Oxygen Supply and Energy-yielding Substrates for Nitrogen Fixation (Acetylene Reduction) by Bacteroid Preparations

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Sucrose and glucose supported acetylene reduction by bacteroids extracted from French-bean, soybean and pea root nodules in the presence of low O₂ concentrations in experiments carried out with or without a gas phase, but not under O₂ tensions usually able to support acetylene reduction with succinate. Addition of leghaemoglobin to the bacteroid suspensions allowed maximum rates of ethylene formation at very low O₂ concentrations (1 to 5 nM) when sucrose or glucose was present in assays with no gas phase. Stimulation of bacteroid O₂ consumption was lower with these carbohydrates than with succinate for a similar acetylene reduction activity. This low O₂ uptake provided O₂ steady-state conditions in gas phase experiments. The optimal O₂ tension required for bacteroid acetylene reduction was lower when nodule age increased.

INTRODUCTION

Bacteroids of legume root nodules have an absolute O₂ requirement for N₂ fixation (Bergersen, 1971), since respiration provides both energy and electron flow for nitrogenase activity. Anaerobic preparations of bacteroids were able to reduce ¹⁵N₂ in the presence of O₂ tensions lower than 10%, which avoid nitrogenase inactivation (Bergersen, 1967). Energy-yielding substrates added to incubations enhanced N₂ fixation by increasing the respiration rate of bacteroids, and organic acids such as succinate and fumarate were particularly efficient (Bergersen & Turner, 1967). In contrast, glucose and sucrose were generally considered as completely inefficient under the same conditions (Bergersen, 1974). Using French-bean bacteroids, we recently observed the ability of glucose to act as an energy-yielding substrate for acetylene reduction when low O₂ tensions were used (Trinchant & Rigaud, 1979). Since sucrose and glucose are the main products translocated to the root nodules (Bach *et al.*, 1958; Streeter & Bosler, 1976; Singh *et al.*, 1980), whereas succinate has not been detected in the host cells (Antoniw & Sprent, 1978), it was of interest to investigate the role of these sugars in N₂ fixation.

In this paper we describe acetylene reduction in the presence of sucrose and glucose, in comparison with succinate, by bacteroids isolated from different legumes. An O₂ concentration-dependent efficiency of these substrates was demonstrated in experiments carried out with a gas phase, or with no gas phase and leghaemoglobin as O₂ carrier.

METHODS

Production of nodules and preparation of bacteroids. Nodules were produced on the roots of French-bean (*Phaseolus vulgaris* L. cv. Contander) inoculated with *Rhizobium phaseoli* strain 9–6, on soybean (*Glycine max* Merr. cv. Altona) inoculated with *Rhizobium japonicum* strain 1809, and on pea (*Pisum sativum* L. cv. Centurion) inoculated with *Rhizobium leguminosarum* strain FH16. Plants were grown in a glasshouse as described previously (Rigaud & Puppo, 1975).

Bacteroids were prepared anaerobically from 25 to 30 g (fresh wt) of French-bean and soybean nodules, or from 15 to 18 g (fresh wt) of pea nodules, by the method of Bergersen & Turner (1973). After washing in 40 ml phosphate buffer (50 mM, pH 7.4) containing sucrose (0.3 M), bacteroids were resuspended in phosphate buffer (25 mM, pH 7.4) to give a final concentration of about 40 mg dry wt ml⁻¹.

Leghaemoglobin. The red supernatant obtained by centrifugation of nodule homogenates was purified according to Appleby (1969), using the conditions described by Puppo & Rigaud (1975). Oxyleghaemoglobin, prepared after Na₂S₂O₄ treatment and chromatography on a 25 × 2.5 cm column of Sephadex G-15 (Wittenberg *et al.*, 1974), was concentrated to 400 to 600 µM over an Amicon UM10 membrane; 1 ml samples were stored in liquid N₂ until used (Bergersen & Turner, 1979). Leghaemoglobin concentrations were determined from pyridine haemochromogen assays (Bergersen *et al.*, 1973).

Measurement of O₂ concentration. For low concentrations of dissolved O₂, O₂ consumption by bacteroids was assayed by the deoxygenation of oxyleghaemoglobin measured at 576 nm (the α peak of the oxyhaemoprotein) and 562 nm (the trough between the α and β peaks) (Bergersen & Turner, 1975, 1979). Assays (4 ml) were made in glass cuvettes (10 mm light path) closed with rubber serum stoppers. The cuvettes were flushed with Ar and then completely filled with the reaction solution which contained 50 to 100 µM-leghaemoglobin and dissolved O₂. Bacteroids (1 mg dry wt) were added using a microsyringe and the cuvettes, containing two glass beads, were vigorously shaken. The reaction (15 min) was followed by recording, during 1 min, the absorbance at 562 or 576 nm alternately in a Varian Techtron spectrophotometer (model 635) with a temperature-controlled sample chamber maintained at 25 °C. The determination of free dissolved O₂ concentrations from the oxygenation state of leghaemoglobin and the constants used in the calculations were as described by Bergersen & Turner (1979).

O₂ concentration was also determined in experiments carried out with a gas phase in 24 ml rubber-capped vials containing 2 ml reaction medium. At intervals, reactions were terminated by injecting 0.1 ml of 2.5 M-HCl. Samples (0.2 ml) were then transferred, under Ar, by microsyringe to an O₂ electrode chamber containing 1.8 ml Ar-saturated phosphate buffer (25 mM, pH 7.4). The O₂ electrode (Rank Bros., Bottisham, Cambs.) was equipped with an amplifier and a recorder (Bergersen & Turner, 1975).

Nitrogenase activity. Incubations with a gas phase were performed in duplicate; bacteroid nitrogenase activity was measured by the acetylene reduction technique (Hardy *et al.*, 1968), using the conditions previously described (Rigaud, 1976). Experiments with no gas phase were done in triplicate in glass cuvettes, using a reaction medium equilibrated with a gas mixture containing 15 to 20% (v/v) acetylene (120 to 140 mmHg). At 5 min intervals during O₂ consumption (15 min), samples were slowly withdrawn from the reaction mixture with a syringe and injected into evacuated 15 ml 'Venocject' tubes (Bergersen & Turner, 1975). The evolved gas was analysed by gas chromatography and the amount of ethylene was determined.

RESULTS

Acetylene reduction by bacteroids in the presence of glucose and sucrose

Experiments were carried out with a gas phase containing different partial pressures of O₂. With French-bean bacteroids, nitrogenase activity initially increased with increasing *p*O₂ when glucose or sucrose was added (Fig. 1*a*). The activity reached an optimum at a *p*O₂ of 20 to 25 mmHg and then sharply declined. No acetylene reduction was observed with O₂ tensions higher than 40 mmHg. In contrast, with succinate, a usual substrate for bacteroid incubations, acetylene reduction activity increased linearly with *p*O₂ between 10 and 60 mmHg. Bacteroids extracted from soybean (Fig. 1*b*) or pea nodules gave similar results.

The dissolved O₂ concentration remained fairly constant, throughout a 15 min experiment, for the different initial values of *p*O₂ in the presence of glucose (Fig. 2) or sucrose. Thus, in experiments performed with a gas phase in the presence of these two substrates, it was possible to provide O₂ steady-state conditions for bacteroid respiration. In contrast, O₂ consumption was stimulated by succinate and the concentration of free dissolved O₂ dropped rapidly, reaching about 50% of the initial concentration after 15 min. In the presence of glucose, acetylene reduction proceeded linearly with time during 15 min for the lowest O₂ tensions (Fig. 2). Acetylene reduction did not occur when the *p*O₂ was 50 mmHg.

Acetylene reduction in the presence of leghaemoglobin

The changes in absorption spectra occurring during the deoxygenation of leghaemoglobin by bacteroids from French-bean (Fig. 3*a*) and soybean (Fig. 3*b*) in experiments performed with no gas phase showed that deoxygenation was a sigmoidal phenomenon both for the

Fig. 1. Acetylene reduction by French-bean (a) and soybean (b) bacteroids as a function of pO_2 in the gas phase. Incubation mixtures (2 ml) contained 50 mM glucose (○), sucrose (●) or succinate (□) and bacteroids (20 mg dry wt) in phosphate buffer (25 mM, pH 7.4). The vials were shaken ($140 \text{ rev. min}^{-1}$) at 30°C for 10 min.

Fig. 2. Time course of acetylene reduction by French-bean bacteroids (—) and dissolved O_2 concentration in the incubation mixtures (---). The initial pO_2 values in the gas phase were 10 (●), 25 (○) and 50 (▲) mmHg; the corresponding dissolved O_2 concentrations were 16, 39 and 78 μM , respectively. Incubation mixtures (2 ml) contained 50 mM-glucose and bacteroids (19 mg dry wt) in phosphate buffer (25 mM, pH 7.4). The vials were shaken ($140 \text{ rev. min}^{-1}$) at 30°C .

increase in absorbance at 562 nm and the decrease at 576 nm. The leghaemoglobin deoxygenation rate was always lower in the presence of glucose or sucrose than with succinate.

The results of a typical experiment conducted with French-bean bacteroids at different low concentrations of dissolved O_2 , with leghaemoglobin, are given in Fig. 4. In the presence of glucose or sucrose, acetylene reduction activity was stimulated with free dissolved O_2 in the range to 1 to 5 nM and strongly inhibited at higher concentrations (Fig. 4a). With succinate, ethylene formation increased at O_2 concentrations between 5 and 10 nM and then remained relatively constant at higher concentrations. The same optimal acetylene reduction rate [5 to $6 \text{ nmol ethylene min}^{-1} (\text{mg dry wt})^{-1}$] was reached with $100 \text{ nM-}O_2$ in the presence of

Fig. 3. Time course of leghaemoglobin deoxygenation by French-bean (a) and soybean (b) bacteroids monitored spectrophotometrically at 562 and 576 nm. Reaction mixtures (4 ml), in stoppered cuvettes, contained 10 mM glucose (—), sucrose (---) or succinate (···), 75 μ M-leghaemoglobin and bacteroids (1 mg dry wt) in phosphate buffer (25 mM, pH 7.4). The initial free O_2 concentration was 1 μ M.

Fig. 4. Acetylene reduction (a) and O_2 consumption (b) by French-bean bacteroids at low dissolved O_2 concentration. The reaction solution (4 ml) contained 10 mM glucose (●), sucrose (○) or succinate (■), 82 μ M-leghaemoglobin and bacteroids (1.2 mg dry wt) in phosphate buffer (25 mM, pH 7.4).

Fig. 5. Acetylene reduction (a) and O_2 consumption (b) by soybean bacteroids at low dissolved O_2 concentration. Experimental conditions were as described in Fig. 4.

Fig. 6. Effect of pO_2 on acetylene reduction activity of French-bean bacteroids extracted from 12-d-old (▲) or 24-d-old (△) nodules. Incubation mixtures (2 ml) contained 50 mM glucose (a) or succinate (b) and bacteroids (20 mg dry wt) in phosphate buffer (25 mM, pH 7.4). The vials were shaken (140 rev. min^{-1}) at 30 °C for 10 min.

succinate as with 5 nM- O_2 in the presence of glucose or sucrose. In all cases the rate of acetylene reduction remained linear with time for at least 10 min. In the range of 1 to 100 nM- O_2 , respiration was enhanced 3-fold with glucose or sucrose (Fig. 4b); in contrast, O_2 uptake increased 8.5-fold in the presence of succinate when the O_2 concentration was increased from 5 to 100 nM. Similar results were obtained with bacteroids extracted from soybean root nodules (Fig. 5 a, b).

Bacteroid O_2 sensitivity in relation to nodule age

The effect of pO_2 on acetylene reduction activity was studied with French-bean bacteroids isolated from 12- and 24-d-old nodules. Glucose or succinate was added as respiratory substrate in experiments with a gas phase (Fig. 6). In both cases, acetylene reduction activity was lower for the older bacteroids, but the decline was always less marked with glucose than with succinate. Moreover, the O_2 tension required for optimal nitrogenase activity was shifted to a lower pO_2 with both glucose (from 30 to 15 mmHg) and succinate (from 60 to 45 mmHg). For both substrates, the O_2 tensions which gave the highest ethylene formation with young bacteroids completely inhibited the activity of old bacteroids.

French-bean bacteroids extracted from 25-d-old nodules and kept in liquid N_2 for 15 d showed a similar increase in sensitivity to O_2 .

DISCUSSION

Sucrose and glucose were able to provide energy for acetylene reduction by bacteroids isolated from root nodules of different legumes. This property was observed with bacteroids belonging to both the fast- and slow-growing groups of *Rhizobium*. Low O_2 tensions were required with both these carbohydrates for acetylene reduction and were critical for optimal activity. These results are similar to those of Rigaud *et al.* (1973) concerning the ability of glucose to support N_2 fixation in anaerobic conditions with NO_3^- as the terminal electron acceptor. They confirm the suggestion of Bergersen (1977) about a role for hexoses in providing energy and reductant for nitrogenase activity in vivo, although glucose was generally considered to be a poor substrate for bacteroids. Several authors have concluded that different preparations of bacteroids are unable to oxidize glucose (Ronson & Primrose, 1979; Vries, 1980; Ratcliffe *et al.*, 1980). The presence of a substantial amount of sucrose, provided by the plant, associated with the low O_2 tension (3 to 30 nM) in the nodules

(Wittenberg *et al.*, 1974), creates optimal conditions for this substrate to play a major role in N_2 fixation in vivo. Bacteroids oxidizing these carbohydrates had a lower O_2 requirement than those oxidizing succinate for the same level of N_2 fixation. The presence of leghaemoglobin in experiments with no gas phase confirmed this result in that significant acetylene reduction activity was observed for O_2 concentrations (1 to 5 nM) lower than those reported in experiments with soybean bacteroids and succinate (Bergersen & Turner, 1979, 1980). These O_2 concentrations are in the range of those determined in the nodules (quoted above). Thus, sucrose and glucose could play a complementary role to leghaemoglobin in the O_2 economy of nodules.

When succinate was used as carbon source, the experimental procedure using bacteroid incubations with a gas phase did not generally provide O_2 steady-state conditions (Trinchant & Rigaud, 1979), except during a short period of time (Laane *et al.*, 1978). The low bacteroid O_2 consumption observed in the presence of glucose or sucrose permitted an equilibrium between O_2 uptake and O_2 dissolution from the gas phase to be maintained for a significant period of time. Thus, in giving an O_2 steady-state, glucose and sucrose provided accurate conditions for O_2 utilization by bacteroids in gas phase assays which were more convenient for ethylene determination.

To explain the discrepancy between our results and those commonly reported in the literature about sugar utilization by bacteroids in vitro, a major role for O_2 may be suggested. Sucrose and glucose were able to support acetylene reduction in a very narrow range of O_2 concentrations in assays conducted with a gas phase (7.5 to 39 μM) or with no gas phase after addition of leghaemoglobin (1 to 5 nM). Higher O_2 concentrations completely abolished N_2 fixation but were optimal when succinate was used. This loss of activity observed with carbohydrates was not caused by an inactivation of nitrogenase by O_2 , since acetylene reduction was completely restored by subsequent addition of succinate (Trinchant & Rigaud, 1979).

The bacteroid concentration was another critical factor. On average, 1 mg (dry wt) of bacteroids required 1.5 μM dissolved O_2 for optimal acetylene reduction when sucrose or glucose was supplied in gas phase experiments. Thus, inadequate conditions such as the dilution of bacteroid suspensions or the use of old bacteroids, in increasing the sensitivity to O_2 , could also explain the previous failure to observe acetylene reduction when sucrose or glucose was used as substrate.

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