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Hydrogen-Mediated Enhancement of Hydrogenase Expression in *Azotobacter vinelandii*†

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Azotobacter vinelandii **cultures express more H_2 uptake hydrogenase activity when fixing N_2 than when provided with fixed N. Hydrogen, a product of the nitrogenase reaction, is at least partly responsible for this increase. The addition of H_2 to NH_4^+-grown wild-type cultures caused increased whole-cell H_2 uptake activity, methylene blue-dependent H_2 uptake activity of membranes, and accumulation of hydrogenase protein (large subunit as detected immunologically) in membranes. Both rifampin and chloramphenicol inhibited the H_2-mediated enhancement of hydrogenase synthesis. Nif⁻ *A. vinelandii* mutants with deletions or insertions in the *nif* genes responded to added H_2 by increasing the amount of both whole-cell and membrane-bound hydrogenase activities. Nif⁻ mutant strain CA11 contained fourfold more hydrogenase protein when incubated in N-free medium with H_2 than when incubated in the same medium containing Ar. N_2-fixing wild-type cultures that produce H_2 did not increase hydrogenase protein levels in response to added H_2.**

In one of their many pioneering studies, P. W. Wilson and colleagues observed that the activity of H_2 uptake hydrogenase in *Azotobacter vinelandii* increased when the cells were grown under N_2-fixing conditions (14), as compared with cells grown with fixed N. This and subsequent studies (11, 15, 20) showed that the activity in N_2-fixing cultures was about three- to fivefold greater than that in cultures grown with fixed N. In *Azotobacter chroococcum*, hydrogenase activity also is enhanced when cells are grown without an added fixed-N source (19).

The activities of uptake hydrogenases in some chemolithotrophic H_2-oxidizing bacteria increase when cells are incubated in the presence of H_2, and some H_2-oxidizing bacteria require incubation with H_2 to obtain hydrogenase (2, 5, 9). The amount of hydrogenase protein (detected immunologically) in the N_2-fixing bacteria *Alcaligenes latus* (8) and *Bradyrhizobium japonicum* (23) is dependent on the addition of H_2 during induction. However, *B. japonicum* does not require H_2 to induce hydrogenase when incubated under free-living N_2-fixing conditions (10). We investigated the possibility that H_2, a product of the nitrogenase reaction, can increase the level of hydrogenase enzyme in *A. vinelandii*.

Cultures were grown in Burk medium (7) containing 2% sucrose. When cells were grown with fixed N, ammonium acetate at 400 μg of N per ml was added. The medium contained Mo (1 μM as Na_2MoO_4) so that strains did not express an alternative nitrogenase system (4).

Cells were incubated in a closed gas system so that the effects of H_2 or Ar could be tested. Baffled flasks (2 liters) containing 400 ml of medium were inoculated with log-phase aerobically grown cells to an optical density of 0.2 at 540 nm (0.1 optical density unit = 2.2×10^7 viable cells per ml). The flasks were tightly stoppered, and 50 ml of H_2 or argon was injected (26). The flasks were incubated without shaking at room temperature for 1 h and then placed on a rotary shaker at 300 cycles per min at 30°C for 4 h. Similar incubations were done to determine the effect of H_2 on whole-cell activities with time (see Fig. 1). Cells were harvested by centrifugation and suspended in 50 mM potassium phosphate, pH 7.0. They were disrupted by passage through a French pressure cell at 10,000 lb/in^2 (25). The membranes were isolated (25) and suspended in 50 mM potassium phosphate, pH 7.0.

Hydrogen uptake activity in whole cells with oxygen as the electron acceptor or in membranes with methylene blue as the acceptor was determined amperometrically (24) as described previously (16, 17). The substrates (O_2, H_2, and methylene blue) were all used at saturation concentrations. N_2-flushed membrane samples were injected into the 5-ml amperometric chamber, and then 10 μl of 10 mM sodium dithionite was injected to remove residual O_2. O_2 was monitored polarographically in the chamber as described previously (16, 17). Methylene blue (50 μl of a 50 mM solution) was added, and then H_2 (75 nmol) was added to initiate the assay. Linear H_2 uptake activities were measured, and amount of uptake per minute was calculated based on the amount produced by the standard 50-μl H_2-saturated solution (37.7 nmol). Protein in membranes was determined by the dye-binding method of Bradford (6).

Hydrogenase protein was quantitated by performing immunoblots on nitrocellulose (22) and measuring the antigen-antibody complex by immunoperoxidase staining with 2,2′-azino-di(3-ethylbenzthiazolinesulfonate)(ABTS)(Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as the chromogenic substrate. Membrane protein samples (20 μg of protein) were loaded onto nitrocellulose strips, and the strips were blocked with BLOTTO (13) and then treated with the anti-*B. japonicum* affinity-purified 65-kilodalton subunit antibody (21) at a dilution of 1:500 for 10 h at 37°C. Antiserum against the *B. japonicum* hydrogenase has been shown to cross-react with *A. vinelandii* hydrogenase (3). After being washed with 50 mM Tris hydrochloride (pH 7.4)–0.9% NaCl, the blots were treated with secondary antibody (peroxidase-conjugated goat anti-rabbit serum at a 1:2,000 dilution in BLOTTO) for 6 h. The strips were then cut, and individual blots were treated with 0.5 ml of ABTS solution according to the instructions of the manufacturer for 2 h. The reaction was stopped by adding 0.5 ml of 10% sodium dodecyl sulfate solution, and the A_{415} was determined with ABTS solution

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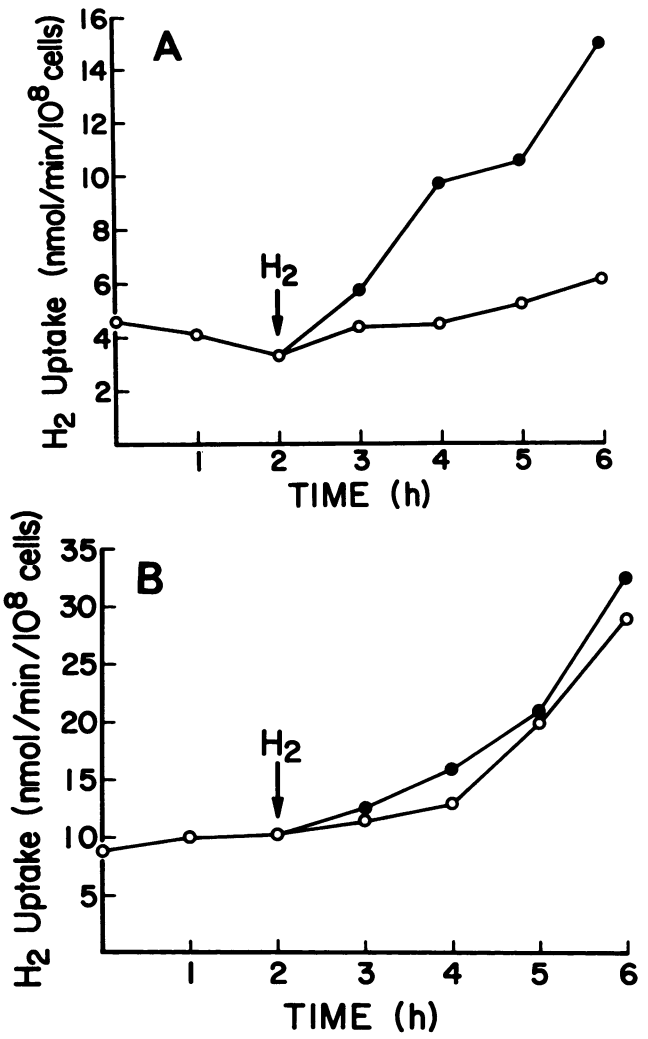

FIG. 1. Effect of H_2 or Ar addition on the O_2-dependent hydrogenase activity of *A. vinelandii* CA growing in ammonium medium (A) or N-free medium (B). Cells were suspended into each of two flasks (2 liters) with 400 ml of medium at about 2.8×10^7 viable cells per ml and incubated as described in the text. After 2 h, H_2 (●) or Ar (○) was injected into the flask, O_2 was injected hourly as needed, and H_2 uptake activity was determined at the times indicated (see text). From the time of injection of H_2 or Ar (arrow) until the final time point (6 h), the viable cell number went from about 4×10^7 to 9×10^7 per ml. Each point before addition of the gas is the average of six values, three from each of the two identical flasks. After the addition of the gas (H_2 or Ar), each hourly point is the average of three samples.

alone as a blank. Western gels (18) of *A. vinelandii* membranes demonstrated predominantly a single reaction with the anti-65-kilodalton antiserum at a molecular mass of approximately 65-kilodaltons. Purified *B. japonicum* hydrogenase was used as a positive control; 0.05 μg of pure hydrogenase had an A_{415} of 0.452 by the ABTS method described above.

Cultures incubated in ammonium medium had hydrogenase activities of about 3 to 6 nmol/min per 10^8 cells. However, when H_2 was added to such cultures (Fig. 1A), activities increased to about 15 nmol/min per 10^8 cells in 4 h. This result indicated that H_2 could play a role in increasing hydrogenase expression. This effect was probably not due to lowering of the O_2 concentration caused by respiration-linked H_2 oxidation, since O_2 was periodically monitored and was injected hourly to maintain O_2 levels at 20% partial pressure. There was considerable variation in hydrogenase activities of N_2-fixing cultures; they had whole-cell hydrogenase activities of about 9 to 30 nmol/min per 10^8 cells. In contrast to the findings with NH_4-incubated cultures, added H_2 did not appreciably increase the hydrogenase activity in N_2-fixing cultures (Fig. 1B), and this result was clear in many experiments. In N_2-fixing cultures, H_2 is produced as a by-product of the nitrogenase reaction; thus, the latter cultures may already have enhanced hydrogenase due to the internally produced H_2. N_2-fixing cultures incubated with either H_2 or Ar increased hydrogenase activity with time (Fig. 1). It is possible that this increase in hydrogenase was due to a limitation of carbon substrates as the cell number increased; regulation (increase in hydrogenase) by C source limitation has been observed for *A. chroococcum* (1). The viable cell number increased more than twofold during the 4-h incubation period in the sealed flask (see Fig. 1 legend).

From Fig. 1, we cannot determine the means by which H_2 increases hydrogenase activity. For example, the effect of H_2 could be due to the activation of preformed hydrogenase by H_2, or it could be at the level of mRNA or protein synthesis. Therefore, the experiment was repeated, but in addition to injection of H_2, at 2 h rifampin or chloramphenicol was also added to inhibit mRNA or protein synthesis, respectively. Both of the inhibitors effectively blocked further increases in hydrogenase activity, whereas without the inhibitors the H_2 stimulation effect was evident (Fig. 2). Therefore, the H_2 enhancement affect appears to be at the transcriptional level.

The results shown in Fig. 1 and 2 indicate that H_2 plays a role in amplifying hydrogenase synthesis. However, in all of

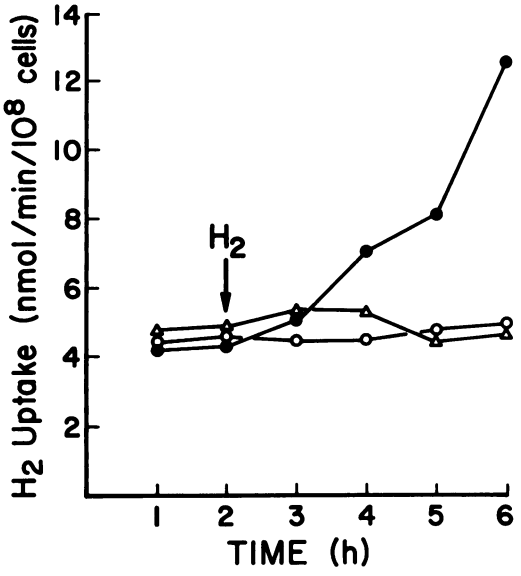

FIG. 2. Effect of addition of H_2, rifampin, or chloramphenicol on H_2-mediated enhancement of O_2-dependent hydrogenase activity. Three cultures in ammonium medium were set up as described in the legend to Fig. 1 and the text. H_2 (50 ml) was injected into all three 2-liter flasks along with 100 μg of rifampin per ml (△), 50 μg of chloramphenicol per ml (○), or no other addition (●) at 2 h. O_2 was injected hourly as needed, and H_2 uptake rates were determined (see text). From the 2-h point (arrow) until 6 h, the viable cell number went from about 5.5×10^7 to 1.4×10^8 per ml in the control culture without inhibitors.

TABLE 1. Hydrogenase activities and hydrogenase protein (large subunit) levels in wild-type strain CA and mutant strains CA11 and CA30 grown under N_2-fixing conditions[a]

Strain	Addition	Hydrogenase activity		ABTS reaction (A_{415})[d]
		Whole cells (nmol/min per 10^8 cells)[b]	Membranes (nmol/min per mg of protein)[c]	
CA	H_2	26.0 ± 3.7	47.4 ± 9.6	0.27 ± 0.06
	Ar	18.4 ± 1.4	38.1 ± 6.5	0.30 ± 0.08
CA11	H_2	68.1 ± 4.4	143.8 ± 11.2	0.47 ± 0.07
	Ar	37.3 ± 3.6	51.2 ± 8.1	0.12 ± 0.03
CA30	H_2	8.4 ± 0.8	26.5 ± 4.3	ND
	Ar	5.1 ± 0.9	11.2 ± 1.9	ND

[a] Strains were incubated with H_2 or Ar for 4 h in Burk N-free medium as described in the text. Whole cells and membranes were assayed with O_2 and methylene blue, respectively, as electron acceptors. The A_{415} was determined from immunoblots on nitrocellulose treated with ABTS as the chromogenic substrate (see text). Purified *B. japonicum* hydrogenase (0.05 μg) had an A_{415} of 0.45 as determined by the ABTS method described in the text.

[b] Mean ± standard deviation for six samples.

[c] Mean ± standard deviation for eight samples (two from each of four different membrane preparations).

[d] Mean ± standard deviation from four separate immunoblots. ND, Not done.

those experiments, the assay for hydrogenase was O_2-dependent H_2 uptake. This measures the complete H_2 oxidation pathway. Methylene blue is an excellent acceptor for the purified hydrogenase; therefore, we measured methylene blue-dependent activity in membranes from cells harvested at the 6-h points of experiments like those shown in Fig. 2. From five membrane samples of cells harvested at 6 h, the methylene blue-dependent H_2 uptake activity of the culture receiving H_2 alone at 2 h was 35.6 ± 1.8 nmol/min per mg of protein (mean ± standard deviation). The cultures receiving argon alone and rifampin (100 μg/ml) plus H_2 at 2 h had membrane activities at 6 h of 12.4 ± 1.4 and 11.0 ± 1.6 nmol/min per mg of protein, respectively. Therefore, the enhancement effect of H_2 appears to be on the level of the hydrogenase enzyme itself rather than on that of the associated electron transport chain. Green and Wilson (11) observed greater methylene blue-dependent H_2 oxidation of extracts from N_2-fixing cultures than from ammonium-grown cultures of *A. vinelandii*.

Strains CA, CA11, and CA30 were supplied by Paul Bishop, North Carolina State University. Strain CA11 is a *nif* deletion mutant (4), and strain CA30 is a Tn*5* insertion strain (12) that lacks Nif activity due to a deficiency in the FeMo cofactor. Both mutants have undetectable whole-cell acetylene-reducing activity even in N-free medium with molybdenum added (4, 12). Therefore, they probably do not produce H_2. The Nif$^-$ mutants of *A. vinelandii* with deletions or insertions in known *nif* genes were used to test the effect of H_2. When incubated under N_2-fixing conditions with added H_2, the wild-type strain CA had whole-cell H_2 uptake activities, methylene blue-dependent H_2 uptake activities of membranes, and large-subunit hydrogenase protein levels similar to those of Ar-incubated cultures (Table 1). However, the Nif$^-$ mutant strains CA11 and CA30 responded to H_2 addition; they exhibited significantly greater whole-cell and membrane activities in the presence of H_2 than when incubated with Ar. The level of the hydrogenase large subunit as detected immunologically was nearly fourfold greater for strain CA11 in H_2 than for CA11 in Ar (Table 1). Both the wild type and mutant CA11 responded to H_2 addition in ammonium-containing medium (Table 2), conditions under which neither strain produced H_2. The levels of whole-cell activity, membrane activity, and 65-kilodalton protein all increased about two- to threefold for both strains. The results indicate that H_2, either exogenously added or as a product of the nitrogenase reaction, enhances hydrogenase synthesis in *A. vinelandii*.

TABLE 2. Hydrogenase activities and hydrogenase protein (large subunit) levels of wild-type strain CA and mutant strain CA11 grown with ammonium ions[a]

Strain	Addition	Hydrogenase activity		ABTS reaction (A_{415})[d]
		Whole cells (nmol/min per 10^8 cells)[b]	Membranes (nmol/min per mg of protein)[c]	
CA	H_2	21.1 ± 4.4	31.9 ± 6.8	0.52 ± 0.16
	Ar	8.6 ± 1.2	10.1 ± 2.1	0.25 ± 0.02
CA11	H_2	21.1 ± 5.1	26.2 ± 4.3	0.59 ± 0.19
	Ar	10.6 ± 2.3	8.6 ± 1.0	0.23 ± 0.10

[a] All conditions and assays were as described in the text and in Table 1, footnote *a*.

[b] Mean ± standard deviation for six samples.

[c] Mean ± standard deviation for eight samples (two from each of four different membrane preparations).

[d] Mean ± standard deviation from four separate immunoblots.

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