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Molecular Properties of Lupin and Serradella Leghaemoglobins

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1. Leghaemoglobins were extracted from the root nodules of lupin (*Lupinus luteus* L.) and serradella (*Ornithopus sativus* Brot.) plants and fractionated into different leghaemoglobin components on DEAE-cellulose–acetate columns. 2. The first two fractions eluted from columns loaded with either lupin or serradella leghaemoglobins were in the Fe^{3+} oxidation state. 3. These components have protohaem IX as the prosthetic group and glycine as the *N*-terminal amino acid. 4. Other properties are: lupin component I, p*I* 5.08, molecular weight 19000; lupin component II, p*I* 5.13, molecular weight 20600; serradella component I, p*I* 5.00, molecular weight 17500; serradella component II, p*I* 5.05, molecular weight 19100. 5. Leghaemoglobins are thus heterogeneous with respect to size and charge.

Two dissimilar legumes, yellow lupin (*Lupinus luteus* L.) and serradella (*Ornithopus sativus* Brot.) can each be effectively nodulated with the same strain of *Rhizobium lupini*. By using the techniques of chromatography on DEAE-cellulose and electrophoresis in polyacrylamide gels, Dilworth (1969) found that extracts from lupin nodules contained four distinct leghaemoglobins and that serradella nodules contained only three. The leghaemoglobins produced in the two types of nodules were shown to be different. When strains of *R. lupini* which are known to be antigenically different were used to inoculate either plant, no change in the chromatographic or electrophoretic pattern of leghaemoglobins could be demonstrated. It was therefore suggested that the DNA specifying leghaemoglobin was located in the plant, a finding since extended to other legumes by other workers (Peive *et al.*, 1970; Cutting & Schulman, 1971; Broughton & Dilworth, 1971).

Leghaemoglobins usually exist in one of two different oxidation states, the ferrous form which is capable of associating with oxygen and the ferric form. Most of the leghaemoglobin *in vivo* is in the ferrous state (Appleby, 1969*a*). However, leghaemoglobins are commonly extracted under normal atmospheric conditions, a process that leads to oxidation at pH values below 7. Much of the leghaemoglobin is thus converted into the Fe^{3+} state, the form in which the bulk of these leghaemoglobins are eluted from DEAE-cellulose–acetate columns at pH 5.2 (Dilworth, 1969). The first two leghaemoglobin fractions eluted from DEAE-cellulose–acetate columns loaded with either lupin or serradella leghaemoglobins are in the Fe^{3+} state, and we have named them (in order of elution) lupin leghaemoglobins I and II, and serradella leghaemoglobins I and II respectively.

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Each of these leghaemoglobins can be produced in quantities sufficient for further analysis. It is the purpose of this communication to present the results of investigations into the molecular basis of the different chromatographic and electrophoretic behaviour displayed by lupin and serradella leghaemoglobins. This was accomplished by measuring terminal amino acids, isoelectric points, and the physical properties as revealed in the analytical ultracentrifuge.

Experimental

Chemicals

Ampholines (pH4–6) were from LKB-Produkter AB, Bromma, Sweden; horse heart myoglobin from Miles Laboratories, Kankakee, Ill., U.S.A.; 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dns chloride) from the Sigma Chemical Company, St. Louis, Mo., U.S.A. Dns chloride was prepared for use as follows: 1g was Soxhlet-extracted with acetone, filtered and concentrated by rotary evaporation. The amount in solution was determined from $\epsilon_{375}=3000$. The solution was stored at −20°C. Haemin (97%, w/w, by iron analysis) was from George T. Gurr Ltd., London, U.K.

Isolation of leghaemoglobin

Leghaemoglobins were isolated from nodules of lupin and serradella plants (inoculated with *R. lupini* strain WU 8) as follows: a minimum of 100g of nodules was homogenized in 0.1M-potassium phosphate buffer, pH6.8, at 4°C in an MSE AtoMix (ratio of tissue to buffer 1:4). The homogenate was filtered through cheesecloth and centrifuged at 10000*g* for 20min, and the supernatant fractionated

with solid $(NH_4)_2SO_4$. The portion precipitating between 50 and 80% saturation was dissolved in a small volume of buffer and dialysed against 0.1mM-EDTA, pH6.8, for 16h. This material was centrifuged at 25000*g* for 15min before being loaded on the ion-exchange columns. Fractionation was achieved on either 40cm×2cm diameter columns of DEAE-cellulose or DEAE-Sephadex A-25. Both were equilibrated and eluted with 0.013M-sodium acetate *I*, 0.010, pH5.25. In general DEAE-Sephadex gave better resolution of the two lupin components, whereas the less expensive DEAE-cellulose could be used for the serradella components. Each of the first two leghaemoglobin peaks eluted from the columns was pooled and concentrated either by ultrafiltration on a Diaflo UM 10 membrane or by dialysis against solid CM-cellulose (Aquacide II, Calbiochem, Australia). All operations were performed at 4°C. Yields of unfractionated leghaemoglobin were about 1mg/g fresh weight.

Analytical techniques

Sedimentation and diffusion coefficients were determined on a Beckman analytical ultracentrifuge (Spinco model E) equipped with schlieren optics as described by Schachman (1957). Sedimentation velocity runs were performed at 59780rev./min and diffusion runs at 15200rev./min. The samples were dialysed against 0.1M-potassium phosphate buffer, pH6.8, and artificially layered with a portion of the diffusate in a rubber-valve type synthetic-boundary cell at approx. 8000rev./min. Photographs were taken at 8–16min intervals on Kodak Tri/X sheet film by using a deep-red filter. Diffusion coefficients were calculated by plotting the square of the second moment against time, and the partial specific volume from the amino acid composition (Schachman, 1957).

Isoelectric points were calculated after electrofocusing leghaemoglobins on polyacrylamide gels (Catsimpoolas, 1968). A minimum of three separate determinations was made, linear regressions were fitted to the results, and standard errors derived from an analysis of variance.

Iron was determined by atomic-absorption spectroscopy (Broughton & Dilworth, 1971), and leghaemoglobin as the pyridine haemochromogen (Paul *et al.*, 1953).

N-terminal amino acid analysis

Leghaemoglobin (0.1μmol dissolved in 7.4M-urea) was treated with dansyl chloride as described by Gray (1967). The labelled protein was collected by centrifugation, washed three times with 2ml of 50% (v/v) acetone and freeze-dried. Then 0.5ml of 6M-HCl was added and hydrolysis performed for 16h *in vacuo* in a boiling-2-methylpropan-1-ol bath (108°C). The ampoule was opened, the supernatant removed and dried by rotary evaporation. In preparation for chromatography, the contents of the rotary evaporator flask were dissolved in 25μl of ethyl acetate or aq. 1M-NH_3. T.l.c. was performed on commercial silica gel sheets (Eastman Chromogram Sheet No. 6061) by using the following solvents: benzene–pyridine–acetic acid (40:10:1, by vol.) (Morse & Horecker, 1966), chloroform–ethanol–acetic acid (38:4:3, by vol.) and chloroform–benzyl alcohol–acetic acid (70:30:3, by vol) (Deyl & Rosmus, 1965).

Preparation of haem

Leghaemoglobin preparations were treated with acetic acid–acetone and the haem was extracted as described by Jackson & Evans (1966). Similar results were obtained with the method of Cutting & Schulman (1969). The haem was dried by rotary evaporation, and redissolved in the appropriate solvent for chromatography or spectral analysis. The chromatographic conditions and solvents used are described in Table 1. Each chromatogram was examined under a Chromatolite u.v. lamp before spraying with a benzidine reagent (Connelly *et al.*, 1958). Absorption spectra were recorded on a Unicam SP.700 recording spectrophotometer.

Results

Identification of the prosthetic group

Two different criteria were used to identify the haem moiety of lupin and serradella leghaemoglobins. First, the visible absorption spectra of protohaem IX was identical with that of the haems from lupin and serradella leghaemoglobins when measured as either the oxidized or reduced pyridine haemochromogen (Fig. 1). The absorption spectra of lupin and serradella leghaemoglobins in both the oxidized and reduced states were similar to those of horse heart myoglobin. This suggests that the prosthetic group is protohaem IX. Confirmation is given in Table 1. The chromophore obtained from the leghaemoglobins of both plants co-chromatographs with authentic protohaem IX in a variety of solvents on paper, thin-layers and a reverse-phase partition system. Minor components, such as those found in crude soya-bean extracts (Falk *et al.*, 1959) were not detected. It is concluded that lupin and serradella leghaemoglobins, like their soya-bean counterparts (Ellfolk & Sievers, 1965), have protohaem IX as the prosthetic group.

Isoelectric points and N-terminal amino acids

Isoelectric focusing of purified leghaemoglobins produced a multiplicity of bands, of which seven were quite distinct. At least four of the bands were green in

colour, the others various hues of red. Obviously, the different colours represent different leghaemoglobin oxidation states, yet the presence of so many bands in a protein which is otherwise homogeneous is disconcerting. Up to four bands from the one haemoglobin can be accounted for on the basis that leghaemoglobins may exist in two different molecular orbital forms as well as two different oxidation states (Appleby, 1969*b*). It is necessary therefore to invoke either complex formation between leghaemoglobins and Ampholines as outlined by Frater (1970), or deamidation of the protein as reported in peptides (McKerrow & Robinson, 1971), and proteins (Flatmark, 1966, 1967). Presumably the latter is the more likely, as older leghaemoglobin extracts bind to DEAE-cellulose–acetate columns more firmly than those which have been freshly prepared. It would seem that the isoelectric point decreases on standing, and satisfactory separation of plant components can only be achieved with an extremely rapid technique (48h from homogenization of the nodules to elution from DEAE-cellulose-acetate columns).

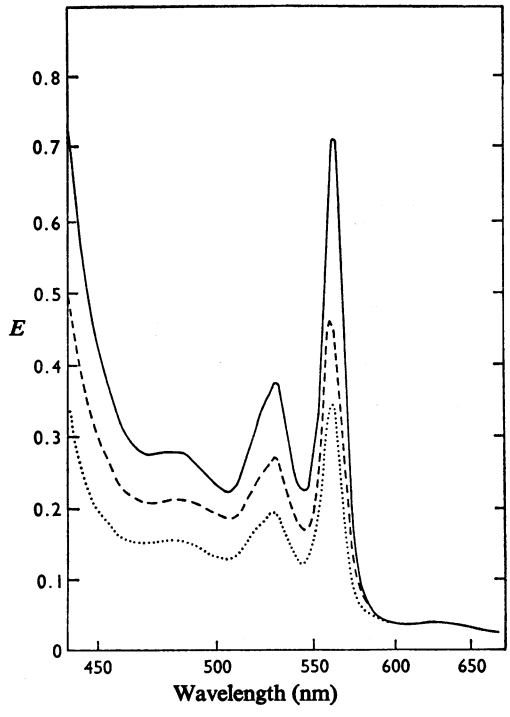

Fig. 1. *Visible spectra of the reduced pyridine haemochromogens (Paul et al., 1953) of protohaem IX (——) and the haems isolated from leghaemoglobins of serradella (– – –) and lupin (· · · ·)*

Despite the difficulties in interpreting much of the electrofocusing results, one band from each component was present at an intensity of roughly ten times the others. We suggest that this band is the native leghaemoglobin. Isoelectric points of lupin components I and II, and serradella components I and II differ by 0.05pH units (Table 2) and this difference is statistically significant ($P < 0.05$).

Dansylation of lupin and serradella leghaemoglobins, followed by acid hydrolysis, and chromatography of the residue, yielded fluorescent spots, corresponding to Dns-OH, Dns-NH_2, Dns-glycine, and traces of *O*-Dns-tyrosine and ε-Dns-lysine. This pattern was the same regardless of the leghaemoglobin used, and addition of authentic Dns-glycine to the hydrolysate before chromatography did not yield more spots. Thus glycine is *N*-terminal on each of the leghaemoglobins.

Molecular properties

Sedimentation and diffusion coefficients were determined in the analytical ultracentrifuge at a leghaemoglobin concentration of 0.3% (w/v). Solutions of this concentration strongly absorb visible light, and it was necessary to use a deep-red filter to visualize the schlieren peak. Single symmetrical peaks were obtained in both sedimentation and diffusion runs, and it was possible to follow the spread of the schlieren peak at 15200rev./min for periods of up to 80min. Molecular-weight determinations were performed twice, and the individual determinations

Table 1. *R_F values of the chromophores obtained from lupin and serradella leghaemoglobins on paper, thin-layer and reverse-phase chromatographic systems*

Authentic protohaem IX co-chromatographed with the compound from lupin and serradella leghaemoglobin in each of the systems listed.

Chromatographic system	Solvent	R_F	Reference
Paper (reverse phase)	Propan-1-ol–pyridine–water (1:4:55, by vol.)	0.3	Chu & Chu (1955)
Paper	2,6-Lutidine–water (11:9, v/v)	0.7	Chu & Chu (1955)
Paper	2,6-Lutidine–collidine–water (1:1:2, by vol.)	0.6	Foster *et al.* (1964)
Thin layer (silica gel G)	Chlorobenzene–acetic acid (20:3, v/v)	0.2	Miyauchi (1968)
Thin layer (silica gel G)	Nitrobenzene–acetic acid (20:3, v/v)	0.2	Miyauchi (1968)

Table 2. *Isoelectric points of purified lupin and serradella leghaemoglobins*

Results are given ± S.E.M. Leghaemoglobins were isolated by passage through DEAE-cellulose–acetate columns as described in the text.

Lupin I			Lupin II		
Slice no.	Colour	p*I*	Slice no.	Colour	p*I*
14	Red–brown	5.00±0.02	13	Red–brown	5.04±0.03
15	Red–brown	5.08±0.03*	14	Red–brown	5.13±0.02*
16	Green	5.16±0.03	15	Green	5.21±0.02
17	Green	5.24±0.04	16	Green	5.30±0.02
17–18	Green	5.32±0.04	17	Green	5.35±0.04
18–19	Green	5.37±0.04	18	Green	5.40±0.04
19	Green	5.43±0.04	19	Green	5.54±0.03
Serradella I			**Serradella II**		
Slice no.	Colour	p*I*	Slice no.	Colour	p*I*
10	Green	4.71±0.02	11	Green	4.73±0.01
11	Orange	4.75±0.03	12	Orange	4.77±0.02
12	Orange	4.81±0.03	12–13	Orange	4.83±0.02
12–13	Red–brown	4.88±0.03	13–14	Red–brown	4.88±0.02
13	Red–brown	4.94±0.03	14–15	Red–brown	5.00±0.02
13–14	Red–brown	5.00±0.02*	15	Red–brown	5.05±0.01*
16	Green	5.17±0.04	17	Green	5.22±0.01

* Major leghaemoglobin components.

agreed within 10%. Errors on reading the photographs were less than 1% for sedimentation runs and between 1 and 3% for diffusion runs.

Lupin and serradella leghaemoglobins have sedimentation coefficients ranging from 1.88 S (lupin I) to 1.99 S (serradella II), whereas diffusion coefficients range from 11.2×10^{-7} cm^2/s (serradella I) to 9.7×10^{-7} cm^2/s (lupin II). This suggests that among leghaemoglobins, either the shape of the molecule or the extent of solvation varies, a suggestion supported by other results. Frictional coefficients for both lupin components was 1.21 (Table 3), whereas f/f_0 was 1.11 for serradella I and 1.15 for serradella II.

Molecular weights were calculated by two independent procedures. The first involved combination of sedimentation and diffusion results, with the partial specific volume of the protein and the density of the solvent. Partial specific volumes were calculated from the amino acid composition results of W. J. Broughton & M. J. Dilworth (unpublished work), which yielded values higher than those normally obtained for proteins (Table 3), and slightly higher than the value of 0.740 ml/g reported for soya-bean leghaemoglobins (Ellfolk, 1960). Ellfolk (1960) determined this value by pycnometry and if the partial specific volume is determined from the amino acid composition results reported later by Ellfolk (1961), values of 0.748 ml/g are obtained. This suggests that the haem moiety contributes −0.008 ml/g to the partial specific volume, and we may confidently assume that partial specific volume results reported in Table 3 should be decreased by a similar amount.

The molecular weights calculated with these results range from 17500 (serradella I) to 20600 (lupin II). They are all higher than other values for leghaemoglobins (Broughton & Dilworth, 1971; Ellfolk, 1960, 1961), yet they agree well with results derived independently from iron determinations. A minimum molecular weight can be calculated by measuring the iron content of a weighed, dried sample. Minimum molecular weights calculated in this manner are also reported in Table 3. Generally the agreement between measured molecular weights is good, suggesting that there is only one haem group per molecule. Only in the case of serradella I is the difference between the two molecular weight values significant, and other results suggest that the lower value is the more likely (W. J. Broughton & M. J. Dilworth, unpublished work).

Discussion

The original impetus for this work came when Dilworth (1969) reported that yellow lupins con-

Table 3. *Molecular properties of lupin and serradella leghaemoglobins*

Property	Lupin I	Lupin II	Serradella I	Serradella II
Sedimentation coefficient ($s^{0,3\%}_{20,w}$)	1.88S	1.97S	1.97S	1.99S
Diffusion coefficient ($D^{0,3\%}_{20,w}$)	10.0	9.7	11.2	10.4
Partial specific volume ($\bar{v}$) (ml/g)	0.748	0.747	0.744	0.746
Molecular weight				
From diffusion and sedimentation results	19000	20600	17500	19100
From iron determinations	18800	20300	16500	18600
Frictional ratio (f/f_0)	1.21	1.21	1.21	1.15
Stokes radius (a)	2.15nm	2.22nm	1.92nm	2.07nm

tained different leghaemoglobins from those in French serradella. One question, which then arose, was how different are the leghaemoglobins from the two plants. The constituent leghaemoglobins of lupin nodules were separated into four distinct components by ion-exchange chromatography on DEAE-cellulose–acetate columns, and the leghaemoglobins of serradella nodules into three (Dilworth, 1969). Up to six leghaemoglobins could be resolved by co-chromatography of pooled lupin and serradella leghaemoglobin extracts. Polyacrylamide-gel disc electrophoresis of the same leghaemoglobin extracts produced only two haemoglobin bands from each plant, and one lupin and one serradella band possessed similar migration rates. These results suggest that lupin and serradella leghaemoglobins definitely have different isoelectric points, and possibly different molecular weights.

Isoelectric points are indeed different, ranging from 5.00 to 5.54 for the lupin leghaemoglobins and 4.71 to 5.22 for the serradella leghaemoglobins. p*I* values of the major components from each plant differ by 0.05 units, and lupin leghaemoglobins I and II and serradella leghaemoglobins I and II all have different isoelectric points. Molecular weights are also different, ranging from as low as 16500 to 20600. In his pioneering leghaemoglobin work, Ellfolk (1960) reported the molecular weights of the soya-bean components as 15400 and 16800. Lupin and serradella leghaemoglobins are obviously larger than this. One of the soya-bean components has valine as the *N*-terminal amino acid whereas the other has glycine (Ellfolk & Levin, 1961). Glycine is *N*-terminal on both lupin and serradella leghaemoglobins.

In conclusion it seems reasonable to suggest that a certain heterogeneity exists among leghaemoglobins. This includes both intra- and inter-plant components and extends to many major properties of the molecule such as sedimentation and diffusion coefficients, *N*-terminal amino acids (though this only applies to one of the soya-bean leghaemoglobins), and isoelectric points. Differences in conformation of the molecules can be deduced from frictional-coefficient results, and one would expect that widely different plants (i.e. plants from different families of the Leguminales) would have very different leghaemoglobins in their root nodules.

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