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Occurrence and Localization of Two Distinct Hydrogenases in the Heterocystous Cyanobacterium *Anabaena* sp. Strain 7120

JEFFREY P. HOUCHINS† AND ROBERT H. BURRIS*

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

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Two distinct types of hydrogenase occur in *Anabaena* 7120 and are distinguishable in whole filaments by the application of selective assay methods. A reversible hydrogenase occurs both in heterocysts and vegetative cells and can be selectively assayed by measuring H_2 evolution from reduced methyl viologen. Activities in aerobically grown filaments were low but could be increased by 2 to 3 orders of magnitude by growing cells microaerobically. The presence of the reversible hydrogenase was independent of the N_2-fixing properties of the organism, and activity did not respond to added H_2 in the culture. Illumination was necessary during derepression of the reversible hydrogenase, and addition of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea increased the amount of enzyme that was synthesized. An uptake hydrogenase occurred only in heterocysts of aerobically grown filaments, but a small amount of activity also was present in the vegetative cells of filaments grown microaerobically with 20% H_2. It was assayed selectively by measuring an oxyhydrogen reaction at atmospheric levels of O_2. Additional uptake hydrogenase could be elicited by including H_2 or by removing O_2 from the sparging gas of a culture.

In the first report of hydrogen metabolism in cyanobacteria, Frenkel et al. (9) demonstrated the ability of a species of *Synechococcus* to carry out H_2-supported CO_2 fixation after a period of anaerobic adaptation. Interest in the H_2 metabolism of cyanobacteria has increased recently because of the relationship of H_2 metabolism to N_2 fixation and biological solar energy conversion.

Investigators of hydrogenase in heterocystous cyanobacteria have identified an uptake hydrogenase (3, 6, 12) that is induced by H_2 (21) and can couple to O_2 uptake via a respiratory chain to produce ATP (19) or can provide electrons for acetylene reduction (5). This enzyme has been demonstrated in isolated heterocysts (19), but there is disagreement over its existence in vegetative cells (8, 20, 22). This enzyme works only in the H_2 uptake direction and generally is believed to recycle H_2 evolved from nitrogenase (6, 19, 22).

The demonstration of hydrogenase-catalyzed H_2 evolution from reduced methyl viologen has prompted some workers to propose the existence of a reversible hydrogenase distinct from the uptake hydrogenase (7, 11, 22). This activity also was reported to be induced by H_2 (21). It is present in ammonia-grown filaments that lack heterocysts (7, 14) and has been partially purified (11). In spite of this evidence, the occurrence of two distinct hydrogenases has been questioned (4), and until this problem is resolved, confusion will persist in the interpretation of H_2 metabolism in the heterocystous cyanobacteria.

We have compared properties of the hydrogenases in cell-free extracts and confirmed the existence of two separate enzymes (11a). Here we report methods for distinguishing between these two hydrogenases in whole filaments. These selective assay methods were used to measure the cellular localization of these enzymes and to determine the conditions affecting the occurrence of these enzymes.

MATERIALS AND METHODS

Batch cultures of *Anabaena* sp. strain 7120 (ATCC 27893, *Nostoc muscorum*) were grown in the medium of Allen and Arnon (1) buffered with 4 mM phosphate if N_2-fixing cells were desired. When NH_4^+-grown cells were required, the medium contained 6 mM NH_4Cl and 8 mM phosphate. Three-liter cultures were sparged at 1 liter/min with the gases described in the text. All sparging gases contained 0.5% CO_2. Temperature was maintained at 30°C, and illumination was provided by two 150-W reflector flood lamps with water filters (600 μEinsteins$\cdot m^{-2} \cdot s^{-1}$ on each side).

To separate vegetative cells from heterocysts, freshly harvested filaments were subjected to a mild sonication treatment to break the attachment between

† Present address: Biology Department, Brookhaven National Laboratory, Upton, Long Island, NY 11973.

heterocysts and vegetative cells. The sonication treatment yielded individual heterocysts and short filaments of vegetative cells. The detached cell types were separated from one another on colloidal silica density gradients (18; J. P. Houchins and R. H. Burris).

Uptake of H_2 was measured with an H_2 electrode (12) in the dark at 30°C with an initial H_2 concentration of 20 μM. Unless otherwise noted, the electron acceptor was O_2 at atmospheric levels. Evolution of H_2 was measured in the presence of 5 mM methyl viologen and 10 mM sodium dithionite. The H_2 produced was quantitated either with the H_2 electrode or with a gas chromatographic unit equipped with a thermal conductivity detector.

Chlorophyll (Chl) was extracted into 80% acetone, and its concentration was determined by applying the extinction coefficient at 665 nm reported by Vernon (24).

RESULTS

Evolution of H_2 in the presence of reduced methyl viologen is catalyzed by hydrogenase, and this is clearly demonstrated by the data in Table 1. Unlike nitrogenase-catalyzed H_2 evolution, the activity was inhibited by CO and was not affected by C_2H_2. The same conclusion was reached by others (7, 14). Uptake hydrogenase can catalyze H_2 evolution from reduced methyl viologen at a rate less than 0.2% of its H_2 uptake rate (in preparation). Therefore, H_2 evolution from reduced methyl viologen is a specific assay for reversible hydrogenase since the negligible H_2 production by uptake hydrogenase does not interfere with the measurements. An exception to this conclusion is possible when reversible hydrogenase activity is very low relative to uptake hydrogenase activity. The oxyhydrogen reaction catalyzed by uptake hydrogenase is tolerant of high levels of O_2 (8). Whole filaments lacking uptake hydrogenase but containing reversible hydrogenase also catalyzed uptake of hydrogen, a reaction that functioned only at very low O_2 concentrations. Figure 1 shows the inactivation of reversible hydrogenase by increasing oxygen levels. Rapid inactivation occurred at O_2 concentrations as low as 1 μM. Thus this reaction is a specific assay for uptake hydrogenase at O_2 concentrations above about 1 μM, as higher levels inactivate reversible hydrogenase but not uptake hydrogenase.

Reversible hydrogenase was present but scarce in aerobically grown filaments. Activities from 0.03 to 1 $nmol \cdot h^{-1} \cdot \mu g^{-1}$ of Chl were measured with different batches of aerobically grown cells. Table 1 records the responses of the cell types from aerobically grown filaments that were separated and assayed for reversible hydrogenase. The data demonstrate that activity was present in both cell types, with heterocysts having several-fold more activity than vegetative cells. If filaments were sparged with an anaerobic gas phase for 24 h before harvest, reversible hydrogenase activity increased by about 2 orders of magnitude as shown in Fig. 2 (note that activity is expressed in minutes in Fig. 2 and in hours in Table 1). Table 2 shows that after derepression of reversible hydrogenase, activity was present in the two cell types with roughly equal specific activities.

Although discontinuing illumination resulted

TABLE 1. *Localization of reversible hydrogenase in aerobically grown Anabaena 7120*

Treatment	H_2 evolved ($nmol \cdot h^{-1} \cdot \mu g^{-1}$ of Chl)	
	Heterocysts	Vegetative cells
No additions[a]	1.7	0.42
Minus methyl viologen	0.12	0.04
Plus 30% CO	0.36	0.08
Plus 18% C_2H_2	1.6	0.47

[a] H_2 evolution was measured in sealed serum bottles. The complete assay mixture contained, in 1 ml of liquid: 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 10 mM $Na_2S_2O_4$, 5 mM methyl viologen, and either 34 μg of Chl as isolated heterocysts or 75 μg of Chl as isolated vegetative cells. The gas phase was initially Ar with the indicated additions. A 1-ml gas sample was analyzed for H_2 content after 2 h.

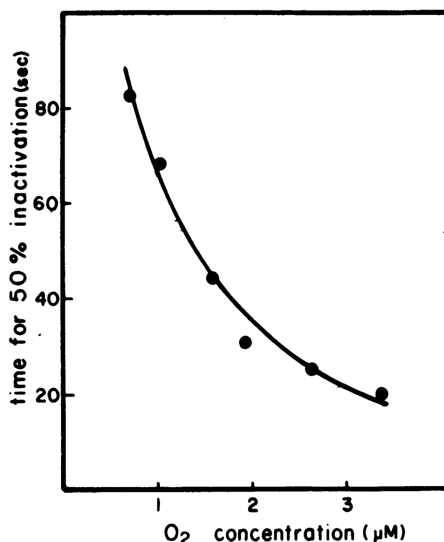

FIG. 1. *O_2 inactivation of reversible hydrogenase in whole filaments of Anabaena 7120. Cells were grown on NH_4^+ and derepressed for reversible hydrogenase. Hydrogenase activity was followed with the H_2 electrode by measuring the rate of H_2 uptake coupled to 1 mM benzoquinone. O_2-saturated buffer was added in varying amounts, and the time required for 50% inactivation of the initial activity was determined.*

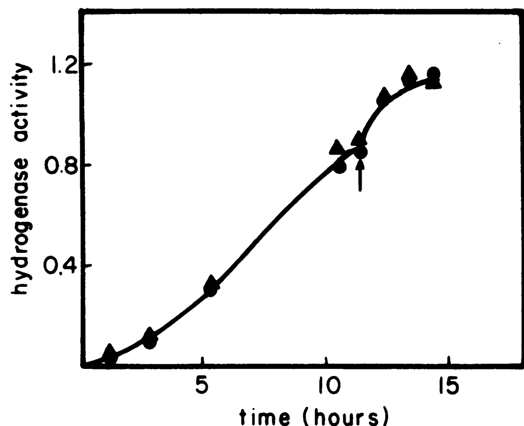

FIG. 2. *Effect of H_2 on reversible hydrogenase activity during derepression. At time zero a 3-liter NH_4^+-grown culture was divided into two equal parts. Illumination (600 μEinsteins·m^{-2}·s^{-1}) was continued; one culture was sparged with N_2:CO_2 (●) (99.5:0.5) and the other was sparged with N_2-H_2-CO_2 (▲) (96.5:3.0:0.5) at a rate of 0.5 liter/min. Samples were taken periodically, and hydrogenase activity was measured. Illumination was discontinued at the arrow. Activity is expressed in nanomoles of H_2 evolved per minute per microgram of Chl.*

TABLE 2. *Localization of reversible hydrogenase in derepressed cells*[a]

Cell type	Hydrogenase activity (nmol of H_2 evolved·h^{-1}·μg^{-1} of Chl)
Whole filaments	45
Vegetative cells	37
Heterocysts	27

[a] N_2-fixing cells were sparged with N_2-CO_2 (99.5:0.5) during growth. Cell types were assayed for H_2 evolution with an H_2 electrode.

in a distinct increase of reversible hydrogenase activity in derepressed *Anabaena* as shown in Fig. 2, illumination was necessary during derepression (Fig. 3). A culture which was sparged anaerobically in the dark developed only a small amount of activity, and the activity plateaued after 10 h. Illumination appears to be necessary to provide a continued source of energy during derepression. If 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea was included in a culture during derepression, O_2 production was prevented, and the amount of reversible hydrogenase activity expressed was enhanced in response to the reduced O_2 concentration in the culture (Fig. 3).

Table 3 gives a comparison of the localization of uptake hydrogenase in aerobically grown cell types with cells grown microaerobically in the presence of 20% H_2. Because induction of an oxyhydrogen reaction has been reported in a number of cyanobacteria grown under an anaerobic gas phase containing H_2 (8), these cells were examined to determine whether this procedure induced uptake hydrogenase in vegetative cells. Activity with O_2 and some artificial acceptors was measured. In isolated heterocysts from aerobically grown filaments, 2,6-dichlorophenol-indophenol reacted rapidly with uptake hydrogenase, methylene blue reacted slowly, and benzyl viologen was unreactive. Uptake hydrogenase was confined entirely to heterocysts in aerobically grown filaments. In microaerobically grown cells the relatively high reactivity of whole filaments and isolated vegetative cells with methylene blue was due to derepression of the reversible hydrogenase. This was demonstrated more clearly by the ability of these cells to take up H_2 in the presence of benzyl viologen, which does not react with uptake hydrogenase.

The oxyhydrogen reaction, which was measured at O_2 levels high enough to inactivate reversible hydrogenase, showed that vegetative cells had a specific activity about 1% that of heterocysts. Since a microscopic cell count revealed that only 0.2% of the cells in this vegetative cell preparation were contaminating heterocysts, it appeared that a small amount of uptake hydrogenase was induced in vegetative cells

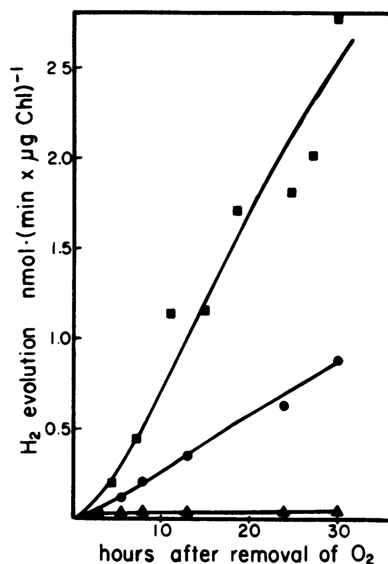

FIG. 3. *Effect of illumination and 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) on reversible hydrogenase derepression. In 1-liter batch cultures the sparging gas was changed at time zero from air-CO_2 to N_2-CO_2. Illumination, when present, was provided by one 150-W reflector flood lamp (600 μEinsteins·m^{-2}·s^{-1}). DCMU, when present, was at a concentration of 15 μM. Samples were taken periodically and assayed for reversible hydrogenase. (▲) Dark; (●) illuminated; (■) illuminated plus 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.*

TABLE 3. *Localization of H_2 uptake in isolated cell types of Anabaena 7120*[a]

Growth	Cell type	H_2 uptake ($nmol \cdot min^{-1} \cdot \mu g^{-1}$ of Chl)			
		O_2[b]	1 mM DCPIP[c]	1 mM methylene blue	1 mM benzyl viologen
Aerobic	Whole filaments	0.30	0.12	0.056	—[d]
	Isolated heterocysts	2.6	2.0	0.36	<0.005
	Isolated vegetative cells	0.0036	<0.002	<0.002	<0.002
Microaerobic + 20% H_2	Whole filaments	0.34	0.33	0.25	0.048
	Isolated heterocysts	1.6	1.0	0.28	0.047
	Isolated vegetative cells	0.017	0.069	0.22	0.21

[a] N_2-fixing cells were sparged during growth either with air-CO_2 (99.5:0.5) or with N_2-H_2-CO_2 (79.5:20:0.5). All H_2 uptake assays were conducted with an H_2 electrode in 50 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 6.8) with the indicated electron acceptor.

[b] The oxyhydrogen reaction was measured at the optimal O_2 concentration for each cell preparation. Filaments were assayed at 250 μM O_2, and isolated vegetative cells and heterocysts were assayed at 70 μM O_2.

[c] DCPIP, 2,6-Dichlorophenol-indophenol.

[d] —, Not tested.

grown microaerobically in the presence of H_2.

The nitrogen source for cell growth has been reported to influence hydrogenase activity in heterocystous cyanobacteria (7, 8, 20). In agreement with others, reversible hydrogenase was derepressed in cells grown on N_2, NO_3^-, or NH_4^+, and the nitrogen source had little effect on the ultimate activity achieved (7, 14). On the other hand, NH_4^+-grown cells had no measurable uptake hydrogenase activity whether grown aerobically or microaerobically plus H_2.

Figure 4 illustrates the effect of H_2 and microaerobic conditions on the levels of activity of the two hydrogenases in a culture of *Anabaena* 7120. Addition of H_2 to the gas phase of an aerobically grown culture caused a small increase in the level of uptake hydrogenase. The reversible hydrogenase did not respond to added H_2 in aerobically grown cells. Figure 2 shows that inclusion of H_2 in the sparging gas during derepression of reversible hydrogenase had no effect on the level of this enzyme. If the sparging gas was made anaerobic by replacing air with N_2, an increase occurred in the levels of both enzymes. This effect was most dramatic for reversible hydrogenase, although oxyhydrogen reaction activity also increased by about 30%.

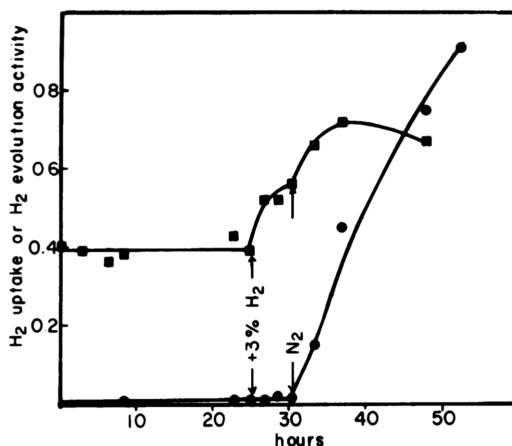

FIG. 4. *Effect of H_2 and microaerobic conditions on the levels of hydrogenase in Anabaena 7120. A 1-liter N_2-fixing culture was continuously sparged at 500 ml/min during growth. The sparging gas was initially air-CO_2 (99.5:0.5). At the first arrow, 3% H_2 was added to the sparging gas. At the second arrow, air was replaced with N_2. Samples were taken periodically, and the oxyhydrogen reaction (■) and H_2 evolution from reduced methyl viologen (●) were measured. Hydrogenase activity is expressed in nanomoles per minute per microgram of Chl.*

DISCUSSION

Daday et al. (7) demonstrated that H_2 evolution from reduced methyl viologen was catalyzed exclusively by hydrogenase and that nitrogenase did not contribute to the H_2 evolution observed in their assay. In this report we have demonstrated that H_2 evolution in the presence of reduced methyl viologen is catalyzed by reversible hydrogenase and that uptake hydrogenase does not contribute appreciably to H_2 evolution. The potent inhibition by O_2 of reversible hydrogenase (Fig. 1) is in sharp contrast to the relative stability of uptake hydrogenase to O_2 (8). Thus, uptake hydrogenase may be specifically assayed at O_2 concentrations that inhibit reversible hydrogenase.

The cellular localization of hydrogenase in heterocystous cyanobacteria has been controversial largely because of the failure of investigators to distinguish between the two hydrogenases. Tel-Or et al. (22) first produced evidence that H_2 uptake and H_2 evolution may be cata-

lyzed by different enzymes when they found that heterocysts had 5 to 10 times as much H_2 uptake activity as vegetative cells, but that H_2 evolution was distributed almost equally between the two cell types. Eisbrenner et al. (8) assumed that H_2 evolution and the oxyhydrogen reaction were catalyzed by the same enzyme, and they measured H_2 evolution from reduced methyl viologen as an assay for total hydrogenase; based on an incorrect assumption, they concluded that hydrogenase is found equally in both cell types. Peterson and Wolk (20) assayed hydrogenase in crude extracts by measuring H_2 uptake coupled to ferricyanide. They concluded that hydrogenase was confined to heterocysts in aerobically grown filaments but that up to 21% of the activity of microaerobically grown filaments was present in vegetative cells. Because both hydrogenases are detected by the assay employed, the vegetative cell activity of microaerobically grown filaments probably was attributable to derepression of reversible hydrogenase. Peschek (16, 17) reported that *Anacystis nidulans* has two distinct particulate hydrogenases, and Tetley and Bishop (23) found both photosynthetic and respiratory H_2 uptake in *Anabaena* 7120. We found that reversible hydrogenase resided about equally in the two cell types. However, uptake hydrogenase was confined to heterocysts in aerobically grown filaments, and slight activity was detectable in the vegetative cells of filaments grown microaerobically in the presence of H_2. The fact that both nitrogenase and uptake hydrogenase are confined to heterocysts in aerobically grown filaments provides additional support for the suggestion that this enzyme functions specifically to recapture H_2 evolved from nitrogenase (6, 19, 22).

The influence of combined nitrogen on the two hydrogenases is understandable in terms of their cellular localizations. Ammonia-grown cultures lack heterocysts and also have greatly decreased activity in the oxyhydrogen reaction. Heterocyst-free cultures possess H_2 evolution activity, however, because the reversible-hydrogenase activity is found in vegetative cells as well as in heterocysts.

Cyanobacteria differ greatly in the conditions required to elicit their reversible hydrogenases. Activity appears to be constitutive in some organisms (2), whereas in others activity is partially (10, 14) or entirely (9, 13) dependent on a dark anaerobic adaptation period. We found that the appearance of reversible hydrogenase was dependent on reduced levels of O_2 in a culture, although illumination (producing O_2 photosynthetically) was necessary during derepression. Little activity developed during a dark, anaerobic incubation. 3-(3',4'-Dichlorophenyl)-1,1-dimethylurea, which eliminates endogenous O_2 evolution in the light, further enhanced hydrogenase activity in the light. Tel-Or et al. reported that reversible hydrogenase activity was induced by growth under an anaerobic gas phase containing H_2 (21). We found that the increase of activity was due to reduced levels of O_2 and that H_2 has no influence on the amount of reversible hydrogenase activity expressed.

Uptake hydrogenase activity responded to the addition of H_2 within a few hours, in agreement with previous reports (8, 21). The observed increase in activity after removal of O_2 from the culture may also have reflected a response to H_2, because nitrogenase activity, and thus endogenous H_2 production, increases at reduced levels of O_2 (15).

Uptake hydrogenase and reversible hydrogenase differ in their cellular localizations and in their responses to O_2, H_2, and combined nitrogen. A thorough understanding of these differences should aid in the study of the functions and capabilities of these enzymes.

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