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Flavin-mediated reduction of ferric leghemoglobin from soybean nodules*

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Abbreviations and terminology: FLbR= ferric leghemoglobin reductase; Hb^{2+}/Hb^{3+}= hemoglobin containing Fe^{2+}/Fe^{3+}; Lb^{2+}/Lb^{3+}= leghemoglobin containing Fe^{2+}/Fe^{3+}; Lb^{3+}·nicotinate/acetate= Lb in which nicotinate or acetate are complexed to Lb^{3+}; $Lb^{2+}·O_2$/CO/NO/ nicotinate= Lb in which O_2, CO, NO or nicotinate are complexed to Lb^{2+}; Rfl= riboflavin; SOD= superoxide dismutase (EC 1.15.1.1)

Abstract. The reduction of ferric leghemoglobin (Lb^{3+}) from soybean (*Glycine max* [L.] Merr.) nodules by riboflavin, FMN and FAD in the presence of NAD(P)H was studied in vitro. The system NAD(P)H + flavin reduced Lb^{3+} to oxyferrous ($Lb^{2+}\cdot O_2$) or deoxyferrous (Lb^{2+}) leghemoglobin in aerobic or anaerobic conditions, respectively. In the absence of O_2 the reaction was faster and more effective (i.e. less NAD(P)H is oxidized per mol Lb^{3+} reduced) than in the presence of O_2; this phenomenon was probably because O_2 competes with Lb^{3+} for reductant, thus generating activated O_2 species. The flavin-mediated reduction of Lb^{3+} did not entail production of superoxide or peroxide, indicating that NAD(P)H-reduced flavins were able to reduce directly Lb^{3+}. The NAD(P)H + flavin system also reduced the complexes $Lb^{3+}\cdot$nicotinate and $Lb^{3+}\cdot$acetate to $Lb^{2+}\cdot O_2$, Lb^{2+} or $Lb^{2+}\cdot$nicotinate, depending on the concentrations of ligands and of O_2. In the presence of 200 μM nitrite most Lb remained as Lb^{3+} in aerobic conditions but the nitrosyl complex ($Lb^{2+}\cdot NO$) was generated in anaerobic conditions. The above-mentioned characteristics of the NAD(P)H + flavin system, coupled with its effectiveness in reducing Lb^{3+} at physiological levels of NAD(P)H and flavins in soybean nodules, indicate that this mechanism may be especially important for reducing Lb^{3+} in vivo.

Key words: Flavins - *Glycine* (nitrogen fixation)- Leghemoglobin - Nitrogen fixation - Oxygen (activated) - Pyridine nucleotides - Symbiosis (legume-*Rhizobium*)

Introduction

The function of leghemoglobin (Lb) in legume root nodules is to bind O_2 and facilitate the transport of O_2 to the N_2-fixing bacteroids at a low but stable concentration that is compatible with both bacteroidal nitrogenase activity and respiration. Although higher oxidation states of Lb, namely ferric Lb (Lb^{3+}) and ferryl Lb (Lb IV), do exist, only the ferrous form (Lb^{2+}) is functional in binding O_2. In vitro Lb^{2+} is readily oxidized to Lb^{3+} or to Lb IV by exposure to nodule metabolites, such as superoxide, nitrite or peroxide (Rigaud and Puppo 1977; Aviram et al. 1978; Puppo et al. 1981). Also, autoxidation of $Lb^{2+}.O_2$ to Lb^{3+} probably occurs in vivo favored by the presence of trace amounts of transition metals or, especially, when there is a decrease in nodule pH that usually accompanies senescence (Pladys et al. 1988). Levels of the ferric forms of O_2-carrying hemoproteins, such as hemoglobin (Hb) in erythrocytes (Jaffé 1981), myoglobin in muscle tissue (Koizumi and Brown 1972; Hagler et al. 1979) or Lb in legume nodules (Shearman et al. 1986), are usually below 1%, probably because of enzymatic and nonenzymatic processes for reducing the hemoproteins back to their functional ferrous forms.

Maintenance of Lb in a functional ferrous state in nodules is therefore important for active N_2-fixation by legumes. In a previous paper Becana and Klucas (1990) described four mechanisms that could be operative for keeping Lb as Lb^{2+} in soybean nodules. These included Lb^{3+} reduction by *(i)* Lb^{3+} reductase (FLbR); *(ii)* NAD(P)H, ascorbate or other endogenous reductants; *(iii)* NAD(P)H-reduced flavins; and *(iv)* an as yet unidentified compound, B. For any of the postulated mechanisms to be germane, it must function at physiological levels of O_2 and relevant metabolites within the nodules. Mechanisms *(i), (ii)* and probably *(iv)* required O_2 to function (Saari and Klucas 1987; Becana and Klucas 1990; Ji 1990), but it is unclear if the free O_2 concentration in the cytosol of nodule infected cells, of less than 20 nM (King et al. 1988), can support these reactions.

In contrast, preliminary results obtained in the laboratory of one of us (R.V.K.) indicated that Lb^{3+} reduction by NADH + flavins was greater in buffers previously saturated with CO, i.e. in microaerobic conditions, and also that levels of flavins, presumably riboflavin (Rfl), were very high in soybean nodules. Extracts from fresh or frozen nodules prepared routinely for enzyme purification had a bright yellow color after removal of proteins by gel filtration and exhibited high fluorescence with excitation at 450 nm and emission at 520 nm, typical of flavins. These observations strongly suggested to us that mechanism *(iii)* involving NAD(P)H and flavins could be biologically very important, and prompted us to study this mechanism using physiological levels of flavins and NAD(P)H as well as microaerophilic conditions. We also used physiological concentrations of some important ligands of Lb^{3+}, such as nicotinate and nitrite, to determine if they could interfere in vivo with the reduction of Lb^{3+} by the NAD(P)H + flavin system.

The results presented in this paper clearly demonstrate that the flavin-mediated reaction is an important mechanism for reducing Lb^{3+} under conditions of very low O_2 tension (King et al. 1988), continuous NAD(P)H supply and high Rfl concentration as expected in soybean nodules. The system NAD(P)H + flavin is faster and consumes less NAD(P)H per mol Lb^{3+} under anaerobic than aerobic conditions, does not involve generation of superoxide or peroxide [and therefore it is not subjected to control or inhibition by nodule superoxide dismutase (SOD) or peroxidases], proceeds at physiological levels of NAD(P)H and flavins, and actively reduces Lb^{3+} when complexed with known nodule metabolites such as nicotinate, nitrite or acetate.

Materials and methods

Chemicals and reagents. All chemicals were of reagent grade or better and were purchased from Sigma Chemical Co. (St. Louis, Mo., USA) or Fisher Scientific (NJ, USA). Hb^{3+} (bovine) was used directly since no contamination by endogenous Hb^{3+} reductase was detected. CuZn-superoxide dismutase (SOD; EC 1.15.1.1) from bovine erythrocytes and catalase (EC 1.11.1.6) from bovine liver were also used without further purification. Materials for column chromatography were obtained as follows: Hydroxylapatite (Bio-Gel HTP; Bio-Rad, Richmond, Ca., USA), Sephadex G-25 (Pharmacia, Piscataway, NJ, USA), and DEAE-cellulose (DE-52; Whatman, Clifton, NJ, USA). Mega-Pure (Corning, NY, USA) water was used throughout the study.

Biological material and growth conditions. Soybean (*Glycine max* [L.] Merr. cv. Hobbit) seeds were surface-disinfected with 70% ethanol, pre-germinated in Petri dishes for 2-3 d, and transferred to 15 cm (diameter) plastic pots containing 1:1 (v/v) perlite:vermiculite wetted with the nutrient solution indicated below. Seedlings between 5 d and 7 d of age were inoculated with *Bradyrhizobium japonicum* strain 3I1b110, which was grown as described previously (Becana and Salin 1989). Two to 3 d before inoculation, seedlings were watered once with 1 mM NH_4NO_3. Plants were provided alternatively with water (to avoid salt accumulation) and with a nutrient solution consisting of: (in mM) 3.97 KH_2PO_4, 0.52 K_2HPO_4, 3.25 $MgSO_4$ and 2.90 $CaSO_4$ and (in μM) 2.50 $MnSO_4$, 0.25 $CuSO_4$, 0.25 $ZnSO_4$, 12.53 H_3BO_3, 25.24 NaCl, 0.13 Na_2MoO_4 and 0.05 $CoCl_2$. Fe was added to the nutrient solution as 15 mg/l of Sequestrene 330 (10% Fe; Ciba-Geigy, Greensboro, NC, USA) and the pH was adjusted to 6.3 ± 0.2 with KOH.

Plants were grown at 25/15°C (day/night) with a photon flux density of 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ (400-700 nm) for a 16-h photoperiod. Plants of the three species were

harvested when between 35 and 40 d of age, all of them being at the vegetative growth stage. Nodules were stored at -70°C until purification of Lbs^{3+}.

Purification of Lbs. Soybean Lbs were purified and resolved into components as described by Appleby and Bergersen (1980), with some modifications (G. Sarath, H.K. Jun and F. Wagner, Univ. of Nebraska, Lincoln, USA; personal communication), as follows. All operations were conducted at 0-4°C. After removal of bound nicotinate by chromatography on Sephadex G-25 at pH 9.2, the preparation was loaded onto a hydroxylapatite column (15 x 2.5 cm) and the fraction of proteins eluting with 50 mM K-phosphate pH 7.0 was collected. This fraction was shown previously to be free of FLbR (Becana and Klucas 1990; Ji 1990). The fraction was concentrated over a YM-10 membrane (Amicon) and the buffer was changed to 20 mM Tris-HCl pH 8.0. Components of Lbs were separated on a DE-52 column pre-equilibrated with the same buffer, applying a linear gradient of 0 to 100 mM NaCl. The components were concentrated (changing the buffer to 100 mM K-phosphate pH 7.0), quantified by the pyridine-hemochrome assay (Appleby and Bergersen 1980), and stored at -70°C. The three isoproteins of soybean Lb*c* (c_1, c_2 and c_3) were not further resolved, and Lb*c* was thus used unfractionated. Components Lb*a* $^{3+}$ and Lb*c* $^{3+}$ were used indistinctly in the experiments because they were reduced by NAD(P)H plus flavins at similar rates in preliminary tests and exhibited identical extinction coefficients (see below).

Aerobic and anaerobic assay of Lb^{3+} and Hb^{3+} reduction. For the aerobic, microaerobic and anaerobic assays of hemoprotein^{3+} reduction, the buffer (100 mM K-phosphate, pH 7.0) was equilibrated with, in the sequence stated, air, CO or N_2. The reaction mixtures (1 or 2 ml) consisted of 70-90 mM K-phosphate, pH 7.0, 30 μM hemoprotein^{3+} (soybean Lb*a* $^{3+}$or Lb*c* $^{3+}$, bovine Hb^{3+}), 10 μM Rfl,

FMN or FAD, and 600 μM NADH or ascorbate. Reactions were allowed to proceed at 22±2°C in the dark, unstirred, and were routinely monitored for 20 min, following an initial period of 5-7.5 min required for the system to stabilize. After this initial period, reactions were linear for at least the 20-min incubation period, and most were linear for at least 60 min.

For the anaerobic assay, buffer and Rfl were placed into a 1-ml cuvette, capped with a serum stopper, and saturated with high-purity N_2 (Lincoln Welding Co., Lincoln, Neb., USA). The hemoproteins were transferred to the cuvette and N_2 was passed through the gas space of the cuvette for 1-2 h to make it anaerobic and avoid protein denaturation. Finally, an aliquot of an anaerobic stock solution of NADH, to give a final concentration of 600 μM, was injected into the cuvette to start the reaction.

For all aerobic, microaerobic and anaerobic assays, standard errors were typically within 10% of the means.

The following extinction coefficients ($\Delta\varepsilon$, $mM^{-1}cm^{-1}$) were used for calculations: $Hb^{2+}{\cdot}O_2$ minus Hb^{3+} (576 nm)= 11.7 (Riggs 1981); $Hb^{2+}{\cdot}CO$ minus Hb^{3+} (562 nm)= 8.4 (this report); Hb^{2+} minus Hb^{3+} (555 nm)= 7.6 (this report); $Lb^{2+}{\cdot}O_2$ minus Lb^{3+} (574 nm)= 10.2 (Saari and Klucas 1984); $Lb^{2+}{\cdot}CO$ minus Lb^{3+} (562 nm)= 8.3 (Becana and Klucas 1990); Lb^{2+} minus Lb^{3+} (555 nm)= 7.4 (Appleby and Bergersen 1980).

Stoichiometry and oxygen consumption. The stoichiometry of NADH oxidized to $hemoprotein^{3+}$ reduced was determined using a reaction mixture (1 ml) containing: 80-90 mM K-phosphate, pH 7.0, 30 μM soybean Lba^{3+} or bovine Hb^{3+}, 5 μM Rfl, and 300 μM NADH. These concentrations allowed the measurement of changes in absorbance both at 340 nm for NADH oxidation and at the wavelengths of the corresponding a absorption bands (indicated above) for

hemoprotein^{3+} reduction. Reactions were permitted to run for the same period and under the same conditions as described above, but using two separate measurements for NADH oxidation and hemoprotein^{3+} reduction. For each of these two measurements, the reactions were repeated until standard errors were within 10% of the means, and the ratios of the means were obtained. At the concentrations used, changes in absorbance of Rfl and hemoproteins at 340 nm (as compared to that caused by NADH oxidation) or at the wavelengths of the α absorption bands of hemoproteins were found to be negligible. However, the ratios calculated in anaerobic conditions may be in minor error because traces of O_2 might have been present at the initial stages of the reaction.

Oxygen consumption was measured with a polarographic Clark-type electrode (Model 5300 biological oxygen monitor; YSI, Yellow Springs, Oh., USA). The reaction mixture contained (2 ml): 70-80 mM K-phosphate, pH 7.0, 60 μM soybean Lb*a* $^{3+}$ or bovine Hb^{3+}, 10 μM Rfl, and 600 μM NADH. Reactions were followed at 25°C as indicated above.

Spectroscopic characteristics of Lb^{3+} complexes and their reduction by NAD(P)H plus flavins. Absorption maxima in the visible region of soybean Lb*a* and Lb*c* were taken from the literature (Dilworth and Appleby 1974) or determined using 1-ml cuvettes containing 5 μM Lb (350-450 nm) or 50 μM Lb (450-650 nm) in 100 mM K-phosphate pH 7.0 or 100 mM Na-acetate pH 7.0 (Lb^{3+}·acetate complexes). Complexes of Lb^{2+} with nicotinate and NO were formed by a few crystals of nicotinate or KNO_2 to Lb^{3+} and then adding a trace of $Na_2S_2O_4$ (final concentration, less than 1 mg/ml). Complexes of Lb^{3+}·acetate were formed by incubating Lb^{3+} in 100 mM Na-acetate pH 7.0 overnight. All spectra were obtained with a Cary 219 (Varian, Palo Alto, Cal., USA) spectrophotometer

interfaced to an Apple II plus microcomputer (Cupertino, Cal., USA), which allowed the automatic recording of scans and calculation of peaks.

The reduction of Lb^{3+} in the presence of various concentrations of nicotinate, nitrite and acetate was studied in aerobic and anaerobic conditions by scanning between 450 and 650 nm at time 0, 1 and 2 h. In some cases, the absorption in the Soret region (350-450 nm) was also recorded. The reaction mixtures (1 ml) contained 70-80 mM K-phosphate, pH 7.0, or 80 mM Na-acetate, pH 7.0 (only in the assay with acetate), 30 μM soybean Lbc^{3+}, nicotinic acid (200 μM or 2 mM) or KNO_2 (20 μM or 200 μM), 10 μM Rfl, and 600 μM NADH. Incubations were at 25°C and scans were done with a Cary 2200 (Varian) spectrophotometer.

Determination of flavins in nodules. Extraction of free flavins Rfl, FMN and FAD was done as described by Cerletti and Giordano (1971), with minor modifications (Becana and Klucas 1990), and analyzed by HPLC according to Light et al. (1980).

Standardization of reagents. Concentrations of pyridine-nucleotides and flavins were determined using the following extinction coefficients ($mM^{-1} \cdot cm^{-1}$): NADH (340 nm)= 6.22; NADPH (340 nm)= 6.20; Rfl (450 nm)= 12.20; FMN (450 nm)= 12.20; and FAD (450 nm) = 11.30 (Dawson et al. 1986). Purities of flavins purchased from Sigma, as assessed by analytical high-performance liquid chromatography (Becana and Klucas 1990), were, in the order given, 100%, 79% and 82% for Rfl, FMN and FAD. These values were taken into account when calculating flavin concentrations. Concentrations of bovine Hb^{3+} were determined using an extinction coefficient at 630 nm of 3.9 $mM^{-1} \cdot cm^{-1}$, similar to that described for human Hb^{3+} (Riggs 1981).

Results

In the absence of flavins, NADH reduced Lb^{3+} or Hb^{3+} only very slightly and at high concentrations (Fig. 1). However, addition of Rfl at low micromolar concentrations to the reaction mixture resulted in elevated and steady rates of hemoprotein^{3+} reduction. This reduction was dependent on the concentration of both NADH and flavin. Values of about 0.40 μM hemoprotein^{3+} reduced · min^{-1} were attained in CO-saturated buffers, using 10 μM Rfl and 800 μM NADH. The effect of various other physiological reductants and flavins on the reduction of Lb^{3+} and Hb^{3+} was investigated using fixed concentrations of 600 μM for the reductants and 10 μM for the flavin coenzymes (Table 1). Lb^{3+} reduction by NADPH alone was 56% of that by NADH, but Hb^{3+} reduction was two-fold greater with NADPH than with NADH. Addition of Rfl to the reaction mixtures containing the reductants enhanced Lb^{3+} reduction rates by 0.36 and 0.47 μM Lb^{3+} reduced · min^{-1} for NADH and NADPH, respectively; the corresponding increase for Hb^{3+} reduction was 0.32 μM Hb^{3+} reduced · min^{-1} for both pyridine-nucleotides (Table 1). In the presence of NADH, reduction rates of Lb^{3+} and Hb^{3+} were 70% and 50% with FMN and 30% and 45% with FAD, respectively, relative to the values with Rfl (Table 1). As expected, flavins did not reduce hemoproteins^{3+} in the absence of NAD(P)H (data not shown). Ascorbate, another important reductant in nodules (Dalton et al. 1986), only slightly reduced Lb^{3+}, and this reaction was independent of flavins. In contrast, ascorbate reduced Hb^{3+} at measurable rates as compared to controls (Table 1).

The effect of O_2, SOD and catalase on the rates of hemoprotein^{3+} reduction is shown in Table 2. The data indicate that: *(i)* rates of hemoprotein^{3+} reduction mediated by Rfl were augmented 2-3 fold by excluding O_2; and *(ii)* the flavin-mediated reaction was not significantly inhibited by elevated amounts of SOD or

catalase. The relatively small inhibition (3-27%) caused in some cases by SOD or catalase (Table 2) is not significant and probably attributable to the high concentration of SOD used or to nonspecific effects of the proteins for several reasons. Firstly, 60 μg/ml of SOD should have suppressed the reaction. Indeed, Winterbourn (1985) indicated that, if superoxide radical is mediating a reaction involving Hbs, exogenous SOD should consistently inhibit the reaction at rather low concentrations (< 5 μg/ml when using 40 μM Hb^{3+}). Secondly, an increase in the concentration of catalase added to the reaction solution from 6 to 12 μg/ml did not further inhibit the reactions. Thirdly, small inhibitions (2-20%) were also observed in anaerobic conditions (Table 2). In these conditions, and even though traces of O_2 were still present initially in the reaction mixtures, formation of superoxide- or peroxide-like intermediates and the consequent inhibition of the reactions by SOD or catalase should not occur. In fact, addition of SOD and catalase increased the reaction rates in some cases (Table 2).

Reduction of Lb^{3+} by NADH and Rfl proceeds to completion in the absence of O_2 (Fig. 2). During this reaction only two Lb species appear to be present, namely Lb^{3+} and Lb^{2+}, as indicated by the sharp isosbestic points at 522 and 598 nm. The reaction product was identified as Lb^{2+} by its α-absorption band at 555 nm (Dilworth and Appleby 1974). The ratio of NADH oxidized to $hemoprotein^{3+}$ reduced was calculated in aerobic and anaerobic conditions by following the changes in absorbance at 340 nm and at the corresponding α absorption bands of the hemoproteins (Table 3). The ratios decreased from 4 to 1.5-0.8 as conditions were changed from aerobic to anaerobic. These ratios are useful only for comparison between aerobic and anaerobic conditions because the values may depend on the concentrations of NADH and Rfl used. The O_2 consumption rate associated with the reactions decreased from 0.25 to 0.15 $\mu M \cdot min^{-1}$ for Lb^{3+} and

from 0.13 to 0.07 μM · min^{-1} for Hb^{3+}, as conditions were changed from aerobic to microaerobic (CO-saturated buffers) (Table 3).

We also investigated whether the system NADH + Rfl reduces Lb^{3+} bound to some ligands of physiological interest (Table 4). To this end, nicotinate, nitrite and acetate were added at different concentrations to solutions containing Lb^{3+} (and Rfl) and the resulting Lb species were identified spectrophotometrically (Dilworth and Appleby 1974). Complete formation of the Lb^{3+}·nicotinate was observed after addition of 2 mM nicotinate to 30 μM Lb^{3+} but not when 200 μM nicotinate was added. Particularly noteworthy was the observation that nitrite, when present at milimolar concentrations, combines with Lb^{3+} to form a Lb^{3+}·nitrite complex, distinctively different from the nitrosyl derivative of Lb^{2+}, formed either by reacting Lb^{2+} with NO directly, or by adding a very small amount of $Na_2S_2O_4$ to a mixture of Lb^{3+} and nitrite. The complex Lb^{3+}·nitrite exhibits peaks at 410.5 nm (Soret) and 538 nm (β) and a shoulder at 559 nm (α), but no charge-transfer absorption bands were seen. Formation of the Lb^{3+}·acetate complex also required milimolar concentrations of acetate (Table 4).

After identifying the dominant initial complexes of Lb^{3+} the reactions were started by adding NADH, and the products were identified after 1 and 2 h by spectrophotometry. In aerobic conditions the major product of Lb^{3+} reduction after 2-h incubation was Lb^{2+}·O_2, except when 200 μM nitrite was present; in this case, the amount of Lb^{3+} exceeded that of Lb^{2+}·O_2 (Table 4). Traces of Lb^{3+} (slight absorption at 626 nm) were also evident in the cases of 200 μM and 2 mM nicotinate. Therefore, O_2 displaces nicotinate from Lb^{2+} at pH 7.0. In anaerobic conditions the only product formed in most cases was Lb^{2+} (Table 4). However, in the presence of 2 mM nicotinate, Lb^{3+}·nicotinate was reduced to Lb^{2+}·nicotinate, and, in the presence of 200 μM nitrite, Lb^{3+} was reduced to the nitrosyl complex (Lb^{2+}·NO). This complex was unequivocally identified on the basis of

its α, β and γ peaks at 570, 544 and 413.5 nm (Fig. 3), in agreement with the values reported for the $Lb^{2+}{\cdot}NO$ complex generated from Lb^{3+}, nitrite and $Na_2S_2O_4$ (Dilworth and Appleby 1974; Maskall et al. 1977; Kanayama and Yamamoto 1990).

To emphasize the biological significance of the in vitro system described here for reducing Lb^{3+}, we present in Table 5 data obtained by us or, in other cases, available in the literature, on levels of various metabolites in soybean nodules. By far, Rfl constitutes the most abundant free flavin in soybean nodules, with an estimated concentration of 70 to100 μM, whereas its coenzyme forms, FMN and FAD, were only present at levels of 3 to 7 μM. Large variation for values of NAD(P)H and nitrite are reported in the literature (Table 5). Levels of NADH + NADPH were in the range from 50 to 300 μM, and those of nitrite in nodules from plants supplied with moderate-high nitrate concentrations were in the order of 30 to 300 μM. Nicotinate, extracted under conditions that prevented NAD(P) degradation to nicotinamide and nicotinate, was found at levels ranging from 12 to 25 μM in soybean nodules (Table 5). The concentrations of NAD(P)H, Rfl, nicotinate and nitrite used in this study were within the physiological expected levels, when these were extrapolated to micromolar concentrations (see Table 5). For example, Lb^{3+} was reduced at substantial rates by 200 μM NADH and 10 μM Rfl (cf. Fig. 1, Table 5), and much greater rates are expected to occur in a highly anaerobic system such as the cytosol of nodule infected cells (cf. Tables 2, 5). Likewise, nicotinate and nitrite were used at concentrations in the low micromolar range, similar to those found in nodules (cf. Tables 4, 5).

Discussion

Active N_2 fixation by legumes requires that Lb be maintained in the functional, ferrous state. Enzymes and small molecules such as flavins may be involved in the reduction of Lb^{3+} to Lb^{2+} (Table 6). Flavins are intermediate one-electron carriers between NAD(P)H and Lb^{3+} or Hb^{3+} (Michelson 1977; Tables 1 and 2). The reduction of Lb^{3+} is faster and more efficient in terms of NADH oxidized per mol Lb^{3+} reduced in anaerobic than in aerobic conditions (Tables 3, 4). The absence of an O_2 requirement for the flavin-assisted reduction contrasts with the strict requirement for O_2 for the Lb^{3+} reduction by NADH alone and by soybean FLbR (Table 6). In the latter case, the stoichiometry of NADH oxidation to Lb^{3+} reduced was found to vary between 2 and 15 depending on NADH and O_2 concentrations (Ji 1990). Koizumi and Brown (1972) also observed a high ratio of NADH oxidized to ferric myoglobin reduced by NADH in the presence of EDTA. Apparently, an excess of NAD(P)H is needed to reduce hemoproteins^{3+} in aerobic conditions, because O_2 competes for the reductant and generates activated O_2 species. Under aerobic conditions, and in the absence of flavins, NADH may reduce Hb^{3+} and Lb^{3+} by way of superoxide radical and-or peroxide, as the reactions are greatly inhibited by SOD and catalase (Michelson 1977; Saari and Klucas 1987). Neither activated O_2 species is formed anaerobically. In the presence of flavins as intermediate electron carriers, more electrons are channelled to hemoprotein^{3+} reduction and less to O_2, and thus molar ratios of NADH oxidized to hemoprotein^{3+} reduced decline (Table 3). Since the flavin-dependent reduction of Lb^{3+} apparently does not require O_2, the O_2 consumed during the aerobic reaction is used in the formation of $Lb^{2+}\cdot O_2$ and in the oxidation of NADH to generate active O_2 species. The O_2 consumed in the microaerobic reaction is

mostly used in NADH oxidation because CO, by trapping all Lb^{2+} produced, eliminates the formation of $Lb^{2+}\cdot O_2$.

The reduction of Lb^{3+} by NAD(P)H + flavins does not proceed via generation of superoxide- or peroxide-like intermediates because they are not inhibited by anaerobiosis or exogenous SOD and catalase (Fig. 2, Table 2). This is in contrast again with the reduction of Lb^{3+} by NADH alone (inhibited both by SOD and catalase), FLbR (inhibited by catalase) or compound B (inhibited by SOD)(Table 6). This lack of inhibition by antioxidant enzymes represents a physiological advantage of the flavin system because the cytosol of soybean nodules contain CuZn-SOD and perhaps ascorbate peroxidase (see review by Becana and Rodríguez-Barrueco 1989), which otherwise would inhibit or, at least, control the rates of reaction. Similarly, Koizumi and Brown (1972) found that reduction of ferric myoglobin by FMN does not involve peroxide, and Michelson (1977) concluded that reduction of Hb^{3+} and ferric cytochrome *c* by flavins does not involve superoxide radical. Such observations along with those described here suggest that the flavin-mediated mechanism of Fe^{3+} reduction is similar for all hemoproteins.

The NAD(P)H + flavin was also able to reduce Lb^{3+} in the presence of physiological concentrations of some ligands of Lb^{3+}, namely, nicotinate, nitrite and acetate (Tables 4, 5). On the contrary, this reduction was not observed using NADH alone or FLbR (Table 6). Reduction of Lb^{3+} by flavins in the presence of micromolar concentrations of nitrite is particularly interesting. In aerobic conditions and with 200 μM nitrite, Lb^{3+} was the major form of Lb even after 2 h of incubation, indicating that the reduction rate was lowered by nitrite or that the $Lb^{2+}\cdot O_2$ formed was oxidized back to Lb^{3+} by nitrite, in agreement with the results of Rigaud and Puppo (1977). In anaerobic conditions the system NADH + Rfl reduced nitrite to NO, as shown by the formation of $Lb^{2+}\cdot NO$ (Table 4). This

complex has been detected by electron-paramagnetic-resonance spectroscopy (Maskall et al. 1977) or spectrophotometry (Kanayama and Yamamoto 1990) of nodule extracts from plants fed with nitrate. Recently, Kanayama and Yamamoto (1990) have put forward the hypothesis that the accumulation of $Lb^{2+}\cdot NO$ in nodules may be responsible for the inhibition of N_2 fixation in nitrate-treated plants (Kanayama and Yamamoto 1990). Conclusive proof of this hypothesis would require the finding of a mechanism able to generate NO in vivo, presumably from nitrite. We have shown that nitrite can be reduced to NO by NAD(P)H and flavins, but ascorbate is also effective in reducing nitrite to NO (Di Iorio 1981). In both cases anaerobic conditions are required.

Conclusions

The following conclusions are pertinent to the reduction of Lb^{3+} in vivo. *(i)* The system NAD(P)H + flavin does not require O_2 and is more effective in its absence (Table 2). Since the free O_2 concentration within the central zone of nodules is very low, in the range of 10-20 nM (King et al. 1988), this mechanism is consistent with Lb^{3+} reduction in nodules. By contrast, the reduction of Lb^{3+} by the previously reported FLbR and compound B as well as by NAD(P)H alone require O_2 (Table 6); *(ii)* Substantial rates of Lb^{3+} reduction are obtained with 200 μM NAD(P)H and 5-50 μM flavins, and even higher rates are expected to occur in nearly anaerobic conditions (Tables 2, 5; Fig. 1). These concentrations are similar to the levels of NAD(P)H and flavins of healthy legume nodules (Table 5), and therefore reduction of Lb^{3+} may proceed efficiently in the nodule environment by this pathway. *(iii)* The reaction does not involve generation of activated O_2, and thus it would not be inhibited by nodule SODs or ascorbate peroxidase (Becana and Rodríguez-Barrueco 1989). This contrasts with other postulated mechanisms

for Lb^{3+} reduction, which are inhibited by one or both antioxidant enzymes (Table 6); *(iv)* The system NAD(P)H + flavin reduces Lb^{3+} to Lb^{2+} in the presence of micromolar (nicotinate, nitrite) or milimolar (nicotinate, acetate) concentrations of ligands (Table 4). Levels of nicotinate in soybean nodules are in the range of 12-25 μM, and those of nitrite in the range of 30-300 μM in advanced stages of nitrate-induced nodule senescence (Table 5). Consequently Lb^{3+} reduction would probably continue to occur at physiological concentrations of nicotinate and nitrite, and it is unlikely that these ligands would play a role in maintaining Lbs as Lb^{3+} under normal or senescent conditions.

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Legends to figures

Fig. 1. Effect of NADH and Rfl concentration on rates of reduction of soybean Lb*a* $^{3+}$ and bovine Hb^{3+}. Reactions proceeded under microaerobic conditions (CO-saturated buffer). Composition of the reaction mixture, except for Rfl, and incubation time were as in *Materials and methods*. Concentrations of Rfl were 0 (Δ), 5 (o) and 10 μM (•).

Fig. 2. Reduction of soybean Lb*a* $^{3+}$ to Lb*a* $^{2+}$ by the system NADH + Rfl under anaerobic conditions. Scans were taken at 2, 20, 40 and 60 min. After 60 min, a few crystals of $Na_2S_2O_4$ were added and a new scan was taken (D). The characteristic absorption bands of Lb^{3+} at 626 nm and of Lb^{2+} at 555 nm are indicated.

Fig. 3. Reduction of soybean Lb*c* $^{3+}$ to Lb*c* $^{2+}$·NO by the system NADH + Rfl under anaerobic conditions. Scans were taken at 0, 1 and 2 h. The characteristic absorption bands of Lb^{3+} at 626 nm and of Lb^{2+}·NO at 544 and 570 nm are indicated.