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Hydrogenase Activity in *Azospirillum brasilense* Is Inhibited by Nitrite, Nitric Oxide, Carbon Monoxide, and Acetylene

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Nitrite, NO, CO, and C_2H_2 inhibited O_2-dependent H_2 uptake (H^3H oxidation) in denitrifying *Azospirillum brasilense* Sp7 grown anaerobically on N_2O or NO_3^-. The apparent K_i values for inhibition of O_2-dependent H_2 uptake were 20 μM for NO_2^-, 0.4 μM for NO, 28 μM for CO, and 88 μM for C_2H_2. These inhibitors also affected methylene blue-dependent H_2 uptake, presumably by acting directly on the hydrogenase. Nitrite and NO inhibited H_2 uptake irreversibly, whereas inhibition due to CO was easily reversed by repeatedly evacuating and backfilling with N_2. The C_2H_2 inhibition was not readily reversed, partly due to difficulty in removing the last traces of this gas from solution. The NO_2^- inhibition of malate-dependent respiration was readily reversed by repeatedly washing the cells, in contrast to the effect of NO_2^- on H_2-dependent respiration. These results suggest that the low hydrogenase activities observed in NO_3^--grown cultures of *A. brasilense* may be due to the irreversible inhibition of hydrogenase by NO_2^- and NO produced by NO_3^- reduction.

Azospirillum brasilense is an N_2-fixing bacterium which occurs in high numbers in the rhizosphere of various tropical grasses (26). *A. brasilense* is very versatile in its nitrogen transformations since, in addition to fixing N_2 under microaerobic conditions (8, 13, 15), it denitrifies under anaerobic conditions (9, 13, 14) and assimilates NH_4^+, NO_3^- (8), or NO_2^- (14).

A. brasilense is one of the N_2-fixing bacteria with more than sufficient H_2 uptake hydrogenase activity to recycle the H_2 produced by nitrogenase (7) and is capable of H_2-dependent C_2H_2 reduction (16). The H_2 uptake system is sensitive to O_2 (16), but its O_2 optimum is significantly higher than that for nitrogenase activity (23), suggesting that H_2 uptake may have a limited ability to support respiratory protection of the even more O_2-sensitive nitrogenase. In *A. brasilense*, hydrogenase expression requires microaerobic or anaerobic conditions, is apparently enhanced by electron donor limitation, does not require exogenous H_2, and is independent of nitrogenase expression (23, 24).

We have previously shown that H_2 uptake activity is 50 to 100 times higher in denitrifying cultures of *A. brasilense* when the terminal electron acceptor for growth is N_2O rather than NO_3^- and that H_2 uptake is supported by NO_2^- only in cells grown with NO_3^- (24). As part of an attempt to explain these phenomena, we now present observations on the inhibition of H_2 uptake by NO_2^-, NO, CO, and C_2H_2.

MATERIALS AND METHODS

Bacterial strain, growth medium, and culture conditions. *A. brasilense* Sp7 (ATCC 29145) was grown in the defined medium previously described (13) and modified (23, 24), which contained 3.7 mM L-malic acid and had an initial pH of 6.8. All of the media used in the present experiments contained 18.7 mM NH_4Cl as the nitrogen source to repress nitrogenase synthesis. The medium was supplemented with 71 μM and 0.5 mM KNO_3 for N_2O-grown and NO_3^--grown cultures, respectively. Culture conditions were as previously described (23, 24) except for the following changes. The 900-ml batch cultures grown microaerobically were sparged at 450 ml min^{-1} with a mixture of 0.75% O_2 in N_2 with or without 5% H_2. Cultures grown anaerobically were sparged at 240 or 120 ml min^{-1} with 10% N_2O in N_2 or 5% H_2 in N_2 for N_2O-grown or NO_3^--grown cultures, respectively. The gases were obtained from Union Carbide Corp., New York, N.Y., or Liquid Carbonic Canada, St. Laurent, Quebec. Aerobic cultures were grown in 50 ml of medium (containing 7.5 mM L-malic acid) in 125-ml flasks on a gyratory shaker (300 rpm) at 30°C.

Uptake hydrogenase assay. Hydrogen uptake was measured by the H^3H oxidation method previously described (23, 24), usually in 50-ml serum-stoppered Erlenmeyer flasks with 5-ml culture samples. Assays were started by the addition of 2.6% H_2 and 0.4% H^3H. The H^3H (specific activity, 17.0 μCi ml^{-1} as of August 1983) was obtained and stored in a lecture bottle (Matheson Canada, Whitby, Ontario). Oxygen (12 μM), N_2O (1 mM), and methylene blue (5 mM) were used as electron acceptors.

The reversibility of NO_2^- inhibition was tested by exposing 35-ml culture samples in anaerobic serum-stoppered centrifuge tubes to 1 mM KNO_2 for 15 min and then anaerobically washing the cells three times with N_2-sparged carbon- and nitrogen-free medium (pH 7.0). The centrifugation steps were performed at 5,900 × *g* for 10 min. The complete removal of NO_2^- by the wash treatment was later confirmed by the method described below. Control culture samples were also subjected to the anaerobic wash treatment and then assayed for H_2 uptake in the presence and absence of NO_2^- by using 5-ml cell suspensions.

For the gas reversibility experiments, 8-ml serum bottles with 1-ml culture samples were used. The bottles were capped with gray butyl rubber stoppers which had been reinforced with silicone rubber. The culture samples were exposed to the inhibitor for 15 min, and then the serum bottles were evacuated and backfilled with N_2 nine times. The gas phases were then checked for residual inhibitor by gas chromatography. Control culture samples were also subjected to evacuation and backfilling and then assayed for H_2 uptake in the presence and absence of inhibitors. Assays were started by adding 4% H^3H (giving 30 μM in solution).

The reaction vessels were incubated on a gyratory water

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bath shaker (250 rpm) at 30°C. Aqueous samples of 0.2 or 1.0 ml were removed and prepared for liquid scintillation counting as previously described (23). All H_2 uptake data are expressed as means of triplicate flasks ± the standard error.

Respiration studies. Oxygen uptake was measured by using a YSI 4004 Clark oxygen probe (Yellow Springs Instruments, Yellow Springs, Ohio) in a 2.2-ml reaction chamber (Gilson Medical Electronics, Middleton, Wis.) at 30°C. The reversibility of the NO_2^- inhibition of O_2 uptake in N_2O-grown cells was tested with the same cells used for the NO_2^- inhibition of H_2 uptake. After washing, 2.2 ml of the anaerobic cell suspension was added to the N_2-flushed reaction chamber, and the capillary bore stopper was inserted. L-Malic acid was added to give a final concentration of 3.7 mM. When the cell suspension was completely anaerobic, as determined from the recorder trace, a spike of O_2-saturated carbon- and nitrogen-free medium was added to give a final concentration of 15 μM, and the rate of O_2 uptake was determined.

To test for the reversibility of NO_2^- inhibition of O_2 uptake in air-grown cells, four 50-ml aerobic cultures were grown to early stationary phase, combined, washed three times with carbon- and nitrogen-free medium, and suspended in 100 ml of the same medium. The cells were kept aerobically on a gyratory water bath shaker (250 rpm) at 30°C until they were assayed. A portion of the cell suspension was incubated in the presence of 2 mM KNO_2 for 8 h and then washed three times with carbon- and nitrogen-free medium. Samples of these suspensions were added to the reaction chamber, the capillary bore stopper was inserted, and each assay was started by adding 3.7 mM L-malic acid (final concentration) to the air-saturated cell suspension.

Analyses. Acetylene and CO were measured by the same gas-chromatographic systems as for C_2H_4 (23) and O_2 (6), respectively. Protein was determined by a modification of the Lowry method (23) with bovine serum albumin as the standard. Colorimetric tests for NO_3^- and NO_2^- were as previously described (24), except that cells were removed by centrifugation at 15,600 × *g* for 4 min with an Eppendorf 5414 microcentrifuge (Brinkmann Instruments Inc., Rexdale, Ontario) instead of by filtration. Ostwald coefficients (28) were used to calculate the concentrations of gases in solution. Nitric oxide and C_2H_2 were obtained from Matheson Canada, Whitby, Ontario, and CO was obtained from Canlab, Montreal, Quebec.

RESULTS

Inhibition of O_2-dependent H_2 uptake. Since we had previously shown that NO_2^- alone does not support and apparently inhibits H_2 uptake in N_2O-grown *A. brasilense* (24), it was of interest whether NO_2^- or any of the other intermediates of denitrification inhibited O_2-dependent H_2 uptake. We found that NO as well as NO_2^- inhibited H_2 uptake, whereas N_2O and NO_3^- had little effect (Table 1). The results were not due to pH effects because the neutral pH of the reaction mixtures was not affected by the addition of NO_2^- and only slightly decreased by NO.

TABLE 1. Inhibition of O_2-dependent H_2 uptake in N_2O-grown *A. brasilense*[a]

Treatment (concn)	H_2 uptake[b]	% Inhibition
1% O_2 (12 μM)	311 ± 15	
1% O_2 + N_2O (1 mM)	293 ± 10	11
1% O_2 + NO_3^- (0.5 mM)	252 ± 29	24
1% O_2 + NO_2^- (0.5 mM)	16 ± 2	95
1% O_2 + NO (0.1 mM)	4 ± 1	99

[a] Stationary-phase culture containing 89 μg of protein ml^{-1}.
[b] Activities expressed as nanomoles of H_2 per milligram of protein min^{-1}. Assays were 15 min long.

TABLE 2. Apparent K_i values for the inhibition of O_2-dependent H_2 uptake in *A. brasilense*[a]

Inhibitor	Apparent K_i (μM)[b]	No. of determinations
NO	0.4 ± 0.1	3
NO_2^-	20 ± 3	3
CO	28 ± 3	3
C_2H_2	88 ± 19	4

[a] Stationary-phase cultures grown anaerobically with N_2O or microaerobically.
[b] Determined from plots of percent inhibition versus log of inhibitor concentration at the point of 50% inhibition. Assays were 15 min long except for two of the C_2H_2 determinations, which were 24 min long. Results are means ± standard error for the number of determinations indicated.

Carbon monoxide and C_2H_2 were included in our studies because they have previously been shown to strongly inhibit H_2 uptake in *A. brasilense* (7). The inhibitor concentrations resulting in 50% inhibition (apparent K_i values) of O_2-dependent H_2 uptake (Table 2) indicate that NO (K_i = 0.4 μM) had the greatest affinity for the enzyme system of the inhibitors tested, followed by NO_2^- (K_i = 20 μM), CO (K_i = 28 μM), and C_2H_2 (K_i = 88 μM).

Nitrite (1 mM), NO (0.1 mM), CO (0.3 mM), and C_2H_2 (1 mM) also inhibited N_2O-dependent H_2 uptake in N_2O-grown cells by 88, 100, 74, and 75%, respectively. This suggested that there was at least one site of inhibition common to the electron transport pathways leading to N_2O and O_2.

Inhibition of methylene blue-dependent H_2 uptake. Methylene blue is assumed to accept electrons directly from hydrogenase and has been used to measure hydrogenase activity specifically (16). Nitrite, NO, CO, and C_2H_2 inhibited methylene blue-dependent H_2 uptake (Table 3), suggesting that they acted directly against the hydrogenase. Only the CO inhibition was easily reversed. The inhibition due to C_2H_2 was not readily reversed, partly because of difficulty in removing the last traces of C_2H_2 from solution. However, the amount remaining after repeatedly evacuating and backfilling (3 μM) was well below the K_i (88 μM) and therefore probably not enough to explain all of the inhibition observed. The inhibitory effects of NO_2^- and NO were irreversible.

Inhibition of malate-dependent respiration. To determine whether NO_2^- could inhibit electron transport in the absence of H_2, we studied malate-dependent O_2 uptake in cells that possessed hydrogenase activity (N_2O grown) and in those that did not (air grown). Nitrite (1 mM and 2 mM for

TABLE 3. Inhibition and reversibility of inhibition of methylene blue-dependent H_2 uptake in N_2O-grown *A. brasilense*[a]

Treatment	% Inhibition[b] with:	
	Inhibitor present	Inhibitor removed
NO_2^- (1 mM)	95	88
NO (0.1 mM)	100	100
CO (0.3 mM)	97	8
C_2H_2 (4 mM)	82	45

[a] Stationary-phase cultures containing 64 μg of protein ml^{-1} after washing for the NO^- treatments and 98 μg of protein ml^{-1} for the gas treatments.
[b] The uninhibited H_2 uptake rates were 58 ± 2 and 430 ± 30 nmol of H_2 per mg of protein min^{-1} for NO_2^- and gas treatments, respectively. Assays were 18 min long for the NO_2^- treatments and 30 min long for the gas treatments.

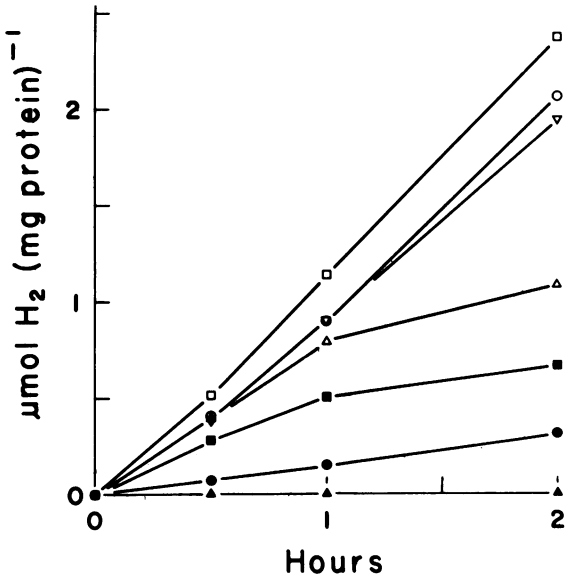

FIG. 1. Inhibition of methylene blue-dependent H_2 uptake in NO_3^--grown *A. brasilense*. Samples were taken when there was no detectable NO_3^- or NO_2^- in the 900-ml culture and the protein concentration was 68 μg ml^{-1}. Culture samples were assayed in the presence of methylene blue (5 mM) alone (○) and supplemented with 0.5 mM NO_3^- (□), 1 mM N_2O (▽), 0.5 mM NO_2^- (△), 3.4 mM C_2H_2 (■), 44 μM CO (●), or 90 μM NO (▲). For liquid scintillation counting, 0.2-ml samples were removed by an N_2-flushed syringe from the assay flasks at the times indicated and immediately dispensed into 10 ml of scintillation fluid.

N_2O- and air-grown cells, respectively) inhibited malate-dependent O_2 uptake in both types of cells. The inhibition was reversible; about 90% of the original activity was restored by washing the cells repeatedly.

Nitrate-grown cells. Nitrite, when present as the sole electron acceptor, can support H_2 uptake in NO_3^--grown cells (24). However, methylene blue-dependent (Fig. 1) or O_2-dependent (data not shown) H_2 uptake in NO_3^--grown cells was inhibited by NO_2^-, NO, CO, and C_2H_2. The inhibition was followed over 2-h time courses because of the low hydrogenase activities in NO_3^--grown cells.

DISCUSSION

We previously observed that in denitrifying cultures of *A. brasilense*, uptake hydrogenase activity was much higher when the terminal electron acceptor for growth was N_2O rather than NO_3^- (24). Our present results show that two of the intermediates of the denitrification pathway, NO_2^- and NO, inhibit hydrogenase activity irreversibly and have low apparent K_i values, 20 and 0.4 μM, respectively. These are very low concentrations compared with the 0.5 mM NO_2^- to which NO_3^--grown cultures were at times exposed (24). Therefore, all of the hydrogenase synthesized in NO_3^--grown cultures may not have been expressed due to irreversible inhibition by NO_2^- and NO.

Nitrate-grown cells possess high levels of all the nitrogen oxide reductases (Lalande and Knowles, unpublished data) and can use NO_2^- as the electron acceptor for H_2 oxidation (24). However, we have now shown that NO_2^- inhibits O_2- or methylene blue-dependent H_2 uptake in NO_3^--grown cells. Nitrogen oxide reductases in whole cells are generally immediately or gradually inhibited by O_2, possibly due to competition for electrons rather than to an inactivation of the enzyme itself by O_2 (2, 10). Therefore, in the presence of O_2 or methylene blue the rate of NO_2^- reduction was probably decreased, leaving NO_2^- to exert inhibitory effects on the cells.

Nitrite inhibits a variety of processes in bacteria, including active transport, O_2 uptake, oxidative phosphorylation, and nitrogen fixation (18, 25). These results are consistent with the oxidation of ferrous iron of the electron carriers to ferric iron (18) or the reaction of iron-sulfur proteins with added NO_2^- to form inactive iron-nitric oxide complexes (17).

We have shown that in *A. brasilense*, NO_2^- inhibits malate-dependent respiration reversibly, as in *Pseudomonas aeruginosa*, in which the inhibitory effect of NO_2^- is readily reversed by washing the cells (18). Hydrogen-dependent respiration in *A. brasilense*, on the other hand, is inhibited irreversibly by NO_2^-, which apparently directly inactivates the iron-sulfur protein hydrogenase.

Iron compounds, both inorganic and organic, are known to form complexes with NO (21). Hydrogenase in *Proteus vulgaris* is irreversibly inhibited by NO, suggesting that it is an iron-containing enzyme (11). In *A. brasilense*, hydrogenase is also irreversibly inhibited by NO.

Since the pH of the reaction mixtures was near 7, there should have been little abiotic conversion of NO_2^- to HNO_2 and NO, which is favored by low pH (20). Nitrite inhibited H_2 uptake in microaerobically grown cells and malate respiration in aerobically grown cells, conditions which do not favor derepression of the nitrogen oxide reductases. Therefore, NO_2^- would seem to have inhibitory effects of its own apart from any possible production of NO. The increasing effect of NO_2^- with time on H_2 uptake in NO_3^--grown cells may have been due to the reduction of NO_2^- to NO, but this process had to compete for electrons with O_2 or methylene blue in the reaction mixtures and therefore probably did not occur at a high rate. Hydrogenase inactivation by NO_2^- could also explain the results. In any case, even if some of the NO_2^- was reduced to NO, our conclusion remains unchanged. The low hydrogenase expression in NO_3^--grown cells may be due to inhibition of activity by the NO_2^- and NO produced in these cultures.

Almost all purified hydrogenases are inhibited by CO, and this inhibition is completely reversed by flushing the reaction mixture with an inert gas to remove the CO (1). Carbon monoxide inhibits H_2 uptake in whole cells of *A. brasilense*, and nitrogenase-mediated H_2 evolution can be observed only in the presence of CO (3, 7). Our data complement these results by showing that the CO inhibition is readily reversed by repeatedly evacuating and backfilling the reaction vessels with N_2 and that the apparent K_i is 28 μM.

Acetylene has no effect on hydrogenases in soybean or pea root nodule bacteroids (4, 19) or in free-living *Rhizobium japonicum* grown chemolithotrophically with NO_3^- as the terminal electron acceptor (12). However, in *Azotobacter chroococcum*, C_2H_2 inhibits H_2 uptake irreversibly (27), and a pretreatment involving exposure to 40% C_2H_2 and its subsequent removal is used to increase the observable rate of H_2 evolution from nitrogenase. Chan et al. (7) found that 10% C_2H_2 (3.3 mM in solution) almost completely inhibited H_2 uptake but that a C_2H_2 pretreatment was not effective in increasing H_2 evolution by *A. brasilense*, suggesting that the inhibition is reversible. Our results show that the C_2H_2 inhibition of H_2 uptake has an apparent K_i of 88 μM and is not readily reversed, although the possibility remains that if all of the C_2H_2 could have been removed, the inhibition would have been reversed.

The relative sensitivity of uptake hydrogenase in whole cells of *Anabaena cylindrica* is similar to that of *A. brasilense*; the K_i for CO (0.5 mM) is lower than that for C_2H_2 (15 mM) (5). The *Azotobacter chroococcum* uptake hydrogenase seems to be more sensitive to C_2H_2 than to CO (22), although no comparative K_i values have been reported.

Our results, which include apparent K_i values and reversibility data, more clearly define the nature of the inhibition of hydrogenase activity in *A. brasilense* by CO and C_2H_2. The results also suggest that the low hydrogenase activities in NO_3^--grown cultures may simply be due to the irreversible inhibition of hydrogenase by NO_2^- and NO rather than to some more complex regulatory mechanism.

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