ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 254, No. 1, April, pp. 263-271, 1987

Sesbania rostrata Root and Stem Nodule Leghemoglobins: Purification, and Relationships among the Seven Major Components

DIDIER BOGUSZ,*†[1] ALEXANDER A. KORTT,‡ AND CYRIL A. APPLEBY*

*CSIRO Division of Plant Industry, Canberra, Australia 2601, †ORSTOM Department F, 213 rue La Fayette, 75480 Paris, France, and ‡CSIRO Division of Protein Chemistry, Parkville, Australia 3052

Received September 9, 1986, and in revised form December 16, 1986

By anion-exchange chromatography, the nitrogen fixing photosynthetic stem nodules and nonphotosynthetic root nodules of *Sesbania rostrata* are shown to contain the same seven major components of leghemoglobin (Lb), numbered LbI–LbVII in order of elution, although in different proportions. No novel component was found in photosynthetic nodules. All components of *Sesbania* Lb are monomeric, with molecular weights varying between 15,000 and 17,000, and at least six of them are separate gene products. It is suspected that variable conjugation with nonprotein moieties might be partially responsible for the molecular weight differences and anomalous behavior observed between isoelectric focusing and anion-exchange chromatography.

Leghemoglobin (Lb)[2] is the oxygen binding protein found in nitrogen fixing root nodules developed by legume plants in symbiotic association with *Rhizobium* bacteria (1). Lb facilitates oxygen flow to the respiring, nitrogen fixing bacteria at a stabilized free oxygen concentration too low to damage the bacterial nitrogenase enzymes (1, 2). These nitrogen fixing nodules are generally found only on plant roots. However, a few legumes such as *Sesbania rostrata* (3) and *Aeschynomene* species (4, 5) are characterized by profuse stem nodulation as well. In contrast with root nodules, the stem nodules harbor chloroplasts in their cortex (3–5), with consequent potential for oxygen evolution adjacent to the *Rhizobium* domain. So, oxygen control might be more demanding and more complex in stem nodules, and preliminary reports have described the apparent presence of an extra Lb component, or very different proportions of the same components, in *Sesbania* (6) and *Aeschynomene* (4) stem nodules compared with root nodules. Apart from the possibility that these "extra" Lb components might have different oxygenation properties from root Lb, this apparent tissue-specific expression of Lb genes has generated interest among molecular biologists. However, it cannot be assumed that every Lb component isolated from nodule extracts represents a separate gene product. Of the eight Lb components separable from soybean root nodule extracts by isoelectric focusing (7), four represent gene products (8) and four arise by post-translational modification (in this instance, N-terminal acetylation) (9). Also, spurious species may be seen (4, 7) unless Lb mixtures are maintained as a single valence state and ligand complex during purification. Accordingly, we have reinvestigated the separation of *Sesbania* Lb components by anion-exchange chromatography and isoelectric focusing. We gave detailed at-

[1] The contribution of D.B. to this work was partly supported by Grant TSD-081-SC(MR) from the European Economic Community to ORSTOM.

[2] Abbreviations used: Lb, leghemoglobin; *Sesbania* LbI–VII, the seven major components of Lb isolated from *Sesbania rostrata* root or stem nodules, numbered in order of elution from DEAE-Sephacel (Pharmacia) anion-exchange columns; SDS, sodium dodecyl sulfate.

0003-9861/87 $3.00

tention to the recognition and removal of artifacts or naturally modified species, which might otherwise have given a false impression of extra complexity to either stem or root nodule Lb.

MATERIALS AND METHODS

Nodule growth. Sesbania rostrata plants were grown during the rainy season in a sandy soil (10) at the ORSTOM Bel Air Station in Dakar, Senegal. For root nodule production the soil was inoculated at sowing with the specific *Rhizobium* strain ORS571. For stem nodule production stems were inoculated by spraying with *Rhizobium* ORS571 45 days after sowing. Nodules were harvested when nitrogen fixing activity was highest (30 days from sowing for root nodules; 20 days from inoculation for stem nodules).

Purification of total ferric Lb. All steps were carried out at 4°C under identical conditions for root and stem nodules, and we describe a typical experiment with stem nodules. These were macerated in a Waring blender with 0.1 M sodium phosphate, pH 6.8 (3 ml buffer/g nodule fresh wt), containing insoluble Sigma polyvinylpolypyrrolidone (0.3 g/g nodule fresh wt). The homogenate was centrifuged (10,000*g*, 30 min) and the supernatant was fractionated with ammonium sulfate. The pooled 55-80% saturation precipitates from 1065-g nodules were dissolved in and dialyzed overnight against two changes of 3 mM potassium phosphate, pH 7, then centrifuged at 80,000*g* for 10 min. The 98 ml of viscous supernatant was loaded onto a 17 × 5 cm column of DEAE-cellulose (Whatman DE 52) equilibrated with the same 3 mM phosphate buffer (11), and colorless, viscous material was washed through the column with 400 ml of buffer. Then, all Lb was eluted with 50 mM potassium phosphate, pH 7, and concentrated by pressure filtration over an Amicon YM10 membrane to give 27 ml of low viscosity solution. After addition of 3 ml of 1 M sodium acetate, pH 5.2, this solution was dialyzed for 3 h against 10 mM sodium acetate, pH 5.2, then centrifuged (100,000*g*, 45 min) to give a small gray pellet and clear red supernatant. The Lb in this pH 5.2 supernatant was fully oxidized by stirring in 4 M equivalents of powdered potassium ferricyanide (111 mg) and loaded (33 ml of 2.58 mM ferric Lb) onto a Sephadex G-25 medium (Pharmacia) column, 50 × 5 cm, also equilibrated with 10 mM sodium acetate, pH 5.2, and eluted with this buffer. Fractions containing Lb were pooled and concentrated over an Amicon YM10 membrane to give a clear green-brown solution of ferric Lb acetate (58 ml of 1.26 mM).

DEAE-Sephacel chromatography. Twenty-two milliliters of this ferric Lb acetate solution was loaded onto a DEAE-Sephacel (Pharmacia) column, 12.5 × 5 cm, equilibrated with 10 mM sodium acetate, pH 5.2. After washing with 250 ml of buffer the column was developed at 4 ml/min (24-ml fractions) with a linear acetate gradient (10 mM sodium acetate, pH 5.2, 2 liters; 100 mM sodium acetate, pH 5.2, 2 liters), (12). The eluate was monitored using a Uvicord III (LKB) dual-wavelength flow photometer set at 277 and 405 nm, with 0.5-mm optical path cuvettes, and conductivity of selected effluent fractions was measured with a Radiometer CDM2 meter. For small scale DEAE-Sephacel chromatography a 15 × 1.6 cm column was used with proportionate (1/10) volumes of all reagents.

Isoelectric focusing. Preparative scale focusing was performed according to Fuchsman and Appleby (7) in the presence of nicotinate, except that the ampholyte used was 2% Pharmalyte, pH 4.5-5.4 (Pharmacia). After 2 h prefocusing, ferric Lb solution from Sephadex G-25 chromatography (70 mg in 3 ml of 1 mM nicotinic acid, pH 5.2) was added via a sample applicator near the cathode and focusing continued for 15-16 h at 8 W limiting power, with cathode electrode strips (soaked in 0.1 M NaOH, 0.1 M nicotinic acid, pH 5.2) replaced every 3 h. Isoelectric points of the separated components were measured at 2°C as already described (7). Analytical scale focusing was also performed in the presence of nicotinate as in (7) except that 0.5-mm gels were cast in an Ultramold (LKB) following the manufacturer's instructions, using 2% Pharmalyte, pH 4.5-5.4 (Pharmacia) as ampholyte. After 1 h prefocusing, 15- to 20-μl samples containing Lb in 1 mM nicotinic acid, pH 5.2, were applied on paper wicks (usually) near the cathode and focusing was continued for 2 h at 8-15 W constant power, with cathode strips (as above) changed every 30 min.

Molecular weight determination. The separate components of *Sesbania* ferric Lb as eluted from DEAE-Sephacel were converted to LbO_2 by adjustment to pH 7.4 with K_2HPO_4 solution, reduction with dithionite under argon, and passage through small Sephadex G-25 columns (Pharmacia type PD10) equilibrated with air-saturated 50 mM potassium phosphate, pH 7.4. The molecular weights of these native *Sesbania* LbO_2 components were determined by gel filtration of 5-ml samples through a 60.5 × 1.6 cm column of Sephacryl S200 (Pharmacia) in 50 mM potassium phosphate, pH 7.4. The column was calibrated with Blue Dextran (Pharmacia), native soybean Lba, M_r 15,876 (13); native lupin LbII, M_r 17,274 (14), native dimeric *Parasponia* HbI, M_r 38,638 (15), and ovalbumin, M_r 42,000. The molecular weights of *Sesbania* Lb denatured subunits were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in an 18% linear gel. Calibration was with the protein subunits of soybean Lba, M_r 15,242; yellow lupin LbII, M_r 16,640, and *Parasponia* HbI, M_r 18,685.

Protein sequence analysis. Amino terminal sequences of the intact, native Lb components I-VII were determined with a gas phase sequencer (Applied Biosystems) using 1 nmol of protein.

Leghemoglobin concentration and absorption spectra. Concentrations were determined as protoheme by the pyridine hemochrome procedure (16), and spectra were recorded using Hitachi-Perkin-Elmer Model PE557 spectrophotometer.

RESULTS AND DISCUSSION

Purification of total leghemoglobin. The redissolved 55–80% ammonium sulfate saturation fraction from root or stem nodule extracts was extremely viscous, so we applied the Dilworth procedure (11) in which Lb was strongly adsorbed to Whatman DE52 cellulose in 3 mM phosphate, pH 7. Much viscous colorless material was washed through, then total Lb eluted at higher salt strength (50 mM phosphate) as a low viscosity solution. The optical absorption spectrum of this total Lb, with the α peak at 570 nm of much lower intensity than the β peak at 539 nm (not illustrated), suggested that it was a mixture of LbO_2 and ferric Lb (cf. Fig. 3 below). When a sample was reduced with dithionite the spectrum of the product was more complex than that of unligated ferrous Lb, the species expected to be formed from both LbO_2 and ferric Lb. Besides the 429 nm shoulder and 556 nm peak characteristic of ferrous Lb, we observed an extremely sharp 417 nm peak and 540 nm shoulder, reminiscent of ferrous LbNO (17). This tentative assignment was confirmed by adding a trace of sodium nitrite to the reaction cuvette. In the presence of dithionite, nitric oxide was generated and a substantial further conversion of ferrous Lb to LbNO was observed.

For the Lb eluted from this DE52 cellulose column the usual procedure of Lb oxidation by ferricyanide at pH 9.2 (12) proved to be slow and incomplete, with ferrous LbNO a particularly persistent complex. Fortunately, ferricyanide oxidation in 10 mM acetate, pH 5.2, was rapid (<5 min at 0°C) and the product after Sephadex G-25 chromatography at this pH was exclusively ferric Lb acetate (cf. Ref. (18)). The optical spectrum of this product, after reduction with dithionite, revealed only unligated ferrous Lb (cf. Fig. 3). Hence, the ferric Lb from Sephadex G-25 was judged to be free of contaminating ligands, and suitable for the separation of components by anion-exchange chromatography or isoelectric focusing.

DEAE-Sephacel chromatography. Elution of *Sesbania* root or stem Lb by a linear

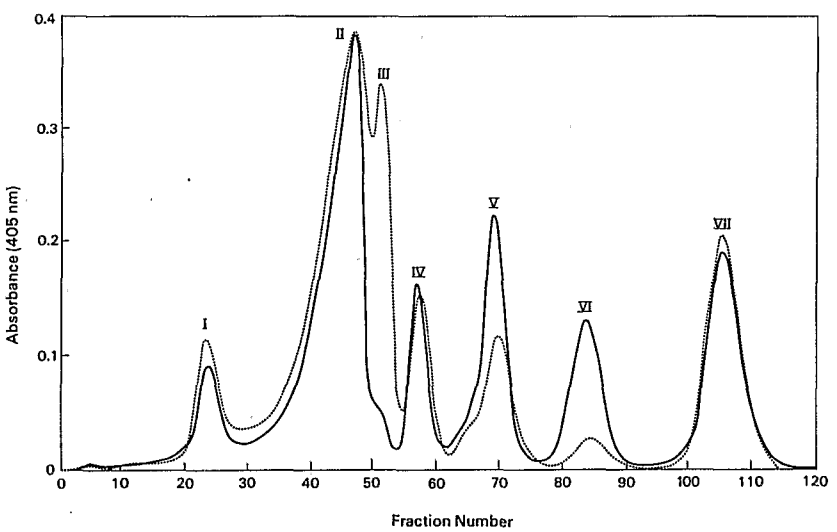

FIG. 1. DEAE-Sephacel elution profiles (405 nm) of *Sesbania* ferric Lb acetate from root (---) and stem (—) nodules, using a linear gradient of sodium acetate, pH 5.2 (see Materials and Methods). The gradient front emerged at fraction 25; at fraction 120 when all Lb components (numbered I–VII) had been eluted the salt strength was ~50 mM acetate.

acetate gradient gave the same seven components of ferric Lb acetate (no other ligand complex was detected) as judged from the identical positions of peaks I–VII in elution profiles (Fig. 1) and near-identical conductivities of peak eluant fractions (Table I). The resolution of LbII and LbIII was somewhat unsatisfactory, but these two components could be completely separated by not starting the salt gradient (see Materials and Methods) until all LbII had emerged. A disadvantage of this alternative procedure is that LbI and LbII are eluted as dilute solutions.

Although unable to repeat the earlier observation of an Lb component *exclusively* present in photosynthetic stem nodules (6), we did find (Table I) that LbVI was much more abundant in stem nodules, LbV a little more abundant and LbIII less abundant. Fuchsman and Appleby (7) had earlier shown the proportions of soybean Lb to be dependent on nodule age so we had been careful to use, in this study, mature (as judged by maximum nitrogenase activity) root and stem nodules. Unfortunately this meant that plant ages were, respectively, 30 and 65 days when root and stem nodules were harvested. Hence, it is possible that plant age or physiological status might have been responsible for the observed difference in proportions of LbIII, LbV, and LbVI between root and stem nodules (Table I) and for the previously reported absence of one component from *Sesbania* root nodules (6).

TABLE I

PERCENT COMPOSITION AND CONDUCTIVITY OF *Sesbania* Lb COMPONENTS FROM ROOT AND STEM NODULES, AS ELUTED FROM DEAE-SEPHACEL

Leghemoglobin component	% Composition[a]		Conductivity (mmho at 2°C)	
	Root	Stem	Root	Stem
I	6.3	5.3	476	511
II	47.4	40.4	920	1016
III	11.3	2.6	1031	1111
IV	7.4	8.3	1162	1262
V	9.3	14.8	1413	1444
VI	2.1	10.4	1714	1765
VII	16.1	18.1	2111	2103

[a] The total yield (23.4 nmol) of root Lb represented 84% of the amount applied to the DEAE-Sephacel column and the total yield of stem Lb (23 nmol) represented 83% of the amount applied.

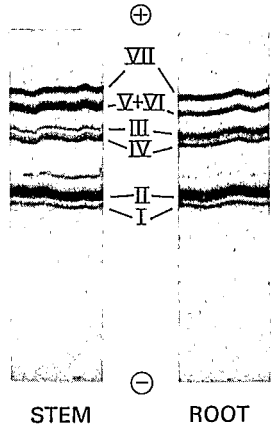

FIG. 2. Preparative scale isoelectric focusing patterns of *Sesbania* ferric Lb nicotinate from root and stem nodules in gel slurries containing 2% Pharmalyte, pH 4.5–5.4 (Pharmacia). The component numbering is explained in Table II.

Isoelectric focusing. From soybean nodule extracts, preparative scale focusing (7) of total ferric Lb as the very stable nicotinic acid complex (19) had offered significant advantages over anion-exchange chromatography (12). Better resolution of components had been achieved, and the procedure could be directly adapted to an analytical scale suitable for plant age studies. Unfortunately, the preparative scale focusing (Fig. 2) or analytical scale focusing (not illustrated) of *Sesbania* root and stem ferric Lb nicotinate proved unsatisfactory. Although there were obvious differences in proportions of Lb components between root and stem, only six major bands were seen instead of the seven separable by anion exchange (Fig. 1). After the rerunning of components eluted from DEAE-Sephacel by analytical scale focusing, and of bands eluted from preparative scale focusing gels on small DEAE-Sephacel columns, we established the relationship shown in Table II. The components separable as LbV and LbVI by anion exchange on DEAE-Sephacel remained merged during focusing, and

TABLE II

RELATIONSHIP AMONG *Sesbania* Lb COMPONENTS

Order of elution from DEAE-Sephacel anion-exchange columns	Isoelectric focusing position[a]	Isoelectric point[b]
I ⟷	I	5.08
II ⟷	II	5.04
III ⟷	IV	4.84
IV ⟷	III	4.81
V ⟷	V and VI	4.70
VI ⟷		
VII ⟷	VII	4.61

[a] Component I is positioned closest to the cathode.
[b] Measured as ferric Lb nicotinate at 2°C, as eluted in water from preparative scale focusing gels. The corresponding Lb components from root and stem nodules had exactly the same isoelectric points.

the anion-exchange components LbIII and LbIV reversed their positions during focusing. A satellite band, often observed close to LbII after isoelectric focusing of nodule extracts (e.g., Fig. 2) or of LbII from DEAE-Sephacel columns, remained merged with LbII or appeared as a shoulder during DEAE-Sephacel chromatography. This minor component, tentatively designated LbIIa, is demonstrably different from LbIII (cf. Figs. 1 and 2). Also, a satellite band of LbIV, tentatively designated LbIVa, could be seen clearly after focusing (Fig. 2) but not after DEAE-Sephacel chromatography (Fig. 1). Our cyclic rerunning of all Lb components, and especially of LbIII-VI, established that none were generated or destroyed during either separation procedure. Because of the lesser discrimination of major components during focusing, we elected to number all components (Figs. 1 and 2; Table II) according to their elution order from DEAE-Sephacel.

The six *Sesbania* ferric Lb nicotinate components separable by focusing from root and stem nodules had, respectively, the same isoelectric points (Table II). This reinforces our conclusion from anion-exchange chromatography (Fig. 1; Table I) that *Sesbania* root and stem nodule Lb compositions are qualitatively similar.

Optical spectra. The uv and visible absorption spectra of *Sesbania* LbII (Figs. 3A and 3B) and of the other components (not illustrated) were not demonstrably different from each other and closely resembled those of soybean Lb (20) and of the nonleguminous hemoglobins from *Parasponia* (21) and *Casuarina* (22). Hence there is no reason to suppose, a priori, that *Sesbania* Lb might differ in oxygen transporting properties from other plant hemoglobins, nor one component of *Sesbania* Lb from another.

Amino terminal amino acid sequences of Sesbania Lb components I-VII. A common cause of heterogeneity within isolated proteins, including Lb (9), is post-translational modification or conjugation. Nevertheless, sequencer analysis of the amino terminal region of each component of *Sesbania* Lb showed that none had blocked amino terminals and that at least six of the seven represented different polypeptide chains (Table III). Hence, they are separate gene products. Only LbVI and LbVII were the same up to residue 25; it is possible that complete analysis will reveal sequence differences between these two, also. Evidence of microheterogeneity was revealed for LbIII, with Glu and Asp present in approximately equal amount at residue position 23, and for LbIV, where ~10-20% of protein molecules had an extra Gly at the amino terminal (Table III).

Molecular weights of Sesbania Lb components. Electrophoresis on SDS-polyacrylamide gels (Fig. 4) produced a single major band, and sometimes minor bands, from each of the seven *Sesbania* Lb components which had been isolated by DEAE-Sephacel chromatography then further purified by elution as symmetrical bands of presumed homogeneous components from Sephacryl S200 columns. The molecular weights of these SDS-denatured, heme-free *Sesbania* Lb major components were estimated to lie between 16,000 and 16,800 (Fig. 4; Table IV). By Sephacryl S200 chromatography, molecular weights of the

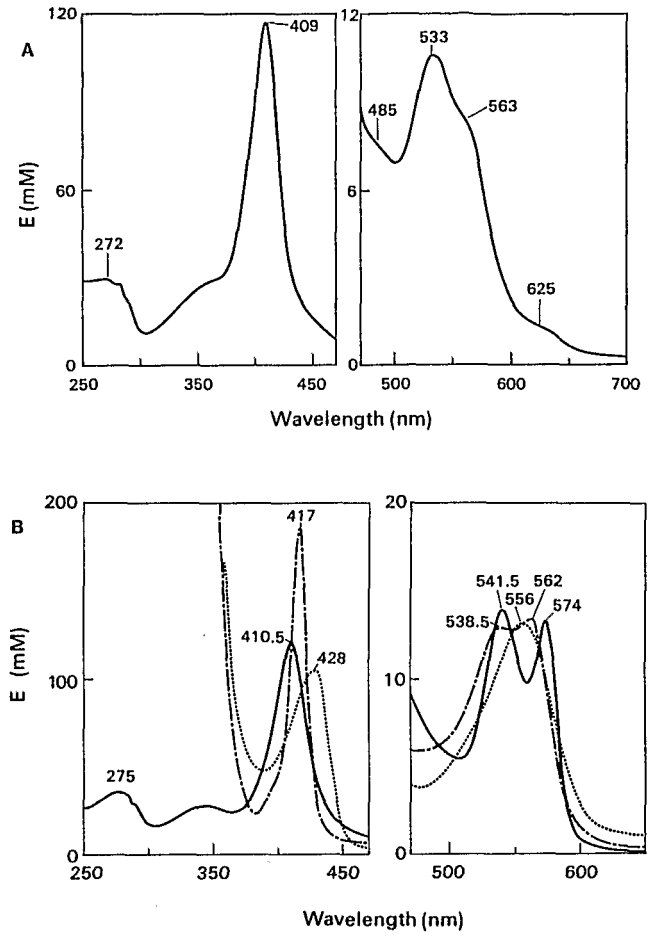

FIG. 3. Optical absorption spectra of *Sesbania* total Lb (A) in the ferric form and (B) as deoxyferrous Lb (---), LbO_2 (—), and LbCO (-·-·-). All spectra were recorded at 20°C in 0.1 M potassium phosphate, pH 6.8, on samples prepared as described elsewhere (21). E (mM) is the millimolar extinction coefficient.

native, heme-containing *Sesbania* LbO_2 components had been estimated to lie between 14,800 and 16,600 (Table IV). While these results clearly showed all native *Sesbania* Lb components to be monomers, they revealed a greater apparent molecular weight diversity than generally observed among the components of other leghemoglobins. For instance, the calculated molecular weights of native soybean Lb components vary only from 15,876 (Lba) to 16,084 (Lbc_3) (13, 23).

It may be that this degree of molecular weight diversity among the major components of *Sesbania* Lb simply reflects their origins as separate gene products (cf. Table III) and that the microheterogeneity apparent within certain components (Fig. 4) reflects the inability of our DEAE-Sephacel purification procedure (Fig. 1; cf. Fig. 2) to separate closely related gene products. Another reason for the large variation in apparent molecular weight among the components of *Sesbania* Lb, between the native and denatured proteins (Table IV), and within separated components (Fig. 4), might be that some or all of these components are conjugated proteins. It is known, for instance, that glycoproteins often behave anomalously on SDS-polyacrylamide gels (24). Increasing glycosylation, by lowering the amount of SDS bound and so de-

TABLE III

AMINO TERMINAL AMINO ACID SEQUENCES OF *Sesbania* Lb COMPONENTS[a]

	5					10					15					20					25				
LbI	G	F	T	D	K	Q	E	A	L	V	N	A	S	Y	E	A	F	K	(K)	N	L	P	G	N	S	V	L	F	Y
LbII	G	F	T	D	K	Q	E	A	L	V	N	A	S	Y	E	A	F	K	K	N	L	P	G	Ⓗ	S	V	L		
LbIII	G	F	T	Ⓔ	K	Q	E	A	L	V	N	A	S	Y	E	A	F	K	Ⓚ	N	L	Ⓢ	Ⓟ	Ⓛ	Ⓝ				
LbIV	Ⓖ	Ⓐ	F	T	Ⓔ	Ⓢ	Q	E	A	L	V	Ⓣ	Ⓢ	S	Y	E	Ⓣ	F	K	Ⓚ	N	L	Ⓐ	( )	Ⓛ	S	V	L		
LbV		G	F	T	D	K	Q	E	A	L	V	N	A	S	Y	E	A	F	K	Ⓘ	N	L	P	G	Ⓢ					
LbVI		G	F	T	Ⓔ	K	Q	E	A	L	V	N	A	S	Y	E	A	F	K	Ⓚ	N	L	P	G	N	S	V	L	F	
LbVII		G	F	T	Ⓔ	K	Q	E	A	L	V	N	A	S	Y	E	A	F	K	Ⓚ	N	L	P	G	(N)	S				

[a] Residues differing from those in LbI are circled; doubtful or unidentified residues are in parentheses.

creasing the charge–mass ratio, causes slower running on electrophoresis gels and so indicates falsely high molecular weights. We are investigating the possibility that *Sesbania* Lb is a family of glycoproteins.

Anomalous behavior between isoelectric focusing and anion-exchange chromatography. We were able to eliminate, quite simply, several of the possible causes for the merging or position reversal of Lb components apparent between isoelectric focusing and anion exchange (Table II). First, the differences were not due to ferric Lb nicotinate being the focused species and ferric Lb acetate being the chromatographed species. When DEAE-Sephacel chromatography was repeated (see above) with 1 mM nicotinate added to root and stem Lb samples and buffers, thereby converting all Lb components to ferric Lb nicotinate, the elution order of components remained as in Fig. 1. The only difference was a shift in position of root and stem LbII closer to LbI (incidentally this provided a second alternative procedure for the clean separation of LbII and LbIII). Second, we were able to discard the possibility that specific ampholyte combination with specific Lb component(s) might have caused isoelectric point distortion. In analytical focusing runs, where wicks containing stem or root Lb samples were placed in various positions between anode and cathode, thereby exposing the migrating Lb to different components of the ampholyte mixture, invariably the same patterns were obtained as with preparative scale focusing (Fig. 2). Third, after demonstrating their monomeric nature (Table IV) we were able to discard the possibility that natural polymer formation by certain *Sesbania* Lb components might have caused the observed position anomalies.

Sesbania LbIV, the component with the highest apparent molecular weight (Table IV) is also the component which most changes its behavior between anion exchange and focusing. If LbIV were also the most conjugated component (see above) then such conjugation could, from a combination of charge masking, conformational change and steric effects sensed differently by anion exchange and focusing,

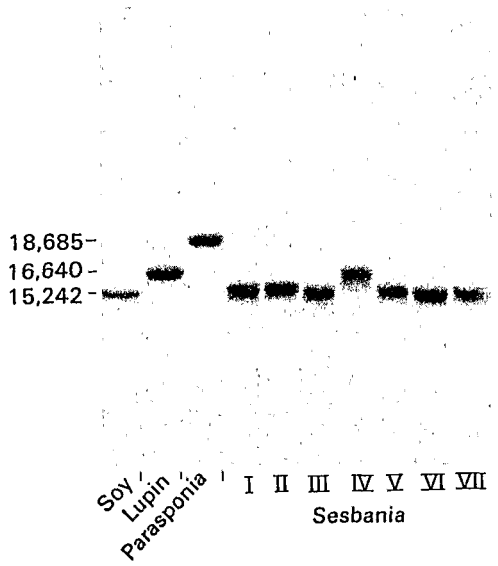

FIG. 4. SDS-polyacrylamide gel electrophoresis of *Sesbania* Lb components I–VII, with soybean Lba (M_r 15,242), lupin LbII (16,640), and *Parasponia* HBI subunits (18,685) as markers, in an 18% polyacrylamide gel (see Materials and Methods).

be the cause of reversed band positions (Table II).

CONCLUSIONS

Despite an exhaustive search we did not find any extra Lb components in the oxygen-evolving photosynthetic stem nodules of *S. rostrata*, as compared with nonphotosynthetic root nodules. Nevertheless, the observed different proportions of the seven common components means that we cannot reject the idea that light or photosynthetic oxygen evolution had caused the differential expression of *Sesbania* Lb genes. The sequencer identification of most major components as separate gene products with unblocked amino terminals (Table III), means that we can reject post-translational modification as a principal cause of the complexities within *Sesbania* Lb. With this identification of six or seven separate gene products the *Sesbania* Lb family is the largest so far characterized (cf. Ref. (1)).

TABLE IV

MOLECULAR WEIGHTS OF *Sesbania* Lb COMPONENTS[a]

Lb component	Native protein[b]	Denatured[c]
I	14,800	16,000
V	14,800	16,000
VI	15,100	16,200
III	15,500	16,000
VII	16,200	16,200
II	16,600	16,500
IV	16,600	16,800

[a] Arranged in order of increasing size.
[b] Measured as LbO_2 on a Sephacryl S200 column.
[c] The molecular weight shown is for the major component as detected by SDS-polyacrylamide gel electrophoresis (cf. Fig. 4).

ACKNOWLEDGMENTS

D.B. thanks the Division of Plant Industry for hospitality and some financial support during a February–April, 1985 visit when this joint study was initiated. Mr. A. Agostino, CSIRO Division of Plant Industry, is thanked for his advice and guidance in respect to SDS-polyacrylamide gel electrophoresis, and Mr. P. M. Strike, CSIRO Division of Protein Chemistry, for the gas phase sequencer analyses.

REFERENCES

1. APPLEBY, C. A. (1984) *Annu. Rev. Plant Physiol.* **35**, 443–478.
2. WITTENBERG, J. B., BERGERSEN, F. J., APPLEBY, C. A., AND TURNER, G. L. (1974) *J. Biol. Chem.* **249**, 4057–4066.
3. DREYFUS, B. L., AND DOMMERGUES, Y. R. (1981) *FEMS Microbiol. Lett.* **10**, 313–317.
4. LEGOCKI, R. P., EAGLESHAM, A. R. J., AND SZALAY, A. A. (1983) *in* Molecular Genetics of the Bacteria—Plant Interaction (Pühler, A., Ed.), pp. 210–219, Springer-Verlag, Berlin.
5. ALAZARD, D. (1985) *Appl. Environ. Microbiol.* **50**, 732–734.
6. BOGUSZ, D. (1984) *in* Advances in Nitrogen Fixation Research (Veeger, C., and Newton, W. E., Eds.), p. 534, Nikoff/Junk, The Hague.
7. FUCHSMAN, W. H., AND APPLEBY, C. A. (1978) *Biochim. Biophys. Acta* **579**, 314–324.
8. BOJSEN, K., ABILDSTEN, D., JENSEN, E. Ø., PALUDAN, K., AND MARCKER, K. A. (1983) *EMBO J.* **2**, 1165–1168.
9. WHITTAKER, R. G., LENNOX, S., AND APPLEBY, C. A. (1981) *Biochem. Int.* **3**, 117–124.
10. RINAUDO, G., DREYFUS, B., AND DOMMERGUES, Y. (1983) *Soil Biol. Biochem.* **15**, 111–113.
11. DILWORTH, M. J. (1980) *in* Methods in Enzymology (San Pietro, A., Ed.), Vol. 69, Part C, pp. 812–823, Academic Press, Orlando, FL.

12. APPLEBY, C. A., NICOLA, N. A., HURRELL, J. G. R., AND LEACH, S. J. (1975) *Biochemistry* **14**, 4444–4450.
13. HYLDIG-NIELSEN, J. J., JENSEN, E. Ø., PALUDAN, K., WIBORG, O., GARRETT, R., JØRGENSEN, P., AND MARCKER, K. A. (1982) *Nucleic Acids Res.* **10**, 689–701.
14. EGOROV, T. A., KAZAKOV, V. K., SHAKHPARONOV, M. I., AND FEIGINA, M. Y. (1980) *Bioorg. Chim.* **6**, 666–683.
15. LANDSMANN, J., DENNIS, E. S., HIGGINS, T. J. V., APPLEBY, C. A., KORTT, A. A., AND PEACOCK, W. J. (1986) *Nature (London)* **324**, 166–168.
16. OHLSSON, P. I., AND PAUL, K. G. (1976) *Acta Chem. Scand. B* **30**, 373–375.
17. MASKALL, C. S., GIBSON, J. F., AND DART, P. J. (1977) *Biochem. J.* **167**, 435–445.
18. ELLFOLK, N. (1961) *Acta Chem. Scand.* **15**, 975–984.
19. APPLEBY, C. A., WITTENBERG, B. A., AND WITTENBERG, J. B. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 564–568.
20. APPLEBY, C. A. (1974) *in* The Biology of Nitrogen Fixation (Quispel, A., Ed.), pp. 521–554, Elsevier, New Holland/New York.
21. APPLEBY, C. A., TJEPKEMA, J. D., AND TRINICK, M. T. (1983) *Science* **220**, 951–953.
22. FLEMING, A. I., WITTENBERG, J. B., WITTENBERG, B. A., DUDMAN, W. F., AND APPLEBY, C. A. (1987) *Biochim. Biophys. Acta* **911**, 209–220.
23. WIBORG, O., HYLDIG-NIELSEN, J. J., JENSEN, E. Ø., PALUDAN, K., AND MARCKER, K. A. (1982) *Nucleic Acids Res.* **10**, 3487–3494.
24. PODUSLO, J. F. (1981) *Anal. Biochem.* **114**, 131–139.