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Oxygen protection of nitrogenase in *Frankia* sp. HFPArI3

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Abstract. O_2 protection of nitrogenase in a cultured *Frankia* isolate from *Alnus rubra* (HFPArI3) was studied in vivo. Evidence for a passive gas diffusion barrier in the vesicles was obtained by kinetic analysis of in vivo O_2 uptake and acetylene reduction rates in response to substrate concentration. O_2 of NH_4^+-grown cells showed an apparent K_m O_2 of approximately 1 μM O_2. In N_2-fixing cultures a second K_m O_2 of about 215 μM O_2 was observed. Thus, respiration remained unsaturated by O_2 at air-saturation levels. In vivo, the apparent K_m for acetylene was more than 10-fold greater than reported in vitro values. These data were interpreted as evidence for a gas diffusion barrier in the vesicles but not vegetative filaments of *Frankia* sp. HFPArI3.

Key words: *Frankia* – Nitrogenase – Oxygen protection – *Alnus rubra* isolate

Nitrogenase from actinorhizal root nodules, in common with nitrogenase from other sources (Brill 1980), is irreversibly inactivated by oxygen both in vitro (Benson et al 1979) and in vivo (Skeffington and Stewart 1976). Nitrogenase activity in root nodules of actinorhizal plants, however, is strictly O_2 dependent (Bond 1960; Waughman 1972; Fessenden et al. 1973; Wheeler et al. 1979), presumably because oxidative phosphorylation is required to generate the large amounts of ATP needed to support enzyme activity. The O_2 relationships in the nodule are thus complex and must be highly regulated; sufficient O_2 must be available for ATP production but too much O_2 denatures nitrogenase.

The development of nitrogenase activity in most actinorhizal root nodules coincides with the differentiation of terminal swellings of the vegetative filaments, termed vesicles (Mian and Bond 1978; Becking 1977). The localization of reducing conditions with tetrazolium dyes (Akkermans 1971) and the isolation of vesicle clusters capable of acetylene reduction (Van Straten et al. 1977) have led to the proposal that these specialized cells are the site of nitrogen fixation. This hypothesis was supported by the correlation of vesicle differentiation with induction of nitrogenase activity in response to nitrogen limitation in several *Frankia* isolates cultured under aerobic conditions (Tjepkema et al. 1980, 1981; Gauthier et al. 1981; Burggraaf and Shipton 1983; Murry et al. 1984). Since in all of the isolates tested, maximum acetylene reduction occurs near ambient pO_2 levels, the vesicles presumably are capable of protecting nitrogenase from inactivation by O_2.

Structural studies of cultured *Frankia* have revealed a multi-laminate cell envelope surrounding the vesicle and possibly extending along the vesicle stalk, to its point of attachment to the vegetative filament (Torrey and Callaham 1982). The laminated structure of the vesicle envelope bears a striking resemblance to the laminated glycolipid envelope surrounding the heterocysts of cyanobacteria (Lang and Fay 1971), which has been postulated to limit the ingress of O_2 into the site of nitrogen-fixation (Lambein and Wolk 1973). By analogy, a comparable protective function was suggested for the laminated layer of *Frankia* vesicles (Torrey and Callaham 1982).

In the present study, the nature of the O_2 protection mechanism of nitrogenase in free-living *Frankia* isolate HFPArI3 was explored. The presence of a gas diffusion barrier in the vesicle was indicated by a high in vivo K_m for acetylene and diffusion-limited kinetics for O_2 uptake in nitrogen-fixing, but not NH_4^+-grown cells.

Materials and methods

Cultivation of organism

Axenic cultures of *Frankia* sp. HFPArI3, isolated from the root nodules of *Alnus rubra* (Berry and Torrey 1979) were grown on defined medium using propionate as the sole carbon source (Murry et al. 1984). Undifferentiated cells for respiratory studies were grown on ammonia to mid-log phase in 1-l air-sparged cultures. Vesicles and nitrogenase were induced aerobically in 300- or 600-ml batch cultures as described earlier (Murry et al. 1984). Acetylene reduction and respiration were measured from 4 to 6 days following initiation of induction.

Assays

Nitrogenase activity was measured with the acetylene reduction assay in standardized 10-ml serum vials. Cells were buffered to pH 6.7 with 50 mM MOPS (3-N-morpholinopropanesulfonic acid) and 1 mM $NaHCO_3$ was added to supply CO_2, except when CO_2 evolution was measured concurrently. Cells were sparged with argon, or a mixture of argon + 5% O_2 for 15 min. Replicate 2-ml samples were withdrawn through the out-gas tube using an argon-flushed syringe fitted with a canula and were injected into serum vials containing argon and O_2 at the desired concentration. Cells were incubated at 28°C on a rotary shaker (70 rpm)

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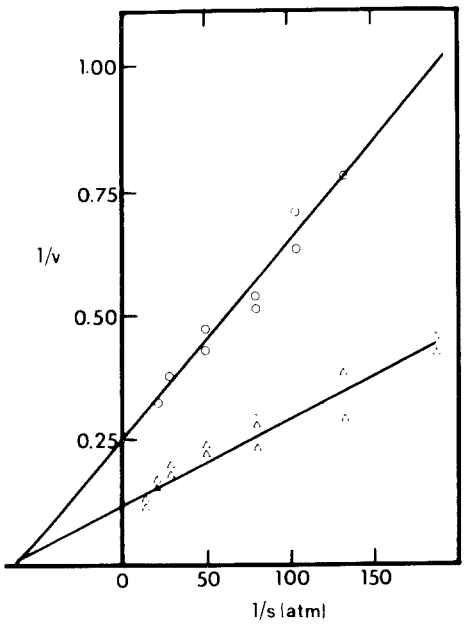

Fig. 1. Lineweaver-Burk plots of in vivo acetylene reduction activity in response to acetylene concentration in *Francia* sp. ArI3 assayed under 20% (△) and 5% (○) O_2 (balance argon). Cells (10.7 μg protein/ml) were assayed for 70–75 min at 30°C, 1/v = nMol C_2H_4/ml/min; 1/s = atm C_2H_2

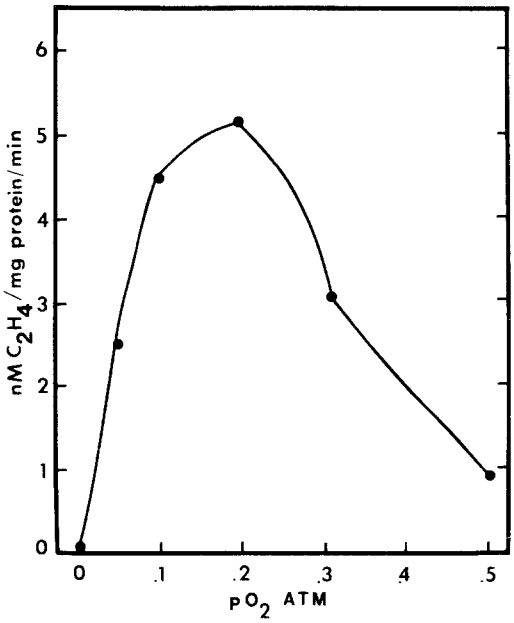

Fig. 2. Response of acetylene reduction activity in *Frankia* sp. ArI3 to O_2 tension of the assay mixture

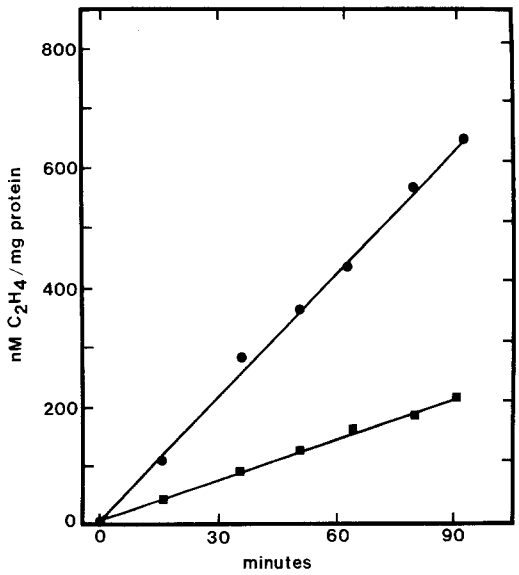

Fig. 3. Time course of acetylene reduction activity in *Frankia* sp. ArI3 incubated under 20% O_2 (●) and 5 % O_2 (■) (balance argon)

for 15–20 min before initiating the assay by introducing acetylene at 10% vol/vol, except for the K_m measurements. Samples of the gas phase (0.1-ml) were removed periodically and analyzed for ethylene production (Murry et al. 1984).

O_2 and CO_2 concentration in the gas phase of the assay mixture were measured in duplicate by analyzing 0.5 ml gas samples with a gas chromatograph equipped with a thermal conductivity detector (Murry et al. 1984). Overall precision of the assays was about 5%. Oxygen uptake was measured polarographically with a YSI Model 5331 O_2 electrode (Yellow Springs, OH, USA) in a 2-ml water jacketed chamber maintained at 30°C. Current was measured with Keithly Model 610C electrometer. Cells were concentrated to 40–60 μg protein/ml. Vesicles were detached by progressively sonicating a 20 ml aliquot of unwashed cells (10–12 μg protein/ml) with a Braunson Model 1510 sonicator (100 W) equipped with a microtip. Cellular protein was measured by the Bradford procedure (1976). The apparent K_m and V_{max} for acetylene and O_2 were determined from the intercepts obtained by linear regression analysis.

Results

Effect of acetylene concentration on acetylene reduction activity

In vivo acetylene reduction activity in HFPArI3 was measured under 5 and 20% O_2 and a range of external acetylene concentrations. At both O_2 tensions, acetylene reduction activity was saturated by 0.1 atm of C_2H_2. Lineweaver-Burke plots of the data show that the V_{max} (8 nM C_2H_4/mg protein/min) at 20% O_2 was about twice that measured at 5% O_2 but that the apparent K_m for acetylene was similar (Fig. 1). The mean apparent K_m value of five separate experiments was 0.0175 + SD 0.0051 atm C_2H_2 (r^2 ranged from 0.933 to 0.997). This value is similar to the apparent K_m for C_2H_2 of excised *Myrica cerifera* nodules (0.02 atm) reported by Sloger (1968) and much greater than the overall average of 0.006 atm summarized for a variety of N_2-fixing organisms (Hardy et al. 1973).

Effect of O_2 concentration on acetylene reduction

Acetylene reduction activity in ArI3 was measured with the gas phase containing different partial pressures of O_2 (Fig. 2). Acetylene reduction in aerobically induced ArI3 was dependent on O_2. No activity could be detected when cells were incubated under argon for 20 min prior to addition of acetylene. Under these conditions, trace levels of contaminating O_2 are respired, providing anaerobic conditions.

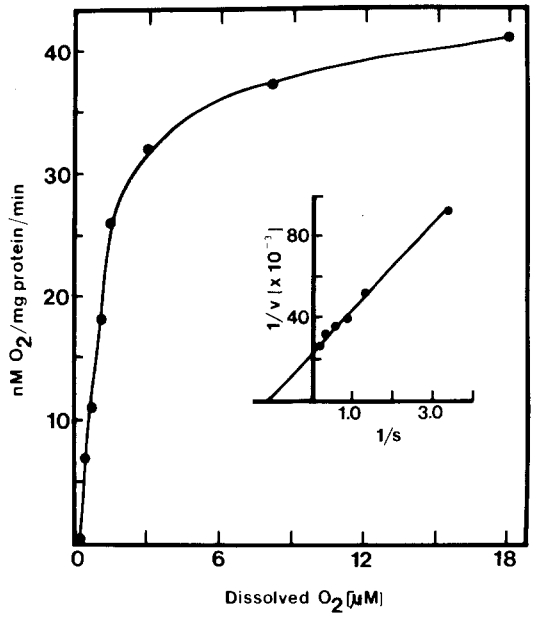

Fig. 4. Response of respiration (O_2 uptake) to dissolved O_2 concentration in NH_4^+-grown *Frankia* sp. ArI3 filaments. Inset shows Lineweaver-Burk plots of the same data

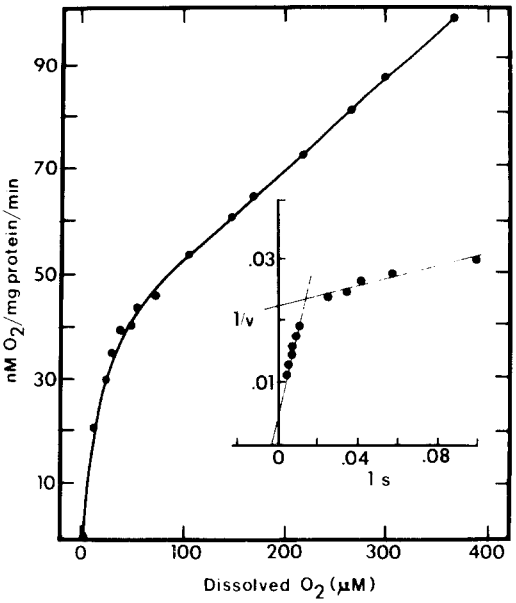

Fig. 5. Response of respiration to dissolved O_2 concentration in N_2-fixing, vesicle-containing filaments of *Frankia* sp. ArI3. Inset shows Lineweaver-Burk plots of the same data

Nitrogenase activity initially increased with increasing pO_2; maximum activity was reached at about 0.2 atm pO_2. pO_2 levels above 0.2 atm were inhibitory to acetylene reduction. Similar results were reported for the *Frankia* isolate CpI1 (Tjepkema et al. 1981) and strains D11 and G2 isolated from *Casuaria* (Gauthier et al. 1981). Acetylene reduction was linear for more than 90 min under these assay conditions (Fig. 3). No significant change in O_2 concentration in the gas phase was detected after 90 min even at low (5% O_2) initial concentrations.

Effect of O_2 concentration on respiration

The response of respiration (O_2 uptake) to dissolved O_2 tension was markedly different in NH_4^+ and N_2-grown cells. Respiration of NH_4^+-grown, undifferentiated filaments was saturated by 18 μM O_2 (Fig. 4) and showed an apparent K_m O_2 of about 1 μM O_2 (see inset). Respiration in N_2-fixing cells with intact vesicles was still not saturated at dissolved O_2 tensions above 350 μM (Fig. 5). A double reciprocal plot of the same data (see insert) shows a biphasic response to O_2 tension. Table 1 compares the apparent K_m O_2 and V_{max} determined from the intercepts of the two regression lines obtained with vesicle containing filaments with these parameters determined in NH_4^+-grown cells. At low O_2 tensions, the apparent K_m O_2 and V_{max} of N_2- and NH_4^+-grown cells are similar. At higher O_2 tensions, a second, much greater K_m for O_2 is observed in N_2- fixing, but not NH_4^+-grown cells. The V_{max} was nearly 5-fold greater in N_2-fixing cells than the V_{max} determined in NH_4^+-grown cells.

Brief sonication (50 s) of N_2-fixing cells results in the detachment of about 95% of the vesicles from the vegetative hyphae and a complete loss of acetylene reduction under air. Only a slight loss (5 to 15%) of CO_2 evolution occurs when all the vesicles are detached; however, the respiratory response to dissolved O_2 is markedly different from that in cells with intact vesicles (Fig. 6). O_2 uptake is saturated at low (about 18 μM) O_2 tensions as was observed in NH_4^+-grown filaments. However, at O_2 tensions between 150 to 250 μM, respiration again becomes limited by O_2 and rates increase until O_2 uptake is saturated at approximately 300 μM O_2.

Table 1. Respiration (O_2 uptake) in *Frankia* sp ArI3: Apparent K_m O_2 and V_{max} in NH_4^+- and N_2-grown cultures[a]

Growth conditions	Range of pO_2 during assay	Apparent K_m O_2 (μM)	V_{max} (nM O_2/ μg · min)
NH_4^+-grown	0–500 μM O_2	0.810 + 0.27 (n = 4)	43.47 + 14.36
N_2-grown	0– 43.6 μM	0.982 + 0.42 (n = 4)	46.5 + 19.8
	100–400 μM	169.8 + 28.4 (n = 3)	215.5 + 36.05

[a] ArI3 was grown aerobically in batch culture on the indicated N source with propionate as the carbon source. Cells were harvested in log-phase and concentrated to 40–60 μg protein/ml. O_2 uptake was determined polarographically at a range of dissolved O_2 levels. The apparent K_m O_2 and V_{max} values were determined using linear regression analysis

Discussion

The mechanism of O_2 protection of nitrogenase in actinorhizal nodules is apparently complex and probably involves several different interacting mechanisms provided by the host and by the endophyte. The experiments described here suggest that at least one mechanism is in operation in the cultured endophyte: a passive gas-diffusion barrier. The in vivo kinetics of acetylene reduction and respiration in response to substrate concentration provide experimental evidence in support of the idea of a gas-diffusion barrier, which was predicted by structural observations of a

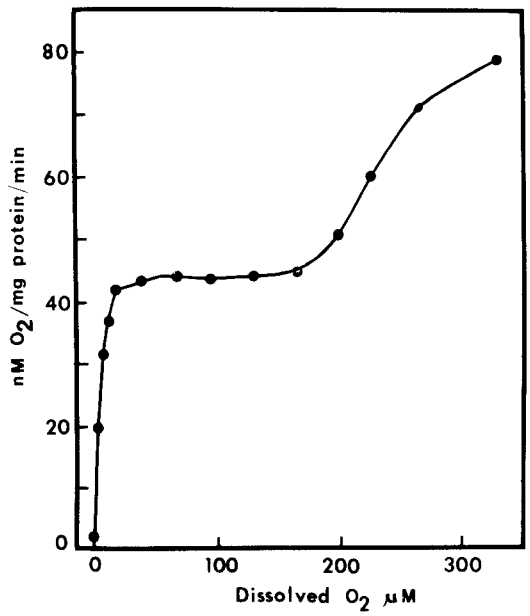

Fig. 6. Response of respiration to dissolved O_2 concentration in N_2-fixing *Frankia* sp. ArI3 filaments that were briefly sonicated (50 s) to detach the vesicles from the vegetative hyphae

complex, multilaminate envelope surrounding the vesicle (Torrey and Callaham 1982). The discrepancy between the high apparent K_m values for acetylene determined here for ArI3 in vivo and earlier in intact *Myrica cerifera* nodules (Sloger 1968), and the smaller in vitro values (Hardy et al. 1973), may indicate that the intracellular level of acetylene is much lower within the vesicle than the external concentration. If the affinity of nitrogenase for acetylene is the same in vivo and in vitro, then a difference in apparent K_m may provide an indirect measurement of the resistance of the vesicle wall to gas-diffusion. Although K_m values for nitrogenase can be influenced by a variety of factors (Bergersen and Turner 1973; Davis and Wang 1980), the in vivo apparent K_m for acetylene observed for ArI3 was remarkably constant even when the V_{max} was altered by the pO_2 of the assay system. These results are consistent with the combined diffusion/enzyme kinetics model of gas exchange in nodules tested by kinetic analysis of acetylene reduction in *Alnus rubra* (Winship and Tjepkema 1983) and soybean nodules (Denison et al. 1983). A 10-fold difference between the apparent K_m for acetylene determined in vivo and in vitro has also been observed in the heterocystous cyanobacterium *Anabaena cylindrica*. Since these values were not significantly different in a non-heterocystous species, this variation was attributed to the presence of a diffusion barrier in the heterocysts of *Anabaena* (Hallenbeck et al. 1979).

Comparison of oxygen uptake rates as a function of oxygen concentration in NH_4^+-versus N_2-grown ArI3 also supports the presence of a gas-diffusion barrier in the vesicles. O_2 uptake in undifferentiated NH_4^+-grown filaments approximates Michaelis-Menten enzyme kinetics. Respiration is saturated at low (18 µM) dissolved O_2 concentrations and shows an apparent K_m for O_2 of about 1 µM. In vesicle-containing cells, a second kinetic component is observed which shows a linear increase in respiratory rate with O_2 tensions from 20 µM to more than 300 µM O_2. This pattern approximates Blackman kinetics characteristic of diffusion-limited processes (Lommen et al. 1971). Diffusion-limited kinetics of dark respiration have also been described in nitrogen-fixing cyanobacteria and were associated with the heterocysts (Rhoades 1981; Jensen and Cox 1983).

The apparent K_m O_2 value (1 µM O_2) corresponding to vegetative cell respiration reported for *Anabaena variabilis* (Jensen and Cox 1983) was similar to the apparent K_m O_2 determined here for the high affinity activity seen in vesicle-containing ArI3 (and for NH_4^+-grown cells). However, the apparent K_m O_2 of the low affinity activity in vesicle-containing HFPArI3 was nearly three times the K_m O_2 (69 µM O_2) associated with the heterocysts in *A. variabilis* (Jensen and Cox 1983). This may indicate that the vesicles provide more resistance to O_2 diffusion than the intercalary heterocysts of *A. variabilis*. According to the calculations of Rhoades (1981), the diffusion component of O_2-uptake in filaments containing terminal heterocysts (analogous to the terminal vesicles of ArI3) would be much more pronounced than the limitaion by diffusion of O_2-uptake in intercalary heterocysts.

The kinetics of O_2 uptake were markedly altered when vesicles were detached from the vegetative hyphae. The kinetics of O_2 uptake were similar to those of NH_4^+-grown cells except that when O_2 concentrations above 200 µM were reached, respiration again became dependent on O_2 tension. Since this was never observed in NH_4^+-grown filaments, even at O_2 concentrations above 500 µM, unsaturated respiration at very high O_2 levels must be associated with the vesicles. Whether these kinetics indicate diffusion limitation (since presumably most of the O_2 reaching the interior of the vesicle must still diffuse through the pore channel of the vesicle stalk), or the operation of alternate oxidases as described in *Rhizobium* (Wittenberg et al. 1974; Bergersen and Turner 1980) and *Azotobacter* (Yates and Jones 1974), perhaps in combination with diffusion limitation, is unclear. Comparison of the kinetics of O_2-uptake associated with the presence of vesicles (Fig. 5) and the response of acetylene reduction to O_2 concentration (Fig. 2) shows that although respiration was unsaturated above pO_2 of 0.2 atm (240 µM dissolved O_2), acetylene reduction was inhibited. Whether this indicates a loss of energy conservation associated with modification of the cytochrome system in ArI3, an important aspect of respiratory protection in *Azotobacter* (Robson and Postgate 1980) or simply, that the diffusion barrier of the vesicle is insufficient at higher pO_2 and that nitrogenase is inactivated by excess intracellular O_2, is unclear from the data.

In conclusion, the results presented here provide experimental evidence that the protection from O_2 afforded nitrogenase by its localization in the vesicles in cultured *Frankia* sp. ArI3 involves a passive barrier to O_2.

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