Physiological Reactions of the Reversible Hydrogenase from
Anabaena 7120

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ABSTRACT

The reversible hydrogenase from Anabaena 7120 appeared when O2 was continuously removed from a growing culture. Activity increased further when cells were incubated under argon in the dark or in the light plus 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Hydrogenase existed in an inactive state during periods of O2 evolution. It could be reductively activated by exposure to reduced methyl viologen or by dark, anaerobic incubation. Hydrogenase-containing cells evolved H2 slowly during dark anaerobic incubations, and the rate of H2 evolution was increased by illumination with low intensity light. Light enhancement of H2 evolution was of short duration and was eliminated by the ferredoxin antagonist disalcyclidene diaminopropane. Physiological acceptors that supported H2 uptake included NO3-, NO2-, and HSO3-, and light had a slight influence on the rate of H2 uptake with these acceptors. Low levels of O2 supported H2 uptake, but higher concentrations of O2 inactivated the hydrogenase. Hydrogen uptake with HCO3- as acceptor was the most rapid reaction measured, and it was strictly light-dependent. It occurred only at low light intensities, and higher light intensities restored normal O2-evolving photosynthesis. It is suggested that hydrogenase is present to capture exogenous H2 as a source of reducing equivalents during growth in anaerobic environments.

A number of eucaryotic algae possess hydrogenase activity that can be induced by a period of dark, anaerobic adaptation (13). Once activated, the hydrogenase catalyzes both the production and consumption of H2. The function of hydrogenase in eucaryotic algae, however, is still uncertain. Blue-green algae also are capable of metabolizing H2 as first demonstrated by Frenkel et al. (6). They found that Synechococcus and Synechocystis could photoreductively evolve CO2 in a hydrogen-dependent reaction.

Heterocystous blue-green algae have two distinct hydrogenases (12). A particulate hydrogenase is confined to heterocysts and acts to recycle H2 evolved from nitrogenase (4, 21, 25). This enzyme works only in the uptake direction and donates electrons either to the respiratory or the photosynthetic electron transport chain (5). A soluble hydrogenase, which can be specifically assayed by measuring H2 evolution from reduced methyl viologen, is present in both heterocysts and vegetative cells (in preparation). Little is known about possible functions for this enzyme. This report examines the physiological reactions catalyzed by reversible hydrogenase in whole filaments of Anabaena 7120.

MATERIALS AND METHODS

Organism and Growth Conditions. Anabaena 7120 (A.T.C.C. 27893, Nostoc muscorum) was grown photoautotrophically in 3-liter batches illuminated with two 150-w reflector flood lamps (200 w-m-2). The medium of Allen and Arnon (1) was supplemented with 6 mm NH4Cl and was buffered with 8 mm phosphate. Under these conditions, heterocyst differentiation did not occur, and uptake hydrogenase and nitrogenase activities were absent (12). Cultures were continuously sparged with air/CO2 (99.5:0.5) at a rate of 1 liter min-1 and were harvested at cell densities of 3 to 5 µg Chl-m1. To achieve derepression of hydrogenase, the sparging gas was changed to N2/CO2 (99.5:0.5) for 24 h before harvesting the cells.

Manometric Measurements of H2 Exchange. Unless otherwise noted, H2 uptake and H2 evolution were measured manometrically in Gilson submarine volumeters in an illuminated bath at 30 C. Twenty-m1 reaction flasks contained 3 ml concentrated cell suspension in growth medium in the main chamber (pH 7.8). A CO2 trap (20% KOH on a filter paper wick in the center well) was used in all assays except when HCO3- acted as the electron acceptor. H2 uptake was measured under 100% H2, and the gas phase for H2 evolution was initially 100% Ar.

Chl Determinations. Chlorophyll was extracted into 80% acetone and the concentration was determined by applying the extinction coefficient at 665 nm reported by Vernon (26).

RESULTS

We have previously demonstrated that little reversible hydrogenase activity is present in aerobically grown Anabaena 7120, but that activity increases greatly if the O2 concentration is reduced during periods of illumination (12). Figure 1 shows the effect of light and dark anaerobic incubations on the levels of reversible hydrogenase activity in aerobically grown cells and in derepressed cells. Aerobically grown cells had no detectable reversible hydrogenase activity initially, and a dark, anaerobic incubation of the type used to adapt several species of eucaryotic and procaroctyotic algae (14) produced only a small amount of activity. However, a considerable amount of hydrogenase activity developed when aerobically grown cells were incubated in the light in the presence of the PSI1 inhibitor DCMU. Illumination is probably necessary to provide a continuous source of energy for synthesis of hydrogenase. A dark anaerobic incubation resulted in significant activity by about 2-fold in cells previously derepressed for hydrogenase, and illumination again produced additional activity.

We have shown that reversible hydrogenase is an O2 stable enzyme, but that after exposure to O2 it exists in a reversibly inactive state and can be reactivated within 1 min by exposure to
dithionite (12). When H₂ evolution from reduced methyl viologen is measured, as in Figure 1, the inactive form of the enzyme is reactivated by the excess reductant in the assay mixture after a brief lag. Therefore, this method cannot be used to determine the state of activation of the enzyme in vivo. If H₂ uptake is used as a measure of hydrogenase activity, then no reductive activation occurs during the assay, and only the enzyme active at the beginning of the assay will contribute to the reaction. Figure 2 shows that in lieu of exogenously added dithionite, endogenous reductant can reactivation the reversible hydrogenase in derepressed cells that had been exposed to O₂. Although hydrogenase activity could be followed during the 24-h microaerobic derepression period by measuring H₂ evolution from reduced methyl viologen, the H₂ uptake assay revealed that the enzyme was kept in the inactive form by the low level of photosynthetically produced O₂ in the culture.

Like the uptake hydrogenase from this organism, reversible hydrogenase will catalyze an oxyhydrogen reaction but only at much lower O₂ concentrations. We find the inactivation of reversible hydrogenase occurs at O₂ concentrations above 1 μM (12). Figure 3 shows data obtained from a combination H₂-O₂ electrode (13). When several small portions of O₂-saturated water were added to a suspension of cells to maintain the O₂ concentration at a low level, H₂ was taken up. However, when an equivalent amount of O₂ was added at one time, the O₂ concentration rose to an inhibitory level and inactivated the hydrogenase.

The ability of a number of physiological acceptors to support H₂ uptake is recorded in Figure 4. Under an atmosphere of 100% H₂, H₂ uptake proceeded slowly in the dark in the absence of any exogenous electron acceptor. The dark rate of H₂ uptake was enhanced by NO₂⁻, NO₃⁻, or HSO₄⁻, all of which are reduced in ATP-independent, ferredoxin-linked reactions (18, 23). When light was provided at a low intensity so that O₂ evolution was not appreciable, the rates of H₂ uptake were enhanced. Only HCO₃⁻-supported H₂ uptake was strictly dependent upon illumination. This reaction has been termed photoreduction and occurs in a number of procaroytic and eucaryotic algae (14). In the absence
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Fig. 4. H₂ uptake in the presence of HCO₃⁻, NO₃⁻, NO₂⁻, and HSO₃⁻. Concentrated suspensions of derepressed Anabaena 7120 cells were assayed manometrically under 100% H₂ (see "Materials and Methods") for H₂ uptake. Acceptors were added at the first arrow and illumination of 5 w·m⁻² was provided at the second arrow. Acceptors and other additions were as follows: ( ), no acceptor; ( ), no acceptor plus 20 μM CCCP; ( ), 10 mM HCO₃⁻; ( ), 20 mM NO₃⁻; ( ), 2 mM NO₂⁻; ( ) 1 mM HSO₃⁻. Results are averages of six experiments. Numbers in parentheses are rates of H₂ uptake in nmol H₂ (h·μg Chl)⁻¹.

Fig. 5. Effect of light intensity on the rate of photoreduction by Anabaena 7120. O₂ evolution and H₂ uptake were measured manometrically; HCO₃⁻ was the acceptor. Concentrated suspensions of algae contained 140 μg Chl·ml⁻¹. Tungsten lamps were used, and intensities are given in w·m⁻².

Table I. Effect of Inhibitors on Rate of Photoreduction of HCO₃⁻ by Anabaena 7120

<table>
<thead>
<tr>
<th>Inhibitor and Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>DCMU, 15 μM</td>
<td>0</td>
</tr>
<tr>
<td>CCCP, 20 μM</td>
<td>100</td>
</tr>
<tr>
<td>DSPD, 0.5 mM</td>
<td>15</td>
</tr>
<tr>
<td>Antimycin A, 50 μM</td>
<td>5</td>
</tr>
</tbody>
</table>

Table II. Rates of Hydrogen Uptake in Whole Filaments of Anabaena 7120 in the Presence of Artificial Electron Acceptors

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Eν</th>
<th>H₂ Uptake Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ν</td>
<td>mmol μg⁻¹ Chl⁻¹</td>
</tr>
<tr>
<td>Naphthoquinone, 10 mM</td>
<td>0.143</td>
<td>1.3</td>
</tr>
<tr>
<td>Benzoquinone, 10 mM</td>
<td>0.293</td>
<td>2.7</td>
</tr>
<tr>
<td>Methylene blue, 4 mM</td>
<td>0.011</td>
<td>3.3</td>
</tr>
<tr>
<td>Benzyl viologen, 5 mM</td>
<td>-0.360</td>
<td>0.42</td>
</tr>
<tr>
<td>DCPIP, 3 mM</td>
<td>0.217</td>
<td>0.0</td>
</tr>
<tr>
<td>Methyl viologen, 3 mM</td>
<td>-0.440</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The abilities of a number of artificial acceptors to support H₂ uptake in whole filaments of Anabaena 7120 are recorded in Table II. Rates of H₂ uptake with these acceptors were unaffected by illumination at low light intensities.

When hydrogenase-containing cells were incubated under 100% Ar, H₂ was evolved slowly in the dark as shown in Figure 6. If

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of an exogenous acceptor, the uncoupler CCCP³ prevented light enhancement of H₂ uptake, thus demonstrating that light enhances H₂ uptake by providing energy to an ATP-dependent reductive process. The probable electron acceptor for this reaction is residual HCO₃⁻ that has not been removed by the KOH trap in the center well of the flask. Anabaena reportedly has the ability to accumulate HCO₃⁻ actively against a gradient (2). If the light-enhanced H₂ uptake rates in the presence of NO₃⁻, NO₂⁻, and HSO₃⁻ are corrected for the light enhancement occurring in the control reaction, it becomes apparent that illumination exerts only a slight effect on the rates of H₂ uptake with NO₃⁻, NO₂⁻, and HSO₃⁻ as acceptors. Addition of the uncoupler CCCP prevented any light enhancement of the rates of H₂ uptake in the presence of these acceptors.

With increasing light intensity, the rate of photoreduction of HCO₃⁻ increased until the light intensity was great enough to support net O₂ evolution. At this point, hydrogenase was inactivated by O₂ and H₂ uptake ceased. The response of the H₂ uptake rate to light intensity is shown in Figure 5. A number of photosynthetic inhibitors were tested to determine their effect on photoreduction and the results are shown in Table I. The reaction was completely inhibited by the uncoupler CCCP. The absence of an effect with DCMU indicates that PSI was not involved. The ferredoxin antagonist DSPD and antimycin A, which blocks cyclic photophosphorylation in chloroplasts (24), had little effect on photoreduction of HCO₃⁻.

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When hydrogenase-containing cells were incubated under 100% Ar, H₂ was evolved slowly in the dark as shown in Figure 6. If

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³ Abbreviations: CCCP, carbonyl cyanide m-chlorophenyl hydrazone; DSPD, diallylidenem-1,3-diaminopropane; DMSO, dimethyl sulfoxide; Pipes, piperoxide-N,N'-bis-2-ethane sulfonic acid; DCPIP, 2,6-dichlorophenol indophenol.
Anabaena of shortations recorderpendent H2 uptake by Anabaena 7120 in growth medium containing 69 μg Chl·ml−1 was placed in the hydrogen electrode chamber, and H2 evolution (upward on strip chart recorder traces shown) and uptake was followed continuously. Illumination, when present, was 0.8 W·m−2; †, light on; ‖, light off. Inhibitors were initially absent and were added where indicated. The inhibitor concentrations were as in Table 1.

determined that PSII was not involved. The inhibition of H2 uptake in the light by CCCP demonstrates that H2 uptake is an ATP-dependent reaction. Endogenously produced CO2 was probably acting as the electron acceptor for this reaction. If cells were incubated in the presence of DSPD, H2 evolution in the dark was only slightly inhibited, but the light-dependent burst was completely eliminated. DSPD did not affect the subsequent light-dependent H2 uptake. When light-dependent H2 uptake was eliminated by the addition of CCCP, photoproduction of H2 could be followed for longer times. The light enhancement of H2 evolution was of short duration, and the rate quickly returned to its dark level (Fig. 6A). Sustained photoproduction of H2 of the type reported in eucaryotic algae (14) apparently does not occur in Anabaena 7120 even in the presence of an uncoupler. Hallenbeck et al. (11) recently reported slow hydrogenase-catalyzed H2 evolution under similar conditions, and they found that light inhibited H2 evolution. Figure 6 suggests that this inhibition is due to diversion of electrons to an ATP-dependent reductive process.

A number of sugars and organic acids enhance dark H2 evolution in eucaryotic algae (14). In Anabaena 7120, H2 evolution in the dark was not enhanced by addition of glucose, fructose, pyruvate, lactate, or succinate.

Several attempts were made to demonstrate H2-supported photoautotrophic or chemooautotrophic cell growth in Anabaena, but all attempts were unsuccessful. DCMU-treated cells did not grow in the light under an anaerobic gas phase containing H2 plus CO2. Hydrogenase-containing cells also failed to grow in the dark under an anaerobic gas phase of H2 plus CO2 in the presence of any of the following electron acceptors: NO3−, NO2−, fumarate, SO42−, Fe3+, DMSO, S2O32−, or a low level of O2.

**DISCUSSION**

A number of aerobically grown blue-green algae contain reversible hydrogenase even before an anaerobic adaptation period (3, 8, 17). Aerobically grown Anabaena 7120 contains very little reversible hydrogenase activity, and dark, anaerobic adaptation under Ar increases activity only slightly. Significant activity was obtained only when cells were illuminated and the level of O2 was decreased either by continuously removing O2 from a growing culture or by illuminating under Ar in the presence of DCMU. After hydrogenase had been derepressed, the activity increased 2-fold during a dark, anaerobic incubation. Similar results have been observed with Anabaena cylindrica (16). It has been suggested that during anaerobic incubation in the dark, there is a reductive activation of an inactive form of hydrogenase in eucaryotic algae. If this is responsible for the additional activity observed in Figure 1, it must be different from the reductive activation caused by dithionite (12), because the additional activity elicited by anaerobic incubation in the dark is not detectable initially even in the presence of methyl viologen and dithionite.

Exogenously provided sugars and organic acids enhance H2 evolution in eucaryotic algae (14). Gogotov and Kosyak (8) reported that dark H2 evolution in Anabaena variabilis is dependent upon the addition of formate, pyruvate, or glucose. Anabaena 7120, however, is an obligate autotroph (22) and probably is incapable of using exogenous substrates to enhance H2 evolution. Although photoproduction of H2 in Anabaena 7120 (Fig. 6), light enhancement was of short duration and rate increases were not sustained suggesting that only a small pool of electrons is available for light-enhanced H2 evolution. In contrast, photoproduction of H2 by A. variabilis continues for several hours (9).

Anabaena hydrogenase does not react with ferredoxin in vitro (10), nor does ferredoxin appear to mediate hydrogenase reactions in vivo, as the antagonist DSPD inhibited H2 evolution in the dark or photoreduction of H2 only slightly. However, DSPD eliminated the light-dependent burst of H2 evolution, suggesting that this burst is mediated by electrons passing from PSI through the ferredoxin pool, and that these electrons then are contributed to the pool of redundant supporting H2 evolution.

Light enhancement of H2 uptake occurred only in ATP-dependent reactions. No enhancement was observed with ATP-independent physiological acceptors or with artificial acceptors. The uptake hydrogenases of Anacystis nidulans (20) and Anabaena 7120 (12) are incapable of providing electrons directly for biosynthetic reductions. Additional energy input from PSI is required before low potential carriers can be reduced by these uptake hydrogenases. Reversible hydrogenase, however, appears to contribute electrons directly for reductive reactions without the mediation of PSI.

Reversible hydrogenase has a $K_a$ for H2 of 2.3 μM (12), and this high affinity suggests that its physiological function is to consume rather than to evolve H2. Because exogenous H2 is present only in anaerobic or microaerobic environments, hydrogenase would be expected to operate only under conditions of decreased O2 concentration. Reversible hydrogenase is synthesized in response to decreased O2 in the environment (12), and strictly anaerobic conditions are required before the enzyme is activated. The extreme O2 sensitivity of active hydrogenase (inactivation at 1 μM O2) indicates that the enzyme functions only in anaerobic environments. Several blue-green algae have the ability to use alternative electron donors under anaerobic conditions (7, 19).

In eucaryotic algae incubated under dark, anaerobic conditions, O2 evolution is inhibited upon illumination, especially at low light intensities. If algae contain hydrogenase, however, photosynthesis starts immediately upon illumination. Therefore, Kessler has proposed (15) that hydrogenase functions to evolve H2 upon illumination, and thereby achieve “an oxidative activation of their...
photosynthetic electron transport system in the absence of molecular oxygen." We measured lag times for O2 evolution in Anabaena 7120 under a wide range of light intensities (data not shown) and found no difference in lag times for Anabaena before and after derepression of hydrogenase. This proposed function probably does not apply to Anabaena 7120.

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LITERATURE CITED