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Regulation of Two Nickel-Requiring (Inducible and Constitutive) Hydrogenases and Their Coupling to Nitrogenase in *Methylosinus trichosporium* OB3b

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Two uptake hydrogenases were found in the obligate methanotroph *Methylosinus trichosporium* OB3b; one was constitutive, and a second was induced by H_2. Both hydrogenases could be assayed by measuring methylene blue reduction anaerobically or by coupling their activity to nitrogenase acetylene reduction activity in vivo in an O_2-dependent reaction. The H_2 concentration for half-maximal activity of the inducible and constitutive hydrogenases in both assays was 0.01 and 0.5 bar (1 and 50 kPa), respectively, making it easy to distinguish these enzymes from one another both in vivo and in vitro. Hydrogen uptake was shown to be coupled to ATP synthesis in methane-starved cells. Methane, methanol, formate, succinate, and glucose all repressed the H_2-mediated synthesis of the inducible hydrogenase. Furthermore, this enzyme was only expressed in N-starved cultures and was repressed by NH_4^+ and NO_3^-; synthesis of the constitutive hydrogenase was not affected by excess N in the growth medium. In nickel-free, EDTA-containing medium, the activities of these two enzymes were negligible; however, both enzyme activities appeared rapidly following the addition of nickel to the culture. Chloramphenicol, when added along with nickel, had no effect on the rapid appearance of either the constitutive or inducible activity, indicating that nickel is not required for synthesis of the hydrogenase apoproteins. These observations all suggest that these hydrogenases are nickel-containing enzymes. Finally, both hydrogenases were soluble and could be fractionated by 20% ammonium sulfate; the constitutive enzyme remained in the supernatant solution, while the inducible enzyme was precipitated under these conditions.

In obligate methanotrophic bacteria, the energy for biosynthetic processes comes primarily form methane through the sequential oxidation of the one-carbon intermediates derived from this molecule (2, 7). Indications that H_2 can provide methanotrophs with a supplemental energy source is seen from the fact that H_2 oxidation can support nitrogen fixation activity (8–10, 28). This means that not only does H_2 generate a strong reductant in these microorganisms, but its oxidation is also coupled to ATP synthesis, since ATP is required for nitrogenase activity. H_2-dependent nitrogenase activity has been reported for *Azotobacter chroococcum* (30), *Rhizobium* bacteroids (11, 13), and *Anabaena* spp. (4, 24), and in each case it has been associated with the presence of a membrane-bound hydrogenase. Dixon (11, 12) showed that one of the functions of this hydrogenase was to increase the efficiency of nitrogen fixation by recycling H_2 produced by the reduction of protons at the active site of nitrogenase. The characteristics of uptake hydrogenases have been described in recent review articles by Cammack et al. (5), Vignais et al. (29), and Hausinger (16). They all appear to be iron-sulfur-nickel-containing, oxygen-stable enzymes, several of which are induced by H_2. The regulation of hydrogenase synthesis is complex but seems to be related to the internal redox environment of the cell (14).

Soluble constitutive hydrogenases have been demonstrated by Kawamura et al. (17) to evolve H_2 from formate in *Methylosinus trichosporium* OB3b and *Methylomonas albus* BG8. In both species an NAD-dependent formate dehydrogenase coupled electron flow to hydrogenase. In this communication we report the properties and regulation of two nickel-requiring uptake hydrogenases in *M. trichosporium* OB3b, one of which is synthesized constitutively and the other of which is induced by H_2.

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MATERIALS AND METHODS

Growth of cells. *M. trichosporium* OB3b (kindly provided by Mary Lidstrom, University of Wisconsin, Milwaukee) was grown on the MS medium of Dalton and Whittenbury (8). This was supplemented with a growth-limiting level (1.0 mM) of either ammonium chloride (LAMS medium) or sodium nitrate (LNMS medium). These nitrogen sources were increased to 10 mM when cells without nitrogenase were needed. Phosphate buffer (pH 6.8) was added to a final concentration of 25 mM after autoclaving. Batch cultures (50 ml) were grown in 125-ml Erlenmeyer flasks fitted with serum bottle stoppers for gassing. The gas phase was 50% methane and 50% air; the cultures were regassed daily for best growth. Cultures were grown at 32°C on a New Brunswick Gyrotory shaker (model 2A) at 200 rpm. All in vivo measurements of nitrogenase and hydrogenase activity were made from cells grown in batch culture having an absorbance (A_{600}) of 0.5 to 0.7.

To induce synthesis of the uptake hydrogenase in mature cultures of *M. trichosporium*, two conditions had to be met. The culture had to be free of NH_4^+ (LAMS or LNMS medium was used routinely, which also derepressed nitrogenase activity), and the CH_4 had to be removed from the gas phase and H_2 added. The usual mixture was 50% H_2–5% air–45% argon.

H_2 uptake assays. (i) Inducible hydrogenase. Three methods were used to assay the activity of the inducible hydrogenase in *M. trichosporium* OB3b. Following induction under H_2, the activity was measured indirectly by coupling it to nitrogenase acetylene reduction activity (ARA) in vivo. Activity was measured by incubating cells under an atmo-

sphere of 2% H_2–4% air–90% argon–4% acetylene and monitoring ethylene formation (as described below).

The inducible hydrogenase was also assayed spectrophotometrically by monitoring the reduction of methylene blue (MB) in detergent-treated permeabilized cells and cell extracts. The assay mixture (in a final volume of 2 ml) contained methylene blue (0.05 mM), hexadexyltrimethylammonium bromide (0.2 mg), and 1.8 ml of cells. The assays were carried out anaerobically in stoppered cuvettes under a 9-ml gas phase of 89% argon and 11% H_2. The rate of MB reduction was determined by following its loss of absorbance at 570 nm in a Perkin-Elmer (Lambda 1) recording spectrophotometer. The extinction coefficient of MB used at this wavelength was 12.8 cm^{-1} mM^{-1}. Hydrogenase activity of cell extracts was assayed in a reaction mixture that contained phosphate buffer (50 mM; pH 6.8), MB (0.05 mM), and water to 1.9 ml. The reaction was started by adding 0.1 ml of the cell extract (50 to 100 μg of protein) to the argon-saturated reaction mixture in a cuvette (as above) having a gas phase of 89% argon and 11% H_2. At this H_2 concentration, the constitutive hydrogenase showed only very low activity.

(ii) Constitutive hydrogenase. Two methods were used to measure the activity of the constitutive hydrogenase. Activity was coupled to nitrogenase in vivo as described for the inducible enzyme, except the H_2 in the gas phase was raised to 92%. The constitutive enzyme was also measured in extracts by the reduction of MB, and except for the gas phase, which was 100% H_2, the assay mixture was the same as that described above for the inducible enzyme.

Anaerobic preparation of cell extracts. Extracts were prepared from cells which were suspended in 2 volumes of argon-saturated 50 mM Tris hydrochloride buffer (pH 6.8). The cells were placed in a flask, which was then closed with a serum bottle stopper, evacuated, and flushed with argon several times. Cells were held on ice until transferred to the sonication vessel. Depending on the volume of the cell suspension, the sonication vessel was a 20-ml glass beaker or a short test tube covered with Parafilm (American Can Co., Greenwich, Conn.). A slit was cut in the Parafilm just large enough to insert the sonicator probe, after which the closed vessel was purged with argon for 5 min. The cell suspension was transferred to the vessel anaerobically by syringe and disrupted by sonic oscillation with a Sonic 2000 (B. Braun Instruments, Burlingame, Calif.) at full output for two 15-s periods. The gas flow through the vessel was maintained during the sonication period. The broken cells were transferred by syringe to a capped, degassed tube and centrifuged at 13,000 × g for 10 min. The pellet was discarded, and the supernatant crude extract was used as a source of hydrogenase or subjected to further purification. Centrifugation at 200,000 × g for 1 h pelleted the cell membranes, leaving a straw-yellow supernatant solution.

Nitrogenase assays. Nitrogenase activity in whole cells of *M. trichosporium* was assayed as ARA. Ethylene (the product) was quantitated with a Varian model 1440 gas chromatograph (Varian Associates, Palo Alto, Calif.) equipped with a flame ionization detector and a 2-m activated alumina column operated at 185°C. Peak areas were integrated on a Hewlett-Packard 3390 integrator. Assays were carried out in 6-ml Fernback flasks containing 1.5 ml of cells incubated with periodic shaking at 32°C. The energy source with either H_2 or formate (12.5 mM), and the gas phase is described in the legends to the figures.

Intracellular ATP pools. Following incubation of the cells with either argon (energy starved) or O_2 plus H_2 as the gas phase, 100 ml of culture (40 Klett units) was rapidly cooled to 0°C and harvested by centrifugation. Ten milliliters of hot (100°C) 80% ethanol was immediately added to the cell pellet and mixed. ATP was extracted from this mixture and quantified by high-pressure liquid chromatography as described by Li et al. (19).

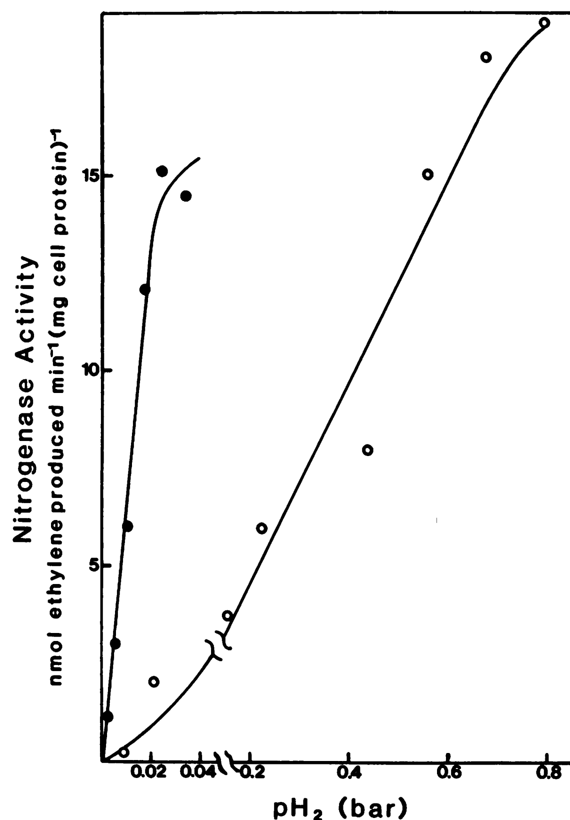

FIG. 1. Effect of the partial pressure of H_2 (pH_2) on hydrogenase-linked nitrogenase ARA in *M. trichosporium* OB3b. The constitutive hydrogenase was assayed in nitrogenase-derepressed cells without further treatment (○). A second hydrogenase was induced in these cells by removing the methane and incubating them for 5 h under an atmosphere of 50% H_2, 5% air, and 45% argon (●). Both hydrogenases (linked at ARA) were assayed in cells with gas phases of 5% air, H_2 as indicated, and the balance as argon.

Protein determinations. Protein was determined by the method of Lowry et al. (20) with bovine serum albumin as a standard. Protein in whole cells was determined by boiling them for 3 min in 1 N NaOH before adding the color reagents.

RESULTS

H_2-driven nitrogenase activity. The observations of Dalton and Whittenbury (8) and Toukdarian and Lidstrom (28) that obligate methanotrophs can use H_2 as an energy source to support nitrogenase activity were confirmed. In *M. trichosporium* OB3b nitrogenase ARA was supported by a constitutive hydrogenase that required a partial pressure of H_2 (pH_2) of approximately 0.8 bar (ca. 80 kPa) for maximal activity (Fig. 1). High rates of H_2-driven nitrogenase ARA were also observed with much lower levels of H_2 (0.02 bar), but only if the cells had previously been incubated under a gas phase containing H_2 and <10% air. Methane also had to be removed from the gas phase for hydrogenase induction,

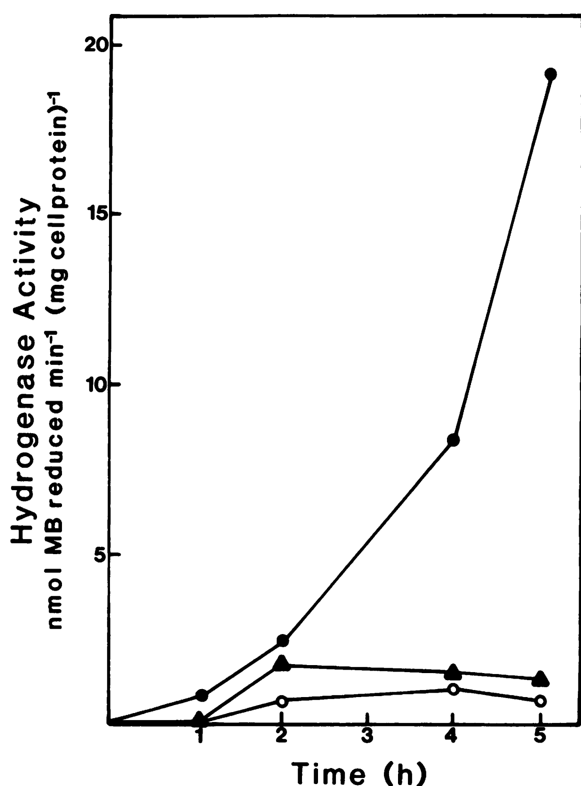

FIG. 2. Effect of H_2, CH_4, and chloramphenicol on hydrogenasase induction in *M. trichosporium* OB3b. Cells grown on LNMS medium were divided into three 20-ml aliquots, which were placed in bottles that were closed with stoppers and evacuated to remove any residual methane from the solution. The atmosphere was replaced with 10% air, 15% argon, and either 75% H_2 (●), 75% argon (instead of H_2) (○), or 75% H_2 plus 25 μg of chloramphenicol per ml (▲). The time course of hydrogenase induction was monitored by removing 2.0-ml portions of cells and monitoring MB reduction in the presence of detergent as described in the text.

and this will be discussed below. As seen in Fig. 1, the constitutive hydrogenase would support only low levels of ARA at 0.02 bar of H_2; it therefore interfered only to a minor extent with assays of the low-H_2-requiring inducible hydrogenase. Controls showed that formate-dependent nitrogenase activity remained constant during hydrogenase induction, suggesting that nitrogenase levels did not influence the coupled reaction. The partial pressure of O_2 required for optimum H_2-driven ARA was approximately 0.01 bar regardless of whether the constitutive or inducible hydrogenase was being assayed (data not shown).

H_2 induction of hydrogenase. With the requirements of H_2 and O_2 established for H_2-driven ARA, the kinetics of hydrogenase induction were easily monitored. Hydrogenase activity (coupled to ARA) in the presence of 0.02 bar of H_2 was first detected 2.5 h after H_2 was added to the gas phase of the cells; this induced activity increased for about 4 or 5 h and then leveled off (data not shown).

To examine hydrogenase induction directly rather than through a coupled reaction, an MB reduction assay was used with detergent-permeabilized cells. The kinetics of H_2-mediated hydrogenase induction were shown (Fig. 2) to be very similar to those observed in the nitrogenase-coupled assay. Omitting H_2 from the gas phase of the culture and replacing it with argon showed the need for exogenous H_2 for synthesis of this uptake hydrogenase, and addition of chloramphenicol with the H_2 completely suppressed hydrogenase synthesis. Incubation of a previously induced culture with chloramphenicol had no effect on hydrogenase activity. These results indicate that H_2 was not activating a previously formed enzyme, but was inducing the synthesis of new protein. Finally, the H_2 concentration required for half-maximal activity of the inducible and constitutive hydrogenases coupled to MB reduction was 0.01 and 0.5 bar, respectively (data not shown), which were very similar to those measured in the nitrogenase-coupled assay.

Repression of hydrogenase by carbon and nitrogen compounds. In *M. trichosporium* OB3b, H_2-mediated hydrogenase synthesis was strongly repressed by methane and to a lesser extent by methanol and formate (Table 1). In addition, succinate inhibited this induction, as did glucose. The latter is difficult to explain, since there is no evidence that this obligate methanotroph utilizes glucose. Nitrate and ammonia in the culture medium also prevented synthesis of the inducible hydrogenase. The appearance (derepression) of nitrogenase activity in N-limited medium (LNMS or LAMS) served as an indicator of when the nitrogen levels had been reduced sufficiently to allow hydrogenase induction, provided that CH_4 was removed and H_2 was added. Finally, the constitutive hydrogenase was present in *M. trichosporium* grown with either full NO_3^- or NH_4^+ (10 mM) as a nitrogen source.

Electron carrier specificity. Only artificial electron acceptors were found to react with the inducible hydrogenase in crude extracts (Table 2). Of these, MB was most reactive, followed by benzyl and methyl viologen. Low but reproducible activity was observed with NAD, but since crude extracts were used, these results may not be meaningful. Flavin adenine dinucleotide and flavin mononucleotide did not stimulate NAD reduction, as shown with *Nocardia opaca* hydrogenase (1), nor were these flavins themselves reduced.

The constitutive hydrogenase was measured in extracts prepared from cells that had not been exposed to H_2. Only when the gas phase was increased above 50% was the constitutive enzyme measurable with MB as an acceptor (Table 2). No other artificial carriers were tested, but NAD reduction was observed, albeit at a low rate. Again, we are

TABLE 1. Effect of carbon and nitrogen compounds on H_2-mediated induction of hydrogenase in *M. trichosporium* OB3b

Addition to culture[a]	Hydrogenase activity[b] (nmol of MB reduced/min per mg of cell protein)
None	18.3
Methane	1.7
Methanol	5.0
Sodium formate	3.8
Sodium succinate	0
Glucose	0
Potassium nitrate	1.8
Ammonium chloride	1.8

[a] After the culture had developed on methane in LNMS medium and was demonstrated to have ARA, the methane was removed and the carbon (10 mM) and nitrogen (5 mM) compounds listed were added to cell samples (for 12 h) along with an H_2-containing atmosphere (50% H_2, 45% argon, 5% air). When methane was added back to the gas phase, it was 30%, with the remainder being air (5%) and argon (65%). Hydrogenase was assayed in situ (in detergent-treated cells) by the MB reduction assay as described in the text.

[b] Mean of three determinations. The standard deviations were all <20% of the mean.

TABLE 2. Reduction of electron acceptors by hydrogenase extracts from *M. trichosporium* OB3b

Hydrogenase[a]	Acceptor[b] and concn	Activity[c] (nmol of acceptor reduced/min per mg of protein)
Inducible	None	1.2
	MB (0.05 mM)	90.1
	Methyl viologen (3.3 mM)	20
	Benzyl viologen (3.3 mM)	83.3
	NAD (5 mM)	3.2
	NADP (5 mM)	0
	FMN (3.3 mM)	0
	FAD (3.3 mM)	0
	NAD + FMN + FAD	0
Constitutive	MB (0.05 mM)	0.01 (165.3)
	NAD (5 mM)	ND[d] (<15)

[a] Assayed spectrophotometrically with 50 μg of protein as described in the text.
[b] FMN, Flavin mononucleotide; FAD, flavin adenine dinucleotide.
[c] Values in parentheses indicate activity in the presence of a 100% H_2 atmosphere.
[d] ND, Not done.

not prepared to place much credence in this activity (NAD reduction) until the enzyme has been purified.

Intracellular ATP pools enhanced by H_2. Because nitrogenase activity requires ATP as a substrate, the H_2-driven, O_2-requiring nitrogenase activity strongly suggests that *M. trichosporium*, like *A. chroococcum* and *Bradyrhizobium* (formerly *Rhizobium*) *japonicum* (11, 13), carries out an H_2-dependent oxidative phosphorylation to support this activity. This was confirmed by direct analysis of ATP; cells starved for energy had only half the intracellular level of ATP (0.8 nmol/mg of cell protein) as did cells that were starved and then incubated under H_2 and O_2 (1.6 nmol/mg of cell protein). Methane-grown cells (the control) had intracellular ATP pools of 1.4 nmol/mg of cell protein.

Nickel requirement of activity. The addition of 10 μM EDTA to nickel-free LNMS medium had no effect on the growth of *M. trichosporium*, but in the presence of this chelating agent neither the constitutive nor the inducible hydrogenase was expressed (Fig. 3). The addition of 7.5 μM $NiCl_2$ to the medium activated the constitutive hydrogenase immediately (no lag period), even in the presence of 50 μg of chloramphenicol per ml (Fig. 3A). These results indicate that protein synthesis was not required for hydrogenase activity to appear after nickel was added to the medium. When $NiCl_2$ was added back in 1 μM increments, we observed rapid activation of constitutive hydrogenase activity between 4 and 8 μM, with inhibition occurring above this level (data not shown). Excess EDTA in the medium probably accounts for the lack of hydrogenase activity until 3 or 4 μM $NiCl_2$ had been added.

To induce the second hydrogenase in a nickel-free environment, H_2 was included in the gas phase of cells grown in the same nickel-free EDTA-containing medium. After 3 h, no hydrogenase activity could be detected until nickel was added, and then it was expressed without an additional lag period (Fig. 3B). Chloramphenicol did not inhibit this rapid appearance of activity. In a control in which chloramphenicol was added to the cells along with the H_2 at time zero, nickel did not stimulate hydrogenase activity (data not shown), indicating that chloramphenicol had inhibited synthesis of apohydrogenase. These observations indicate that in *M. trichosporium* nickel plays no role in the synthesis of either the constitutive or inducible hydrogenase apoprotein

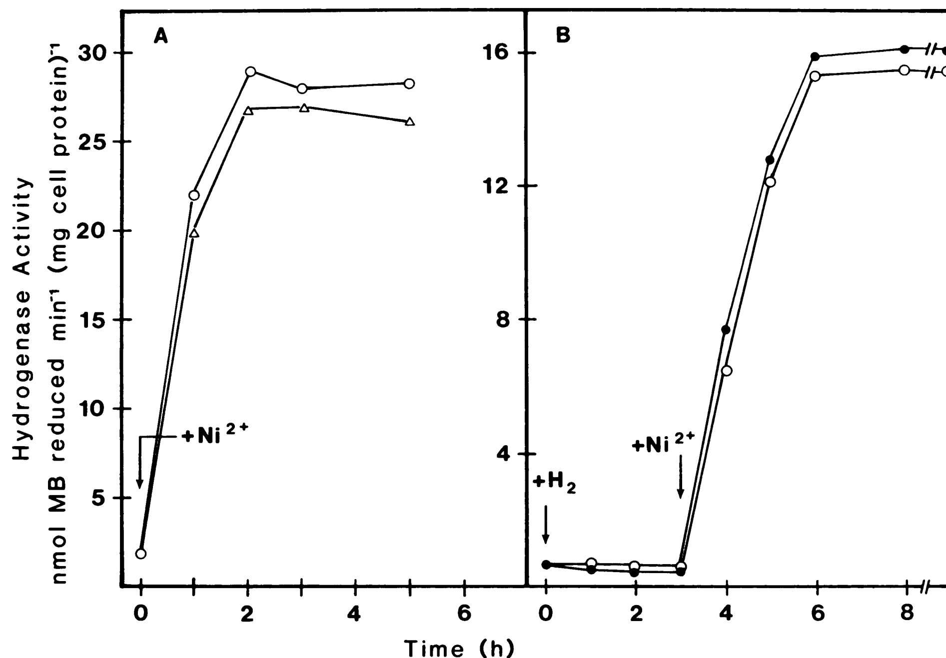

FIG. 3. Effect of nickel and chloramphenicol on hydrogenase activity. *M. trichosporium* OB3b was grown on methane in LNMS medium made up without $NiCl_2$ but with 10 μM EDTA added. Hydrogenase activity was assayed by measuring MB reduction in 2-ml portions of detergent-treated cells (see text). (A) Constitutive activity. $NiCl_2$ (7.5 μM) was added to two cultures; chloramphenicol (50 μg/ml) was added to one at the same time (△), and to the other no chloramphenicol was added (○). (B) Inducible activity. Hydrogen (50%) (along with 45% argon and 5% air) was added to the gas phase of two cultures at time zero, one with chloramphenicol (50 μg/ml) added at 3 h along with the nickel (○) and one without chloramphenicol (●). $NiCl_2$ (7.5 μM) was added to both cultures at the time indicated.

TABLE 3. Fractionation of *M. trichosporium* constitutive and inducible hydrogenase activities with ammonium sulfate

Conditions (gas phase prior to cell harvest)	H_2 (%) in assay vessel[a]	Hydrogenase activity (nmol of MB reduced/min per mg of protein) in 20% ammonium sulfate fraction:	
		Supernatant	Precipitate
Noninducing (95% argon, 5% air)	100	354.5	17.0
	11	2.1	7.4
Inducing (50% H_2, 5% air, 45% argon)	100	300.9	5.9
	11	4.9	364.0

[a] A hydrogen atmosphere of >80% was required for maximal activity of the constitutive hydrogenase. The 11% concentration of hydrogen was sufficient for maximal activity of the inducible hydrogenase.

and that the addition of $NiCl_2$ to a nickel-free growth medium activates these enzymes with no further protein synthesis being required. A final control showed that 50 μg of chloramphenicol per ml was sufficient to inhibit cell growth, another indicator of protein synthesis.

Fractionation of constitutive and inducible hydrogenase activities. Both hydrogenase activities in *M. trichosporium* were found in the supernatant fraction following centrifugation of a crude extract at 240,000 × *g* for 1 h. In contrast, in other aerobes such as *Azotobacter*, *Nocardia*, *Bradyrhizobium*, *Pseudomonas*, and *Alcaligenes* spp. and the facultative anaerobe *Escherichia coli*, all the nickel hydrogenases were membrane bound (see reviews by Arp [3] and Hausinger [16]). That the constitutive and inducible hydrogenases from *M. trichosporium* are really two distinct enzymes is seen from the fact that the constitutive enzyme remained soluble in the presence of 20% ammonium sulfate, whereas the inducible (i.e., low H_2 requiring) hydrogenase was found in the precipitate following centrifugation at 25,000 × *g* for 10 min (Table 3). Purification of these enzymes will be the subject of a later report.

DISCUSSION

The data presented here demonstrate that H_2 oxidation in *M. trichosporium* OB3b is readily coupled to N_2 fixation by two cytoplasmic hydrogenases, one that is maintained constitutively and a second that is induced by H_2. The inducible enzyme is repressed by CH_4 (and other oxidizable carbon substrates) and by common nitrogen compounds (NH_4^+ and NO_3^-). The repression of nickel hydrogenases by carbon compounds is similar to that found in *Alcaligenes eutrophus* (14, 25), *Bradyrhizobium japonicum* (21), and *Rhodobacter capsulatus* (formerly *Rhodopseudomonas capsulata*) (6). Thus, the inducible hydrogenase in *M. trichosporium* was only found in cells starved for an energy source and derepressed for nitrogenase. The complex relationship among H_2, CH_4, and NH_4^+ metabolism on expression of the inducible hydrogenase gene(s) remains to be elucidated. The inhibition of this hydrogenase by NH_4^+ and NO_3^- suggests that its synthesis may be linked to that of nitrogenase; however, we have no additional data to support this premise. Maier et al. (21) found that nitrogenase expression in *B. japonicum* was not necessary for H_2 uptake by free-living cells, and Colbeau et al. (6) also found that the synthesis of these two enzymes in *R. capsulatus* was independent. Siefert and Pfennig (26) concluded, however, from a study of *Rhodopseudomonas acidophilia* mutants that a regulatory linkage exists between nitrogenase and the uptake hydrogenase. The physiological roles of these methanotrophic hydrogenases remain to be determined, but the inducible enzyme is readily synthesized in resting-cell suspensions and could provide energy for cell maintenance under these conditions. This function is possible because of the ability of the enzyme to couple H_2 uptake to ATP synthesis.

Constitutive hydrogenases in general are believed to arise in bacteria as an adaptation to growth in ecosystems where low concentrations of H_2 are constantly generated. However, with the high level of H_2 needed to saturate the uptake activity of the constitutive hydrogenase from *M. trichosporium* (Fig. 1), it is not likely that this uptake function has any physiological significance. The relationship between the constitutive MB-reducing uptake hydrogenase reported here and the constitutive NAD-linked H_2-evolving enzyme reported by Kawamura et al. (17) is not yet clear, but it is possible they are the same enzyme. We confirmed (in whole cells) their observations of formate-dependent H_2 evolution and further found that if *M. trichosporium* was grown on a limiting N substrate where nitrogenase was induced, the H_2 evolved from formate was nil compared with that observed in cells grown on full NH_4^+ (91.4 nmol of H_2 per h per mg of cell protein). We also found that when *M. trichosporium* was grown on full nitrate medium, the intact cells were incapable of evolving H_2 from formate. These results suggest that both nitrogenase and nitrate reductase compete more effectively for reducing power generated by formate oxidation than does the hydrogenase.

Nickel is required for hydrogenase activity in *M. trichosporium*, but it is not required for the synthesis of these enzymes. This was seen by the fact that chloramphenicol had no effect on hydrogenase activation following the addition of nickel to nickel-depleted medium. This result is in contrast to that observed for the inducible hydrogenases from *A. eutrophus* (15), *B. japonicum* (18, 27), *A. chroococcum* (23), and *R. capsulatus* (6), in which nickel was required for derepression of hydrogenase. The activation by nickel of *M. trichosporium* hydrogenase in vivo and *N. opaca* hydrogenase in vitro has certain similarities, but the direct association of nickel with the enzymes from this methylotroph has not yet been demonstrated.

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LITERATURE CITED

1. **Aggag, M., and H. G. Schlegel.** 1974. Studies on a gram-positive hydrogen bacterium, *Nocardia opaca* 1b. III. Purification, stability and some properties of the soluble hydrogen dehydrogenase. Arch. Microbiol. **100**:25–39.
2. **Anthony, C.** 1981. Electron transport in methylotrophic bacteria, p. 220–230. *In* H. Dalton (ed.), Microbiol growth on C_1 compounds. Leyden and Son, London.
3. **Arp, D. J.** 1985. H_2-oxidizing hydrogenases of aerobic N_2-fixing micro-organisms, p. 121–131. *In* P. W. Ludden and J. E. Burris (ed.), Nitrogen fixation and CO_2 metabolism. Elsevier Science Publishing Inc., New York.
4. **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.
5. **Cammack, R., D. O. Hall, and K. K. Rao.** 1985. Hydrogenases: structure and applications in hydrogen production, p. 75–102. *In* R. K. Poole and C. S. Dow (ed.), Microbial gas metabolism. Academic Press, Ltd., London.
6. **Colbeau, A., B. C. Kelley, and P. M. Vignais.** 1980. Hydrogen-

ase activity in *Rhodopseudomonas capsulata*: relationship with nitrogenase activity. J. Bacteriol. **144**:141–148.

7. **Dalton, H., and D. J. Leak.** 1985. Methane oxidation by bacteria, p. 173–200. *In* R. K. Poole and C. S. Dow (ed.), Microbial gas metabolism. Academic Press, Ltd., London.
8. **Dalton, H., and R. Whittenbury.** 1976. The acetylene reduction technique as an assay for nitrogenase activity in the methane oxidizing bacterium *Methylococcus capsulatus* strain Bath. Arch. Microbiol. **109**:147–151.
9. **DeBont, J. A. M.** 1976. Hydrogenase activity in nitrogen-fixing methane-oxidizing bacteria. Antonie van Leeuwenhoek J. Microbiol. Serol. **42**:255–259.
10. **DeBont, J. A. M., and E. G. Mulder.** 1974. Nitrogen fixation and co-oxidation of ethylene by a methane-utilizing bacterium. J. Gen. Microbiol. **83**:113–121.
11. **Dixon, R. O. D.** 1972. Hydrogenase in legume root nodule bacterioids: occurrence and properties. Arch. Microbiol. **85**:193–201.
12. **Dixon, R. O. D.** 1976. Hydrogenases and efficiency of nitrogen fixation in aerobes. Nature (London) **262**:173.
13. **Emerich, D. W., T. Ruiz-Argueso, M. C. Te, and H. J. Evans.** 1979. Hydrogen-dependent nitrogenase activity and ATP formation in *Rhizobium japonicum* bacteriods. J. Bacteriol. **137**: 153–160.
14. **Friedrich, C. G.** 1982. Derepression of hydrogenase during limitation of electron donors and derepression of ribulosebisphosphate carboxylase during carbon limitation of *Alcaligenes eutrophus*. J. Bacteriol. **149**:203–210.
15. **Friedrich, B., E. Heine, A. Finck, and C. G. Friedrich.** 1981. Nickel requirement for active hydrogenase formation in *Alcaligenes eutrophus*. J. Bacteriol. **145**:1144–1149.
16. **Hausinger, R. P.** 1987. Nickel utilization by microorganisms. Microbiol. Rev. **51**:22–42.
17. **Kawamura, S., J. G. O'Neil, and J. F. Wilkinson.** 1983. Hydrogen production by methylotrophs under anaerobic conditions. J. Ferment. Technol. **61**:151–156.
18. **Klucas, R. V., F. J. Hanus, S. A. Russell, and H. J. Evans.** 1983. Nickel: a micronutrient element for hydrogen-dependent growth of *Rhizobium japoncium* and for expression of urease activity in soybean leaves. Proc. Natl. Acad. Sci. USA **80**:2252–2257.
19. **Li, J., C. Z. Hu, and D. C. Yoch.** 1986. Changes in amino acid and nucleotide pools of *Rhodospirillum rubrum* during switch-off of nitrogenase activity initiated by NH_4^+ or darkness. J. Bacteriol. **169**:231–237.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein determination with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
21. **Maier, R. J., F. J. Hanus, and H. J. Evans.** 1979. Regulation of hydrogenase in *Rhizobium japonicum*. J. Bacteriol. **137**:824–829.
22. **Murrell, J. C., and H. Dalton.** 1983. Nitrogen fixation in obligate methanotrophs. J. Gen. Microbiol. **129**:205–218.
23. **Partridge, C. D. P., and M. G. Yates.** 1982. Effect of chelating agents on hydrogenase in *Azobacter chrococcum*. Biochem. J. **204**:339–344.
24. **Peterson, R. B., and R. H. Burris.** 1978. Hydrogen metabolism in isolated heterocysts of *Anabaena* 7120. Arch. Microbiol. **116**:125–132.
25. **Schlegel, H. G., and U. Eberhardt.** 1972. Regulatory phenomenon in the metabolism of knoll gas bacteria. Adv. Microb. Physiol. **7**:205–242.
26. **Siefert, E., and N. Pfennig.** 1978. Hydrogen metabolism and nitrogen fixation in wild type and Nif⁻ mutants of *Rhodopseudomonas acidophila*. Biochimie **60**:261–265.
27. **Stults, L. W., E. B. O'Hara, and R. J. Maier.** 1984. Nickel is a component of hydrogenase in *Rhizobium japonicum*. J. Bacteriol. **159**:153–158.
28. **Toukdarian, A. E., and M. E. Lidstrom.** 1984. Nitrogen metabolism in a new obligate methanotroph 'Methylosinus' strain 6. J. Gen. Microbiol. **130**:1827–1837.
29. **Vignais, P. M., A. Colbeau, J. C. Willison, and Y. Jouanneau.** 1985. Hydrogenase, nitrogenase and hydrogenase metabolism in the photosynthetic bacteria. Adv. Microb. Physiol. **26**:155–234.
30. **Walker, C. C., and M. G. Yates.** 1979. The hydrogen cycle in nitrogen-fixing *Azotobacter chroococcum*. Biochimie **60**:225–231.