Nitrate Dependent Anaerobic Acetylene-reduction and Nitrogen-fixation by Soybean Bacteroids

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SUMMARY

Bacteroids isolated from nodules produced by one strain of *Rhizobium japonicum* (CC705) had strong nitrate-reducing activity and reduced C_2H_2 to C_2H_4 and N_2 to NH_3 anaerobically with nitrate. Bacteroids of another strain (CB1809) were much less active nitrate reducers and reduced little C_2H_2 anaerobically. Nitrite, which accumulated in the medium in anaerobic assays, was an inhibitor of C_2H_2 reduction in both aerobic and anaerobic conditions. Succinate, at about 25 mM, stimulated both nitrate reduction and C_2H_2 reduction under aerobic conditions. Glucose stimulated C_2H_2 reduction up to 120 mM but nitrate reduction was inhibited in the presence of glucose. In terms of electrons transferred, the aerobic pathway appeared to be about 2.5 times more efficient than the anaerobic pathway in supporting nitrogenase activity of CC705 bacteroids.

INTRODUCTION

The utilization of nitrate for respiration by some strains of *Rhizobium japonicum* under low O_2 tension was suggested by Murphy & Elkan (1965). Daniel & Appleby (1972) reported that *R. japonicum* (strain CC705) grew anaerobically with nitrate; they also found high levels of nitrate reductase activity in bacteroids isolated from soybean nodules as had been reported previously (Chenae & Evans, 1960; Bergersen, 1961). N_2 fixation by intact nodules and by bacteroid suspensions is normally aerobic (Bergersen & Turner, 1967), but nitrate respiration might produce ATP for the support of N_2 fixation by soybean nodule bacteroids under anaerobic conditions. We now report anaerobic, nitrate-dependent reduction of C_2H_2 to C_2H_4 and of N_2 to NH_3 by bacteroid suspensions. The effects of substrate and nitrate concentrations were also investigated and comparisons were made between two strains of *Rhizobium japonicum* and between nitrate-dependent and O_2 -dependent reactions.

METHODS

Nodules. Soybeans (*Glycine max* Merr., cv. Lincoln), inoculated with *Rhizobium japonicum* strain CB1809 or CC705 (syn. Wisconsin 505), were grown as previously described (Bergersen, 1970) and collected about 35 days after sowing (nodule age 28 to 29 days), when nitrate reductase activity in the bacteroids was high; it declined in older nodules (cf. Bergersen, 1961).

Bacteroid suspensions. Nodules (about 20 to 40 g fresh wt) were homogenized in a Sorvall Omni-mixer under argon, in 100 ml potassium phosphate buffer (0.1 M, pH 7.0) containing sodium ascorbate (0.2 M) and polyvinyl pyrrolidone (50 g) and the homogenate filtered, as

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described previously (Bergersen & Turner, 1970, 1973). The filtrate was centrifuged and the sedimented bacteroids were washed twice in phosphate buffer (25 mM, pH 7.4) and their dry weight recorded.

Assays of nitrogenase activity. In most cases, activity was measured by C_2H_2 reduction (Hardy, Holsten, Jackson & Burns, 1968). In anaerobic conditions the assays were done in duplicate in rubber-capped glass vials (13.5 ml capacity) containing, in 5 ml of phosphate buffer (25 mM, pH 7.4), glucose or sodium succinate as substrate at various concentrations, and KNO_3 (8 mM). The gas phase contained 25 % (v/v) C_2H_2 or 70 % $^{15}N_2$ in argon. The reactions were started by the injection of bacteroids (40 to 50 mg dry wt/assay) and the vials were agitated on a reciprocating shaker at 100 c.p.m. at 30 °C. The reactions were terminated by the injection of 1 ml of 2.5 N-HCl containing 1 % (w/v) sulphanilamide.

In aerobic conditions, Warburg vessels (15 ml capacity) with rubber-capped sidearms were used, containing in 2 ml of phosphate buffer (25 mM, pH 7.4), sodium succinate (50 mM) unless otherwise stated, and approximately 20 mg (dry wt) of bacteroids. The gas phase contained 5 % (v/v) O_2 and 25 % (v/v) C_2H_2 or 70 % $^{15}N_2$ in argon and the final pressure was 700 mmHg. Flasks were shaken at 150 c.p.m. at 30 °C; these conditions were critical for optimum activity in aerobic assays with CC705 bacteroids. Reactions (in duplicate) were started by tipping the bacteroids from the sidearms and were terminated by the injection of 0.3 ml of 10 % (w/v) trichloroacetic acid.

C_2H_2 reduction was measured by analysing 200 μ l samples of gas from reaction vessels as described previously (Bergersen, 1970). N_2 fixation was measured by determining the ^{15}N content of NH_3 , recovered by steam distillation of the reaction mixture supernatants, using 50 μ g of unlabelled NH_3 -N as a carrier (Bergersen & Turner, 1968) and saturated Na-borate, pH 10.5. In all experiments, there was little variability between duplicates (e.g. Table 1) and in the Figures only mean values are shown.

Determination of nitrite. The colorimetric method of Nicholas & Nason (1957) was used for the determination of nitrite in supernatants after centrifugation of reaction mixtures.

Measurement of O_2 uptake. Aerobic respiration was measured by analysis of gas samples collected from reaction vessels, using an Atlas M 86 mass spectrometer, calibrated for the various gases.

RESULTS

The relationship between anaerobic C_2H_2 reduction and nitrate reductase activity

The results of a typical experiment with CC705 bacteroids are given in Fig. 1. Acetylene reduction was stimulated by succinate and glucose and the rates declined after 10 to 15 min. Anaerobic C_2H_2 reduction was absolutely dependent upon the presence of nitrate and the rate increased with increasing nitrate concentrations up to 4 mM (Fig. 2). The time course of nitrate reduction was different from that of C_2H_2 reduction; there was an initial lag, followed by a period in which the rates were constant. It also appeared that the rates of nitrate reduction with different substrates were not related to rates of C_2H_2 reduction, since glucose (60 mM) produced the highest rate of C_2H_2 reduction, but the lowest rate of nitrate reduction. Also, C_2H_2 reduction was highest in each treatment in the first 10 min when nitrate reduction rates were lowest. There was substantial endogenous C_2H_2 reduction and nitrate reduction (Fig. 3).

Identical experiments with CB1809 bacteroid suspensions produced little activity in either C_2H_2 reduction or nitrate reduction (for example in 30 min 0.3 nmol C_2H_4 mg^{-1} and 8.4 nmol NO_2^- mg^{-1} were produced), although aerobic C_2H_2 reduction was very active (86.7 nmol C_2H_4 mg^{-1} in 30 min).

Fig. 1. Time course of C_2H_2 reduction (a) and nitrate reduction (b) by bacteroid suspensions, in anaerobic conditions. Incubation mixtures (5 ml) contained KNO_3 (8 mM), bacteroids, strain CC705 (42 mg dry wt). The substrate was glucose (▲—▲, 60 mM), succinate (●—●, 25 mM), nil (+—+). The shaking rate was 100 c.p.m. at 30 °C. For other details see text.

Fig. 2. The effect of nitrate concentration upon C_2H_2 reduction by bacteroid suspensions, in anaerobic conditions. Incubation mixtures (5 ml) contained glucose (50 mM), KNO_3 (0.2 to 8 mM), bacteroids strain CC705 (45 mg dry wt). Assayed for 10 min at 30 °C with shaking.

Fig. 3. The effect of glucose and sodium-succinate upon C_2H_2 reduction (a, c) and nitrate reduction (b) by bacteroid suspensions, in anaerobic conditions. Bacteroids were resuspended in phosphate buffer, 25 mM, pH 7.4 (a, b) and substrates were sodium-succinate (●—●) or glucose (▲—▲). Bacteroids resuspended (c) in 15% (w/v) dextran 40 (△—△), and in 30% (w/v) dextran 40 (+—+) in the same buffer with glucose as substrate. Assayed at 30 °C for 20 min with shaking.

Fig. 4. Effect of glucose upon C_2H_2 reduction by bacteroid suspensions in aerobic conditions. Incubation mixtures (2 ml) contained sodium-succinate (50 mM), glucose (0 to 100 mM), bacteroids strain CC705 (22 mg dry wt). Assayed for 30 min at 30 °C with shaking.

Fig. 5. Inhibitory effects of nitrite upon C_2H_2 reduction by bacteroid suspensions. (a) Acetylene-reduction velocities at 10 and 20 min were derived from tangents drawn to the curves of Fig. 1. These rates are plotted as a function of nitrite concentration at those times. ●—●, Succinate; ▲—▲, glucose; +—+, without additions. (b) The effect of nitrite added to anaerobic incubation mixtures (5 ml) containing glucose (50 mM), KNO_3 (8 mM) and 17 mg (dry wt) of CC705 bacteroids. The vessels were shaken at 30 °C for 30 min. The nitrite concentrations are initial values. (c) The effect of nitrite added to aerobic incubation mixtures (2 ml) containing sodium succinate (50 mM) and 17 mg (dry wt) of strain CB1809 bacteroids (■—■) or CC705 bacteroids (●—●). The vessels were shaken at 30 °C for 30 min.

Effects of substrate concentration

Succinate stimulated both nitrate reductase and C_2H_2 reduction at about 25 mM, but higher concentrations were inhibitory (Fig. 3*a, b*). The effects of glucose concentration were quite different. Concentrations up to 120 mM stimulated C_2H_2 reduction and higher concentrations were inhibitory. However, glucose inhibited nitrate reduction at all concentrations used. The high optimum glucose concentration for C_2H_2 reduction and its different effect on the two reactions suggested that this sugar may be exerting an osmotic effect,

Table 1. Nitrogen fixation by bacteroid suspensions in aerobic and in anaerobic conditions (strain CC705)

Incubation mixtures (2 ml) in aerobic conditions contained: sodium-succinate (50 mM), bacteroids 25 mg (dry wt) in phosphate buffer (25 mM, pH 7.4). In anaerobic conditions, the medium composition was: KNO₃ (8 mM), glucose (0.1 M), bacteroids 50 mg (dry wt) in phosphate buffer (25 mM, pH 7.4) for a final volume of 5 ml. The gas phase was: N₂, 68.6% (¹⁵N, 87.6 atoms %), A, 26.6% and O₂ 4.8%. Duplicate preparations were incubated for 30 min at 30 °C with shaking. For further details see text.

Preparations		NO ₃ ⁻ (mM)	NH ₃ -N (μg per vessel)*	¹⁵ N excess (atoms %)	Fixed NH ₃ -N (nmol mg ⁻¹)
Aerobically	1	0	47.3	2.157	2.92
	2	0	47.3	2.006	2.71
Anaerobically	3	8	55.4	1.602	1.27
	4	8	55.4	1.565	1.24
	5	0	45.0	0.000	0.00

* Includes carrier NH₃-N.

similar to that of sucrose in aerobic assays (Bergersen & Turner, 1967). The effect of glucose concentration was therefore tested in the presence of 15% and 30% (w/v) dextran 40 (Pharmacia, Uppsala, Sweden). In these conditions, the optimum glucose concentration for C₂H₂ reduction was lower (Fig. 3*a, c*). In contrast, in aerobic assays, glucose did not stimulate C₂H₂ reduction by strain CC705 bacteroids. However, with succinate, slight stimulation of C₂H₂ reduction by glucose (up to about 50 mM) was observed. Above 75 mM, glucose was sharply inhibitory (Fig. 4): these concentrations (75 to 120 mM) stimulated anaerobic C₂H₂ reduction.

The effect of nitrite

Nitrite is a strong inhibitor of C₂H₂ reduction by bacteroids under both aerobic and anaerobic conditions. Fig. 5(*a*) shows that the rates of C₂H₂ reduction decrease as nitrite accumulates. Rates of C₂H₂ reduction, obtained by drawing tangents to the curves of Fig. 1(*a*) at 10 and 20 min, are plotted against nitrite concentration at those times. With the exception of the point derived from the 10 min assay without additions, all points fall on a typical inhibition curve. Similar results were obtained when nitrite was added to anaerobic or aerobic assays (Fig. 5*b, c*). In the case of the anaerobic experiment the curve is flattened. This is due to the accumulation of nitrite from nitrate reduction; in Fig. 5(*b*) activities are plotted against the initial nitrite concentrations. In the curves of Fig. 5(*a*) and (*c*) 50% inhibition was obtained at 0.5 to 0.8 mM-nitrite. In the absence of added nitrite, concentrations of 2–3 mM-nitrite were usual after 40 min in experiments with succinate as substrate (Fig. 1*b*).

¹⁵N₂ experiments

All of the results described so far utilize C₂H₂ reduction as the assay for nitrogenase activity. Using ¹⁵N₂ under anaerobic conditions with nitrate, glucose and CC705 bacteroids, 1.2 nmol of NH₃/mg of bacteroids was fixed in 30 min (Table 1). Aerobically, with succinate as substrate, 2.8 nmol NH₃/mg was fixed.

Fig. 6. Nitrate reduction and O_2 uptake during the C_2H_2 reduction by bacteroid suspensions (CC705) in anaerobic (a) and in aerobic (b) conditions. All assays with sodium-succinate (25 mM) as substrate, shaking at 30 °C. Anaerobically, incubation mixtures (5 ml) contained KNO_3 (8 mM), bacteroids (40 mg dry wt); ○—○, C_2H_2 reduction; +—+, nitrate reduction. Aerobically, incubation mixtures (2 ml) contained bacteroids (20 mg dry wt); ○—○, C_2H_2 reduction; ▲—▲, O_2 uptake.

Comparison of O_2 and nitrate respiration

In order to compare the relative efficiencies of O_2 and nitrate respiration for the support of C_2H_2 reduction by bacteroids, experiments such as the one illustrated in Fig. 6 were conducted. Nitrite production was used to assay nitrate reduction since nitrite reduction was negligible (Daniel & Appleby, 1972). Uptake of O_2 was measured by analysis of the gas phase. In the experiment shown (Fig. 6), after 15 min, when all reactions were proceeding linearly with time, O_2 -uptake and nitrate-reduction rates were the same (14 nmol/min/mg). However, the C_2H_2 -reduction rate was 1.85 nmol C_2H_4 /min/mg, which was about 5 times higher than the anaerobic rate (0.38 nmol C_2H_4 /min/mg). In terms of electrons transferred, the aerobic pathway was thus approximately 2.5 times as effective as the anaerobic pathway. This result is similar to that obtained in the ^{15}N experiments (Table 1), where the ratio of the amounts of NH_3 produced aerobically versus anaerobically was about 2.3, although the substrates were different in this experiment.

DISCUSSION

Soybean bacteroid nitrogenase requires ATP for activity (Koch, Evans & Russel, 1967). The results presented above show that sufficient ATP is produced by anaerobic nitrate respiration to support nitrogenase activity in bacteroids which have an active nitrate reducing system. However, nitrate respiration is apparently 2.5 times less efficient than the aerobic pathway of respiration. The exact pathway(s) of terminal respiration in bacteroids is not known although the constituent haemoproteins (Appleby, 1969) and some of the other components have been described (Evans & Russel, 1971).

The role of glucose as a substrate for bacteroids is uncertain. Burris & Wilson (1939) found that glucose was a poor substrate for aerobic respiration by soybean bacteroids and this has been general experience since (see Bergersen, 1971). D. K. Kidby and C. A. Parker (private communication) have proposed that a glucose permease may be damaged during the preparation of bacteroids since enzymes for glucose metabolism are found in bacteroid

extracts. The present results suggest that glucose may be utilized as an energy source by bacteroids, because C_2H_2 reduction is stimulated (Fig. 1*a*, 3*a*, 4). It is puzzling that glucose stimulates C_2H_2 reduction but not nitrate reduction. If it was being metabolized as a source of reducing power, glucose would be expected to stimulate both activities. Apparent osmotic effects concerned with the conservation of endogenous substrate have been reported previously for sucrose (Bergersen & Turner, 1967). It is possible that glucose exerts a similar effect because the optimum glucose concentration for anaerobic C_2H_2 reduction is quite high (120 mM, Fig. 3*a*) and is less in the presence of dextran (Fig. 3*c*) or when succinate is also present in aerobic assays (Fig. 4). This question would be resolved by a radiorespirometric study of glucose effects.

Inhibition by nitrite seemed to be superficially related to the different effects of glucose on C_2H_2 reduction and nitrate reduction. With glucose as substrate, nitrite production was always low and this seemed to allow higher rates of C_2H_2 reduction than with endogenous substrates. Fig. 1(*a*) and 6(*a*) show the curved time course of nitrate-supported C_2H_2 reduction. In contrast, rates of C_2H_2 reduction under aerobic conditions were linear for 20 min (Fig. 6*b*). Also, Fig. 1 shows that succinate, which gave the highest rate of nitrate reduction, supported the briefest period of C_2H_2 reduction. These observations are consistent with the inhibitory effects of nitrite. However, other factors must also be involved in the declining rates because the changes in rates of C_2H_2 reduction between 20 and 30 min (Fig. 1*a*) were not directly related to rates of nitrite accumulation in the assays. I. R. Kennedy (private communication) has observed inhibition by nitrite of nitrogenase in cell-free preparations from lupin bacteroids. The inhibitory effects of nitrite accumulating in the nodules of plants exposed to nitrate may explain in part the inhibitory effects of nitrate upon N_2 fixation by legumes (Nutman, 1956).

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