Hydrogen-uptake Hydrogenase Activity in Nitrogen-fixing
Azospirillum brasilense

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N₂-fixing Azospirillum brasilense possesses an H₂-uptake hydrogenase activity capable of supporting H₂-dependent acetylene reduction by whole cells starved of carbon metabolites. H₂ did not support acetylene reduction in carbon-sufficient bacteria, although carbon substrates did not inhibit H₂-dependent respiration. H₂-dependent respiration was extremely O₂ sensitive and could not usually protect nitrogenase against inhibition by O₂. H₂ partly inhibited nitrogenase activity at sub-optimal O₂ concentrations: this may be because H₂-dependent respiration has a greater affinity for O₂ but is less efficiently coupled to ATP production than is carbon-dependent respiration.

INTRODUCTION

Several aerobic N₂-fixing bacteria possess an H₂-uptake hydrogenase which may recycle H₂ produced by nitrogenase in vivo (Bothe et al., 1977; Walker & Yates, 1978; Emerich et al., 1979). Dixon (1972) postulated that the uptake hydrogenase of N₂-fixing bacteria could respire H₂ to: (a) protect nitrogenase against inactivation by O₂; (b) recover by oxidative phosphorylation some of the energy lost through ATP-dependent H₂ production by nitrogenase; and (c) prevent inhibition of N₂ reduction by H₂. Hydrogenase activity can support acetylene reduction by nitrogenase and protect the enzyme against inactivation by O₂ in Azotobacter chroococcum (Walker & Yates, 1978), Rhizobium japonicum bacteroids (Emerich et al., 1979) and Anabaena cylindrica (Bothe et al., 1977), thus providing evidence that roles (a) and (b) for the H₂-uptake hydrogenase occur in vivo. Walker & Yates (1978), however, showed that H₂ produced by nitrogenase in A. chroococcum dispersed too rapidly to inhibit N₂ reduction.

Azospirillum species (Tarrand et al., 1978) are aerobic N₂-fixing bacteria which also contain an H₂-uptake hydrogenase activity (Stephan et al., 1981). In the present paper we present evidence that H₂ can support nitrogenase activity (acetylene reduction) of Azospirillum brasilense only under carbon-limited conditions, although carbon substrates do not inhibit H₂-dependent respiration. However, H₂ cannot support respiratory protection of nitrogenase because O₂-dependent H₂-uptake is itself extremely sensitive to inhibition by O₂.

METHODS

Organism and growth. Azospirillum brasilense Sp7 (ATCC 29145) was grown under N₂-fixing conditions at 37 °C in the medium described by Okon et al. (1976) supplemented with biotin (100 µg l⁻¹) and sodium lactate (1 g l⁻¹) or sodium malate (5 g l⁻¹) (Nfb medium). The partial pressure of O₂ in the medium was monitored with a
sterilizable oxygen electrode connected to an FL 3 oxygen meter (Western Biological Equipment Ltd, Sherbourne, Dorset, U.K.) and maintained at 0.003 atm. The bacteria were harvested after 16 h by centrifuging under N₂ (6000 g, 30 min), resuspended in a minimum volume of C- and N-free medium without added carbon substrate and stored in liquid N₂.

**Hydrogenase activity.** H₂ uptake by hydrogenase was measured by Warburg manometry at 37 °C with methylene blue (30 mM) as the electron acceptor, in 100 mM-Tris/HCl buffer (pH 8.5) or 100 mM-sodium phosphate buffer (pH 6.8) containing EDTA (3 mM) and NaF (100 mM) (Walker & Yates, 1978). Controls indicated no gas consumption in the absence of methylene blue.

Alternatively, hydrogenase activity was measured by the rate of H₃H uptake with methylene blue (10 mM) as an electron acceptor. H²H gas was produced by reacting sodium borohydride (from Amersham) with distilled water, then diluted 5000-fold with H₂. The resulting mixture (1-0 to 1.5 µCi ml⁻¹; 37 to 55 kBq ml⁻¹) was used as a stock. The reaction mixtures (1 ml), as described above, were incubated in 7 ml serum bottles under Ar containing H₂ + H₃H (1 ml) at 37 °C for 30 min. The reaction was stopped by removing the Subaseal to let the H₂ disperse. Incorporation of H into the aqueous phase was estimated in 40 µl samples using a Nuclear Enterprises scintillation counter with NEN250 (10 ml) as the scintillation fluid. A correction was made for quenching by the chromophore.

O₂-dependent H₂ uptake. Cells were suspended in C- and N-free Nfb medium to an A₅₆₀ of 0.3 to 0.4 and preincubated under Ar/O₂ (99:1, v/v) at 37 °C for 1 h to decrease endogenous carbon levels. Serum bottles (7 ml) containing bacteria (1 ml) under an atmosphere of Ar with 15% (v/v) H₂ + H₃H and different O₂ concentrations were shaken at 37 °C for 1 h. The reaction was stopped with 0.1 ml 40% (w/v) KOH and incorporation of H into the aqueous phase was measured in 0-2 ml samples as described above.

Anaerobic H₃H uptake. Cultures (1 ml) were incubated at 37 °C in the side arm of a 25 ml conical flask under Ar/H₂ + H₃H (9:1, v/v) for 1 h. The main compartment of the flask contained 100 mM-Na₂S₂O₄ (5 ml) to scavenge traces of O₂. H₃H uptake was determined as above.

Nitrogenase activity. Bacterial suspensions were prepared as above and the assay was as for O₂-dependent H₃H uptake except that acetylene (8%, v/v) replaced H₂ + H₃H. Ethylene was measured in a Pye 104 gas chromatograph fitted with a flame ionization detector.

O₂ sensitivity of hydrogenase. Cell suspensions (1 ml) were exposed to different concentrations of O₂ for 1 h under the conditions described above, without acetylene. The O₂ was then replaced by Ar and hydrogenase activity determined by H₃H incorporation with methylene blue or O₂ as the electron acceptor at pH 6-8. Alternatively, a cell suspension (10 ml) was exposed to air for 1 h and then centrifuged; the cells were resuspended in 0.5 M-sodium phosphate buffer (pH 8.0), and hydrogenase activity was measured manometrically with methylene blue as the electron acceptor.

Protein was estimated by the Lowry method with bovine serum albumin as standard.

**RESULTS**

**pH optima**

The pH optima for O₂-dependent H₃H uptake and acetylene reduction by nitrogenase *in vivo* were similar (pH 6-8 and 7-3, respectively) and close to the pH of the cell during growth (pH 6-8 to 7-0). The hydrogenase activity *in vitro* had a sharp optimum at pH 8.5, considerably higher than the growth pH. The specific activity of hydrogenase *in vitro* [45 µmol H₂ absorbed (mg protein)⁻¹ h⁻¹] was approximately 11 times greater than that of O₂-dependent H₃H uptake [3.9 µmol (mg protein)⁻¹ h⁻¹] at their respective pH optima, and four times greater at pH 6-8.

**O₂-dependent H₃H uptake**

Nitrogenase catalyses an N₂-dependent H₃H (and presumably H₃H) exchange, usually measured by mass spectrometric determination of H₂H. However, according to Burgess *et al.* (1980) this exchange does not result in the formation of H₂HO (H₃HO). Replacing N₂ by Ar, or adding CO (1-5%, v/v), to inhibit the nitrogenase-catalysed H₃H exchange, had no effect on O₂-dependent H₃H uptake by *A. brasilense*. The latter reaction is therefore a measure of hydrogenase-mediated respiration and is not catalysed by nitrogenase.

**O₂ sensitivity of hydrogenase**

O₂-dependent H₃H uptake was very sensitive to inhibition by O₂ (Fig. 1). This was partly due to damage to some component of the H₂-linked respiratory system, since only 62% of
Hydrogenase in Azospirillum

Fig. 1. O₂-dependent H₂ uptake by A. brasilense. H³H uptake was measured in N₂-fixing whole cells (0·26 mg protein ml⁻¹) over a range of O₂ concentrations. The cells were preincubated in 1% (v/v) O₂ for 1 h to diminish the concentration of endogenous carbon substrate, then flushed with N₂ for 30 min to remove traces of O₂. ■ Control; ▲ control + 50 mM-succinate; ■ control + 50 mM-malate.

Fig. 2. Effect of O₂ pretreatment on hydrogenase and O₂-dependent H³H uptake by A. brasilense. To determine hydrogenase activity (■), cells were exposed to different concentrations of O₂ for 1 h, and hydrogenase activity was then measured anaerobically at pH 6·8 by the rate of H³H uptake with methylene blue as the electron acceptor. To determine O₂-dependent H₂ uptake (□, ○), cells were treated with 4% (v/v) O₂ (□) or Ar (○) for 1 h, flushed with Ar to remove O₂, and then tested for O₂-dependent H³H uptake at pH 6·8.

O₂-dependent H³H uptake was retained after exposing the cells to inhibitory (4%, v/v) O₂ for 1 h (Fig. 2). The O₂-sensitive component was probably not hydrogenase because the enzyme activity in vitro was unaffected by a similar O₂ treatment when methylene blue was the electron acceptor (Fig. 2). Addition of either malate (on which the bacteria were grown) or succinate, which were the most active respiratory substrates of several tested, protected to some degree against inhibition by O₂ (Fig. 1). These substrates did not inhibit O₂-dependent H³H uptake, which would be expected if they were competing successfully for the small amount of O₂ available.

Effect of KCN

The lack of inhibition by malate or succinate of H₂-dependent respiration suggests two different respiratory pathways. Sensitivity to KCN is commonly used to distinguish different terminal oxidases. H₂-dependent respiration was inhibited by KCN (Kᵢ 20 μM) at 0·5% (v/v) O₂ (optimum), whereas malate-dependent respiration was unaffected by KCN at 0·5% O₂ but inhibited under air (Kᵢ 32 μM). Hydrogenase activity in vitro was only slightly sensitive to KCN (Kᵢ ~ 3 mM).

H₂-dependent acetylene reduction

Nitrogenase activity with either H₂ (Fig. 3) or carbon substrate (Fig. 4) as the electron donor was also very sensitive to inhibition by O₂. The pO₂ optima for maximum acetylene reduction in both the control, without exogenous carbon substrates, and in H₂-supported bacteria were very similar (Fig. 3). H₂ therefore failed to support respiratory protection of nitrogenase in carbon-starved bacteria. This is consistent with the evidence in Fig. 1 that O₂-dependent H³H uptake is either as sensitive or more sensitive than nitrogenase to inhibition by O₂. H₂ at 5% (v/v) [apparent Kₘ for H₂ uptake is 3% (v/v)] failed to support
significant acetylene reduction, possibly because acetylene inhibits O$_2$-dependent H$_2$ uptake at low H$_2$ concentrations (Chan et al., 1980).

When the bacteria were not pre-incubated to decrease the endogenous carbon supplies, they showed no H$_2$-supported acetylene reduction (Fig. 4). Apparently the endogenous carbon substrate concentration was sufficient to almost saturate the energy and reductant requirement for nitrogenase activity since addition of malate, succinate or lactate (all at 50 mM) stimulated the endogenous activity only slightly (0 to 15%). H$_2$ actually inhibited the rate of acetylene reduction slightly at partial pressures of O$_2$ below the optimum (Figs 3 and 4). This inhibitory effect was very marked in one batch culture where the optimum pO$_2$ for acetylene reduction was also shifted from 1.0 to 1.5% (v/v) O$_2$ by the presence of H$_2$ (results not shown). Presumably the hydrogenase in this particular culture was less O$_2$-sensitive and less well coupled to ATP production than normally.

**Anaerobic H$^3$H uptake**

Berlier & Lespinat (1980) observed H$_2$ and H$^3$H evolution by *A. brasilense* under anaerobic conditions with $^3$H$_2$, which suggested that reversible hydrogenase activity was present. Our *A. brasilense* cultures showed H$^3$H uptake in the absence of added O$_2$ even when sodium dithionite was present to scavenge the last traces of O$_2$. This H$^3$H uptake was less than 5% of the H$^3$H uptake activity at optimum pO$_2$ values. Whether it reflects 'exchange' activity by a second, reversible hydrogenase, or a degree of reversibility by the H$_2$-uptake hydrogenase, is not known.

**DISCUSSION**

The relationship between O$_2$, nitrogen fixation and the uptake hydrogenase of aerobic bacteria is as follows. Nitrogenase is an O$_2$-sensitive enzyme which requires ATP produced by respiratory activity. It also produces H$_2$ which is recycled by the uptake hydrogenase to provide energy and reducing power.
H₂ supported acetylene reduction by nitrogenase in _A. brasilense_ by means of H₂-uptake hydrogenase activity similar to that in _Azotobacter chroococcum_, _Anabaena cylindrica_ and soybean bacteroids. However, _A. brasilense_ differs from the other organisms in that the hydrogenase usually failed to support respiratory protection of nitrogenase activity, presumably because O₂-dependent H₂ uptake was itself extremely sensitive to inhibition by O₂. By contrast, O₂-dependent H²H uptake in _A. chroococcum_ is less sensitive than nitrogenase activity to inhibition by O₂ (Walker et al., 1981).

H₂ did not stimulate acetylene reduction by _A. brasilense_ unless the cells were first starved of endogenous carbon substrate. In this respect _A. brasilense_ differed from _A. chroococcum_, where H₂ stimulated acetylene reduction even at saturating mannitol concentrations (Walker & Yates, 1978). Again, this lack of stimulation may reflect sensitivity to O₂ of the hydrogenase respiratory system if O₂-dependent H₂ uptake is more sensitive to O₂ than C-dependent nitrogenase activity. Alternatively, hydrogenase may provide reducing power for respiration and ATP production but not for nitrogenase, and endogenous carbon substrate may have saturated the electron requirement for nitrogenase in our experiments.

Malate and succinate, which were actively respired by malate-grown _A. brasilense_, did not inhibit O₂-dependent H³H uptake, suggesting that this process has a higher affinity for O₂ than has C-dependent respiration and that H₂-linked respiration possesses a different terminal oxidase of high O₂ affinity. This is supported by the results in Fig. 4: if the inhibition by H₂ of C-dependent acetylene reduction at sub-optimum O₂ concentrations occurs because H₂ is less efficiently linked to ATP production than is C-dependent respiration, then H₂ must be preferentially metabolized at low O₂ concentrations. A similar effect of H₂ on acetylene reduction by soybean bacteroids at low O₂ concentrations was reported by Emerich et al. (1979), who also found that H₂ inhibited ATP production by C-dependent respiration. This may be due to less efficient ATP production by H₂-dependent respiration (T. Ruiz-Argüeso, personal communication). In this respect, _A. brasilense_ and soybean bacteroids differ from _Azotobacter_, where H₂- and NADH-linked respiration are equally efficient in ATP production (Laane et al., 1979). A second possibility, that H₂ links to the carbon-respiratory chain but by-passes a rate-limiting electron transfer step associated with carbon-substrate respiration, is unlikely, since saturating concentrations of malate or succinate offered some degree of respiratory protection to O₂-dependent H³H uptake. This could not be so if their respiration was controlled by a rate-limiting step slower than that associated with H₂-dependent respiration.

The different sensitivities to inhibition by KCN also support the suggestion that the H₂- and C-linked respiratory chains are different and separate with no common electron carrier as in a branched electron transport chain. However, this conclusion is based on the assumption that the only site of KCN action is at the terminal oxidase and that the internal pH of the cell is the same during C- or H₂-dependent respiration, since the intracellular concentration of CN⁻ is pH dependent.

The specific activity of hydrogenase in _A. brasilense_ in the methylene blue assay was higher at pH 6.8 than that of O₂-dependent H³H uptake. Therefore, as in other hydrogenase-containing organisms, a component or components other than hydrogenase activity limit the rate of uptake. Nevertheless, O₂-dependent H³H uptake is a useful and sensitive qualitative test for the presence of hydrogenase.

**REFERENCES**


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