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Enzymatic and nonenzymatic mechanisms for ferric leghemoglobin reduction in legume root nodules

(flavins/ferric leghemoglobin reductase/nitrogen fixation/physiological reductants)

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ABSTRACT **Evidence is presented for the operation in nodules of at least four systems for restoring functional ferrous leghemoglobin (Lb^{2+}) from its inactive, ferric form. (*i*) Reduction of ferric leghemoglobin (Lb^{3+}) by a reductase. The enzyme is a flavoprotein of 100 kDa with two equally sized subunits and exhibits a K_m of 9 μM for soybean Lb^{3+} component *a* and a K_m of 51 μM for NADH. NADPH is only 30% (initial velocities) as effective as NADH. Lb^{3+} reductase converts 215 nmol of Lb^{3+} to $Lb^{2+}\cdot CO$ (or $Lb^{2+}\cdot O_2$) per mg of protein per min and does not require an exogenous electron carrier. The enzyme shows similar affinity for soybean, bean, and cowpea Lb^{3+}, but different V_{max} values. The reductase is inactive when Lb^{3+} is bound to nicotinate or NO_2^-. (*ii*) Direct reduction of Lb^{3+} by NAD(P)H, ascorbate, and cysteine. Reduction by NAD(P)H is greatly stimulated by trace amounts of metals such as Mn^{2+}. (*iii*) Reduction of Lb^{3+} by the flow of electrons from NAD(P)H to free flavins to Lb^{3+}. The reaction does not occur via $O_2^{\cdot-}$ or H_2O_2, and thus NAD(P)H-reduced flavins can directly reduce Lb^{3+}. The efficiency of the reaction follows the order riboflavin > FMN > FAD. (*iv*) Reduction of Lb^{3+} by an unknown compound, B, of nodules. B has a molecular mass < 1 kDa and is heat-stable. The reaction mediated by B differs from those mediated by flavins and metals in several ways, requires NAD(P)H, and generates $O_2^{\cdot-}$.**

Only the ferrous forms of hemoglobin (Hb), myoglobin (Mb), and leghemoglobin (Lb) bind O_2. Oxidation of these hemoproteins to the ferric form is readily observed *in vitro*, but the proportions of the ferric forms are remarkably low *in vivo*. For example, erythrocytes from reptiles and mammals have a steady-state level of 1–3% Hb^{3+} (1). In human erythrocytes enzymatic and nonenzymatic mechanisms exist for reducing Hb^{3+} to Hb^{2+} (2). The contribution of each system to Hb^{3+} reduction is estimated to be 67% NADH:Hb^{3+} reductase (also named NADH:cytochrome b_5 reductase), 5% NADPH:flavin reductase, 16% ascorbate, and 12% reduced glutathione (2). Analogous systems may exist in skeletal muscles for the reduction of Mb^{3+} (3, 4).

In leguminous nodules a steady-state level of Lb^{3+} is also believed to result from the autoxidation of $Lb^{2+}\cdot O_2$, which is favored by low pH values (5). Several nodule metabolites, such as $O_2^{\cdot-}$, NO_2^-, and H_2O_2, may contribute to the oxidation of Lb^{2+} and $Lb^{2+}\cdot O_2$ (6). Detection of Lb^{3+} in intact or minimally disturbed nodules is difficult due to the inherent light scattering by nodules, the low extinction coefficient of the diagnostic absorption band of Lb^{3+} at ≈625 nm, and the existence in nodules of several ligands, such as nicotinate (7), whose complexes with Lb^{3+} do not exhibit the 625-nm band.

The observation that chemically generated Lb^{3+} is rapidly reduced in soybean nodule slices suggests that nodules are equipped with mechanisms for restoring functional Lb^{2+} (8). Proteins with Lb^{3+} reductase (FLbR) activity were reported in lupin (9) and soybean (10, 11) nodules. Lupin FLbR is very similar to cytochrome b_5 reductase from erythrocytes (9). Puppo *et al.* (10) partially purified an FLbR-like enzyme from soybean nodules, but their preparation showed very low activity and this was not corrected for nonenzymatic Lb^{3+} reduction. Saari and Klucas (11) also purified a FLbR from soybean nodules that was shown to be a homodimer of 100 kDa and, therefore, unlike lupin FLbR. They also reported the existence of small, thermostable molecules in nodules that reduced Lb^{3+} upon addition of NADH and interfered with the purification of FLbR (11). The identification of these compounds was not attempted and their efficacy for reducing Lb^{3+} was not compared with that of FLbR.

In this paper we describe several mechanisms for the reduction of Lb^{3+} to Lb^{2+} that may be functional in legume nodules: (*i*) a specific enzyme (FLbR), (*ii*) endogenous reductants, (*iii*) NAD(P)H-reduced flavins, and (*iv*) a nonflavin unknown compound that also requires NAD(P)H for activity.

MATERIALS AND METHODS

Materials. Equipment for FPLC (fast protein liquid chromatography) and HPLC were purchased from Pharmacia and Waters, respectively. Reagents and chemicals were obtained as follows: hydroxylapatite (Bio-Gel HPT), Bio-Gel P-6DG, and protein assay reagent from Bio-Rad; Sephadex G-25 from Pharmacia; DEAE-cellulose (DE-52) from Whatman; ammonium sulfate and chemicals for HPLC from Baker; and sodium amobarbital from Lilly (Indianapolis, IN). All other chemicals were from Sigma. Mega-Pure (Corning) water was used throughout the study.

Bacterial and Plant Culture. *Rhizobium leguminosarum* bv. *phaseoli* 3622, *Bradyrhizobium japonicum* 3I1b110, and *Bradyrhizobium* spp. (*Vigna*) BR7301 were used to elicit root nodules on seedlings of bean (*Phaseolus vulgaris* L. cv. Canadian Wonder), soybean [*Glycine max* (L.) Merr. cv. Hobbit], and cowpea [*Vigna unguiculata* (L.) Walp. cv. California Blackeye]. Bacteria and plants were grown as indicated previously (12) except that the nutrient solution for plants had 15 mg of Sequestrene 330Fe (10% Fe; Ciba-Geigy) per liter instead of ferric citrate. Nodules were harvested from plants at the late vegetative growth stage: bean, 35–40 days; soybean, 30–35 days; and cowpea, 40 days.

Purification of Lbs. All operations were conducted at 0–4°C. Nodules (50 g or 100 g) were homogenized with an ice-cold Sorvall Omni-mixer (3 × 1 min; maximum setting) in 3 ml of 50 mM KP_i (pH 7.0) per g and 0.3 g of polyvinylpolypyrrolidone per g. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 30,000 × *g* for

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Abbreviations: DCIP, 2,6-dichloroindophenol; Lb, leghemoglobin; $Lb^{2+/3+}$, ferrous/ferric Lb; Lb*a*, -*b*, -*c*, and -*d*, different Lb isoproteins or components from the same legume species; FLbR, Lb^{3+} reductase; SOD, superoxide dismutase.

60 min. The clear supernatant was applied to a hydroxylapatite column (15 × 2.5 cm) previously equilibrated with 10 mM KP_i (pH 7.0). Three protein fractions were eluted by sequentially washing the column with 2–3 column volumes of 50 mM, 200 mM, and 700 mM KP_i (pH 7.0), respectively. The fraction that was eluted with 50 mM KP_i buffer was free of FLbR and was used for further purification of Lbs by DE-52 chromatography with sodium acetate at pH 5.2 (soybean; ref. 13) or with Tris·HCl at pH 8.0 (bean and cowpea; G. Sarath, H. K. Jun, and F. Wagner, personal communication).

Concentrations of Lb^{3+} (purified as above), whale skeletal muscle Mb^{3+} (Sigma), and bovine Hb^{3+} (Sigma) were determined by the pyridine–hemochrome method (13).

Purification of Soybean FLbR. The enzyme was purified according to the procedure of Saari and Klucas (11), as revised by Ji (14). The entire purification process was carried out at 0–4°C and required less than 4 days to complete. FLbR was purified from the 700 mM KP_i fraction mentioned above by several steps of FPLC, involving anion-exchange, gel-filtration, and hydrophobic columns (14). Protein concentration was determined by the Bio-Rad microassay (Bio-Rad Bulletin), with bovine serum albumin as a standard.

Assay of Diaphorase Activity. FLbR was routinely assayed during the purification by using its diaphorase activity as a convenient assay (15). One unit of activity was defined as the amount of enzyme that reduced 1 nmol of 2,6-dichloroindophenol (DCIP) per min (15).

Assay of FLbR Activity. FLbR activity was measured by following the conversion of Lb^{3+} to Lb^{2+}·CO at 562 nm. The reaction was linear for at least 15 min. The reaction mixture (1 ml) contained 60–85 mM KP_i (CO-saturated) at pH 7.0, 50 μM Lb^{3+} (from soybean, bean, or cowpea), 3 μg of enzyme, and 700 μM NADH to initiate the reaction. One unit of FLbR activity was defined as the amount of enzyme that produced 1 nmol of Lb^{2+}·CO per min. All FLbR activities were corrected for nonenzymic reduction of Lb^{3+} by NADH and were determined at 23 ± 2°C with a Cary 219 (Varian) spectrophotometer.

Kinetic Parameters of FLbR. A linear relationship between initial velocities (V_0) and enzyme concentration was observed at least in the range of 1–8 μg of protein. K_m values of soybean FLbR for several Lbs and NADH were determined essentially as indicated above during the first 5–10 min. Values of K_m and V_{max} were calculated from Eadie–Hofstee plots.

Effect of Metabolites, Inhibitors, and Other Modulators on FLbR Activity. FLbR activity was assayed as described above except 50 μM soybean Lb isoform *c* (Lb*c*) was used, and the relevant compound at the concentrations indicated in Table 1 was added to the reaction solutions. All tested compounds were also assayed in the absence of enzyme to correct for nonenzymatic reduction of Lb^{3+} by the compound(s) alone. Reactions were followed by sequential scanning (450–650 nm) at 0 (100% Lb^{3+}), 0.5, 1, and 4 hr. After the last scan, a few crystals of dithionite were added to the cuvette and CO was gently bubbled through the cuvette for a few seconds. A new scan was run after 1 min, which corresponded to 100% Lb^{2+}·CO. Percentages of Lb^{2+}·CO formed were then calculated with the 0% and 100% values of A_{562}. Although FLbR activity is similar with air- and CO-saturated buffers (11), CO was preferred for long incubations to avoid problems of Lb^{2+} autoxidation.

Lb^{3+}·nicotinate and Lb^{3+}·nitrite were produced by adding a few crystals of nicotinic acid and KNO_2 just prior to the addition of the enzyme and NADH. Inhibitors were preincubated with the enzyme in buffer at 23°C for 1 hr.

Flavin Content of Nodules. Free flavins were extracted from nodules essentially as described by Cerletti and Giordano (16), at 0°C in the dark. Nodules (0.3 g) were extracted twice with ice-cold trichloroacetic acid, and to the pooled supernatants 2 M KP_i (pH 7.0) was added to give a final pH of 6.1. Aliquots were stored at −70°C until further analysis (2–10 days later) of flavins according to Light *et al.* (17).

Extraction of Low Molecular Mass Compounds from Nodules. These were prepared either from the supernatant after the 55–85% ammonium sulfate fractionation (13) used for Lb purification (for experiment in Table 3) or directly from the nodule cytosol (for experiment in Table 4). Both the supernatant and the cytosol were filtered sequentially through YM10 (10-kDa nominal cutoff) and YM2 (1-kDa nominal cutoff) membranes (Amicon).

Extinction Coefficients. The following ε or Δε values ($mM^{-1}cm^{-1}$) were used for calculations. For diaphorase activity: DCIP (600 nm), 21 (15). For FLbR activity: soybean Lba^{2+}·CO minus Lba^{3+} (562 nm), 8.26; bean Lba^{2+}·CO minus Lba^{3+} (562 nm), 5.87; cowpea Lbb^{2+}·CO minus Lbb^{3+} (562 nm), 6.44. For ferric hemoprotein-reducing activity of small molecules: Lb^{2+}·O_2 minus Lb^{3+} (574 nm), 10.2 (11); Mb^{2+}·O_2 minus Mb^{3+} (581 nm), 11.9 (18); Hb^{2+}·O_2 minus Hb^{3+} (576 nm), 11.7 (19). For concentrations of pyridine nucleotides and flavins (20): NADH (340 nm), 6.22; NADPH (340 nm), 6.20; riboflavin (450 nm), 12.20; FMN (450 nm), 12.20; FAD (450 nm), 11.30.

RESULTS AND DISCUSSION

Purification of Lb Components. Soybean, bean, and cowpea Lbs were separated by anion-exchange chromatography (DE-52) into four, two, and three components, respectively. These components were named according to their order of elution. Bean and cowpea Lbs were extracted from fresh nodules and the relative abundances of the components were calculated from the peak areas. The proportions were as follows: bean Lb*a* (87%) and Lb*b* (13%); cowpea Lb*a* (2%), Lb*b* (83%), and Lb*c* (15%).

FLbR: Purification, Molecular Mass, and Kinetic Characteristics. A protein that catalyzes the reduction of Lb^{3+} to Lb^{2+} using NADH has been purified to homogeneity from soybean nodules, as judged by a single protein band of 55 kDa on silver-stained gels after SDS/PAGE. Likewise, the molecular mass of the native enzyme, determined by gel filtration on a Superose-12 column, was 100 kDa, in agreement with an earlier report (11). Small molecules that are present in the nodule cytosol and facilitate reduction of Lb^{3+} and DCIP precluded a reliable determination of specific activities at the initial stages of purification. The maximal specific activity achieved was 2000 nmol of DCIP reduced per mg of protein per min or 215 nmol of Lb^{3+} reduced per mg of protein per min. The kinetic parameters of soybean FLbR were calculated using soybean Lb*a* as well as closely related Lbs from bean and cowpea to determine whether the enzyme can distinguish natural and extraneous substrates in terms of affinity and catalytic activity. The K_m values of FLbR for Lb^{3+} from soybean, bean, and cowpea nodules were very similar, ranging from 8.8 to 13.3 μM, but significant differences were found in the V_{max} values for Lbs from different species. Relative to the V_{max} with soybean Lb*a*, V_{max} values were 42% and 45% for bean Lb*a* and cowpea Lb*b*, respectively. The K_m value for NADH was 51 μM with soybean Lb*a* as the other substrate. If a steady-state proportion of ≈1% Lb^{3+} exists in soybean nodules, as occurs for Hb^{3+} in human erythrocytes (2), Lb^{3+} concentrations in nodules would be 10–30 μM. This range of values would be consistent with the postulated function of FLbR *in vivo*.

FLbR: Effect of Physiological Reductants, Enzyme Inhibitors, and Lb Ligands. The effect of NAD(P)H and various inhibitors on the reaction catalyzed by FLbR was studied over 4-hr incubation periods (Table 1). FLbR can use NADPH instead of NADH as a reductant, but the activity was only 54% and 80% that using NADH, after 0.5 hr and 4 hr, respectively (Table 1). This is consistent with a previous

Table 1. Effect of physiological reductants, inhibitors, and Lb ligands on FLbR activity of soybean nodules

Addition(s)	% Lb^{2+}·CO formed		
	0.5 hr	1 hr	4 hr
NADPH (700 μM)	8.1	13.7	31.8
NADH (700 μM)	14.9	23.2	39.8
+ iodoacetamide (1 mM)	0	0	2.0
+ *p*-hydroxymercuribenzoate (500 μM)	21.2	24.9	34.1
+ quinacrine (500 μM)	10.5	16.3	22.7
+ amobarbital (1 mM)	9.8	14.8	8.1
+ SOD* (50 μg)	17.0	19.7	31.4
+ catalase (6 μg)	3.0	4.4	7.7
+ Lb^{3+}·nicotinate (50 μM)	1.1	1.3	0
+ Lb^{3+}·nitrite (50 μM)	1.5	1.6	0

*Superoxide dismutase.

report comparing V_0 values of FLbR with NADH or NADPH (11). Incubation of FLbR with the thiol reagent iodoacetamide (1 mM) resulted in 95–100% loss of activity, whereas *p*-hydroxymercuribenzoate (0.5 mM) initially (0.5 hr) enhanced and finally (4 hr) slightly decreased FLbR activity (Table 1). Quinacrine (0.5 mM), an inhibitor of flavoproteins (20), inhibited FLbR by 30–40%, and amobarbital (1 mM), an inhibitor of NADH-oxidizing enzymes and other flavoproteins (20), also inhibited FLbR by 35% after 0.5–1 hr, but by 80% after 4 hr (Table 1). These results suggest that thiol groups are important for FLbR activity and that the enzyme is a flavoprotein. To confirm the latter point the enzyme was digested with trypsin/chymotrypsin at pH 7.5 and 37°C for 2 hr. HPLC analysis showed unequivocally that the enzyme contains FAD (data not shown).

The effect of exogenous SOD and catalase on the reaction was analyzed to identify possible intermediates. Addition of high amounts of SOD (50 μg) had little effect on the rate of Lb^{3+} reduction, but catalase (6 μg) inhibited FLbR activity by 80% throughout the incubation period (Table 1). This suggests, but does not prove, that H_2O_2, and not $O_2^{\cdot-}$, may be an intermediate in the reaction.

The capacity of FLbR to reduce some complexes of Lb^{3+} was also investigated. Nicotinate binds tightly to Lb^{2+} and Lb^{3+} at neutral and low pH values (7). We have found that NO_2^- also ligates Lb^{3+} *in vitro*, forming a distinctive complex exhibiting α, β, and γ absorption bands at 558–560, 537.5–538.5, and 410–410.5 nm, respectively, for Lbs from different species. These spectral characteristics obviously differ from those of Lb^{2+}·NO (7, 21). To our knowledge, this is the first time a complex between Lb^{3+} and NO_2^- has been described, but a similar complex has been reported for Hb^{3+} (22). The complexes of Lb^{3+} with nicotinate and NO_2^- are not reduced by FLbR (Table 1), and this fact may be of physiological interest because both compounds are common metabolites of nodules (7, 23).

Direct Lb^{3+} Reduction by Physiological Reductants. The ability of various electron donors commonly present in plant and animal cells to nonenzymatically reduce Lb^{3+} was also examined. At 700 μM, NADPH was only 30% (0.5–4 hr) as effective as NADH, but cysteine (200 μM) and ascorbate (1 mM) reduced Lb^{3+} about 3-fold and 7-fold faster than NADH (700 μM) after 0.5 hr. Percentages of Lb^{2+}·CO formed from Lb^{3+} after 4 hr of incubation were 18%, 5%, 25%, and 34% for NADH, NADPH, cysteine, and ascorbate, respectively. Concentrations of cysteine and ascorbate used in this study are within the physiological range for plant tissues (24, 25) whereas those of NAD(P)H are 2- to 3-fold higher than those reported *in vivo* (26, 27, *). Cysteine and ascorbate might contribute directly to the reduction of Lb^{3+} in the nodules, but NAD(P)H probably not. This possibility cannot, however, be ruled out, because several factors, including trace amounts of transition metals, can enhance the NADH-dependent reduction of hemoproteins (ref. 3 and this report). Glutathione (1 mM) reduced Lb^{3+} only slightly but interacted with the protein, since the charge-transfer absorption band at 625 nm did not disappear upon addition of dithionite (data not shown).

*Shearman, L. L. & Klucas, R. V., 75th Annual Meeting of the American Society of Agronomy, Aug. 14–19, 1983, Washington, p. 98 (abstr.).

Table 2. Reduction of Lb^{3+} isoproteins from soybean, bean, and cowpea nodules by NADH

Isoprotein	% Lb^{2+}·CO formed*		
	0.5 hr	1 hr	4 hr
Soybean Lb*a*	0.8 a	1.8 a	7.3 a
Soybean Lb*c*	2.7 b	5.2 b	17.2 ab
Soybean Lb*d*	6.1 c	10.7 c	34.3 bc
Bean Lb*a*	2.4 ab	4.5 b	16.0 ab
Bean Lb*b*	7.1 c	12.7 d	42.6 c
Cowpea Lb*b*	10.9 d	17.0 e	73.2 d
Cowpea Lb*c*	12.2 d	18.6 e	81.4 d

The reaction mixtures (1 ml) contained 45–85 mM KP_i (CO-saturated) at pH 7.0, 50 μM Lb^{3+}, and 700 μM NADH.
*Values denoted by the same letter within each column did not significantly differ at $P = 0.05$ based on Duncan's multiple range test.

Finally, we investigated the relative effectiveness of NADH to reduce Lb^{3+} forms isolated from different legumes (Table 2). We observed large differences in the reduction of Lb^{3+} forms between and within legume species. Maximal differences in yield of Lb^{2+}·CO were noted between soybean Lb*a* and cowpea Lb*c*; 0.8% and 12.2% at 0.5 hr, and 7% and 82% at 4 hr, respectively. Differences between components within species were also apparent for soybean and bean (Table 2). The differences in the rates of Lb^{3+} reduction among components over long periods of time may have significance *in vivo*, as the relative proportion of components varies during aging of nodules (28).

Lb^{3+} Reduction Mediated by Flavins. As expected, flavins alone were totally unable to reduce Lb^{3+}, but the system NADH plus flavins brought about the virtually complete reduction of Lb^{3+} in 1 hr (riboflavin) or 4 hr (FMN and FAD) (Fig. 1). The order of effectiveness was riboflavin > FMN > FAD, and no significant effect, except a slight diminution for FAD, was observed upon boiling the flavins for 10–15 min. Addition of FLbR (3 μg) to the reaction mixture enhanced the rates of Lb^{3+}-reducing activity for all three flavins, although almost maximal values were already obtained with riboflavin during the first 0.5 hr (Fig. 1).

The high activities obtained with flavins prompted us to investigate whether flavins are involved in Lb^{3+} reduction *in vivo*. Flavins are likely candidates for the physiological reduction of Lb^{3+}. NAD(P)H reduces riboflavin, FMN, and FAD nonenzymatically, although this reaction may require catalytic amounts of metals; reduced flavins, in turn, reduce Fe^{3+}, whether free in solution or in hemoproteins such as cytochrome *c*, Mb, and Hb (3, 29).

The concentrations of riboflavin, FMN, and FAD in soybean nodules were determined by HPLC using fluorescence detection (17) and were corrected for recovery. Average recoveries were 78% for FAD and 65% for FMN and riboflavin. Riboflavin was by far the most abundant free flavin in soybean nodules; 1 g of fresh nodules contained 60 nmol of riboflavin but only 5.5 nmol of FMN or FAD. The corresponding concentrations in nodules were estimated to be 69 μM for riboflavin and 6 μM for the coenzymes (see legend to Fig. 2). Concentrations of NAD(P)H in soybean nodules (26, 27, *) were estimated to range from 11 to 67 μM for NADH and from 36 to 110 μM for NADPH (see legend to Fig. 2).

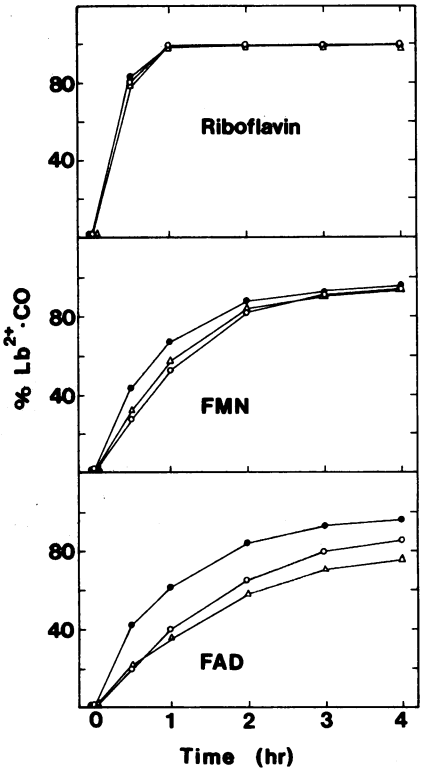

FIG. 1. Time course of Lb^{3+} reduction (% Lb^{2+}·CO formed) by flavins of physiological interest. The reaction mixture (1 ml) contained 50–80 mM KP_i (CO-saturated) at pH 7.0, 50 μM soybean Lbc^{3+}, 50 μM flavin, and 700 μM NADH. ○, Flavin; ●, flavin plus 3 μg of FLbR; △, flavin boiled for 10 min.

These values may represent underestimations because bacteroids have a very active deamidase that can degrade nucleotides to nicotinamide and nicotinate during extraction.* In any case, Lb^{3+} was reduced when flavins and NAD(P)H were used at the purported physiological concentrations, albeit at lower rates (cf. Figs. 1 and 2). The rate of Lb^{2+}·CO formed, however, began to decline after 30 min, probably due to NAD(P)H depletion because the reaction continued when the supply of NAD(P)H was reestablished (Fig. 2).

The physiological implications of flavins as intermediates in the reduction of Lb^{3+} in nodules are important. The reduction for Lb^{3+} by soybean FLbR (14) or by NADH alone (30) requires O_2, whereas the system NAD(P)H → flavins → Lb^{3+} does not; further, this reaction proceeds faster and more efficiently (i.e., less NADH consumed per Lb^{3+} reduced) under anaerobic conditions, with complete reduction of Lb^{3+} to deoxy-Lb^{2+} (M.B., M. L. Salin, and R.V.K., unpublished data). Therefore, the microaerophillic conditions inside the infected cells of nodules, with an estimated free O_2 concentration of ≈10 nM (28), should be conducive for the flavin-mediated reduction of Lb^{3+}. Another conclusion from Figs. 1 and 2 is that the NAD(P)H/riboflavin ratio is critical for Lb^{3+} reduction. High ratios (e.g., ≥6) accelerate Lb^{3+} reduction, whereas low ratios (e.g., 1–3) could cause Lb^{2+} reoxidation after NAD(P)H depletion. Very likely, the situation *in vivo* is much more favorable because of a constant level of NAD(P)H and of nearly anaerobic conditions. However, the NAD(P)H/riboflavin ratio in nodules is expected to decline sharply under stress conditions, as the concentration of NAD(P)H decreases (27) and that of riboflavin increases (31).

Lb^{3+} Reduction Mediated by an Unknown Compound of Nodules and by Metals. Nodules from soybean, bean, and cowpea plants contain a small molecule(s) (<1 kDa), which

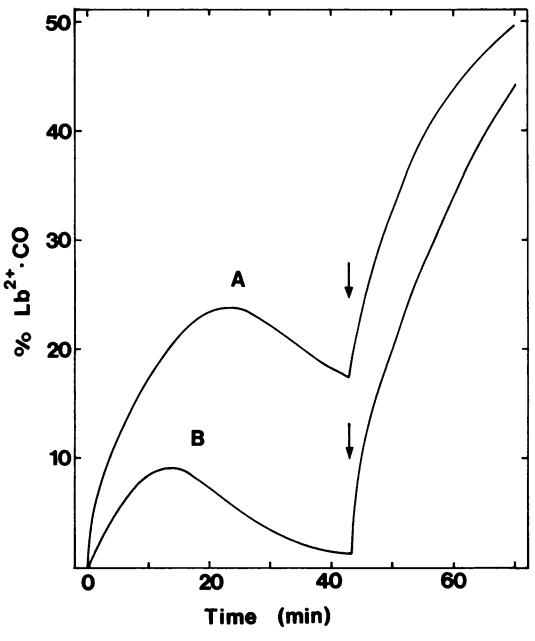

FIG. 2. Time course of Lb^{3+} reduction (% Lb^{2+}·CO formed) by the presumptive concentrations of flavins and NAD(P)H in soybean nodules. Given the large variation of NAD(P)H concentrations reported in the literature, a minimum (curve A; *) and a maximum (curve B; ref. 29) set of data were considered. Also, maximum and minimum concentrations of flavins were used, considering those of fresh (curve A) and frozen (curve B) nodules (see text). Concentrations (μM) were estimated from nmol per g of fresh nodules by assuming an 85% nodule water content (23) and a homogeneous distribution of compounds throughout nodules. The reaction mixtures (1 ml) contained 60–80 mM KP_i (CO-saturated) at pH 7.0 and 40 μM soybean Lbc^{3+}, plus (in μM) 69 riboflavin, 6.2 FMN, 6.5 FAD, 67 NADH, and 110 NADPH (curve A) or 28 riboflavin, 6.1 FMN, 5.9 FAD, 11 NADH, and 36 NADPH (curve B). Arrows mark addition of NADH to a final concentration of 400 μM.

we have called compound B, that (*i*) is very efficient at facilitating the reduction of Lb^{3+} at rates much higher than the enzymatic system, (*ii*) requires NADH (NADPH is similarly effective; data not shown), and (*iii*) is heat-stable (Table 3). Thus, compound B is not NAD(P)H, ascorbate, glutathione, or other typical biological reductant. Compound B could be a flavin, since flavins are relatively thermostable and are needed only at low micromolar concentrations to reduce Lb^{3+} in the presence of NADH (Fig. 2). To test this possibility, the fractions containing B were depleted of flavins by phenol extraction. No flavins were detected in the phenol-extracted fractions. In nondepleted fractions, flavin concentrations were 0 (FMN), 0.1 μM (FAD), and 2.2 μM (riboflavin), which appear to be too low to reduce Lb^{3+} after

Table 3. Reduction of Lb^{3+} by compound B of soybean, bean, and cowpea nodules

	% Lb^{2+}·CO formed					
	Soybean		Bean		Cowpea	
Addition(s)	0.5 hr	1 hr	0.5 hr	1 hr	0.5 hr	1 hr
NADH	7.4	8.8	1.3	3.0	16.1	30.3
B	0	0	0	0	0	0
B + NADH	66.3	85.6	74.9	88.9	68.5	85.5
B^b	0	0	0.6	1.8	0	2.4
B^b + NADH	64.5	84.6	76.2	92.3	75.5	86.4

The reaction mixtures (1 ml) contained 75–90 mM KP_i (CO-saturated) at pH 7.0, 50 μM Lb^{3+} (soybean Lb*a*, bean Lb*a*, or cowpea Lb*b*), 10–50 μl of B (30 μl soybean, 50 μl bean, 10 μl cowpea), and 700 μM NADH (where indicated). B^b, B boiled for 10 min.

their subsequent (10- to 100-fold) dilution in the reaction solution. Because the two types of fractions were nearly equally active in reducing Lb^{3+} (data not shown), B is not a flavin. Compound B is not an artifact arising from the extraction procedure with ammonium sulfate, KP_i buffer, and polyvinylpolypyrrolidone, because nodules extracted with distilled water (Mega-Pure) yielded activities of B that were comparable to those extracted with ammonium sulfate (cf. Tables 3 and 4).

To identify possible intermediates in the reduction of Lb^{3+} mediated by B, SOD and catalase were added in the reaction mixtures. Catalase (10 or 30 μg) had little effect on the reducing activity of B, whereas SOD (10 μg) fully suppressed the reaction (Table 4). When 10 μg of boiled SOD was added, values remained near the control (data not shown). These results clearly indicate that the reaction is mediated by $O_2^{\cdot-}$ but probably not by H_2O_2. This pattern of inhibition differs from that of Lb^{3+} reduction by NAD(P)H alone (inhibited both by SOD and catalase; ref. 30) and by reduced flavins (not inhibited by SOD or catalase; M.B., M. L. Salin, and R.V.K., unpublished data).

The possibility of B being a metal was also examined. The activity of B was unaffected when the nodule extract was passed through a Chelex-100 (Bio-Rad) column, and only a 13% inhibition was observed upon addition of 0.5 mM EDTA (Table 4). Transition metals such as Mn^{2+} reduce Mb^{3+} (3). We found that Mn^{2+} (10 μM) actively reduced Lb^{3+} (55% $Lb^{2+}\cdot O_2$ formed in 0.5 hr) in the presence of NADH (Table 4), but not in its absence. The reaction was inhibited by SOD but not by catalase. The inhibition by SOD was not as effective as for compound B; 10 μg and 30 μg of SOD inhibited the Mn^{2+}-mediated reaction by 31% and 56%, respectively. In contrast, EDTA (0.5 mM) stimulated the metal-mediated reduction by 22% (Table 4). Reactions mediated by B and Mn^{2+} were distinctly different when other hemoproteins were used as substrates. After correcting the values of hemoprotein-reducing activity of Mn^{2+} and B for reduction by NADH alone, we observed that (*i*) Mn^{2+} reduced Mb^{3+} (22% $Mb^{2+}\cdot O_2$) and Hb^{3+} (7.3% $Hb^{2+}\cdot O_2$), though much less than Lb^{3+} (49.2% $Lb^{2+}\cdot O_2$); and (*ii*) compound B formed only 8.5% $Mb^{2+}\cdot O_2$ and did not reduce Hb^{3+} (Table 4). We conclude that an unknown compound, B, of legume nodules may participate as intermediate in a system NAD(P)H → B → Lb^{3+}. Compound B is thermostable and generates $O_2^{\cdot-}$ upon addition of NAD(P)H, but the reaction differs in several aspects from those mediated by flavins or metals.

Table 4. Reduction of Lb^{3+}, Mb^{3+}, and Hb^{3+} by compound B and Mn^{2+}

Protein	Addition(s)	% hemoprotein·O_2 formed in 0.5 hr		
		Control	B	Mn^{2+}
Lb	None/B/Mn^{2+}	5.8	61.4	55.0
	+ SOD (10 μg)		0	38.1
	+ SOD (30 μg)		ND*	24.3
	+ catalase (10 μg)		57.9	52.9
	+ catalase (30 μg)		52.3	60.2
	+ EDTA (0.5 mM)		53.6	67.1
Mb	None/B/Mn^{2+}	15.5	24.0	37.5
Hb	None/B/Mn^{2+}	4.0	2.3	11.3

The reaction mixtures (1 ml) contained 70–85 mM KP_i (air-equilibrated) at pH 7.0; 30 μM Lb^{3+} (soybean Lbc^{3+}), Mb^{3+}, or Hb^{3+}; and 700 μM NADH with no further additions (control) or with 50 μl of B or 10 μM Mn^{2+} (as $MnSO_4$) plus SOD, catalase, or EDTA as indicated.
*Not determined.

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