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Crystalline Leghemoglobin

III. Amino Acid Composition of the Two Main Components

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The amino acid composition of the two main components of leghemoglobin has been determined by the chromatographic technique of Stein and Moore. Separate determinations have been made for tryptophan and amide ammonia. The electrophoretically faster component is shown to contain the following amino acid residues: Asp_{12}, Thr_7, Ser_{11}, Glu_{14}, Pro_5, Gly_8, Ala_{21}, Val_{13}, $Ileu_6$, Leu_{12} Tyr_3, Phe_8, Lys_{13}, His_2, Arg_1, Try_3, $(-CONH_2)_8$ and the electrophoretically slower component: Asp_{11}, Thr_6, Ser_{10}, Glu_{12}, Pro_5, Gly_6, Ala_{24}, Val_{14}, $Ileu_4$, Leu_{12}, Tyr_3, Phe_7, Lys_{13}, His_2, Arg_2, Try_3, $(-CONH_2)_6$. Small but significant differences in the amino acid content of the two components involve: Ala, Arg, Glu, Gly, Ileu, and Val. No significant differences are found in the other amino acids of the two main components. The molecular weight of the faster component calculated on the basis of the amino acid content is 16 695 and for the slower one 15 429. The electrophoretic behavior of the two components can be satisfactorily explained on the basis of the amino acid composition. Leghemoglobin differs from other hemin proteins in showing a very low histidine content and the absence of methionine and cysteine. The results are discussed.

Information on the amino acid composition of leghemoglobin (Lhb) has been limited to one report on the histidine content of an unresolved preparation which was found to be one fourth of that of cow hemoglobin [7]. No other studies on the amino acid content of Lhb have been performed.

The present study reports the amino acid composition of the two main components of Lhb as evaluated mainly by the chromatographic method of ion exchange resin developed by Moore and Stein.

EXPERIMENTAL

Material. The two main components of Lhb used in these studies were prepared as described previously [2,3]. The electrophoretic homogeneity of the components were checked.

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Apoproteins of the two components of Lhb were prepared by adding one volume of cold, salt-free water solution of the two chromoproteins slowly with stirring to twenty volumes of acetone-hydrochloric acid solution (2 ml of N HCl/l of acetone) at −13°C. The precipitate was centrifuged in the cold (4 000 *g*, 5 min.) and dissolved in cold water and dialyzed in the cold against a dilute sodium bicarbonate solution (50 mg/l). A small amount of denaturated protein precipitated and was removed by high-speed centrifugation.

Amino acid analyses

Hydrolysis. 2.5 mg of the two main components of Lhb were hydrolyzed in sealed evacuated tubes in 1 ml 6 N HCl. The hydrolysis was conducted in an oven at 110°C for 20 and 70 h. The cooled tubes were opened and the contents evaporated over NaOH pellets *in vacuo* at room temperature. To complete the removal of hydrochloric acid, small portions of water were occasionally added.

Chromatography. The chromatographic separation of the amino acids in the hydrolysates were performed on columns of Amberlite IR 120 (< 200 mesh) with use of the 0.9 × 150 cm column for the acidic and neutral amino acids and 0.9 × 15 cm column for the basic ones as described by Moore *et al.*[4] The extent of recovery of amino acids from each column was determined by use of a synthetic mixture of amino acids (Schwarz Laboratories Inc) and hydrolysates of the following synthetic peptides: HCl·H·Leu—Phe—Pro·OMe, HCl·H·Gly—Gly—Phe·OEt, H·His—Leu—Leu·OH and HCl·H·Leu—Leu—Leu—Phe—Pro·OMe (Ciba Ltd.).

Determination of amino acids. The new photometric ninhydrin method of Moore and Stein [5] was used.

Determination of tryptophan [6] was carried out by the spectrophotometric analysis of a sample of the apoproteins of the two components in 0.1 N sodium hydroxide.

Determination of cyst(e)ine by oxidation to cysteic acid [7]. 5 mg freeze dried protein was dissolved in 1 ml of cold formic acid (98—100 %) and 0.06 ml of 30 % hydrogen peroxide was added. The reaction was terminated after one hour by addition of 2 ml of distilled water. The oxidized preparation was repeatedly dried *in vacuo* at room temperature over NaOH in order to remove traces of hydrogen peroxide, after which the preparation was hydrolyzed with HCl as above and analyzed for cysteic acid by the chromatographic procedure [4].

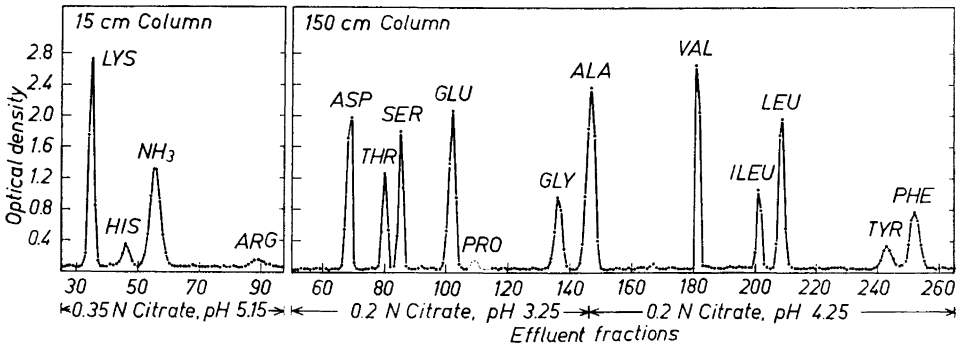

Fig. 1. Eluation patterns given by a 70 h hydrolysate of the electrophoretically faster component of leghemoglobin on Amberlite IR—120 columns. The data are given in optical densities at 570 mμ without corrections for base-line colors and for different color yields of the individual amino acids. The dashed curve for proline gives the optical density at 440 mμ with the base line corrected to that of the other amino acids. The abscissa shows the number of 2-ml fractions collected. The amount of hydrolysate put on the columns corresponded to 1.627 mg of protein.

Determination of amide ammonia. The procedure used was essentially the same as that described by Laki *et al.*[8] The Conway microdiffusion technique was employed and ammonia was determined by the photometric ninhydrin method of Moore and Stein [5]. The protein was heated in 2 ml of 1 N HCl for 4 h in a stoppered tube in a water bath at 100°C. From the hydrolysates, aliquots were added to Conway vessels containing 0.01 N H_2SO_4 in the inner chamber. Saturated sodium tetraborate solution was added to the outer chamber (200 ml sodium tetraborate and 50 ml 2 N NaOH). After standing over night, the solution of the inner chamber was analyzed for ammonia with the ninhydrin method. In order to get an accurate value for the traces of ammonium salts present in the protein sample, diffusion was allowed to continue as described above for periods of 5 to 24 h with the unhydrolysed protein. Under these conditions a slight liberation of amide ammonia occurred. Since the diffusion of ammonia was completed within 3 h, a linear correction for the liberation of amide ammonia could easily be applied.

RESULTS

Typical elution patterns, from the long and short ion exchange columns, obtained with 70 h hydrolysates of the two main components of Lhb are shown in Figs. 1 and 2. The most striking feature of these patterns is the absence of methionine or methionine sulfoxide as well as cysteine or cystine.

Table 1 lists the results of the duplicate experiments for the various amino acids in the hydrolysates of the two components which had been hydrolysed for 20 and 70 h. The variation is expressed as the average deviation. The data show that the recovery of the greater number of amino acids is independent of time of hydrolysis. Extensive decomposition with increasing time of hydrolysis occurred with tryptophan, threonine and serine only. The destruction of tryptophan was complete and therefore this amino acid was determined spectrophotometrically. The average molar ratio of tyrosine to tryptophan for the intact apoproteins of the two components was found to be 1.03 for the faster component and 0.98 for the slower one.

The concentration of serine and threonine were calculated by extrapolation to zero time of hydrolysis.

A comparison of lenghemoglobin with other proteins in regard to the destruction of various amino acids shows a slightly different behavior. Decreases in

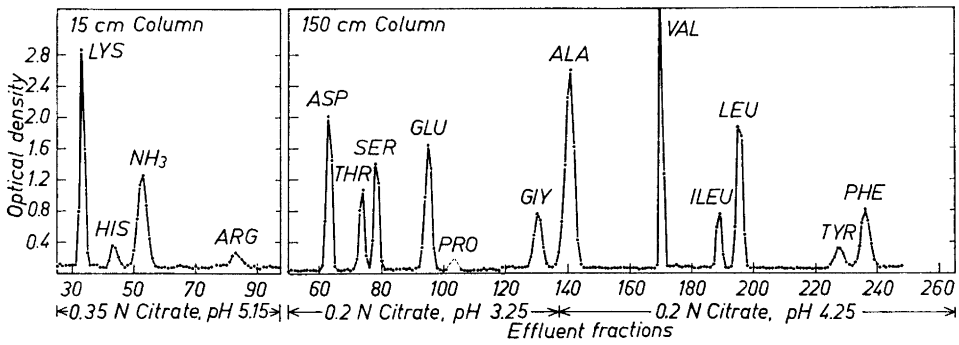

Fig. 2. Eluation patterns given by a 70 h hydrolysate of the electrophoretically slower component of leghemoglobin. The conditions are the same as in Fig. 1. The amount of hydrolysate put on the columns corresponded to 1.519 mg of protein.

Table 1. Amino acid recoveries from the two components of leghemoglobin. The results are in terms of grams of amino acid residue per 100 g of protein.

Amino Acid	Fast component			Slow component		
	Time of hydrolysis		Average or extrapolated value	Time of hydrolysis		Average or extrapolated value
	20 h	70 h		20 h	70 h	
Aspartic acid	8.03 ± 0.11	8.05 ± 0.09	8.04	7.98 ± 0.15	8.03 ± 0.07	8.03
Threonine	4.20 ± 0.05	3.74 ± 0.11	4.38 (a)	4.02 ± 0.00	3.79 ± 0.08	4.10 (a)
Serine	5.43 ± 0.07	4.64 ± 0.10	5.73 (a)	5.35 ± 0.13	4.79 ± 0.22	5.57 (a)
Glutamic acid	10.38 ± 0.11	10.62 ± 0.26	10.62 (b)	9.63 ± 0.32	9.84 ± 0.10	9.84 (b)
Proline	2.94 ±	2.79 ± 0.03	2.88	2.83 ± 0.11	3.04 ± 0.08	3.04 (b)
Glycine	2.61 ± 0.09	2.59 ± 0.00	2.60	2.28 ± 0.00	2.38 ± 0.04	2.38 (b)
Alanine	8.91 ± 0.08	9.04 ± 0.23	9.04 (b)	10.28 ± 0.12	10.89 ± 0.10	10.89 (b)
Valine	6.34 ± 0.14	7.45 ± 0.01	7.45 (b)	7.26 ± 0.05	8.74 ± 0.08	8.74 (b)
Methionine	0.00	0.00	0.00	0.00	0.00	0.00
Isoleucine	3.63 ± 0.07	3.97 ± 0.11	3.97 (b)	2.68 ± 0.05	2.85 ± 0.07	2.85 (b)
Leucine	8.18 ± 0.18	8.28 ± 0.15	8.28 (b)	8.11 ± 0.03	8.56 ± 0.03	8.56 (b)
Tyrosine	2.82 ± 0.04	3.07 ± 0.02	2.95	3.18 ± 0.51	3.25 ± 0.06	3.21
Phenylalanine	6.98 ± 0.25	7.24 ± 0.26	7.11	6.91 ± 0.11	7.10 ± 0.01	7.01
Lysine	9.74 ± 0.22	9.91 ± 0.24	9.83	10.50 ± 0.11	10.52 ± 0.09	10.51
Histidine	1.84 ± 0.09	1.7 ± 0.12	1.77	1.76 ± 0.06	1.95 ± 0.10	1.85
Arginine	0.76 ±	1.3 ± 0.02	1.03	2.13 ± 0.027	2.16	2.15
Cystine	0.00	0.00 (c)	0.00	0.00	0.00 (c)	0.00

(a) These values were obtained by extrapolation to zero time of hydrolysis.
(b) The 70 h value only.
(c) The same result was obtained when cyst(e)ine was determined as cysteic acid in a 50 h hydrolysate of performic acid oxidized protein.

aspartic acid, glutamic acid, arginine and lysine during the acid hydrolysis of carboxypeptidase and papain have been reported by Smith and coworkers [9,10]. Hirs, Stein and Moore have reported decreases in tyrosine, aspartic acid, glutamic acid, proline and arginine during acid hydrolysis of ribonuclease [11]. In the present study none of these amino acids were found to decrease during the period between 20—70 h of hydrolysis.

The yield of valine and *iso*leucine was slightly higher in the 70 h hydrolysates. From earlier work it was expected that the recovery of *iso*leucine and valine was incomplete on short hydrolysis. Peptides in which the carboxyl groups of *iso*leucine or valine are coupled in peptide linkages have shown a great resistance to hydrolysis [9–13]. This depends evidently on a steric hindrance through the branched alkyl residues [14,15]. This phenomenon is not known with leucine. Hence the increase of leucine yield on prolonged hydrolysis evidently depends on the formation of leucine peptides with valine and *iso*leucine. Only 70 h hydrolyses have been used in the final calculation of these amino acids.

Both components seem to lack the sulfur-containing amino acids, although traces of methionine could be detected on some chromatograms. No cystine or cysteic acid could be found in any of the unoxidized preparations. A single run, made after performate oxidation followed by hydrolysis for 50 h, was made

Table 2. Composition and molecular weight of the electrophoretically faster component of leghemoglobin.

Amino Acid	Grams of amino acid residue per 100 g of protein	Minimum molecular weight (d)	Assumed number of residues per molecule	Calculated molecular weight (e)	Calculated numbers of residues for $M = 16\,695$
Aspartic acid	8.04	1 431	12	17 172	11.7
Threonine	4.38 (a)	2 308	7	16 156	7.2
Serine	5.73 (a)	1 520	11	16 720	11.0
Glutamic acid	10.67	1 215	14	17 010	13.7
Proline	2.88	3 371	5	16 855	5.0
Glycine	2.60	2 193	8	17 544	7.6
Alanine	9.04	786	21	16 506	21.2
Valine	7.45	1 330	13	17 290	12.6
Methionine	0.00	—	—	—	—
Isoleucine	3.97	2 848	6	17 088	5.9
Leucine	8.28	1 366	12	16 392	12.2
Tyrosine	2.95	5 528	3	16 584	3.0
Phenylalanine	7.11	2 069	8	16 552	8.1
Lysine	9.83	1 303	13	16 939	12.8
Histidine	1.77	7 745	2	15 490	2.2
Ammonia (NH_2)	0.74 (b)	2 162	8 (b)	17 296	7.7 (b)
Arginine	1.03	15 153	1	15 153	1.1
Cystine	0.00	—	—	—	—
Tryptophan	3.27 (c)	5 691	3	17 073	2.9
Hemin	3.70	—	—	—	—
Totals	92.65		139	16 695	138.2

(a) These values were obtained by extrapolation to zero time of hydrolysis.
(b) These values omitted from the total.
(c) An estimated value based upon a tyrosine/tryptophan ratio of 1.03/1.
(d) (Molecular weight of amino acid residue × 100)/percent of amino acid residue in the protein.
(e) Minimal molecular weight × the nearest integral number of residues.

on both the components (hydrolysates of 2.1 mg of the faster component and 2.7 mg of the slower component were put on the column). No cysteic acid could be detected in these oxidized preparations. As a control, total sulfur was determined on dialysed preparations of the two components after passing them over a column of Lewatit in the OH form in order to remove traces of sulfate. The faster component showed an average sulfur content of 0.055 % S, corresponding to 0.28 atoms of sulfur per protein molecule. The slower component contained 0.15 % S, corresponding to 0.72 atoms of sulfur per protein molecule. In both cases the sulfur content is considerably lower than the minimum required for one sulfur-containing amino acid per molecule of the protein.

The amount of amide nitrogen of the two components was estimated by determining the release of ammonia at 100°C in 1 N HCl. An amide ammonia content of 0.79 % was found for the faster component and 0.66 % for the slower one. These values represent an amide residue content of 0.74 % for the faster

and 0.62 % for the slower component and correspond to 7.7 amide groups per mole for the faster component and 6.0 amide groups per molecule for the slower one.

The present estimates of the composition of the two main components of Lhb are summarized in Tables 2 and 3. For the most stable amino acids the values represent the average of four determinations.

The required limits of accuracy for the estimation of the number of residues of the amino acids in the minimum molecule depends upon the number of residues of any particular amino acid. For the purpose of calculation it has been assumed that if n is the number of residues of the amino acid in question then the maximum permitted variation would be $n \pm 0.4$ and accuracy therefore [16] $\pm 0.4/n$. The limits required for alanine, which is present in leghemoglobin in the largest molar amount, were calculated to be $\pm$ 1.90 % for the

Table 3. Composition and molecular weight of the electrophoretically slower component of leghemoglobin.

Amino Acid	Grams of amino acid residue per 100 g of protein	Minimum molecular weight (d)	Assumed number of residues per molecule	Calculated molecular weight (e)	Calculated number of residues for M = 15 429
Aspartic acid	8.03	1 433	11	15 763	10.8
Threonine	4.10 (a)	2 466	6	14 796	6.3
Serine	5.57 (a)	1 563	10	15 630	9.9
Glutamic acid	9.84	1 317	12	15 804	11.7
Proline	3.04	3 193	5	15 965	4.8
Glycine	2.38	2 398	6	14 388	6.4
Alanine	10.89	652	24	15 648	23.7
Valine	8.74	1 134	14	15 876	13.6
Methionine	0.00	—	—	—	—
Isoleucine	2.85	3 968	4	15 872	3.9
Leucine	8.56	1 321	12	15 852	11.7
Tyrosine	3.21	5 236	3	15 708	2.9
Phenylalanine	7.01	2 098	7	14 686	7.4
Lysine	10.51	1 219	13	15 847	12.7
Histidine	1.85	7 410	2	14 820	2.1
Ammonia (NH_3)	0.62 (b)	2 581	6 (b)	15 486	6.0 (b)
Arginine	2.15	7 260	2	14 520	2.1
Cystine	0.00	—	—	—	—
Tryptophan	3.57 (c)	5 212	3	15 636	3.0
	92.30				
Hemin	3.40				
Totals	95.70		134	15 429	133

(a) These values were obtained by extrapolation to zero time of hydrolysis.
(b) These values omitted from the total.
(c) An estimated value based upon a tyrosine/tryptophan ratio of 0.98/1.
(d) (Molecular weight of amino acid residue × 100)/percent of amino acid residue in the protein.
(e) Minimal molecular weight × the nearest integral number of residues.

faster component ($n = 21$) and $\pm$ 1.60 % for the slower one ($n = 24$). Lysine in the faster component ($n = 13$) requires an accuracy of $\pm$ 3.08 % and in the slower one ($n = 14$) requires an accuracy of $\pm$ 2.86 %. If the analytical data are accurate within 3 %, it should be possible to calculate with assurance the relative number of residues to the nearest integer of each of the amino acids except alanine.

The average molecular weight calculated from all the amino acid residues was found to be 16 695 for the faster component with a standard deviation of $\pm$ 651. For the slower component the average molecular weight was found to be 15 429 with a standard deviation of $\pm$ 548. The molecular weight of 16 800 for the faster component and 15 400 for the slower one obtained by the physical measurements [17] are in good agreement with the recoveries from the Amberlite IR 120 columns. The high degree of accuracy attending the determinations of several amino acids which have been used in the calculations of the minimum molecular weight, adds considerable support to these values. Moreover these estimations are independent of any complications arising from instability of these components, which evidently is the reason for the rather low iron content particularly for the slower component.

When the number as well as the dissociation constants of the various amino acids that make up the protein are known, the theoretical net charges can be calculated as a function of pH. It is assumed that all groups of a given type have the same intrinsic dissociation constant. Although along a peptide chain this may not be true, it nevertheless will provide a value of the proper order of magnitude. The pK values of Cohn and Edsall [18] have been used in this calculation. The terminal amino and carboxyl groups are not considered since they neutralize each other. By subtracting the amount of amide groups from the total amount of carboxyl groups, the theoretical titration curves for both the components could be calculated (Fig. 3). The isoionic point was at pH 4.5 for the apoprotein of the electrophoretically faster component and at pH

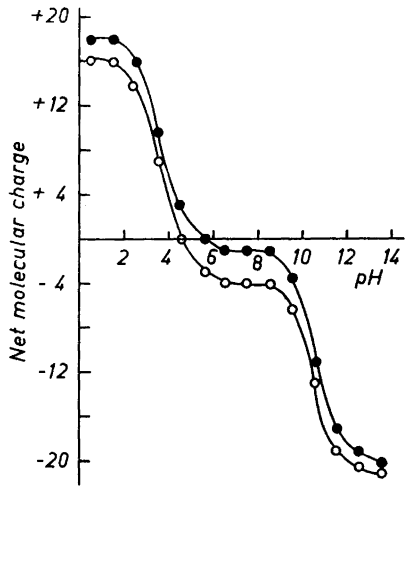

Fig. 3. Charge — pH relationship of the apoprotein of the electrophoretically faster (○) and slower (●) components of leghemoglobin (calculated from the amino acid content on the basis of the following pK's: γ- and δ-carboxyl 3.5, imidazole 5.6, phenol 9.5, ε-amino 10.5, and guanidinium 12.5).

Table 4. Comparative amino acid composition of the electrophoretically faster component of leghemoglobin and some other hemin proteins. The results are in terms of grams of amino acids per 100 g of protein.

Amino Acid	Human Hb A	Human Mb	*Aplysia* Mb	Japanese radish peroxidase	Lhb faster component
Aspartic acid	9.99	8.27	12.71	12.34	9.30
Threonine	5.48	2.85	1.70	5.93	5.16
Serine	4.77	4.43	8.29	9.83	6.93
Glutamic acid	6.89	16.17	9.58	7.06	12.11
Proline	5.22	5.40	0.98	6.58 *	3.42
Glycine	4.46	6.08	4.60	0.76	3.43
Alanine	9.33	5.82	11.66	10.43	11.30
Half cystine		0.00	0.00	2.18	0.00
Valine	10.44	4.64	6.00	4.30	8.81
Methionine	1.25	2.69	1.88	0.97	0.00
Isoleucine	0.02	5.27	4.89	3.62	4.61
Leucine	13.49	13.67	8.73	8.62	9.60
Tyrosine	3.05	2.19	1.48	1.07	3.27
Phenylalanine	7.33	8.22	11.75	5.51	7.80
Histidine	8.32	7.79	1.22	1.19	2.00
Lysine	9.00	19.09	6.89	1.78	11.22
Arginine	3.02	2.47	4.57	3.36	1.15
Tryptophan	2.0	3.40		0.92	3.59
Ammonia (NH_3)		1.22		1.55	0.79

* Proline + hydroxyproline.

5.6 for the apoprotein of the slower one. The two ferri porphyrin carboxyl groups present in the whole leghemoglobin components are omitted from the calculations. These carboxyl groups are expected to contribute somewhat to the acid character of these two proteins. The measured values for the isoelectric points 4.4 for the faster component and 4.7 for the slower one [19], are in good agreement with the isoionic points calculated above for the apoproteins of the two components.

A comparison of Lhb (represented by the electrophoretically faster component) and some other hemin proteins of rather low molecular weight is made in Table 4. These proteins are human (white) hemoglobin A [20] and human myoglobin [21], myoglobin from Mediterranean molluscs (*Aplysia Depilans* and *Aplysia Limacina*) [22] and Japanese radish peroxidase [23].

DISCUSSION

Comparison of the amino acid composition of Lhb with that of hemin proteins of various origins reveals a rather individual pattern of amino acid composition for Lhb (Table 4). Human myoglobin, *Aplysia* myoglobin and leghemoglobin are all in the same low molecular weight range. Lhb and *Aplysia* Mb are rather acidic proteins, whereas human Mb is a neutral protein. The differences in isoelectric points of these proteins are clearly correlated with the major differences in the arginine and lysine contents. In fact, in respect

to nearly all of the amino acids the relative composition of the four proteins are widely different. The most striking difference between Lhb and the other hemin proteins is the absence of the sulfur containing amino acids. This is of special interest because there is now much experimental evidence that all hemoglobins contain SH-groups and that these groups are related to the oxygenation process [24]. Lhb shows in this respect similarities to Mb which also contains no SH-groups.

The complete absence of disulfide bridges in these hemin proteins raises the question of the nature of the tertiary structure of these proteins. Present views ascribe to the α-helix a major role in maintaining coiled peptide chains. By its nature, however, the α-helix is rigid and straight. A molecule made up of the 134—139 amino acids in Lhb all in the form of a single α-helix would have an axial ratio of twenty, whereas in fact both components are nearly spherical. Smith [25] suggests that the covalent bonds involving thiol, imidazole, phenol and possibly other groups linked to carboxyl groups play an important role in maintaining the globular structure. In a protein like myoglobin, which is completely lacking in cysteine or cystine, it is suggested that histidine is implicated in maintaining the regular, globular structure [25].

Looking for some tentative hint of structural relationships among the different hemin proteins, their histidine content deserves special attention particularly as it is generally assumed that histidine functions as a hemelinked group in several hemin proteins. However, the histidine contents of the different proteins seem to vary considerably, even in the proteins containing only one heme per molecule. Lhb shows similarities to *Aplysia* Mb only, which also has a low histidine content.

It is apparent from the data on the amino acid analyses that the two main components definitely represent individual proteins. Several amino acids are present in equal concentrations, within experimental error. The differences in alanine, arginine, glutamic acid, glycine, threonine and valine, although not large, are considered significant. These differences in amino acid content of the two main components seem to satisfactorily explain the differences between the two components in electrophoretic mobility and their behavior on the ion exchange column.

The differences between the two components are rather small and are comparable to those observed in several biologically active proteins where certain amino acids from one species are replaced by other amino acids in another species, without changing the biological properties of the protein. The physical differences are slight because the replacements usually involve a pair of closely related amino acids. The amino acid exchanges most frequently encountered are those first observed in insulin from different species, namely the interchange of valine and *iso*leucine, of serine and glycine, and of alanine and threonine [26,27]. In the case of another peptide hormone, vasopressin, the molecules from the pig and ox are identical except for the presence of an arginine residue in the ox which is replaced by a lysine residue in the pig [28,29]. Of special interest are the amino acid interchanges in the hem-peptide fragments from partial hydrolysates of cytochrome c isolated from different species, namely the interchange of serine and alanine [30], of alanine and glutamic acid [31], and of alanine and leucine [32].

An examination of the differences in amino acid composition between the two main components of Lhb in light of the above examples on interchangeable amino acids suggests that some form of genetic control must be exerted over the synthesis of the globins of the two components.

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