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 Na₂S₂O₅ treatment and chromatography on a 25 × 2-5 cm column of Sephade G-15 (Wittenberg et al., 1974), was concentrated to 400 to 600 μM over an Amicon U101 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 oxylaemoprotein) 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 "Venoject" 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 pO₂ when glucose or sucrose was added (Fig. 1a). The activity reached an optimum at a pO₂ 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 pO₂ between 10 and 60 mmHg. Bacteroids extracted from soybean (Fig. 1b) or pea nodules gave similar results.

The dissolved O₂ concentration remained fairly constant, throughout a 15 min experiment, for the different initial values of pO₂ 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 pO₂ 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. 3a) and soybean (Fig. 3b) in experiments performed with no gas phase showed that deoxygenation was a sigmoidal phenomenon both for the
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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 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 \( \mu \)M, 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 rev. min\(^{-1}\)) at 30 °C.

increase in absorbance at 562 nm and the decrease at 576 nm. The leghaemoglobindeoxygenation 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 nmol ethylene min\(^{-1}\) (mg dry wt\(^{-1}\))] was reached with 100 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 μM-leghaemoglobin and bacteroids (1 mg dry wt) in phosphate buffer (25 mM, pH 7.4). The initial free O₂ concentration was 1 μM.

Fig. 4. Acetylene reduction (a) and O₂ consumption (b) by French-bean bacteroids at low dissolved O₂ concentration. The reaction solution (4 ml) contained 10 mM glucose (●), sucrose (○) or succinate (■), 82 μM-leghaemoglobin and bacteroids (1.2 mg dry wt) in phosphate buffer (25 mM, pH 7.4).

Fig. 5. Acetylene reduction (a) and O₂ consumption (b) by soybean bacteroids at low dissolved O₂ concentration. Experimental conditions were as described in Fig. 4.
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Fig. 6. Effect of pO₂ 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.

Bacteroid O₂ sensitivity in relation to nodule age

The effect of pO₂ 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 O₂ tension required for optimal nitrogenase activity was shifted to a lower pO₂ with both glucose (from 30 to 15 mmHg) and succinate (from 60 to 45 mmHg). For both substrates, the O₂ 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 N₂ for 15 d showed a similar increase in sensitivity to O₂.

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 O₂ 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 N₂ fixation in anaerobic conditions with NO₃⁻ 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 O₂ tension (3 to 30 nm) in the nodules...
N, fixation in vivo, Bacteroids oxidizing these carbohydrates had a lower O2 requirement than those oxidizing succinate for the same level of N2 fixation. The presence of leghaemoglobin in experiments with no gas phase confirmed this result in that significant acetylene reduction activity was observed for O2 concentrations (1 to 5 nM) lower than those reported in experiments with soybean bacteroids and succinate (Bergersen & Turner, 1979, 1980). These O2 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 O2 economy of nodules.

When succinate was used as carbon source, the experimental procedure using bacteroid incubations with a gas phase did not generally provide O2 steady-state conditions (Trinchant reported in experiments with soybean bacteroids and succinate (Bergersen literature about sugar utilization by bacteroids in vitro, a major role for O2 may be suggested. For ethylene determination.

Acetylene reduction activity was observed for O2 concentrations in assays conducted with a gas phase (7.5 to 39 uM) or with no gas phase after addition of leghaemoglobin (1 to 5 nM). Higher O2 concentrations completely abolished N2 fixation but were optimal when succinate was used. This loss of activity observed with carbohydrates was not caused by an inactivation of nitrogenase by O2, 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 uM dissolved O2 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 O2, could also explain the previous failure to observe acetylene reduction when sucrose or glucose was used as substrate.

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REFERENCES

BERGERSEN, F. J. & TURNER, G. L. (1979). Systems...


