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Comparative Characterization of Two Distinct Hydrogenases from *Anabaena* sp. Strain 7120

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Two distinct hydrogenases, hereafter referred to as "uptake" and "reversible" hydrogenase, were extracted from *Anabaena* sp. strain 7120 and partially purified. The properties of the two enzymes were compared in cell-free extracts. Uptake hydrogenase was largely particulate, and although membrane bound, it could catalyze an oxyhydrogen reaction. Particulate and solubilized uptake hydrogenase could catalyze H_2 uptake with a variety of artificial electron acceptors which had midpoint potentials above 0 mV. Reversible hydrogenase was soluble, could donate electrons rapidly to electron acceptors of both positive and negative midpoint potential, and could evolve H_2 rapidly when provided with reduced methyl viologen. Uptake hydrogenase was irreversibly inactivated by O_2, whereas reversible hydrogenase was reversibly inactivated and could be reactivated by exposure to dithionite or H_2. Reversible hydrogenase was stable to heating at 70°C, but uptake hydrogenase was inactivated with a half-life of 12 min at this temperature. Uptake hydrogenase was eluted from Sephadex G-200 in a single peak of molecular weight 56,000, whereas reversible hydrogenase was eluted in two peaks with molecular weights of 165,000 and 113,000. CO was competitive with H_2 for each enzyme; the K_i's for CO were 0.0095 atm for reversible hydrogenase and 0.039 atm for uptake hydrogenase. The pH optima for H_2 evolution and H_2 uptake by reversible hydrogenase were 6 and 9, respectively. Uptake hydrogenase existed in two forms with pH optima of 6 and 8.5. Both enzymes had very low K_m's for H_2, and neither was inhibited by C_2H_2.

Heterocystous cyanobacteria are reported to contain three separate enzymes capable of metabolizing H_2 (13). Nitrogenase evolves H_2 unidirectionally in an ATP-dependent reaction. An uptake hydrogenase has been identified (3) that can deliver electrons to O_2 with the electron transfer coupled to ATP synthesis (21) or that can donate electrons to nitrogenase through photosystem 1 (5, 22). Tel-Or et al. (25) examined the cellular localization of H_2 uptake and H_2 evolution in *Anabaena cylindrica* and *Nostoc muscorum*, and their work suggests that uptake and evolution may be catalyzed by different enzymes. Recently Hallenbeck and Benemann (13) reported the partial purification and characterization of a reversible hydrogenase from *A. cylindrica*. Nonetheless, suggestions persist that only 1 hydrogenase exists in *Anabaena* and that the H_2-evolving capability may be a non-physiological artifact of cell disruption (4, 10).

The following report compares the properties of the uptake hydrogenase with those of the reversible hydrogenase in cell-free preparations from *Anabaena* sp. strain 7120 in an effort to distinguish between these two enzymes.

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MATERIALS AND METHODS

Organism and growth conditions. *Anabaena* sp. strain 7120 (ATCC 27893, *Nostoc muscorum*) was grown photoautotrophically in 80-liter batches in modified Allen and Arnon medium (1) with continuous sparging with air at a rate of 10 liters/min. The sparging gas was supplemented with 0.5% CO_2. The algae were harvested with a Sharples centrifuge and stored in liquid N_2 until used. If uptake hydrogenase was desired, the cells were grown in the absence of combined nitrogen, and the medium contained 4 mM phosphate. If reversible hydrogenase was desired, the cells were grown on 6 mM NH_4Cl, and the medium was buffered with 8 mM phosphate. Ammonia represses heterocyst formation and the associated activities of nitrogenase and uptake hydrogenase that otherwise may complicate experimental interpretation (8, 10). To derepress the reversible hydrogenase the sparging gas was changed to N_2-CO_2 (99.5:0.5) for 24 h before harvest.

Reversible hydrogenase preparation. To prepare partially purified reversible hydrogenase, 50 g of frozen NH_4^+-grown cell paste was thawed in 150 ml of

HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (40 mM, pH 7.5) and sonicated at full power for 10 min (Heat Systems-Ultrasonics Inc., standard probe, 230 W power input; 30 min of pulsed sonication on 33% duty cycle). The extract from broken cells was centrifuged for 1 h at 16,000 × *g*, and acetone (precooled to −20°C) was added slowly with stirring to the supernatant to a final concentration of 33% (vol/vol). The acetone-treated supernatant was centrifuged at 30,000 × g for 30 min. This supernatant was applied to a column (4.5 by 14 cm) of DEAE-cellulose (Whatman DE52) which had been preequilibrated with 0.1 M KCl in 20 mM Tris, adjusted to pH 7.4 with HCl. The column was washed with 0.1 M KCl in Tris, and then hydrogenase was eluted with a gradient of 0.1 to 0.3 M KCl in Tris. Hydrogenase was eluted by approximately 0.22 M KCl. Active fractions were pooled, precipitated by adding solid $(NH_4)_2SO_4$ to a concentration of 70% saturation at 0°C, and suspended in HEPES buffer. At this stage reversible hydrogenase was purified about 10-fold and had a specific activity of approximately 300 nmol of H_2 evolved per mg of protein per min. Preparations were stored at −20°C and retained activity for several months.

Heterocyst isolation and preparation of uptake hydrogenase. All steps in the preparation of uptake hydrogenase were conducted under an anaerobic gas phase, and 1 mM $Na_2S_2O_4$ was included in all steps after the initial heterocyst isolation. Fifty grams of cell paste from N_2-fixing cells was combined with 50 ml of Tris-EDTA buffer (20 mM Tris, 20 mM EDTA, pH 7.5) and 200 mg of lysozyme. The suspension was incubated at 30°C for 1 h and then was sonicated in 30-ml batches (90-W power input for 1.5 min, 3 min on 50% duty cycle) to break vegetative cells. The remaining intact heterocysts were washed by centrifuging three times for 5 min at 150 × *g* in HEPES buffer. They finally were suspended in 30 ml of HEPES containing 1 mM $Na_2S_2O_4$. To obtain particulate cell-free preparations of uptake hydrogenase, the heterocyst suspension was passed twice through a French press at 20,000 lb/in^2. The broken-cell preparation then was centrifuged at 40,000 × *g* for 1 h, and the pellet was suspended in HEPES buffer plus 1 mM $Na_2S_2O_4$. Soluble preparations were obtained by sonicating 30 ml of isolated heterocysts at full power. A sonication time of 22 sec/ml of heterocysts broke most of the heterocysts and partially solubilized the hydrogenase. Continuing sonication for 40 s/ml almost completely solubilized the uptake hydrogenase. The sonicated extract was centrifuged at 40,000 × *g* for 1 h, and this supernatant was used as soluble uptake hydrogenase.

Hydrogenase assays. Unless otherwise noted, the reversible hydrogenase was assayed by measuring H_2 evolution in 50 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)] (pH 6.8) containing 5 mM methyl viologen plus 10 mM $Na_2S_2O_4$. The H_2 produced was analyzed either in an H_2-O_2 electrode (15) or on a gas chromatograph equipped with a thermal conductivity detector. Unless otherwise noted, H_2 uptake was measured by following H_2 consumption with the H_2 electrode in PIPES plus 20 μM H_2 with 1 mM methylene blue as electron acceptor.

pH profiles. pH profiles were measured in a buffer system containing 30 mM glutamate, 30 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), and 30 mM *N*-2-hydroxyethylpiperazine-*N'*-propanesulfonic acid (HEPPS) titrated to the proper pH between 4 and 10. All assays were conducted with the H_2 electrode as described. The true pH was measured after addition of all reactants.

O_2 inactivation. To measure irreversible inactivation by O_2, 1-ml active samples of uptake and reversible hydrogenase, previously stored under Ar in the presence of 1 mM $Na_2S_2O_4$, were placed in 6-ml serum bottles and exposed to the atmosphere. Samples were continuously shaken at 150 strokes per min at 30°C and were periodically assayed for activity. Reversible hydrogenase was injected into a H_2 evolution assay mixture, and H_2 evolution was measured. Uptake hydrogenase was injected into the H_2 electrode chamber containing buffer plus 0.2 mM $Na_2S_2O_4$. After a 30-s preexposure to dithionite, 1 mM methylene blue was added, and H_2 uptake was measured.

Carbon monoxide inhibition. Carbon monoxide inhibition was measured in stoppered cuvettes containing 2 ml of PIPES buffer (pH 6.8) plus 100 μM methylene blue. The K_m for methylene blue for each enzyme is below 10 μM. The head space of each cuvette was approximately 13 ml and contained the desired gas phase. H_2 was present at from 2 to 4%, CO was present at from 0 to 6% for reversible hydrogenase and from 0 to 40% for uptake hydrogenase, and the balance was Ar. After addition of enzyme, methylene blue reduction was followed at 600 nm, and it proceeded until all H_2 in solution was consumed. Velocity at several different H_2 concentrations was determined by drawing tangents to the progress curve. Data were computer-fitted according to Cleland (7).

RESULTS

When isolated heterocysts are broken with a French press, most of the uptake hydrogenase activity is recovered in the particulate fraction. Table 1 shows the distribution of activity with O_2 and benzoquinone as acceptors. O_2 is activated by a terminal oxidase to react with electrons transferred from H_2 through an electron transport chain (21). Because this transport chain is no longer coupled to hydrogenase when hydrogenase is released from the membrane, only particulate hydrogenase reacts with O_2. Reactivity with hydrophilic acceptors increases when hydrogenase is solubilized; activity with 1 mM methlyene blue increased fivefold upon solubilization of uptake hydrogenase by sonication or with 0.5% Triton X-100 (data not shown). Therefore, to measure accurately the distribution of activity between particles and the soluble fraction, a lipophilic acceptor is required. Benzoquinone is such an acceptor, and it reacts equally well with hydrogenase before and after cell breakage and solubilization.

Reversible hydrogenase appears in the soluble fraction after cells are broken by sonication.

When an extract from NH_4^+-grown cells was centrifuged at 144,000 × *g* for 90 min, 94% of the hydrogenase activity was recovered in the supernatant (data not shown). Sonication times of 40 s/ml of cell suspension were required to solubilize uptake hydrogenase. In contrast to this, only 2.7 s/ml of cell suspension was required to disrupt NH_4^+-grown filaments to yield a soluble extract of reversible hydrogenase. This suggests that reversible hydrogenase is soluble in vivo and does not require a solubilization treatment.

Table 2 shows the relative reactivities and reversibilities of preparations of the two hydrogenases with a variety of electron acceptors. Reversible hydrogenase can consume H_2 in the presence of electron acceptors covering a wide range of midpoint potentials. It also effectively catalyzes H_2 evolution from reduced methyl or benzyl viologens. Other mediators tested with the reversible hydrogenase that were totally unreactive in supporting either H_2 evolution or H_2 uptake included ferredoxin from spinach chloroplasts or *Anabaena* sp. strain 7120, F_{420} (low-potential deazaflavin-type e^- carrier from methanogens), and pyridine nucleotides. Flavins (0.5 mM flavin adenine dinucleotide or flavin mononucleotide) supported uptake at rates approximately 11 to 12% the rates with 1 mM methylene blue. Uptake hydrogenase, whether particulate or solubilized by sonication, reacted rapidly only with acceptors of positive midpoint potential. Once hydrogenase was solubilized, its ability to couple H_2 uptake to O_2 consumption was lost. Neither particulate nor solubilized uptake hydrogenase evolved H_2 at an appreciable rate when provided with reduced methyl viologen.

TABLE 1. *Distribution of uptake hydrogenase activity after breaking heterocysts*[a]

Fraction	% Activity with acceptor:	
	35 μM O_2	1 mM benzoquinone
Whole heterocysts	100	0
Total pellet	62	64
Supernatant	0	25

[a] Heterocysts isolated from frozen cells were broken by two passes through a French press. The extract was centrifuged at 5,000 × *g* for 10 min, and the supernatant from this step was centrifuged at 160,000 × *g* for 2 h. The activity of the two pellets was summed and is reported here as "total pellet."

Although solubilized uptake hydrogenase could react with ferricyanide and dichlorophenol-indophenol, these acceptors rapidly inactivated the enzyme during the course of an assay. The half time of inactivation was between 15 s and 1 min for these acceptors, and reactivities could be determined only by measuring the initial rate of H_2 uptake with the H_2 electrode. Inactivation was accelerated above pH 7.5, and it is for this reason that H_2 uptake was measured at pH 6.8 rather than at the pH optimum of 8.5. Reactions were linear at pH 6.8 with methylene blue as electron acceptor, and much slower inactivation occurred at higher pH. Neither particulate uptake hydrogenase nor reversible hydrogenase was inactivated by these electron acceptors during H_2 uptake assays.

A pH profile for H_2 evolution from reduced methyl viologen and for H_2 uptake to 1 mM methylene blue catalyzed by reversible hydrogenase is shown in Fig. 1A. With solubilized uptake hydrogenase, the pH optimum was 8.5, but the portion of the curve between 5 and 7 varied considerably with different preparations of the enzyme. Figure 1B shows two extremes of this type of behavior obtained from the same preparation of enzyme before and after several days of storage in liquid N_2. The apparent pres-

TABLE 2. *Relative reaction rates with cell-free preparations of reversible and uptake hydrogenases*[a]

Mediator	$E^{0'}$ (V)	Relative reaction rate of hydrogenase:					
		Reversible		Solubilized uptake		Particulate uptake	
		Evolution	Uptake[b]	Evolution	Uptake[c]	Evolution	Uptake[d]
10 mM methyl viologen	−0.44	63	24	0.14	0.0	0.18	0.0
5 mM benzyl viologen	−0.34	17	47	0.13	0.5	—	0.0
1 mM methylene blue	+0.01	—[e]	100	—	37	—	25
1 mM DCPIP[f]	+0.22	—	70	—	22	—	100
1 mM ferricyanide	+0.34	—	68	—	100	—	83
30 μM O_2	+0.81	—	0	—	0	—	30

[a] All hydrogen uptake assays were conducted with the H_2 electrode with 20 μM H_2 at pH 6.8. H_2 evolution was also measured at pH 6.8, and $Na_2S_2O_4$ was provided at a concentration of 10 mM.

[b] 100% = 3,855 nmol of $H_2 \cdot min^{-1} \cdot ml^{-1}$ of extract.

[c] 100% = 1,460 nmol of $H_2 \cdot min^{-1} \cdot ml^{-1}$ of extract.

[d] 100% = 401 nmol of $H_2 \cdot min^{-1} \cdot ml^{-1}$ of extract.

[e] —, Not run.

[f] DCPIP, Dichlorophenol-indophenol.

ence of two forms of the enzyme having pH optima at 6 and 8.5 will be examined more closely below.

There was about 15% loss of activity in 20 min when reversible hydrogenase, previously maintained anaerobically and stored in the presence of 1 mM $Na_2S_2O_4$, was exposed to air. This small amount of activity was lost irreversibly upon initial exposure to air, and activity was stable thereafter at about 80% of initial activity. Once exposed to O_2 the reversible hydrogenase exists in a reversibly inactive state. It can be reactivated by removal of O_2 and addition of 1 mM $Na_2S_2O_4$. Figure 2 shows the results of hydrogen uptake and evolution assays before and after preactivation with dithionite. Enzyme exposed to O_2 is inactive and incapable of catalyzing H_2 uptake. It can catalyze H_2 evolution only after a lag during which the excess dithionite in the H_2 evolution assay mixture activates the enzyme. If the enzyme is first activated with dithionite, it can evolve H_2 without a lag and can catalyze H_2 uptake.

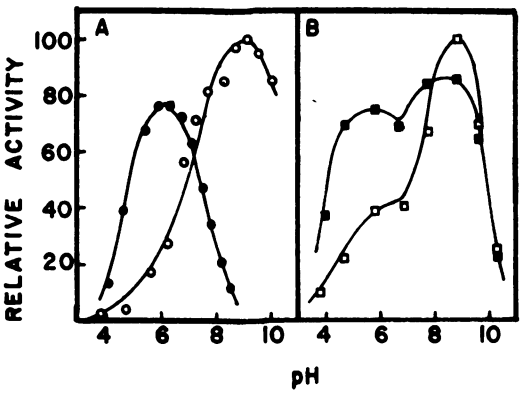

FIG. 1. *(A) pH profiles of H_2 evolution (●) and H_2 uptake (○) catalyzed by reversible hydrogenase (100 = 2,480 nmol of $H_2 \cdot min^{-1} \cdot ml^{-1}$ of extract). (B) pH profiles for solubilized uptake hydrogenase (100 = 1,340 nmol of $H_2 \cdot min^{-1} \cdot ml^{-1}$ of extract). The soluble enzyme obtained after disruption of heterocysts by a French press was assayed for H_2 uptake immediately (■) or after 3 days of storage in liquid N_2 (□). Assay conditions were as described in the text.*

If reversible hydrogenase is incubated anaerobically under H_2, autoactivation will occur. If methyl viologen is included in the solution, the autoactivation process can be followed spectrophotometrically by measuring the appearance of reduced methyl viologen. Lag times between 50 and 125 min were observed and were found to be inversely proportional to the hydrogenase activity in the assay system. Addition of 10 mM dithiothreitol, 50 mM glucose plus 1 mg of glucose oxidase per ml, or 1 mM NADH decreased the lag to about one-third of the value in the control lacking these O_2-scavenging or reducing agents.

While in whole filaments, the uptake hydrogenase catalyzing the oxyhydrogen reaction is resistant to atmospheric O_2 levels (10); however, inactivation becomes marked with increasing disruption of the system. O_2 inactivation began to occur in isolated heterocysts during assays with O_2 levels above 120 μM. With cell-free particulate hydrogenase, inactivation began to occur at about 30 μM O_2. Once uptake hydrogenase was solubilized, the sensitivity of the enzyme to O_2 increased dramatically. Figure 3 shows a semilogarithmic plot of the time course of inactivation of uptake hydrogenase upon exposure to atmospheric O_2 levels. If the enzyme was assayed at pH 6.8, inactivation was clearly biphasic, with half times of inactivation of 2 min and 14 min. Approximately 70% of the activity was lost in the initial rapid phase. If activity was assayed at pH 8.5, more than 95% of the activity

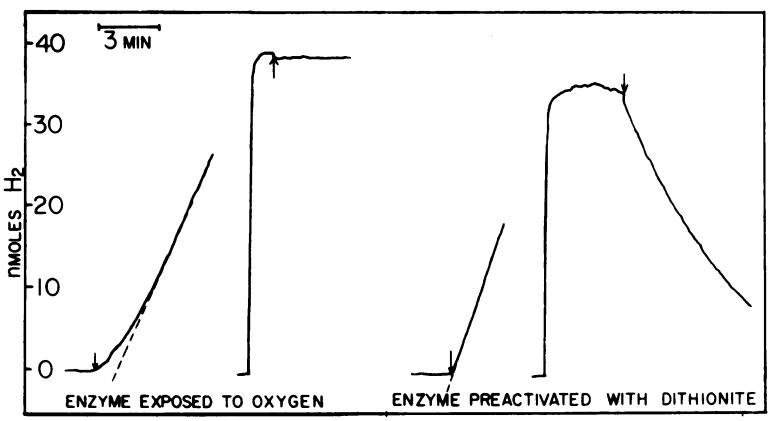

FIG. 2. *H_2 electrode and strip chart recorder tracings of H_2 evolution and H_2 uptake. Enzyme was added at the arrows.*

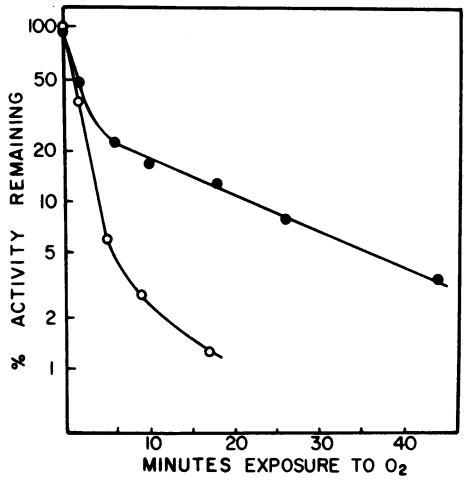

FIG. 3. *Loss of activity upon exposure of uptake hydrogenase to atmospheric O_2. An exposed sample of enzyme was assayed in 50 mM PIPES, pH 6.8 (closed circles), or in 50 mM Tricine, pH 8.5 (open circles), (100% = 2,430 nmol of $H_2 \cdot min^{-1} \cdot ml^{-1}$ of extract).*

was lost in the rapid phase. These data, coupled with the pH profiles (Fig. 1B), suggest that there are two forms of uptake hydrogenase: one with a pH optimum of 8.5 which is inactivated by O_2 with a half-life of 2 min, and another with a pH optimum of 6 which is inactivated by O_2 with a half-life of 14 min. Before O_2-exposed samples were assayed, they were preincubated with 0.2 mM $Na_2S_2O_4$ as described in Materials and Methods. Considerably less activity was observed when there had been no preexposure to dithionite; this reveals some reversible inhibition by O_2 as well as the irreversible inactivation shown in Fig. 3.

Uptake and reversible hydrogenases differ in their thermal stabilities. Reversible hydrogenase retained complete activity for 1 h at 70°C. Uptake hydrogenase that had been solubilized either by sonication or with 5% Triton X-100 was inactivated with a half-life of 12 min at 70°C.

The molecular weights of reversible hydrogenase and solubilized uptake hydrogenase were determined by chromatography on calibrated Sephadex columns. Uptake hydrogenase was eluted as a single peak with a molecular weight of 56,000. A small amount of hydrogenase activity also was eluted in the void volume of Sephadex G-100 or G-200 columns. This void-volume activity was not inactivated by ferricyanide during H_2 uptake assays, and this identifies it as uptake hydrogenase still bound to particles. When reversible hydrogenase was chromatographed on Sephadex G-200, two separate peaks of activity were eluted with molecular weights of 113,000 and 165,000; about two-thirds of the activity resided in the 165,000 peak. If reversible hydrogenase first was heated to 70°C for 1 h, a third peak carrying activity appeared at a position corresponding to a molecular weight of 55,000. The catalytic and physical properties of this 55,000 unit were similar to those of the other reversible hydrogenase peaks and differed from those of the uptake hydrogenase of 56,000 molecular weight in the ways previously described.

Plots of carbon monoxide inhibition are shown in Fig. 4. For each enzyme, inhibition by CO was competitive versus H_2. The reversible hydrogenase was more sensitive to CO inhibition than the uptake hydrogenase and showed a K_i of 0.0095 atm compared to a K_i of 0.039 atm for uptake hydrogenase. The K_m's for H_2 also were determined from the data in Fig. 4. Reversible hydrogenase had a K_m of 2.3 μM, and uptake hydrogenase had a K_m of 0.92 μM.

Although C_2H_2 has been reported to inhibit uptake hydrogenase in *Anabaena* (6), we failed to observe any inhibition of the oxyhydrogen reaction in whole filaments or isolated heterocysts by up to 0.6 atm C_2H_2. Purified extracts of reversible hydrogenase also were examined for C_2H_2 inhibition and, in agreement with the reports of others (13), no inhibition was observed at C_2H_2 levels up to 0.6 atm.

DISCUSSION

Several particulate uptake hydrogenases have been reported from N_2-fixing organisms. Hyndman et al. (14) recovered a cell-free particulate preparation of hydrogenase from *Azotobacter vinelandii*. Dixon (9) compared the particulate hydrogenases of *Rhizobium* bacteroids and *Azotobacter* and found that each enzyme could react only in the uptake direction and was unable to reduce low potential acceptors. In preparations from *N. muscorum* and *A. cylindrica* (25) most of the H_2-consuming activity was recovered in the particulate fraction with 10-fold greater activity in heterocysts as compared with vegetative cells. In isolated heterocysts (21) and crude extracts (4) prepared from *Anabaena* this uptake hydrogenase was found to couple only slowly to low potential acceptors. The distribution of uptake hydrogenase between soluble and particulate fractions reported in Table 1 is similar to that observed in *Rhizobium japonicum* bacteroids by Arp and Burris (2). It is uncertain whether the soluble form of the uptake hydrogenase is present in vivo or is an artifact of cell breakage. The fact that reactivity with hydrophilic but not with hydrophobic electron acceptors increases several fold upon solubilization

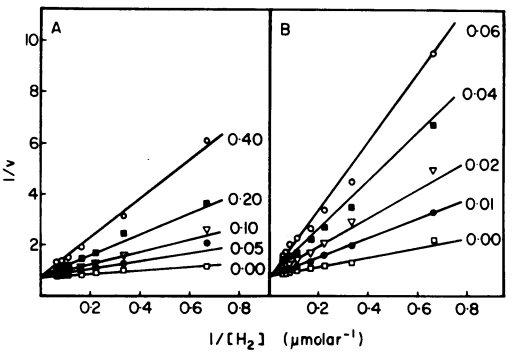

FIG. 4. *Double-reciprocal plot of methylene blue reduction by (A) solubilized uptake hydrogenase or (B) reversible hydrogenase in the presence of fixed levels of CO (indicated at right-hand margin in atmospheres). Velocity is expressed in micromoles of H_2 consumed per minute per milliliter of extract.*

suggests that the active site may be partially buried in the membrane. Because this enzyme presumably couples with a membrane-bound electron transport chain, such a possibility is not surprising.

There are only a few reports of soluble reversible hydrogenases from cyanobacteria. The enzyme from *Spirulina maxima* (18) was partially purified and characterized after extraction from frozen cells without mechanical disruption. Fujita et al. (12) obtained a reversible, soluble hydrogenase after acetone extraction of anaerobically adapted *A. cylindrica*. Tel-Or et al. (25) found H_2 evolution activity in *N. muscorum* and *A. cylindrica* to be confined mainly to the soluble fraction after cell disruption. Hallenbeck and Benemann (13) obtained a soluble hydrogenase from *A. cylindrica* by sonication. We used a similar method to prepare extracts of reversible hydrogenase from *Anabaena* sp. strain 7120. In addition to being reversible this enzyme also differs from uptake hydrogenase in that it can react with electron acceptors covering a wide range of midpoint potentials.

Whereas Hallenbeck and Benemann reported a molecular weight of 230,000 for reversible hydrogenase from *A. cylindrica* (13), we observed two peaks that eluted from Sephadex G-200 at positions corresponding to molecular weights of 113,000 and 165,000. These could be a dimer and trimer of the 55,000 peak that is eluted from Sephadex G-200 after heat treatment; however, proof of this requires preparation of a homogeneous enzyme. It is possible that proteolytic enzymes present as contaminants degraded a polymeric form of hydrogenase. We have observed no larger aggregation forms. Although the 55,000 form of reversible hydrogenase, appearing after a heat treatment, has a molecular weight similar to that of the solubilized uptake hydrogenase (56,000), this form of the enzyme still differs from uptake hydrogenase in its reversibility, thermal stability, O_2 sensitivity, and electron acceptor specificity. One cannot reconcile these results by suggesting that there is a single hydrogenase subunit with properties modified by the addition of secondary subunits.

Reversible hydrogenase from *Anabaena* sp. strain 7120 can be added to a growing list of hydrogenases that are stable to O_2 and can be purified aerobically (16, 23, 24, 28); however, this enzyme is not catalytically active until O_2 is removed. Exhaustive degassing will not reactivate the enzyme, and this suggests that inactivation may be due to oxidation of an allosteric site or to inhibition by a very tightly bound O_2 molecule. The shortening of the lag for autoactivation by a glucose-glucose oxidase O_2 trap lends support to the latter proposal. Similar behavior has been observed for the soluble (24) and particulate hydrogenases (23) of *Alcaligenes eutrophus*.

Unlike reversible hydrogenase, uptake hydrogenase from *Anabaena* sp. strain 7120 is irreversibly inactivated by O_2. The biphasic inactivation profiles coupled with the pH profiles for uptake hydrogenase suggest that two forms of this enzyme exist which differ in pH optima and stability to O_2. Other reports of biphasic time courses for inactivation of hydrogenases by O_2 (17, 27) also have been explained as supporting two forms of the enzyme with differing O_2 sensitivities. Evidence recently reported by Peschek (19, 20) supports the existence of two functionally distinct particulate hydrogenases catalyzing photosynthetic and respiratory H_2 uptake in *Anacystis nidulans*. Photosynthetic and respiratory H_2 uptake also have been reported in *Anabaena* sp. strain 7120 (26). The two forms of solubilized uptake hydrogenase may be separate enzymes catalyzing functionally distinct reactions or may simply arise from a conformational change that occurs upon removal of the enzyme from the membrane.

In the H_2 uptake assay, CO was competitive with H_2; the reversible and the uptake hydrogenase of *Anabaena* sp. strain 7120 had K_i's of 0.0095 atm and 0.039 atm, respectively. Competitive inhibition also has been reported for hydrogenase from *Clostridium pasteurianum* with a K_i of 0.0066 atm (11).

It is generally assumed that a low K_m for H_2 indicates that the physiological function of the hydrogenase is in the uptake direction (4). The low K_m's measured here for both the reversible and uptake hydrogenases suggest that these enzymes are both well suited to consume H_2 in vivo. In contrast, *C. pasteurianum* hydrogenase,

an enzyme known to evolve H_2 physiologically, has a K_m of 380 μM for H_2 (corresponds to 0.49 atm at 25°C) (11). The situation in *Anabaena* sp. strain 7120 is similar to that reported in *Alcaligenes eutrophus*, an organism that also possesses a soluble reversible hydrogenase (24) and an irreversible membrane-bound enzyme (23), both of which have low K_m's for H_2 and consume H_2 physiologically.

When attempting to determine the capabilities of *Anabaena* for H_2 metabolism or the properties of its hydrogenases, it is essential to recognize the existence of multiple hydrogen-metabolizing enzymes. Although the data strongly indicate that two hydrogenases are present in *Anabaena* sp. strain 7120, further purification of the enzymes and tests with antigen-antibody techniques and genetic techniques could furnish confirming information. The present comparison of the hydrogenases should prove useful in distinguishing between these enzymes and in elucidating their metabolic roles and capabilities.

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LITERATURE CITED

1. **Allen, M. B., and D. I. Arnon.** 1955. Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen-fixation by *Anabaena cylindrica* Lemm. Plant Physiol. **30**:366–372.
2. **Arp, D. J., and R. H. Burris.** 1979. Purification and properties of the particulate hydrogenase from the bacteroids of soybean root nodules. Biochim. Biophys. Acta **570**:221–230.
3. **Benemann, J. R., and N. M. Weare.** 1974. Nitrogen fixation by *Anabaena cylindrica*. III. Hydrogen-supported nitrogenase activity. Arch. Microbiol. **101**:401–408.
4. **Bothe, H., E. Distler, and G. Eisbrenner.** 1978. Hydrogen metabolism in blue-green algae. Biochimie **60**:277–289.
5. **Bothe, H., J. Tennigkeit, and G. Eisbrenner.** 1977. The utilization of molecular hydrogen by the blue-green alga *Anabaena cylindrica*. Arch. Microbiol. **114**:43–49.
6. **Bothe, H., J. Tennigkeit, G. Eisbrenner, and M. G. Yates.** 1977. The hydrogenase-nitrogenase relationship in the blue-green alga *Anabaena cylindrica*. Planta **133**:237–242.
7. **Cleland, W. W.** 1979. Statistical analysis of enzyme kinetic data. Methods Enzymol. **63**:103–138.
8. **Daday, A., G. R. Lambert, and G. D. Smith.** 1979. Measurement *in vivo* of hydrogenase catalyzed hydrogen evolution in the presence of nitrogenase enzyme in cyanobacteria. Biochem. J. **177**:139–144.
9. **Dixon, R. O. D.** 1972. Hydrogenase in legume root nodule bacteroids: occurrence and properties. Arch. Microbiol. **85**:193–201.
10. **Eisbrenner, G., E. Distler, L. Floener, and H. Bothe.** 1978. The occurrence of the hydrogenase in some blue-green algae. Arch. Microbiol. **118**:177–184.
11. **Erbes, D. L., and R. H. Burris.** 1978. The kinetics of methyl viologen oxidation and reduction by the hydrogenase from *Clostridium pasteurianum*. Biochim. Biophys. Acta **525**:45–54.
12. **Fujita, Y., H. Ohama, and A. Hattori.** 1964. Hydrogenase activity of cell-free preparation obtained from the blue-green alga, *Anabaena cylindrica*. Plant Cell Physiol. **5**:305–314.
13. **Hallenbeck, P. C., and J. R. Benemann.** 1978. Characterization and partial purification of the reversible hydrogenase of *Anabaena cylindrica*. FEBS Lett. **94**: 261–264.
14. **Hyndman, L. A., R. H. Burris, and P. W. Wilson.** 1953. Properties of hydrogenase from *Azotobacter vinelandii*. J. Bacteriol. **65**:522–531.
15. **Jones, L. W., and N. I. Bishop.** 1976. Simultaneous measurement of oxygen and hydrogen exchange from the blue-green alga *Anabaena*. Plant Physiol. **57**:659–665.
16. **Kakuno, T., N. O. Kaplan, and M. D. Kamen.** 1977. Chromatium hydrogenase. Proc. Natl. Acad. Sci. U.S.A. **74**:861–863.
17. **Lappi, D. A., F. E. Stolzenbach, N. O. Kaplan, and M. D. Kamen.** 1976. Immobilization of hydrogenase on glass beads. Biochem. Biophys. Res. Commun. **69**:878–884.
18. **Llama, M. J., J. L. Serra, K. K. Rao, and D. O. Hall.** 1979. Isolation and characterization of the hydrogenase activity from the non-heterocystous cyanobacterium *Spirulina maxima*. FEBS Lett. **98**:342–346.
19. **Peschek, G. A.** 1979. Anaerobic hydrogenase activity in *Anacystis nidulans*. H_2-dependent photoreduction and related reactions. Biochim. Biophys. Acta. **548**:187–202.
20. **Peschek, G. A.** 1979. Aerobic hydrogenase actvity in *Anacystis nidulans*. The oxyhydrogen reaction. Biochim. Biophys. Acta **548**:203–215.
21. **Peterson, R. B., and R. H. Burris.** 1978. Hydrogen metabolism in isolated heterocysts of *Anabaena* 7120. Arch. Microbiol. **116**:125–132.
22. **Peterson, R. B., and C. P. Wolk.** 1978. High recovery of nitrogenase activity and of ^{55}Fe-labeled nitrogenase in heterocysts isolated from *Anabaena variabilis*. Proc. Natl. Acad. Sci. U.S.A. **75**:6271–6275.
23. **Schink, B., and H. G. Schlegel.** 1979. The membrane-bound hydrogenase of *Alcaligenes eutrophus*. I. Solubilization, purification, and biochemical properties. Biochim. Biophys. Acta **567**:315–324.
24. **Schneider, K., and H. G. Schlegel.** 1976. Purification and properties of soluble hydrogenase from *Alcaligenes eutrophus* H16. Biochim. Biophys. Acta **452**:66–80.
25. **Tel-Or, E., L. W. Luijk, and L. Packer.** 1978. Hydrogenase in N_2-fixing cyanobacteria. Arch. Biochem. Biophys. **185**:185–194.
26. **Tetley, R. M., and N. I. Bishop.** 1979. The differential action of metronidazole on nitrogen fixation, hydrogen metabolism, photosynthesis and respiration in *Anabaena* and *Scenedesmus*. Biochim. Biophys. Acta **546**: 43–53.
27. **Toai, C. D., S. D. Varfolomeev, I. N. Gogotov, and I. V. Berezin.** 1976. Kinetic principles of the inactivation of bacterial hydrogenases. Mol. Biol. (USSR). **10**:368–374.
28. **Van der Westen, H. M., S. G. Mayhew, and C. Veeger.** 1978. Separation of hydrogenase from intact cells of *Desulfovibrio vulgaris*. FEBS Lett. **86**:122–126.