ATP production coupled to the denitrification of nitrate in *Rhizobium japonicum*, grown in cultures and in bacteroids from *Glycine max*

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The denitrification of nitrate in membrane fractions of *Rhizobium japonicum* CC705 grown in culture solutions with nitrate and those from bacteroids of *Glycine max* was coupled to ATP production. The ATP/$2e^-$ ratios for electron transfer from succinate and NADH to NO_3^- and O_2 in membrane fractions of *R. japonicum* grown with nitrate were: succinate to O_2, 2.0; succinate to nitrate, 1; NADH to O_2, 3; NADH to nitrate, 2. In membrane fractions of bacteroids the value for succinate to nitrate was also 1.

Rhizobium japonicum *Bacteroid* *Denitrification* *ATP production*

1. INTRODUCTION

Although it is well known that a nitrate reductase enzyme is present in bacteroids of a variety of leguminous plants, its physiological role is uncertain [1]. Bacteroids derive ATP for dinitrogen reduction primarily from oxidative phosphorylation coupled to electron transport to O_2 [2]. A supply of ATP under these conditions of low partial pressure for O_2 in the nodules is likely to be rate limiting for symbiotic fixation of dinitrogen. Authors in [3] reported that soybean bacteroids are able to carry out acetylene reduction when also reducing nitrate under anaerobic conditions. There is however no direct experimental evidence for ATP synthesis during denitrification of nitrate in rhizobia. We show here that membrane fractions of cells of *Rhizobium japonicum* either grown in cultures with nitrate or derived from bacteroids of *Glycine max* produce ATP when nitrate is denitrified to nitrite. Comparisons are made between ATP/$2e^-$ ratios produced with succinate and NADH as donors with NO_3^- and O_2 as terminal acceptors in these membrane fractions.

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Abbreviations: BV, benzyl viologen; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCPIP, 2,6-dichlorophenolindophenol; DNP, 2,4-dinitrophenol; HOQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; MV, methyl viologen; PMS, phenazine methosulfate

2. MATERIALS AND METHODS

2.1. *Culture of bacterium*

R. japonicum strain CC705 was kindly supplied by Dr F.J. Bergersen, CSIRO Division of Plant Industry, Canberra. This was maintained on yeast extract–mannitol slopes at 30°C [4]. The liquid cultures were grown aerobically with 5 mM KNO_3 (1 l in 2-l Erlenmeyer flasks) in the shake culture as in [5] and semi-aerobically (5% O_2/95% N_2) by sparging the gas mixture into the cultures via rotameters. The cells harvested at 15000 × *g* for 20 min at 4°C in a RC 5B centrifuge (Sorvall, Norwalk, USA) were washed 3 times with 50 mM

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Tris–HCl buffer (pH 7.5). The washed cells were then suspended in the same buffer. All buffers and solutions were sparged with argon for 20 min before use.

2.2. *Preparation of bacteroids*

Seeds of *G. max* (L.) Merr (cv Clark 63) were supplied by D.L. Chase, Agricultural Research Station, Leeton, NSW. Plants of *G. max* were inoculated with *R. japonicum* CC705 grown in culture solution with 5 mM KNO_3 as a nitrogen source [6] and bacteroids from 3-week-old root nodules were prepared as in [7] except that 50 mM Tris–Cl buffer (pH 7.5) was used. Bacteroids were washed with 100 mM Tris–Cl buffer (pH 7.5) and resuspended in the same buffer.

2.3. *Preparation of membrane fractions*

Cells of *R. japonicum* and a suspension of bacteroids were disrupted using an ultrasonic probe (Branson, Sonifer B-12, CT) for 1 min (80 W, 15-s intervals) at 4°C and then centrifuged at 500 × *g* for 10 min. The supernatant fraction was centrifuged at 240000 × *g* for 45 min at 4°C in a Beckman ultracentrifuge (Model L8-70, 65 Rotor). The membrane fraction (P_{240}) was washed in 50 mM Tris–Cl buffer (pH 7.5) and then recentrifuged at 240000 × *g* for 45 min. The P_{240} membrane fractions obtained from cells of *R. japonicum* and bacteroids were suspended in 50 mM Tris–HCl buffer (pH 7.5).

2.4. *Nitrate reductase activity*

Nitrate reductase was assayed using various electron donors as follows: Glass tubes (78 × 8 mm) containing 100 μl electron donor (as indicated), 10 μl cysteine (2.5 μmol) and 100 μl P_{240} membrane fraction (0.2 mg protein) in a final volume of 500 μl of 50 mM Tris–Cl buffer (pH 7.5) were fitted with Suba-seals and after evacuating for 1 min, via needles, the tubes were filled with argon. The reaction was started by adding 25 μl containing 2.5 μmol KNO_3 and 10 μl containing 2.5 μmol $Na_2S_2O_4$ in 1% (w/v) $NaHCO_3$ (as indicated). Control reactions were included without electron donors and nitrate. Nitrate reductase activity was determined at zero time and after 10 min incubation at 30°C by measuring nitrite production [8]. The enzyme assays recorded in table 1 did not include cysteine.

2.5. *Effects of inhibitors and uncouplers*

Nitrate reductase activity with or without inhibitors was assayed at zero time and after 10 min incubation by measuring nitrite production as follows: Glass tubes (3 ml) fitted with Suba-seals contained 50 μl P_{240} membrane fraction (0.2 mg protein from either cells of *R. japonicum* or bacteroids) and 10–30 μl inhibitor (as indicated) in a final volume of 0.5 ml of 50 mM Tris–HCl buffer (pH 7.5) containing 2.5 μmol cysteine. After evacuating with a two-stage pump for 1 min via insertion needles, the tubes were filled with argon. After incubation with the inhibitor for 10 min at 30°C the reaction was started by adding 25 μl KNO_3 (2.5 μmol) and either 50 μl sodium succinate or NADH (0.5 μmol).

2.6. *Determination of ATP*

The production of ATP was assayed by the luciferase method in a reaction mixture as described for nitrate reductase in section 2.5, except that in addition 1 μmol each of ADP, $MgCl_2$ and K_2HPO_4 was included. Samples (50 μl) taken at zero time and after 10 min incubation at 30°C were injected into Eppendorf centrifuge tubes (1.5 ml) containing 50 μl of 5% (v/v) ice-cold perchloric acid. The tubes were agitated on a vortex mixer and the reaction mixtures neutralised with 0.1 M KOH were then diluted to 250 μl with 50 mM Tris–HCl buffer (pH 7.5) and centrifuged at 10000 × *g* for 2 min (Eppendorf microfuge, Hamburg). The supernatant fractions were kept on ice. ATP was determined in samples diluted 10 times with ice-cold glass-distilled water by the firefly method in [9]. Internal ATP standards (10–100 pmol) were employed and control reactions without an electron donor were always included.

2.7. *O_2 uptake and protein determination*

O_2 uptake was measured as in [10] and protein according to [11] using bovine serum albumin (Sigma fraction V) as standard.

3. RESULTS

A particulate nitrate reductase from cells of *R. japonicum* utilized a variety of artificial electron donors, namely, reduced forms of DCPIP, PMS, MV and BV (reduced by $Na_2S_2O_4$, table 1). The

Table 1

Electron donors for nitrate reductase activity of a membrane fraction (P_{240}) of cells of *Rhizobium japonicum* grown in air with 5 mM KNO_3

Electron donor (mM)	Nitrate reductase activity (nmol NO_2^-/min per mg protein)
Sodium succinate (5)	15
NADH (1)	7
$Na_2S_2O_4$ (5)	215
$FMNH_2$ (0.5)	468
$FADH_2$ (0.5)	381
Reduced cytochrome *c* (0.5)	260
Ubiquinone (0.5)	265
β-Hydroxybutyrate (5)	31
$FADH_2$ (0.5) + ubiquinone (0.5)	400
$FMNH_2$ (0.5) + ubiquinone (0.5)	602
Reduced DCPIP (0.5)	349
Reduced PMS (0.5)	500
Reduced 8-dimethylamino-2,3-benzophenoxazine (0.5)	580
MVH (0.5)	694
BVH (0.5)	878

Experimental details: see section 2. Enzyme assays were performed in the absence of cysteine under argon. All compounds except sodium succinate, NADH and β-hydroxybutyrate were reduced by adding 5 mM $Na_2S_2O_4$ in 1% (w/v) $NaHCO_3$. In the absence of cells, nitrate reduction by $Na_2S_2O_4$ plus viologen dyes was 16 nmol NO_2^- produced/min

most effective reductant was BVH. Among the physiological electron donors, $FMNH_2$ was most effective and in combination with ubiquinone, enzyme activity was considerably enhanced. Although succinate and NADH functioned as reductants, their activities were much less when compared with the other reducing systems used (table 1). The results in table 3 indicate that nitrate reductase activity with either succinate or NADH as a donor was about 8-times greater when cells were grown under semi-aerobic conditions (5% O_2/95% N_2). The addition of cysteine at 5 mM resulted in a marked increase in NADH-linked nitrate reductase activity (table 2). The data in table 3 indicate that electron transfer from NADH to nitrate was inhibited by rotenone and amytal, but these did not affect succinate-linked nitrate reductase activity. Antimycin A and HOQNO, especially at higher concentrations (1.5 mM), markedly inhibited both NADH- and succinate-dependent nitrate reduction. The inhibitions recorded for azide and KCN are probably associated with their effects on the Mo moiety of nitrate reductase.

ATP synthesis was coupled to electron transfer from either NADH or succinate to O_2 and nitrate, respectively, as terminal electron acceptors (table 4). The ATP/$2e^-$ ratios were considerably higher with O_2 than with NO_3^-. Inhibitors of NADH dehydrogenase, namely, rotenone and amytal, also restricted ATP synthesis but were without effect

Table 2

Effect of cysteine on nitrate reductase activity of a membrane fraction (P_{240}) of cells of *Rhizobium japonicum*

Growth conditions	Electron donor (mM)	Nitrate reductase activity (nmol/min per mg protein)	
		− cysteine	+ cysteine (5 mM)
Aerobic (air)	Succinate (5)	15	18
	NADH (1)	7	12
Semi-aerobic (5% O_2/95% N_2)	Succinate (5)	120	134
	NADH (1)	54	102

Experimental details: see section 2. Enzyme assays were carried out under strictly anaerobic conditions under argon

Table 3

Effects of inhibitors on nitrate reductase activity of a membrane fraction (P_{240}) of cells of *Rhizobium japonicum* grown with 5 mM KNO_3 under 5% O_2/95% N_2

Electron donor (mM)	Inhibitor (mM)										
	Azide	KCN		Rotenone		Amytal		HOQNO		Antimycin A	
	1	0.02	1	0.1	0.3	0.3	3.0	0.1	1.5	0.1	1.0
NADH (1)	82	0	78	30	45	45	61	15	50	30	58
Succinate (5)	86	16	81	10	10	0	14	0	21	23	66

Experimental details: see section 2. Enzyme assays were conducted under strictly anaerobic conditions. Except for azide and KCN, all inhibitors were dissolved in 95% (v/v) ethanol. Appropriate controls with equivalent volumes of ethanol were included for each inhibitor, dissolved in ethanol. Results are expressed as % inhibition

Table 4

ATP production coupled to electron transfer from succinate and NADH to O_2 and NO_3^- as terminal acceptors in membrane fraction (P_{240}) of cells of *Rhizobium japonicum*

Growth conditions	Electron acceptor	Electron donor (mM)	O_2 uptake	NO_2^- production	ATP production	Ratio ATP/2e$^-$
Aerobic (air)	O_2	succinate (5)	18	–	66	1.8
		NADH (1)	6	–	36	3.0
Semi-aerobic (5%O_2/95% N_2)	NO_3^-	succinate (5)	–	132	125	0.9
		NADH (1)	–	108	204	1.9

Experimental details: see section 2. Enzyme assays were done with 5 mM cysteine in the reaction mixtures under strictly anaerobic conditions. ATP production by semi-aerobic cells (a) without NO_3^- with either NADH or succinate or (b) without both nitrate and electron donor was 50 nmol/min per mg protein. This value has been subtracted from ATP production recorded for NO_3^- plus either NADH or succinate. Values of O_2 uptake, and NO_2^- and ATP production are given as nmol/min per mg protein

Table 5

Effects of inhibitors and uncouplers on ATP production in a membrane fraction (P_{240}) of cells of *Rhizobium japonicum* grown with 5 mM KNO_3 under 5% O_2/95% N_2

Electron donor (mM)	Inhibitor (mM)					
	Rotenone (0.3)	Amytal (3.0)	HOQNO (1.5)	Antimycin A (1.0)	CCCP (0.02)	DNP (0.8)
NADH (1)	100	100	80	95	60	60
Succinate (5)	15	20	95	100	58	56

Experimental details: see section 2. Enzyme assays were performed under strictly anaerobic conditions. All inhibitors were dissolved in 95% (v/v) ethanol. Appropriate controls with equivalent volumes of ethanol were included for each inhibitor. ATP production with succinate and NADH, was 88 and 143 nmol ATP/min per mg protein, respectively. Values are expressed as % inhibition

Table 6

ATP production coupled to NO_3^- reduction in membrane fraction (P_{240}) of bacteroids prepared from root nodules of *Glycine max*

Electron donor (mM)	Exp. no.	NO_2^- production	ATP production	Ratio ATP/$2e^-$
Succinate (5)	1	35	31	0.9
	2	37	33	0.9
	3	40	36	0.9

Experimental details: see section 2. Enzyme assays were carried out under strictly anaerobic conditions. ATP production without NO_3^- plus succinate or without both NO_3^- and succinate was 16 nmol/min per mg protein and this value has been subtracted from the ATP production recorded with NO_3^- plus succinate. Values of NO_2^- and ATP production are expressed as nmol/min per mg protein

when succinate was the donor (table 5). Antimycin A and HOQNO, inhibitors of electron transfer usually at the cytochrome *b* site, blocked ATP synthesis coupled to oxidation of both NADH and succinate. Both CCCP and DNP uncoupled ATP synthesis without affecting nitrate reduction.

In a particulate fraction (P_{240}) prepared from bacteroids of nodules of *G. max* (inoculated with the same strain of *R. japonicum*) biosynthesis of ATP was recorded (ATP/$2e^-$, 1) coupled to oxidation of succinate and reduction of nitrate (table 6). A similar result was recorded for membrane fractions (P_{240}) of *R. japonicum* grown semi-aerobically in culture solution with nitrate (table 4).

4. DISCUSSION

Leghaemoglobin present in nodules is known to protect bacteroid nitrogenase against inactivation by O_2. It has been shown [12] that oxyhaemoglobin also stimulates oxidative phosphorylation in bacteroids and that this ATP is utilized for dinitrogen fixation. Very high nitrate reductase activity has often been reported in bacteroids, that also fix dinitrogen [3,13]. The physiological function of this nitrate reductase has not been determined. Our results (table 4,6) indicate that under semi-anaerobic conditions prevalent in nodules [2] nitrate can function as an alternative terminal electron acceptor to O_2, generating ATP for dinitrogen fixation [3]. In free-living rhizobia, denitrification of NO_3^- to NO_2^-, N_2O and N_2 has been reported [14]. In bacteroids, however, the further metabolic fate of NO_2^- produced from NO_3^- is not known with certainty. Authors in [13] did not detect nitrite reductase activity in bacteroids of *R. japonicum*, however, in those of cowpea the production of $^{15}N_2O$ from $^{15}NO_3^-$ was recorded [15]. It is also possible that nitrite may be metabolized in the nodule cytosol by the plant nitrite reductase enzyme.

Inhibition of nitrate reduction and associated oxidative phosphorylation in the membrane fractions of *R. japonicum* indicates that electron transfer from both NADH and succinate is associated with nitrate respiration in rhizobia (table 3,5). This is also confirmed by the uncoupling effects of CCCP and DNP (table 5). The branch point where electrons are directed to nitrate reductase and to cytochrome oxidase and O_2 is likely to be at the cytochrome *b* site.

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REFERENCES

[1] Bergersen, F.J. (1961) Biochim. Biophys. Acta 52, 206–207.
[2] Bergersen, F.J. (1971) Annu. Rev. Plant Physiol. 22, 121–140.
[3] Rigaud, J.F., Bergersen, F.J., Turner, G.L. and Daniel, R.M. (1973) J. Gen. Microbiol. 77, 137–144.
[4] Dalton, H. (1980) in: Methods for Evaluating Biological Nitrogen Fixation (Bergersen, F.J. ed) pp.13–64, John Wiley, Chichester.
[5] Bhandari, B. and Nicholas, D.J.D. (1983) Arch. Microbiol., in press.
[6] Vairinhos, F., Bhandari, B. and Nicholas, D.J.D. (1983) Planta 159, 207–215.

[7] Bishop, P.E., Guevara, J.G., Engelke, J.A. and Evans, H.J. (1976) Plant Physiol. 57, 542–546.
[8] Nicholas, D.J.D. and Nason, A. (1957) Methods Enzymol. 3, 981–984.
[9] Stanley, P.E. and Williams, S.G. (1969) Anal. Biochem. 29, 381–392.
[10] Bhandari, B. and Nicholas, D.J.D. (1979) Arch. Microbiol. 122, 249–255.
[11] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[12] Appleby, C.A., Turner, G.L. and Macnicol, P.K. (1975) Biochim. Biophys. Acta 387, 461–474.
[13] Daniel, R.M. and Appleby, C.A. (1972) Biochim. Biophys. Acta 275, 347–354.
[14] O'Hara, G.W., Daniel, R.M. and Steele, K.W. (1983) J. Gen. Microbiol. 129, 2405–2412.
[15] Zablotowicz, R.M. and Focht, D.D. (1979) J. Gen. Microbiol. 111, 445–448.