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Review

Oxidation and Reduction of Leghemoglobin in Root Nodules of Leguminous Plants[1]

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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 (Lb^{2+}) or its oxygenated form (LbO_2) to nonfunctional ferric Lb (Lb^{3+}) and ferryl Lb. Because Lb^{3+} is nearly nonexistent in nodules and undergoes observable reduction *in vivo*, mechanisms must operate in nodules to maintain Lb in the Lb^{2+} state. Redox reactions of Lb are mediated, for the most part, by activated oxygen species: (a) oxidation of LbO_2 to Lb^{3+} involves superoxide; (b) excess peroxide oxidizes LbO_2 and Lb^{3+} 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 Lb^{3+} requires active flavin and thiol groups and involves formation of peroxide; and (d) direct reduction of Lb^{3+} 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 Lb^{3+}, 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 N_2 fixation by legume nodules, but its role is obviously indirect because isolated bacteroids fix N_2 in the absence of Lb. Bacteroids are microaerobes and require O_2 for respiration and N_2 fixation but rapidly lose nitrogenase activity if exposed to atmospheric levels of O_2. In legume nodules, O_2 supply to the central, infected zone is controlled by a variable O_2 diffusion barrier located in the inner cortex. After O_2 passes through the barrier and reaches the central zone of nodules, Lb facilitates diffusion of O_2 from the plasmalemma of infected cells to the peribacteroid membrane. Apparently, free O_2 then diffuses through the peribacteroid space, which lacks Lb, to ultimately reach the efficient terminal oxidases of bacteroids. Facilitative O_2 diffusion by Lb and high respiration rates by bacteroids ensure a rapid flux of O_2 through the plant cytoplasm and avoid sudden changes in O_2 supply that otherwise would be detrimental to nitrogenase. To facilitate diffusion of O_2, Lb must bind O_2 reversibly, exhibit a high affinity for O_2, and be present at a concentration that is much higher than that of free O_2. Data on O_2 status of some nodules and kinetic constants of typical Lbs and other plant Hbs are presented in Table I. The high affinity ($K_{dissociation} = K_m = k_{off}/k_{on}$) of all plant Hbs for O_2 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 O_2 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 Lb^{2+} binds O_2, 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 O_2 exhibit the absorption peaks characteristic of LbO_2 (541 and 575 nm) and Lb^{2+} (555 nm). In intact nodules, Y, *i.e.* the proportion of LbO_2 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 LbO_2 (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; Lb^{2+}, ferrous Lb; LbO_2, oxygenated Lb^{2+}; Lb^{3+}, ferric Lb; Lb^{IV}, ferryl Lb; Hb, hemoglobin; HbO_2, oxygenated ferrous Hb; Hb^{3+}, ferric Hb; Mb, myoglobin; Mb^{3+}, ferric Mb; Mb^{IV}, ferryl Mb; FLbR, Lb^{3+} 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}	k_{off}	K_m	Y	O_i[a]
		$\mu M^{-1} s^{-1}$	s^{-1}	nM	%	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+}·NO). Extracts of nodules from soybean and cowpea plants grown on nitrate have substantial levels of Lb^{2+}·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:

$$HbO_2 \rightarrow Hb^{3+} + O_2^- \quad (1)$$

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+}:

$$HbO_2 + O_2^- + 2H^+ \rightarrow Hb^{3+} + O_2 + H_2O_2 \quad (2)$$

$$Hb^{3+} + O_2^- \rightarrow HbO_2 \quad (3)$$

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 LbO_2 to Lb^{3+}, 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 Mb^{3+} by H_2O_2 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 Mb^{3+} can be accomplished by some physiological reductants such as glutathione and ascorbate. A continuous cycling between Mb^{IV} and Mb^{3+} can be established if both glutathione or ascorbate and H_2O_2 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, Lb^{2+}, LbO_2, and Lb^{3+} yield Lb^{IV} upon reaction with H_2O_2 or organic hydroperoxides (3, 29). Lb^{IV} does not form complexes with common ligands of Lb^{2+} or Lb^{3+} and has no known function in nodules. Interestingly, the $Lb^{2+}\cdot$nicotinate complex is oxidized by H_2O_2 to $Lb^{3+}\cdot$nicotinate, but the latter does not react further with H_2O_2, *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 H_2O_2 to LbO_2 or Lb^{3+} induced generation of hydroxyl radical as a result of the release of Fe^{2+} from heme and subsequent reduction of H_2O_2 to hydroxyl radical by Fe^{2+}, apparently outside the protein (25).

Another property of the heme group is its ability to decompose H_2O_2 to H_2O 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 Lb^{3+} 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 Lb^{3+}, but not soybean Lb^{3+}, is reduced to LbO_2 by glycine at alkaline pH with concomitant oxidation of glycine to glyoxylate and production of H_2O_2. The resulting LbO_2 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 Lb^{3+} from a variety of legumes with high concentrations ($\approx$ 10 mM) of ascorbate under aerobic conditions initially yields LbO_2, but this is rapidly broken down to biliverdin-like pigments (19). Degradation of heme from LbO_2, 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 Lb^{3+} to Lb^{2+} 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 Lb^{2+} and LbO_2 to Lb^{3+} inside the cells; after the nodule slices were washed to remove excess hydroxylamine, Lb^{3+} was gradually reduced to Lb^{2+} as shown by the decrease in the 625-nm absorption band with the concurrent increase in absorption at 550 nm. Formation of Lb^{3+} by hydroxylamine was confirmed by complexing Lb^{3+} with acetate, fluoride, or nicotinate, and formation of Lb^{2+} from Lb^{3+} by complexing the resulting Lb^{2+} with nicotinate. These observations indicate that nodule cells can efficiently reduce Lb^{3+} to Lb^{2+} (15).

RESTORATION OF THE FUNCTIONAL STATE OF Lb BY ENZYMES

Several research groups have suggested that a system to reduce Lb^{3+} should exist in nodules. In 1969, Appleby (1) found that bacteroids can slowly reduce Lb^{3+} to Lb^{2+} 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 μmol 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 μM for NADH and 10 μM for Lb^{3+} (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 Mb^{3+} 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 Lb^{3+} reduction *in vivo*. A positive correlation exists between symbiotic performance (rates of N_2 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 Lb^{3+} 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 Lb^{3+} 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 O_2, but not an intermediate electron carrier, are required for activity. Apparent K_m values for Lb^{3+}, NADH, and O_2 are 9.5, 37, and 7 μM, respectively. Soybean FLbR reduces all eight components of Lb^{3+} at similar rates, but the reaction is partially or totally inhibited if Lb^{3+} is bound to acetate, nicotinate, or nitrite (4, 28). Exogenous catalase also inhibits Lb^{3+} 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 O_2 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.

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^{-1}[b]	0.5 μmol min^{-1} mg^{-1}
K_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.

RESTORATION OF THE FUNCTIONAL STATE OF Lb BY SMALL MOLECULES

Nodules contain many potential reductants of Lb^{3+} including NAD(P)H, ascorbate, reduced glutathione, and cysteine. Estimates of "physiological" levels of these reductants to determine their relative effectiveness in reducing Lb^{3+} 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 Lb^{3+} (4). Excess ascorbate (*e.g.* 10 mM), however, initially reduces Lb^{3+} to LbO_2 but then induces heme degradation with formation of activated O_2 species (19). Reduction of Lb^{3+} by small molecules may be affected by the presence of metal ions and by changes in pH. For example, addition of Mn^{2+} markedly stimulates reduction of Lb^{3+} by NADH, and this reaction is mediated by superoxide radical (4).

Flavins are intermediate electron carriers between NAD(P)H and Lb^{3+} (4). In the presence of NAD(P)H, free flavins efficiently reduce Lb^{3+} without formation of superox-

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 Lb^{3+}. Because the reaction is greatly stimulated in anaerobic or nearly anaerobic conditions, flavins may play an important role in reducing Lb^{3+} 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, Lb^{2+} is oxidized back to Lb^{3+} (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 Lb^{3+} in nodules. (b) Although various enzymatic and nonenzymatic processes for the reduction of Lb^{3+} 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 O_2 transport, but levels of nicotinate in nodules from other legume species have not been investigated. Although $Lb^{2+}\cdot NO$ 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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