate, and isocitrate (all 5 mM), when exogenously supplied, had no effect on *in vivo* hydrogenase activity in *Anabaena cylindrica*. It is likely that at least glucose was assimilated [18]. Addition of 50 μM CCCP (carbonyl cyanide m-chlorophenyl hydrazone) caused a two-fold stimulation of H_2 evolution.

Discussion

Our results provide evidence for *in vivo* H_2 evolution by cyanobacteria that can be attributed to a reversible hydrogenase, hitherto characterized only *in vitro*. This hydrogenase appears to be unlike the hydrogenase of green algae in several respects. Illumination (within the range tested) did not lead to a photo-induced evolution of H_2; rather, photoinhibition was found, and DCMU failed to alleviate this inhibition. Thus, at least in *A. cylindrica*, reductant generated directly by photosynthesis does not seem to be available to hydrogenase. This is consistent with the lack of interaction between the *A. cylindrica* ferredoxin and this hydrogenase *in vitro* [12, 14]. It is possible that the photoinhibition was due to a stimulation of H_2 consumption, but at the very low partial pressure of H_2 in these experiments, the effect of H_2 consumption activity would be rather minor [11].

The dark metabolic pathways leading to H_2 evolution are unknown at present. Addition of several carbon compounds – glucose, isocitrate, and succinate – failed to stimulate hydrogenase activity. The *in vivo* rates of H_2 evolution without added electron donors were only one-tenth or less of the rates obtained *in vitro* or *in vivo* with dithionite and methyl viologen. These results indicate that hydrogenase activity *in vivo* is severely limited by reductant supply. At present, it is not clear whether this limitation is at the level of reductant or electron carrier to the hydrogenase.

Heterocyst-containing cultures had three- to four-fold higher rates of H_2 evolution both *in vivo* and *in vitro* than did cultures grown on ammonia which repressed heterocyst formation. Heterocyst-containing cultures were more resistant to O_2 inhibition than cultures without heterocysts, and the magnitude of the decrease in H_2 evolution by heterocystous cultures at low O_2 concentrations is the same as that observed with heterocyst-less cultures. These results are consistent with heterocysts containing a proportionately larger amount of hydrogenase than vegetative cells. Previous studies with isolated heterocysts and vegetative cell fragments have indicated that H_2 evolution is equally concentrated in both cell types [8, 11]. However, isolated heterocysts leak some of their soluble contents [19], possibly accounting for some of this difference.

Recently, hydrogenase activity supported by dithionite-reduced methyl viologen was reported in both heterocystous and heterocyst-less cultures of *A. cylindrica* [15]. The rate of H_2 production by air-grown cultures incubated with dithionite and methyl viologen in that study was 1.2 μl/mg · h which is very similar to the value (0.98 μl/mg · h) obtained *in vitro* in this study (Table I). However, no *in vivo* H_2 evolution in the absence of added reductant was observed by the workers probably because the cultures were not anaerobically adapted, which is shown in the present study to be necessary for the development of *in situ* H_2 evolution.

In conclusion, the results presented here have established that heterocystous blue-green algae can evolve H_2 by a hydrogenase-catalyzed reaction which appears to be localized primarily, but not wholly, in heterocysts. It was also demonstrated that some of the characteristics of this activity are unlike the hydrogenase activity that has been observed in green algae. The *in vivo* rates of H_2 evolution reported here are much lower than rates of H_2 evolution by nitrogenase but are significant in that the observed H_2 evolution indicates a form of cyanobacterial metabolism that has not been previously described. Maximizing *in vivo* hydrogenase activity will depend on gaining information on activating the supporting reductant-generating mechanisms. The use in biophotolysis of hydrogen evolution catalyzed by the hydrogenase of cyanobacteria would also require that the hydrogenase content of the cultures be substantially increased.

Acknowledgements

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[1] E. Kessler, in: Algal Physiology and Biochemistry (W. D. P. Stewart, ed.), pp 456–473, Blackwell Scientific, London 1974.
[2] P. C. Hallenbeck and J. R. Benemann, in: Topics in Photosynthesis, **Vol. 3** (J. Barber, ed.), pp. 332–364, Elsevier, North Holland 1979.
[3] J. R. Benemann and N. M. Weare, Science **184,** 174–175 (1974).
[4] H. Bothe, J. Tennigkeit, G. Eisbrenner, and M. G. Yates, Planta **133,** 237–242 (1977).
[5] A. Daday, R. A. Platz, and G. D. Smith, Appl. Environ. Microbiol. **34,** 478–483 (1977).
[6] G. R. Lambert and G. D. Smith, FEBS Letters **83,** 159–162 (1977).
[7] J. R. Benemann and N. M. Weare, Arch. Microbiol. **101,** 401–408 (1974).
[8] G. Eisbrenner, E. Distler, L. Floener, and H. Bothe, Arch. Microbiol. **118,** 177–184 (1978).
[9] R. B. Peterson and C. P. Wolk, Plant Physiol. **61,** 688–691 (1978).
[10] H. Bothe, E. Distler, and G. Eisbrenner. Biochimie **60,** 277–289 (1978).
[11] E. Tel-Or, L. W. Luijk, and L. Packer, Arch. Biochem. Biophys. **185,** 185–194 (1978).
[12] Y. Fujita, H. Ohama, and A. Hattori, Plant Cell Physiol. **5,** 303–314 (1964).
[13] M. A. Ward, Phytochemistry **9,** 259–266 (1970).
[14] P. C. Hallenbeck and J. R. Benemann, FEBS Letters **94,** 261–264 (1978).
[15] A. Daday, G. R. Lambert, and G. D. Smith, Biochem. J. **177,** 139–144 (1979).
[16] J. C. Weissman and J. R. Benemann, Appl. Environ. Microbiol. **33,** 123–131 (1977).
[17] F. P. Healey, Plant Physiol. **45,** 153–159 (1970).
[18] M. Lex and N. G. Carr, Arch. Microbiol. **101,** 161–167 (1974).
[19] C. P. Wolk and E. Wojcivch, J. Phycol. **7,** 339–344 (1971).