Nitrate Reductase from Anaerobically Grown *Rhizobium japonicum*

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SUMMARY

The activity of nitrate reductase in *Rhizobium japonicum* is controlled by oxygen tension, and not by nitrate. The enzyme from *R. japonicum* grown anaerobically in the presence of nitrate resembles that from bacteroids in having a molecular weight of about 69000 daltons; the enzyme from aerobically grown cells has a molecular weight of about 170000 daltons. Both types of enzyme have similar K_m values, but differ in their sensitivity to KCN.

INTRODUCTION

Free-living *Rhizobium japonicum* strain 505 (Wisconsin) grown anaerobically with nitrate as an electron acceptor has a number of features in common with the symbiotic, N_2 -fixing bacteroid form of this organism, including a nitrate reductase level which is about five times that found in free-living *R. japonicum* grown aerobically (Daniel & Appleby, 1972). Since the ability of *Rhizobium* to fix N_2 depends upon environmental conditions, rather than being a unique feature of an obligate symbiosis with legumes (Pagan *et al.*, 1975; Kurz & LaRue, 1975; McComb, Elliot & Dilworth, 1975), a study of the effect of environmental conditions is of some interest. In particular, a study of nitrate reductase may be important in understanding N_2 fixation, since there is evidence for a common genetic determinant for nitrate reductase and nitrogenase (Kondorosi *et al.*, 1973), and they may share a common subunit (Evans & Russel, 1971). Some aspects of the nitrate reductase from both the aerobically grown (Lowe & Evans, 1964) and bacteroid (Evans, 1954; Cheniae & Evans, 1959; Kennedy, Rigaud & Trinchant, 1975) forms of *R. japonicum* have been studied, and this paper presents complementary work on the enzyme from cells grown anaerobically.

METHODS

Growth and preparation of free-living bacteria and bacteroids. *Rhizobium japonicum* strain CC705 (Wisconsin 505) was grown as described by Daniel & Appleby (1972). Bacteroids were isolated and purified from N_2 -fixing soybean root nodules (*Glycine max* Merr., cv. Lincoln) inoculated with *R. japonicum* strain CC705 and grown as described by Appleby (1969a).

Preparation of nitrate reductase. All operations were carried out at 0 to 4 °C, using buffers flushed with N_2 or argon. Anaerobic conditions were maintained where necessary, by using a stream of N_2 or argon.

Washed organisms, suspended in 0.1 M-sodium-potassium phosphate buffer pH 6.8, were ruptured in a French pressure cell at about 10^8 Pa. The exuded material was centrifuged at 10000 g for 15 min; and then the supernatant was re-centrifuged at 250000 g for 1 h.

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The final supernatant was concentrated, where necessary, by pressure filtration over a Diaflow PM 10 membrane (Amicon Corporation, High Wycombe, Buckinghamshire). The pellet from the second centrifugation was washed once in 0.1 M-sodium-potassium phosphate buffer pH 6.8, and the membrane particles were resuspended to a final protein concentration of 30 to 50 mg ml⁻¹.

Assay of nitrate reductase. In the presence of nitrite reductase, nitrate reductase was assayed by nitrate removal, using a method for nitrate determination based on that of Szekely (1967). Assays were run in duplicate in conical 15 ml centrifuge tubes at 25 °C. When using electron donors other than dithionite, the tubes were stoppered and the reaction was run under argon. The reaction mixture contained 100 mM-sodium-potassium phosphate buffer pH 6.8, 70 µg methyl viologen/ml and 1.2 mM-KNO₃. The enzyme suspension in 0.1 ml was added to 0.5 ml reaction mixture, and the reaction was started by adding 0.1 ml 30 mM-Na₂S₂O₄. After incubating for 5 to 30 min, the reaction was stopped by agitating the tube on a vortex mixer for about 90 s, and then immediately adding 0.5 ml 20 % (v/v) trichloroacetic acid. The precipitated protein was centrifuged for 15 min at 6000 g, the supernatant was decanted and cooled to 0 °C, about 2 mg NaCl was added, and 6 ml ice-cold diaminodiphenylsulphone reagent (see below) was added dropwise with thorough mixing and cooling in an ice bath. When the addition was complete, the tube was kept in ice until the reagent had been added to all tubes. The mixtures were then incubated, with standards, in a water bath at 15 °C for 1 h and their extinctions at 610 nm were read against a reagent blank. The time and temperature of this incubation were critical. Shorter incubations and/or lower temperatures gave greater sensitivity but erratic results; longer incubations and/or higher temperatures gave linear results over a wider range of nitrate concentrations but lower sensitivity.

For the diaminodiphenylsulphone reagent, 50 mg diphenylamine and 2 g *p*-diaminodiphenylsulphone were dissolved in 225 ml double-distilled water, 600 ml 18 M-H₂SO₄ (BDH Aristar grade) was added slowly, the solution was cooled, and 100 ml 9M-H₃PO was added. The mixture was then made up to 1 l with 18 M-H₂SO₄, and was colourless or pale blue. An intense blue colour indicated excessive contamination with nitrate.

In the absence of nitrite reductase, it was more convenient to assay nitrate reductase by nitrite production. The reaction mixture and procedures were as described above, except that after stopping the reaction by agitating the tube on a vortex mixer, nitrite was determined as described by Nicholas & Nason (1957).

In whole cells, nitrate reductase was determined as described by Daniel & Appleby (1972).

Molecular-weight determination and partial purification of soluble nitrate reductase. A portion (3 ml) of the 250000 g supernatant containing 110 to 130 mg protein was applied to an 85 × 2.5 cm column of Sephadex G-150 (Pharmacia). The column was eluted with N₂-saturated 25 mM-sodium-potassium phosphate buffer pH 6.8, and 5 ml fractions were collected under N₂. All procedures were carried out at 0 to 4 °C. The column was calibrated for molecular-weight determinations using crystalline proteins (mol. wt. 247000 to 12000 daltons) as described by Andrews (1964). Cytochrome *c* (Appleby 1969*b*; Daniel & Appleby 1972), and nitrite reductase when present (Daniel & Appleby, 1972; Daniel, unpublished results), were used as internal molecular-weight markers.

RESULTS AND DISCUSSION

The level of activity of nitrate reductase in free-living *R. japonicum* depended on the O₂ tension (Fig. 1), as suggested by earlier work (Daniel & Appleby, 1972). This explains the

Fig. 1. The effect of oxygen tension during growth on the nitrate reductase activity of whole cells of *R. japonicum*. Cells were grown and nitrate reductase activity determined as described by Daniel & Appleby (1972). The point for zero O_2 was determined from cells grown only in the presence of 6 mM- KNO_3 . Cells grown at all other O_2 tensions gave identical levels of nitrate reductase activity in both the presence and absence of 6 mM- KNO_3 .

Table 1. *Distribution and purification of nitrate reductase in anaerobically- and aerobically-grown R. japonicum*

Assays were carried out anaerobically by measuring the removal of nitrate as described in Methods.

	Vol. (ml)	Total protein (mg)	Specific activity [nmol NO_3^- removed min^{-1} (mg protein) $^{-1}$]	Purification
Anaerobically-grown cells				
Cell-free extract	16	1410	74	1
Membrane particles	6.2	552	34	—
Supernatant	12.2	537	127	1.7
Sephadex G-150 eluate (peak fraction)	6	1.6	650	8.8
Aerobically-grown cells				
Cell-free extract	15	1040	14.3	1
Membrane particles	6.8	273	8.1	—
Supernatant	13.4	555	26	1.8
Sephadex G-150 eluate (peak fraction)	6	0.8	80	5.6

similar high levels of activity of the enzyme in the anaerobically grown and bacteroid forms of *R. japonicum* (Daniel & Appleby, 1972), since the O_2 tension within the root nodule is very low (Appleby 1962, 1969c).

As previously reported for aerobically grown cells and the bacteroid form of *R. japonicum* (Cheniae & Evans, 1959; Lowe & Evans, 1964; Kennedy *et al.*, 1975), nitrate reductase activity was present in both particulate and soluble fractions of anaerobically grown cells (Table 1). Although the ratio of total soluble nitrate reductase activity to total membrane-bound activity was 3.5 for anaerobically grown cells and 6.5 for aerobically grown cells, this difference may not be significant, particularly as the ratios of the specific activities of the soluble and membrane-bound fractions in the two types of cells are the same. Any variation in the fractionation characteristics of the two types of cells could alter the relative yields of the membrane fraction.

Only dithionite is an effective reductant of the soluble partially-purified nitrate reductase

Table 2. *Effect of different electron donors on the activity of the partially-purified nitrate reductase from anaerobically-grown R. japonicum*

Nitrate reductase activity was assayed by measuring the removal of nitrate as described in Methods, except that the assays were carried out under argon. All assay mixtures contained 0.1 ml of column eluate (80 µg protein).

Electron donor	Specific activity [nmol NO ₃ ⁻ removed min ⁻¹ (mg protein) ⁻¹]
None	0
Na ₂ S ₂ O ₄	58
Na ₂ S ₂ O ₄ + benzyl viologen (70 µg ml ⁻¹)	130
Na ₂ S ₂ O ₄ + methyl viologen (70 µg ml ⁻¹)	145
Na ₂ S ₂ O ₄ + FAD (40 µM)	82
Na ₂ S ₂ O ₄ + 1-methylnicotinamide (140 µM)	152

NaBH₄, succinate, NADH and NADPH all gave zero specific activity in both the presence and absence of the above carriers.

from anaerobically grown *R. japonicum* (Table 2). The effectiveness of 1-methylnicotinamide as an electron donor (whose use we have not seen described before) is useful in situations where a colourless reaction mixture is required, e.g. in relieving CO inhibition by light.

The elution pattern from Sephadex G-150 columns showed that only a single nitrate reductase was present in each of the three types of cell, and indicated that the nitrate reductase from anaerobically grown cells had the same molecular weight as that from bacteroids (about 69000 daltons). The molecular weight of the nitrate reductase from aerobically grown cells was much higher (about 170000 daltons), which disagrees with the work of Kennedy *et al.* (1975) who found that the nitrate reductase from bacteroids was of the same molecular weight as that from aerobically grown cells, i.e. 66000 to 70000 daltons. This may be due to the lower aeration applied during growth of aerobically grown cells by Kennedy *et al.* (1975), or to their use of sonication for cell breakage. Our attempts to induce low molecular weight nitrate reductase by growth under low O₂ tension were only partially successful, and other treatments, such as altering the nitrate levels in the growth medium or treating the 250000 g supernatant, were unsuccessful. Application of a mixture of 250000 g supernatants from aerobically and anaerobically grown cells to the Sephadex column yielded two peaks at elution volumes corresponding to about 70000 daltons and 170000 daltons, as expected.

The soluble nitrate reductase from anaerobically grown cells had a very similar *K_m* to that from aerobically grown cells; values were 106 µM and 91 µM respectively. These values are somewhat lower than the 330 µM for aerobically grown cells and higher than the 16 µM for bacteroids reported by Kennedy *et al.* (1975).

The inhibition of nitrate reductase by cyanide was assayed by measuring nitrate removal. KCN at 0.55 mM inhibited nitrate reductase from aerobically grown cells by about 50 %, but had no effect on that from anaerobically grown cells. At 5.5 mM, it totally inhibited the former but resulted in only 25 % inhibition of the latter. Assays for the inhibition of nitrate reductase by CO were carried out in closed test-tubes under argon, using 1-methylnicotinamide instead of methyl viologen. At 3.6 µM, CO inhibited nitrate reductase from aerobically grown cells by 80 % and that from anaerobically grown cells by 90 %. In the presence of light, the inhibition was only 35 % in both cases, indicating the involvement of a haemoprotein.

As noted by Lowe & Evans (1964) and Kennedy *et al.* (1975), nitrate reductase from

R. japonicum is sensitive to air. Exposure of a 2 mm layer of 250000 g supernatant to a stream of air for 5 min decreased the nitrate reductase activity from anaerobically grown cells by 52 %, and that from aerobically grown cells by 14 %, compared with exposure to N₂.

The similar K_m values of the nitrate reductases from aerobically and anaerobically grown cells taken together with the findings of Kennedy *et al.* (1975) and Lowe & Evans (1964) suggest that the nitrate reductases from *R. japonicum* grown under different conditions (root nodule bacteroids, anaerobically and aerobically grown free-living cells) are identical. However some findings reported here (O₂ sensitivity, cyanide inhibition, and molecular weight) indicate differences between the nitrate reductases from aerobically and anaerobically grown cells. These differences may be due to sub-unit interactions.

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