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[原著] Amino Acid Sequence of Avian Hemoglobins and Its Evolutiomary Implication at仙e Molecular Level 1. Partial Amino Acid Sequence of the $ \alpha $ Chain of Japanese Quail QII Hemoglobin

メタデータ	言語: English 出版者: 琉球大学医学部 公開日: 2010-06-30 キーワード (Ja): キーワード (En): $ \alpha $ chain, amino acid sequence, hemoglobin, Japanese quail, evolution 作成者: Takei, Hiroshi, Eguchi, Yukinori メールアドレス: 所属:
URL	http://hdl.handle.net/20.500.12000/0002015753

Ryukyu Med. J., 7(2): 79~89, 1984.

Amino Acid Sequence of Avian Hemoglobins and Its Evolutionary Implication at the Molecular Level 1. Partial Amino Acid Sequence of the α Chain of Japanese Quail QII Hemoglobin

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Key words : α chain, amino acid sequence, hemoglobin, Japanese quail, evolution

Abstract

Amino acid sequence of the α chain of Japanese quail (*coutournix coutournix japonica*) hemoglobin QII was investigated and determined except its 'core' portion. The carboxymethylated α chain was hydrolysed with trypsin and 13 tryptic peptide fragments were purified by using ion exchange chromatography, Sephadex gel filtration, etc.

Ten peptides of them, T-1, T-2, T-3, T-5, T-7, T-8, T-10, T-11, T-12 and T-13 were short ones and their amino acid sequence was directly determined mainly by Edman degradation method.

Since peptides T-4 and T-6 were longer ones, they were further hydrolysed with chymotrypsin and the amino acid sequence of the chymotryptic peptides purified was respectively determined by DNS-Edman or Edman degradation method. The peptide T-9 was also a longer one and was subjected to digestion with thermolysin and the amino acid sequence of the thermolytic peptides purified was determined by Edman degradation method.

The amino acid sequence of the soluble fraction of the α chain of Japanese quail QII hemoglobin thus determined was compared to that of chicken AII hemoglobin. Their homology was so high with each other that the percentage of amino acid differences was 10.9 and the amino acid residues placed in the important position involved in hemoglobin function was less variable.

Introduction

Amino acid sequences of several avian hemoglobins have been so far determined.[1),2),3)] We have investigated the amino acid sequence of the α chain of the major component (QII) of Japanese quail (*coutournix coutournix japonica*) hemoglobins in order to study the mechanism of evolution at the molecular level.

In this paper we here report on the partial amino acid sequence of the α chain of

the QII component. The Japanese quail hemoglobins consist of two components, the major component (QII) and the minor one (QI), each of which is homologous in amino acid sequence to the chicken corresponding components, AII and AI, respectively.

According to the animal classification table, the quail and the chicken belong to the same suborder, *galli*. By comparing the amino acid sequence of the hemoglobins from Japanese quail with those from chicken, we are going to examine the phylogenetic relationships between these two bird species at the molecular level and to approach the evolutionary implication of the two different molecular species, the major and the minor components.

Materials and Methods

I. Preparation of hemolysates

Blood was collected by cutting cervical veins into a beaker containing 3.8% sodium citrate as an anticoagulant and centrifuged at 3,000 r. p. m. for 5 min to separate the erythrocytes from the plasma and the other components. The preparation of hemolysates was performed in principle according to the Drabkin's method.[4),5)]

II. Preparation of QII component from the hemolysates

2 ml of the hemolysates thus purified was dialysed against 0.01 M phosphate buffer containing 0.01% (w/v) KCN (pH 6.85) and applied on a CM-52 (Whatman, England) column (1.0 × 17 cm) which had previously been equilibrated with the same buffer (pH 6.85) as above. The elution was performed by forming a linear gradient between the two buffers, the starting 0.01 M phosphate buffer (pH 6.85) and 0.02 M phosphate buffer containing 0.01% (w/v) KCN (pH 9.35). Optical density was measured at 415 nm.

III. Cellulose acetate membrane electrophoresis

The electrophoresis on cellulose acetate membrane (Chemetron, Italy) was carried out in 0.12 M Tris-0.30 M glycine buffer (pH 8.9) at 200 V for 1 hr and the membrane was stained with 0.5% amino black solution.

IV. Separation of the α and β chains

Amberlite CG-50 (Type 2) column (2.5 × 21 cm) was prepared after the resin was activated and then equilibrated with 0.01 N HCl.

We modified the previous method described elsewhere in a preceding paper for separation of the α and β chains.[6)]

The lyophilized QII hemoglobin (400 mg) was dissolved in 40 ml of 4 M urea-HCl buffer (pH 2.3) with 2-mercaptoethanol added at 0.5 ml/dl and allowed to stand stirring at room temperature for 50 min. Approximately 40 ml of the precipitated resin previously equilibrated and suspended in 0.05 N HCl was then added to the above hemoglobin solution, which was mixed another 30 min to adsorb the globin on the resin. The mixture was applied on the above prepared Amberlite CG-50 column and the elution was performed by starting with 4 M urea-HCl buffer (pH 2.3) and forming a urea concentration gradient with a 8 M urea-HCl (pH 2.3) in a supply bottle. Both of the buffers contained 2-

mercaptoethanol in the volume ratio of 0.5 ml/dl. Fifteen ml fraction was collected and the absorbance at 280 nm was measured. The fractions corresponding to the α polypeptide chain was pooled and dialysed against deionzed water and lyophilized.

V. Carboxymethylation of the α polypeptide chain

500 mg of the α chain was dissolved in 60 ml of 8 M urea-0.35 M Tris-0.004 M EDTA-HCl buffer and the pH of the solution was adjusted to 8.2 by 1.0 N HCl.

The carboxymethylation of the α chain was performed according to the method described elsewhere.[7] The purified sample by a sephadex G-25 column (3 × 95 cm) was then lyophilized.

VI. Tryptic digestion of the carboxymethylated α chain

The carboxymethylated QII α chain (780 mg) was dissolved in 100 ml of deionized water and the tryptic digestion of the solution was performed according to the method described elsewhere.[8]

The digest solution was then centrifuged at 5,000 r. p. m. for 20 min to remove the undigested fraction and the pH of the supernatant was adjusted to 6.4 by the addition of 1 N HCl. The pH 6.4 solution was then allowed to stand overnight in a cold room and centrifuged at 5,000 r. p. m. for 20 min. The supernatant was named 'soluble fraction' and the precipitates, "core".

VII. Gel filtration of the tryptic digests

Sephadex G-50 was used for gel filtration and the column size was 5 × 140 cm. 0.05 M ammonium bicarbonate buffer was used for the gel filtration. The fractions collected were 15 ml and the optical density of each fraction was measured at 280 and 230 nm.

VIII. Ion exchange chromatography for separation of the peptides

Chromo Beads P (Technicon, U. S. A.) was used as an ion exchange resin. The column size was 0.9 × 15 cm and the column temperature was kept at 50°C. The elution of the sample by forming a pyridine concentration gradient from 0.05 M to 2.0 M by using pyridine-acetate buffer and the photometric measurement of the eluates at 570 nm after ninhydrin reaction were carried out automatically by using an automatic peptide analyzer (Technicon, U. S. A.).

IX. Paper chromatography of the peptides

Toyo filter paper No. 50 (40 × 40 cm) was used and the descending method was employed. The upper layer of the mixture of n-butanol, acetic acid and water (4 :1 : 5 by volume) was taken off and used as a developer and the paper chromatography was run for 15 hr. The peptides developed on the paper were detected by spraying 0.2% ninhydrin-butanol solution.

X. High voltage paper electrophoresis of the peptides

High voltage paper electrophoresis was carried out according to Offord's method.[8] Toyo filter paper No. 51 (60 × 60 cm) was used and a mixture of pyridine, acetic acid and water (25 : 1 : 225 by volume, pH 6.5, $\mu \fallingdotseq 0.07$) was prepared as the buffer for electrophoresis. The electrophoresis was run at 3,000 V for 45 hr and the peptides on the paper were detected with 0.2% ninhydrin-butanol solution.

XI. Amino acid analysis

Approximately 0.1 μmol of each purified peptide was placed in a test tube, 1 ml of 6 N HCl was added to it and the peptide was hydrolysed at 110°C for 20 hr. The hydrolysates were analyzed by an automatic amino acid analyzer (JEOL JLC-6AH).

XII. Amino acid sequence analysis

A. Edman's method

Each peptide sample, the amount of which was equivalent to approximately 0.1 μmol for one step, was taken into a test tube. The coupling reaction of the peptide with PITC (phenyl isothiocyanate) and the splitting reaction with TFA (trifluoro acetic acid) were performed according to the Edman's method.[9] After the reactions, the TFA was lyophilized, the dried sample was dissolved in 1 ml of deionized water and the solution was washed with 2 ml of ethylacetate 2 times. After the ethylacetate layer was removed with pipet, aliquot of the water layer was taken and hydrolysed with 6 N HCl or dansylated for determination of amino acid sequence.

B. Dansylation

Aliquot of the water solution containing approximately 2 nmol of a peptide was placed in a test tube and lyophilized, after which 10 μl of 0.2 M $NaHCO_3$was added and again lyophilized. 10 μl of deionized water and 10 μl of 5 mM of dansyl chloride (Wako Chemicals)-acetone solution were added to the dried sample and the solution was kept at 37°C for 1 hr and lyophilized. 50 μl of 6 N HCl was then added to the dried sample and hydrolysed at 105°C for 18 hr. After the hydrolysis, the dansylated amino acid was extracted with 50 μl of ethylacetate 2 times and subjected to polyamido thin layer chromatography to identify the dansylated amino acid.[10),11)]

Results

In this paper, we determined the amino acid sequence of the soluble fraction of the α chain of the major component (QII) of Japanese quail hemoglobins.

The QII component of the quail hemoglobins was separated by using CM-52 according to the procedure described in the preceding section. Optical density was measured at 415 nm and a chromatography pattern as shown in Fig 1 was obtained. In this figure two components QI and QII were obtained and the ratio of the amount QI to QII was about 1 to 2.

Polypeptide chain separation into α and β was performed by using Amberlite CG-50 (Type 2) according to the procedure as described above in the preceding section and a chromatography pattern as shown in Fig 2 was obtained. The shaded portion on the chromatograph corresponded to the α polypeptide chain.

After the α polypeptide chain of the QII component was carboxymethylated and subjected to the tryptic digestion according to the procedure described in the preceding section, the soluble fraction of the tryptic digests of the α chain was prepared. The sample thus prepared was passed through the column of a Sephadex G-50 and 5 fractions named T-I, T-II, T-III, T-IV and T-V respectively were obtained as shown in Fig 3. T-II fraction was purfied by rechromatography on the Sephadex G-50 column of the same scale as above.

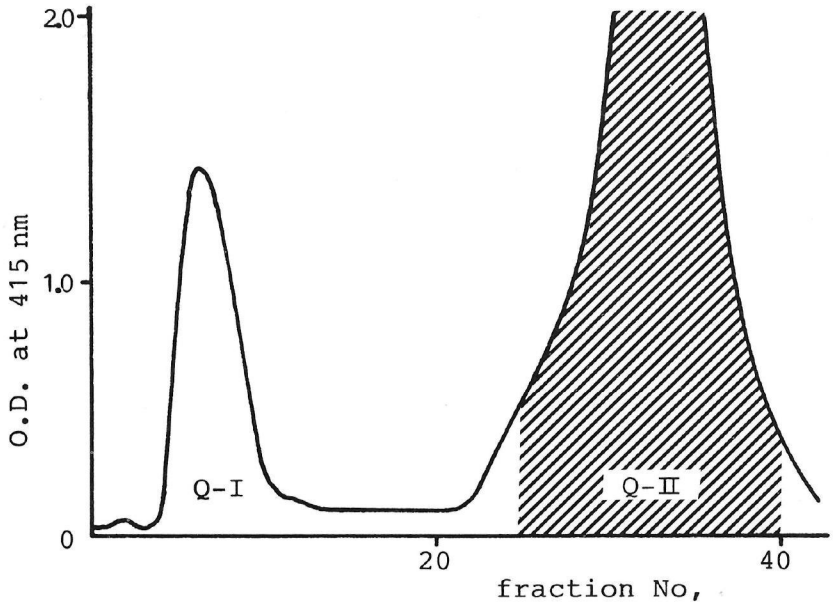

Fig. 1 CM-52 column chromatogram of quail hemolysates.

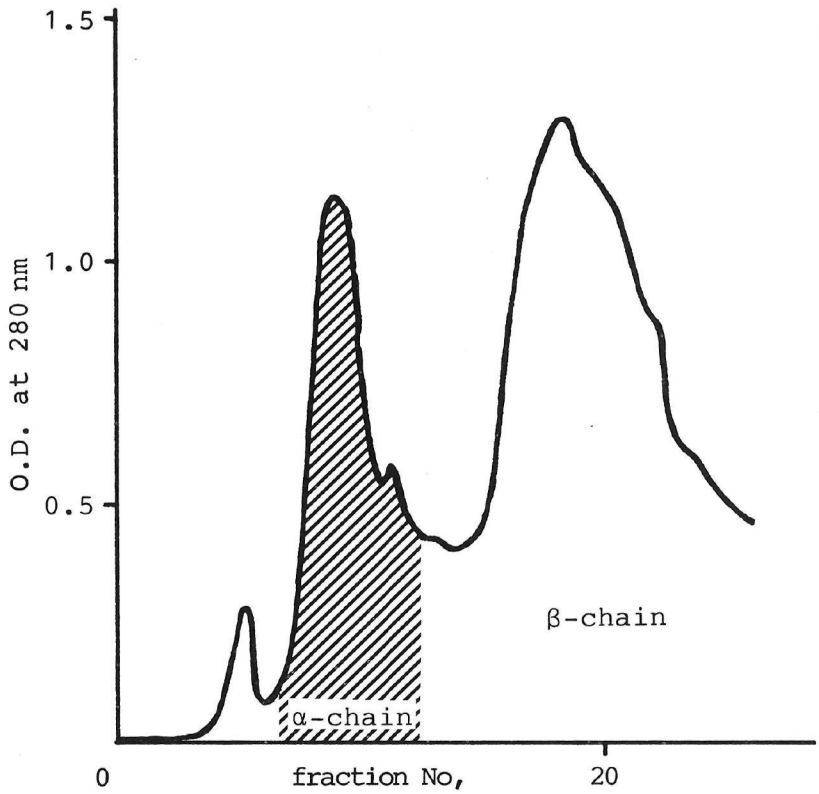

Fig. 2 Purification of Q-II α-chain on Amberlite CG-50.

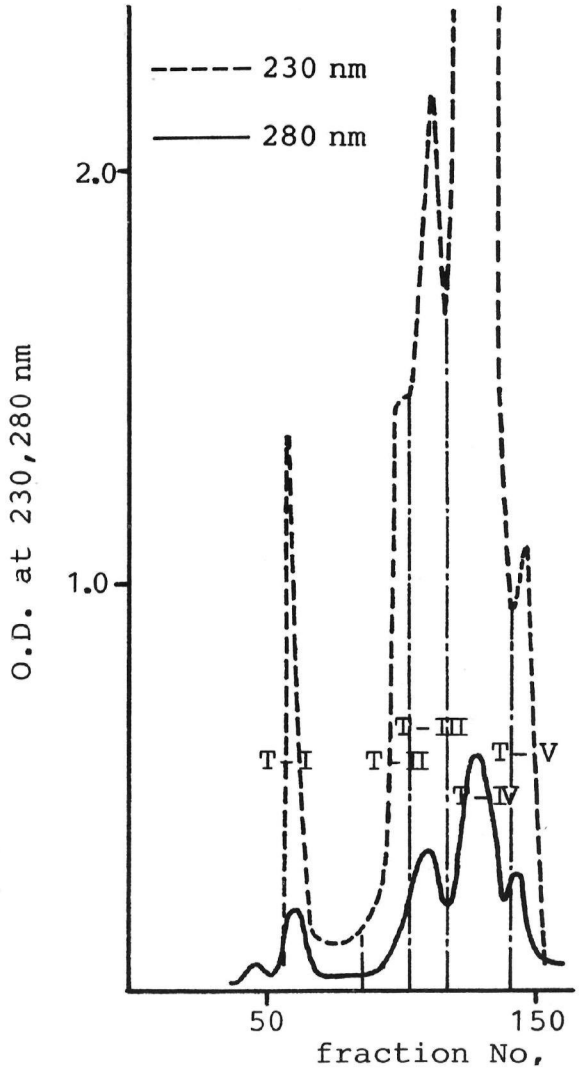

Fig. 3 Purification of tryptic peptides on Sephadex G-50.

The fractions T-III, T-IV and T-V were chromatographed on the column of Chromo Beads P and the purity of each peptide was examined by paper chromatography or high voltage paper electrophoresis and if necessary, the peptides were subjected to re-chromatography or reelectrophoresis (Fig 4).

Each peptide thus purified was subjected to amino acid analyses and the amino acid composition of each peptide was obtained as Table 1.

Amino acid sequence of each peptide was determined mainly by using DNS-Edman's method, some of the peptide, determined by using subtractive method (Table 2).

The distinction between acid and acid amide of amino acids was determined by high voltage paper electrophoresis.

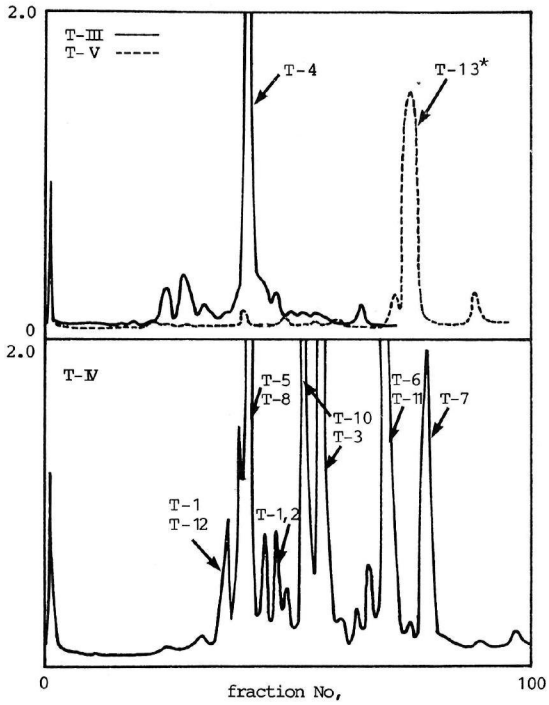

Fig. 4 Purification of tryptic peptides on Chromo Beads P.*) Number of order of tryptic peptides from NH_2-terminus

Table 1 Amino acid composition of tryptic peptides of α-chain from quail hemoglobin.
*) purified by paper chromatography and high-voltage paper electrophoresis

	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8	T-9	T-10	T-11	T-12	T-13
	IV-3-E	IV-7	IV-10-E	III-7-A	IV-5-D	IV-14-C	IV-16-A	IV-4-A	T-II	IV-9-B	IV-14-D	IV-3-F	V-8-B*
Asp	1.41(1)	2.43(2)		0.70(1)		1.01(1)			2.68(3)	0.85(1)		2.10(2)	
Thr		0.94(1)		2.14(2)	3.09(3)	1.02(1)			1.07(1)				
Ser	0.65(1)	0.84(1)				2.00(2)			0.86(1)	0.98(1)			
Glu				2.43(2)	0.91(1)	1.00(1)			1.34(1)	0.97(1)			
Pro				0.64(1)	1.27(1)	1.10(1)						1.02(1)	
Gly			1.13(1)	1.35(1)		1.15(1)	1.97(2)		1.10(1)				
Ala	2.12(2)	1.93(2)	0.98(1)	2.65(3)		1.12(1)			6.09(6)	0.93(1)			
Val	0.87(1)	2.10(2)				0.95(1)			1.89(2)			1.87(2)	
Met					0.47(1)								
Ile			0.80(1)	0.76(1)		0.99(1)			1.86(2)				
Leu	0.94(1)	1.17(1)		0.76(1)					2.10(2)	2.27(2)			
Tyr				0.96(1)	0.96(1)	0.77(1)					1.00(1)		1.00(1)
Phe			1.09(1)		0.81(1)	1.92(2)						1.02(1)	
Lys	1.00(1)	2.00(2)	1.00(1)		1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.06(1)	1.02(1)		1.00(1)	
His				1.00(1)		2.00(2)	1.00(1)		0.94(1)	0.95(1)			
Arg				1.00(1)							1.00(1)		1.00(1)

Table 2 Edman degradation and carboxypeptidase digestion of tryptic peptides. Underlined parts show the amino acids substracted. The arrow (→) represents Edman degradation and the arrow (←), carboxypeptidase digestion.

T-1 (Ⅳ-3-E)

		Step 1	2	3	4	5	6
Asp	1.41	1.26	1.30	1.21	0.84	1.00	0.00
Ser	0.65	0.81	0.79	0.49			
Ala	2.12	1.91	1.91	1.78	1.17	0.55	
Val	0.87	0.00					
Leu	0.94	1.02	0.18				
Lys	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Val-Leu-Ser-Ala-Ala-Asp-Lys
→ → → → → →

T-2 (Ⅳ-7)

		Step 1
Asp	1.00	1.00
Thr	1.00	0.45
Val	0.80	0.90
Lys	1.20	1.10

Thr-Asn-Val-Lys
→ ← ←

T-5 (Ⅳ-5-D)

		Step 1	2	3	4	5	6
Thr	3.09	2.84	3.09	2.24	1.28	1.12	1.12
Glu	0.91	1.24	1.00	1.06	1.05	0.88	0.88
Pro	1.27	0.89	1.12	0.70	0.88	0.55	0.39
Met	0.47	0.00					
Tyr	0.96	1.00	0.77	0.70	1.00	0.05	
Phe	0.81	1.24	0.00				
Lys	1.00	1.00	0.79	1.27	1.00	1.00	1.00

Met-Phe-Thr-Thr-Tyr-Pro-Glu-Thr-Lys
→ → → → → → ← ←

T-7 (Ⅳ-16-A)

		Step 1	2	3
Gly	1.97	1.20	1.25	0.65
Lys	1.00	1.00	1.00	1.00
His	1.00	1.00	0.40	

Gly-His-Gly-Lys
→ → → →

T-10 (Ⅳ-9-B)

		Step 1	2	3	4
Asp	0.85	0.85	0.83	0.36	
Ser	0.98	0.98	0.36		
Glu	0.97	0.97	0.84	0.81	1.03
Ala	0.93	0.93	1.39	1.30	0.97
Leu	2.27	1.07	0.94	0.89	0.48
His	0.95	0.95	1.03	1.02	1.22
Lys	1.02	1.02	0.97	0.98	0.78

Leu-Ser-Asp-Leu-His Ala Glu Lys
→ → → →

T-11 (Ⅳ-14-D)

		Step 1	2
Leu	1.00	0.00	
Arg	1.00	1.00	0.00

Leu-Arg
→ →

T-12 (Ⅳ-3-F)

		Step 1	2	3
Asp	2.10	1.93	1.27	1.14
Pro	1.02	1.02	1.01	0.47
Val	1.86	1.03	0.85	0.92
Phe	1.02	1.02	0.88	0.94
Lys	1.00	1.00	1.00	1.00

Val-Asp-Pro-Val Asn Phe Lys
→ → →

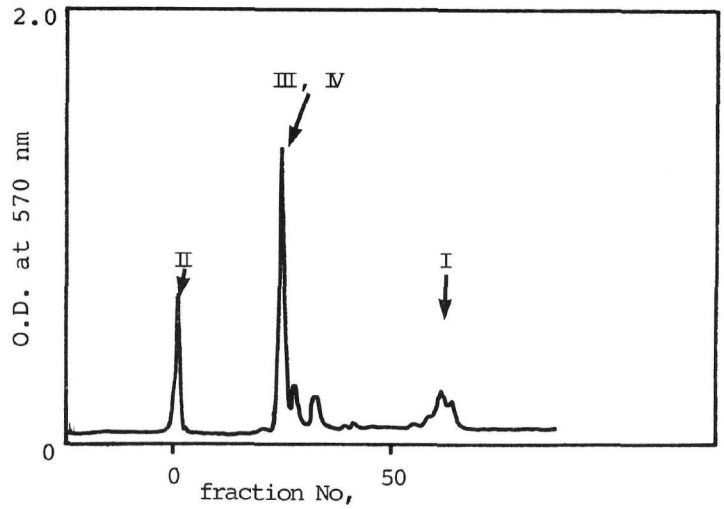

Fig. 5 Purification of chymotryptic peptides from T-6 on Chromo Beads P

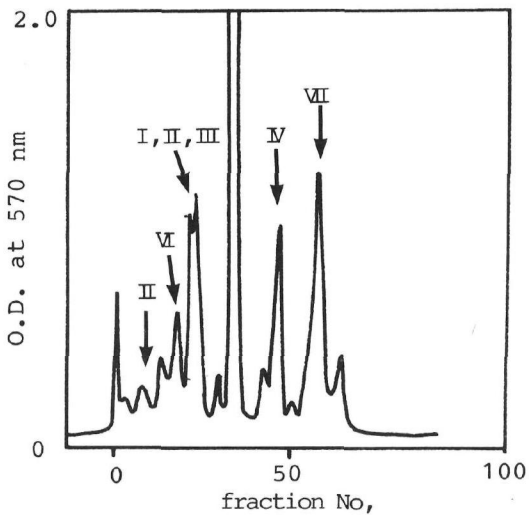

Fig. 6 Purification of thermolytic peptides from T-9 on Chromo Beads P

Longer tryptic peptides, T-4 and T-6 were hydrolysed further with chymotrypsin (SIGMA Chemical Co.) (Fig 5) and another longer tryptic peptide, T-9 was hydrolysed with thermolysin (SEIKAGAKU KOGYO Co. LTD) (Fig 6). The smaller peptide thus hydrolysed were purified and amino acid sequence of each peptide was determined by the same procedure as described above (Fig 7).

Sequencing of the tryptic peptides in the α chain was made by the aid of its homology with other avian α chains.

Fig. 7 Sequence of longer tryptic peptides
The arrow (→) represents DNS-Edman and the arrow (→), Edman degradation.
The arrow (←) represents carboxypeptidase digestion.

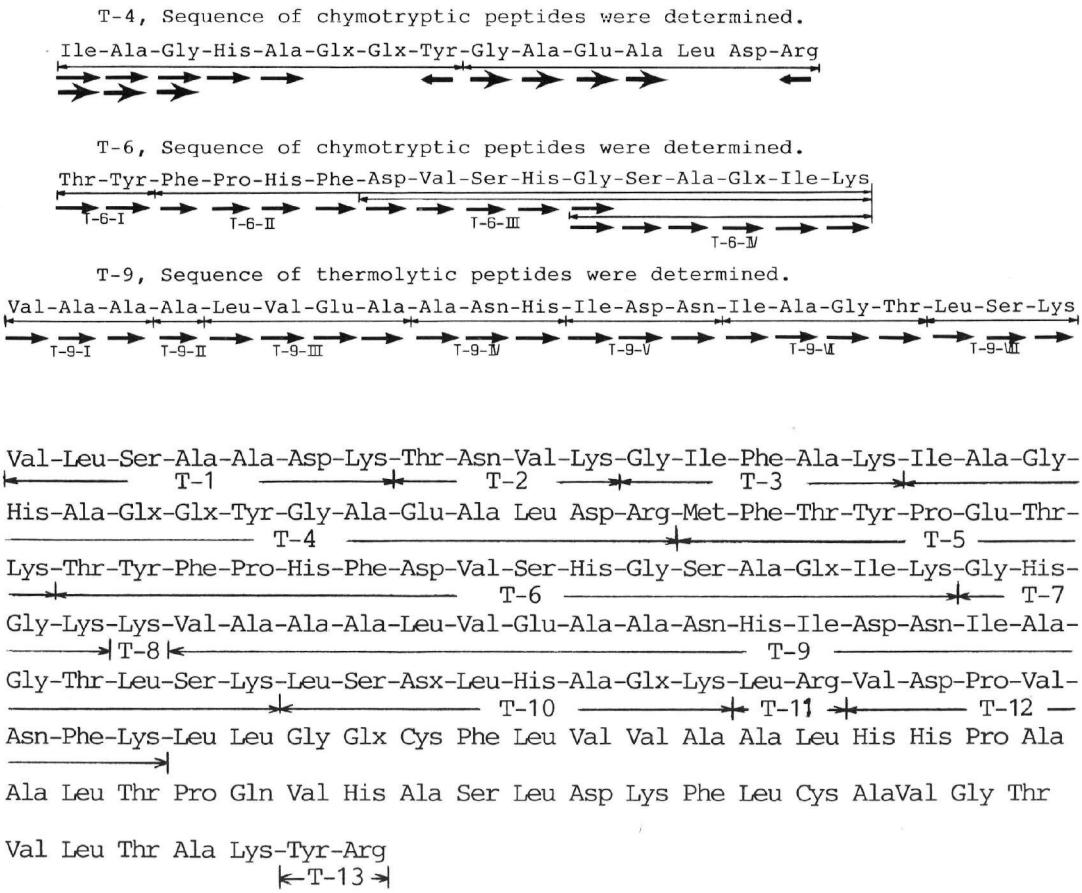

Fig. 8 Amino acid sequence of soluble fraction from Q-II α-chain

Discussion

The amino acid sequence of the α polypeptide chain of the quail QII hemoglobin determined in this paper is shown in Fig 8. By examining its homology with α chain of chicken AII hemoglobin, we can deduce the following facts. The sequence of the QII α chain begins with N-terminal Val-Leu and terminates in C-terminal Tyr-Arg. These N and C-terminal sequences coincide with those of the chicken AII α chain. In comparing the sequences in heme contact regions the critical amino acid residues involved in oxygen binding, λ 87 proximal His, α 58 distal His and α 62 Val are all unvaried.

15 amino acid residues placed in heme contact regions including the three residues described above, which are determined in these experiments, coincide completely with

those of the chicken AII α chain. Only one amino acid substitution out of 10 residues which are placed in α_1-$\beta_2$contact region was observed.

The amino acid residues in the α_1-β_1 contact region have not been determined yet because most of them are included in the core fraction.

In short, the amino acid differences between sequences of the soluble fraction of α chains of chicken AII and quail QII are 10.9%, i. e. 89.1% of the amino acid sequence of the α chain is common to both species which shows high homology to each other, the amino acid residues placed in the important position involved in hemoglobin function being less variable.

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