Characterization and Improved Separation of Soybean Leghemoglobins†

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ABSTRACT: An improved separation procedure is described for isolating five leghemoglobin components from the nodules of soybean plants. After a preliminary oxidation with ferricyanide, and separation from endogenous nicotinate at pH 9.2, the ferrileghemoglobins are separated by DEAE-cellulose chromatography using gradient elution with acetate buffer (pH 5.2). The components have been characterized by their acetate and nicotinate binding affinities, gel electrophoretic, visible, and circular dichroic spectra in the ultraviolet, Soret and visible regions. Two formerly unresolved components of leghemoglobin c have indistinguishable circular dichroic, electrophoretic, and ligand binding properties, but differ in their spin states as judged by their visible spectra, their amino acid analyses, and their tryptic maps.

The red pigment of Rhizobium-infected nitrogen-fixing legume root nodules was first characterized as an oxygen-binding hemoprotein by Kubo (1939), named leghemoglobin by Virtanen et al., (1945), and extensively studied by Ellfolk (1972). Many views have been expressed on its primary or secondary function in symbiotic nitrogen fixation (see, e.g., Virtanen et al., 1947, Bergersen, 1960; Abel et al., 1963; Bergersen and Turner, 1967; Koch et al., 1967); though it now seems likely that leghemoglobin acts by facilitating the diffusion and delivery of oxygen to the bacteroid surface at a stable low oxygen tension (see, e.g., Smith, 1949; Yocum, 1964; Appleby, 1969a, 1974; Tjepkema and Yocum, 1970; Bergersen et al., 1973; Wittenberg et al., 1974; Appleby et al., 1975a,b; Bergersen and Turner, 1975).

Leghemoglobins qualitatively resemble vertebrate myoglobins in their oxygen and ligand-binding properties, molecular weights (15,000–20,000), and noncovalent protein–heme linkage. The evolutionary and structural relationships between Lb's[1] and Mb's have therefore attracted attention. An X-ray analysis of the crystal structure of lupin leghemoglobin at 5-Å resolution (Vainshtein et al., 1974) suggests that it has essentially the same folded structure as myoglobin, although the C and D helices were not clearly delineated. The amino acid sequence of leghemoglobin a from soybean has been elucidated by Ellfolk and Sievers (1971), that of kidney bean leghemoglobin by Lehtovaara and Ellfolk (1974) and of broadbean leghemoglobin I by Richardson et al., (1975). They have been shown to have homologies with myoglobins and hemoglobin chains (Barker and Dayhoff, 1972; Dayhoff et al, 1972; Ellfolk, 1972; Lehtovaara and Ellfolk, 1974) though it is not yet clear whether this relationship indicates a common genetic origin or a functional convergence producing similar essential structural features more than once during evolution (Appleby, 1974). In either case, it becomes important to establish the structural and functional relationships between these two classes of heme proteins.

Most of the legumes examined have several chromatographically distinguishable leghemoglobins in their root nodule system. Several procedures for separating and purifying these components have been evolved (see, e.g., Thorogood and Hanania, 1963; Cutting and Schulman, 1969; Peive et al., 1967) but the most satisfactory preparative procedures involve chromatography on DEAE-cellulose or DEAE-Sephadex (Ellfolk, 1960; Appleby, 1969a,b; Melik-Sarkisyan et al., 1969; Broughton and Dilworth, 1971, 1972; Peive et al., 1972). Problems of fully resolving component leghemoglobins have been caused by the formation of multiple bands due to different oxidation states of the same species, formation of nicotinic acid hemochromes during extraction (Appleby, 1969b; Appleby et al., 1973a,b), and nonoptimal conditions for stepwise or gradient elution. The present paper describes an improved method for ion-exchange chromatography of soybean leghemoglobins after oxidation to ferrileghemoglobin and separation from nicotinic acid. This achieves a clean separation not only into the two major (*a* and *c*) and two minor (*b* and *d*) fractions but also separates leghemoglobin *c* into two components. The two Lb *c* components have been shown to differ in their chromatographic behavior, spectroscopic properties, amino acid composition, and tryptic peptide maps. The leghemoglobins have also been characterized by gel electrophoresis, circular dichroism and absorption spectroscopy of their derivatives, and their ligand binding properties.

Materials and Methods

Chromatographic Separations. The growth of the soybean plants (*Glycine max* cultivar "Lincoln"), and the harvesting and extraction of the nodules have been described

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[1] Abbreviations used are: Lb, leghemoglobin; Mb, myoglobin; Lb(III), ferrileghemoglobin; $Lb(II)O_2$, oxy-ferroleghemoglobin; Mes, 2-(*N*-morpholino)ethanesulfonic acid; BAWP, butanol–acetic acid–water–pyridine, 30:6:24:20 (v/v); fluorescamine, 4-phenylspirofuran-2(3H), 1′-phthalan-3,3′-dione as supplied by Hoffmann-La Roche; DEAE-, diethylaminoethyl-; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

elsewhere (Appleby, 1969b). The ammonium sulfate (80% saturated) precipitate from 767 g of fresh nodules was taken up in a minimum volume of Tris-HCl buffer (0.1 *M*, pH 7.7) containing EDTA (10^{-4} *M*). The solution was dialyzed against the buffer for 5 hr to remove salt and centrifuged at 100,000*g* for 20–30 min and the supernatant concentrated over a Diaflo UM10 membrane.

The leghemoglobin was oxidized fully to the Fe(III) state and thereby stabilized, using 4 equiv of potassium ferricyanide at pH 9.2 as follows. Tris buffer (0.1 *M*, pH 9.2, 15 ml) was cooled to 2° and added to the Lb solution (2.67 m*M*, 50 ml) at 2°. Potassium ferricyanide (176 mg) was added and the solution gently stirred until oxygen evolution had ceased. To remove the oxidant and also the endogenous nicotinic acid which is strongly bound to Lb at pH 5.2 (Appleby et al., 1973a,b), the 50 ml of solution was loaded onto a G-15 Sephadex column (55 × 5 cm equilibrated with Tris-HCl buffer 0.1 *M*, pH 9.2 at 2°) at 2 ml/min and 20-ml fractions were collected at this rate using the Tris buffer as eluent. A red Lb(III) band ran well ahead of a compact yellow band of excess ferricyanide and displaced nicotinic acid, which in turn moved ahead of a slow purple band. The pooled Lb peak fractions were tested for Lb(III) nicotinate by diluting samples tenfold with Mes buffer (0.1 *M*, pH 5.2) and determining the visible spectrum. The ratio *R* 560/620 nm for the nicotinate complex is 8.1. The pooled fractions (140 ml) had an *R* 560/620 nm of 1.70 which is characteristic of nicotinate-free ferrileghemoglobin. Using ϵ_{mM} 8.5 at pH 5.2, the absorbance value A_{496} of 7.08 provided an estimate of 0.83 m*M* for the concentration and 116 μmol for the total amount of Lb.

After concentrating the solution to 20 ml over a UM10 Diaflo membrane (Amicon Corporation, Cambridge, Mass.) and dialyzing for 36 hr against two changes of acetate buffer (0.01 *M*, pH 5.20, 4 l.) at 2°, it was clarified by centrifugation, then the 30 ml of clear green supernatant was diluted to 50 ml with acetate buffer (0.01 *M*) to give a 2.15 m*M* solution and loaded onto a DE-52-cellulose column (25 × 5 cm). This column was packed with Whatman DE-52-cellulose which had been preequilibrated with sodium acetate buffer (0.5 *M*, pH 5.2) and washed with glass-distilled water (8 l.) until the effluent conductivity and pH approached that of acetate buffer (0.01 *M*, pH 5.2). This latter buffer (3 l.) was used for final equilibration of the packed column at 2°. The flow rate for development was 120 ml/hr and a linear gradient of sodium acetate buffer (0.01 *M*, pH 5.2, 4 l. to 0.10 *M*, pH 5.2, 4 l.) was applied. The eluate was monitored using a Uvicord III flow photometer (LKB-Produkter AB., Sweden) set at 280 and 546 nm. Peak fractions were pooled, concentrated in a Diaflo cell over a UM 10 membrane, and exchanged into phosphate buffer (0.01 *M*, pH 7.0) to give final volumes of 5–10 ml per peak. Concentrations were estimated by the pyridine-hemochrome method (de Duve, 1948) using ϵ_{mM} at 556 nm of 34.6 (Paul et al., 1953), criteria for complete reaction being a major peak at exactly 556 nm and a ratio of the major peak to the first trough of ≥3.5.

Refractionation of the mixed Lb (c_1 + c_2) components was carried out using a smaller DE-52-cellulose column (36 × 1.5 cm) and linear buffer gradients, either with (i) sodium acetate buffers at pH 5.30 (0.02 *M*, 200 ml to 0.04 *M*, 200 ml) or (ii) Mes buffers at pH 5.3 (0.02 *M* Mes/NaOH, 200 ml to 0.02 *M* Mes-NaOH–0.02 *M* NaCl, 200 ml), or (iii) potassium phosphate buffers at pH 6.5 (0.02 *M*, 1 l. to 0.02 *M* with 0.2 *M* NaCl, 1 l.) at 3–4° and flow rates of 50–70 ml/hr. In each case the molarities refer to anion molarity. Further purification of the Lb c_1 and Lb c_2 fractions from the main Lb fractionation were also carried out using systems (i) and (ii).

Ligand Binding Curves for Purified Lb c_1 and Lb c_2. Stock solutions of Lb c_1 and Lb c_2 (4–5 m*M*, in 0.01 *M* phosphate (pH 7)) from the fractionation of Figure 1 were diluted to ≃66 μ*M* with water. Solutions of constant Lb concentration and pH but increasing acetate concentration were made up by mixing Lb (≃66 μ*M*, 250 μl), water (750 down to 450 μl), and acetate buffer (0.1 *M*, pH 5.30, 0 up to 300 μl). Difference spectra were run in the visible range against a blank solution (250 μl of Lb and 750 μl of H_2O) at 15°.

The difference spectrum peak at 620 nm representing the concentration of ferrileghemoglobin acetate provided an estimate of the mean fractional saturation of Lb binding sites (α) and this was recorded for each concentration (A) of acetate ion calculated from the ratio [HOAc]/[OAc^-] at pH 5.30.

Binding of nicotinate to each of the six Lb components was measured spectroscopically using Lb(III) solutions (1 μ*M*) in Mes buffer (0.05 *M*, pH 5.30) at 20° (Appleby et al., 1973a) using the difference spectrum peak at 416 nm as a measure of mean fractional saturation of Lb binding sites (α) with nicotinate.

Gel Electrophoresis. For electrophoresis at pH 8.9 the Tris-glycine system of Davis (1964) and Ornstein (1964) was used, at pH 5.2, the creatinine–acetic acid system of Richards et al. (1965), and for pH 7.0 sodium dodecyl sulfate, the continuous phosphate–sodium dodecyl sulfate system of Weber and Osborn (1969). The staining method used was the Coomassie Brilliant Blue sulfosalicylic acid system of Vesterberg (1971) which gives rapid visualization of protein bands with virtually no background.

Amino Acid Analysis and Peptide Mapping. Apoproteins of Lb c_1 and c_2 were made by the acid–acetone procedure of Ellfolk (1961a) and concentrations estimated by differential refractometry using a specific refractive increment of 0.188 ml/g. Salt-free apoprotein (≃50 nmol of the main amino acids) and norleucine standard (50 nmol) were freeze-dried, dissolved in HCl (6 *M*, 250 μl containing 1% phenol), and hydrolyzed at 110° for 24, 48, and 72 hr in evacuated sealed tubes. Amino acid analyses were performed with a Beckman 120B amino acid analyzer. Tryptophan analyses were performed by two methods. Hydrolysates produced using *p*-toluenesulfonic acid (3 *M*, 110°, 24 hr) in the presence of 3-indoleacetic acid (0.2%) were analyzed using a 20-cm resin column (Liu and Chang, 1971); yields from tryptophan standards were 86%. Analyses without prior hydrolysis were carried out using the method of Opieńska-Blauth et al. (1963) as modified by Dalby and Tsai (1975). The latter method gave 2.1 residues per mol for myoglobin and 1.9 for leghemoglobin a.

For tryptic digestion, the apoprotein (approximately 5 mg in 4 ml of glass-distilled water, dialyzed against 100 mg/l. of $NaHCO_3$) was digested in the pH-Stat at pH 8.2 and 38° with trypsin (Sigma diphenylcarbamyl chloride treated Lot 78B-8260, stock solution 5 mg in 1 ml of 1 m*M* HCl) using 1% total enzyme to protein, added in several aliquots. The reaction was allowed to proceed under N_2 for 1.5 hr after NaOH (0.1 *M*) uptake ceased and the reaction mixture freeze-dried.

Apoprotein tryptic hydrolysates (1 mg for analytical and 5 mg for preparative maps, at 1 mg/cm) were electrophor-

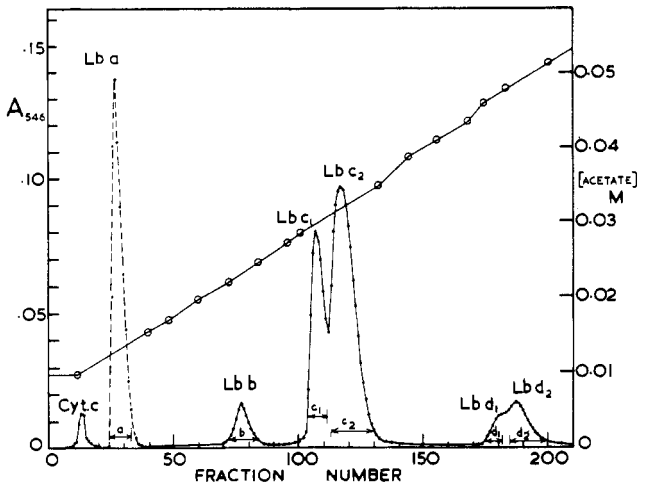

FIGURE 1: Elution profile showing the separation of soybean leghemoglobins on a DE-52-cellulose column, using an acetate gradient at pH 5.17 and 2° (see text): (○) acetate gradient molarity determined conductimetrically on effluent fractions; (●) A_{546} for 3-mm flow cell. The dashed line for Lb a represents $A_{546}/2$. The fractions pooled for recovery of components are indicated by horizontal arrows.

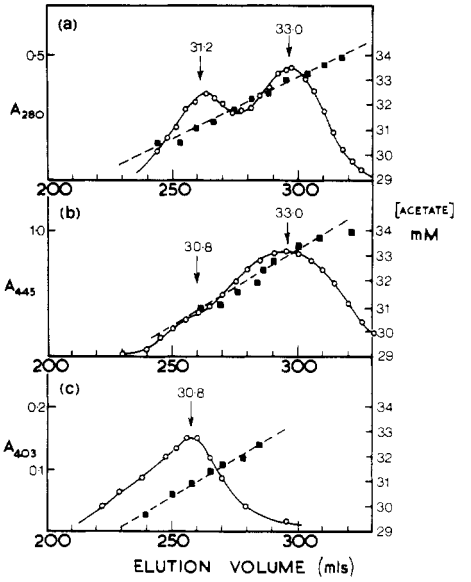

FIGURE 2: Rechromatography of pooled Lb c fractions on the same DE-52-cellulose column, using an acetate gradient (■) at pH 5.30 and 3-4° (see text). (a) Total Lb ($c_1 + c_2$), (○) A_{280}; (b) partially purified Lb c_2, (○) A_{445}; (c) partially purified Lb c_1, (○) A_{403}. The molarity of acetate at each elution peak is indicated by an arrow.

esed at pH 6.5 (pyridine–acetic acid–water, 100:3:897) on Whatman 3MM paper (1 kV for 15 min then 3 kV for 40 min) and the strip was dried and sewn to a new sheet for chromatography at right angles in BAWP. After drying, the paper was stained with fluorescamine (0.005% in acetone) and/or Cd-ninhydrin (0.01% in acetone) for preparative maps and 1% Cd-ninhydrin for analytical maps. Spots from the preparative maps were cut out and washed three times with acetone to remove excess ninhydrin and the peptides eluted with NH_4OH (0.1 *M*) for analysis.

Circular Dichroism (CD) and Absorption Spectra. CD measurements on Lb c_1 and c_2 were made in sodium acetate buffer (0.1 *M*, pH 5.3). Concentrations and cell path lengths were 0.17–0.18% and 0.1 mm (far-ultraviolet), 0.08–0.09% and 5 mm (near-ultraviolet), and 0.32–0.35% and 0.2 mm (Soret region). The circular dichrometer was a Jasco J5 which had been modified optically and electronically to the equivalent of a J20 model. Dry nitrogen was used to purge the instrument. Path lengths and instrument sensitivity were calibrated with *d*-10-camphorsulfonic acid. Scanning speed was 50 nm/hr, absorbancies of samples were less than 2, and ellipticities are reported only in the wavelength range where there was a linear relationship between concentration and response.

Absorption spectra of Millipore-filtered solutions were recorded on a Beckman DK2 or (for definitive measurements) a Cary 14 spectrophotometer at 20°.

Results

Leghemoglobin Fractionation. The fractionation of soybean Lb's using DE-52-cellulose and an acetate gradient at pH 5.2 and 0° typically gave the elution profile shown in Figure 1. After a small peak due to cytochrome *c* (identified by its characteristic absorption spectrum with a peak at 550 nm after reduction with sodium dithionite) six Lb(III) peaks may be distinguished with no complications due to Lb(II)O_2 species or ferric hemochromes (Ellfolk, 1960; Appleby, 1969b). The two Lb c components are well resolved and the twin peaks cannot be attributed to a separation of Lb c(III) and Lb c(II)O_2 since (i) both have the spectral characteristics of the ferric protein and (ii) two separate small peaks corresponding to Lb c_1(II)O_2 and Lb c_2(II)O_2 can be seen at higher elution volumes when ferricyanide oxidation is omitted or incomplete. There is no clear separation between Lb d_1 and Lb d_2. The estimated yields of the six pooled components are shown in Table I, together with their molar extinction coefficients at 280 nm and in the Soret region and the ratios of absorbancies *R* Soret/280 and *R* 495/530. The total recovery of 95.8 μmol in pooled peak fractions represents 84% of the leghemoglobin applied to the column. Components c_2 and d_2 have Soret peaks at slightly but consistently higher wavelengths compared with the other four components which have their maxima at 403 nm.

Table I: Yields and Spectral Characteristics for six Soybean Leghemoglobin Components.[a]

Component	Yield (μmol)	A_{280} (m*M*)	λmax (Soret)	A Soret (m*M*)	*R* Soret/280	*R* 495/530
Lb a	41.5	28.9	403	157	5.43	1.146
Lb b	2.2	32	403	154.5	4.83	1.153
Lb c_1	15.9	29.4	403	155	5.27	1.139
Lb c_2	31.4	29.0	404	146	5.03	1.088
Lb d_1	1.3	29.5	403	145	4.92	1.139
Lb d_2	3.5	29.5	403.5	141.5	4.80	1.071

[a] For spectroscopic examination, the Lb solutions (which were in phosphate buffer (0.01 *M*, pH 7.0)) were diluted 100–1000-fold with Mes buffer (0.10 *M*, pH 5.2).

The results of refractionating the mixed c_1 and c_2 fractions depended upon the buffer system used. Both acetate and Mes at pH 5.3 gave similar elution profiles, with incompletely resolved but clearly defined double peaks (Figures 2a and 3b, respectively). The arrows above the peaks indicate that in both cases, the anion concentration at which components c_1 and c_2 emerge (approximately 31 and 33 m*M*, respectively) are similar. Figure 3a shows that the two peaks are unresolved when separation is attempted with phosphate at pH 6.5. Rechromatography of the two separated (c_1 and c_2) components obtained from the experiment recorded in Figure 1, and concentrated prior to running in

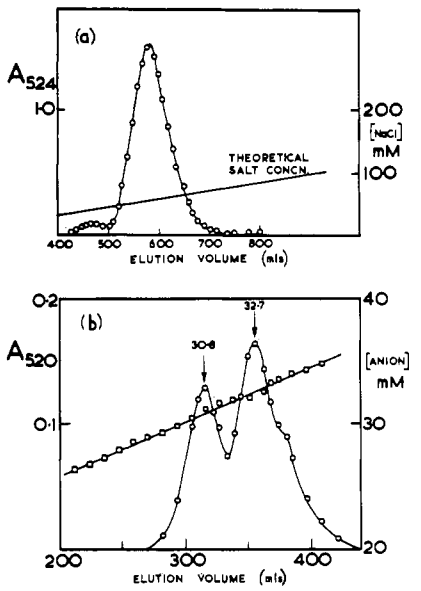

FIGURE 3: Rechromatography of pooled Lb c fractions (a) on a DEAE-A-50-Sephadex column using a phosphate–NaCl gradient at pH 6.5 and 3–4°; flow rate, 50 ml/hr; (b) on a DE-52-cellulose column using a Mes–NaCl gradient at pH 5.3 and 3–4°; flow rate, 72 ml/hr. (○) A_{520}; (□) anion concentration.

the acetate/pH 5.3 system, gave the two profiles in Figure 2b and c. Each component runs as a substantially single peak, emerging at the same acetate molarity as in the initial separation (Figure 2a). Refractionation of components Lb a and Lb b under the same conditions as Lb c showed that only one component was present in each case (not shown). Refractionation of the partly separated components Lb d_1 and Lb d_2 (cf. Figure 1) did not give complete resolution (not shown).

The visible spectra (400–700 nm) for Lb c_1 and c_2 in Mes buffer (0.1 *M*, pH 5.2) are shown in Figure 4a. They indicate that, at the same pH and temperature (20°), Lb c_1 is in a slightly higher spin state (Brill and Williams, 1961) than Lb c_2, since the charge transfer bands near 495 and 625 nm are relatively higher in Lb c_1. This conclusion is borne out also by the absorption spectra in the Soret region (Figure 4b). In this respect, the spectra of Lb a and Lb b are identical with that of Lb c_1, while those of Lb d_1 and d_2 show the same differences as noted between Lb c_1 and Lb c_2 (Table I).

Ligand Binding Data. Data from the spectroscopic method of determining acetate binding to Lb c_1 and c_2 showed that the proteins were indistinguishable in their binding behavior, giving straight line Hill plots with the same binding constant of 2.89×10^{-3} M^{-1}. Scatchard plots gave 3.03×10^{-3} M^{-1}. The linearity of both Hill and Scatchard plots and the value of the slope for the Hill plot ($n = 1.11$) indicate that if there is more than a single spectroscopically demonstrable binding site, the sites are noninteracting. The calculations assume that the ligand responsible for the spectral change is acetate anion (C. A. Appleby, unpublished) and the method of measuring binding takes no cognizance of any acetate ions which might bind without effect on the visible spectrum.

Titration of the ferrous forms of all the Lb's with nicotinic acid produced changes in the visible spectrum of the kind observed by Appleby (1973b). Scatchard plots for the binding of nicotinate to the six soybean ferri Lb fractions (cf. Table I) at pH 5.3 and 20° gave association constants

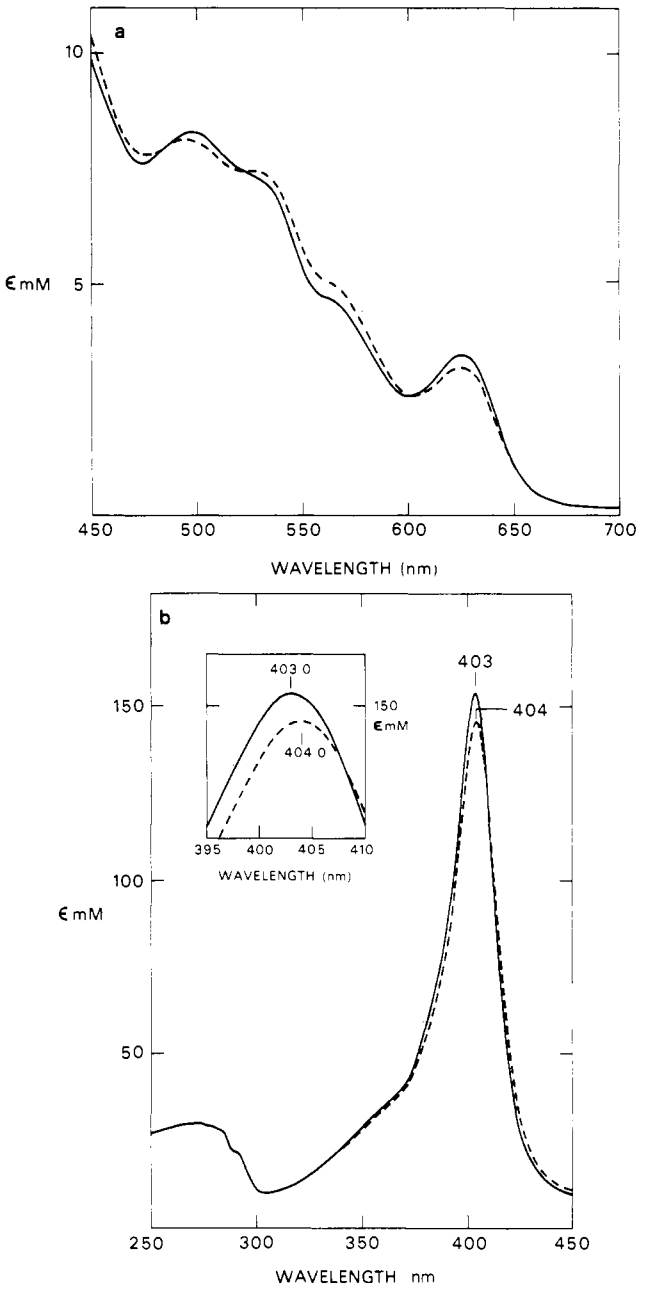

FIGURE 4: (a) Visible absorption spectra for (- - -) Lb c_1 (also Lb a and Lb b); (—) Lb c_2 (also unfractionated Lb ($d_1 + d_2$) measured in Mes buffer (0.1 *M* pH 5.2) at 20°. (b) Soret absorption spectra for (—) Lb c_1 and (- - -) Lb c_2.

($\times 10^{-6}$ M^{-1}) of 1.0 (Lb a), 1.3 (Lb b), 1.2 (Lb c_1), 1.3 (Lb c_2), 0.90 (Lb d_1), and 0.95 (Lb d_2). Hence, as found for acetate binding there was no significant difference in nicotinate binding affinity for the components Lb c_1 and Lb c_2.

Gel Electrophoresis. Sodium dodecyl sulfate electrophoresis of Lb a, b, c_1, c_2, and d, run with markers of known molecular weight, gave closely similar molecular weights for all, in the range 15,000–20,000. Isoelectric focussing of each separated component of soybean Lb gave multiple colored bands as noted by Broughton et al. (1972) for lupin and serradella Lb's due presumably to formation of Lb–ampholine complexes (Frater, 1970). Gel electrophoresis at pH's 5.2, 7, and 8.9 of the components from a fractionation such as that in Figure 1 showed that Lb a, Lb c_1, Lb c_2, and Lb ($d_1 + d_2$) each had one major component even at heavy loadings, while Lb b showed a minor component esti-

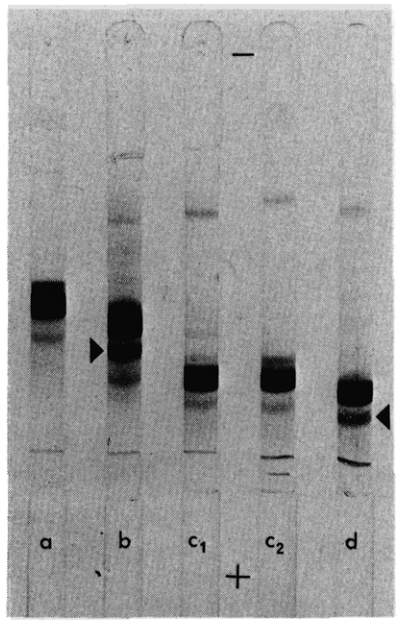

FIGURE 5: Gel electrophoresis patterns for heavy loadings of Lb a, Lb b, Lb c_1, Lb c_2, and pooled Lb d (from top to bottom) at pH 8.9.

mated at 7% of the total by scanning the gel spectrophotometrically. Figure 5 shows the gel patterns obtained at pH 8.9. The minor bands running toward the anode ahead of the main bands in Lb b and Lb d were heme-containing but the other trace contaminant bands visible in Figure 5 showed up only after staining. Lb c_1 and c_2 were electrophoretically indistinguishable either at pH 5.2, 7.0 (sodium dodecyl sulfate), or 8.9.

Amino Acid Analysis and Peptide Mapping of Lb c_1 and Lb c_2. The amino acid analyses for Lb c_1 and c_2 are shown in Table II, together with the literature values (Ellfolk, 1961a) for unfractionated Lb c. Lb c_2 appears to have one isoleucine residue additional to those in Lb c_1 but at least one aspartic acid, one glutamic acid, four alanine, three valine, and one leucine residue less than Lb c_1. Both proteins contain two tryptophan residues and this was confirmed by analyses on the intact protein which gave values of 2.0 for both Lb c_1 and Lb c_2. The mean values obtained for Lb c_1 and Lb c_2 agree with the literature value for unfractionated Lb c (Ellfolk, 1961a) for all but the serine, glycine, valine, and tryptophan data.

In addition to the origin spot there are about 14–18 peptide spots reactive to ninhydrin, though some spots are very weak in intensity (Figure 6). Resolution of all the possible peptides is incomplete under these conditions but is adequate for demonstrating differences between proteins. The peptide maps show some definite amino acid differences between Lb c_1 and Lb c_2. Peptides 14 and 15 in Lb c_2 do not occur in Lb c_1. Peptides 16 and 17 are weak and present only in Lb c_2. pH 3.5 electrophoresis of the neutral peptides indicated one further peptide difference between Lb c_1 and Lb c_2.

Circular Dichroism of Lb c_1 and Lb c_2. The CD spectra of the acetate complexes of the two Lb c components are recorded in Figure 7. Figure 7a shows the far-ultraviolet CD spectra and indicates that the secondary structure of Lb c_1 and c_2 is essentially the same; about 52% helix and 48% unordered structure. CD spectra for Lb c_1 and c_2 in the near-ultraviolet, which reflect the environments around the aromatic amino acids of proteins, are shown in Figure 7b

Table II: Amino Acid Analyses[a] of Lb c_1 and Lb c_2.

Amino Acid	Lb c_1 Mean	Lb c_1 Nearest Integer	Lb c_2 Mean	Lb c_2 Nearest Integer	Lb c[b]
Lys	13.05 (0.52)	13	12.79 (0.23)	13	13
His	2.19 (0.11)	2	1.97 (0.17)	2	2
Arg	1.00 (0.03)	1	0.96 (0.04)	1	1
Asp	12.55 (0.19)	12	11.04 (0.18)	11	12
Thr	6.92*	7	7.03*	7	7
Ser	9.80*	10	10.10*	10	11
Glu	15.14 (0.27)	15	13.75 (0.20)	14	14
Pro	4.61 (0.34)	5	4.65 (0.32)	5	5
Gly	7.00 (0.04)	7	7.26 (0.25)	7	8
Ala	22.88 (0.07)	23	19.19 (0.60)	19	21
Val	12.8*	13	10.21 (0.18)	10	13
Ile	4.84 (0.18)	5	5.85 (0.05)	6	6
Leu	12.94 (0.24)	13	12.16 (0.27)	12	12
Tyr	2.90*	3	2.90*	3	3
Phe	7.80 (0.39)	8	8.18 (0.09)	8	8
Trp	2.01	2	1.85	2	3

[a] The apoproteins of Lb c_1 and Lb c_2 (0.5 mg) were hydrolyzed (100 μl of 6 *M* HCl and 1% phenol, 110°, 24, 48, and 72 hr in vacuo) and analyzed with a Beckman 120 B analyzer. The values marked with an asterisk have been corrected for destruction of Ser, Thr, and Tyr and incomplete release of Val. Standard deviations are shown in parentheses. [b] Literature values for unfractionated Lb c (Ellfolk, 1961a).

and are indistinguishable from each other. Differences in the Soret region (Figure 7c) are barely significant. The possibility that one component is a denatured form or conformational variant of the other may therefore be discounted.

Discussion

Prior oxidation of the leghemoglobin extract with ferricyanide converted all heme protein components to their ferric forms. Subsequent ion-exchange chromatography then showed no evidence of the $Fe(II)O_2$ components which otherwise complicate the elution profile. Elimination of nicotinate (called X by Appleby, 1969b; Appleby et al., 1973a,b) from crude Lb by gel filtration at pH 9.2 also eliminated the double bands due to partial formation of Lb nicotinate hemochrome. Previously (Appleby, 1969b) these bands had complicated the ion-exchange elution profile. The use of a continuous, shallow acetate gradient with DE-52-cellulose then achieved a good resolution, not only of the previously reported components (Lb's a, b, c, d) but also demonstrated that Lb c and Lb d each have two components. Unlike the Lb d components, those of Lb c (c_1 and c_2) were quite well separated by the initial ion-exchange chromatogram. Further purification was achieved by a second run under similar conditions with a shallow acetate gradient. It was found important to maintain a constant low pH of 5.30 in both separations; for example, no resolution of the two Lb c components was observed in phosphate buffers at either pH 6.5 or 7.8. It is interesting to note that the resolution attainable in this ion-exchange chromatographic procedure is superior to that achieved in polyacrylamide gel electrophoresis even in the presence of acetate. In Figure 5 it is seen that the components Lb c_1 and Lb c_2 cannot be distinguished by gel electrophoresis at pH 8.9, or at pH 5.2 or 7.0 (not shown).

It has been conclusively shown by Cann and Goad (1964) and Cann (1969) that electrophoresis of proteins in carboxylic acid buffers can produce illusory additional components due to complexing between the protein and undissociated

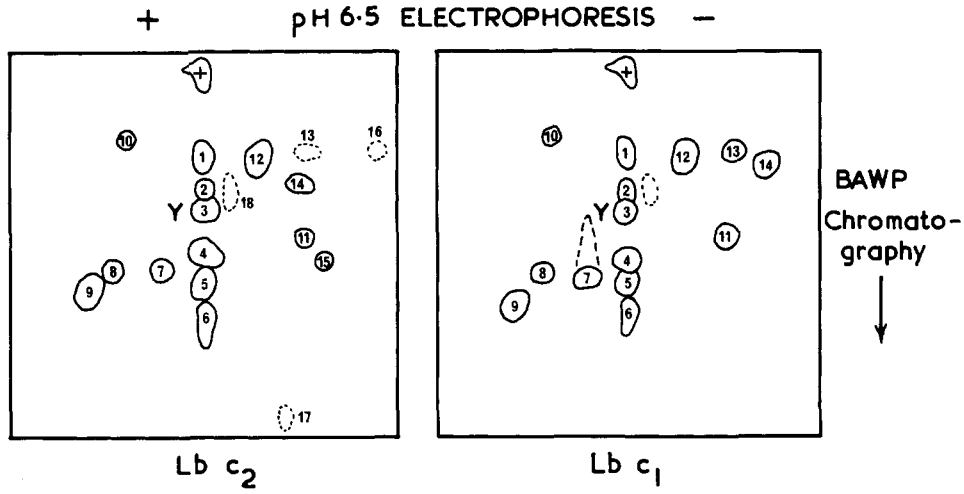

FIGURE 6: Tryptic "fingerprint" maps for apo Lb c_1 and c_2. The origin spot is shown as +. After running first at pH 6.5 electrophoretically, then by descending chromatography in BAWP, each map was sprayed with fluorescamine or Cd-ninhydrin. The spots indicated by broken lines are of low intensity.

buffer acids. The latter may cause subtle structural changes which increase the net positive charge. Similar arguments and cautions are applicable to ion-exchange chromatography.

The possibility that the separation of Lb c into two subfractions is an artefact of the buffer system and that the two components refer to a single leghemoglobin species may be dismissed on several grounds. Rechromatography of each component under identical conditions to the initial separation showed no evidence of reequilibration to the two original components. The anion concentration at which each component was released from the column was independent of the buffer species (acetate or Mes) at the same pH and resolution was equally complete in the noninteracting buffer, Mes.

The difference in properties between the two subcomponents of either Lb c or Lb d is not reflected in any difference in their binding affinities for acetate or nicotinate. Since all available evidence (Ellfolk, 1961b; Wittenberg et al., 1973; Appleby et al., 1973a) suggests that both ligands bind in the heme pocket and close to or at the heme iron we conclude that these subcomponents probably have similar structures at their active centers.

The CD spectra of the acetate complexes of the Lb c_1 and Lb c_2 components in the far-ultraviolet show no measurable differences in conformation, and have similar helical contents. In the near-ultraviolet, the CD spectra are nearly identical, with aromatic side chain environments which are very similar to those in Lb a (N. A. Nicola et al., in preparation). Nor do the CD characteristics in the Soret region show any evidence of significant differences between the heme environments of the two proteins. We do, however, observe slight differences in spin state between the unligated subcomponents ferric Lb c_1 and Lb c_2 and also between ferric Lb d_1 and Lb d_2 (Table I; Figure 4) which suggest slight differences in heme environment.

More significant differences between the two proteins are, however, detectable in their amino acid analyses and their tryptic peptide maps. The two subfractions differ significantly in their content of six amino acids. It is also significant that Lb c_1 appears to have nine or ten amino acid residues per molecule more than Lb c_2. The differences are borne out by the significant differences between the tryptic peptide maps of the two proteins. The maps presented here

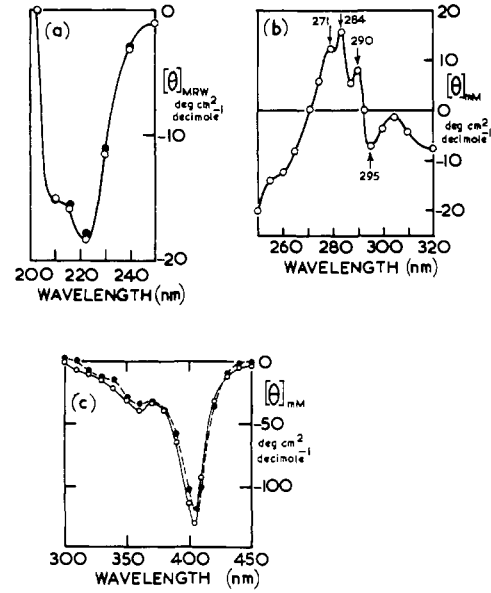

FIGURE 7: Circular dichroism spectra for Lb c_1 (○) and Lb c_2 (●) in acetate buffer (0.1 *M*, pH 5.30): (a) far-ultraviolet, (b) near-ultraviolet, and (c) Soret region. In (b) the two spectra are identical and only open circles are therefore shown.

(Figure 7) are not fully resolved but show a different number and distribution of basic peptides (at pH 6.5) in Lb c_1 than Lb c_2. Preliminary resolutions of the "neutral" peptides at pH 3.5 show further significant differences. Complete separations and analyses of these peptides as well as immunochemical studies are in progress (N. A. Nicola et al., unpublished) and bear out the contention that Lb c_1 and Lb c_2 are coded for by different genes.

The changes in amino acid composition and surface charge which allow the separation of closely related leghemoglobins from the same plant species, apparently do not necessarily cause significant changes in their biological (Appleby, 1962; Wittenberg et al., 1972), ligand-binding, or overall conformational properties.

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