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Characteristics of Modified Leghemoglobins Isolated from Soybean (*Glycine max* Merr.) Root Nodules[1]

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Hemoprotein derivatives of an abundant soybean (*Glycine max* Merr.) root nodule leghemoglobin, Lba, were studied for their modified spectral characteristics and physical properties. Three modified hemoprotein derivatives of Lba (Lb*am*$_1$, Lb*am*$_2$, and Lb*am*$_3$) were purified by preparative isoelectric focusing. The ferric forms of these pigments were green and exhibited anomalous spectra in the visible region as compared to the Lba^{3+} forms. These modified pigments showed a hypochromic shift of 10 nm for the charge transfer absorption maximum; however, differences were not apparent in the Søret region. Upon binding with nicotinate, the α and β bands were shifted significantly into the red region as compared to the Lba^{3+} nicotinate complex. The three Lb*am* fractions were reduced by dithionite or by NADH in the presence of riboflavin. Lb*am*$^{2+}$ also bound nicotinate and displayed absorption spectra indistinguishable from those of Lba^{2+} nicotinate. In contrast to Lba^{2+}, Lb*am*$^{2+}$ displayed aberrant spectra when bound with either O_2 or CO. These complexes exhibited a prominent charge transfer band at approximately 620 nm and failed to exhibit spectra characteristic of Lba$^{2+}O_2$ and Lba^{2+}CO. The protein moiety of these modified pigments was intact because their tyrosine/tryptophan ratios and their amino acid compositions were identical with those of Lba, nor were differences observed in the peptide profiles resulting from trypsin digests of purified Lba and Lb*ams*. Automated Edman degradation of selected peaks further confirmed the intactness of the protein backbone including the absence of deamination. Pyridine hemochromogen for heme from Lb*ams* could be formed, and the spectra displayed distinct differences compared to those of Lba. A new peak at 580 nm and a loss of a peak at 480 nm were observed for all three Lb*ams*.

Lbs are monomeric hemoproteins that facilitate O_2 diffusion within legume root nodules (Appleby, 1984). Like other O_2-carrying hemoproteins, such as myoglobin and hemoglobin, Lbs display reversible oxidation and reduction of the heme iron. To bind O_2 reversibly, Lbs must be reduced to their ferrous forms. Whereas studies have shown that soybean (*Glycine max* Merr.) Lb levels decline with the onset of nodule senescence, whether induced or natural (Klucas, 1974; Pfeiffer et al., 1983; Sutton, 1983), the biochemical events surrounding this decline are unknown. It is also unclear whether Lbs undergo irreversible changes during repeated oxidation-reduction cycles and whether inactive Lbs are present in appreciable quantities in functional root nodules.

An analysis of soybean Lbs by ion-exchange HPLC revealed the presence of a hemoprotein that appeared to be very similar to Lb*a* but with somewhat different properties (Sarath et al., 1986). The ratio of the levels of this protein, termed Lb*am* (Wagner and Sarath, 1987), to Lb*a* increased when nodulated soybean plants were subjected to dark stress. Lb*am* was the major hemoprotein detectable in nodules at advanced stages of senescence (Wagner and Sarath, 1987). Preliminary spectroscopic data indicated that in the ferric state Lb*am* was different from Lb*a*, although this Lb*am*$^{3+}$ preparation underwent normal reduction (Wagner and Sarath, 1987). These observations suggested that Lb*am* was a potential inactive form of Lb*a* that accumulated in senescent nodules. However, the small amounts of Lb*am*, purified by HPLC, precluded a detailed investigation of its properties.

Recently, a method was developed to separate and purify Lb*ams* by ion-exchange chromatography, followed by preparative IEF (Jun, 1993). This procedure results in the separation of a crude Lb*am* fraction into three distinct proteins resolvable by IEF. All three Lb*ams* possessed an NH_2-terminal sequence identical with that of Lb*a* and only appeared to differ in their isoelectric point (Jun, 1993), suggesting that subtle changes had occurred in these molecules. In this report, we demonstrate that these proteins exhibit characteristic spectral properties and show anomalous binding of O_2 and CO.

MATERIALS AND METHODS

Protein Purification

Root nodules were harvested from 40- to 50-d-old *Gycine max* Merr. cv Hobbit plants nodulated with *Bradyrhizobium japonicum* strain 61A89. Lbs were purified from soybean root nodules according to the method of Jun (1993). Briefly, root nodule extracts were prepared in 20 mM Na-phosphate buffer (pH 6.8) and fractionated with ammonium sulfate. Proteins

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Abbreviations: Lb, leghemoglobin; Lb^{2+}, ferrous leghemoglobin; Lb^{3+}, ferric leghemoglobin; Lb*a*, leghemoglobin component *a*; Lb*am*$_x$, modified components of Lb*a* (x = 1, 2, or 3); Lb*b*, leghemoglobin component *b*.

precipitating between 35 and 90% saturation were dialyzed against 10 mM Na-phosphate (pH 6.8) and applied to an hydroxylapatite column equilibrated and washed with the same buffer. The column effluent containing Lbs was concentrated and dialyzed against 20 mM Tris-HCl (pH 9.2). Lb samples were subsequently oxidized with an excess of ferricyanide (Appleby et al., 1975) and chromatographed in a Sephadex G-75 column with 20 mM Tris-HCl (pH 9.2) containing 0.6 M NaCl. Fractions containing Lbs were concentrated, dialyzed against 20 mM Tris-HCl (pH 8.0), and applied to a DEAE-cellulose (Whatman DE-52) column. This column was developed isocratically with 20 mM Tris-HCl (pH 8.0). Two major Lb fractions, Lb*a* and Lb*am*s, were collected. Each of these fractions was further purified as Lb^{3+} nicotinate complexes by preparative IEF gels containing 2% of pH 4 to 6 ampholytes (Jun, 1993). The catholyte was 0.1 M β-Ala, and the anolyte was 0.1 M acetic acid. The Lb*am* fraction from the DEAE-cellulose column was resolved into three major proteins, namely Lbam_1, Lbam_2, and Lbam_3. The resolved proteins were eluted from macerated gel pieces with 20 mM Tris-HCl (pH 9.2). Pooled fractions were concentrated, oxidized with an excess of ferricyanide (Appleby et al., 1975), and passed over a PD-10 column (Pharmacia Fine Chemicals) equilibrated and developed with 20 mM Tris-HCl (pH 9.2). Recovered Lb^{3+} samples were concentrated and buffer exchanged into 50 mM K-phosphate buffer (pH 7.0) using a PD-10 column. Lbs were stored in a concentrated form (1–4 mM) at −20°C. All samples were concentrated using YM-10 Centricon concentrators (Amicon Corp.).

The concentrations of both Lb*a* and Lb*am*s possessing a single band in SDS-PAGE were determined by using the molar absorptivity of Lb*a* at 280 nm (29 cm^{-1} mM^{-1}) (Appleby et al., 1975).

Spectroscopic Methods

Spectra of purified proteins were performed in 50 mM K-phosphate buffer (pH 7.0). Visible spectra were collected using a Hitachi U-2000 spectrophotometer interfaced to a microcomputer or on a Cary 2200 spectrophotometer.

Lb^{3+} samples were either reduced with a few crystals of dithionite (Becana and Klucas, 1990) or with a mixture of riboflavin and NADH (Becana et al., 1991). Reduced Lb samples were mixed with O_2-saturated or CO-saturated buffers to obtain these liganded forms. Pyridine-hemochromogen spectra were obtained as described earlier (Riggs, 1981).

Protein Analyses

Apo-Lbs were generated by extraction of acidified protein samples with 2-butanone (Ascoli et al., 1981). To avoid protein precipitation, apo-Lb samples were neutralized immediately to pH 7.0 by the addition of a 50-μL aliquot of 1 M K-phosphate (pH 7.0), followed by dialysis against 50 mM K-phosphate (pH 7.0) to remove all traces of 2-butanone. Concentrated apo-Lb samples were used for the determination of their Tyr/Trp ratios (Edelhoch, 1967).

Trypsin Digestion

N-tosyl-L-Phe chloromethyl ketone-treated trypsin (Sigma) was dissolved in 0.5 mM HCl. An 8-μg aliquot of trypsin was added to 400 μL of Lb sample (1 $\mu g/\mu L$) in 20 mM Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$. After an initial incubation for 2 h at 37°C, an additional 8-μg aliquot of trypsin was added and digestion was allowed to proceed for an additional 3 h. Enzymic reactions were terminated by the addition of 8 μL of TFA.

Released peptides were analyzed by HPLC (Sarath et al., 1986). Peptides were resolved on a 3.9- × 150-mm C_{18}-μBondapak column (Waters Associates) at room temperature using gradients with acetonitrile. Multiple injections of the same samples were performed using an automated sample injection (WISP 715, Waters Associates), and the column eluate was analyzed at A_{215} and subsequently at A_{290}. Two peptides with high A_{290} were further analyzed by automated Edman degradation on a Millipore 6600 protein sequencer using protocols supplied by the manufacturer.

RESULTS AND DISCUSSION

Lb^{3+} Spectra

The absorption spectra for the Lba^{3+} and $Lbam^{3+}$s are shown in Figure 1. Several features characteristic of the $Lbam^{3+}$ were readily apparent. The charge transfer bands in the Lb^{3+} state were shifted between 13 and 20 nm into the blue region for the Lb*am*s. In addition, the α band was pronounced between 551 and 555 nm, and the β band at 530 nm was absent. The $Lbam^{3+}$s showed lower absorptivities in their Søret bands compared to Lba^{3+}. The nicotinate complexes of $Lbam^{3+}$s exhibited significantly different α and β bands. Both bands were shifted bathochromically over 20 nm in the α band and over 14 nm in the β band. $Lbam_1{}^{3+}$ and $Lbam_3{}^{3+}$ exhibited a 4-nm red shift in the Søret region compared to the spectra observed with Lba^{3+}. These spectral differences could arise from changes to either the globin component or to the heme. The fact that nicotinate would bind to Lb*am*s shows that the sixth coordination site on the iron is accessible to the solvent and, therefore, to ligands. However, the spectral shifts and the anomalous spectra of $Lbam^{3+}$ indicate that some changes to the heme or heme environment had taken place that did not involve a new ligand.

Lb^{2+} Spectra and Ligand-Binding Properties

Spectra of the $Lbam^{2+}$ and $Lbam^{2+}$-nicotinate were very similar to those of Lb*a* (Fig. 2), indicating that the heme in the Lb*am* molecules could undergo reduction and binding of nicotinate in a manner similar to those of Lb*a*. Analyses of $Lbam^{2+}$s in the presence of either O_2 or CO did not reveal the characteristic appearance of strong absorption bands at 574 and 540 nm of the $Lba^{2+}O_2$ complex or the 562- and 538-nm bands of the $Lba^{2+}CO$ complex (Fig. 3). Instead, the three Lb*am*s displayed a strong charge transfer band at approximately 618 to 625 nm and minor bands centered at approximately 570 and 535 nm but failed to exhibit a clearly defined peak in the Søret region. Earlier data that indicated

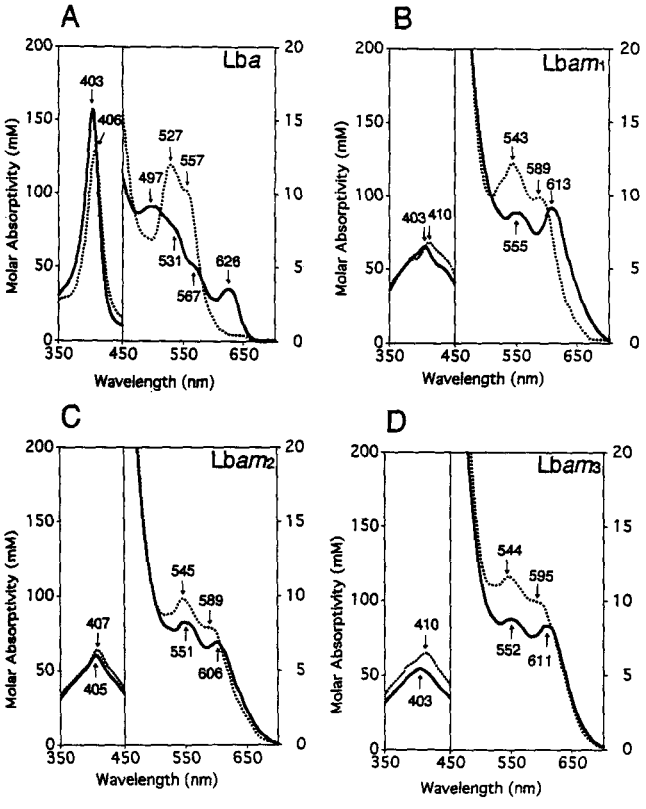

Figure 1. Visible absorption spectra of Lb^{3+} and Lb^{3+} nicotinate complexes for Lba and Lbams. Samples purified from preparative IEF gels were oxidized with a 4-fold excess of ferricyanide, desalted by gel filtration at pH 9.2, concentrated, and buffer exchanged in 50 mM K-phosphate (pH 7.0). Nicotinate complexes were formed by incubating Lb^{3+} samples with a final nicotinate concentration of 0.5 mM. Samples were scanned from 350 to 450 nm (8 μM Lb in 500 μL total volume) and from 450 to 700 nm (50 μM Lb in 500 μL total volume) with a Hitachi U-2000 spectrophotometer. The spectra were obtained after 20 min of equilibration at 22°C. Solid line, Lb^{3+}; dotted line, Lb^{3+} nicotinate; A, Lba; B, $Lbam_1$; C, $Lbam_2$; D, $Lbam_3$.

CO binding with an Lbam fraction (Wagner and Sarath, 1987) can probably be ascribed to the presence of Lba in those Lbam samples. It is also possible that Lbams isolated from senescent root nodules might have slightly different properties.

The apparent aberrant binding of Lbams with O_2 could have important effects on nodule metabolism. If such proteins become predominant, and as our data suggest have impaired capacity to reversibly bind O_2, the flux of O_2 to the bacteroids could decrease, which is in accord with studies showing an increase in the amounts of Lbams with the onset of stress (Wagner and Sarath, 1987).

Protein Structure Studies

The apparent changes in the binding of O_2 and CO suggested that a subtle modification of Lba had taken place to form the Lbams. To evaluate the causes for this change, a detailed examination of the globin backbone was undertaken. Lehtovaara (1978) had suggested that a specific Tyr residue near the COOH terminus in bean Lb appeared to be essential in protecting the heme from hydroxylation reactions. The loss of this Tyr appeared to be correlated with the formation of green pigments under alkaline conditions in the presence of ambient O_2. However, incubation of soybean Lba under similar conditions did not result in the appearance of green pigments (Lehtovaara, 1978). Spectrally, the green pigments obtained from alkaline oxidation of bean Lb were quite different from those reported in this study. Soybean Lbs, however, contain a conserved Tyr^{133} residue near the COOH terminus, which may participate in oxidation reactions (Davies and Puppo, 1992). Also, this residue interacts with the heme (Ollis et al., 1983). Oxidation of Tyr^{133} could lead to perturbation of the heme pocket with resultant changes in ligand binding. Furthermore, any chemical changes in the Tyr ring structure would be expected to affect its absorption in the UV and could thus be detected in the apo-protein. Lba contains two Trp and three Tyr residues (Hyldig-Nielsen et al., 1982), and, therefore, the expected ratio of Tyr to Trp is 1.5. A spectroscopic investigation of the Tyr to Trp ratios in apo-Lba and apo-Lbams revealed no differences for any of the samples analyzed (Table I). The lack of differences would indicate that there was no chemical change to these residues,

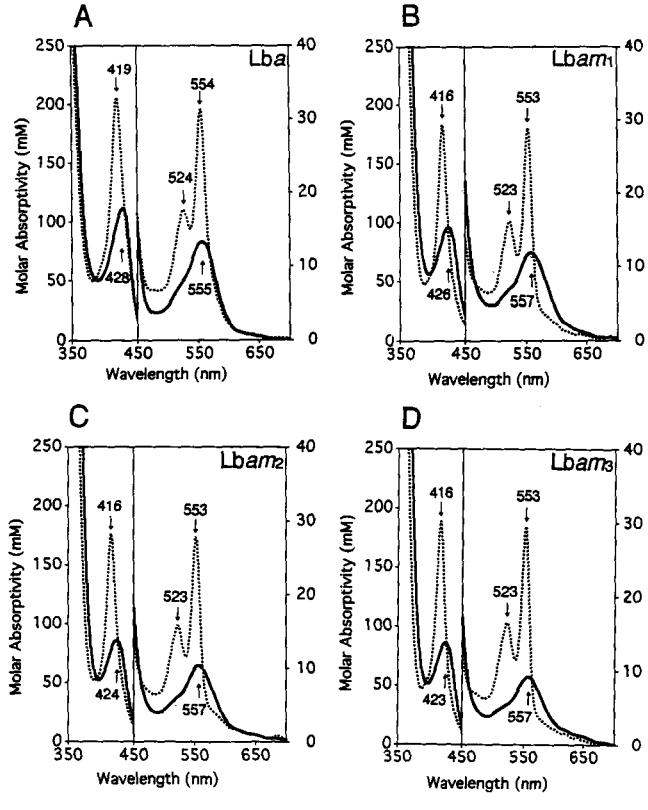

Figure 2. Visible absorption spectra of Lb^{2+} and Lb^{2+} nicotinate complexes for Lba and Lbams. Conditions of analyses were as described in Figure 1, except Lb samples were reduced as described in "Materials and Methods." Lb^{2+} nicotinate complexes were formed by the addition of 0.5 mM (final concentration) of nicotinate to the reduced Lb samples.

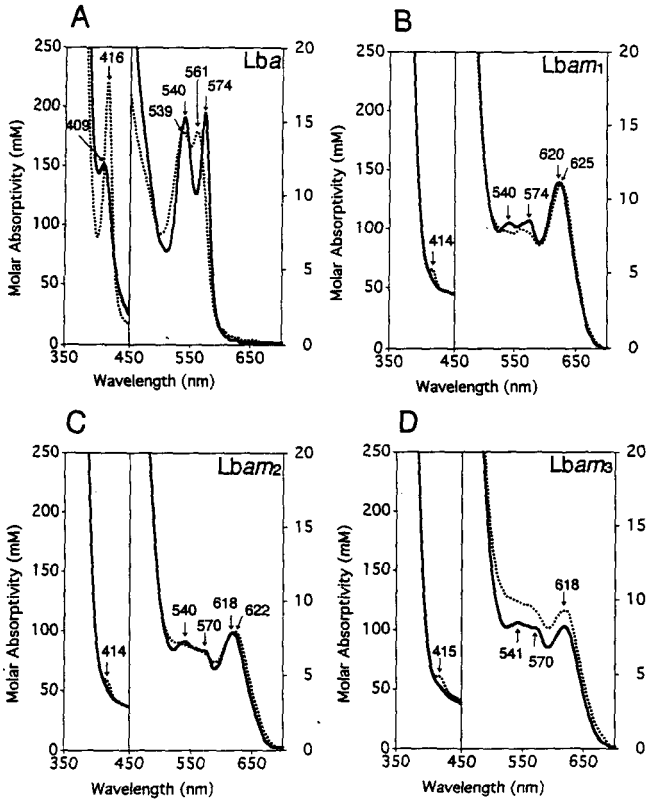

Figure 3. Visible absorption spectra for $Lb^{2+}O_2$ and $Lb^{2+}CO$ complexes for Lba and Lbams. Conditions were as described in Figures 1 and 2, except reduced Lb samples were incubated in O_2- or CO-saturated buffer at 22°C for 20 min.

especially to Tyr. These measurements are based on differences in the extinction coefficients as a function of pH (Edelhoch, 1967), and it is conceivable that oxidation of Tyr could have been reversed during sample handling. Attempts to chemically acylate accessible Tyr residues on Lb*a* and Lb*ams* with a variety of reagents (Riordan et al., 1965; Cuatrecasas et al., 1968) did not yield useful data. Such chemical reactions were not sufficiently specific and resulted in a progressive denaturation of the intact proteins (data not shown).

In addition to Tyr^{133}, Leu^{134} and Ile^{139} near the COOH terminus also interact with the heme of soybean Lb (Ollis et al., 1983); these residues form a hydrophobic end to the heme pocket. The results of earlier studies had indicated that there was no apparent change to the NH_2 terminus of the Lb*am* proteins (Jun, 1993) and that proteolysis might be involved in the conversion of Lb*a* to Lb*am* (Wagner and Sarath, 1987). Thus, proteolytic cleavage of residues from the COOH terminus might result in changing the environment of the heme pocket with resultant effects on ligand binding. To determine fully the nature of any of such modifications, IEF-purified Lbs were subjected to trypsin digestion. Peptide profiles resulting from tryptic digestion are shown in Figure 4. The overall profiles and the relative ratios of the peaks were generally similar for all of the proteins analyzed. To determine the residues near the COOH terminus, peptide peaks exhibiting high A_{290} were collected and subjected to automated Edman degradation. The results of this analysis identified the COOH terminal fragments from residues 121 to 127 (peptide labeled B in Fig. 4) and from residues 128 to 141 (peptide labeled A in Fig. 4). Conceivably, deamidation of Asn and Gln residues in Lb*a* could result in changing the isoelectric point of such modified molecules (Jun, 1993). In addition, such changes might have an effect on the physical and chemical characteristics of the deamidated proteins. However, the similar peptide profiles of Lb*ams* and Lb*a* observed upon tryptic digestion (Fig. 4) do not support such a change.

Overall, these results ruled out any gross modification of the protein backbone in the Lb*am* molecules and suggested that there might be changes to the heme.

Pyridine Hemochromogen Spectra

Pyridine hemochromogen spectra of the heme from Lb*a* and Lb*ams* provided strong evidence for changes to the heme (Fig. 5). As expected, Lb*a* hemochromogen spectra were

Table I. *Spectrophotometric determination of the ratio of Tyr to Trp in lysozyme, sperm whale apomyoglobin, and apoleghemoglobin*

Apoproteins were prepared by 2-butanone method (Ascoli et al., 1981) and dissolved in 20 mM K-phosphate buffer (pH 7.0) containing 6 M guanidine chloride at a concentration of 20 mM. The pH 12.0 was obtained by adding 5 μL of 10 N KOH to the neutral apoprotein solutions. The ratio of Tyr to Trp for these samples was determined using the spectrophotometric procedure of Edelhoch (1967).

	$A_{280,pH7}$	$A_{300,pH7}$	$A_{300,pH12}$	Ratio of Tyr to Trp	
				Calculated	Expected
Lysozyme[a]	0.699	0.149	0.276	0.51	0.5
SWMb[b]	0.320	0.097	0.234	1.42	1.5
Lba[c]	0.316	0.109	0.247	1.45	1.5
$Lbam_1$	0.321	0.097	0.237	1.45	
$Lbam_2$	0.296	0.105	0.234	1.45	
$Lbam_3$	0.299	0.123	0.252	1.44	

[a] Lysozyme from egg white contains three Tyr and six Trp. [b] SWMb, sperm whale myoglobin, contains three Tyr and two Trp. [c] Soybean Lba contains three Tyr and two Trp.

identical with those of free hemin with absorption peaks at 556 and 525 nm and with a small peak at 480 nm. Spectra obtained from Lb*am* samples also had absorption peaks at 556 and 525 nm that were of reduced amplitude, none at 480 nm, and a new peak at 580 nm. These spectral differences suggested that the heme in Lb*am*s was modified in some way. However, the presence of apparent α and β bands at 556 and 525 nm, respectively, in the Lb*am*s indicated that the macroporphyrin ring was intact, and the native spectra indicated that the heme contained iron. Major changes to the porphyrin ring would result in the release of iron as well as a failure to detect the α and β bands at 556 and 525 nm, respectively. Degradation products of heme, such as biliverdin, exhibit a linear pyrrole ring structure and show a broad absorption spectra between 400 and 750 nm with a peak centered at 665 nm (Heirwegh et al., 1991). In animal systems, heme degradation is known to occur by hydroxylation of the methene bridge (Schmid and McDonagh, 1975) with further catabolism of the free heme catalyzed by the enzyme heme oxygenase (Brown et al., 1990). Heme destruction can also be initiated by reductants such as ascorbate in the presence of O_2 (Buldain et al., 1986), by thiol compounds that result in the formation of sulfur-heme adducts (Luiz-Dafré and Reischl, 1990), or by H_2O_2 under physiological conditions (Prasad et al., 1989). Root nodules contain an abundance of ascorbate and reduced thiols such as GSH (Dalton et al., 1991). Ascorbate in the presence of O_2 will convert bean Lbs into inactive forms; however, this reaction requires nonphysiological conditions (Lehtovaara, 1978). The pH of functional root nodules is near neutral and becomes more acidic during senescence (Klucas, 1974; Pladys et al., 1988), suggesting that heme destruction might involve other mechanisms, such as reactions with the hydroxyl radical that is produced during stress (Becana and Klucas, 1992). Our data suggest that functional Lbs can be converted in vivo into proteins exhibiting aberrant binding of O_2; however, the nature of this change is unknown.

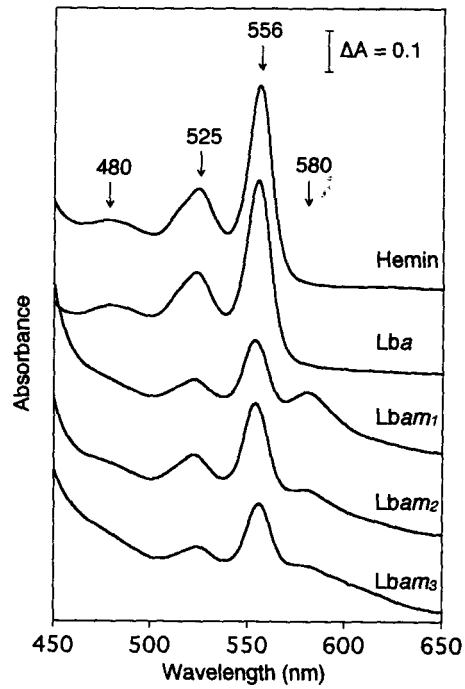

Figure 5. Pyridine hemochromogen spectra of Lb samples. Lb samples (1 nmol) or hemin (0.1 mL) were prepared in 0.3 mL of alkaline pyridine solution composed of 33.3% (v/v) pyridine and 0.1 N NaOH. The sample mixtures (0.4 mL) were reduced with an excess of solid sodium dithionite (a few crystals). Numbers on the graphs indicate wavelength of the peaks.

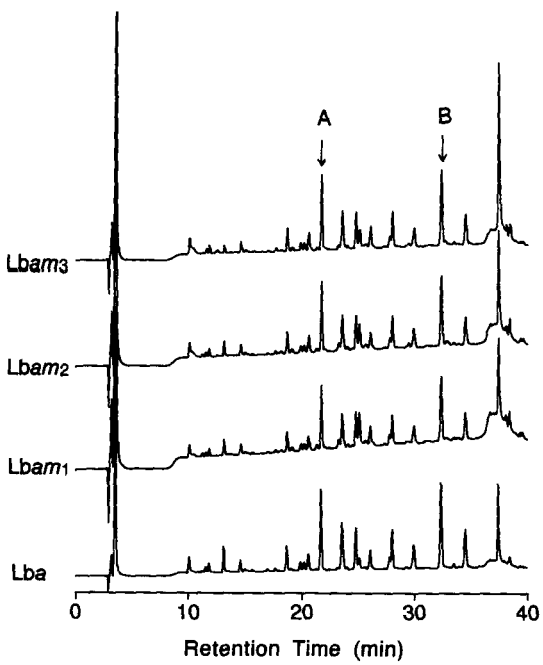

Figure 4. Reversed phase HPLC chromatograms of trypsin-digested Lb samples. Lb samples (0.4 mg) were digested initially with an 8-μg aliquot of *N*-tosyl-L-Phe chloromethyl ketone-treated trypsin in 0.4 mL of 20 mM Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$ for 3 h at 37°C, followed by a second 8-μg aliquot of trypsin for additional 2 h. The digestion mixtures were separated by HPLC on a C_{18}-μBondapak column (250 × 4.6 mm), and the eluate was monitored at 215 nm. Peaks labeled A and B were further subjected to automatic Edman degradation. Conditions: solvent A, 0.1% (v/v) TFA; solvent B, 90% (v/v) acetonitrile in 0.1% (v/v) TFA; flow rate, 1 mL min^{-1}. The gradient parameters were 0 to 30 min, 0 to 40% solvent B in a linear gradient; 30 to 35 min, 40 to 100% in a linear gradient; 35 to 40 min, 100% isocratic; 40 to 45 min, 100 to 0% in a linear gradient.

Lb*am*s appear to have sustained some form of modification to their heme. These hemoproteins do not bind O_2 with characteristics similar to Lb*a* and may be functionally impaired. Since these molecules have been shown to become predominant during aging or stress-induced senescence of soybean root nodules, it would appear that Lb*am*s are generated in vivo from Lb*a*. Thus, Lb*am*s could represent an early degradation product of Lb*a* and might be used as a marker for the physiological status of the root nodule.

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