Nitric oxide detoxification in the rhizobia–legume symbiosis

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Abstract

NO (nitric oxide) is a signal molecule involved in diverse physiological processes in cells which can become very toxic under certain conditions determined by its rate of production and diffusion. Several studies have clearly shown the production of NO in early stages of rhizobia–legume symbiosis and in mature nodules. In functioning nodules, it has been demonstrated that NO, which has been reported as a potent inhibitor of nitrogenase activity, can bind Lb (leghaemoglobin) to form LbNOs (nitrosyl–leghaemoglobin complexes). These observations have led to the question of how nodules overcome the toxicity of NO. On the bacterial side, one candidate for NO detoxification in nodules is the respiratory Nor (NO reductase) that catalyses the reduction of NO to nitrous oxide. In addition, rhizobial fHbs (flavohaemoglobins) and single-domain Hbs which dioxygenate NO to form nitrate are candidates to detoxify NO under free-living and symbiotic conditions. On the plant side, sHbs (symbiotic Hbs) (Lb) and nsHbs (non-symbiotic Hbs) have been proposed to play important roles as modulators of NO levels in the rhizobia–legume symbiosis. In the present review, current knowledge of NO detoxification by legume-associated endosymbiotic bacteria is summarized.

Introduction

Rhizobia are Gram-negative soil bacteria with the unique ability to establish a dinitrogen (N2)-fixing symbiosis on legume roots and on the stems of some aquatic legumes. During this interaction, bacteroids, as rhizobia are called in the symbiotic state, are contained in intracellular compartments, the symbiosomes, within a specialized organ, the nodule, where they fix N2. Rhizobia within the symbiosomes differentiate and induce a variety of new enzyme systems, i.e. nitrogenase. Nitrogenases are complex metalloenzymes with conserved structural and mechanistic features that catalyse the biological reduction of N2 to ammonia [1,2]. In the nodule, maintenance of nitrogenase activity is subject to a delicate equilibrium. A high rate of oxygen (O2) respiration is necessary to supply the energy demands of the N2-reduction process, but, on the other hand, O2 also irreversibly inactivates the nitrogenase complex. These conflicting demands are reconciled by control of O2 flux through a diffusion barrier in the nodule cortex, which greatly limits permeability to O2 [3]. O2 is then delivered to the symbiosomes by the plant O2-carrier Lb (leghaemoglobin).

The gas NO (nitric oxide; nitrogen monoxide) is well known for its major role in many physiological systems. The relationship between the biological activity of NO and its chemistry is particularly complex, as NO will react in the cellular environment to give a range of products with different reactivities and biological effects (for a review, see [4]). Several studies have clearly shown the production of NO in early stages of symbiosis. It has been reported that a rapid and transient NO production occurs in Lotus japonicus roots inoculated with Mesorhizobium loti [5] and that modulation of NO levels are involved in the establishment of the symbiosis [6]. Furthermore, NO is involved in the auxin-signalling pathway controlling indeterminate nodule formation [7]. A recent transcriptomic study of NO-responsive genes in Medicago truncatula revealed that NO may regulate important processes of nodule development and functioning [8]. NO production in mature nodules has also been shown and it has been associated with the bacteroid-containing cells of the fixing zone in alfalfa nodules [9]. Rhizobial denitrification in the symbiosomes is a likely source of NO in nodules [10,11]. NO may also originate from the root tissue through the action of plant enzymes such as nitrate reductase and/or NOS (NO synthase). In fact, an NOS-like activity that produces NO from arginine has been identified in nodules of Lupinus albus [12]. Furthermore, it has been suggested that an NOS-like enzyme may participate in NO production in M. truncatula nodules [9].

A direct inhibition of nitrogenase activity by NO has been demonstrated in vitro [13]. The application of artificial NO donors decreased N2 fixation activity in L. japonicus [14,15] and Alnus firma root nodules [16]. Several authors have indeed observed that a decrease in NO production in root nodules results in an increase in N2-fixation activity [11,15,17], which suggests that adequate concentrations of NO might be necessary for nitrogenase activity. It has also been observed that NO formed by Bradyrhizobium japonicum

Key words: Bradyrhizobium japonicum, denitrification, haemoglobin, nitric oxide, nodule, soya bean.

Abbreviations used: fHb, flavohaemoglobin; Lb, leghaemoglobin; LbNO, nitrosyl-leghaemoglobin complex; LjHb1, Lotus japonicus nsHb-1; NR, nitrate reductase; Nor, nitric oxide reductase; NOS, nitric oxide synthase; nsHb, non-symbiotic Hb; nsHb-1, class 1 nsHb; sHb, single-domain Hb; sbHb, symbiotic Hb; trHb, truncated Hb.

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bacteroidal denitrification in soybean nodules in response to flooding and nitrate has a negative effect on both nitrogenase activity and expression of the nifH and nifD genes, which encode the Fe protein and the α-chain of the FeMo protein from the nitrogenase complex [11]. As NO has been shown to inhibit nitrogenase, its steady-state concentration should be kept low at the bacteroid level. In the present article, the mechanisms by which nodules (bacteroids and plant tissue) detoxify NO are reviewed briefly.

**Role of rhizobial Nor (NO reductase)**

In denitrifying bacteria, one candidate for NO detoxification is the respiratory Nor that catalyses the two-electron reduction of two molecules of NO to the greenhouse gas N₂O (nitrous oxide) through the denitrification process. At present, three different Nors have been characterized: cNor, qNor and qCu₅₂Nor. The best studied are the cNors encoded by norCBQD genes which receive electrons from either membrane or soluble c-type cytochromes or small blue copper proteins (azurin and pseudoazurin). The qNors use ubihydroquinone (QH₂) or menahydroquinone (MQH₂) as electron donors and are found not only in denitrifying archaea and soil bacteria but also in pathogenic micro-organisms that do not denitrify. The third type, qCu₅₂Nor, has so far been found in the Gram-positive bacterium Bacillus azotoformans and uses both MQH₂ and a specific cytochrome c (c₅₃₁) as the electron donor. It was suggested that the MQH₂-linked activity of qCu₅₂Nor has a detoxifying role (cf. cNor) and the c₅₃₁ pathway has a bioenergetic function (cf. cNor) [for reviews, see [18–20]]. In addition to the above-mentioned systems, the non-haem di-iron-containing flavohbredoxin (NorV) along with its cognate reductase (NorW) catalyses the reductive detoxification of NO to N₂O under microaerobic or anaerobic conditions in enterobacteria [21–23].

*B. japonicum* USDA110 is a rhizobial strain that forms a symbiotic association with soybean plants. It contains a cNor encoded by the gene cluster norCBQD [24], which is expressed in nodules of soybean plants grown in the presence or absence of nitrate [25]. Under free-living denitrifying conditions, *B. japonicum* cNor is physiologically important for the detoxification of NO since a norC mutant is defective in anaerobic growth and accumulates NO [24]. Inoculation of nitrate-treated soybean plants with the norC mutant does not affect the level of NO and LbNOs (nitrosyl- and haemoglobin complexes) within nodules [26]. These authors have proposed that Nor is not solely responsible for NO detoxification within nodules of soybean plants treated with nitrate, leading to the suggestion that other systems may be involved in NO detoxification. However, when plants are subjected to flooding, a significant increase in NO and LbNO is observed in norC nodules compared with WT nodules, indicating that Nor is involved in NO reduction in nodules under flooding stress conditions [11]. In *Sinorhizobium meliloti* 1021, the symbiont of alfalfa, it has been recently shown that nor gene expression is up-regulated by NO [27]. However, the function of these genes in the NO response under both free-living and symbiotic conditions is still unknown in this bacterium.

**Role of rhizobial Hbs**

In recent years, research into microbial Hbs has revealed that there are three main classes of Hbs in bacteria: fHbs (flavohaemoglobins), sdHbs (single-domain Hbs) and trHbs (trimeric Hbs). Enteric bacteria, such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium use an fHb (Hmp) to detoxify NO. This enzyme consists of two domains, an N-terminal haem-binding globin domain and a C-terminal FAD- and NAD(P)H-binding reductase domain, which are both required for NO detoxification. Hmp protects against NO both aerobically and anaerobically. Under anaerobiosis, fHbs have a slow Nor activity which might not be sufficient to protect against NO [28,29]. In the presence of O₂, the enzyme, using an electron from NAD(P)H delivered via the flavin protein, catalyses a denitrosylase (‘dioxygenase’) reaction in which NO is stoichiometrically converted into nitrate ion (NO₃⁻) [30].

Some bacteria, such as *Campylobacter jejuni*, do not synthesize fHbs, but possess sdHbs that exhibit high-sequence homology with and high structural similarity to the globin domain of fHbs lacking the FAD-containing domain. It has been found recently that an sdHb, Cgb, performs a major role in NO detoxification in *C. jejuni* [31,32]. TrHbs also lack the reductase domain, but their globin domain is 20–40 residues shorter than the sdHbs. On the basis of phylogenetic analysis, trHbs can be further divided into three subgroups: trHb-I, trHb-II and trHb-III. Intriguingly, the various classes of microbial Hbs may coexist in the same organism. For example, *Mycobacterium tuberculosis* contains a trHb-I (trHbN) and a trHb-II (trHbO), *C. jejuni* contains a trHb-III (trCtb) and an sdHb (Cgb), and *Mycobacterium avium* contains three trHbs, one from each subgroup [29,33].

A search for Hb-like sequences in the *S. meliloti* 1021 genome revealed that *smal191* gene encodes a Hmp-like fHb protein [34]. More recently, it has been reported that an *S. meliloti* *hmp* mutant displays a higher sensitivity towards NO in culture and leads to a reduced N₂ fixation efficiency *in planta*, suggesting that fHb and NO have a role during the interaction between *S. meliloti* and alfalfa or the model plant *M. truncatula* [27]. A transcriptomic study in *S. meliloti* 1021 identified *hmp* among approx. 100 bacterial genes whose expression is up-regulated by NO [27]. A BLAST search of the *B. japonicum* USDA110 genome sequence using Hmp from *S. meliloti* revealed a gene (*bll3766*) which encodes a protein that shares 35, 40 and 40% identity with the *S. meliloti* 1021, *E. coli* and *S. enterica* serovar Typhimurium Hmp proteins respectively. Surprisingly, the homology is restricted to the FAD/NAD(P)H-binding C-terminal domain of the protein. In contrast with *S. meliloti*, the Hmp-like protein from *B. japonicum* lacks the N-terminal globin domain containing the b-type haem. Instead, the predicted amino acid sequence shows the presence of an MOSC (molybdenum cofactor sulfatase C-terminal) domain that binds the MOCO.
Figure 1 | Sequence alignment of *B. japonicum* sdH6 with other bacterial Hbs

Sequence alignment of *B. japonicum* USDA110 sdHb (Bjgb, Q89RG5) with sdHbs from *V. stercoraria* (Vgb, P04252) and *C. jejuni* (Cgb, Q9PM89), and the haem domain of Hbs from *E. coli* (EcHb, P24322), *S. enterica* serovar Typhimurium (SefHb, P26353) and *S. meliloti* 1021 (SmfHb, Q7WUM4). Identical amino acids are shown by an asterisk. Proximal F8 histidine (His81) and the CD1 phenylalanine (Phe42) residues are shown in bold. Sequences were obtained from UniProt database (http://www.uniprot.org/) using the accession numbers given in parentheses above. Sequences were aligned by using the ClustalW algorithm (http://www.ebi.ac.uk/).

(molybdenum cofactor) sulfurase and several other proteins from prokaryotes and eukaryotes.

A search for Hb-like sequences in the *B. japonicum* USDA110 genome allowed the identification of a putative sdHb (we designate Bjgb) encoded by the *blr2807* gene. The predicted protein shares 37 and 32 % amino acid identity with sdHbs from *Vitreoscilla stercoraria* and *C. jejuni* respectively (Vgb and Cgb respectively) which have been implicated in NO detoxification processes [31,32,35,36] (Figure 1). Bjgb also showed 34, 35 and 33 % identity with the haem domain in fHbs from *E. coli*, *S. enterica* serovar Typhimurium and *S. meliloti* respectively (Figure 1), but it does not possess the binding sites for FAD and NAD(P)H seen in these three fHbs. The sequence alignment revealed that Bjgb possesses the proximal F8 histidine (His81) and the CD1 phenylalanine (Phe42) residue (Figure 1) which are considered to be key residues that are highly conserved in bacterial and non-bacterial Hbs [28]. *B. japonicum* *blr2807* is included in a gene cluster where the *blr2803–blr2805* genes have been annotated as an ATP-binding cassette nitrate transporter, *blr2806* as a nitrite extrusion protein, *blr2808* as a putative assimilatory NiR (nitrite reductase) and *blr2809* as the large catalytic subunit, NasA, from the assimilatory nitrate reductase containing binding domains for a molybdenum cofactor (MobisMGD) and an iron–sulfur cluster [4Fe–4S] cluster (http://genome.kazusa.or.jp/rhizobase/) (Figure 2). Bacterial assimilatory NiRs are cytoplasmic enzymes that catalyse the six-electron reduction of nitrite (NO$_2^-$) to ammonia (NH$_4^+$) which contain a sirohaem and a [4Fe–4S] cluster as prosthetic groups [37]. Two types of NiRs, according to the electron donor specificity, have been described: the ferredoxin-dependent NiRs and the NAD(P)H-dependent NiRs. The NAD(P)H-NiRs also contain non-covalently bound FAD. Notably, *B. japonicum* *blr2808* encodes a protein that lacks the binding domains for sirohaem and [4Fe–4S] cluster, but shows the presence of a FAD/NAD(P)H-binding domain. It might be possible that this protein, instead of being a NiR, is involved in electron transfer to the Bjgb (Blr2807) and to the NasA subunit (Blr2809), since the small subunit (NasC) predicted to be an NADH oxidoreductase (diaphorase) that bears FAD and electron transfer to NasA is not present in the gene cluster (Figure 2B). The role of *blr2807* and *blr2808* genes in NO detoxification within *B. japonicum* free-living cells and soya bean nodules is currently being investigated.

**Role of plant Hbs**

Since the first discovery of plant Hbs in soya bean root nodules [38], Hbs have been identified in a range of plants and are now believed to exist in all plants. There are three different types of Hbs in plants: sHbs (symbiotic Hbs), nsHbs (non-symbiotic Hbs) and trHbs. The nsHbs are divided into nsHb-1s (class 1 nsHbs), which have a very high affinity for O$_2$, and nsHb-2s (class 2 nsHbs),
which have lower affinity for O₂ and are similar to the sHbs [39]. Plant nsHbs, especially nsHb-1s, are differentially expressed under different stresses, especially hypoxic stress. It has been reported that hypoxic conditions increased NO formation from nitrate in plants, and nsHb induction functioned to protect from this nitrosative stress [39]. More research is needed to understand the involvement of nsHbs in other plant stresses such as osmotic, nutrient deprivation, cold, nitrosative and oxidative stresses. Plant nsHbs appear to catalyse NO-related detoxification in a manner similar to bacterial fHbs and to form S-nitrosohemoglobin in a manner reminiscent of nematode worm and human Hbs [40].

Several works have demonstrated that nsHb-1s are involved in the modulation of NO levels in the rhizobia-legume symbiosis. Shimoda et al. [5] demonstrated that a LjHb1 (L. japonicus nsHb-1s) and NO have important roles in stress adaptation and in the early stages of rhizobia-legume symbiosis. Nagata et al. [6] demonstrated that expression of LjHb1 and NO production were induced transiently in L. japonicus roots after inoculation with M. loti. In contrast, when plants were inoculated with pathogens, continuous NO production was observed in roots, but induction of LjHb1 did not occur. These authors concluded that modulation of NO levels and expression of nsHb-1s are involved in the establishment of the symbiosis. In addition, overexpression of nsHb-1s in transformed A. firma and L. japonicus hairy roots enhances symbiotic N₂ fixation activity by removing NO [15,16]. It has been proposed that, as for other known nsHb-1s, AHEb1 and LjHb1 act as dioxygenases to convert NO into nitrate [15].

The sHb known as Lb exists abundantly in the root nodules of legumes. It binds O₂ with high affinity and is thought to fulfil two functions: (i) to limit O₂ concentration in the root nodule to a level at which the O₂-sensitive nitrogenase can function, and (ii) to deliver O₂ to the respiring bacteroids to meet the high ATP demands of N₂ fixation [40]. Despite a high affinity for O₂, only approx. 20–30% of Lb is in the oxy form [Lb(Fe³⁺)O₂⁻], with the remainder believed to be deoxyLb. Like all Hbs, Lb can also bind NO with high affinity. Indeed, by using EPR spectroscopy, LbNOs have been found in intact nitrate-free soya bean nodules [41]. When soya beans are supplied with nitrate and subjected to hypoxic conditions, a great induction of LbNO in nodules is observed [10,11]. It has been proposed that NO could interfere with N₂ fixation by competing with oxygen for Lb-binding sites [42]. By contrast, other authors have proposed that the presence of large amounts of Lb in root nodules in which only a minimum proportion is bound to O₂ and which has a high affinity for NO can act as a NO scavenger, modulating NO bioactivity [43]. Supporting this hypothesis, it has recently been shown that increase of LbNOs in nodules in response to flooding conditions does not impair N₂ fixation [11], suggesting an additional role for Lb in root nodules, i.e. scavenging NO and nitrite produced by bacteroidal denitrification in response to flooding conditions and protecting nitrogenase activity. OxyLb (oxylegohaemoglobin) is also able to scavenge peroxynitrite (ONO Ok−), which is likely to be produced from the reaction of NO with superoxide (O₂−) formed in nodules, precluding any damaging effect of this species [43]. In both cases, the metLb [Lb(Fe³⁺)] generated can be reduced by a methaemoglobin reductase, which is present in root nodules [44].

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**References**


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