

Review

Oxidation and Reduction of Leghemoglobin in Root Nodules of Leguminous Plants¹

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ABSTRACT

Reactions involving changes that affect the function of leghemoglobin (Lb) are reviewed. The chemical nature of Lb and conditions inside nodules, such as slightly acid pH and the presence of metal ions, chelators, and toxic metabolites (nitrite, superoxide radical, peroxides), are conducive for oxidation of ferrous Lb (Lb²⁺) or its oxygenated form (LbO₂) to nonfunctional ferric Lb (Lb³⁺) and ferryl Lb. Because Lb³⁺ is nearly nonexistent in nodules and undergoes observable reduction *in vivo*, mechanisms must operate in nodules to maintain Lb in the Lb²⁺ state. Redox reactions of Lb are mediated, for the most part, by activated oxygen species: (a) oxidation of LbO₂ to Lb³⁺ involves superoxide; (b) excess peroxide oxidizes LbO₂ and Lb²⁺ to ferryl Lb and may cause breakdown of heme, release of iron, and generation of hydroxyl radicals (protein radicals may be formed in this process); (c) enzymatic reduction of Lb³⁺ requires active flavin and thiol groups and involves formation of peroxide; and (d) direct reduction of Lb³⁺ by NADH is mediated by superoxide and peroxide. Transition metal ions and certain small molecules of nodules such as flavins may act as intermediate electron carriers between NADH and Lb³⁺, increasing the rate of reaction, which then proceeds via superoxide or flavin radicals, respectively.

Lb,² a hemoprotein of about 16 kD, is an essential component for N₂ fixation by legume nodules, but its role is obviously indirect because isolated bacteroids fix N₂ in the absence of Lb. Bacteroids are microaerobes and require O₂ for respiration and N₂ fixation but rapidly lose nitrogenase

activity if exposed to atmospheric levels of O₂. In legume nodules, O₂ supply to the central, infected zone is controlled by a variable O₂ diffusion barrier located in the inner cortex. After O₂ passes through the barrier and reaches the central zone of nodules, Lb facilitates diffusion of O₂ from the plasmalemma of infected cells to the peribacteroid membrane. Apparently, free O₂ then diffuses through the peribacteroid space, which lacks Lb, to ultimately reach the efficient terminal oxidases of bacteroids. Facilitative O₂ diffusion by Lb and high respiration rates by bacteroids ensure a rapid flux of O₂ through the plant cytoplasm and avoid sudden changes in O₂ supply that otherwise would be detrimental to nitrogenase. To facilitate diffusion of O₂, Lb must bind O₂ reversibly, exhibit a high affinity for O₂, and be present at a concentration that is much higher than that of free O₂. Data on O₂ status of some nodules and kinetic constants of typical Lbs and other plant Hbs are presented in Table I. The high affinity ($K_{\text{dissociation}} = K_m = k_{\text{off}}/k_{\text{on}}$) of all plant Hbs for O₂ is a consequence of a fast k_{on} coupled to a moderately fast k_{off} . The abundance of Hbs in nodules (0.1–3 mM) and the very high affinity of the terminal oxidase of the bacteroids (or of *Frankia*, the endophyte in actinorhizal plants such as *Casuarina*) indicate that a likely important function of these proteins is facilitation of O₂ diffusion (1, 2). Recently, Hbs or mRNAs coding for Hbs have been detected also in the roots of other plants, including *Parasponia* and its nonnodulating relative *Trema*, which has led to the proposal that Hbs may be widespread in the plant kingdom and may have a more general function in all plants (2).

OXYGENATION OF Lb *IN VIVO*

Although only Lb²⁺ binds O₂, various other forms of Lb do exist *in vitro* and possibly *in vivo*. Ligated forms of Lb can be identified by spectrophotometry because they show distinct absorption peaks in the visible region. Spectra of a variety of minimally disturbed and intact nodules (*e.g.* see refs. 8, 14, 15) exposed to various levels of O₂ exhibit the absorption peaks characteristic of LbO₂ (541 and 575 nm) and Lb²⁺ (555 nm). In intact nodules, Y, *i.e.* the proportion of LbO₂ relative to total Lb, has been estimated by spectroscopic methods. Values of Y and K_m of Lb permit the calculation of the free O_i. For example, if 37% of Lb in soybean is present as LbO₂ (14), then O_i is equal to 28 nM (Table I). O_i decreases in plants subjected to stress presumably because of the increase in

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² Abbreviations: Lb, leghemoglobin; Lb²⁺, ferrous Lb; LbO₂, oxygenated Lb²⁺; Lb³⁺, ferric Lb; Lb^{IV}, ferryl Lb; Hb, hemoglobin; HbO₂, oxygenated ferrous Hb; Hb³⁺, ferric Hb; Mb, myoglobin; Mb³⁺, ferric Mb; Mb^{IV}, ferryl Mb; FLbR, Lb³⁺ reductase; SOD, superoxide dismutase; O_i, oxygen concentration in the infected cells of nodules; Y, fractional oxygenation of Lb; k_{on} , loading rate constant; k_{off} , unloading rate constant; NO, nitric oxide; FAD, flavin adenine dinucleotide; pI, isoelectric point.

Table I. Oxygen Status of Some Nodules from Leguminous and Nonleguminous Plants and Kinetic Constants of their Hemoglobins

Data taken from refs. 7, 9, 14, 15, and 21.

Plant	Hb	K_{on} $\mu M^{-1} s^{-1}$	K_{off} s^{-1}	K_m nM	Y %	O_i^a nM
Soybean	Lba	120	5.6	48	37	28
Pea	Lb I	250	16	65	25	22
Sweet clover				48 ^b	5	3
Lotus				48 ^b	20	12
Casuarina	Hb II	41	6	135		
Parasponia	Hb I	165	15	89		

^a Data calculated from the corresponding K_m and Y values. ^b Assuming the same K_m value as for soybean Lba.

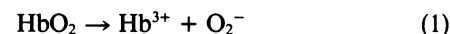
resistance of the O_2 diffusion barrier (7). Assuming a concentration of 3 mM for total Lb in soybean nodules (1), then the ratio [bound O_2]/ O_i is equal to 50,500. Reported values for other legumes were Y = 5% and O_i = 3 nM for sweet clover nodules and Y = 25% and O_i = 22 nM for pea nodules (Table I). A general observation from many determinations with sweet clover nodules suggests that the average O_i is much higher in older nodules than in younger nodules and, for a given nodule, in the older, proximal region of the nodule than in the younger, distal region (15).

INACTIVATION OF Lb BY OXIDATION

From the chemical nature of Lb, Lb^{3+} is expected to be continually formed *in vivo* by oxidation of Lb^{2+} and LbO_2 . Lb^{3+} can be formed by a typical electron oxidation of Lb^{2+} with redox potentials of around +0.22 V at pH 7 and around +0.27 V at pH 6. The soluble fraction of nodules extracted in air invariably shows the presence of at least traces of Lb^{3+} . Although most of this Lb^{3+} is undoubtedly an artifact of the extraction procedure, it does suggest that Lb^{3+} is readily formed from other Lb forms present in the nodules. Spectrophotometric detection of low levels of any form of Lb, and particularly of Lb^{3+} , in intact nodules is difficult because of the inherent light scattering of nodules. Using old, intact soybean nodules attached to roots and exposed to air or 100% O_2 , Klucas and coworkers (unpublished data) have detected a small absorption peak at 625 nm that, upon exposure of nodules to fluoride, shifts to around 610 nm. This observation supports the supposition that Lb^{3+} is present in legume nodules. A similar treatment with nicotinate resulted in a decrease in the 625-nm peak with the concomitant increase in absorption at 557 and 526 nm, indicating formation of the Lb^{2+} -nicotinate complex. A number of ligated forms of Lb^{3+} could also exist *in vivo*, including those formed with carboxylic acids such as formate, acetate, propionate, butyrate, valerate, and nicotinate (8). Although nicotinate has greater affinity for Lb^{3+} than for Lb^{2+} at acid pH, and this may tend to shift the redox equilibrium of Lb toward Lb^{3+} formation (8), most nicotinate detected in soybean nodules is probably an artifact originating from degradation of pyridine nucleotides during the extraction procedure, and actual levels of nicotinate in nodules may be clearly insufficient to affect binding of Lb^{2+} to O_2 (16).

A number of metabolites in nodules, including nitrite (27), superoxide radical (24), and peroxides (3), may oxidize Lb^{2+} . *In vitro* nitrite induces the oxidation of Lb^{2+} to Lb^{3+} (27), whereas NO binds tightly to Lb^{2+} and LbO_2 , displacing O_2 and forming nitrosyl-Lb ($Lb^{2+} \cdot NO$). Extracts of nodules from soybean and cowpea plants grown on nitrate have substantial levels of $Lb^{2+} \cdot NO$ (13), but uncertainty exists on whether or not this complex exists in the nodule or is an artifact arising from extraction. In nodules, nitrite may be formed by reduction of nitrate by plant and/or bacteroid nitrate reductases. In turn, NO might originate by nonenzymatic reduction of nitrite by reduced flavins or ascorbate; the reactions require nearly anaerobic conditions, such as those prevailing in nodules.

The superoxide radical is involved in many redox reactions of hemoproteins. For example, spontaneous autoxidation of HbO_2 in erythrocytes generates superoxide, in a reaction stimulated by H^+ and certain anions:



The superoxide radical can act also both as an oxidant of HbO_2 and as a reductant of Hb^{3+} , depending mainly on concentrations of HbO_2 and Hb^{3+} :



As expected, autoxidation of LbO_2 (Eq. 1) also generates superoxide radical (24). The rate of autoxidation of soybean LbO_2 , which is monitored by the decrease in absorption at 574 nm or the increase in absorption at 625 nm, varies with pH, temperature, and concentrations of Lb, certain anions, metal ions, and chelators. Puppo *et al.* (24) suggested that superoxide radicals are involved in autoxidation of Lb *in vivo* and that autoxidation occurs in the nodule. Direct evidence for this is lacking, but conditions in soybean nodules, such as high LbO_2 concentration (approximately 1 mM; refs. 1, 14) and slightly acid pH values (approximately 6.4 in younger nodules, dropping to 5.5 in older or stressed nodules; ref. 22), are conducive to oxidation. Oxidation of LbO_2 to Lb^{3+} by a flux of superoxide radicals generated artificially (Eq. 2) and re-reduction of Lb^{3+} by superoxide radicals (Eq. 3), although at a much slower rate, were also observed (24). Detailed

mechanisms of both reactions were not provided but may be similar to those of Hb. Because exogenously added SOD inhibited oxidation of LbO₂ to Lb³⁺, it was proposed that nodule SOD protects Lb against inactivation (24).

Hemoproteins react with peroxides to yield a higher oxidation state of the heme Fe, called the oxoferryl complex, whose structure can be described as HX-Fe^{IV}-OH or HX-Fe^{IV}=O (5). HX represents an amino acid residue that can become "radicalized" during the reaction, forming the oxoferryl radical (\cdot X-Fe^{IV}-OH or \cdot X-Fe^{IV}=O). This amino acid residue has been identified in Mb as tyrosine-151 by spin-trapping electron paramagnetic resonance (20). Apparently, oxidation of Mb³⁺ by H₂O₂ or lipid hydroperoxides generates both Mb^{IV} and its transient globin free radical (\cdot Mb^{IV}). The molecular mechanism of this oxidation is still uncertain but apparently does not involve hydroxyl radical, a highly oxidizing free radical that reacts with most biomolecules at nearly diffusion-controlled rates (5). Reduction of Mb^{IV} or \cdot Mb^{IV} to Mb³⁺ can be accomplished by some physiological reductants such as glutathione and ascorbate. A continuous cycling between Mb^{IV} and Mb³⁺ can be established if both glutathione or ascorbate and H₂O₂ are initially present in the reaction mixture, and, therefore, Mb reacts similarly to glutathione or ascorbate peroxidases (5).

As occurs with other hemoproteins, Lb reacts with peroxides to form toxic products. Thus, Lb²⁺, LbO₂, and Lb³⁺ yield Lb^{IV} upon reaction with H₂O₂ or organic hydroperoxides (3, 29). Lb^{IV} does not form complexes with common ligands of Lb²⁺ or Lb³⁺ and has no known function in nodules. Interestingly, the Lb²⁺·nicotinate complex is oxidized by H₂O₂ to Lb³⁺·nicotinate, but the latter does not react further with H₂O₂, *i.e.* does not form Lb^{IV} (3). As expected, "activated" Lb, *i.e.* Lb treated with peroxides, also catalyzes lipid peroxidation, but Lb^{IV} probably is not the chemical species initiating the process (26). Addition of excess H₂O₂ to LbO₂ or Lb³⁺ induced generation of hydroxyl radical as a result of the release of Fe²⁺ from heme and subsequent reduction of H₂O₂ to hydroxyl radical by Fe²⁺, apparently outside the protein (25).

Another property of the heme group is its ability to decompose H₂O₂ to H₂O in the presence of suitable artificial electron donors, such as guaiacol, *o*-dianisidine, or 4-chloro-1-naphthol (29). This pseudoperoxidatic activity of Lb and other hemoproteins follows first-order kinetics and has been used to locate Lb *in situ* or to visualize hemoproteins after electrophoresis. However, its physiological relevance is uncertain because only Lb³⁺ exhibits pseudoperoxidatic activity (29), and no electron donors for this reaction are known in the nodules.

Oxidative breakdown of heme in Lb yields green pigments *in vitro* and *in vivo*. At least two of them may be important *in vivo*. Choleglobin originates by oxidative attack of the porphyrin ring without loss of heme Fe, and biliverdins result from heme degradation with release of Fe and CO. Pigments with characteristics of choleglobin and biliverdins were partially purified from senescent pea nodules (1). However, green pigments are not well characterized and most information about them come from studies of heme degradation *in vitro*. Common bean Lb³⁺, but not soybean Lb³⁺, is reduced to LbO₂ by glycine at alkaline pH with concomitant oxidation of glycine to glyoxylate and production of H₂O₂. The resulting

LbO₂ is then rapidly degraded to form a green product that still contains Fe and exhibits a characteristic absorption peak at 697 nm (18). The physiological relevance of this finding is not clear but, interestingly, heme breakdown and consequent formation of green pigment is stimulated by previous digestion of Lb with carboxypeptidase and partially inhibited by SOD and catalase (18). "Coupled oxidation" of Lb³⁺ from a variety of legumes with high concentrations (\approx 10 mM) of ascorbate under aerobic conditions initially yields LbO₂, but this is rapidly broken down to biliverdin-like pigments (19). Degradation of heme from LbO₂, as monitored by a decrease in absorbance at 575 nm and an increase at 675 nm, produces different biliverdin isomers depending on the methine bridge that is cleaved: pea and vetch Lbs yield almost exclusively isomer β , but soybean and common bean Lbs are degraded to a mixture of approximately 30% isomer α , 50% isomer β , and 20% isomer δ (19).

FERRIC Lb IS REDUCED *IN VIVO*

Functioning of Lb requires the maintenance of its heme in the reduced form. Reduction of Lb³⁺ to Lb²⁺ in nodule slices can be monitored by diffuse reflectance or by direct transmission spectrophotometry (15). Treatment of soybean nodule slices with hydroxylamine caused oxidation of Lb²⁺ and LbO₂ to Lb³⁺ inside the cells; after the nodule slices were washed to remove excess hydroxylamine, Lb³⁺ was gradually reduced to Lb²⁺ as shown by the decrease in the 625-nm absorption band with the concurrent increase in absorption at 550 nm. Formation of Lb³⁺ by hydroxylamine was confirmed by complexing Lb³⁺ with acetate, fluoride, or nicotinate, and formation of Lb²⁺ from Lb³⁺ by complexing the resulting Lb²⁺ with nicotinate. These observations indicate that nodule cells can efficiently reduce Lb³⁺ to Lb²⁺ (15).

RESTORATION OF THE FUNCTIONAL STATE OF Lb BY ENZYMES

Several research groups have suggested that a system to reduce Lb³⁺ should exist in nodules. In 1969, Appleby (1) found that bacteroids can slowly reduce Lb³⁺ to Lb²⁺ under anaerobic conditions, suggesting that an enzyme is involved in the process. In 1977, Kretovich and coworkers reported the partial purification of a protein with FLbR activity from lupine nodules (reviewed in ref. 17). The enzyme was assayed routinely with NADH as the electron donor, ferric Cyt *c* as the electron acceptor, and methylene blue as an intermediate electron carrier. Cyt *b*₅ from rabbit liver microsomes could replace methylene blue, but the rates were much lower. Recently, they purified the enzyme from the cytosol of lupine nodules about 3000-fold by a four-step procedure to a specific activity of 235 μ mol min⁻¹ (mg protein)⁻¹, as determined with methylene blue as an intermediate electron carrier in the enzyme assay (10, 11). The enzyme contains FAD and active thiol groups but no catalytic metals, has a molecular mass of 60 kD, and exhibits *K*_m values of 8.7 μ M for NADH and 10 μ M for Lb³⁺ (Table II). The purified preparations of lupine FLbR were nearly homogeneous as shown by a single protein band after SDS-PAGE and staining with Coomassie blue. Isoelectric focusing revealed a single band with a pI value of

5.8 (Table II). In some respects, the physical and enzymatic properties of lupine FLbR resemble those of NADH:Cyt *b*₅ reductase from erythrocytes and Mb³⁺ reductase from red muscle. These three enzymes are flavoproteins with active thiol groups and use NADH as a reductant. It is possible that lupine FLbR is involved in Lb³⁺ reduction *in vivo*. A positive correlation exists between symbiotic performance (rates of N₂ fixation, Lb content) and FLbR activity during lupine growth; conversely, FLbR activity is low in inefficient or water-stressed nodules (30). However, direct evidence is lacking for the postulated physiological role of FLbR in lupine nodules.

Subsequent to the finding of a protein with FLbR activity in lupine nodules, several groups (23, 28, 30) reported an enzyme with similar activity in soybean nodules. Puppo *et al.* (23) observed three peaks of reductase activity upon fractionation of soybean nodule cytosol with ammonium sulfate (20–40%) and column chromatography on Sephadex G-200. The activity associated with proteins of intermediate molecular mass had specific activities of 1.2 and 15 nmol Lb³⁺ reduced min⁻¹ (mg protein)⁻¹ at pH 7.5 and 4.8, respectively, with NAD(P)H as a reductant.

A protein with FLbR activity has been purified to homogeneity from soybean nodules (Table II). Most of this enzymatic activity (91%) was localized in the plant fraction of nodules, presumably in the cytosol, whereas the remaining activity (9%) was in the bacteroids (28). The purification protocol involved several steps of fast protein liquid chromatography, based on protein separation by anion exchange, gel exclusion, and hydrophobic interaction (12). The purification achieved was 2000-fold with a 15% yield and a specific activity of 500 nmol Lb³⁺ reduced min⁻¹ (mg protein)⁻¹. A single band of protein was observed after electrophoresis and silver-staining of SDS-polyacrylamide gels. The protein has a molecular mass of 110 kD and is composed of two identical subunits. Isoelectric focusing of the native protein revealed three equal protein bands with pI values of 5.5, 5.6, and 5.7, which suggest slightly modified or different proteins. The

purified protein has FLbR, diaphorase, and NADH-oxidase activities with ratios of 1:10:2.5 under saturating conditions. Reductant and O₂, but not an intermediate electron carrier, are required for activity. Apparent *K*_m values for Lb³⁺, NADH, and O₂ are 9.5, 37, and 7 μM, respectively. Soybean FLbR reduces all eight components of Lb³⁺ at similar rates, but the reaction is partially or totally inhibited if Lb³⁺ is bound to acetate, nicotinate, or nitrite (4, 28). Exogenous catalase also inhibits Lb³⁺ reduction by FLbR, whereas exogenous SOD has no effect, indicating that peroxide-type intermediates are involved in the reaction (4).

Recently Ji *et al.* (12) sequenced the first 50 N-terminal amino acids of FLbR. The amino acid sequence showed a high homology with the enzyme family of flavin-pyridine nucleotide-disulfide oxidoreductases, which are dimeric proteins with FAD and active site disulfides. The greatest homology was with dihydrolipoamide dehydrogenase: the partial sequence of FLbR had 54, 70, 78, and 78% identity with the comparable sequences of *Escherichia coli*, yeast, pig heart, and human liver, respectively. In addition, this limited sequence of FLbR had all the amino acid residues necessary for the disulfide active site and all conserved amino acid residues in the FAD-binding domain (12). Complete sequencing of the soybean enzyme is important to gather information on structural aspects and reaction mechanisms of FLbR, including ascertaining the role of the active disulfides, FAD, and O₂ in the reaction, and locating the three different isoenzymes or modified forms of FLbR within the nodule cells. As in the case of lupine FLbR, a direct role for the FLbR from soybean nodules has not been demonstrated.

RESTORATION OF THE FUNCTIONAL STATE OF Lb BY SMALL MOLECULES

Nodules contain many potential reductants of Lb³⁺ including NAD(P)H, ascorbate, reduced glutathione, and cysteine. Estimates of "physiological" levels of these reductants to determine their relative effectiveness in reducing Lb³⁺ can be made assuming a water content of 85% in nodules and no compartmentation of metabolites within the nodules. Extraction of reductants from nodules in buffers that preserve organelle integrity to determine concentrations of metabolites in different nodule compartments is likely to introduce artifacts; conversely, use of extractants that inactivate enzymes will certainly disrupt cell organelles. With these limitations in mind, published levels of reductants in mature nodules are in the order of 150 to 250 μM for NADH + NADPH (4), 200 μM for cysteine (4), 40 to 150 μM for reduced glutathione (6), and 1 to 2 mM for ascorbate (6). At approximately similar concentrations, only ascorbate and cysteine significantly reduced Lb³⁺ (4). Excess ascorbate (*e.g.* 10 mM), however, initially reduces Lb³⁺ to LbO₂ but then induces heme degradation with formation of activated O₂ species (19). Reduction of Lb³⁺ by small molecules may be affected by the presence of metal ions and by changes in pH. For example, addition of Mn²⁺ markedly stimulates reduction of Lb³⁺ by NADH, and this reaction is mediated by superoxide radical (4).

Flavins are intermediate electron carriers between NAD(P)H and Lb³⁺ (4). In the presence of NAD(P)H, free flavins efficiently reduce Lb³⁺ without formation of superox-

Table II. Comparison of Biochemical Properties of FLbR from Lupine and Soybean Nodules

Properties	Lupine	Soybean
Molecular mass	60 kD	100 kD
Subunits	1	2 (equal)
Active -SH groups	ND ^a	+
Flavins	FAD	FAD
Diaphorase activity	+	+
Specific activity	235 μmol min ⁻¹ mg ^{-1b}	0.5 μmol min ⁻¹ mg ⁻¹
<i>K</i> _m for substrates		
NADH	8.7 μM	37 μM
Lb ³⁺	10 μM	9.5 μM
Reductant	NADH	NADH, NADPH (30% as effective)
pI values of isoforms	5.8	5.5, 5.6, 5.7
Subcellular location	Cytosol	Cytosol
References	10, 11	4, 12, 28

^a Not determined for the pure enzyme. ^b Calculated using methylene blue as intermediate electron carrier.

ide or peroxide because the reaction is not substantially affected by exogenous SOD or catalase, and therefore reduced flavins or flavin radicals are able to directly reduce Lb³⁺. Because the reaction is greatly stimulated in anaerobic or nearly anaerobic conditions, flavins may play an important role in reducing Lb³⁺ in the microaerophilic conditions inside the nodules (4). The abundance of free flavins, especially riboflavin, in nodules also supports this contention; however, for the mechanism to be operative, the supply of NAD(P)H should be relatively high and constant. Otherwise, *i.e.* with excess of flavins and deficit of NAD(P)H, Lb²⁺ is oxidized back to Lb³⁺ (4).

PROSPECTS FOR FUTURE RESEARCH

Some specific issues pertaining to Lb reactivity *in vivo* that must be resolved are: (a) Definitive proof is needed for the presence of Lb³⁺ in nodules. (b) Although various enzymatic and nonenzymatic processes for the reduction of Lb³⁺ have been proposed and shown to occur *in vitro*, definitive evidence is lacking for any of the processes *in vivo*. (c) Very little information is known about Lb degradation in nodules. For example, is Lb oxidatively attacked before it is degraded by proteases? Is the Lb heme moiety oxidatively attacked before the apoprotein? Are these oxidative steps reversible? (d) Speculation exists about the physiological relevance of Lb ligands, such as nicotinate and NO, which interfere with Lb function *in vitro*. Levels of nicotinate in soybean nodules apparently are too low to impair O₂ transport, but levels of nicotinate in nodules from other legume species have not been investigated. Although Lb²⁺·NO has been shown to exist in nodule extracts, does it exist *in vivo*? (e) Oxidation and reduction of Lbs have been studied in only a few species of legumes and, thus, it would be of interest to expand the studies to other species of legumes and other plants containing Hb.

Most of the information on the redox reactions of Lb in leguminous nodules has been obtained through *in vitro* experiments and, of these, only a few have simulated *in vivo* conditions. Insufficient information about the reactions of Lb *in vivo* has given rise to speculations and extrapolations from data on the chemical behavior of Lb *in vitro*. Questions regarding the existence, compartmentalization, and levels of proposed enzymes, metabolites, and free-radicals within nodules must be considered in future research.

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