The contribution of bacteroidal nitrate and nitrite reduction to the formation of nitrosylleghaemoglobin complexes in soybean root nodules

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It is becoming recognized that leghaemoglobin constitutes an important buffer for the cytotoxic nitric oxide radical (NO[•]) in root nodules, although the sources of this NO[•] within nodules are unclear. In *Bradyrhizobium japonicum* bacteroids, NO[•] can be produced through the denitrification process, during which nitrate is reduced to nitrite by the periplasmic nitrate reductase Nap, and nitrite is reduced to NO[•] by the respiratory nitrite reductase NirK. To assess the contribution of bacteroidal denitrification to the NO[•] within nitrate-treated soybean nodules, electron paramagnetic resonance and UV–visible spectroscopy were employed to study the presence of nitrosylleghaemoglobin (LbNO) within nodules from plants inoculated with wild-type, *napA* or *nirK* *B. japonicum* strains. Since it has been found that hypoxia induces NO[•] production in plant root tissue, and that plant roots can be subjected to hypoxic stress during drought and flooding, the effect of hypoxic stress on the formation of LbNO complexes within nodules was also investigated. Maximal levels of LbNO were observed in nodules from plants treated with nitrate and subjected to hypoxic conditions. It is shown that, in the presence of nitrate, all of the LbNO within normoxic nodules arises from nitrate reduction by the bacteroidal periplasmic nitrate reductase, whereas Nap activity is only responsible for half of the LbNO within hypoxic nodules. In contrast to Nap, NirK is not essential for LbNO formation under any condition tested.

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INTRODUCTION

Bradyrhizobium japonicum is a Gram-negative soil α -proteobacterium that has the ability to form dinitrogen-fixing symbiotic nodules with soybean plants (*Glycine max*). Within these nodules, vegetative cells differentiate into bacteroids, which then synthesize nitrogenase, the enzyme responsible for the reduction of dinitrogen to ammonia. Nitrogenase requires both a low partial oxygen pressure and a high dose of ATP for its activity. These conflicting demands are met by the control of oxygen flux through a diffusion barrier in the inner cortex of the nodules (Minchin, 1997), and by leghaemoglobin (Lb), a high-affinity carrier protein, which binds oxygen and delivers it to the bacteroids (Appleby, 1992). To cope with the micro-oxic conditions (3–22 nM pO₂) prevailing in the infected plant tissue, dinitrogen-fixing bacteroids use a *cbb₃*-type high-affinity terminal cytochrome oxidase

encoded by the *fixNOQP* operon (Delgado *et al.*, 1998; Preisig *et al.*, 1996).

Nitric oxide (NO[•]) is both a gaseous free radical and a versatile cell-signalling effector that plays important roles in diverse (patho)physiological processes in plants (Lamotte *et al.*, 2005). In nodules, NO[•] is produced (Baudouin *et al.*, 2006) and can bind Lb to form nitrosylleghaemoglobin (LbNO) complexes, the presence of which has been observed within nodules using UV–visible (Kanayama *et al.*, 1990; Kanayama & Yamamoto, 1990a) and electron paramagnetic resonance (EPR) (Mathieu *et al.*, 1998) spectroscopy. Recently, it has been proposed that oxyleghaemoglobin (LbO₂) is able to scavenge any NO[•] formed in functional nodules (Herold & Puppo, 2005), contributing to the protection of nitrogenase, which is rapidly inactivated by this reactive species. However, although the formation of LbNO complexes has been demonstrated in nodules, the sources of the NO[•] in these complexes are still unclear.

In nodules, NO[•] may originate from the plant tissue or from the bacteroids. Two possible sources of NO[•] in plant

Abbreviations: EPR, electron paramagnetic resonance; Lb, leghaemoglobin; Lb²⁺, ferrous leghaemoglobin; Lba, soybean leghaemoglobin a; LbNO, nitrosylleghaemoglobin; LbO₂, oxyleghaemoglobin; MV, methyl viologen; NOS, nitric oxide synthase; WT, wild-type.

tissue are nitrite and arginine. The plant nitrate reductase (Yamasaki & Sakihama, 2000; Yamasaki *et al.*, 1999) and root mitochondria (Gupta *et al.*, 2005) have been shown to reduce nitrite to NO[•], although whether these processes have a role in NO[•] production in nodules has yet to be addressed. NO[•] may also be produced in plant tissue from arginine by a nitric oxide synthase (NOS), the activity of which has been identified in the nodules of *Lupinus albus* (Cueto *et al.*, 1996), and the gene for which, *AtNOS1*, has been identified in *Arabidopsis thaliana* (Guo *et al.*, 2003). Whether *AtNOS1* is present in soybean nodules is unknown.

In *B. japonicum* bacteroids, denitrification, the sequential reduction of nitrate or nitrite to dinitrogen via the intermediate compounds NO[•] and nitrous oxide, is a source of NO[•]. In *B. japonicum*, denitrification depends on the *napEDABC* (Delgado *et al.*, 2003), *nirK* (Velasco *et al.*, 2001), *norCBQD* (Mesa *et al.*, 2002) and *nosRZDYFLX* (Velasco *et al.*, 2004) genes, encoding nitrate-, nitrite-, nitric oxide- and nitrous oxide-reductase enzymes, respectively. Expression of *B. japonicum* denitrification genes has been reported in isolated bacteroids and in soybean root nodules by *in situ* histochemical detection of β -galactosidase activity from transcriptional fusions of the *nirK*, *norC* and *nosZ* genes to the reporter gene *lacZ* (Mesa *et al.*, 2004). If the *nap* genes are also expressed within bacteroids, then the reduction of nitrate to NO[•] by Nap and NirK may be a possible route for NO[•] production within nodules. However, nitrate and nitrite metabolism in nodules could be very complex, as the *B. japonicum* genome has genes that potentially encode cytoplasmic nitrate (*nasA*) and nitrite reductases (*nirB*; Kaneko *et al.*, 2002). The gene products of *nasA* and *nirB* are potentially involved in nitrate/nitrite assimilation, but the regulation of the expression and activity of the NasA and NirB enzymes is currently unknown.

Recently it has been found that NO[•] is produced in plant root tissue when it is subjected to hypoxic stress (Dordas *et al.*, 2003, 2004). It has been proposed that nitrate reduction by the plant nitrate reductase is responsible for this hypoxia-induced NO[•] increase, as the presence of nitrate has been shown to enhance NO[•] production in response to hypoxic stress (Dordas *et al.*, 2004). Since plant roots can be subjected to hypoxic stress during drought and flooding, the latter causing waterlogging of the soil that can be a particular problem for soybean crops (Linkemer *et al.*, 1998), hypoxia may cause an increase in LbNO formation within soybean root nodules. If this were to occur, and since there is a bacteroidal component in nodules, then nitrate and nitrite reduction by the bacteroids may contribute to hypoxia-induced NO[•] production. This has been assessed in the present study by using EPR and UV-visible spectroscopy to investigate the presence of LbNO within nodules from plants inoculated with the wild-type (WT), *napA* (Delgado *et al.*, 2003) or *nirK* (Velasco *et al.*, 2001) *B. japonicum* strains.

METHODS

Bacterial strains, plasmids and growth conditions. *B. japonicum* USDA110 (US Department of Agriculture, Beltsville, MD), and *napA* GRPA1 (Delgado *et al.*, 2003) and *nirK* GRK308 (Velasco *et al.*, 2001) mutant derivative strains were used in this study. *B. japonicum* strains 0602 and 2498 are USDA110 derivatives that contain a chromosomally integrated transcriptional *lacZ* fusion to the promoter region of the *napEDABC* genes (Robles *et al.*, 2006) or *nirK* gene (Mesa *et al.*, 2003), respectively, which have been used to monitor *nap* and *nirK* expression in bacteroids. *B. japonicum* strains were routinely grown in peptone-salts-yeast extract (PSY) medium (Regensburger & Hennecke, 1983) at 28 °C. Antibiotics were added to *B. japonicum* cultures at the following concentrations ($\mu\text{g ml}^{-1}$): chloramphenicol, 15; spectinomycin, 200; streptomycin, 200; tetracycline, 100.

Plant growth conditions. Soybean (*Glycine max* L. Merr., cv. Williams) seeds were surface-sterilized with 96% ethanol (v/v) for 30 s, immersed in H₂O₂ (15%, v/v) for 8 min, then washed thoroughly with sterile water and germinated in darkness at 28 °C. Selected seedlings were planted in autoclaved 1 l Leonard jar assemblies filled with vermiculite. Plants (two per jar) were inoculated at sowing with 1 ml of a single bacterial strain (10^8 cells ml⁻¹), supplied with a nitrogen-free mineral solution (Rigaud & Puppo, 1975), and grown in a controlled environmental chamber under conditions previously described (Delgado *et al.*, 1998). After growth for 28 days, one set of plants was provided with the same mineral solution supplemented with 4 mM KNO₃, while the other set was maintained solely under N₂-fixing conditions. A nitrate concentration of 4 mM was used, as it has previously been demonstrated that although treatment of plants with 4 mM KNO₃ does result in denitrification activity, it does not inhibit nodule formation or nitrogenase activity (Mesa *et al.*, 2004). Seven days before nodule harvesting, a set of nitrate-treated plants and a control set of untreated plants were subjected to hypoxia by transferring 33-day-old plants from the Leonard jars into bottles containing nutrient solution. The bottles were bunged with cotton wool and made hypoxic by sparging with argon for 15 min, and then silicone was spread on top of the cotton wool to seal the bottles. Another two sets of control plants and nitrate-treated plants were maintained under atmospheric conditions for one additional week before nodule harvesting.

Bacteroid isolation. Nodules were harvested from 40-day-old plants and bacteroids were prepared as previously described (Mesa *et al.*, 2004). In brief, 1.5 g of fresh nodules was ground in 7.5 ml Tris/HCl (pH 7.5) supplemented with 250 mM mannitol. The homogenate was filtered through four layers of cheesecloth and centrifuged at 250 g at 4 °C for 5 min to remove nodule debris. The resulting supernatant was recentrifuged at 12 000 g at 4 °C for 10 min to pellet the bacteroids. The bacteroids were washed twice with 50 mM Tris/HCl (pH 7.5) and resuspended in a final volume of 2.5 ml.

Analytical methods. For determination of β -galactosidase activity, bacteroids were resuspended in 100 mM sodium phosphate buffer (pH 7.0) to an OD₆₀₀ of ~0.6. β -Galactosidase activities were determined from permeabilized bacteroids from at least three independent preparations, as described previously (Miller, 1972). Bacteroids were not kept micro-oxic but were used immediately for assays. All media and materials used for incubation were sterilized at 120 kPa and 110 °C for 30 min before use.

Methyl viologen (MV)-dependent nitrate reductase and nitrite reductase activities were analysed essentially as described by Delgado *et al.* (2003). Nitrite concentration was estimated after diazotization by adding the sulfanilamide/naphthylethylene diamine dihydrochloride reagent (Nicholas & Nason, 1957).

The protein concentration of bacteroids was estimated by using the Bio-Rad assay, with a standard curve of varying BSA concentrations.

For determination of nitrite accumulation in nodules, 0.5 g of nodules was homogenized with 2 ml 1 M zinc acetate and centrifuged at 12 000 *g* at 4 °C for 5 min. The resulting supernatant was mixed with 1 vol. 100 % ice-cold ethanol and centrifuged for a further 5 min. The nitrite concentration of the final supernatant was determined as described above.

Whole-nodule EPR spectroscopy. Whole-nodule EPR spectroscopy was performed essentially as described by Mathieu *et al.* (1998). Intact frozen nodules were transferred directly to cylindrical EPR tubes to a depth of approximately 4 cm. Although attempts were made to load nodules of similar size to the tubes, the actual amount of tissue within each tube varied within a maximum of 30 %. EPR spectra were recorded at 60 K on a Bruker EMX system X-band spectrometer equipped with an Oxford Instruments ESR-9 liquid helium flow cryostat. The instrument settings were as follows: microwave power 2 mW, modulation amplitude 10 G, modulation frequency 100 kHz, microwave frequency 9.67 GHz and magnetic field scan range from 3000 to 3800 G.

UV-visible spectroscopy. For the quantification of the percentage of Lb bound to NO[•] within nodules, the procedure was as follows. First, Lb extracts were prepared from nodules by homogenizing 0.3 g of nodules with 4 ml Lb extraction buffer [40 mM Na₂HPO₄·2H₂O (pH 7.4); 10 mM NaH₂PO₄·H₂O (pH 7.4); 0.02 % K₃Fe(CN)₆; 0.1 % NaHCO₃], centrifuging at 12 000 *g* at 4 °C for 20 min and retaining the supernatant. As a control, soybean leghaemoglobin *a* (Lba) was purified from an overexpressing *E. coli* strain, as described by Jones *et al.* (1998). Second, since Lb directly extracted from nodules was a mixture of LbNO, ferric leghaemoglobin (Lb³⁺) and LbO₂, nodule extracts were reduced by the addition of excess sodium dithionite to convert the Lb³⁺ and LbO₂ to a single species, ferrous leghaemoglobin (Lb²⁺). Samples of reduced Lba were also prepared through the addition of excess sodium dithionite. Third, all of the Lb in the reduced nodule extracts and purified Lba samples was converted to LbNO by the addition of excess nitrite. Next, absorption spectra from 300 to 700 nm at 20 °C were recorded from 1 ml reduced and nitrosylated nodule extracts and Lba samples using a Hitachi U-3310 spectrophotometer linked to a circulating BC-10 water bath (Fisher Scientific). Finally, absorption measurements (described below) were taken from the spectra to allow quantification of the nodular LbNO using equation (2) below, derived from the following rationale.

Since the reduced nodule extracts contained only Lb²⁺, if LbNO was absent, or a mixture of Lb²⁺ and LbNO, if LbNO was present, then the difference between the absorption spectra from the reduced and nitrosylated extracts would give an indication of the proportion of LbNO within the nodules. The difference between the reduced and nitrosylated spectra was measured as the difference between the absorption of the two spectra at 579 nm ($\Delta A_{579} = A_{579}^{\text{LbNO}} - A_{579}^{\text{Lb}^{2+}}$), the wavelength at which a maximum difference between the two spectra was observed. This difference was then divided by the absorbance at the isosbestic point at 590 nm to nullify the effect of differences in concentration between the samples. To quantify these differences, the difference between the reduced and nitrosylated Lba spectra ($\Delta A_{579}^{\text{Lba}}$) was used as a reference for zero LbNO, as shown in equation (1):

$$(1) \quad 0\% \text{ LbNO} = \Delta A_{579}^{\text{Lba}} / A_{590}^{\text{Lba}} = 0.081 / 0.2865 = 0.28$$

The ratios of $\Delta A_{579} / A_{590}$ for the nodule extracts were determined and used in equation (2) to give the percentage of Lb bound to NO[•] within

the original nodule extracts:

$$(2) \quad \text{Percentage LbNO} = (0.28 - \Delta A_{579} / A_{590}) / 0.28$$

RESULTS

LbNO and nitrite accumulation in normoxic WT nodules

To confirm the observation of Kanayama and others (Kanayama *et al.*, 1990; Kanayama & Yamamoto, 1990a) that LbNO is present within nodules from nitrate-treated plants, spectroscopic techniques were performed on nodules harvested from soybean plants inoculated with the WT *B. japonicum* strain and supplemented with 4 mM nitrate. Whole-nodule EPR spectroscopy was used to detect LbNO non-invasively, and UV-visible spectroscopy was used to quantify the proportion of Lb bound to NO[•] in nodule extracts (see Methods). An EPR signal corresponding to LbNO was observed within these WT nodules (Fig. 1a i). Inspection of the absorption spectra from extracts of these nodules (Fig. 2a) showed that approximately 25 % of the Lb within these nodules was bound to NO[•] (Fig. 3a). In addition, Kanayama & Yamamoto (1990a) have proposed that nodular nitrite accumulation is the source of LbNO within nitrate-treated nodules. To check that nitrite was produced within our nitrate-treated nodules, nitrite accumulation was determined within WT normoxic nodules, which revealed that nitrite had accumulated to a level of approximately 230 nmol (g fresh-weight nodule)⁻¹ (Fig. 3b).

Bacteroidal contribution to LbNO formation within normoxic WT nodules

Nitrite accumulation within nitrate-treated nodules, a possible source of LbNO, has been proposed to arise mainly from the reduction of nitrate by the plant nitrate reductase (Kanayama *et al.*, 1990). However, those authors did not fully explore the possibility that the bacteroids might play a role in this nitrite accumulation and nitrate and nitrite reduction. Since *B. japonicum* cells can reduce nitrate to nitrite through the action of the periplasmic nitrate reductase Nap (Delgado *et al.*, 2003), and reduce nitrite to NO[•] through the action of the respiratory nitrite reductase NirK (Velasco *et al.*, 2004), the expression of the *nap* operon and *nirK* gene was assessed in bacteroids. Plants were inoculated with a *B. japonicum* strain containing a chromosomally integrated P_{napE}-*lacZ* or P_{nirK}-*lacZ* fusion. Bacteroids prepared from nodules of these plants exhibited both *nap* and *nirK* expression (Fig. 4a). In addition, bacteroids from WT nodules showed MV-dependent nitrate reductase activity at a rate of 328 ± 69 nmol NO₂⁻ produced h⁻¹ (mg protein)⁻¹ (Fig. 4b), and nitrite reductase activity at 144 ± 7 nmol NO₂⁻ consumed h⁻¹ (mg protein)⁻¹ (Fig. 4c).

Fig. 1. (a) EPR spectra from intact normoxic *B. japonicum* WT (i), *napA* (iii) and *nirK* (v) nodules or hypoxic WT (ii), *napA* (iv) and *nirK* (vi) nodules. The y axis has the same scale in i–vi. Spectra are representative of EPR spectra recorded from triplicate nodule samples from two or three different nodule harvests. (b) Peak-to-trough amplitudes (mean $\pm$ SEM), presented as fractions of the maximum EPR signal observed, determined from EPR spectra of WT (black bars), *napA* (grey bars) and *nirK* (white bars) nodules from plants treated with nitrate, presented as fractions of the maximum EPR signal observed. Amplitudes were determined from EPR spectra recorded from triplicate nodule samples from at least two different nodule harvests.

Nap activity and LbNO formation in normoxic nodules

Since the *nap* operon is expressed in nitrate-treated nodules, then the periplasmic nitrate reductase may have a role in LbNO formation. To investigate this, plants were inoculated with a *napA* mutant strain and the nodules harvested. To assess the phenotype of this mutant, MV-dependent nitrate reductase activity was measured in bacteroids prepared from the *napA* nodules. As Fig. 4(b) shows, no nitrate reductase activity was observed in the *napA* bacteroids. To see whether

Fig. 2. UV-visible spectra from reduced (solid line) and nitrosylated (dotted line) normoxic *B. japonicum* WT (a), *napA* (c) and *nirK* (e) nodule extracts or hypoxic WT (b), *napA* (d) and *nirK* (f) nodule extracts. The absorbance (Abs) scale is indicated in panel (a) and is the same for all panels. Spectra are representative of UV-visible spectra recorded from triplicate nodule extracts from at least two different nodule harvests.

this lack of bacteroidal nitrate reductase activity in the *napA* nodules affected the level of nitrite accumulation and LbNO within the nodules, nodular nitrite accumulation was determined, and EPR and UV-visible spectra were taken from whole nodules and nodule extracts, respectively. No nitrite accumulation (Fig. 3b) and no LbNO EPR signal was observed within *napA* nodules, only a signal corresponding to the presence of organic radicals remained in the EPR spectrum (Fig. 1a iii). Consistent with this finding, the difference between the reduced and nitrosylated UV-visible spectra from the *napA* nodule extract (Fig. 2c) was similar to that observed between the spectra from purified Lba samples (data not shown), demonstrating the lack of LbNO in *napA* normoxic nodules (Fig. 3a).

NirK activity and LbNO formation in normoxic nodules

Since we have demonstrated that, in nitrate-treated normoxic nodules, nitrate reduction by NapA is the sole source

Fig. 3. LbNO proportion (a) and nitrite accumulation (b) within *B. japonicum* WT (black bar), *napA* (grey bar) and *nirK* (white bar) nodules. LbNO proportion was determined from the UV-visible spectra presented in Fig. 2, using the calculation described in Methods. Data are means $\pm$ SEM from at least two different nodule harvests, assayed in triplicate. FWN, fresh weight nodule.

of LbNO within nodules, then it is possible that the next step in the denitrification pathway, the reduction of nitrite to NO[•] by NirK, may also play a role in LbNO production. To investigate this, bacteroidal nitrite reductase activity and nodular nitrite and LbNO accumulation were determined in nodules from plants inoculated with a *nirK* mutant strain. As Figs 4(c) and 3(b) show, similar levels of bacteroidal nitrite reductase activity and nodular nitrite accumulation were observed between the *nirK* and WT nodules. Observation of the EPR spectra from the WT and *nirK* nodules (Fig. 1a i and v) showed that similar EPR signal amplitudes were observed in both nodule samples (Fig. 1b). Likewise, similar differences between the reduced and nitrosylated nodule extract absorption spectra were observed between the *nirK* and WT nodules (Fig. 2a, e), corresponding to approximately 25 % of the Lb being nitrosylated (Fig. 3a).

Fig. 4. (a) β -Galactosidase activity from a P_{napE} -*lacZ* (grey bar) or P_{nirK} -*lacZ* (white bar) fusion in WT *B. japonicum* bacteroids. MV-dependent nitrate reductase (b) or nitrite reductase (c) activity in bacteroids from WT (black bars), *napA* (grey bars) and *nirK* (white bars) nodules is shown. Bacteroids were prepared from normoxic and hypoxic nodules, as described in Methods. Data are means $\pm$ SEM from at least two different bacteroid isolations, assayed in triplicate.

Hypoxia, LbNO formation and nitrite accumulation in soybean nodules

Recent studies have found that NO[•] levels in plant tissue are elevated when the tissue is subjected to hypoxic stress (Dordas *et al.*, 2003, 2004). To investigate whether hypoxic stress causes an increase in LbNO formation within soybean root nodules, the roots of soybean plants, inoculated with the WT *B. japonicum* strain and supplemented with nitrate, were subjected to hypoxic stress, as described in the Methods. The EPR spectra from whole WT nodules demonstrated that hypoxia caused just over a twofold increase in the EPR LbNO signal (Fig. 1a i and ii, b). The absorption spectra from the hypoxic WT nodule extract (Fig. 2b) showed that the reduced spectrum was similar to the nitrosylated spectrum, suggesting that the original extract contained a higher proportion of LbNO than the

normoxic WT extract (Fig. 2a). In confirmation of this, interpretation of the absorption spectra found that approximately 70 % of the Lb in the hypoxic WT nodules was bound to NO[•] (Fig. 3a), 2.5 times that found in the normoxic nodules. Correlated with this, approximately 2.5 times more nitrite accumulated within the hypoxic WT nodules than in the normoxic nodules (Fig. 3b).

Bacteroidal *nap* and *nirK* expression and nitrate and nitrite reductase activities following hypoxic stress

To investigate the possible role of the bacteroidal Nap and NirK enzymes in this hypoxic increase in LbNO formation, the expression levels of the *nap* operon and *nirK* gene, and the rates of bacteroidal nitrate and nitrite reductase activities, were determined in WT nodules from plants subjected to hypoxic stress. As Fig. 4(a, b) shows, hypoxia caused *nap* expression to increase 1.5-fold, although bacteroidal nitrate reductase activity actually underwent a fivefold increase in response to hypoxic stress. Hypoxia also resulted in a twofold increase in *nirK* expression and nitrite reductase activity in bacteroids (Fig. 4a, c).

The contribution of NapA to LbNO formation in hypoxic nodules

Since *nap* expression and nitrate reductase activity were upregulated in bacteroids from nodules of plants subjected to hypoxic stress, then Nap may have a role in the formation of LbNO within hypoxic nodules. To investigate the contribution of nitrate reduction by Nap to LbNO formation in response to hypoxic stress, bacteroidal nitrate reductase activity and nodular nitrite and LbNO accumulation were observed in hypoxic *napA* nodules. Unlike the normoxic *napA* bacteroids, the hypoxic *napA* bacteroids exhibited some nitrate reductase activity, although at a rate sixfold lower than that in the hypoxic WT bacteroids (Fig. 4b). Likewise, less nitrite accumulation was observed in the hypoxic *napA* nodules compared to the WT nodules (Fig. 3b). Observation of the EPR spectrum from the hypoxic *napA* nodules showed that the LbNO signal was half the magnitude of the WT signal (Fig. 1a iv, b). Similarly, the difference between the hypoxic *napA* reduced and nitrosylated spectra was larger than that between the WT spectra (Fig. 2d), corresponding to less LbNO. Calculating the proportion of LbNO from the absorption spectra showed that the level of LbNO in the *napA* nodules was half that in the WT nodules, at approximately 30 % (Fig. 3a).

The contribution of NirK to LbNO formation in hypoxic nodules

Like *nap* expression and nitrate reductase activity, *nirK* expression and nitrite reductase activity were also upregulated in bacteroids from nodules of plants subjected to hypoxic stress. To investigate the contribution of nitrite reduction by NirK to LbNO formation in response to hypoxic stress, bacteroidal nitrite reductase activity and

nodular nitrite and LbNO accumulation were observed in hypoxic *nirK* nodules. As under normoxic conditions, the levels of bacteroidal nitrite reductase activity and nodular nitrite accumulation in hypoxic *nirK* nodules were similar to the levels in the hypoxic WT nodules (Figs 4c and 3b). Correspondingly, the magnitude of the hypoxic *nirK* EPR LbNO signal (Fig. 1a vi, b) and the differences between the reduced and nitrosylated *nirK* nodule extract absorption spectra (Fig. 2f) were similar to those observed in the hypoxic WT spectra. Determination of the proportion of LbNO within hypoxic *nirK* nodules showed that, as in the hypoxic WT nodules, approximately 70 % of the Lb was bound to NO[•] (Fig. 3a).

DISCUSSION

LbNO has previously been identified in nitrate-treated soybean nodules, where its formation has been proposed to inhibit nitrogenase activity through the decrease of available Lb to bind oxygen, thereby limiting oxygen diffusion to the bacteroids (Kanayama & Yamamoto, 1990b). NO[•] for the formation of LbNO has been proposed to arise from nitrite accumulation within the nodules from nitrate reduction by the plant nitrate reductase (Kanayama & Yamamoto, 1990a). However, we reasoned that NO[•] might also be produced through the activities of the *B. japonicum* denitrifying Nap and NirK enzymes, which can reduce nitrate to nitrite, and nitrite to NO[•], respectively.

We initially established that there was expression of the *nap* operon in bacteroids from normoxic nodules, suggesting that the free oxygen concentration within infected nodules was low enough to allow expression of this denitrifying gene. Bacteroids from normoxic nodules were also found to have MV-dependent nitrate reductase activity, suggesting that nitrate reduction by normoxic bacteroids had a role in LbNO formation. Accordingly, analysis of normoxic nodules formed by *B. japonicum* cells lacking a functional *napA* gene showed that bacteroids from these nodules lacked MV-dependent nitrate reductase activity. No nitrite accumulated and no LbNO formed within these nodules. This established that, under normoxic conditions in the presence of nitrate, nitrite produced by the *B. japonicum* periplasmic nitrate reductase is the major source of LbNO within soybean nodules. This is in contrast to the proposal by Kanayama & Yamamoto (1990a) that LbNO formation within nodules may be caused by nitrite accumulation generated from nitrate reduction by the plant nitrate reductase.

Although we have demonstrated a role for the bacteroidal Nap enzyme in nodular LbNO formation, the role of NirK is less clear. We have confirmed that *nirK* is expressed in normoxic WT bacteroids. However, normoxic *nirK* nodules had a WT rate of bacteroidal nitrite reductase activity. This established the presence of an alternative nitrite reductase to NirK that may be upregulated in *nirK* mutant bacteroids. Whilst the NapA nitrate reductase is a possible candidate for nitrite reduction, we believe this to be unlikely, given that

detailed studies on the highly similar (88 %) NapA of *Paracoccus pantotrophus* show it to be completely non-reactive towards nitrite (Butler *et al.*, 1999). In *B. japonicum*, the published genome sequence (Kaneko *et al.*, 2002) reveals the presence of a *nirB* gene, which may encode a nitrite reductase that reduces nitrite during nitrate assimilation (Lin & Stewart, 1998). If this enzyme were active in the *nirK* bacteroids, it might not be expected to contribute to the formation of LbNO from the reduction of nitrite, since an assimilatory nitrite reductase reduces nitrite to ammonium, not to nitric oxide (Lin & Stewart, 1998), though the release of NO[•] as a side product of this reaction cannot be excluded. The observation of a WT level of LbNO within normoxic *nirK* nodules does, however, demonstrate that NirK-independent routes for the reduction of nitrite to NO[•] are present and that these need not be bacteroidal. For example, nitrite may be reduced to NO[•] by the plant nitrate reductase (Yamasaki *et al.*, 1999) or by non-enzymic reduction (Yamasaki & Sakihama, 2000), which may occur in nodules, due to the acidic environment within symbiosomes (Blumwald *et al.*, 1985) and the presence of high levels of reductant required for nitrogen fixation (Haaker, 1988).

In the transition from normoxia to hypoxia, the levels of nitrite and LbNO within WT nodules both underwent a 2.5-fold increase, suggesting a correlation between nitrite accumulation within nodules and LbNO formation. In addition, *nap* and *nirK* expression and nitrate and nitrite reductase activities were upregulated in bacteroids from these nodules, suggesting that the denitrification system in *B. japonicum* bacteroids may have a role in this hypoxia-induced increase of LbNO. In contrast to normoxic *napA* nodules, some bacteroidal MV-dependent nitrate reductase activity and nodular nitrite and LbNO accumulation were observed in the *napA* hypoxic nodules, although at levels significantly lower than those in the WT hypoxic nodules. A possible candidate for this bacteroidal nitrate reductase activity is an assimilatory nitrate reductase which reduces nitrate to nitrite for biosynthetic purposes (Lin & Stewart, 1998). Inspection of the *B. japonicum* complete genome sequence (Kaneko *et al.*, 2002) does indeed reveal the presence of an ORF, *nasA*, that may encode an assimilatory nitrate reductase. This enzyme may be responsible for the nitrite accumulation observed in the hypoxic *napA* nodules. Further investigation is required as to why such an enzyme would only be expressed under anaerobic conditions in the nodule, but we note that in *Sinorhizobium medicae*, the *nasA* gene (termed *narB* in this species) is under the control of ActR, which regulates nitrogen metabolism in response to low pH and micro-aerobiosis (Fenner *et al.*, 2004). NasA is predicted to have a cytoplasmic location. Since reduced benzyl viologen (BV) is more membrane-permeant than reduced MV, we also attempted to assay the enzyme with this electron donor; however, no activity was detected. Reduced BV is not as strong a reducing agent as reduced MV. Cytoplasmic bacterial assimilatory nitrate reductase operates in a very low-potential domain (Jepson *et al.*, 2004), and although reduced MV is less membrane-permeant than

reduced BV, its stronger reducing power may still make it a more effective electron donor to cytoplasmic assimilatory nitrate and nitrite reductases.

Although the bacteroidal nitrate reductase activity in the hypoxic *napA* nodules may be responsible for nitrite accumulation, nitrate reduction by the plant nitrate reductase may also contribute, since activity of this enzyme is upregulated under hypoxia (Allegre *et al.*, 2004; Glaab & Kaiser, 1993). Furthermore, nitrate has been shown to be a precursor of NO[•] in anoxic maize cell-suspension cultures (Dordas *et al.*, 2004). In addition, it has recently been demonstrated that mitochondria from plant root tissue are also able to reduce nitrite to NO[•], with the highest rates of NO[•] production observed under hypoxia/anoxia (Gupta *et al.*, 2005). However, whether the plant nitrate reductase or root mitochondria contribute to LbNO formation in soybean nodules in response to hypoxia has yet to be addressed.

Under hypoxic stress, nitrite accumulated in the *napA* nodules to 25 % of that in the WT nodules; however, nitrosylation of the Lb was 50 % of that in the WT nodules. This suggests the presence of a nitrite-independent source of NO[•] within nodules, possibly arising from the action of a plant NOS which uses arginine, oxygen and NADPH to produce NO[•] and citrulline (Lamotte *et al.*, 2005). Arginine-dependent NOS activity has been detected in nodules of *Lupinus albus* (Cueto *et al.*, 1996), and the *Arabidopsis* AtNOS1 protein has been implicated in NO[•] synthesis (Guo *et al.*, 2003). Whether a plant NOS is present and functional in soybean nodules is unknown, although recent evidence suggests that NO[•] production by NOS contributes to the NO[•] in *Medicago truncatula* nodules (Baudouin *et al.*, 2006).

As in the normoxic *nirK* nodules, the levels of bacteroidal nitrite reductase activity and nodular nitrite and LbNO accumulation in the hypoxic *nirK* nodules were similar to those observed in the hypoxic WT nodules. In a similar manner to the normoxic nodules, nitrite reduction by the plant nitrate reductase and non-enzymic nitrite reduction may be responsible for the nitrite-derived LbNO formation observed in hypoxic *nirK* nodules, and NirB could be responsible for the bacteroidal nitrite reductase activity detected in the *nirK* mutant. Thus, on the basis of these data, no role can be definitively assigned to NirK in LbNO formation in response to hypoxic stress.

In conclusion, our results clearly demonstrate that the process of nitrate reduction by Nap in bacteroids makes an important contribution to the formation of LbNO in soybean nodules. The relevance of this contribution in nodules from soybean plants subjected to physiological hypoxic conditions, such as drought and flooding, is currently being investigated.

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