Leghemoglobin green derivatives with nitrated hemes evidence production of highly reactive nitrogen species during aging of legume nodules

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Globins constitute a superfamily of proteins widespread in all kingdoms of life, where they fulfill multiple functions such as efficient O₂ transport and modulation of nitric oxide bioactivity. In plants, the most abundant Hbs are the symbiotic leghemoglobins (Lbs) that scavenge O₂ and facilitate its diffusion to the N₂-fixing bacteroids in nodules. The biosynthesis of Lbs during nodule formation has been studied in detail, whereas little is known about the green derivatives of Lbs generated during nodule senescence. Here we characterize modified forms of Lbs, termed Lbaₘ, Lbcₘ, and Lbdₘ, of soybean nodules. These green Lbs have identical globins to the parent red Lbs but their hemes are nitrated. By combining UV-visible, MS, NMR, and resonance Raman spectroscopies with reconstitution experiments of the apoprotein with protoheme or mesoheme, we show that the nitro group is on the 4-vinyl. In vitro nitration of Lba with excess nitrite produced several isomers of nitrated heme, one of which is identical to those found in vivo. The use of antioxidants, metal chelators, and heme ligands reveals that nitration is contingent upon the binding of nitrite to heme Fe, and that the reactive nitrogen species involved derives from nitrous acid and is most probably the nitronium cation. The identification of these green Lbs provides conclusive evidence that highly oxidizing and nitrating species are produced in nodules leading to nitrosative stress. These findings are consistent with a previous report showing that the modified Lbs are more abundant in senescing nodules and have aberrant O₂ binding.

heme nitration / (leg)hemoglobin / legume nodules / reactive nitrogen species
Globins constitute a superfamily of proteins widespread in bacteria, protozoa, fungi, plants, and animals (1). Not surprisingly, they are structurally and functionally diverse. Flavohemoglobins of bacteria and yeast are chimeric Hbs with heme and FAD reductase domains, and are involved in nitric oxide (NO•) metabolism because of its high NO• dioxygenase activity (2). In humans and other vertebrates, Hb and Mb play key roles in efficient O₂ transport and storage but are also involved in NO• homeostasis, whereas the recently discovered neuroglobin and cytoglobin might assist in O₂ transport to the mitochondria and act as NADH oxidases and O₂ sensors (2, 3). Plants contain up to three types of Hbs: symbiotic, nonsymbiotic, and truncated. Symbiotic Hbs, which include leghemoglobins (Lbs) of legumes and some Hbs of actinorhizal plants, scavenge O₂ and facilitate its diffusion to the N₂-fixing microbial symbionts in nodules (4). Nonsymbiotic Hbs are further classified into two groups based on phylogeny and O₂-binding properties. Class 1 Hbs are expressed at ~100 nM in most plant tissues, display extremely high affinity for O₂, and participate in NO• metabolism and in the maintenance of cell energetics under hypoxia (5-7), whereas class 2 Hbs have similar O₂ affinities to Lbs but unknown function (8). Plant truncated Hbs resemble their bacterial counterparts in having a 2/2 helical sandwich secondary structure instead of the canonical 3/3 structure of other Hbs, and have not been assigned any role yet (1).

Legume nodules are an interesting model to study Hb function and regulation as they express the three types of plant globins (9). Specifically, Lbs are present at concentrations of 2-3 mM and maintain a free O₂ concentration of 20-40 nM in the cytosol of host cells (10). This range of O₂ concentration permits an adequate supply of ATP for N₂ fixation while avoiding nitrogenase inactivation (4). In nodules, Lbs are usually found as multiple components whose relative proportions vary with age (11). In soybean nodules, there are four major components (a, c₁, c₂, c₃), encoded by different genes, and four minor components (b, d₁, d₂, d₃), originated by posttranslational modification (11, 12). Considerable progress has been made on elucidating the regulatory pathways of Lb biosynthesis (13, 14, and refs.
therein), whereas the mechanisms implicated in its degradation are virtually unknown. In animals and plants, the conversion of heme to biliverdins is catalyzed by heme oxygenase (15, 16), but can be carried out also nonenzymatically (‘coupled oxidation’) at pH 7.5 in the presence of ascorbate and O₂ (17, 18). In plants, biliverdin-like pigments perform important functions in photosynthesis and photomorphogenesis (15) and are also associated with a decrease of N₂ fixation activity and Lb content in senescent nodules (17, 19). Legume nodule senescence, whether natural or stress-induced, is a complex and poorly studied process with potential agricultural and ecological relevance as it limits the functional lifespan of nodules and thereby N₂ fixation (19-21).

The green proteins derived from Lb in nodules have not yet been characterized. More than 60 years ago, Virtanen and Laine (17) reported the presence in legume nodules of a green pigment similar to animal choleglobin and proposed that it was generated from Lb through the breaking of the tetrapyrrole ring without the release of Fe. Much more recently, a different type of green proteins was isolated from soybean nodules (22). The ‘modified’ proteins, termed Lbaₘ and Lbcₘ, derive from Lba and Lbc (22). Spectroscopic analysis of Lbaₘ, purified by isoelectric focusing (IEF), revealed that this protein has an amino acid sequence identical to Lba but an unknown alteration of the tetrapyrrole ring (22, 23).

Identification of the heme modifications in Lbaₘ and Lbcₘ is important because they are increasingly produced during nodule senescence and exhibit aberrant binding to O₂ (24). In this paper we show that soybean Lbaₘ and Lbcₘ have a 4-nitrovinyl in their heme groups and that these modified hemoproteins can be reproducibly generated in vitro by exposure of functional Lba and Lbc to nitrite (NO₂⁻) via reactive nitrogen species (RNS). This finding reveals that Lbs are a target of nitration in vivo and demonstrates the production of powerful oxidant and nitrating species in nodules, particularly during senescence.

**Results**

**Purification and Identification of Lb Components and Modified Forms.** The major Lb components and their derivatives were purified from soybean nodules by ammonium sulfate
fractionation followed by several chromatographic steps (22). Fractions containing Lbs were further purified by IEF using a narrow range of pH, which allowed us to separate Lba, Lbc1, and Lbc2+c3 from the corresponding green derivatives. It was not possible to fully resolve Lbc2 and Lbc3, as their pI values differ by only 0.01 units (11), and the same occurred with the respective modified forms Lbc2m and Lbc3m. To confirm the identification of Lbs and their modified forms and to detect possible chemical modifications in the polypeptides, all bands containing Lbs were carefully excised from the IEF gels and the proteins were eluted and analyzed by MALDI-TOF/MS. The molecular masses of the apoproteins of Lba, Lbc1, Lbc2, and Lbc3, as well as those of their respective modified forms, were found to be 15241, 15256, 15393, and 15451 Da, respectively, which matched ± 1 Da those predicted from the amino acid sequences excluding the initial Met. The lack of this Met residue is common in Lbs, which usually have Gly or Val at the N-terminus (25). We also purified two fractions containing the Lbd and Lbd_m components. The molecular masses of the apoproteins of Lbd1, Lbd2, and Lbd3 were found to be 15299, 15436, and 15492 Da, which exceed by 42 ± 1 Da those of Lbc1, Lbc2, and Lbc3, respectively. This mass difference was in agreement with the presence of an N-terminal acetylation as confirmed by MALDI-TOF peptide mass fingerprinting of the tryptic digests. As occurred for the other Lb modified forms, the apoproteins of the Lbd_m derivatives have identical molecular masses to those of the parent proteins. We thus conclude that all four minor Lb components of soybean arise from the major components by N-terminal acetylation and that all the green Lb derivatives are affected in the hemes and not in the globins.

**Structural Elucidation of Modified Hemes.** Purified Lba and Lbc_m from soybean nodules were used for comparative structural analyses of the protoheme and the modified heme (Fig. S1) by using UV-visible, MS, NMR, and resonance Raman (RR) spectroscopies. In some cases, Lbc was also used as a model because the spectral properties of Lba, Lbc1, and Lbc2+c3 are almost identical. The major features of the UV-visible spectra of Lba and Lbc_m, as well as those of some representative complexes, are shown in Table S1. In sharp contrast to the ferric form of typical Lbs, ferric Lbc_m exhibits a Soret band at 389 nm with a shoulder
at 436 nm, and a charge-transfer absorption band at 615 nm. The pyridine hemochrome spectrum of Lb\textsubscript{cm} was identical to that of Lb\textsubscript{am} (23), exhibiting prominent absorption bands at 553 nm (\(\alpha\) band) and 522 nm (\(\beta\) band) and a new peak at 580 nm. However, the spectra of the deoxyferrous forms or of the ferrous complexes with NO\textsuperscript{-} or nicotinate were similar for Lba and Lb\textsubscript{cm}. These data indicate that the heme of Lb\textsubscript{cm} is not cleaved and still retains the capacity to bind ligands, but also that it is chemically affected on the tetrapyrrole ring itself, perhaps on the vinyl groups.

To determine precisely the nature of the modification, heme structures were exhaustively analyzed by MS\textsuperscript{n} fragmentation with micro-electrospray ionization-linear ion trap (\(\mu\)ESI-LIT) and with Fourier transform-ion cyclotron (FT-ICR) mass spectrometers. Initial analyses were performed on the isolated modified hemes but they were relatively unstable. Consequently, the whole proteins were directly subjected to MS analysis, which was optimized for maximal yield of the heme molecular ions. The hemes of Lba, Lbc, and Lbd had a \(m/z\) 616, as expected for protoheme, whereas those from Lb\textsubscript{am}, Lb\textsubscript{cm}, and Lbd\textsubscript{m} had a \(m/z\) 661. High-resolution MS of these molecular ions proved that the 45-Da difference was due to the insertion of a NO\textsubscript{2} group (Table S2). The molecular ions were extensively fragmented (MS\textsuperscript{2} to MS\textsuperscript{4}) and the elemental compositions of the most relevant fragments elucidated by high-resolution MS. These analyses revealed that one propionic group, at least the \(\alpha\)-carbon and carboxyl of the other propionic group, and at least three Me groups of the tetrapyrrole were intact in the modified hemes (Table S2). Notably, the fragmentation patterns up to MS\textsuperscript{4} of the Lb\textsubscript{am}, Lb\textsubscript{cm}, and Lbd\textsubscript{m} hemes were identical, thus confirming, together with the UV-visible spectroscopy data, that all of them bear a NO\textsubscript{2} group.

Further structural information on the modified hemes was obtained by \(^1\)H NMR spectroscopy using the the ferric-cyano forms of the unmodified Lba and the modified Lb\textsubscript{cm} proteins rather than the free hemes to avoid problems encountered with instability and artifactual chemical alteration during heme isolation from Lb\textsubscript{cm}. As a standard for comparison, the Lba sample was found to have a 1D \(^1\)H NMR spectrum with identical proton signals to that already published (26), but with assignments, made via the WEFT-NOESY (Water-Eliminated Fourier Transform-Nuclear Overhauser and Exchange Spectroscopy)
spectrum (Fig. S2), that showed the heme to be reversed in vinyl substituent placement within the protein, as reported for the nicotinate complex (27). All resonances of the Lba heme were assigned except for those of the 4-vinyl group and meso-γ-H (Table S3), whose resonances were buried in the protein resonance region. Because the sample of Lbcₘ protein was relatively small and composed of a mixture of Lbc₁ₘ and Lbc₂ₘ+ₙₖ (see above), it was not possible to assign as many of the heme resonances of these Lbcₘ isoproteins (Fig. S3). The chemical shifts of all of the heme Me resonances of the two major species of the Lbcₘ sample were changed by the heme modification, in part because the greater size of the modified substituent changed the heme seating by about 7° (28). The chemical shifts of the 2-vinyl group and the 6-propionate α and β protons were not significantly modified, nor were those of the 7-propionate α protons. The 7β protons could not be unambiguously assigned in this dilute sample; however, because the 7α protons do not show large chemical shifts relative to those of Lba, the modification cannot be at the 7β carbon. Thus, the modification of the heme appears to be at the 4-vinyl substituent. Unfortunately, none of the protons of the 4-vinyl group of Lbcₘ, could be identified, and thus our study was complemented with RR spectroscopy and reconstitution experiments.

The RR spectra of ferric Lba and Lbcₘ are compared in Figure S4. The mode notations and the band assignments are based on refs. 29-31. The high-frequency regions of RR spectra reveal the binding of a NO₂ group to the protoheme of Lbcₘ, with a signature at 1320 cm⁻¹, specific of a nitroaromatic group (30). In the mid-frequency and low-frequency RR spectra, the frequencies of modes involving the peripheral vinyl and Me groups are significantly modified, further indicating binding of a NO₂ group to a vinyl (29, 31). In-plane and out-of-plane porphyrin modes show changes in frequency in accordance with an increased porphyrin distortion upon nitration (32). This increased protoheme distortion likely originates from a minimization of the steric contacts between nitrovinyl and its adjacent Me groups.

**Reconstitution of Lbs with MesoHeme and In Vitro Nitration.** The possibility that the nitrated heme originated by a substitution of a proton by NO₂ on a vinyl group was suggested by previous work on nitriMb (33) and nitriHb (34, 35). These green Mb and Hb derivatives
contain a 2-nitrovinyl group and are generated in vitro by exposing the proteins to excess NO$_2^-$.

Thus, we prepared apoLba and apoLbc, reconstituted the holoproteins with protoheme or mesoheme (heme with ethyl groups replacing vinyls), and attempted to nitrate them (Fig. 1). The apoLb reconstituted with protoheme yielded green protein products having modified visible spectra and heme groups with $m/z$ 661, which had identical fragmentation patterns to the hemes of nitriMb, Lba$_m$, Lbc$_m$, and Lbd$_m$. By contrast, the apoLb reconstituted with mesoheme remained unaffected after the NaNO$_2$ treatment, based on the IEF, Soret and visible spectra, RR, and MS analyses of the protein. The MS analysis showed a molecular ion of $m/z$ 620, characteristic of the Fe-mesoporphyrin lacking NO$_2$ (Fig. 1). Taking these results together with the MS, NMR, and RR data, we conclude that the NO$_2$ group of the modified Lb hemes is on the 4-vinyl and that several structural isomers are produced by nitration of the protoheme. To substantiate the presence of several isomers of Lb hemes, Lba purified from soybean nodules was nitrated with NaNO$_2$ at pH 7.0 or 5.5 at room temperature and the resulting proteins were resolved on preparative IEF gels (Fig. 2). Nitration was faster at pH 5.5 than at pH 7.0, being completed within ~1 d and ~2 d, respectively, when ~200 µM Lb and ~200 mM NO$_2^-$ were employed. At pH 5.5, heme nitration required ~3 d to complete with ~20 mM NO$_2^-$ and was far from completion after 5 d with ~2 mM.

Typically, six Lba derivatives were produced, four of which (LbaN2, LbaN4, LbaN5, and LbaN6) were green (Fig. 2). LbaN6 was low abundant and could not be studied further. All other derivatives had pyridine hemochromes with a 580 nm band that is absent in unmodified Lbs (Table S1). The ferric aquo forms had Soret bands at 391-403 nm with shoulders at 433-436 nm, as well as a charge-transfer band at 615 nm. The Soret bands of LbaN4 and LbaN5 showed the closest match to those of Lba$_m$ or Lbc$_m$ (Table S1). This similarity was confirmed by RR spectroscopy (Fig. S5). Based on the relative intensity of the bands at ~1320 and 1373 cm$^{-1}$, the spectrum of the LbaN4 is the closest one to that of Lbc$_m$.

Also, in the 1400-1700 cm$^{-1}$ region, the spectra of Lbc$_m$ and LbaN4 were most similar in terms of band shape and frequency. This similarity was also found in the mid- and low-frequency regions. Thus, we conclude that LbaN4 has an identical modified heme to Lba$_m$ or Lbc$_m$. 
All LbaN derivatives had hemes with a m/z 661 and identical high-order fragmentation profiles. Likewise, all the apoLbaN derivatives were found to have a molecular mass of 15240 ±1 Da, as determined by MALDI-TOF/MS, and hence do not bear any modification in their amino acid residues. Consequently, the in vitro nitration of Lbs with excess NO₂⁻ can reproducibly generate the modified Lbs found in nodules, as well as several isomers of nitrated hemes.

Involvement of RNS in Heme Nitration. Both NO₂⁻ and NO• are unable to directly nitrate proteins, whereas other RNS derived therefrom can do it in vitro and presumably in vivo. These oxidant and nitrating RNS include peroxynitrite (ONOO'), nitrogen dioxide radical (NO₂•), and nitronium (NO₂⁺) salts (36-38). Experiments were carried out with recombinant or purified soybean Lba and with equine Mb for comparative purposes to elucidate the nature of the RNS and the pathways involved in heme nitration. This task is complicated because ONOO', when present as peroxynitrous acid (ONOOH, pKₐ = 6.8), can undergo homolytic cleavage to NO₂• and hydroxyl radical (•OH), and because nitrous acid (HNO₂, pKₐ = 3.2) can give rise to NO₂⁺ (Fig. 3). We obtained similar results with Mb and Lb. Addition of 10 mM cyanide, a strong ligand of both hemoproteins, completely prevented nitration, indicating that the reaction involves the heme Fe. To examine whether ONOO' was the nitrating agent, we used SIN-1 because synthetic ONOO' is a very short-lived molecule in buffered solutions. Spontaneous decomposition of SIN-1 yields NO• and superoxide anion radicals (O₂•⁻), which react with each other to form ONOO', and thus SIN-1 can mimic a slow exposure of the protein to ONOO' (Fig. 3). Incubation of Mb or Lba with 0.5-1 mM SIN-1 at pH 5.5 or 7.0 for up to 4 h did not nitrate the heme, excluding any contribution of free ONOO' to nitration. Likewise, an exogenous supply of superoxide dismutase (50-100 µg) and/or catalase (50-100 µg) did not prevent nitration and therefore production of O₂•⁻ radicals or H₂O₂ outside the protein are not involved in the reaction. Addition of 30-100 µM H₂O₂ did not promote nitration, confirming that peroxide is apparently not required. By contrast, incubation of Mb or Lba with 1 mM desferrioxamine (DFO) for 2-48 h inhibited nitration substantially (Fig. S6). DFO is a natural Fe chelator commonly used to establish the dependence of biological
reactions on free Fe$^{2+/3+}$ ions, but can also intercept free radicals (39). To gain information on the inhibitory effect of DFO and the role of metals on Lb$\alpha$ nitration, we used ferrioxamine (1 mM), prepared by equimolar mixing of DFO and Fe$^{3+}$ ions, and two powerful metal chelators, diethylenetriamine pentaacetic acid (1 mM) and Chelex resin (5 mg). Neither FO (Fig. S6) nor the other two compounds had any effect on heme nitration when added to the hemoprotein prior to NO$_2^-$. Therefore, free metal ions are not required for the reaction and DFO needs to have the hydroxamic moieties unblocked to inhibit nitration, which is consistent with the high affinity binding of DFO for heme (40).

Discussion

Green pigments and nitrated derivatives have been generated in vitro from animal and plant hemoproteins. Thus, human and equine Mb can be nitrated in the heme group, Tyr$^{103}$, and/or Tyr$^{146}$, depending on the Mb source and on the relative concentrations of NO$_2^-$ and H$_2$O$_2$ (33, 41). In the case of plant hemoproteins, HRP was found to be nitrated on the vinyl groups (42) and a green derivative of Lb has been produced by oxidative reaction with H$_2$O$_2$ (43). The latter authors surmised that the green Lb species was formed at least in part by heme-globin cross-linking. We failed to detect similar compounds in vivo but found instead that the green Lbs of soybean originated by nitration of the heme. Furthermore, spectroscopic and reconstitution analyses of the hemoprotein revealed that the NO$_2$ group is on the 4-vinyl (Fig. S1). The modified Lbs were reproducibly synthesized in vitro by exposing the proteins to excess NO$_2^-$. These findings are fully consistent with a recent study showing that nitration of HRP heme occurs preferentially on the 4-vinyl rather than on the 2-vinyl (42). Nevertheless, nitriMb and nitriHb are nitrated on the 2-vinyl (33-35), suggesting that multiple isomers can be formed during nitration and that the vinyl which is preferentially nitrated may be predicted by its relative availability to nitration reagents within the heme pocket. The regiospecificity of Lb heme nitration is indeed consistent with the crystal structure of soybean Lb$\alpha$ (44), which shows a greater accessibility of the 4-vinyl relative to the 2-vinyl when considering both surface electrostatic charges and steric restrictions for insertion of a NO$_2$ group (Fig. 4).
It also should be noted that the NMR spectra tell us that the Lb protein imposes strict binding of the unsymmetrical heme molecule in only one orientation at equilibrium. Thus, the specific placement of the 2- and 4-vinyl groups, as shown in Figure 4, defines a strong thermodynamic preference for the observed heme orientation, which, along with the accessibility of the 4-vinyl group seen in the structure, dictates that only the 4-vinyl group is attacked. In an early study, however, three Lba\textsubscript{m} derivatives were found to exhibit virtually identical Soret-visible spectra (23) and here we found also different Lba\textsubscript{m} products from in vitro nitration of Lba. In light of the present results, we propose that these products are isomers differing in the site of the NO\textsubscript{2} group on the 4-vinyl, such as the \(\alpha\)- or \(\beta\)-carbons and/or cis- or trans-configuration (Fig. S1).

How was the nitrated heme produced? We used RNS scavengers and releasing compounds, antioxidants, and metal chelators to gain insights on the nature of nitrating molecules (Fig. 3 and Fig. S6). Nitrination of Mb and Lb requires binding of NO\textsubscript{2}\textsuperscript{-} to the heme because it was inhibited by cyanide. The reaction is strongly pH dependent, which points out the implication of a nitrating agent derived from HNO\textsubscript{2} rather than NO\textsubscript{2}\textsuperscript{-} itself. We can exclude a direct involvement of ONOOH formed outside the protein (\textit{pathway 1}) because SIN-1 did not nitrate the Lb heme and superoxide dismutase and catalase did not prevent nitration. The same conclusion can be drawn for an oxidative attack of NO\textsubscript{2}\textsuperscript{+}, which may be formed outside the protein from HNO\textsubscript{2} decomposition (\textit{pathway 2}), because addition of 1-10 mM nitronium tetrafluoroborate (NO\textsubscript{2}BF\textsubscript{4}) did not elicit heme nitration. Two alternative mechanisms, involving oxidation of NO\textsubscript{2}\textsuperscript{-} to NO\textsubscript{2}\textsuperscript{•} by ferryl Lb (\textit{pathway 3}) or by \textsuperscript{•}OH generated via Fenton reactions (\textit{pathway 4}), can be also discarded because nitration did not require H\textsubscript{2}O\textsubscript{2} and was not dependent on free metal ions. A mechanism of protein nitration based on Fenton chemistry with free metals or heme was initially proposed as an alternative to the ONOO\textsuperscript{-} pathway (45). Exogenous H\textsubscript{2}O\textsubscript{2} is not required either for the nitration of HRP heme (42) or for the production of NO\textsubscript{2}-Tyr on a plant Hb (46). In these two cases, the nitrating agent is proposed to be the NO\textsubscript{2}\textsuperscript{•} radical based on the peroxidase activity (\textit{pathway 3}) of the hemoproteins (42, 46). In fact, in these and our own studies, the possibility that H\textsubscript{2}O\textsubscript{2} be generated inside the heme pocket cannot be entirely ruled out. This is unlikely,
however, because addition of up to 100 μM H₂O₂ did not accelerate nitration. Recently, two additional mechanisms have been proposed for nitration of Mb (38, 41) and Hb (34) with a large excess of NO₂⁻. As in our case, these two pathways require binding of NO₂⁻ to the heme. The first one (pathway 5) entails a subsequent reaction of the [heme-NO₂⁻] complex with H₂O₂ to form a heme-bound peroxynitrite [heme-N(O)OO] species (38, 41). The nitration potential of this pathway for Lb could nevertheless be markedly diminished, as ferric Lb isomerizes ONOO⁻ to NO₃⁻ at rates that are 10-fold higher than those for Mb or Hb (47). This pathway would require formation of H₂O₂ inside the heme crevice and probably decomposition of the protonated species [heme-N(O)OOH] to NO₂• radical. The second one (pathway 6) proposes that N₂O₅ is an intermediate (34). In this case, the [heme-NO₂⁻] complex would react with another molecule of HNO₂ giving rise to N₂O₅, which in turn would decompose to NO₂⁺ and NO₃⁻. Our findings that HNO₂ is the precursor of the nitrating agent and that a [heme-NO₂⁻] complex is a prerequisite for nitration are fully consistent with this hypothesis. Specifically, we propose that nitration is mainly a result of an electrophilic attack on the vinyl by the NO₂⁺ generated from HNO₂ inside the heme pocket according to pathway 6, although we cannot discard the simultaneous formation of NO₂• radical by pathway 5 as mentioned before.

The deoxyferrous and oxyferrous forms of Lb are predominant in nodules, but other heme oxidation states and Lb complexes are also present. These include ferric Lb and the ferrous Lb-NO• (nitrosyl) complex that have been detected in intact nodules (48, 49). Ferric Lb can arise from several oxidative reactions, including the autoxidation of oxyferrous Lb or the reaction of NO• with oxyferrous Lb (47). In nodules, NO₂⁻ and NO• are mainly produced as a result of the nitrate reductase activities in the cytosol and bacteroids (50-52). Under natural conditions, nitration reactions are likely to occur because Lb may be exposed to NO₂⁻ over weeks or months and because the pH decreases to 5.5 during nodule senescence (53). The identification of Lbs bearing a nitrovinyl in their hemes provides conclusive evidence that nitrating and oxidizing RNS are produced in nodules. These reactive molecules are increasingly produced during aging or stressful conditions, in accord with the enhanced concentrations of Lbaₘ and Lbcₘ observed in senescing nodules (24). Because these green
proteins appear not to be competent for O$_2$ transport (23, 24), it will be of interest to
determine whether they are unavoidable by-products of Lb-mediated RNS detoxification or
perform as yet unknown functions in legume nodules.

Materials and Methods

Biological Material. Soybean plants (Glycine max cvs Hobbit or Williams x Bradyrhizobium japonicum strains 61A89 or USDA110) were grown under environment controlled conditions until the late vegetative growth stage (22). Nodules were harvested in liquid nitrogen and stored at –80ºC.

Purification of Lbs, Protein Identification, and Molecular Mass Determination. Soybean Lbs were purified using ammonium sulfate precipitation and chromatographic steps in hydroxylapatite, Sephadex G-75, and DE-52 columns (22, 23). Proteins were subjected to in-gel digestion with trypsin using a Digest MSPro (Intavis, Koeln, Germany). Peptide and protein identification was performed by peptide mass fingerprinting in a MALDI-TOF instrument (Applied Biosystems) as described (54). The molecular masses of Lbs were determined by MALDI-TOF/MS. Details of all these procedures are provided in SI Materials and Methods.

Structural Analyses of Hemes and Hemoproteins. Details of equipment and protocols used for MS, NMR, and RR analyses are given in SI Materials and Methods.

Production of Recombinant Soybean Lba. Recombinant Lba was used instead of protein purified from soybean nodules to duplicate nitration experiments, producing identical results. The recombinant protein was expressed using conventional protocols described in SI Materials and Methods.

Reconstitution and In Vitro Nitration of Hemoproteins. The apoproteins of Mb and Lba were obtained by the acid-butanolone method (55). After neutralization of the aqueous phase with phosphate buffer (pH 7.0), the apoproteins were incubated overnight with a 2-fold excess of protoheme or mesoheme, dialyzed, and nitrated. For time-course studies of nitration, hemoproteins (150-200 µM) were treated with NaNO$_2$ (200 mM) in 50 mM phosphate buffer (pH 5.5 or 7.0) for 2 to 48 h at room temperature. The mixtures were dialyzed, concentrated, and resuspended in water (IEF analysis) or in 10 mM NH$_4$HCO$_3$ (MS analysis).

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Figure Legends

**Fig. 1.** In vitro reconstitution and nitration of Lb. (A) ApoLbc was reconstituted with either protoheme (LbP) or mesoheme (LbM) and treated for 24 h at pH 6.5 with a 1000-fold excess of NaNO₂. The products (LbP/N and LbM/N) were loaded on an analytical IEF gel and let to proceed until separation of Lbc₁ (top band) and Lbc₂+c₃ (bottom band). Green nitrated derivatives were formed from the Lb bearing heme with vinyls (LbP/N) and not from the Lb bearing heme with ethyl groups (LbM/N). (B) Soret and visible spectra of aliquot samples of the proteins loaded on the gel. Note that LbM and LbM/N have identical spectra, whereas LbP/N is being converted to green derivatives, with a Soret band of lower intensity and a hypsochromic shift of the 625 nm charge transfer absorption band. (C) Mass spectra of the hemes from Lba reconstituted with protoheme or mesoheme and then nitrated. Note the absence of nitration (m/z 620) in the mesoheme. Experiments (A) and (B) were repeated three times and experiment (C) was repeated twice, each with a different apoLb preparation, producing identical results.

**Fig. 2.** Nitration of Lba and separation of the nitrated products on preparative IEF gels. **Left lane,** mixture of Lba, Lbb, Lbc, and Lbcₙ standards. The two Lbc protein bands correspond to Lbc₁ and Lbc₂+c₃. **Right lane,** Lba (500 µM) purified from soybean nodules was nitrated with NaNO₂ (500 mM) for 48 h in citrate buffer (pH 5.5), yielding six derivatives (LbaN₁ to LbaN₆). **Middle lane,** a similar pattern of LbaN derivatives was obtained when nitration was performed in phosphate buffer (pH 7.0). These experiments were repeated at least twice with identical results.

**Fig. 3.** Mechanisms that may be operative in the nitration of Tyr residues and/or heme groups of hemoproteins. The pathways have been exemplified for Lb but are also extensive to Mb and Hb. Some intermediates are indicated in square brackets only to mean that they are formed inside the heme pocket but, except for the nitrite and peroxynitrite complexes, are not necessarily bound to the heme. Experiments designed to test these pathways are described in the text. Additional abbreviations: Lb³⁺, ferric Lb; Lb⁴⁺=O, ferryl Lb; Lb⁵⁺(nitrovinyl), ferric Lb bearing a vinyl-bound NO₂ group in the heme.

**Fig. 4.** Heme pocket of soybean Lba (PDB accession 1BIN; ref. 44) showing relevant α-helices and amino acid residues. (A) Electrostatic potential surface of the whole protein, showing heme localization. (B) Detail of the heme pocket. Ribbon diagram showing side-chains B, E, and F, with stick representation of the relevant amino acid residues, including proximal His⁹₂ and distal His⁶¹. The vinyl groups of the heme are highlighted in yellow and the propionic groups in pink. Electrostatic potential surface is overlapped as transparency. Molecular structures were inspected, analyzed, and plotted with PyMol (56).
SIN-1 → NO⁻ + O₂⁻

ONO⁻ → NO²⁻ + OH⁻

Lb³⁺ → NO²⁻

NO₂⁻ / HNO₂ → [Lb³⁺-NO₂⁻]

H₂O₂ → [Lb³⁺-N(O)OO]

HNO₂ → [Lb³⁺-N₂O₅]

[Lb³⁺-NO₂⁻] → [Lb³⁺-NO₂⁻] → [Lb³⁺-NO₂⁻]

[Lb³⁺-NO₂⁻] → [Lb³⁺-NO₂⁻]

[H₂O₂] → OH⁻ → NO₂⁻

Lb⁴⁺ = 0

Fe²⁺ → OH⁻ → NO₂⁻