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Crystalline Leghemoglobin

XI. The Amino Acid Sequences of two Histidine-containing Tryptic Peptides of the Slow Component

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A tryptic digest of the slow component of soya leghemoglobin was fractionated on a column of Dowex 1 and two histidine peptides were isolated. The sequences of these two peptides were found to be Ala—Ser—Gly—Thr—Val—Ala—Val—Asp—Ala—Ala—Leu—Gly—Ser—Val—His—Ala—Gln—Lys and Leu—Thr—Gly—His—Ala—Glu—Lys.

The hemin iron of hemoglobin is known to be attached to the globin by a histidine residue on one side of the hemin ring and an iron-bound water molecule by a hydrogen bond to a second histidine residue on the other side.[1,2] It has been assumed that histidine residues are also involved in the binding of the hemin to the globin of leghemoglobin (Lhb), in particular as Lhb has been found to contain only two histidine residues.[3]

The present communication describes the elucidation of the sequence of amino acid residues around the two histidines of the slow component of Lhb.

MATERIALS AND METHODS

Leghemoglobin. The two main components of Lhb were isolated as described previously.[4]

Apoleghemoglobin. The apoprotein of the slow component of Lhb was isolated as before by splitting with acid acetone.[5]

Enzymes. The trypsin used for the digestion of the apoprotein was a crystalline chymotrypsin-free protein salt obtained from Serva AG (Heidelberg, Germany), papain was a twice crystallized preparation from Worthington Biochemical Corporation (Freehold, New Jersey, U.S.A.), carboxypeptidase A was a crystallized DFP-treated preparation and subtilopeptidase A a crystallized preparation, both from Sigma Chemical Company (St. Louis, U.S.A.), and leucine aminopeptidase was a preparation from Worthington Biochemical Corporation (Freehold, New Jersey, U.S.A.).

Digestion of apoprotein with trypsin. Before digestion, the apoprotein was denaturated by incubation at pH 9.5 for 4 min at 95°C. The denaturated protein (250 mg) was digested with 2.5 mg of trypsin for 2 h at 37°C in a pH-stat. An amount of trypsin equal to 0.5 %

of the weight of the substrate was added at 0 and 45 min. The pH was kept at 8.0 by automatic titration with 1 N NaOH. On completion of the hydrolysis, the pH was reduced to pH 5.8 and the slight precipitate that formed was removed by centrifugation. The supernatant solution was lyophilized to dryness.

Column chromatography of the tryptic peptides. A Dowex 1 × 2 column (1.5 × 80 cm) was equilibrated to pH 8.4 at 35°C with a buffer composed of 1 % 2,4,6-collidine and 1 % pyridine adjusted to pH 8.4 with glacial acetic acid.[6] The average rate of flow of the buffer through the column was maintained at 25 ml/h. 380 fractions, each 2.5 ml, were collected. The run was started by adding the sample in a volume of 6 ml, the pH of which had been adjusted to pH 8.82, on to the column. The first 30 fractions were eluted with the pH 8.4 buffer used for equilibration of the column. After fraction 30 had emerged, a gradient elution buffer in respect of pH was applied in the following way: The buffer reservoir was replaced by a 300-ml closed mixing chamber containing the pH 8.4 equilibration buffer and into this a 1 % 2,4,6-collidine — 1 % pyridine buffer of pH 6.5 (adjusted with glacial acetic acid) was permitted to flow. After 169 fractions, the pH 6.5 buffer was replaced by 0.5 N acetic acid, after fraction 225, this was replaced by 1 N acetic acid, and after fraction 252, the 1 N acetic acid was replaced by glacial acetic acid.

Peptides were detected in the effluent by alkaline hydrolysis procedure combined with a slightly modified ninhydrin analysis.[7] 0.1 ml of each sample was incubated with 0.5 ml of 2.5 N NaOH at 90°C for 3 h. The mixture was then neutralized with 0.5 ml of 30 % acetic acid and incubated with 1 ml of ninhydrin solution at 100°C for 15 min, and finally diluted with 5 ml of 50 % ethanol. All of the fractions giving the same peak were pooled and freed of the volatile buffers *in vacuo*. Each peak was tested for histidine with the Pauly reagent.[8]

Further purification of peptides. When necessary, the peptides were purified on Dowex 50 according to Schroeder *et al.*[9]

Paper chromatography. The solvent used in descending paper chromatography was butanol-acetic acid-water (4:1:5) and the paper Schleicher & Schüll MM. The chromatographic mobilities were determined relative to leucine and are denoted by R_{Leu}.

Paper electrophoresis. This electrophoresis was performed on Schleicher & Schüll MM paper. The buffer used contained 5 % pyridine and 0.2 % acetic acid and had a pH of 6.2. The electrophoresis was usually carried out employing a potential gradient of 30 V/cm. The electrophoretic mobilities at pH 6.2 were determined relative to lysine and aspartic acid and are denoted by E_{Lys} and E_{Asp}, respectively. Caffeine was used to measure the electroendosmotic mobility.

Amino acid analyses. The quantitative compositions of the peptides were determined after total hydrolysis in 2 ml of 6 N HCl at 108°C for 18 h in evacuated sealed tubes. After hydrolysis, the amino acids were determined in an automatic amino acid analyzer (Beckman 120 B) as described by Spackman *et al.*[10]

Sequence studies. Peptide T-6 (2 μmoles) was hydrolyzed in 1 ml of 0.2 M pyridine acetate buffer, pH 5.5, to which 2.5 μl of 2,3-dimercaptopropanol and 200 μg of papain had been added. The mixture was incubated at 37°C for 24 h and lyophilized. The formed peptides were separated by ion exchange chromatography on Dowex 50 [9] and purified further by paper electrophoresis at pH 6.2.

The subtilisin digest was carried out in a 0.2 M *N*-ethyl-morpholine—acetic acid buffer, pH 8.0, with a 1:100 molar ratio at 37°C for 16 h. The final concentration of peptide was about 0.75 μmole/ml.

For carboxypeptidase digestion, the peptide (0.05—0.1 μmole) was dissolved in 0.2 ml of 0.2 M $NaHCO_3$, pH 8.3. 10 μl of the carboxypeptidase solution was added and the mixture incubated for the indicated time at 37°C. The carboxypeptidase solution was prepared by adding 5 μl of enzyme suspension (50 mg/ml) to 50 μl of buffer containing 40 μl 2 M NaCl and 10 μl of 1 M disodium hydrogen phosphate, pH 8.0. Hydrolysis was terminated by adding 50 μl of 6 N acetic acid, after which lyophilization was performed. After adding citrate buffer of pH 2.2, the liberated amino acids were determined on the amino acid analyzer.

The leucine aminopeptidase digestion was carried out by incubating at 37°C 0.05 μmole of peptide in 0.1 ml of 0.1 M Tris—HCl buffer, pH 8.5, containing 0.2 M $MgCl_2$ and 10 μl of the enzyme suspension (5.5 mg/ml). After the indicated time, the solution was lyophilized and redissolved in a citrate buffer of pH 2.2 and the amino acids analyzed on the amino acid analyzer.

Sequential degradation of peptides from the amino terminus was performed by the Edman procedure as modified by Konigsberg and Hill using 50 % pyridine containing 1 % triethylamine as the solvent.[11,12]

The nomenclature of the tryptic peptides of Lhb is the same as that used in a forthcoming paper on the tryptic peptides of Lhb.

RESULTS

The histidine-containing fractions I and II are indicated by black bars in the elution diagram of the tryptic peptides of the slow component of Lhb on a Dowex 1 column (Fig. 1).

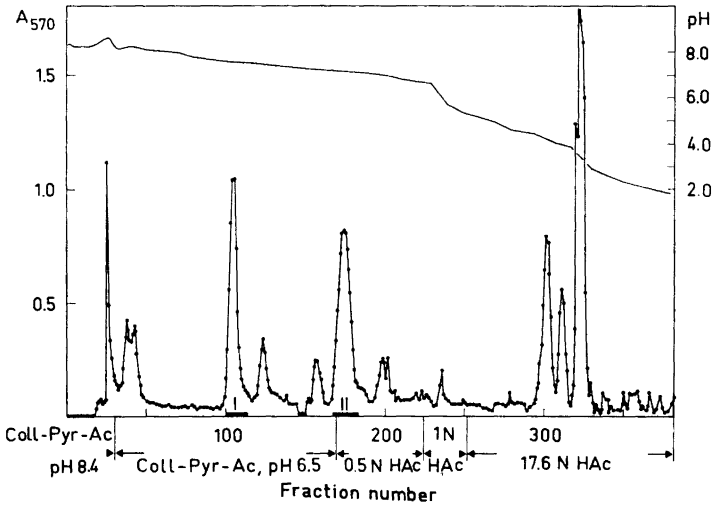

Fig. 1. Elution pattern of the tryptic digest of the slow component of leghemoglobin. Chromatography was performed at 35°C on a column of Dowex 1 × 2 (1.5 × 80 cm, 200—400 mesh). Fractions 2.5 ml in volume were collected at a flow rate of 25 ml/h. The first 30 fractions were eluted with the pH 8.4 buffer, after which the buffer in the reservoir was replaced by a gradient buffer. The closed mixing chamber contained 300 ml of the pH 8.4 buffer. The different gradient buffers are indicated in the figure. The horizontal black bars mark the pooled fractions from which the histidine peptides were isolated.

Fraction I was eluted from the column in the pH range of 8.05—7.95. The fraction was found to consist of a pure peptide, designated T-6, on the basis of paper chromatography and electrophoresis ($R_{Leu}=0.07$, $E_{Lys}=0.15$). The amino acid composition of the peptide is given in Table 1. The peptide was slightly basic when subjected to electrophoresis at pH 6.2; this indicated that at least one of the two dicarboxylic acid residues was present in amide form. The Edman degradation established the amino terminal sequence Ala—Ser— (Table 2). Digestion with leucine aminopeptidase liberated alanine and serine and, in addition glycine and threonine in quantities which confirmed that the sequence of the first four N-terminal amino acids was Ala—Ser—Gly—Thr—.

Table 1. Amino acid composition of the histidine containing tryptic peptides of the slow component of Lhb.

Amino acid	T-6	T-11
Aspartic acid	1.02	
Threonine	0.97	0.99
Serine	1.90	
Glutamic acid	1.05	1.05
Glycine	2.03	1.02
Alanine	5.09	1.04
Valine	2.95	
Leucine	1.00	0.99
Histidine	0.86	0.94
Lysine	1.05	0.97

Peptide T-6 was digested with papain at pH 5.5 and 37°C for 24 h and the products were fractionated by ion exchange chromatography as described in Fig. 2. The compositions of the papain peptides (Pa) are given in Table 3.

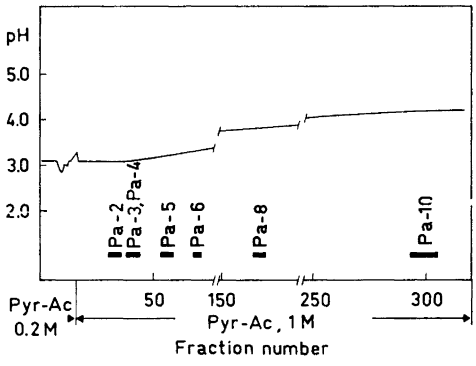

Fig. 2. A diagram showing the elution of the papain peptides from peptide T-6. The chromatography was performed at 35°C on a column of Dowex 50×2 (0.9×55 cm, 200—400 mesh) equilibrated with 0.2 M pyridine acetate buffer, pH 3.1. Gradient elution was begun at fraction 16 by allowing 1.0 M pyridine acetate buffer, pH 5.0, to flow into a closed mixing chamber containing 500 ml of the starting buffer. The flow rate was maintained at 20 ml/h and 2 ml effluent fractions were collected.

Peptide Pa-2, which eluted with the starting buffer, was acidic when subjected to electrophoresis at pH 6.2. The results of the Edman degradation in Table 2 show that the sequence of the peptide is Asp—Ala—Ala—Leu—Gly. This peptide had evidently split into peptides Pa-4 and Pa-6. Peptide Pa-4 is an acidic peptide that emerged from the column together with the neutral peptide Pa-3 from which it was easily separated by paper electrophoresis at pH 6.2. The sequences of Pa-4 and of Pa-6 after one Edman step were found to be Asp—Ala—Ala and Leu—Gly, respectively (Table 2).

Since the NH_2-terminus of T-6 was found to be Ala—Ser—Gly—Thr, it is evident that Pa-3 and Pa-5 both are NH_2-terminal portions of peptide T-6. The results of Edman degradation and analyses of carboxypeptidase A and leucine aminopeptidase digests of Pa-5 are summarized in Table 2. These data show that the sequence of Pa-5 is Ala—Ser—Gly—Thr—Val—Ala.

Table 2. Sequence studies on peptide T-6.

Edman degradation	
Step 1	Residue: Asp 1.05, Thr 0.94, Ser 1.97, Glu 1.16, Gly 2.15, *Ala 4.26*, Val 2.39, Leu 1.08. His and Lys were not estimated.
Step 2	Residue: Asp 1.03, Thr 0.95, *Ser 1.28*, Glu 1.04, Gly 1.93, Ala 3.97, Val 2.13, Leu 0.95. His and Lys were not estimated.
Step 3	Residue: Asp 1.07, Thr 0.84, Ser 1.28, Glu 1.03, *Gly 1.54*, Ala 4.11, Val 1.94, Leu 0.96. His and Lys were not estimated.
Leucine aminopeptidase 30 min	Ala 1.00, Ser 0.94, Gly 0.46, Thr 0.30
Papain peptides	
Pa-2	Asp—Ala—Ala—Leu—Gly
(R_{Leu}=0.64, E_{Asp}=0.45)	
Edman degradation	
Step 1	Residue: *Asp 0.12*, Gly 1.00, Ala 2.00, Leu 0.91
Step 2	Residue: Gly 1.00, *Ala 1.26*, Leu 0.90
Step 3	Residue: Gly 1.00, *Ala 0.81*, Leu 0.84
Step 4	Residue: Gly 1.00, Ala 0.78, *Leu 0.65*
Pa-3	Ala—Ser—(Gly,Thr,2Val,Ala)
(R_{Leu}=0.64, neutral at pH 6.2)	
Edman degradation	
Step 1	Residue: Thr 0.94, Ser 0.95, Gly 1.05, *Ala 1.00*, Val 1.69.
Step 2	Residue: Thr 0.94, *Ser 0.28*, Gly 0.96, Ala 1.00, Val 2.02.
Pa-4	Asp—Ala—Ala.
(R_{Leu}=0.38, E_{Asp}=0.59)	
Edman degradation	
Step 1	Residue: *Asp 0.36*, Ala 2.00.
Pa-5	Ala—Ser—Gly—Thr—Val—Ala
(R_{Leu}=0.25, neutral at pH 6.2)	
Edman degradation	
Step 1	Residue: Thr 0.94, Ser 1.00, Gly 1.05, *Ala 1.16*, Val 1.80.
Step 2	Residue: Thr 0.97, *Ser 0.32*, Gly 1.03, Ala 1.17, Val 1.56.
Step 3	Residue: Thr 0.97, *Gly 0.56*, Ala 1.23, Val 1.62.
Leucine aminopeptidase	
30 min	Ala 1.00, Ser 0.49, Gly 0.22, Thr 0.05.
Carboxypeptidase A	
10 min	Ala 1.00, Val. 0.65.
30 min	Ala 1.00, Val 0.82, Thr 0.19.
Pa-5/S-1	(Ala, Ser, Gly, Thr).
(R_{Leu}=0.22, neutral at pH 6.2)	
Amino acid composition	Thr 0.84, Ser 0.98, Gly 1.02, Ala 1.17, (Val 0.32)
Pa-5/S-2	(Val, Ala).
(R_{Leu}=1.15, neutral at pH 6.2)	
Amino acid composition	(Thr 0.33, Ser 0.45, Gly 0.47), Ala 1.55, Val 1.0.
Pa-6	Leu—Gly.
(R_{Leu}=0.94, neutral at pH 6.2)	

Table 2. Continued.

Edman degradation	
Step 1	Residue: Gly 1.00, *Leu 0.*
Pa-8	(Ala, Gln, Lys).
(R_{Leu}=0.05, E_{Lys}=0.59)	
Pa-10	Ser—Val—His—Ala—Gln—Lys.
(R_{Leu}=0.02, E_{Lys}=0.69)	
Edman degradation	
Step 1	Residue: *Ser 0*, Glu 1.03, Ala 1.04, Val 0.89, His 1.03, Lys 0.33.
Step 2	Residue: Glu 1.00, Ala 1.04, *Val 0*, His 0.97, Lys 0.54.
Step 3	Residue: Glu 1.03, Ala 0.97, *His 0.48*, Lys 0.43,
Step 4	Residue: Glu 1.00, *Ala 0.55*. Lys was not estimated.
Leucine aminopeptidase 40 min	Ser 1.00, Val 0.59, His 0.58, Ala 0.42

Table 3. Compositions of papain peptides from peptide T-6.

Amino acid	Pa-2	Pa-3	Pa-4	Pa-5	Pa-6	Pa-8	Pa-10
Aspartic acid	0.96		0.99				
Threonine		0.96		0.75			
Serine		0.99		1.00			0.91
Glutamic acid						1.11	1.00
Glycine	1.05	1.03		1.00	1.05		
Alanine	2.03	2.02	2.01	1.87		0.89	0.96
Valine		1.51		1.21			0.72
Leucine	0.96				0.95		
Histidine							0.82
Lysine						0.97	1.00
Yield, %	25	51	7	21	11	2	65

Because some Edman degradation experiments with Pa-5 led to losses of alanine and threonine in the first step, which indicated either a lability of the Gly—Thr bond or a possible acyl shift N→O, a confirmative subtilisin digest was performed on Pa-5. The peptides formed were purified by electrophoresis at pH 6.2 and Pa-5/S-1 was sufficiently pure to give a satisfactory amino acid analysis indicating that splitting had occurred at the Thr—Val bond (Table 2).

Lysine was assigned as the COOH-terminal residue of T-6 on the basis of the specificity of trypsin. The peptides Pa-8 and Pa-10 both include the COOH-terminal lysine. The results of the Edman degradation and analysis of the leucine aminopeptidase digest given in Table 2 show that the sequence of Pa-10 is Ser—Val—His—Ala—Gln—Lys. The electrophoretic properties of Pa-8 and Pa-10 require that the glutamic acid residue exists as glutamine in the intact peptide.

The partial sequence work and the compositions of the papain peptides of peptide T-6 permit the complete sequence of the octadecapeptide to be written as shown in Fig. 3.

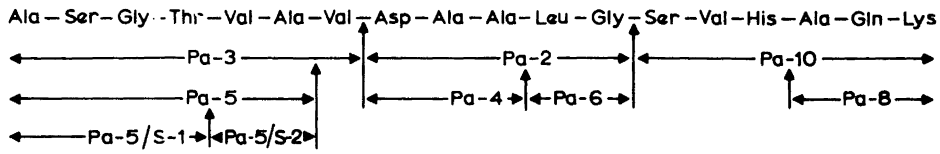

Fig. 3. The amino acid sequence of peptide T-6.

Fraction II was eluted from the Dowex 1 column at a pH near 7.30. It was found to consist of two components in addition to a histidine-containing peptide when examined by paper chromatography. Purification was achieved by ion exchange chromatography on Dowex 50 as shown in Fig. 4. The third

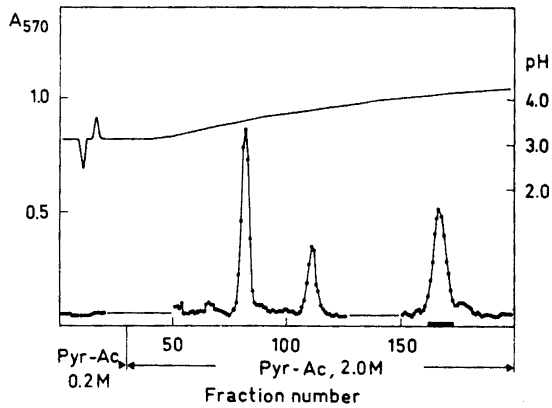

Fig. 4. Elution pattern obtained when fraction II from the Dowex 1 × 2 column was subjected to chromatography on a column of Dowex 50 × 2 (0.9 × 55 cm, 200–400 mesh) at 35°C. The column was equilibrated with 0.2 M pyridine acetate buffer, pH 3.1. Gradient elution was begun at fraction 30 by allowing 2.0 M pyridine acetate buffer, pH 5.0, to flow into a closed mixing chamber containing 500 ml of the starting buffer. Fractions 2.0 ml in volume were collected at a flow rate of 20 ml/h. The solid bar indicated the pooled fractions of the histidine peptide (T-11).

eluted peak from the column gave a positive Pauly test for histidine and was designated peptide T-11. The amino acid composition of the peptide is given in Table 1. The fact that the heptapeptide was found to be slightly basic when subjected to electrophoresis at pH 6.2 indicated one free carboxyl group on the glutamic acid residue ($R_{Leu}=0.11$, $E_{Lys}=0.33$) Edman degradation and the analysis of the leucine amino peptidase digest of the peptide indicated the sequence Leu–Thr–Gly–His–Ala–Glu–Lys (Table 4).

Table 4. Sequence studies on peptide T-11.

Edman degradation	
Step 1	Residue: Thr 0.98, Glu 0.99, Gly 0.97, Ala 1.00, *Leu 0*, His 1.00, Lys 0.54.
Step 2	Residue: *Thr 0.23*, Glu 1.04, Gly 1.01, Ala 1.01, His 0.95, Lys 0.69.
Step 3	Residue: Glu 1.06, *Gly 0.59*, Ala 0.96, His 0.98, Lys 0.45.
Step 4	Residue: Glu 0.98, Ala 1.02, *His 0.49*, Lys 0.36.
Step 5	Residue: Glu 1.00, *Ala 0.80*, Lys was not estimated.
Leucine aminopeptidase 20 min	Leu 1.00, Thr 0.53, Gly 0.17.

DISCUSSION

On the assumption that the two histidine residues of Lhb are involved in binding the hemin to the globin of Lhb, the structures of peptides T-6 and T-11 can be compared with the sequences around the distal and proximal histidine residues in hemoglobin. A general feature of the sequences around these histidines is that residues of neutral amino acids are located on both sides of the histidines.[13] A similar distribution of neutral amino acids was found in peptides T-6 and T-11. Glycine and alanine are generally found in these positions. When the comparison of the Lhb peptides and the sequences around the two histidine residues in hemoglobin is extended further, it seems that T-6 and T-11 are structurally closer to the sequences around the proximal histidine than to those around the distal one, which latter are characterized by the presence of several lysine residues forming the basic center of the peptide chain.

It was recently shown that native Lhb is a hemochrome in which presumably two histidine residues are bound to the hemin iron.[24] It is plausible that the formation of such bonds is particularly favored by the hydrophobic structures around the histidine residues in peptides T-6 and T-11.

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