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Leghemoglobin is nitrated in functional legume nodules in a tyrosine residue within the heme cavity by a nitrite/peroxide-dependent mechanism

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SUMMARY

Protein Tyr nitration is a post-translational modification yielding 3-nitrotyrosine (NO₂-Tyr). Formation of NO₂-Tyr is generally considered as a marker of nitroxidative stress and is involved in some human pathophysiological disorders, but it has been poorly studied in plants. Leghemoglobin (Lb) is an abundant hemeprotein of legume nodules that plays an essential role as O₂ transporter. Liquid chromatography coupled to tandem mass spectrometry was used for a targeted search and quantification of NO₂-Tyr in Lbs. For all Lbs examined, Tyr³⁰, located in the distal heme pocket, is the major target of nitration. Lower amounts were found for NO₂-Tyr²⁵ and NO₂-Tyr¹³³. Nitrated Lb and other as yet unidentified nitrated proteins were also detected in nodules of plants not receiving NO₃⁻ and were found to decrease during senescence. This demonstrates formation of nitric oxide (•NO) and NO₂⁻ by alternative means to nitrate reductase, probably *via* a NO synthase-like enzyme, and strongly suggests that nitrated proteins perform biological functions and are not merely metabolic byproducts. *In vitro* assays with purified Lbs revealed that Tyr nitration requires NO₂⁻ + H₂O₂ and that peroxynitrite is not an efficient inducer

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Peak-base separation of non-nitrated and mono-nitrated peptides of PvLba.

Figure S2. Tandem MS analyses of the molecular ions (*m/z* 661) of nitrated hemes.

(a) Fragmentation profile of heme from the green Lb derivative S6 (terminology according to Figure 7).

(b) Fragmentation profile of nitri-heme (Navascués *et al.*, 2012). Insets are close-up views of the spectra (range *m/z* ~480–660).

Table S1. Compilation of data obtained for Lb derivatives using immunoblots, low-resolution MS and high-resolution MS.

Table S2. MS/MS Transitions monitored for the calculation of the nitration ratios.

of nitration, possibly by isomerizing it to NO_3^- . Nitrated Lb is formed *via* oxoferryl Lb, which generates nitrogen dioxide and tyrosyl radicals. This mechanism is distinctly different from that involved in heme nitration. Formation of NO_2 -Tyr in Lbs is a consequence of active metabolism in functional nodules, where Lbs may act as a sink of toxic peroxy nitrite and may play a protective role in the symbiosis.

Keywords

Glycine max; leghemoglobin; legume nodules; nitrogen dioxide; peroxy nitrite; *Phaseolus vulgaris*; protein tyrosine nitration

INTRODUCTION

The molecular interaction of legumes and soil bacteria (rhizobia) culminates with the formation of nodules, generally on the roots. Legume nodules are unique symbiotic organs where atmospheric N_2 is reduced to ammonia assimilable for the plant in exchange for photosynthetically produced sugars. This biological N_2 fixation is highly beneficial for agriculture and the environment as it allows a reduction in the input of nitrogen fertilizers. Nodule function requires leghemoglobins (Lbs), hemeproteins that occur at concentrations of 1–5 mM in the cytosol of nodule host cells. These proteins transport and deliver O_2 to the symbiosomes at a low but steady concentration that allows efficient bacteroid respiration while preventing nitrogenase inactivation (Appleby and Bergersen, 1980). Generally, there are several Lb isoproteins in the nodules. Soybean nodules contain four major Lbs and bean nodules one major Lb (Fuchsman and Appleby, 1979).

Only the ferrous form of Lbs binds O_2 and under physiological conditions 20% of the ferrous Lb is oxygenated and 80% is deoxygenated. However, nonfunctional forms of Lb may be produced in nodules (Lee *et al.*, 1995) by redox reactions involving reactive oxygen species (ROS). Ferric Lb may be generated by autoxidation of oxygenated Lb, and ferryl Lb ($\text{Lb}^{4+}=\text{O}$, formally oxoFe^{4+}) by oxidation of ferrous or ferric Lbs with H_2O_2 (Aviram *et al.*, 1978; Moreau *et al.*, 1995). *In vitro* studies have shown that the oxidative attack of H_2O_2 on Lbs gives rise to protein radicals, which are then quenched *via* formation of intramolecular (heme-protein) and intermolecular (dimers) cross-links (Moreau *et al.*, 1995). Ferrous, ferric and ferryl Lbs can react also with reactive nitrogen species (RNS). Thus, ferrous Lb binds $\bullet\text{NO}$ avidly to form the nitrosyl complex (LbNO), which has been detected in intact nodules (Mathieu *et al.*, 1998; Meakin *et al.*, 2007). *In vitro* oxygenated ferrous Lb and ferric Lb are able to isomerize ONOO^- to NO_3^- and ferryl Lb is reduced by nitric oxide ($\bullet\text{NO}$) (Herold and Puppo, 2005a,b). Recently, we reported the presence in soybean nodules of green derivatives of Lbs having a nitro (NO_2) group on the 4-vinyl of the heme (nitri-heme) (Navascués *et al.*, 2012). By analogy with similar nitri-hemes found *in vitro* in myoglobin (Mb; Bondoc and Timkovich, 1989) and hemoglobin (Hb; Otsuka *et al.*, 2010), and to avoid confusion with Lb nitrated in Tyr residues, we will refer to those Lbs as nitri-Lbs.

The nitration of Tyr residues in proteins involves the substitution of a H atom by a NO_2 group to yield NO_2 -Tyr. This post-translational protein modification has attracted much attention in studies related to human health as it is involved in diverse pathological

conditions (Schopfer *et al.*, 2003; Radi, 2004). Our knowledge of protein nitration is much more limited in plants, where an increase in the amount of nitrated proteins is associated to nitrooxidative stress (Corpas *et al.*, 2007; Cecconi *et al.*, 2009; Begara-Morales *et al.*, 2013). Many of these studies are based on proteomic approaches, in which the proteins are separated in two-dimensional gels, detected with anti-NO₂-Tyr antibody, and identified (Chaki *et al.*, 2009; Galetskiy *et al.*, 2011; Begara-Morales *et al.*, 2013). In other cases, the plants are treated with •NO or ONOO⁻ donors prior to proteomic analysis (Tanou *et al.*, 2012). Detailed studies using immunoprecipitation and/or semiquantitative assays with antibodies led to the identification of *o*-acetylserine(thiol)lyase (Álvarez *et al.*, 2011) and glutamine synthetase (Melo *et al.*, 2011) as targets of nitration. However, to our knowledge there are no studies showing unequivocally the presence and site of NO₂-Tyr residues in plant proteins *in vivo*. In an incisive work, Lozano-Juste *et al.* (2011) were able to detect a peptide of glyceraldehyde-3-phosphate dehydrogenase with an NH₂-Tyr residue, and concluded that this was generated by reduction of NO₂-Tyr during the mass spectrometry (MS)-based proteomic analysis, emphasizing that Tyr nitration analyses are prone to artifacts.

The major drawbacks for studying protein nitration *in vivo* are indeed the low abundance of nitrated proteins and the potential artifacts that may arise during processing and MS analysis of samples. Because Lb is essential for symbiotic N₂ fixation and participates in multiple reactions involving ROS and RNS, we reasoned that this hemeprotein might be a target of nitration *in vivo* and that its high abundance in nodules would facilitate the quantification of NO₂-Tyr by dedicated MS procedures. Further motivation for undertaking this research was the puzzling observation that nitri-Lbs have their apoproteins intact (Navascués *et al.*, 2012), which suggests that nitration of hemes and of Tyr residues, if any, should obey distinct mechanisms and/or occur at different developmental stages and/or stress conditions of nodules.

In this work, we demonstrate that Lbs bearing NO₂-Tyr are present in legume nodules and that Tyr³⁰, which is located in the heme distal pocket, is the major target of nitration. Nitrated Lb decreases during nodule senescence and is present also in nodules of plants that were not given any NO₃⁻ in the nutrient solution. This indicates that there is a steady-state level of nitrated Lb *in vivo* and that sources other than bacteroid or plant nitrate reductases, probably a •NO synthase (NOS)-like activity, also produce the NO₂⁻ required for nitration. Based on *in vitro* assays that generate nitrating molecules, such as nitrogen dioxide radicals (•NO₂) and ONOO⁻-derived species, we propose a mechanism entailing ferryl Lb and •NO₂, rather than ONOO⁻, that accounts for the Tyr nitration pattern of Lb observed *in vivo*. Finally, we compare the mechanisms involved in heme and Tyr nitration and discuss their biological relevance.

RESULTS

Physiological state of nodules during natural and induced senescence

Two crop legumes, bean and soybean, were chosen to investigate Lb nitration because they have been extensively used by our group and others in studies of nodule natural and induced senescence (Pfeiffer *et al.*, 1983; Evans *et al.*, 1999; Escuredo *et al.*, 1996; Gogorcena *et al.*,

1997). Bean and soybean plants were grown in controlled-environment cabinets and under field conditions, respectively, and nodules were harvested at three developmental stages. Additional experiments were set up with bean plants grown in the complete absence of NO_3^- to compare nitration levels in nodules, as well as with bean plants treated with 10 mM NO_3^- for 4 days to induce senescence of young nodules. The total soluble protein and Lb of host cells are markers of the physiological state of nodules and were found to decrease by 19% and 39% in senescent bean nodules and by 27% and 20% in senescent soybean nodules; however, much larger decreases, 44% for soluble protein and 74% for Lb, were found in NO_3^- -treated bean nodules (Figures 1a,b). The NO_2^- content of nodules was also determined as it is relevant for our nitration studies. Bean nodules contained very low amounts, $\sim 5\text{--}8 \text{ nmol g}^{-1}$ fresh wt (detection limit $\sim 1 \text{ nmol g}^{-1}$ fresh wt), whereas the NO_2^- content of soybean nodules reached $\sim 40\text{--}80 \text{ nmol g}^{-1}$ fresh wt, showing a decreasing trend with aging (Figure 1c).

The protein nitration profiles were examined on immunoblots with an anti- $\text{NO}_2\text{-Tyr}$ antibody (Figure 2a,b). These allowed us to make a number of interesting observations. First, the nitration patterns of bean and soybean nodules were similar during senescence, and in fact nitrated proteins were virtually undetectable in aged or NO_3^- -treated bean nodules and hardly visible in senescent soybean nodules. Second, nitration was more intense in soybean nodules (readily visible bands with 20 μg protein) than in bean nodules (requiring 50 μg protein). Third, nodules of bean plants not receiving NO_3^- had substantial nitration levels. Fourth, comparison of immunoblots and the respective Coomassie-stained gels revealed that a few proteins with lower abundance than Lb were intensely nitrated. These proteins were not identified and had apparent molecular masses of ~ 32 , 38 and 45 kDa in bean nodules and ~ 28 , 34, 38, 48 and 75 kDa in soybean nodules. Finally, nitrated Lb was hardly detectable on immunoblots of bean nodules and not visible on immunoblots of soybean nodules. To verify the presence of nitrated Lb in nodule extracts using immunoblots, a preparative native gel was loaded with high amounts ($\sim 500 \mu\text{g}$) of protein and the bands corresponding to bean Lba (PvLba) and soybean Lba and Lbc (GmLba and GmLbc) were excised, eluted and analyzed on SDS-gels and immunoblots. This demonstrated that nitrated Lb is present in nodules (Figure 2b). Direct in-gel trypsinization of the excised Lb bands and subsequent MS analysis or direct analysis of crude nodule extracts by MS with Orbitrap technology also confirmed the presence of very low levels of nitrated Lb.

Nitration of Lbs occurs in legume nodules and decreases during senescence

The tryptic peptides of Lbs obtained as indicated above were independently analyzed by $\mu\text{LC-ESI-MS/MS}$ and nLC-ESI-MS/MS with similar results. Both methods were used in order to increase the reproducibility and confidence of the identification and quantification of the very low abundant modified peptides. The use of dithiothreitol or β -mercaptoethanol was avoided to prevent reduction of $\text{NO}_2\text{-Tyr}$ residues, which may be mediated by the hemeproteins themselves at 37°C and above (Balanbali *et al.*, 1999; Söderling *et al.*, 2007), as often practised in proteomic analyses. Because Lbs do not contain Cys residues, the alkylating reagent iodoacetamide was also omitted prior to trypsinization. An illustrative example is the tryptic peptide GNIPQYSVVFYTSILEK of PvLba and its mononitrated

species GNIPQY*SVVVFYTSILEK (NO₂-Tyr²⁵) and GNIPQYSVVFY*TSILEK (NO₂-Tyr³⁰). The three peptide species were fully resolved (Figure S1) and unequivocally identified by their MS/MS profiles (Figure 3). Likewise, we found in nodules the nitrated peptides ANIPQYSVVFY*TSILEK (NO₂-Tyr³⁰) of GmLba and ANIPQYSVVFY*NSILEK (NO₂-Tyr³⁰) of GmLbc₁, as well as a small proportion (0.16–0.34%) of the C-terminal peptide AWEVAY*DELAAAIK (NO₂-Tyr¹³³) of GmLba. We were unable to detect in nodules the dinitrated species NO₂-Tyr²⁵ + NO₂-Tyr³⁰ or mononitrated NO₂-Tyr¹³³ in PvLba, and the same occurred for mononitrated NO₂-Tyr²⁵ in GmLba and GmLbc₁.

LC-MS/MS approaches based on targeted precursors and selected reaction monitoring (SRM) were used to determine the proportion of NO₂-Tyr³⁰ in Lbs during natural and NO₃⁻-induced nodule senescence. We chose Tyr³⁰ to quantify nitration because it is the most abundant nitrated Tyr residue and is important for Lb function (Kundu *et al.*, 2004). Figure 4 shows that young nodules contain 0.23% of nitrated PvLba, 0.95% of nitrated GmLba and 2.31% of nitrated GmLbc₁. Similar values of Lb nitration were obtained for nodules of plants than had received 0 or 0.25 mM NH₄NO₃ in the nutrient solution. The proportions of nitrated Lb relative to total Lb decreased during the natural (~30%) and NO₃⁻-induced (~60%) senescence of bean nodules as well as with advanced aging (~70%) of soybean nodules (Figure 4).

Nitration of Tyr residues *in vitro*: in search for a mechanism *in vivo*

The model structure of GmLba shows that Tyr³⁰ is located in the distal heme pocket, Tyr¹³³ is also in the heme pocket but situated laterally, and Tyr²⁵ is outside the heme crevice (Figure 5). Because Tyr³⁰ is the main target of nitration *in vivo*, we surmised that the heme may participate in nitration of this Tyr residue. Considering that NO₂⁻ and •NO lack of sufficient reactivity to cause nitration but may give rise to the nitrating agents •NO₂ and ONOO⁻ in the presence of H₂O₂ (Herold, 2004; Nicolis *et al.*, 2004), we assayed nitration using the estimated concentrations of ferric Lb (10 μM), H₂O₂ (20–100 μM) and NO₂⁻ (100 μM) in nodules or in other plant tissues (Evans *et al.*, 1999; Becana and Klucas, 1992). Also, care was taken to perform all the reactions at physiological conditions of nodules (pH 6.5 and 26C) rather than at conditions commonly used for animal systems (pH 7.4 and 37C). At proportions of Lb:H₂O₂:NO₂⁻ of 1:2:2 and 1:2:10, nitration was less intense than at 1:10:10 or 1:10:100. The two last proportions were chosen for further studies because the high yields of the nitrated products facilitated their separation on preparative gels and the subsequent MS analyses (see below). Treatment of 10 μM of PvLba or GmLba with 100 μM H₂O₂ + 100 μM NO₂⁻ yielded 25–50% NO₂-Tyr³⁰ and ~0.5% NO₂-Tyr²⁵ (except for GmLbc₁, which produced 13.5% NO₂-Tyr²⁵) (Table 1). Increasing NO₂⁻ concentration to 1 mM, while keeping H₂O₂ constant, produced ~70% of NO₂-Tyr³⁰ and 0.4–1.4% NO₂-Tyr²⁵ (Table 1). Thus, NO₂-Tyr³⁰ was the preferred target of Lb nitration by H₂O₂ + NO₂⁻ *in vitro*.

To gain further insights on the mechanism of the nitration reaction, Lbs were treated with H₂O₂ + NO₂⁻ in the presence of potential inhibitors (Bartesaghi *et al.*, 2004, 2006; Castro *et al.*, 2004) and the products were analyzed on immunoblots (Figure 6a). These show that

nitration does not take place when H_2O_2 was omitted and that the reaction was inhibited by deferoxamine mesylate (DFO; powerful Fe^{3+} chelator and $\bullet\text{NO}_2$ scavenger), 4-hydroxyphenylacetic acid (pHPA; hydrophylic structural analog of Tyr that intercepts $\bullet\text{NO}_2$), uric acid (scavenger of radicals derived from ONOO^-) and cyanide (ligand of ferric hemeproteins). The addition of diethylenetriamine-pentaacetic acid (DTPA; metal ion chelator) had no effect. We then examined whether ONOO^- could be also a nitrating agent in our system by treating Lbs with 3-morpholino-sydnimine (SIN-1) (Figure 6b) or with synthetic ONOO^- (Figure 6c) in the presence of 100 μM DTPA to suppress metal-catalyzed nitration. It has been shown that SIN-1 generates nearly equimolar amounts of $\bullet\text{NO}$ and superoxide radicals ($\text{O}_2^{\bullet-}$), which then react with each other producing a flux of ONOO^- for at least 2 h (Álvarez *et al.*, 2002). At pH 6.5 and 26°C, we could only detect weak nitration on immunoblots when 10 μM of GmLba or PvLba were treated with 1 mM SIN-1 for 1 h (6 μg protein) or for 2 h (3 μg protein) (Figure 6b). Nitration was not visible when 1 mM SIN-1 was applied for 1 h and 3 μg of Lb was loaded. Nevertheless, significant nitration occurred when the pH was raised to 7.4 and the temperature to 37°C. To further support the absence of nitration at relevant conditions for nodule cells, Lbs were exposed to pure ONOO^- in the presence of a physiological concentration of CO_2 (5 mM HCO_3^- at pH 6.5) because CO_2 potentiates the nitrating effect of ONOO^- (Gow *et al.*, 1996). We could detect significant nitration only when 30 μM Lb was treated with a bolus of 500 μM ONOO^- and 6 μg protein was loaded on the gels, and nitration was negligible when 60 μM Lb and 3 μg protein were used (Figure 6c). Interestingly, in contrast with the SIN-1 treatment, which produces a controlled flux of ONOO^- over time, only Lb aggregates could be observed with the bolus addition of pure ONOO^- (Figure 6c).

Nitration of Tyr residues *in vitro* at preparative scale

The nitration reaction was scaled up using 1 mM Lb and proportions of Lb: H_2O_2 : NO_2^- of 1:10:10 and 1:10:100. The resulting Lb derivatives were resolved on preparative native gels (Figure 7a). PvLba and GmLba yielded three major products (B1–B3 and S1–S3) when nitration was performed with 10 mM H_2O_2 + 10 mM NO_2^- . The same Lbs produced four major derivatives (B4–B7 and S4–S7) upon nitration with 10 mM H_2O_2 + 100 mM NO_2^- . Some of these protein derivatives are green, indicating modification of the hemes. Because our results showed formation of nitrating species during the reactions, we searched for the presence of nitrated hemes. These can be identified by MS as they have molecular ions of m/z 661 (616 + 45 Da), corresponding to replacement of a H by a NO_2 group. Tandem MS revealed that the m/z 661 ion exhibits a fragmentation pattern identical to that of the nitrated heme (Figure S2), suggesting that the NO_2 is also on a vinyl group (Navascués *et al.*, 2012). This peak was clearly detectable in proteins B1–B3 (red), B4 (green), S2 (red) and S3 (green). Therefore, the nitrated heme in those red proteins is at low levels, and the proteins B5–B7 (green) and S4–S7 (green), with virtually no peak at 661 Da, have other types of modified hemes as a result of the oxidative attack by H_2O_2 .

All the proteins were eluted from the gels and analyzed by immunoblots, low-resolution MS (MALDI-TOF/TOF-MS) and high-resolution MS (Orbitrap nESI-FT-MS). Immunoblot (Figure 7b) and MS (Table S1) analyses revealed that each protein band contains NO_2 -Tyr and is a mixture of monomers, dimers and in some cases trimers (B1, B4, S1 and S4), with

molecular masses in the range of ~15300–15600, ~30500–31900 and ~46400–47900 Da, respectively. High-resolution MS analysis (± 0.2 Da) of the monomers (Table S1) confirmed the presence of $\text{NO}_2\text{-Tyr}$ (+45 Da) in most of the proteins, as well as dinitration (+90 Da) in B4. It also revealed changes in the monoisotopic molecular masses of the monomeric proteins compared with the control proteins (15479 for PvLba and 15233 for GmLba). These changes are +13 Da for B1 and –190 Da for B5, B6 and B7, but were not investigated further as their relevance *in vivo* is uncertain.

DISCUSSION

Legume nodule senescence is characterized by, among other factors, a decline in N_2 fixation, soluble protein and Lb (for reviews see Puppo *et al.*, 2005; Becana *et al.*, 2010). Indeed, we confirmed the senescent stage of nodules harvested from fruiting plants or from young plants supplied with NO_3^- because the total soluble protein and Lb of bean and soybean nodules decreased markedly due to an increase in endoprotease activity (Pfeiffer *et al.*, 1983; Pladys *et al.*, 1988). In particular, treatment of bean plants with 10 mM NO_3^- for 4 days caused a major decrease of Lb and Lb/soluble protein ratio (from 16% to 7%), indicating that Lb is especially prone to degradation (Figures 1a,b). This is consistent with an early observation that proteolytic activity against Lb is augmented at the acid pH of senescent nodules (Pladys *et al.*, 1988).

Bean nodules contained low amounts of NO_2^- but soybean nodules accumulated relatively high levels of this metabolite (Figure 1c). This is readily explained by the fact that bean nodule bacteroids (strain 3622) do not express nitrate reductase, whereas soybean nodule bacteroids (strain USDA110) display relatively high nitrate reductase activity, which is responsible for most NO_2^- produced in soybean nodules (Becana *et al.*, 1989; Meakin *et al.*, 2007). This NO_2^- accumulation may, in fact, be a contributing factor to the high nitration levels of soybean nodules relative to bean nodules (Figure 2a). However, nitration was clearly detectable in bean nodules that had not received any NO_3^- (Figure 2a), evidencing that alternative sources to bacteroid and plant nitrate reductases also exist in nodules to generate $\bullet\text{NO}$ and/or the NO_2^- derived therefrom. In fact, $\bullet\text{NO}$ and NO_2^- are precursors of nitrating RNS and the presence of the LbNO complex has been reported in soybean nodules grown without NO_3^- (Mathieu *et al.*, 1998). A likely source of RNS is a NOS-like activity. In plants, this activity was originally described in lupin (*Lupinus albus*) roots and nodules and measured by the conversion of ^{14}C -Arg into ^{14}C -citrulline at a rate of 150–200 pmol $\bullet\text{NO}$ produced $\text{min}^{-1} \text{mg}^{-1}$ protein (Cueto *et al.*, 1996). We have observed similar values in bean nodules with a highly sensitive and specific assay in which $\bullet\text{NO}$ was detected by ozone chemiluminescence (Corpas *et al.*, 2007). Together, these observations give strong support to the presence of NOS-like activity in legume nodules.

The accumulation of nitrated proteins is assumed to be a marker of nitroxidative stress in animals (Schopfer *et al.*, 2003; Radi, 2004) and plants (Corpas *et al.*, 2007). Our finding that protein nitration decreases with nodule senescence was completely unexpected as nitrated proteins may accumulate during aging of leaves and roots of several plants, including legumes (Begara-Morales *et al.*, 2013). At present we cannot offer an explanation for this other than it may be a feature of nodules and/or that nitrated proteins are preferential

substrates for the nodule proteases induced during senescence. Consistent with this decreasing trend for nitrated proteins of nodules in general, we demonstrate here that Lb nitration occurs *in vivo* and is more intense in young than in senescent nodules (Figure 4). We had anticipated that Lb may be prone to nitration in nodules because it reacts rapidly *in vitro* with ROS and RNS (Becana and Klucas, 1992; Moreau *et al.*, 1995, 1996). However, other still unknown proteins appear to be more nitrated than Lb, for they exhibit intense bands on immunoblots and are considerably less abundant than Lb. Our data also show that a steady-state level of nitrated Lb, roughly estimated in the range of 0.1–1%, occurs as a result of normal nodule metabolism. A few unidentified nitrated proteins have been detected also by immunoblots in other 'control' (unstressed, young) plant tissues (Chaki *et al.*, 2011; Galetskyi *et al.*, 2011; Tanou *et al.*, 2012; Begara-Morales *et al.*, 2013). Thus, the existence of a basal nitration level is a general phenomenon, strongly suggesting that nitrated proteins perform biological functions and are not merely metabolic byproducts.

Previous findings of oxidative damage of crucial biomolecules (Evans *et al.*, 1999) and production of nitri-Lbs (Navascués *et al.*, 2012) in senescing soybean nodules indicate that nitrooxidative stress occurs at very late stages of senescence. We are therefore bound to conclude that NO₂-Tyr in proteins is not a useful marker of nitrooxidative stress, at least in nodules. Also, the decreased content of nitrated Lbs in senescing tissue strongly suggests that the two forms reported so far of modified Lbs, nitri-hemes and nitrated apoproteins, are generated by distinct mechanisms, as we will discuss below.

The mechanism of Tyr nitration in Lbs was investigated. Essentially, three types of mechanisms have been proposed for Tyr nitration of animal globins (Figure 8). (a) Formation of ferryl globins and their radical forms, which oxidize NO₂⁻ to •NO₂ (Figure 8a; Van der Vliet *et al.*, 1997; Herold, 2004; Sakamoto *et al.*, 2004). (b) Fenton reaction, catalyzed by traces of free or protein-associated transition metals, in which the resulting •OH oxidizes NO₂⁻ to •NO₂ (Figure 8b; Thomas *et al.*, 2002). (c) Formation of ONOO⁻ or its derived species, peroxyxynitrous acid (ONOOH) and nitrosoperoxocarbonate (ONOCO₂⁻), which may undergo homolytic cleavage to •OH, •NO₂ and carbonate radical (CO₃⁻•) (Figure 8c; Radi, 2004; Bartsaghi *et al.*, 2004, 2006). Mechanisms (a) and (b) require NO₂⁻ + H₂O₂, whereas mechanism (c) does not. In our experiments with Lb, the failure of DTPA to inhibit Tyr nitration rules out mechanism (b). However, ONOO⁻ might be formed *via* •NO + O₂⁻• (Radi, 2004), and this possibility was examined by treating Lbs with the ONOO⁻-generating compound SIN-1 or with synthetic ONOO⁻. In both cases, we found no significant nitration when the reactions were carried out at pH 6.5 and 26C, physiological conditions of nodules (Pladys *et al.*, 1988). Instead, we did detect nitration when a high flux of ONOO⁻ was generated from SIN-1 at pH 7.4 and 37C for 2 h. Under these conditions, the production and stability of ONOO⁻ are higher and thus 1 mM SIN-1 generates an accumulated level equivalent to ~0.5 mM ONOO⁻ in 1 h (Álvarez *et al.*, 2002). This is an extremely high amount, unlikely to be produced in healthy plant tissues and certainly not in young, actively N₂-fixing nodules. Taking into account that 500 µM of pure ONOO⁻ does not cause appreciable nitration of Lb and that the pH of nodules decreases from 6.5 to 5.5 during nodule senescence, we conclude that direct nitration of Lb by ONOO⁻ is probably irrelevant *in vivo*, although we cannot entirely preclude the possibility

that some minor nitration occurs *via* ONOO⁻. This conclusion is consistent with previous *in vitro* studies showing that horse Mb and human Hb catalyze ONOO⁻ isomerization without being significantly nitrated (Herold and Shivashankar, 2003).

The inhibition of nitration by DFO, pHPA and uric acid (Figure 6a), which preferentially scavenge •NO₂ (Bartesaghi *et al.*, 2004, 2006; Castro *et al.*, 2004), supports mechanism (a). Interestingly, DFO suppressed formation of dimers and other aggregates and decreased nitration of monomeric Lb, consistent with previous work showing that DFO inhibits dimerization and nitration of free Tyr by mechanisms that are independent of metal ions (Bartesaghi *et al.*, 2004). In sharp contrast, cyanide completely inhibited nitration of the monomer, indicating the participation of the heme in the reaction. However, it did not inhibit dimerization, which may be attributed to side reactions of cyanide-derived radicals (Castro *et al.*, 2004).

Studies of Tyr nitration of Mb and Hb with NO₂⁻ + H₂O₂ have led to the proposal that the nitrating compound is •NO₂ and/or heme-bound ONOO⁻, depending of the relative concentrations of NO₂⁻ and H₂O₂ (Herold, 2004; Nicolis *et al.*, 2004). Moreover, in all cases, a tyrosyl radical (•Tyr) must be formed from Tyr, a reaction that cannot be achieved by ONOO⁻ *per se* (Castro *et al.*, 2004; Radi, 2004). We conclude that Tyr nitration of Lb occurs by mechanism (a), requiring ferryl Lb to generate both •NO₂ and •Tyr, which then recombine to form NO₂-Tyr (Figure 8a). The inhibition of Lb nitration by cyanide further supports a ferryl-mediated, heme-dependent mechanism. This would explain why Tyr³⁰, located in the distal heme pocket, is a preferred target for *in vivo* and *in vitro* nitration. However, the oxidation of Tyr³⁰ by the oxoferryl intermediate is unlikely to be direct because the distance between the two structures is >7 Å (Figure 5b,c). Generally, Tyr residues with at least one carbon within 4–5 Å from the oxoferryl are assumed to be amenable for their direct one-electron oxidation to •Tyr (Prasad *et al.*, 2007). Thus, in the case of Lb, Tyr³⁰ oxidation may be mediated *via* intramolecular electron transfer (Petruk *et al.*, 2012). Formation of •Tyr must also occur on Tyr²⁵ and Tyr¹³³, as they were found to be nitrated. Notably, a protein radical was detected by EPR in GmLba treated with H₂O₂ in the absence of NO₂⁻, and the signal was assigned to Tyr¹³³ (Moreau *et al.*, 1996). This observation is not at odds with our results, considering that ferryl Lb is responsible for the H abstraction from Tyr according to mechanism (a).

The nitrated Lbs described here are different from nitri-Lbs (Navascués *et al.*, 2012). Nitrated Lbs contain NO₂-Tyr whereas nitri-Lbs do not, probably as a result of the different free radical mediated reactions implicated in their production. Formation of nitrated Lbs requires H₂O₂ to generate ferryl Lb and ultimately •Tyr, whereas formation of nitri-Lbs requires very high NO₂⁻ concentrations but not H₂O₂, and involves HNO₂ as precursor of the nitrating agent. Treatment of Lbs with NO₂⁻ + H₂O₂ elicited not only Tyr nitration but also heme modifications and protein dimerization and aggregation, as shown by immunoblots (Figure 7b) and MS analysis (Table S1). These Lb derivatives are originated by intermolecular reactions of protein radicals, not necessarily entailing •Tyr. Notably, the dimers and aggregates contain high amounts of NO₂-Tyr relative to monomers. In this respect, we noted that ONOO⁻ also produced aggregates of nitrated Lb, whereas SIN-1 formed mainly monomers (Figures 6b,c). We attribute this to differences in the

concentrations of •Tyr achieved with the different modes of exposure, which are much greater with the bolus addition of ONOO⁻ than with SIN-1. Thus, in the first case, •Tyr radicals are more likely to recombine forming Tyr-Tyr bridges than with SIN-1.

The relative abundance of the two types of modified Lbs during nodule aging is also different. The content of nitrated Lbs is higher in young (very active) nodules, which evidences the production *in vivo* of ferryl Lb, •NO₂ and •Tyr. Nitration of Tyr³⁰ may interfere with Lb function because this amino acid residue allows for a proper delivery of O₂ (Kundu *et al.*, 2004). Our results show that a low steady-state level of nitrated Lbs is fully compatible with nodule activity and that Lb is nitrated by a mechanism involving oxyferryl heme rather than ONOO⁻, most likely as a result of the efficient capacity of Lb to isomerize ONOO⁻ to NO₃⁻ (Herold and Puppo, 2005a). Taking these results together with other *in vivo* observations (Mathieu *et al.*, 1998; Navascués *et al.*, 2012), we propose that, in young nodules, Lbs may act as a sink of •NO and derived RNS such as ONOO⁻ to keep the symbiosis active, preventing rejection of, or toxic effects on, the bacteroids. By contrast, the content of nitri-Lbs is higher in old (less active or inactive) nodules and may be a result of an excessive production of RNS associated to senescence and nitroxidative stress. Further work will be needed to determine if Lb dimers and other aggregates occur *in vivo* during legume nodule development and senescence.

EXPERIMENTAL PROCEDURES

Plant growth

Bean (*Phaseolus vulgaris* L. cv Contender) was inoculated with *Rhizobium leguminosarum* by *phaseoli* strain 3622 and grown in vermiculite-containing pots under controlled environment conditions (Gogorcena *et al.*, 1997) with either 0 or 0.25 mM NH₄NO₃ in the nutrient solution. This low NH₄NO₃ concentration stimulates nodulation of most legumes. Young, mature and senescent nodules were harvested from beans of ~1 month (vegetative stage), ~1.5 months (flowering stage) and ~2 months (fruiting stage), respectively. In another set of experiments, beans of ~1 month were supplied with 10 mM KNO₃ for 4 days to induce nodule senescence. Soybean (*Glycine max* Merr. cv Williams) was inoculated with *Bradyrhizobium japonicum* strain USDA110 and grown in an experimental plot in Sevilla (Spain) from July to September. Young, mature and senescent nodules were harvested from soybeans of ~1 month (vegetative stage), ~2 months (late flowering-early fruiting stage) and ~3 months (fruiting stage), respectively. Bean and soybean nodules were frozen in liquid nitrogen and stored at -80°C.

Physiological state and immunoblot analyses of nodules

Total soluble protein and Lb were used as markers of the physiological state of nodules (Pfeiffer *et al.*, 1983; Escuredo *et al.*, 1996). Nodules (200 mg) were homogenized with 2 × 500 µl 20 mM potassium phosphate (pH 7.0), and the extracts were cleared by centrifugation (20000 g, 20 min). Soluble protein was determined by the dye-binding microassay (Bio-Rad; <http://www.bio-rad.com>) using bovine serum albumin as a standard. Lb concentration was determined by the alkaline pyridine method, based on the differential spectra of the

dithionite-reduced *versus* ferricyanide-oxidized pyridine-hemochromes (556 nm *minus* 539 nm, extinction coefficient 23.4 mM⁻¹ cm⁻¹; Appleby and Bergersen, 1980).

Some biochemical markers relevant to our nitration studies, including NO₂⁻ concentrations and protein nitration profiles, were also determined in nodules. Nitrite was quantified by diazotization after rapid and complete deproteinization of nodule extracts (Becana *et al.*, 1989; Arrese-Igor *et al.*, 1998). Nodules (0.5 g) were ground in a mortar on melting ice with 5 ml of 1 M zinc acetate at 0°C. The precipitated proteins were removed by centrifugation and the supernatant mixed with an equal volume of ice-cold ethanol. After centrifugation, 500 µl of the clean extract was mixed with equal volumes of 1% sulfanilamide in 1.5 M HCl and 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride. Blanks containing sample but 1.5 M HCl instead of sulfanilamide were run in parallel. After 20 min at room temperature, the absorbance at 540 nm was read and NO₂⁻ concentration calculated with a calibration curve (0–20 nmol).

The protein nitration profiles of nodules were obtained using immunoblots. Nodule extracts, SDS-gels and transfer of proteins to polyvinylidene difluoride membranes (FluoroTrans; Pall, <http://www.pall.com>) were performed following standard protocols, but omitting reductants in the sample buffer. Membranes were probed with a monoclonal anti-NO₂-Tyr (Cayman, <https://www.caymanchem.com>) antibody and in some cases with a polyclonal affinity-purified anti-Lb antibody (see below), both at a dilution of 1:1000. The secondary antibodies were, respectively, goat anti-mouse IgG (Bio-Rad) and goat anti-rabbit IgG (Sigma, <http://www.sigmaaldrich.com>) peroxidase conjugates, which were used at a dilution of 1:20000. Immunoreactive proteins were detected by chemiluminescence using the SuperSignal West Pico kit (Pierce, <http://www.piercenet.com>).

The antiserum against PvLba was produced by injecting ~0.8 mg of protein in each of two white rabbits and was used to purify polyclonal monospecific antibodies by chromatography in CNBr-activated Sepharose 4 Fast Flow (GE Healthcare, www.gelifesciences.com). The immunoaffinity column was prepared with ~5 mg of protein. Conventional protocols for immunization and antibody purification were carried out by BioGenes (<http://www.biogenes.de>).

LC-MS/MS

For the identification and quantification of NO₂-Tyr in Lbs *in vivo*, nodules (200 mg) were ground in a ice-cold mortar with a medium (400 µl) consisting of 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 1% polyvinylpyrrolidone-10 and protease inhibitor cocktail (Complete Mini; Roche, <https://lifescience.roche.com/shop/home>). Extracts were centrifuged (13000 g, 10 min) and the soluble proteins subjected to preparative native gel electrophoresis (12.5% acrylamide, 3 mm thick). Samples (400–500 µg of protein per lane) were mixed 1:4 (vol:vol) with loading buffer omitting SDS and reductants. Gels were run at 100 V for 2 h 15 min on a MGV-100 apparatus (CBS, <http://www.cbsscientific.com>). The bands of PvLba, GmLba and GmLbc were carefully excised from the gels, cut into small pieces and kept at –20°C until MS analysis, which usually took place one week later. In-gel digestion was performed with trypsin at 37°C for 8 h with an automatic device (DigestPro MS; Intavis,

<http://www.intavis.com>). For some MS experiments, proteins were eluted overnight with 10 mM NH_4HCO_3 with identical results.

Two types of analyses were used to detect and quantify the low abundant nitrated peptides of Lbs. The first analysis involved liquid chromatography coupled to tandem MS with micro-electrospray ionization ($\mu\text{LC-ESI-MS/MS}$). In this case, the instrument was an LTQ Velos (Thermo Scientific; www.thermofisher.com). Aliquots of the tryptic digest were diluted to 20 μl with 5% methanol and 1% formic acid and loaded onto a chromatographic system consisting of a C_{18} preconcentration cartridge (Agilent Technologies; <http://www.agilent.com>) connected to a C_{18} tip column (15 cm \times 150 μm , 5 μm ; Nikkyo Technos, <http://www.nikkyo-tec.co.jp>). The separation was performed using a 40-min acetonitrile gradient from 3% to 40% (solvent A: 0.1% formic acid; solvent B: acetonitrile + 1% formic acid) at a flow rate of 1.0 $\mu\text{l min}^{-1}$. The HPLC system comprised an Agilent 1200 capillary pump, a binary pump, a thermostatted micro-injector and a micro-switch valve. The MS/MS system was operated in the positive ion mode with a spray voltage of 2 kV. Initial identification of the non-nitrated and nitrated peptides in the chromatograms was carried out in the data dependent mode, acquiring a full scan followed by ten MS/MS scans of the ten most intense signals detected in the MS scan. An exclusion time of 30 s was included to avoid repetitive MS/MS analysis of the dominant MS signals. Peptides were identified by database search of the MS/MS spectra using SEQUEST (Proteome Discoverer 1.3; Thermo Scientific). Search was performed with the following parameters: peptide mass tolerance 5 ppm, fragment tolerance 0.8 Da, enzyme set as trypsin, allowance up to two missed cleavages, and dynamic modification for Met oxidation (+15.995 Da) and Tyr nitration (+44.985 Da). Relative quantification was carried out using SRM. The areas of the signals of the product ions were integrated and the ratios of the areas of the modified to unmodified peptides were calculated. The transitions used to integrate the peak areas are given in Table S2.

For the second analysis, peptide digests were applied to an EASY-Spray PepMap RSLC C_{18} column (15 cm \times 50 μm ; particle size, 2 μm ; Thermo Scientific) coupled to a one-dimensional nano-flow LC (Dionex UltiMate 3000; Thermo Scientific). The peptides were separated using a 60-min acetonitrile gradient from 95% of solvent A (0.1% formic acid) to 50% of solvent B (50% acetonitrile + 0.1% formic acid) at a flow rate of 300 nl min^{-1} . The nLC-ESI-MS/MS analysis was performed on a LTQ Orbitrap Elite (Thermo Scientific) at a resolution of 60,000. The repeat count was set to 1, repeat duration 20 sec, exclusion list size 500, exclusion duration 60 sec and exclusion mass width 10 ppm. Charge state screening was enabled with rejection of unassigned and 1+ charge states. Minimum signal threshold counts were set to 5,000. Peptide identification was carried out with SEQUEST as indicated for the first method. ProtMAX software was used for relative quantification of the identified nitrated peptides with respect to the unmodified ones by the relative abundances calculated from automated extraction of the summed ion intensities of the targeted peptide precursor masses (Egelhofer *et al.*, 2013).

For both analyses, the monitored tryptic peptides with UniProt accession numbers of the proteins are given in Table S2.

Purification of Lbs and *in vitro* nitration

For *in vitro* nitration experiments, bean and soybean Lbs were purified essentially as described (Jun *et al.*, 1994). Nodule extracts were prepared in 50 mM Tris-HCl (pH 7.5), containing 1 mM EDTA and protease inhibitor cocktail. The protein was fractionated with 50–85% ammonium sulfate, resuspended in 10 mM Tris-HCl (pH 9.2), and oxidized with excess ferricyanide. The protein was dialyzed overnight with 50 mM potassium phosphate (pH 7.0), and applied to a hydroxyapatite column (HTP; Bio-Rad). The column effluent was concentrated and the buffer exchanged to 20 mM Tris-HCl (pH 8.0) with Vivaspin devices (10 kDa cutoff; Sartorius, <http://www.sartorius.de>). The protein was loaded onto a weak anion-exchange column (Whatman DE-52 cellulose; GE Healthcare) equilibrated with 20 mM Tris-HCl (pH 8.0). PvLba was eluted with 80 mM NaCl in buffer, GmLba with buffer alone, and GmLbc (Lbc₁ + Lbc₂ + Lbc₃) with a gradient of 0–100 mM NaCl in buffer.

Nitration of pure Lbs at analytical (10 μM ferric Lb) and preparative (1 mM ferric Lb) scales was conducted with relative proportions of Lb: H₂O₂: NO₂⁻ of 1:10:10 and 1:10:100. The assays were carried out in 20 mM potassium phosphate buffer (pH 6.5) at 26°C for 30 min. The proteins were then immediately loaded on SDS-gels and immunoblots performed with anti-NO₂-Tyr and anti-Lb antibodies. At the analytical scale, the effects of some compounds on nitration were tested by adding them in the reaction mix after Lb, but prior to NO₂⁻ + H₂O₂, at the following concentrations: DTPA (100 μM), DFO (100 μM), pHPA (5 mM), uric acid (10 μM) and KCN (10 mM). Nitration of Lbs was also attempted by exposing the proteins to SIN-1 (0.2–1 mM) for 1–2 h or to synthetic ONOO⁻ (100–500 μM) for 5 min, at pH 6.5 and 26°C. For comparison, additional assays were performed by exposing Lbs to SIN-1 at pH 7.4 and 37°C for 1–2 h. Synthetic ONOO⁻ was prepared by the reaction between H₂O₂ and NO₂⁻ at acid pH following standard protocols (Gow *et al.*, 1996; Bartesaghi *et al.*, 2006). The ONOO⁻ preparations had <20% NO₂⁻ contamination but were free of H₂O₂. Any possible effect of contaminating NO₂⁻ was ruled out by control experiments with decomposed ONOO⁻ ('reverse-order addition'; Bartesaghi *et al.*, 2004).

At the preparative scale, the Lb nitration products were excised from gels, eluted with 10 mM NH₄HCO₃ and analyzed by MS to determine molecular masses of the holoproteins and hemes and to quantify NO₂-Tyr. Low-resolution MS was performed using different dilutions of the samples in 0.1% trifluoroacetic acid. The samples were spotted on a MALDI plate with sinapinic acid and analyzed using a MALDI-TOF/TOF mass spectrometer (4800 TOF/TOF; AB Sciex, <http://www.absciex.com>). High-resolution MS was performed by nLC-ESI-MS/MS with an LTQ XL-Orbitrap mass spectrometer (Thermo Scientific). For nESI-FT-MS analysis, 5 μl of sample diluted in 50% MeOH + 0.1% trifluoroacetic acid was loaded in the nanospray needle. Full MS spectra were acquired (50 microscans/sec) at a resolution of 100,000 using an energy on source of 35 (full range of *m/z* 300–1800). For heme analyses, the signals at *m/z* 616 and 661 were selected as precursor masses and product ion spectra were obtained by collision induced dissociation (isolation width 2.5 and collision energy 35). Spectra were deconvoluted using the Xtract tool included in the Xcalibur 2.0 software (Thermo Scientific).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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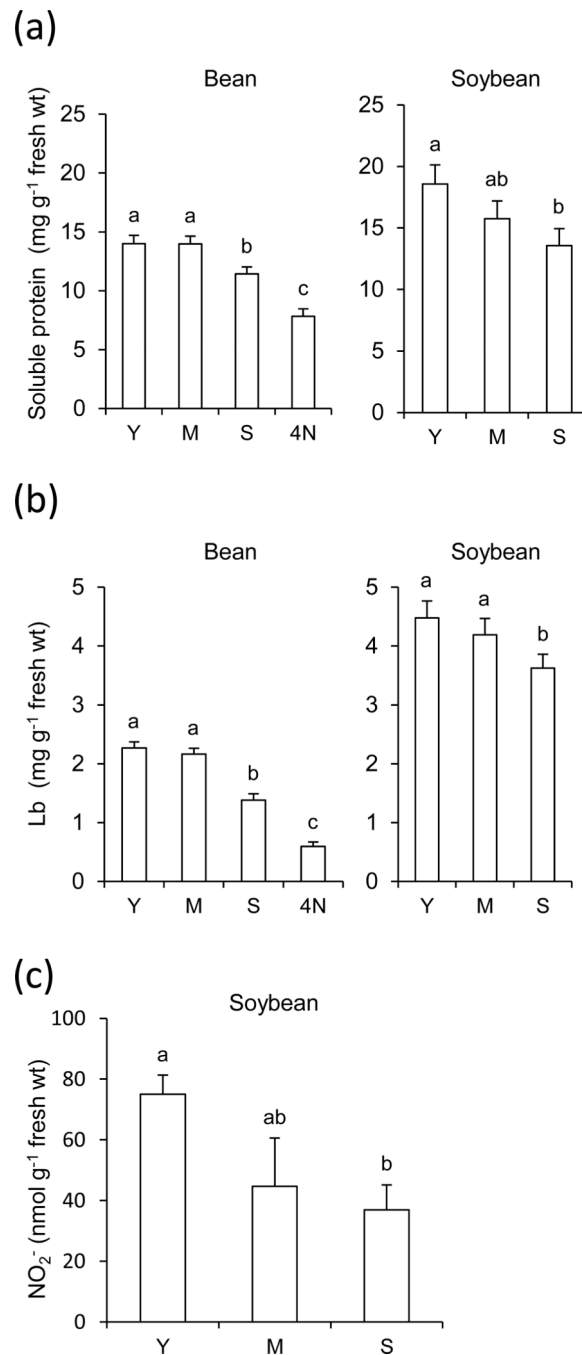


Figure 1. Physiological state of bean and soybean nodules

Nodule contents of total soluble protein (a), Lb (b) and NO₂⁻ (c) during natural and NO₃⁻-induced senescence. The NO₂⁻ content of bean nodules was ~5–8 nmol g⁻¹ fresh wt, close to the detection limit of the assay, and therefore it was not shown in the figure. Y, young nodules; M, mature nodules; S, senescent nodules; 4N, young nodules after 4 days of NO₃⁻ treatment. Data are means ± SE of four to six biological replicates. Means denoted by the same letter do not significantly differ at $P < 0.05$ based on the Duncan's multiple range test.

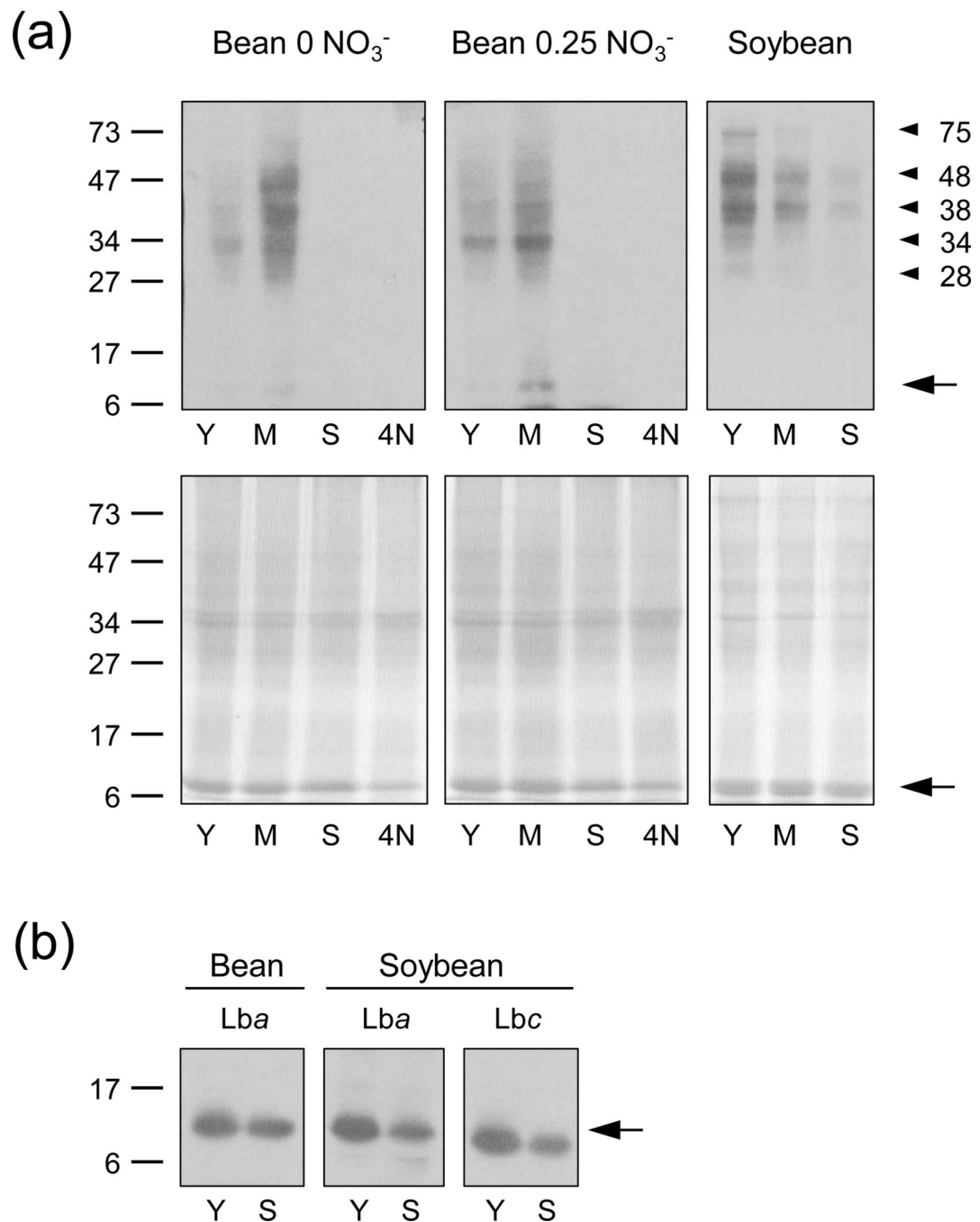


Figure 2. Immunoblots showing the presence of nitrated proteins in bean and soybean nodules (a) Bean plants were grown in the absence of NO₃⁻ or under common conditions with 0.25 mM NH₄NO₃, and soybean plants were grown in the field. Nodule extracts were analyzed on 12.5% SDS-gels. Immunoblots (*up*) and Coomassie-stained gels (*down*) are shown. Protein loaded was 50 µg (bean) or 20 µg (soybean) per lane.

(b) Extracts from similar nodule samples as above were loaded on preparative native gels, then Lb bands were eluted and proteins were resolved on 15% SDS-gels and blotted. Protein loaded was 20 µg per lane.

Y, young nodules; M, mature nodules; S, senescent nodules; 4N, young nodules after 4 days of NO_3^- treatment. Molecular mass (kDa) markers are indicated on the left. Nitrated protein bands and nitrated Lb bands are marked with arrowheads and arrows, respectively. Immunoblots are representative of six (a) or three (b) blots obtained with different nodule samples, except that in a few cases nitration intensity was similar in Y and M nodules of bean plants grown on 0.25 mM NH_4NO_3 .

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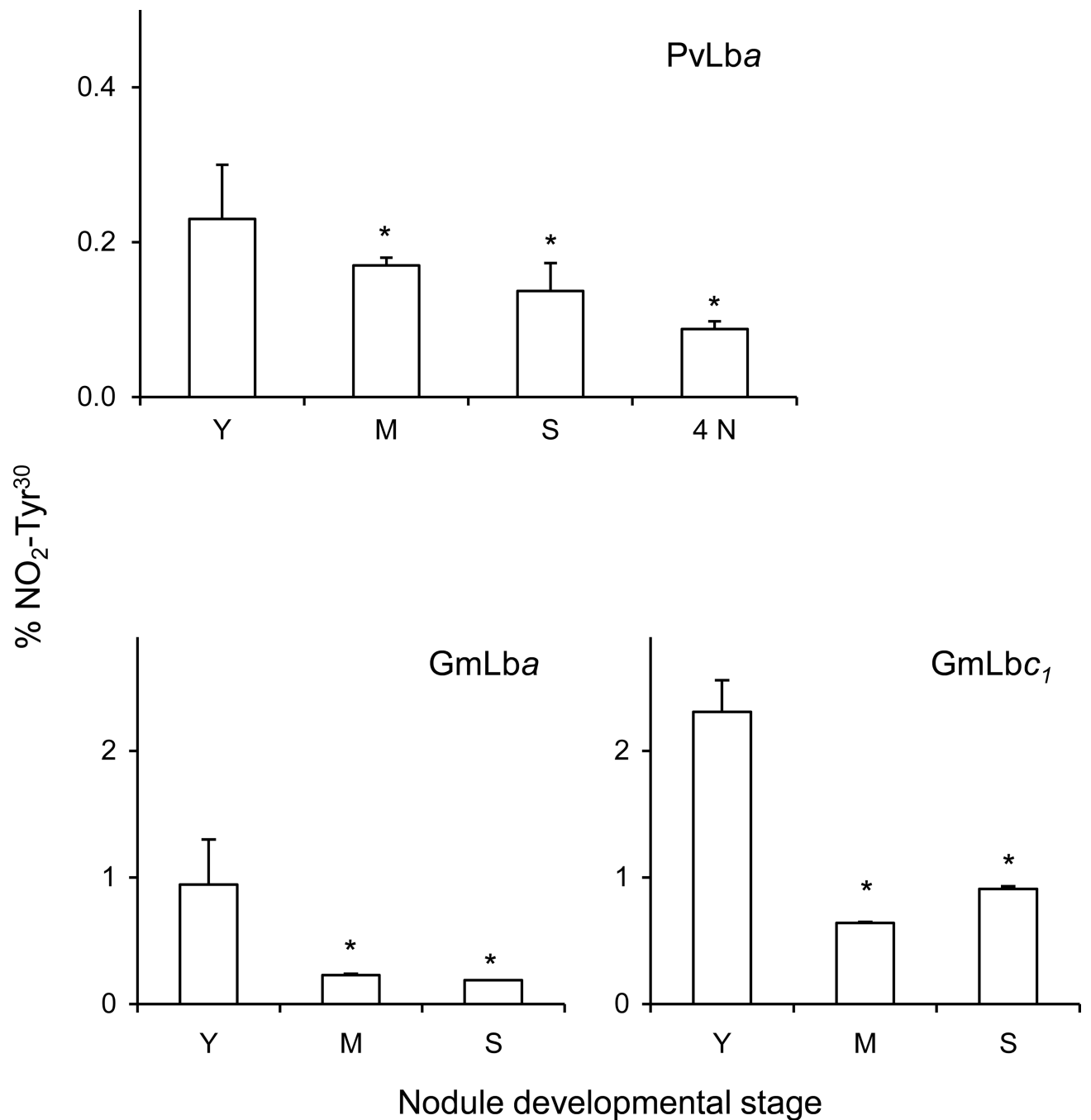


Figure 4. *In vivo* nitration of PvLba, GmLba and GmLbc₁ during natural and nitrate-induced senescence of nodules

Y, young nodules; M, mature nodules; S, senescent nodules; 4N, young nodules after 4 days of NO_3^- treatment. Data are given as percentage of $\text{NO}_2\text{-Tyr}^{30}$ relative to Tyr^{30} , and represent the mean \pm SE of two or three biological replicates corresponding to different protein preparations. Asterisks denote statistical differences relative to young nodules at $P < 0.05$ based on the Student's *t*-test. No differences in the $\text{NO}_2\text{-Tyr}^{30}$ content of PvLba were found in nodules treated with NO_3^- for 1, 2 or 3 days relative to untreated (control) nodules.

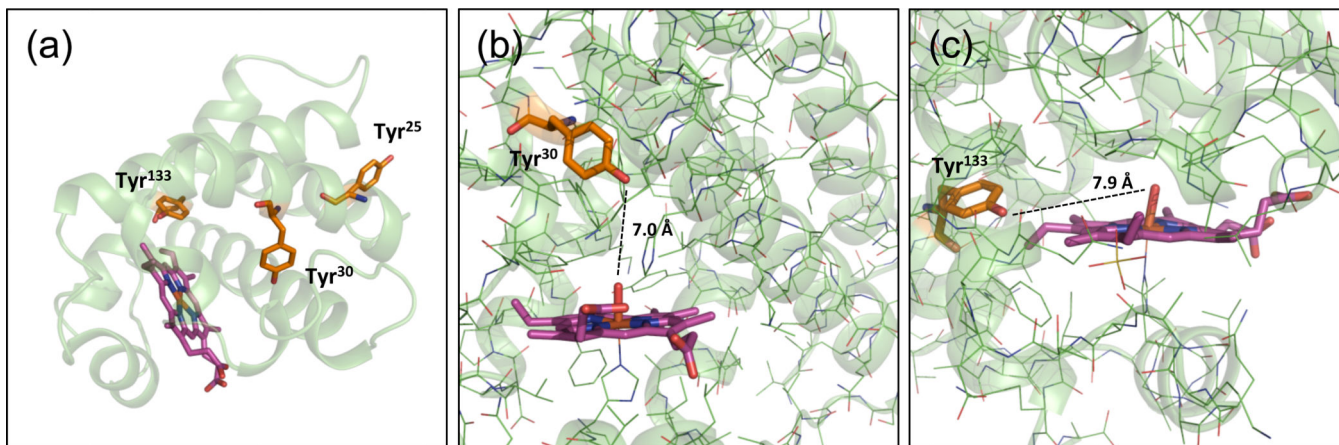


Figure 5. Molecular model of GmLba showing the three Tyr residues

(a) Positions of Tyr²⁵, Tyr³⁰ and Tyr¹³³ in relation to the heme.

(b) Tyr³⁰ located in the distal heme pocket.

(c) Tyr¹³³ in the edge of the heme, showing in both cases the distance between the Tyr hydroxyl and the oxoferryl. The GmLba crystal structure (PDB accession 1BIN) was used for modeling with PyMOL software (www.pymol.org).

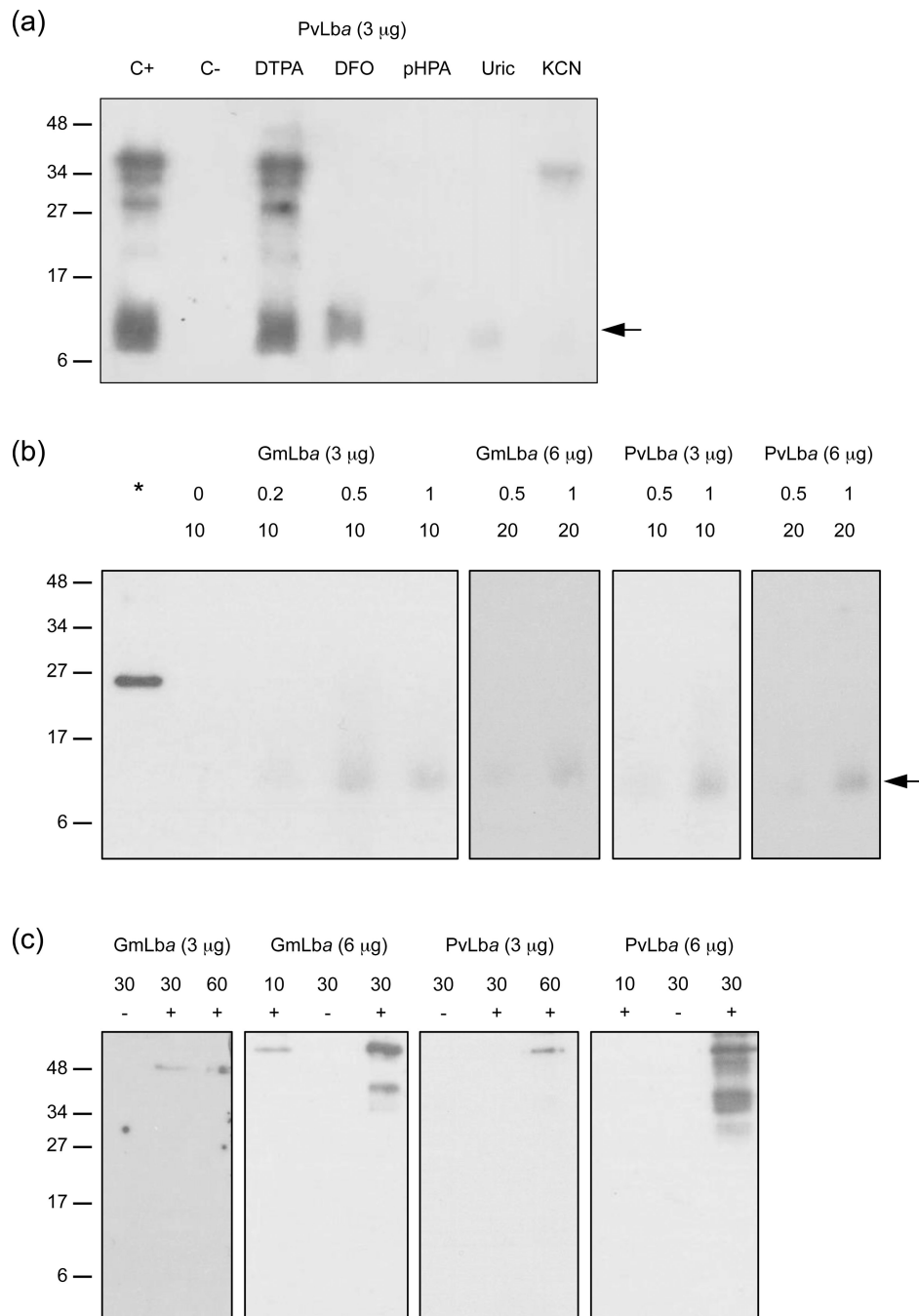


Figure 6. *In vitro* nitration of Lb

(a) PvLba (10 μ M) was treated with H_2O_2 (100 μ M) + NO_2^- (1 mM), then immediately loaded on SDS-gels, blotted and probed with anti- NO_2 -Tyr antibody. (C+) positive control with complete mix. (C-) negative control omitting H_2O_2 . Concentrations of compounds (added to the proteins before inclusion of H_2O_2 + NO_2^-): 100 μ M DTPA, DFO and uric acid; 5 mM pHPA; 10 mM KCN. Similar results were obtained for GmLba.

(b) Effect of SIN-1 on GmLba and PvLba nitration. The asterisk is a positive control of nitrated iron-superoxide dismutase (1 μ g; Castro *et al.*, 2004). Numbers are the

concentrations of SIN-1 (0.2–1 mM) and Lb (10–20 μ M) used for the reactions and the amounts of Lb protein (3–6 μ g) loaded per lane.

(c) Effect of ONOO⁻ on GmLba and PvLba nitration. Lbs were treated with 500 μ M ONOO⁻ + HCO₃⁻ (5 mM) + DTPA (100 μ M) for 5 min. Numbers are the concentrations of Lb (10–60 μ M) used for the reactions and the amounts of Lb protein (3–6 μ g) loaded per lane. Symbols +/- indicate addition or omission of ONOO⁻. In panels (a) and (b), arrows mark the position of the monomeric Lbs. In panels (a) and (c), immunoreactive protein bands with molecular masses >27 kDa correspond to dimers and aggregates of nitrated Lb. For all panels, experiments were performed in 20 mM potassium phosphate buffer (pH 6.5) at 26C, and molecular mass (kDa) markers are shown to the left.

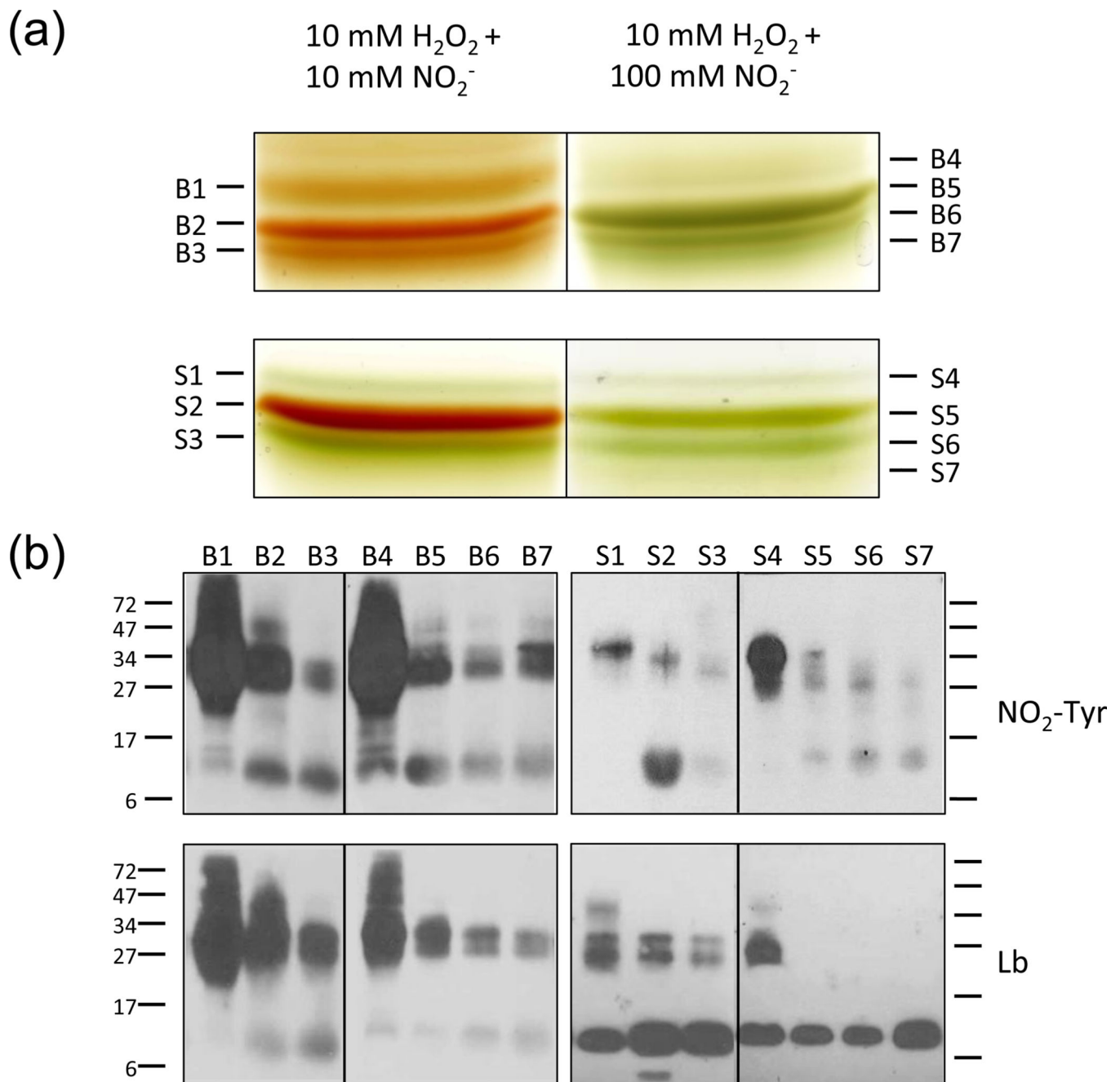


Figure 7. Preparative separation and immunoblot analysis of Lb derivatives generated by nitration

(a) Separation of Lb derivatives on preparative native gels. PvLba (*upper gels*) and GmLba (*lower gels*), both at 1 mM, were treated for 30 min with 10 mM H₂O₂ + 10 or 100 mM NO₂⁻ in 20 mM potassium phosphate buffer (pH 6.5). The protein products (bean, B1 to B7; soybean, S1 to S7) were eluted from gels and analyzed by MS.

(b) Immunoblot analyses of proteins eluted from (a) using antibodies against NO₂-Tyr and Lb. Besides monomeric nitrated Lb, dimers and other Lb aggregates (>27 kDa) were formed with different levels of nitration. GmLba was more resilient to nitration than PvLba, as the

former showed less intense signal with the NO₂-Tyr antibody and maintained most Lb in monomeric form.

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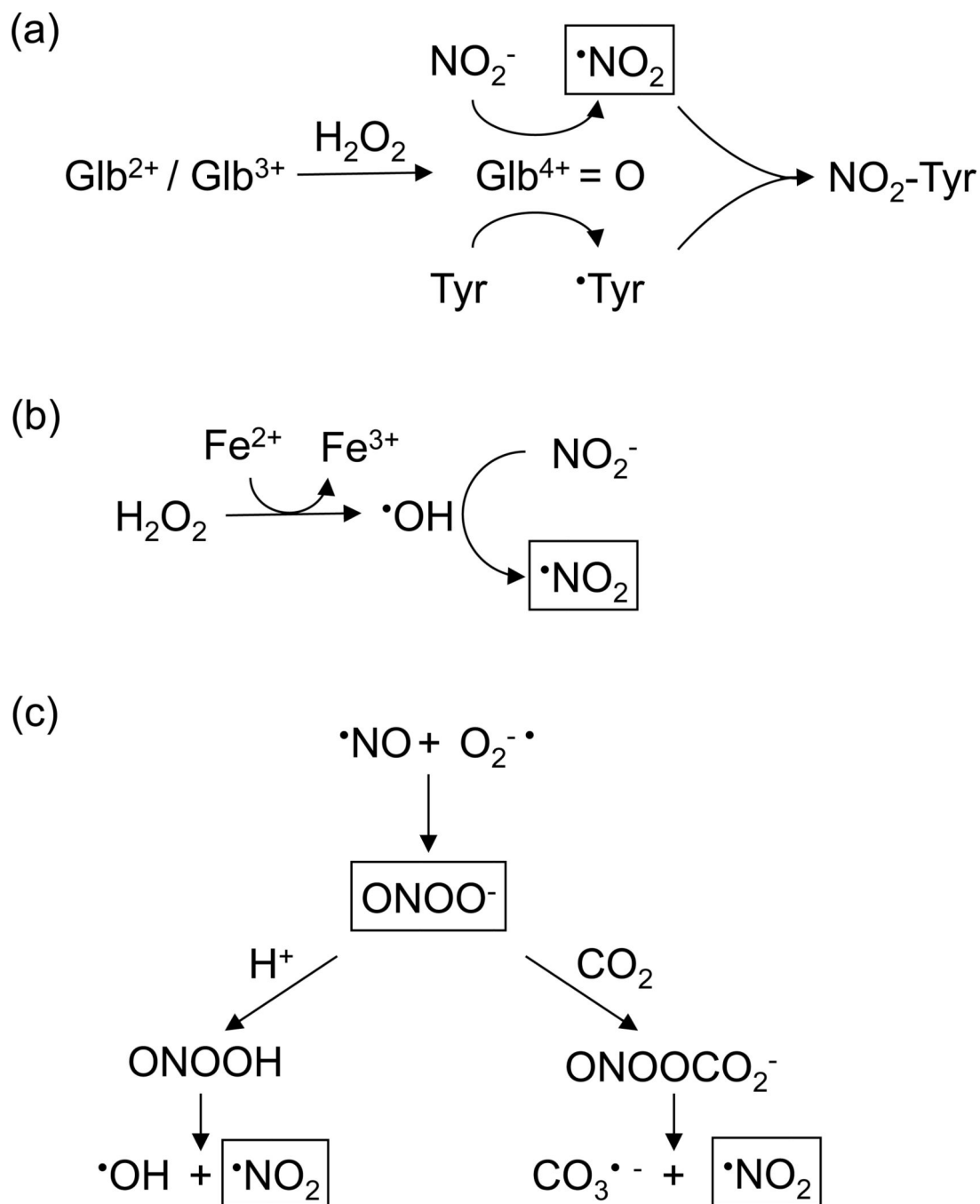


Figure 8. Mechanisms of protein Tyr nitration

(a) Nitration mediated by ferryl globin ($\text{Glb}^{4+}=\text{O}$), which oxidizes NO_2^- to $\bullet\text{NO}_2$. The tyrosyl ($\bullet\text{Tyr}$) radical cannot be generated directly from Tyr by reaction with H_2O_2 or ONOO^- and its formation is very slow with $\bullet\text{NO}_2$ (Radi, 2004).

(b) Iron-catalyzed Fenton reaction followed by $\bullet\text{OH}$ -mediated NO_2^- oxidation.

(c) Nitration mediated by ONOO^- and/or its derived radicals $\bullet\text{NO}_2$, $\bullet\text{OH}$ and $\text{CO}_3\bullet^-$. The nitrating species are indicated within boxes. For further explanation see Discussion.

Table 1*In vitro* nitration of Tyr residues of Lbs

Lb ^a	Tyr	% Nitration ^b	
		0.1 mM H ₂ O ₂ + 0.1 mM NO ₂ ⁻	0.1 mM H ₂ O ₂ + 1 mM NO ₂ ⁻
PvLba	Tyr ²⁵	0.4	1.4
	Tyr ³⁰	54.4	75.9
GmLba	Tyr ²⁵	0.6	1.1
	Tyr ³⁰	49.9	77.7
GmLbc ₁	Tyr ²⁵	13.5	0.9
	Tyr ³⁰	25.3	64.4
GmLbc ₂	Tyr ²⁵	0.5	0.6
	Tyr ³⁰	38.3	65.6
GmLbc ₃	Tyr ²⁵	0.5	0.4
	Tyr ³⁰	32.1	55.2

^a All Lbs were used at a concentration of 10 μM.

^b Percentage of nitration = $[\text{NO}_2\text{-Tyr}/(\text{NO}_2\text{-Tyr} + \text{Tyr})] \times 100$. Values are means of two or three biological replicates with SE < 10%.