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Bile-Pigment Formation from Different Leghaemoglobins

METHINE-BRIDGE SPECIFICITY OF COUPLED OXIDATION

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The coupled oxidation of leghaemoglobins with O_2 and ascorbate yielded oxyleghaemoglobin in the first reaction step, and the second step was the degradation of haem characterized by an A_{675} increase. Leghaemoglobins were degraded to biliverdin isomers specifically, depending on the structure of the protein. The main leghaemoglobin components of *Glycine* (soya bean) and *Phaseolus* (kidney bean) were degraded to biliverdin mixtures containing about 50% of the β-form, about 30% of the α-form and about 20% of the δ-isomer, whereas the leghaemoglobin I components of *Vicia* (broad bean) and *Pisum* (pea) were degraded almost exclusively to the β-isomer, with traces of the α-isomer. The amino acid sequences of *Glycine* and *Phaseolus* leghaemoglobins resemble each other, as do those of *Vicia* and *Pisum*. The site specificity of bile-pigment formation from leghaemoglobins can be tentatively explained by specific differences in the amino acid sequences at those regions of the polypeptide chain that are in the vicinity of the appropriate methine bridges. The ligand-binding site in different leghaemoglobins may be outlined on the basis of the present results, supposing that the haem is degraded when a reduction product of haem-bound O_2 reacts with a methine bridge of the haem, and that the bridge specificity is regulated by hindering amino acid residues that determine the location of the bound O_2. The residue phenylalanine-CD1 appears to be further away from the haem plane or in a markedly more flexible position in leghaemoglobins than in mammalian globins. The haem-bound oxygen atom B, in Fe–O(A)–O(B), seems to be free to rotate in all directions except that of the γ-bridge in *Glycine* and *Phaseolus* leghaemoglobins, but its position in *Vicia* and *Pisum* leghaemoglobin I might be restricted to the direction of the β-methine bridge.

The only known globin-type haemoprotein in the plant kingdom is leghaemoglobin, which is found in the root nodules of leguminous plants during effective, nitrogen-fixing symbiosis of the plant with *Rhizobium* bacteroids. Leghaemoglobins are monomeric proteins of molecular weight about 16000. They contain one protohaem prosthetic group, and their general properties have been reviewed by Ellfolk (1972) and by Appleby (1974). The amino acid sequence has been determined for soya-bean (*Glycine max*) leghaemoglobin *a* (Ellfolk & Sievers, 1971, 1974) and *c* (Sievers *et al.*, 1977), for kidney-bean (*Phaseolus vulgaris*) leghaemoglobin *a* (Lehtovaara & Ellfolk, 1974, 1975), for broad-bean (*Vicia faba*) leghaemoglobin component I (Richardson *et al.*, 1975) and for lupin (*Lupinus luteus*) leghaemoglobin component I (Jegorov *et al.*, 1976). Of plant globins, only the tertiary structure of lupin leghaemoglobin has been determined (Vainshtein *et al.*, 1975, 1977), and it closely resembles that characteristic for all animal globins.

The catabolism of haemoglobins and myoglobins is well documented (reviewed by Jackson, 1974; O'Carra, 1975; Schmid & McDonagh, 1975), but the mechanism of the individual steps involved in haem catabolism are not yet clear. A few works on leghaemoglobin catabolism were published 30 years ago, and possibly the most important result of these was the observation that a biliverdin-type absorption spectrum was obtained after acid treatment of the degradation product of leghaemoglobin (Virtanen & Miettinen, 1949).

O'Carra & Colleran (1969) investigated the breakdown of haemoproteins to biliverdin isomers by coupled oxidation with O_2 and ascorbate, and were led to support the original view of Lemberg (1935), who suggested that the mechanism of the catabolism *in vivo* may be the same as in coupled oxidation. The methine-bridge specificity of haem breakdown to biliverdin isomers α, β, γ and δ (Fig. 1) is, according to present knowledge, determined by the protein to which the haem group is specifically attached during its catabolism, and this protein may either be the haemoprotein itself, as in coupled oxidation *in vitro*, or a haem-cleaving enzyme (O'Carra, 1975; Schimd & McDonagh, 1975).

Random cleavage of the haem to the four biliverdin isomers was observed in coupled oxidation of

Abbreviations used: Hb, haemoglobin; Mb, myoglobin; Lb, leghaemoglobin.

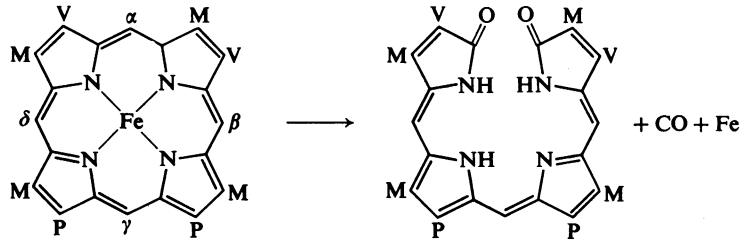

Fig. 1. *Derivation of biliverdin α-isomer by cleavage of protohaem-IX at methine bridge α*
Cleavage at the methine bridge-β gives rise analogously to biliverdin β-isomer etc. Abbreviations used: M, $-CH_3$; V, $-CH=CH_2$; P, $-CH_2CH_2CO_2H$.

protohaem and haem–albumin, as well as of myoglobin and haemoglobin denatured with 8M-urea, whereas native haemoproteins were cleaved site-specifically (O'Carra & Colleran, 1969; O'Carra, 1975). Coupled oxidation of myoglobin yielded only the α-isomer, and a mixture of α- and β-isomers was obtained from catalase and from different haemoglobins. The relative proportions of the α- and β-isomers from haemoglobin breakdown were dependent on the structure of each protein.

It is not clear how the apoproteins determine the bridge specificity of haem breakdown. Differential accessibility of the methine bridges to solvent seems to be ruled out because the γ-bridge is in the most accessible position and the α-bridge is most deeply buried. Differential hydrophobicity around the methine bridges has been considered as a possible factor directing the specificity (O'Carra & Colleran, 1969; O'Carra, 1975). Brown (1976) has recently suggested a hypothesis based on the probable nature of the first intermediates in haem degradation. An 'activated oxygen' seems to be formed when O_2 bound to ferrous haem iron is further reduced by a reductant (O'Carra, 1975), and according to the hypothesis of Brown (1976) the methine bridge that is preferentially attacked is that which is most accessible to the haem-bound O_2. The role of the amino acid residues of the protein would thus be to restrict free rotation of the bound O_2 molecule, and from this it follows that the biliverdin isomers produced in coupled oxidation should give spatial information about the haem-binding site.

In this work the chemical degradation of the haem group of different leghaemoglobins was studied, with the aim of elucidating the largely unknown catabolism of leghaemoglobins and also the mechanisms involved in haem breakdown in general. The plant globins are suitable for the latter purpose because much is known about their molecular structures and also because they are only remotely related to the animal globins so far studied.

Experimental

Proteins

Leghaemoglobin components were isolated and purified from the root nodules of soya bean (*Glycine max*), kidney bean (*Phaseolus vulgaris* var. Kaiser Wilhelm), pea (*Pisum sativum* var. Torstai II) and broad bean (*Vicia faba*). The pea plants were cultivated in open fields in Southern Finland and the other plants in a greenhouse as described earlier (Ellfolk, 1960; Lehtovaara & Ellfolk, 1975). Purification of the leghaemoglobins from the root nodules of the different plants was carried out essentially by the method used for kidney-bean leghaemoglobin (Lehtovaara & Ellfolk, 1975). Details for the final purification of *Pisum* and *Vicia* leghaemoglobins are presented in Fig. 2.

Sperm-whale myoglobin, human haemoglobin and bovine liver catalase were used as reference proteins in the coupled oxidation experiments. They were all obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Protohaem was prepared from fresh blood (Fuhrhopf & Smith, 1975). All reagents were of analytical grade.

Coupled oxidation

Coupled oxidation of protohaem was carried out either in 25% (v/v) pyridine in water, or in pyridine/ethanol/water (3:8:9, by vol.). Both solutions contained 1–2mg of ascorbic acid/ml. Coupled oxidation was more efficient in the latter system, which gave much better yields of the biliverdin isomers. After incubating the protohaem solution (0.6–1.0mg/ml) at 37°C in the dark for 90min, the reaction was stopped by cooling to 0°C and adding 0.3ml of acetic acid and 0.8ml of 5M-HCl to every 1.0ml of the incubation mixture (O'Carra & Colleran, 1969).

Coupled oxidation of haemoproteins was carried out in 0.1M-phosphate buffer, pH7.5, containing

2mg of ascorbic acid/ml. Amounts from 100 to 800nmol of haemoprotein [concentration based on haem content (Paul *et al.*, 1953)] were dissolved in this buffer to a final concentration of 160nmol/ml and incubated aerobically in a beaker in the dark at 37°C for 160min. The reaction was terminated as above.

Isolation and quantitative determination of biliverdin isomers

Biliverdin isomers were isolated and methylated as described by O'Carra & Colleran (1970). The dimethyl esters of biliverdin isomers were separated on thin-layer plates of glass, covered with a 0.25mm layer of silica gel (Camag, Muttenz, Switzerland), which was made 13% (w/w) with respect to calcium sulphate. After three successive developments in the dark at 4°C, with the elution system of O'Carra & Colleran (1970), the blue-green sample bands were quantitatively scraped off and transferred to 1ml centrifuge tubes, extracted with acetone and centrifuged. The combined biliverdin solutions obtained from three successive acetone extractions were evaporated to dryness under N_2 and transferred quantitatively to a microcuvette by rinsing with $3 \times 100\,\mu l$ of acetone. Absorption spectra from 450 to 350nm were immediately recorded with a Cary 14 spectrophotometer, and the amounts of each biliverdin isomer were calculated on the basis of the intensity of the 370nm absorption maximum. All steps of biliverdin isolation, methylation and handling of the esters were carried out at 0–4°C under N_2 and in the dark, with the exception of the drying of the thin-layer plates between the successive developments, which was carried out with cool air of a hair-dryer.

Results

The leghaemoglobins used in this work were: kidney-bean (*Phaseolus vulgaris*) Lb*a*, which is the

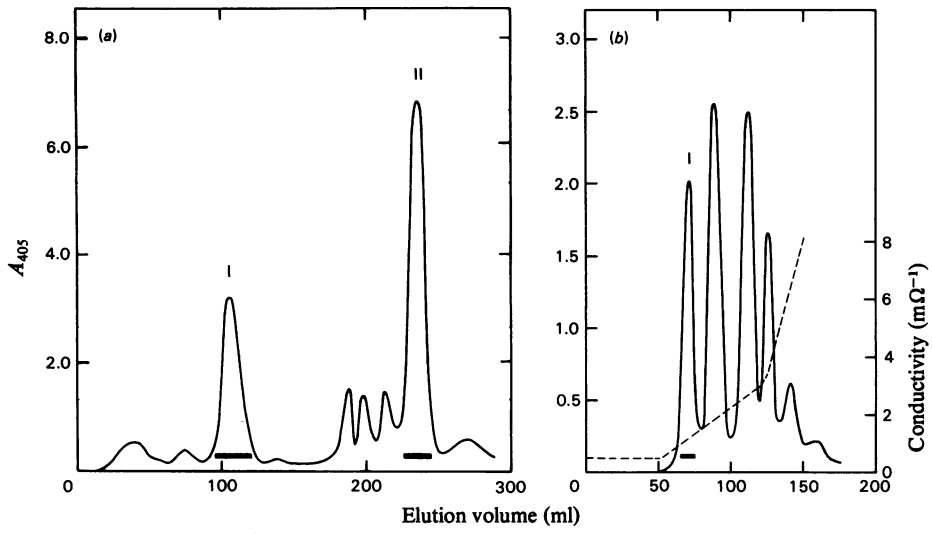

Fig. 2. *Elution diagram for Pisum sativum (a) and Vicia faba (b) leghaemoglobins from DE-cellulose*
About 1 μmol of crude leghaemoglobin, i.e. the fraction precipitated from root-nodule extract between 55 and 80% saturation with $(NH_4)_2SO_4$, was used for chromatography. The DE-52 cellulose (Whatman Biochemicals, Maidstone, Kent, U.K.) column was equilibrated with sodium acetate/acetic acid buffer, pH5.6, which was 10mM with respect to acetate. The column diameter was 1.5cm, and its height was 25cm in (*a*) and 13cm in (*b*). The closed mixing chamber contained 80ml of the equilibration buffer. This was first connected to a buffer reservoir containing the same buffer, and when starting the ionic-strength gradient, to a reservoir containing 100mM-acetate buffer, pH5.6. Elution rate was 20ml/h. The gradient was started in (*a*) when the elution volume was 115ml. In (*b*) it was started at an elution volume of 43ml, and a second gradient, containing 0.5M-acetate buffer, pH5.6, was used when the elution volume reached 90ml. However, the leghaemoglobin components eluted with higher ionic strength than 100mM were presumably denaturation products, as were the small components similarly obtained from pea Lb. The absorbance of the effluent fractions was followed at 405nm in a cuvette of 1cm or 0.1cm light-path. The horizontal bars represent eluate containing LbI and LbII in (*a*) and LbI in (*b*) indicated by the appropriate numeral above the peaks. The broken line in (b) represents conductivity.

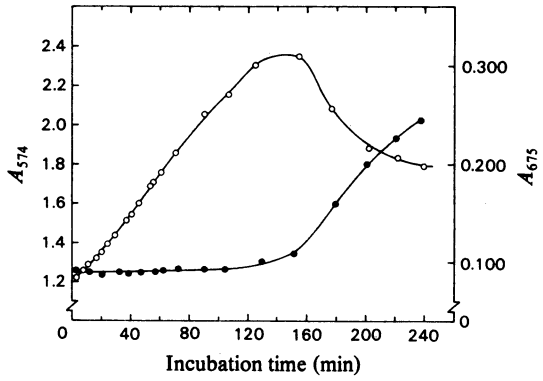

Fig. 3. *Formation of oxyleghaemoglobin* (○) *and bile pigments* (●) *during coupled oxidation of leghaemoglobin*
Ferric Lb*a* from *Phaseolus* (400nmol/2.5ml) was incubated in a cuvette at 23°C in 0.1M-phosphate buffer, pH7.5, containing 2mg of ascorbic acid/ml. The light path was 1cm, and A_{675} (●) and A_{574} (○) were taken from the absorption spectra recorded at time intervals.

Table 1. *Isomer composition of the biliverdin dimethyl esters obtained from different leghaemoglobins*
The isomers formed in coupled oxidation are presented in their order on the thin-layer plate. The values represent the means ± S.E.M. from four experiments, each performed independently from coupled oxidation to biliverdin isomer quantification. The material available from *Pisum* and *Vicia* origin only sufficed for one determination.

	Isomer composition (%)			
	α	δ	β	γ
Glycine max (soya-bean) Lb*a* and Lb*c*	30±4	19±6	51±1	0
Phaseolus vulgaris (kidney-bean) Lb*a*	31±6	23±6	46±6	0
Pisum sativum (pea) LbI	5	0	95	0
Vicia faba (broad-bean) LbI	5	0	95	0

only main leghaemoglobin component of this species; the two main components of soya-bean (*Glycine max*) leghaemoglobin, Lb*a* and Lb*c*; pea (*Pisum sativum*) LbI and LbII; and broad-bean (*Vicia faba*) LbI. The final purification step and identification of the leghaemoglobin components from the two latter species is presented in Fig. 2 to avoid further confusion in the nomenclature. Purification of *Vicia* LbI has been described, though without elution profile (Richardson *et al.*, 1975), but owing to the remarkable degree of polymorphism reported within *Vicia* LbI the possibility of incomplete fractionation cannot be dismissed. The purification of pea leghaemoglobins has not been presented previously.

The coupled oxidation of leghaemoglobins was measured by recording absorption spectra at time intervals. Oxyleghaemoglobin was formed first, as shown by the increase of A_{574} (Fig. 3). Degradation of the haem group, characterized by an absorption increase in the 675nm region and decrease elsewhere, began after a lag period corresponding to the time of oxyleghaemoglobin formation. The experiment presented in Fig. 3 was carried out at room temperature (23°C), whereas the coupled oxidation for preparative purposes was more rapid due to a higher temperature (37°C) and more aerobic conditions, e.g. by performing the reaction in a beaker. Because oxyleghaemoglobin was always formed before the degradation of haem, it seems to be an intermediate in the degradation reaction.

The yield of bile pigments from different leghaemoglobins varied markedly. The ratio of the bile pigment to the remaining undegraded leghaemoglobin was estimated from the absorption spectra, by measuring the ratio A_{657}/A_{574} after coupled oxidation at 37°C for 160min. This absorbance ratio was about 0.2 for *Glycine* Lb*c* and *Pisum* LbII, and about 0.3 for *Phaseolus* Lb*a* and *Glycine* Lb*a*, whereas for *Pisum* LbI it was 1.0 and for *Vicia* LbI 2.1. The ε_{mM} at 675nm was estimated to be 22 on the basis of the spectral changes. Thus the approximate biliverdin yields would be about 20% for *Phaseolus* Lb*a* and for *Glycine* Lb*a*, about 40% for *Pisum* LbI and about 60% for *Vicia* LbI. The coupled oxidation step was critical in view of the yields of methylated biliverdins, as losses in biliverdin isolation and methylation were small.

The bile pigments from coupled oxidation of leghaemoglobins had typical biliverdin absorption spectra during all steps of their purification and esterification. Reference spectra were obtained from biliverdins of haem and myoglobin origin. Some precautions were taken to obtain unequivocal results on the thin-layer plates used for separation and identification of the biliverdin dimethyl esters: samples were applied as bands to improve their separation, and each purified biliverdin isomer of leghaemoglobin origin was finally applied on a second plate over each standard isomer to rule out possible errors arising from the effect of concentration on mobility.

The nature and quantities of the biliverdin isomers from the chemical degradation of leghaemoglobins are presented in Table 1. Bridge specificity of haem breakdown was evident, and the leghaemoglobins belonged to two clearly different groups. The *Pisum* and *Vicia* leghaemoglobins were degraded almost exclusively to biliverdin IX-β, whereas *Glycine* and *Phaseolus* leghaemoglobins were degraded to three

biliverdin isomers. Differences within each of these groups were negligible. Interestingly, the amino acid sequence of *Pisum* LbI (P. Lehtovaara & A. Lappalainen, unpublished work) especially resembles that of *Vicia* LbI, and the primary structure of *Vicia* LbI differs from both *Glycine* and *Phaseolus* leghaemoglobins more than the two latter differ from each other (Richardson *et al.*, 1975). There was thus a clear correlation between similarities of primary structure and site specificity of haem breakdown. The 21% difference between *Glycine* and *Phaseolus* leghaemoglobin sequences was not sufficient to be reflected in the site specificity of haem degradation.

A general feature of the catabolism of leghaemoglobins was the preponderance of the β-isomer, and thus leghaemoglobin breakdown differs from that of animal globins previously studied. In agreement with the results obtained with animal globins, not even traces of the γ-biliverdin isomer were present among the degradation products of leghaemoglobins, but the δ-isomer, so far only reported to be formed in minor amounts from Hb M Iwate (Brown *et al.*, 1977), was formed from *Glycine* and *Phaseolus* leghaemoglobins, where it comprised about 20% of total biliverdins.

Discussion

The degradation of leghaemoglobins to biliverdin isomers has a different specificity than the corresponding degradation of animal globins, which yield either 100% of the α-isomer or mixtures of α- and β-isomers. Leghaemoglobins yield either almost exclusively the β-isomer (*Pisum* and *Vicia* Lb), or about 50% β-, 30% α- and 20% δ-isomer (*Glycine* and *Phaseolus* Lb).

Detailed information concerning the haem environment is available for the following globins: sperm-whale Mb (Kendrew *et al.*, 1961; Takano, 1977), horse Hb α- and β-chains (Perutz *et al.*, 1968), *Chrionomus* (insect) Hb (Huber *et al.*, 1971), lamprey Hb (Hendrickson *et al.*, 1973) and *Glycera* (bloodworm) Hb (Padlan & Love, 1974), in addition to lupin Lb (Vainshtein *et al.*, 1975, 1977). The polypeptide environment of the haem group in animal globins is markedly similar, and the amino acid residues in contact with the haem on the ligand-binding side are at the positions CD1, E7, E11 and G8. The tertiary structure of lupin Lb is closely related to that of animal globins.

The amino acid sequences of different leghaemoglobins were aligned, with lupin Lb as reference, to identify those homologous residues identical within the *Glycine–Phaseolus* group, and also within the *Pisum–Vicia* group, while distinguishing the two groups from each other. In the A helix there are no such positions, and only a few positions in the B and C helices possibly fulfil the condition (the region is partly unknown in the *Pisum* LbI sequence). However, the two leghaemoglobin groups differ from each other in the sequence from CD5 to the beginning of the E helix, indicating that this loop of polypeptide chain far away from the haem may have two different conformations. Moreover, residues E11 and G4 fulfil the condition, whereas the H helix does not seem to contain such residues. It will be demonstrated below how these regions coincide with the regions in contact with the methine bridges α, β and δ.

The residue CD1 is invariably a phenylalanine in all globins, also in all leghaemoglobins, and it is situated almost parallel with the plane of haem in the vicinity of the β-methine bridge in animal globins and in lupin Lb. If the hypothesis of Brown (1976) is correct, the present results indicate that the distance of phenylalanine-CD1 from the haem plane is greater in leghaemoglobins than in haemoglobin and myoglobin, or that the leghaemoglobin molecule is more flexible and allows movement of phenylalanine-CD1. The residue CD5 is lysine in *Pisum* and *Vicia* LbI, yielding 95% of the β-isomer, but alanine in *Glycine* Lb*a* and Lb*c*, and in *Phaseolus* Lb*a*, all yielding only 50% of this isomer. Whether the size and charge of residue CD5 affect the position of CD region and phenylalanine-CD1, must remain unresolved until more detailed reports concerning the tertiary structure of leghaemoglobin are available. In other respects, the amino acid sequences supposed to be in the vicinity of phenylalanine-CD1 on the haem side are closely similar in different leghaemoglobins.

The residue E7 is histidine in mammalian globins and also in all leghaemoglobins so far analysed, and it is in contact with haem near the γ-methine bridge. The fact that cleavage of haem to γ-biliverdin has never been observed in coupled oxidation was explained by Brown (1976) to be due to the steric hindrance of the histidine-E7. No traces of γ-biliverdin were formed in the degradation of the five leghaemoglobins either, and it is possible that the explanation might be the same as in the case of mammalian globins.

The methine bridge δ is hindered from approach of ligands by the residue valine-E11 in myoglobin and haemoglobin, which yield no δ-biliverdin isomer in coupled oxidation. Both *Pisum* LbI and *Vicia* LbI resemble mammalian globins in that they have a valine residue at E11 position and that the methine bridge δ is not degraded. However, degradation of *Glycine* and *Phaseolus* leghaemoglobins yields about 20% of the biliverdin δ-isomer, and the residue E11 in both of these proteins is leucine. In myoglobin the replacement of valine-E11 with leucine, which is longer by one methylene group, should move the hindering methyl groups further away from the methine bridge δ (e.g. Fig. 4.8 of Antonini & Brunori, 1971, or Fig. 3 of Brown, 1976).

Haem contact near the α-methine bridge on the

ligand-binding side is provided by the residue G8, which is isoleucine in myoglobin and in haemoglobin. All leghaemoglobins studied in the present paper have a valine at this position (denoted as G7 by Vainshtein *et al.*, 1977), even though the amount of α-biliverdin obtained from degradation varies from 5% to about 30%. Alignment of the leghaemoglobin sequences reveals considerable identity in the region near the α-methine bridge, with the exception of residue G4 (denoted as G3 in lupin Lb). Those leghaemoglobins that yield only traces of α biliverdin (*Pisum* LbI and *Vicia* LbI) have a histidine residue at this position, whereas those that yield about 30% of this isomer have a glutamic or glutamine residue (*Glycine* Lb*a* and Lb*c*, *Phaseolus* Lb*a*). It is not impossible that the substitution of glutamic acid or glutamine with histidine might affect the position of the G helix relative to the haem so that the distance of valine-G8 to the α-methine bridge comes shorter.

A general correlation was thus observed between the methine-bridge specificity of haem breakdown and the primary structure of the protein. Those leghaemoglobins that were more related with each other were degraded in a similar way. Furthermore, the different accessibilities of the individual methine bridges of each of the five leghaemoglobins to degradation could be tentatively explained by the corresponding differences in primary structure, either at the amino acid residue expected to be in contact with the specific methine bridge or at an amino acid residue in the close vicinity of such a residue. The present results are not in conflict with the hypothesis presented by Brown (1976) for the specificity of haem degradation. Rather, they seem to support it, but a final answer to the question of the role of the protein awaits degradation studies on *Glycera*, *Chironomus* and lamprey haemoglobins.

Supposing that the hypothesis tested in the present paper is correct, the following conclusions can be drawn concerning the ligand-binding site of leghaemoglobins. (1) In all leghaemoglobins phenylalanine-CD1 is either situated further away from the haem or in a markedly more flexible position than in mammalian globins. (2) The iron-bound O_2 molecule, which may be preferentially bound towards the α-methine bridge in mammalian globins, may be preferentially bent towards the β-methine bridge in plant globins. (3) The bound O_2 is bent towards the β-methine bridge in *Pisum* LbI and in *Vicia* LbI, whereas it is relatively free to rotate also in the directions of the α- and δ-bridges in *Glycine* Lb*a* and Lb*c*, and in *Phaseolus* Lb*a*.

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