Effect of O_2 on vesicle formation, acetylene reduction, and O_2-uptake kinetics in *Frankia* sp. HFPCcI3 isolated from *Casuarina cunninghamiana*

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The effect of the partial pressure of oxygen (P_{O_2}) on the formation of vesicles, which are thought to be the site of N_2 fixation in *Frankia*, was studied in HFPCcI3, an effective isolate from *Casuarina cunninghamiana*. Unlike other actinorhizal root nodules, vesicles are not produced by the endophyte in *Casuarina* nodules. However, in culture under aerobic conditions, large, phase-bright vesicles are formed in HFPCcI3 within 20 h following removal of NH_4^+ from the culture medium and reach peak numbers within 72 to 96 h. *In vivo* acetylene reduction activity parallels vesicle formation. Optimum rates of acetylene reduction in short-term assays occurred at 20% O_2 (0.2 atm (1 atm = 101.325 kPa)) in the gas phase. O_2 uptake (respiration) determined polarographically showed diffusion-limited kinetics and remained unsaturated by O_2 until 300 μM O_2. In contrast, respiration in NH_4^+-grown cells was saturated by O_2 between 8 and 10 μM O_2. These results indicate the presence of a diffusion barrier associated with the vesicles. Vesicle development was repressed in cells incubated in N-free media sparged with gas mixtures with P_{O_2} between 0.001 and 0.003 atm. Nitrogenase was induced under these conditions, but acetylene reduction was extremely O_2 sensitive. The kinetics of O_2 uptake as a function of dissolved O_2 concentration in avesicular cells were similar to those in NH_4^+-grown cells indicating the lack of a diffusion barrier. These results demonstrate that vesicle formation and the development of the O_2 protection mechanisms of nitrogenase are regulated by ambient P_{O_2} in HFPCcI3.

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L'effet de la pression partielle d'oxygène (P_{O_2}) sur la formation des vésicules, que l'on a pensé être le site de fixation de N_2 chez le *Frankia*, a été étudié avec un isolat efficace extrait de *Casuarina cunninghamiana*, le HFPCcI3. Contrairement à ce qui fait chez les autres nodules racinaires actinorhiziens, les vésicules ne sont pas produites par l'endophyte dans les nodules de *Casuarina*. Toutefois, en culture et sous conditions aérobiques, de larges vésicules à phase claire sont formées chez le HFPCcI3 en dedans de 20 h après l'enlèvement du NH_4^+ du milieu de culture, atteignant des nombres maximums en dedans de 72 à 96 h. *In vivo*, l'activité de réduction de l'acétylène se fait en parallèle avec la formation de vésicules. Les taux optimums du réduction de l'acétylène dans des essais de courte durée se sont situés à 20% de O_2 (0,2 atm (1 atm = 101,325 kPa)) dans la phase gazeuse. L'absorption de O_2 (respiration), déterminée de façon polarographique, a présenté une cynétique de diffusion limitée, demeurée insaturée par l'O_2 jusqu'à 300 μM de O_2. A l'opposé, la respiration des cellules croissant en présence de NH_4^+ fut saturée de O_2 entre 8 et 10 μM de O_2. Ces résultats indiquent la présence d'une barrière de diffusion associée aux vésicules. Le développement des vésicules fut réprimé chez les cellules incubées dans des milieux sans N mais pourvu d'un mélange gazeux dont la pression en P_{O_2} se situait entre 0,001 et 0,003 atm. Sous ces conditions, de la nitrogénase fut induite, mais le reduction de l'acétylène fut extrêmement sensible à l'O_2. La cinétique de l'absorption de O_2 en tant que fonction d'une concentration de O_2 dissoute dans des cellules avésiculaires s'est comportée de façon similaire à la cinétique des cellules croissant en présence de NH_4^+, ce qui indique l'absence d'une barrière de diffusion. Ces résultats démontrent que la formation de vésicules et le développement des mécanismes de protection de l'O_2 de la nitrogénase sont régularisés par la P_{O_2} ambiante chez HFPCcI3.

[Traduit par le journal]

Introduction

Oxygen lability is a characteristic of all nitrogenases studied to date (Brill 1980), including nitrogenase from actinorhizal root nodules (Skeffington and Stewart 1976; Benson *et al.* 1979). Nitrogenase activity in actinorhizal plants, i.e., the diverse group of woody, dicotyledonous plants nodulated by the filamentous actinomycete *Frankia*, is, however, strictly O_2 dependent (Bond 1961; Waughman 1972; Fessenden *et al.* 1973; Wheeler *et al.* 1979; Zhang and Torrey 1985*a*). Unlike legume nodules where a cortical barrier results in a microaerophilic environment for the bacteroids (Bergersen 1962; Tjepkema and Yocum 1973), the occurrence of lenticels (McVean 1956; Bond 1963) and large intercellular spaces (Wheeler *et al.* 1979; Tjepkema 1979) allows free air diffusion to the region of infected cells in actinorhizal root nodules.

Oxygen protection of nitrogenase in most actinorhizal associations has been attributed to localization of the enzyme in structurally and presumably, biochemically, unique cells of the endophyte, the vesicles. These cells differentiate from the tips of hyphal branches of the *Frankia* endophyte coincidentally with the onset of nitrogenase activity both in the nodule (Becking 1977; Mian and Bond 1978; VandenBosch and Torrey 1983) and, under conditions of combined nitrogen limitation, in pure culture (Tjepkema *et al.* 1980, 1981; Gauthier *et al.* 1981; Burggraaf and Shipton 1983; Fontaine *et al.* 1984; Lopez *et al.* 1984; Murry, Fontaine, and Torrey 1984; Zhang *et al.* 1985). Since maximum acetylene reduction activity in the free-living endophyte occurs near atmospheric partial pressure

of oxygen (P_{O_2}), the endophyte at least in culture, is capable of protecting nitrogenase from inactivation by oxygen.

The oxygen protection mechanisms of nitrogenase in *Frankia* are poorly understood at present but are thought to involve, in part, a gas diffusion barrier associated with the vesicle. A complex multilaminate cell envelope surrounding the vesicle was demonstrated in freeze–fracture preparations of a *Frankia* isolated from *Comptonia peregrina* (Torrey and Callaham 1982). This same layered structure is observed in freeze–fracture electron microscopy (EM) preparations of root nodules of *Alnus* (Lalonde *et al.* 1976). The vesicle envelope which resembles the laminated layer of the heterocyst in cyanobacteria (Lang and Fay 1971) was postulated to provide a barrier to the ingress of O_2 (Torrey and Callaham 1982). Diffusion-limited kinetics of respiration associated with the vesicles of a *Frankia* isolated from *Alnus rubra* provided experimental evidence in support of this view (Murry, Fontaine, and Tjepkema 1984).

Although it is generally accepted that the vesicle is the site of nitrogen fixation in *Frankia*, several structural studies of two related genera of actinorhizal plants in the Casuarinaceae, including *Casuarina* and *Allocasuarina*, have shown that the *Frankia* endophyte in these hosts does not produce vesicular structures (see J. G. Torrey. 1981. Proceedings of the International Workshop on Biological Nitrogen Fixation Technology for Tropical Agriculture, International Centre for Tropical Agriculture, Cali, Colombia. pp. 427–439); however, like *Alnus*-type nodules, *Casuarina* nodules are capable of nitrogen fixation at atmospheric oxygen tensions (Bond 1961; Zhang and Torrey 1985*a*). Thus, the vesicle is not a prerequisite for nitrogenase synthesis and function in at least some actinorhizal symbioses. The recent isolation of effective *Frankia* strains from the nodules of *Casuarina cunninghamiana* (Zhang *et al.* 1984) and *Allocasuarina* (Zhang and Torrey 1985*b*) has furthermore shown that these isolates behave like other *Frankia* strains in culture and produce vesicles concomitant with the induction of nitrogenase activity in response to limitation of combined nitrogen. These observations raise several questions including the following: What is the site of N_2 fixation in *Casuarina* nodules? How is the enzyme protected from inactivation by O_2? and What environmental factors influence vesicle differentiation in *Casuarina* endophytes? In the present study, the effect of ambient P_{O_2} on vesicle differentiation and O_2 protection of nitrogenase activity was studied in an effective *Casuarina* isolate, HFPCcI3, in culture.

Materials and methods

Culture conditions

Axenic cultures of *Frankia* sp. HFPCcI3 (catalog No. HFP020203, referred to here as CcI3), isolated from the root nodules of *Casuarina cunninghamiana* (Zhang *et al.* 1984), were grown at 33°C in defined medium containing 5 m*M* NH_4Cl and 10 m*M* sodium pyruvate as described earlier (Zhang *et al.* 1985). Nitrogenase was induced as before. Briefly, cells were washed free of NH_4^+ and resuspended at a cell density of 10 μg protein · mL^{-1} in N-free BV medium (Zhang *et al.* 1985) buffered at pH 6.3 with either 10 m*M* sodium pyruvate as the sole carbon source or in combination with 40 m*M* glucose as indicated in the text. Cells were cultured in 0.5-L cylindrical bottles with a working volume of 350 mL and sparged continuously (150–200 mL/min) with either air or a defined gas mixture. The defined gas mixtures which contained O_2 (between 0.1 and 5%, v/v), 0.5% CO_2, balance N_2, were produced with a Matheson three-stage gas mixer (Matheson Gas Products, Seacaucus, NJ). When defined gas mixtures were used, 1 m*M* $NaHCO_3$ was added to the medium.

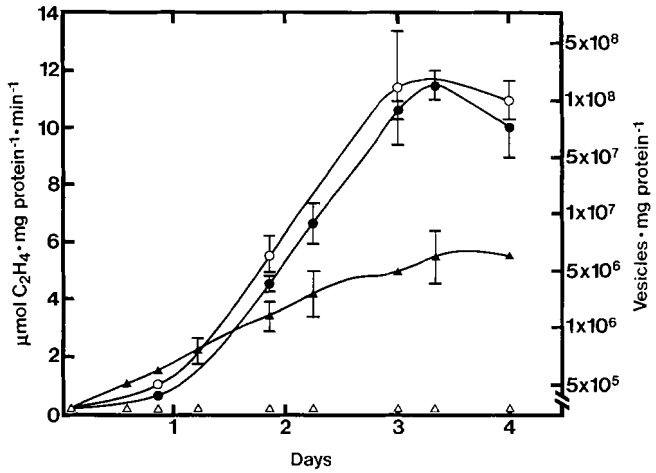

FIG. 1. Time course of acetylene-reducing activity in CcI3 incubated under air (●) and microaerophilic (P_{O_2} = 0.003 atm) conditions (▲). Vesicle frequency under air (○) and under microaerophilic conditions (△) is also shown. Vertical bars represent standard deviations based on two to four replicate samples.

The O_2 concentration in the gas phase was monitored by analyzing 1-mL samples from the out-gas tube of the culture vessel by thermal conductivity gas chromatography (Murry, Fontaine, and Torrey 1984). Since low cell densities were used and the cultures were sparged at a rapid rate, the dissolved O_2 concentration of the medium was assumed to be in equilibrium with the gas phase.

Assays

Acetylene reduction activity was monitored during induction in 10-mL serum vials containing the same P_{O_2} as the induction medium (balance N_2). Replicate 2-mL samples were taken by inserting a N_2-flushed syringe with a 18-gauge needle through a serum-stoppered sample tube that extended to the bottom of the culture bottle and forcing the cell suspension into the sample tube by closing off the out-gas tube. To determine the optimum P_{O_2} for acetylene reduction, the cells were sparged with N_2 for 15–30 min to remove dissolved O_2 from the medium before sampling. The cells were equilibrated at the desired O_2 tension on a reciprocal shaker (80 rpm) at 33°C for 20–30 min before initiating the assay by injecting acetylene at 10% (v/v). Ethylene production, measured as described before (Murry, Fontaine, and Torrey 1984), was linear for more than 90 min under these conditions. The bars shown in the figure legends represent standard deviation of two to four replicate samples.

For O_2-uptake measurements cells were concentrated 10-fold and placed in a 2-mL chamber maintained at 33°C; O_2 uptake was measured polarographically using a YSI model 53 O_2 electrode (Yellow Springs, OH) and a Keithley model 610 C electrometer. A high-sensitivity membrane (YSI) was used when measuring dissolved O_2 below 15 μ*M*.

Vesicles were counted in a Petroff–Hausser chamber at 400× magnification with a phase-contrast microscope.

Cellular protein was estimated by the Bradford procedure (Bradford 1976) using cells that were sonicated (15 s at 100 W) with a Braunsonic model 1510 sonicator and boiled for 10 min in 0.3 *N* NaOH (Drews 1965).

Results

In Fig. 1 the time courses of nitrogenase induction (acetylene reducing activity) in CcI3 incubated under air and under microaerophilic conditions are compared. At time zero, NH_4^+-grown, undifferentiated cells were washed to remove NH_4^+ and incubated in N-free medium that was continuously sparged with the desired gas mixture. Under air, phase-bright vesicles (Fig. 2*a*) were evident within 20 h and increased linearly until maximum

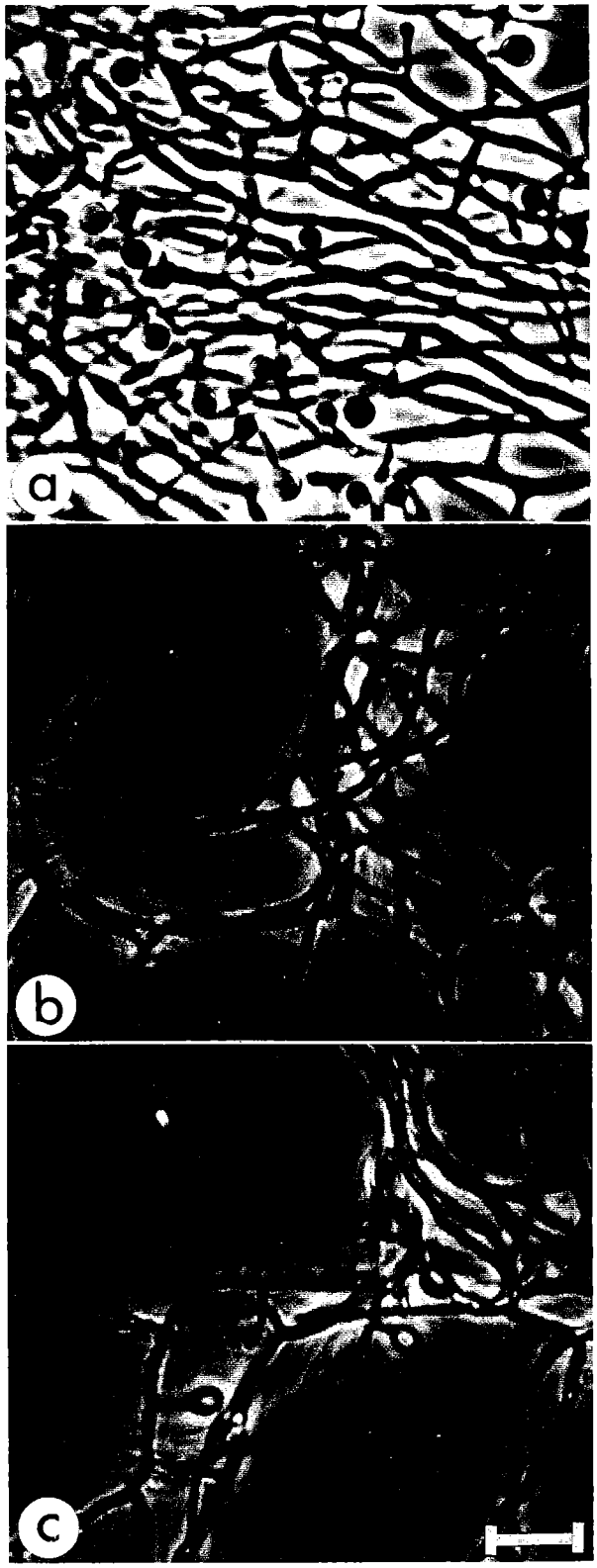

FIG. 2. Phase-contrast micrographs of CcI3 induced to fix N_2 under varying O_2 tensions. Bar equals 10 μm. (*a*) CcI3 induced for 3 days under air; (*b*) CcI3 induced for 3 days under P_{O_2} of 0.003 atm; (*c*) CcI3 induced for 3 days under P_{O_2} of 0.005 atm.

frequency was reached at about 72 h after initiating induction (Fig. 1). *In vivo* acetylene reduction activity (measured under air) developed in parallel with vesicle differentiation. Cellular protein levels increased nearly threefold within 4 days (data not shown; see Zhang *et al.* 1985). This pattern of events is typical

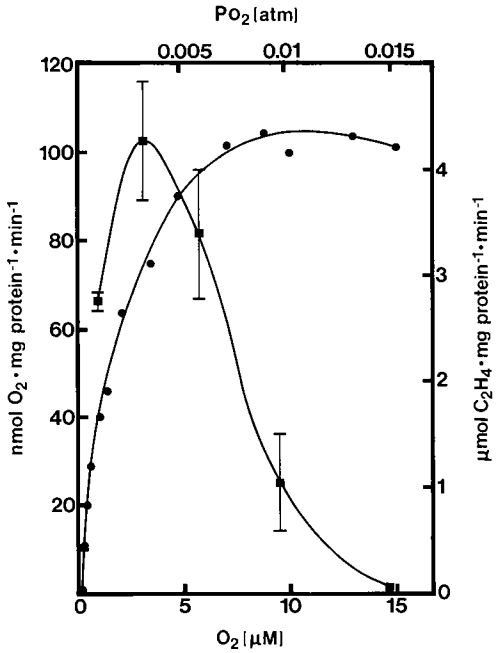

FIG. 3. Effect of O_2 concentration on rates of acetylene reductio (■) and respiration (●) in avesicular cultures of CcI3 induced to fi N_2 for 3 days under a P_{O_2} of 0.003 atm. Vertical bars represer standard deviations based on two to four replicate samples.

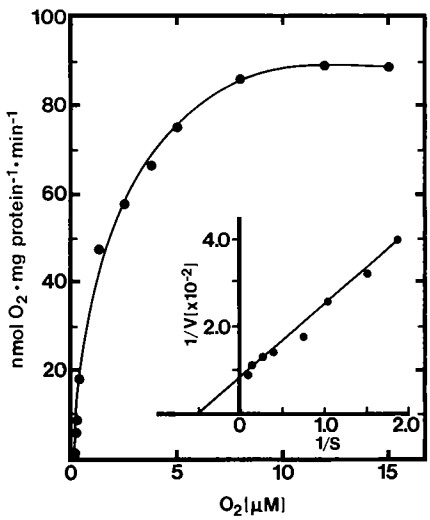

FIG. 4. Response of respiration to dissolved O_2 concentration ir NH_4^+-grown CcI3 filaments. Inset shows Lineweaver–Burk plot o the same data.

of other *Frankia* strains subjected to N limitation under aerobi conditions (Tjepkema *et al.* 1980, 1981; Gauthier *et al.* 1981 Burggraaf and Shipton 1983; Fontaine *et al.* 1984; Lopez *et al* 1984; Murry, Fontaine, and Torrey 1984). However, unde microaerophilic conditions (in Fig. 1, about 0.3% O_2 in th sparging mixture), the cells remained undifferentiated (Figs. and 2*b*) during the course of the experiment, yet nitrogenas activity (measured under 0.3% O_2) was induced and increase linearly for 36 h and then gradually leveled off. There was n significant change in total cellular protein during the course o induction.

Although we have not attempted a thorough survey of nutri tional conditions, maximum rates of acetylene reduction unde microaerophilic conditions were obtained using pyruvate plu glucose as the carbon source. Earlier, we showed that these tw

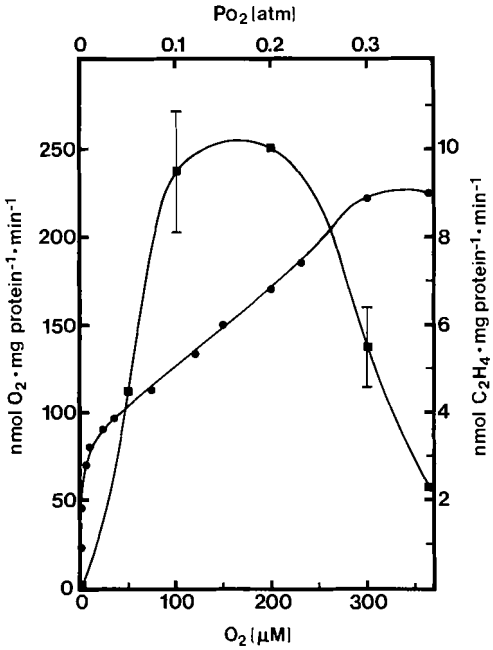

FIG. 5. Effect of O_2 concentration on rates of acetylene reduction (■) and respiration (●) in CcI3 filaments induced to form vesicles and nitrogenase under air for 3 days. Vertical bars represent standard deviations based on two to four replicate samples.

carbon sources together stimulated acetylene reduction but not vesicle formation above pyruvate-supported rates in CcI3 induced aerobically (Zhang *et al.* 1985). The maximum rates of acetylene reduction obtained under microaerophilic conditions were less than 50% of rates normally seen in aerobically induced vesicle-containing cells. It is unclear whether the low rates of acetylene reduction under these conditions indicate nutrient limitations to nitrogenase synthesis and function or whether other factors relating to O_2 availability are involved. In O_2-limited bacteroid suspensions of *Rhizobium japonicum*, the addition of partially oxygenated leghemoglobin caused a great stimulation of acetylene reduction yet a modest rise in respiration. In the absence of leghemoglobin, increasing O_2 concentration stimulated respiration but not acetylene reduction (Bergersen *et al.* 1973; Wittenberg *et al.* 1974).

In vivo acetylene reduction activity was extremely O_2 sensitive in avesicular cultures. Figure 3 shows that maximum acetylene reduction activity in cells induced under a P_{O_2} of 0.003 atm (3 μM O_2) occurred at the same P_{O_2} (i.e., 0.003 atm) as that of the induction medium. Increasing the P_{O_2} of the assay system to 0.01 atm O_2 (10 μM O_2) substantially inhibited acetylene reduction and activity was abolished at a P_{O_2} of 0.015 (15 μM O_2) atm. The kinetics of O_2 uptake in microaerophilically induced cells suggest that the O_2 sensitivity of nitrogenase was due in part to a poorly developed gas-diffusion barrier. Respiration (O_2 uptake) was saturated by dissolved O_2 concentrations of between 8 and 10 μM which corresponds to the P_{O_2} at which acetylene reduction was inhibited by O_2 (Fig. 3). At lower O_2 levels, both respiration and acetylene reduction were limited by O_2.

The kinetics of respiration as a function of dissolved O_2 concentration in microaerophilically induced avesicular cells were similar to those observed on NH_4^+-grown undifferentiated CcI3; O_2 uptake was saturated at about 8 μM O_2 and showed an apparent K_m of about 1.0 μM O_2 (Fig. 4). Reported apparent K_m values for *in vivo* O_2 uptake in bacteria range between 0.006 and 2.4 μM O_2 (Linton *et al.* 1977; Rice and Hempfling 1978; Bergersen and Turner 1980; Jensen and Cox 1983; Murry, Fontaine, and Tjepkemah 1984). In contrast, the kinetics of O_2 uptake as a function of dissolved O_2 concentration in aerobically induced (i.e., vesicle-containing cells) showed a diffusion-limited component: O_2 uptake was dependent on O_2 concentration until 300 μM O_2 which corresponds to a P_{O_2} of about 0.3 atm at 33°C (Fig. 5). In aerobically induced CcI3, maximum rates of acetylene reduction were observed at P_{O_2} between 0.1 and 0.2 atm in the gas phase of the assay. Acetylene reduction was dependent on O_2 which is presumably due to a requirement for ATP provided by oxidative phosphorylation. As respiration became saturated by dissolved O_2, acetylene reduction was progressively inhibited.

Small, usually phase-dark vesicles developed in CcI3 after incubation in N-free media for 2 to 3 days when sparged with gas mixtures with a P_{O_2} between 0.005 to 0.01 atm (Fig. 2*c*).

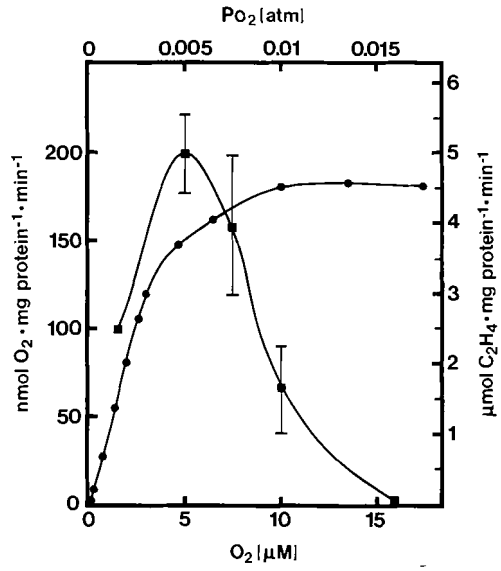

FIG. 6. Effect of O_2 concentration rates on acetylene reduction (■) and respiration (●) in CcI3 induced for 3 days under a P_{O_2} of 0.005 atm. Vertical bars represent standard deviations based on two to four replicate samples.

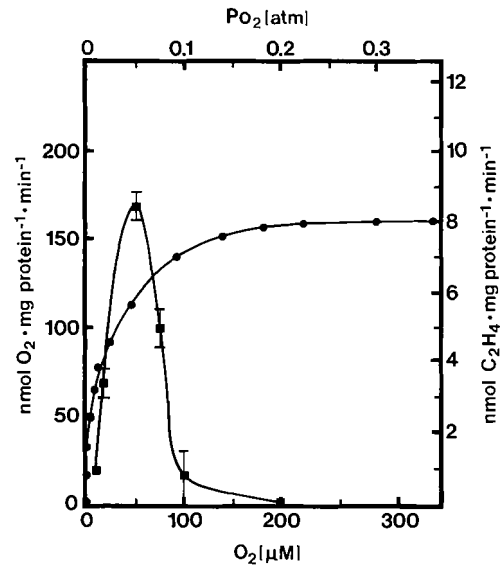

FIG. 7. Effect of O_2 concentration on rates of acetylene reduction (■) and respiration (●) in CcI3 induced for 3 days under a P_{O_2} of 0.05 atm.

However, O_2 protection mechanisms of nitrogenase were poorly developed. Maximum rates of *in vivo* acetylene reduction occurred at the P_{O_2} of the induction medium (0.005 atm in the experiment shown in Fig. 6). As respiration became saturated by dissolved O_2 (about 15 μM), acetylene reduction was sharply inhibited. Vesicles, indistinguishable under phase optics from those developed under aerobic conditions, were produced in cells incubated at a P_{O_2} of 0.05 atm (data not shown). Again, maximum acetylene reduction occurred at the P_{O_2} of the induction medium (0.05 atm) and was progressively inhibited as respiration became saturated by O_2 at about 180 μM (Fig. 7).

Discussion

Nitrogenase activity was induced in the effective *Casuarina* isolate CcI3 in culture without the concomitant development of vesicles when the P_{O_2} of the induction medium was lowered to 0.002 to 0.003 atm following the removal of NH_4^+. *In vivo* nitrogenase activity, however, was extremely O_2 sensitive which was correlated with the absence of a diffusion barrier to gas exchange as indicated by the kinetics of respiration as a function of O_2 concentration. The kinetics of respiration in undifferentiated, microaerophilically induced cells were similar to those of NH_4^+-grown cells. In each case, respiration was saturated at low (5–10 μM O_2) dissolved O_2 concentrations and approximated Michaelis–Menton enzyme kinetics. In vesicle-containing cells, a second kinetic component was observed which, in air-grown cells, showed a linear increase in O_2-uptake rates in response to increasing O_2, until respiration was saturated at about 300 μM. These kinetics approximate Blackman kinetics characteristic of diffusion-limited processes (Lommen *et al.* 1971) and are similar to those observed in vesicle-containing cultures of a *Frankia* strain isolated from *Alnus* (Murry, Fontaine, and Torrey 1984) and a heterocystous cyanobacterium (Jensen and Cox 1983). In each case, the association of Blackman kinetics with the presence of vesicles and heterocysts, analogous structures which are the locus of nitrogen fixation in each organism, was interpreted as evidence for the existence of a gas-diffusion barrier surrounding these specialized cells which retarded the ingress of O_2.

The development of O_2 protection mechanisms is adaptive in CcI3 and allows for maximum acetylene reduction at the P_{O_2} of the induction medium. This result is in part due to the effect of P_{O_2} on the development of the gas-diffusion barrier of the vesicle itself as indicated by the increase in the diffusion-limited component of respiration correlated with increased P_{O_2} of the induction medium. Although vesicles developed in cells incubated at P_{O_2} greater than 0.005 atm O_2, they provided little protection of nitrogenase activity against suprambient O_2 concentrations.

The induction of nitrogenase activity in CcI3 under microaerophilic culture conditions without the concomitant development of vesicles suggests that microaerophilic conditions exist within *Casuarina* nodules. This possibility was suggested by earlier reports in the literature. Davenport (1960) and more recently Tjepkema (1983*a*) reported the occurrence of hemoglobin in actinorhizal nodules, suggesting the presence of zones of low P_{O_2}. In legume nodules, leghemoglobin is thought to reduce the P_{O_2} gradient within the bacteroid-containing cells by facilitating diffusion of O_2 thus maximizing oxidative respiration and keeping nitrogenase inactivation to a minimum (Appleby 1984). Infected cells of *Myrica gale* and *Casuarina cunninghamiana* contain hemoglobin levels comparable with those in legume nodules (Tjepkema 1983*a*). Low levels were also detected in *Comptonia peregrina, Alnus rubra,* and *Elaeagnus umbellata* but not in *Ceanothus* or *Datisca* nodules (Tjepkema 1983*a*). The presence of hemoglobin is correlated to some degree with the presence of suberized cell walls in *Casuarina* (Berg 1983) and *Myrica gale* (VandenBosch and Torrey 1985) nodules. In *M. gale*, microelectrode measurements of O_2 tension within root nodules revealed very low O_2 levels in certain regions corresponding in size and location to infected cells; relatively high O_2 tensions were measured in the rest of the nodules (Tjepkema 1979, 1983*b*). Regions of low O_2 tension could not be detected in *Alnus rubra* nodules (J. D. Tjepkema, personal communication). Thus, there is apparently considerable variation in the strategies of O_2 protection mechanisms within actinorhizal plants. In the *Casuarina* isolate studied here, vesicle differentiation and the development of the gas-diffusion barrier are adaptive and regulated by both combined nitrogen and O_2 availability.

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