RP 102

Hydrogen-uptake Hydrogenase Activity in Nitrogen-fixing *Azospirillum brasilense*

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N_2-fixing *Azospirillum brasilense* possesses an H_2-uptake hydrogenase activity capable of supporting H_2-dependent acetylene reduction by whole cells starved of carbon metabolites. H_2 did not support acetylene reduction in carbon-sufficient bacteria, although carbon substrates did not inhibit H_2-dependent respiration. H_2-dependent respiration was extremely O_2 sensitive and could not usually protect nitrogenase against inhibition by O_2. H_2 partly inhibited nitrogenase activity at sub-optimal O_2 concentrations: this may be because H_2-dependent respiration has a greater affinity for O_2 but is less efficiently coupled to ATP production than is carbon-dependent respiration.

INTRODUCTION

Several aerobic N_2-fixing bacteria possess an H_2-uptake hydrogenase which may recycle H_2 produced by nitrogenase *in vivo* (Bothe *et al.*, 1977; Walker & Yates, 1978; Emerich *et al.*, 1979). Dixon (1972) postulated that the uptake hydrogenase of N_2-fixing bacteria could respire H_2 to: (a) protect nitrogenase against inactivation by O_2; (b) recover by oxidative phosphorylation some of the energy lost through ATP-dependent H_2 production by nitrogenase; and (c) prevent inhibition of N_2 reduction by H_2. Hydrogenase activity can support acetylene reduction by nitrogenase and protect the enzyme against inactivation by O_2 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 H_2-uptake hydrogenase occur *in vivo*. Walker & Yates (1978), however, showed that H_2 produced by nitrogenase in *A. chroococcum* dispersed too rapidly to inhibit N_2 reduction.

Azospirillum species (Tarrand *et al.*, 1978) are aerobic N_2-fixing bacteria which also contain an H_2-uptake hydrogenase activity (Stephan *et al.*, 1981). In the present paper we present evidence that H_2 can support nitrogenase activity (acetylene reduction) of *Azospirillum brasilense* only under carbon-limited conditions, although carbon substrates do not inhibit H_2-dependent respiration. However, H_2 cannot support respiratory protection of nitrogenase because O_2-dependent H_2-uptake is itself extremely sensitive to inhibition by O_2.

METHODS

Organism and growth. Azospirillum brasilense Sp7 (ATCC 29145) was grown under N_2-fixing conditions at 37 °C in the medium described by Okon *et al.* (1976) supplemented with biotin (100 μg l^{-1}) and sodium lactate (3 g l^{-1}) or sodium malate (5 g l^{-1}) (Nfb medium). The partial pressure of O_2 in the medium was monitored with a

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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 N_2 (6000 *g*, 30 min), resuspended in a minimum volume of C- and N-free medium without added carbon substrate and stored in liquid N_2.

Hydrogenase activity. H_2 uptake by hydrogenase was measured by Warburg manometry at 37 °C under H_2 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 H^3H uptake with methylene blue (10 mM) as an electron acceptor. H^3H gas was produced by reacting sodium boro[^3H]hydride (from Amersham) with distilled water, then diluted 5000-fold with H_2. 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 $H_2 + H^3H$ (1 ml) at 37 °C for 30 min. The reaction was stopped by removing the Subaseal to let the H_2 disperse. Incorporation of ^3H 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.

O_2-dependent H_2 uptake. Cells were suspended in C- and N-free Nfb medium to an A_{540} of 0·3 to 0·4 and preincubated under Ar/O_2 (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) $H_2 + H^3H$ and different O_2 concentrations were shaken at 37 °C for 1 h. The reaction was stopped with 0·1 ml 40% (w/v) KOH and incorporation of ^3H into the aqueous phase was measured in 0·2 ml samples as described above.

Anaerobic H^3H uptake. Cultures (1 ml) were incubated at 37 °C in the side arm of a 25 ml conical flask under $Ar/H_2 + H^3H$ (9 :1, v/v) for 1 h. The main compartment of the flask contained 100 mM-$Na_2S_2O_4$ (5 ml) to scavenge traces of O_2. H^3H uptake was determined as above.

Nitrogenase activity. Bacterial suspensions were prepared as above and the assay was as for O_2-dependent H^3H uptake except that acetylene (8%, v/v) replaced $H_2 + H^3H$. Ethylene was measured in a Pye 104 gas chromatograph fitted with a flame ionization detector.

O_2 sensitivity of hydrogenase. Cell suspensions (1 ml) were exposed to different concentrations of O_2 for 1 h under the conditions described above, without acetylene. The O_2 was then replaced by Ar and hydrogenase activity determined by H^3H incorporation with methylene blue or O_2 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 O_2-dependent H^3H 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 H_2 absorbed (mg protein)$^{-1}$ h^{-1}] was approximately 11 times greater than that of O_2-dependent H^3H uptake [3·9 μmol (mg protein)$^{-1}$ h^{-1}] at their respective pH optima, and four times greater at pH 6·8.

O_2-dependent H^3H uptake

Nitrogenase catalyses an N_2-dependent H^2H (and presumably H^3H) exchange, usually measured by mass spectrometric determination of H^2H. However, according to Burgess *et al.* (1980) this exchange does not result in the formation of H^2HO (H^3HO). Replacing N_2 by Ar or adding CO (1·5%, v/v), to inhibit the nitrogenase-catalysed H^2H exchange, had no effect on O_2-dependent H^3H uptake by *A. brasilense*. The latter reaction is therefore a measure of hydrogenase-mediated respiration and is not catalysed by nitrogenase.

O_2 sensitivity of hydrogenase

O_2-dependent H^3H uptake was very sensitive to inhibition by O_2 (Fig. 1). This was partly due to damage to some component of the H_2-linked respiratory system, since only 62% of

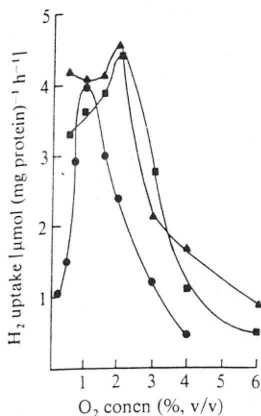

Fig. 1

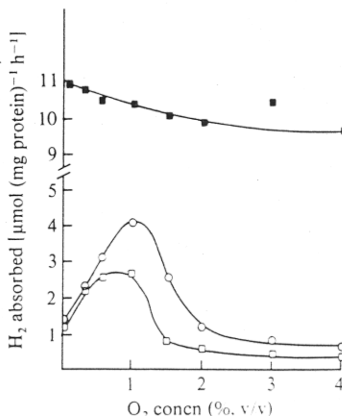

Fig. 2

Fig. 1. O_2-dependent H_2 uptake by *A. brasilense*. H^3H uptake was measured in N_2-fixing whole cells (0·26 mg protein ml^{-1}) over a range of O_2 concentrations. The cells were preincubated in 1% (v/v) O_2 for 1 h to diminish the concentration of endogenous carbon substrate, then flushed with N_2 for 30 min to remove traces of O_2. ●, Control: ▲, control + 50 mM-succinate: ■, control + 50 mM-malate.

Fig. 2. Effect of O_2 pretreatment on hydrogenase and O_2-dependent H^3H uptake by *A. brasilense*. To determine hydrogenase activity (■), cells were exposed to different concentrations of O_2 for 1 h, and hydrogenase activity was then measured anaerobically at pH 6·8 by the rate of H^3H uptake with methylene blue as the electron acceptor. To determine O_2-dependent H_2 uptake (□, ○), cells were treated with 4% (v/v) O_2 (□) or Ar (○) for 1 h, flushed with Ar to remove O_2, and then tested for O_2-dependent H^3H uptake at pH 6·8.

O_2-dependent H^3H uptake was retained after exposing the cells to inhibitory (4%, v/v) O_2 for 1 h (Fig. 2). The O_2-sensitive component was probably not hydrogenase because the enzyme activity *in vitro* was unaffected by a similar O_2 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 O_2 (Fig. 1). These substrates did not inhibit O_2-dependent H^3H uptake, which would be expected if they were competing successfully for the small amount of O_2 available.

Effect of KCN

The lack of inhibition by malate or succinate of H_2-dependent respiration suggests two different respiratory pathways. Sensitivity to KCN is commonly used to distinguish different terminal oxidases. H_2-dependent respiration was inhibited by KCN (K_i 20 μM) at 0·5% (v/v) O_2 (optimum), whereas malate-dependent respiration was unaffected by KCN at 0·5% O_2 but inhibited under air (K_i 32 μM). Hydrogenase activity *in vitro* was only slightly sensitive to KCN ($K_i \sim 3$ mM).

H_2-dependent acetylene reduction

Nitrogenase activity with either H_2 (Fig. 3) or carbon substrate (Fig. 4) as the electron donor was also very sensitive to inhibition by O_2. The pO_2 optima for maximum acetylene reduction in both the control, without exogenous carbon substrates, and in H_2-supported bacteria were very similar (Fig. 3). H_2 therefore failed to support respiratory protection of nitrogenase in carbon-starved bacteria. This is consistent with the evidence in Fig. 1 that O_2-dependent H^3H uptake is either as sensitive or more sensitive than nitrogenase to inhibition by O_2. H_2 at 5% (v/v) [apparent K_m for H_2 uptake is 3% (v/v)] failed to support

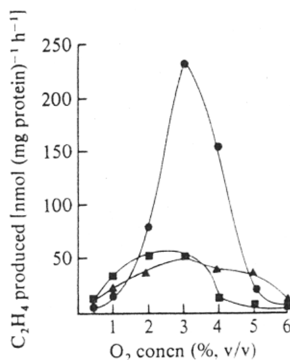

Fig. 3

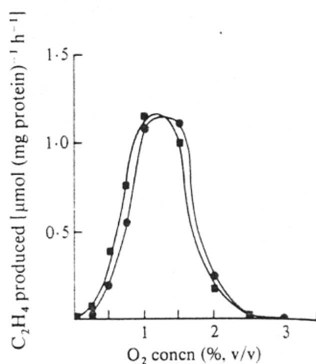

Fig. 4

Fig. 3. H_2-supported nitrogenase activity in C-starved *A. brasilense*. The bacteria were preincubated to deplete endogenous carbon substrates as described for Fig. 1. Acetylene reduction was then measured over a range of O_2 concentrations at 37 °C under 90% (v/v) or 5% (v/v) H_2 with Ar as the normalizing gas. ■, Control (without exogenous carbon substrates); ●, control + 90% H_2; ▲, control + 5% H_2.

Fig. 4. Effect of H_2 on C-dependent acetylene reduction by C-sufficient *A. brasilense*. Malate-grown cells were resuspended (without washing) in C-free Nfb medium. ■, Control; ●, control + 90% (v/v) H_2.

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^3H uptake

Berlier & Lespinat (1980) observed H_2 and H^2H evolution by *A. brasilense* under anaerobic conditions with $^2H_2$, which suggested that reversible hydrogenase activity was present. Our *A. brasilense* cultures showed H^3H uptake in the absence of added O_2 even when sodium dithionite was present to scavenge the last traces of O_2. This H^3H uptake was less than 5% of the H^3H 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.

H_2 supported acetylene reduction by nitrogenase in *A. brasilense* by means of H_2-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 O_2-dependent H_2 uptake was itself extremely sensitive to inhibition by O_2. By contrast, O_2-dependent H^3H uptake in *A. chroococcum* is less sensitive than nitrogenase activity to inhibition by O_2 (Walker *et al.*, 1981).

H_2 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 H_2 stimulated acetylene reduction even at saturating mannitol concentrations (Walker & Yates, 1978). Again, this lack of stimulation may reflect sensitivity to O_2 of the hydrogenase respiratory system if O_2-dependent H_2 uptake is more sensitive to O_2 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 O_2-dependent H^3H uptake, suggesting that this process has a higher affinity for O_2 than has C-dependent respiration and that H_2-linked respiration possesses a different terminal oxidase of high O_2 affinity. This is supported by the results in Fig. 4: if the inhibition by H_2 of C-dependent acetylene reduction at sub-optimum O_2 concentrations occurs because H_2 is less efficiently linked to ATP production than is C-dependent respiration, then H_2 must be preferentially metabolized at low O_2 concentrations. A similar effect of H_2 on acetylene reduction by soybean bacteroids at low O_2 concentrations was reported by Emerich *et al.* (1979), who also found that H_2 inhibited ATP production by C-dependent respiration. This may be due to less efficient ATP production by H_2-dependent respiration (T. Ruiz-Argüeso, personal communication). In this respect, *A. brasilense* and soybean bacteroids differ from *Azotobacter*, where H_2- and NADH-linked respiration are equally efficient in ATP production (Laane *et al.*, 1979). A second possibility, that H_2 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 O_2-dependent H^3H uptake. This could not be so if their respiration was controlled by a rate-limiting step slower than that associated with H_2-dependent respiration.

The different sensitivities to inhibition by KCN also support the suggestion that the H_2- 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 H_2-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 O_2-dependent H^3H uptake. Therefore, as in other hydrogenase-containing organisms, a component or components other than hydrogenase activity limit the rate of uptake. Nevertheless, O_2-dependent H^3H uptake is a useful and sensitive qualitative test for the presence of hydrogenase.

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