Primary Structure of Hb 3, a Major Hemoglobin, of Bonnet Monkey (*Macaca Radiata*)

Bunji WATANABE[1] · Tetsuo MAITA[2] and Genji MATSUDA[2]

Abstract An adult bonnet monkey (*Macaca radiata*) was found to have three major hemoglobin components (Hb 1, Hb 2, and Hb 3) which were separated by carboxymethyl cellulose column chromatography. The α and β chains were isolated from Hb 3, and digested with trypsin. The tryptic peptides were isolated and sequenced by conventional methods. The alignment of these peptides in each chain was deduced from the homology of their sequences with those of human adult hemoglobin. The primary structures thus determined are compared with those of other primate hemoglobins.

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Key Words : Hemoglobin, Amino acid sequence, Bonnet monkey

Introduction

Since Braunitzer *et al.*[1] determined the primary structure of human hemoglobin in 1961, the primary structures of many kinds of hemoglobins have been reported[2] in connection with the molecular evolution of hemoglobin. Interested in the molecular evolution of the hemoglobin and in particular in the rate of evolution and its mechanism, we have dedicated the primary structures of various primate hemoglobins.[3−6] As for the hemoglobin of macaques which belong to Old World monkey, the complete sequences of α and β chains have been reported for the rhesus monkey,[7] the Japanese monkey,[8] the pig-tailed monkey,[9] the stump-tailed monkey,[10] and the Assamese monkey.[11] The former

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two macaques have only one major hemoglobin component. On the other hand, the latter three and the crab-eating monkey are known to have multiple hemoglobin components. A bonnet monkey (*Macaca radiate*) had three major hemoglobin components (Hb 1 to Hb 3). In this paper, we describe the complete sequences of the α and β chains of Hb 3.

Materials and Methods

Materials

Blood was obtained from one adult bonnet monkey (No. MR5) kept in the Primate Reserch Institute, Kyoto University. Hemoglobin solution was prepared by the method of Drabkin.[12] Three kinds of hemoglobins were isolated by ion-exchange chromatography on CM-52 (Whatman Biochemical Co.) under the conditions described in Fig. 1 .

Isolation of α and β chains from Hb 3

The hem was removed from the globin by the method of Anson and Mirsky.[13] Globin was separated into α and β chains by CM-52 column chromatography under the conditions described in Fig. 2. S-Carboxymethylation of each chain was carried out by the method of Crestfield *et al.*[14] Cellogel (Chemetron Co., Italy) electrophoresis of hemoglobin and globin were performed with 30 mM phosphate buffer (pH 8.9) or 20 mM phosphate buffer (pH 6.8) and 10 mM Tris/borate buffer (pH 8.6) containing 8 M urea and 20 mM 2-mercaptoethanol, respectively.

Fragmentations of the α chain, β chain and larger peptides

Procedures for the digestion of the S-carboxymethylated α and β chains with trypsin (treated with (N-tosylphenylalanyl) chloromethane, Worthington Biochemical Co.) and for further fragmentation of larger peptides with chymotrypsin (Sigma Co.), thermolysin (Sigma Co.) and cyanogen bromide were described in previous papers,[15, 16] and the conditions of each procedure are described in Results.

Isolation and sequence analysis of peptides

Chromatographic separation on Chromo Beads P (cation exchange resin, Technicon Co.), AG 1 x 2 (Bio Rad Co.), Sephadex G-50 (Pharmacia Co.) and paper, and paper electrophoresis for the isolation of peptide fragments were carried out as described in the previous papers.[15, 16] Amino acid analysis of these purified peptides was performed with an amino acid analyzer JLC 200A (Jeol Co., Japan) after hydrolysis with 6 M HCl in evacuated sealed tubes at 110℃ for 20-72h. Tryptophan was detected by the Ehrlich color reaction.[17] Sequences of peptides were analysed by manual Edman degradation,[18] and digestion with carboxypeptidases A and B.[19] Phenylthiohydantoin derivatives

of amino acids were identified by thin-layer chromatography on a silica gel plate with the conventional solvent system.[20]

Results

Three major hemoglobin components were detected on Cellogel electrophoresis at pH 6.8 of the hemolysate from the bonnet monkey (the electrophoretogram is not shown). These hemoglobins were isolated by ion exchange column chromatography on CM-52 as shown in Fig. 1. The ratio of mean yields of Hb 1, Hb 2 and Hb 3 were about 0.5 : 1.0 : 0.6. In order to analyse the

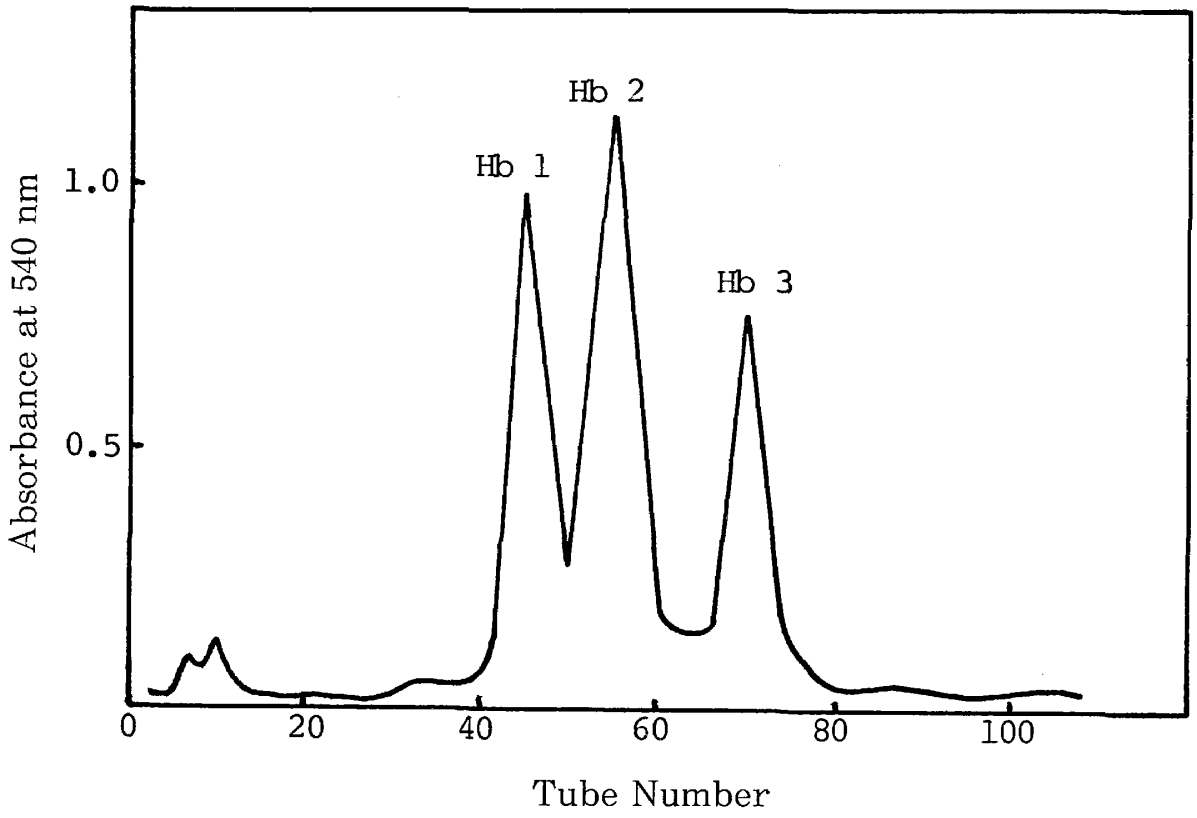

Fig. 1. Elution pattern of three major hemoglobins of the bonnet monkey on CM-52 chromatography.

About 8 ml of hemoglobin solution was applied to a column (2.0 x 20 cm). The hemoglobins were eluted with a linear gradient of 700 ml of 10 mM phosphate buffer (pH 6.7) towards 50 mM phosphate buffer (pH 6.7) at 4℃. Fraction volume : 10 ml.

primary structure, the globin from Hb 3 was separated into α and β chains by chromatography on a CM-52 in the presence of 8 M urea and 2-mercaptoethanol. As shown in Fig. 2, the α and β chains were completely

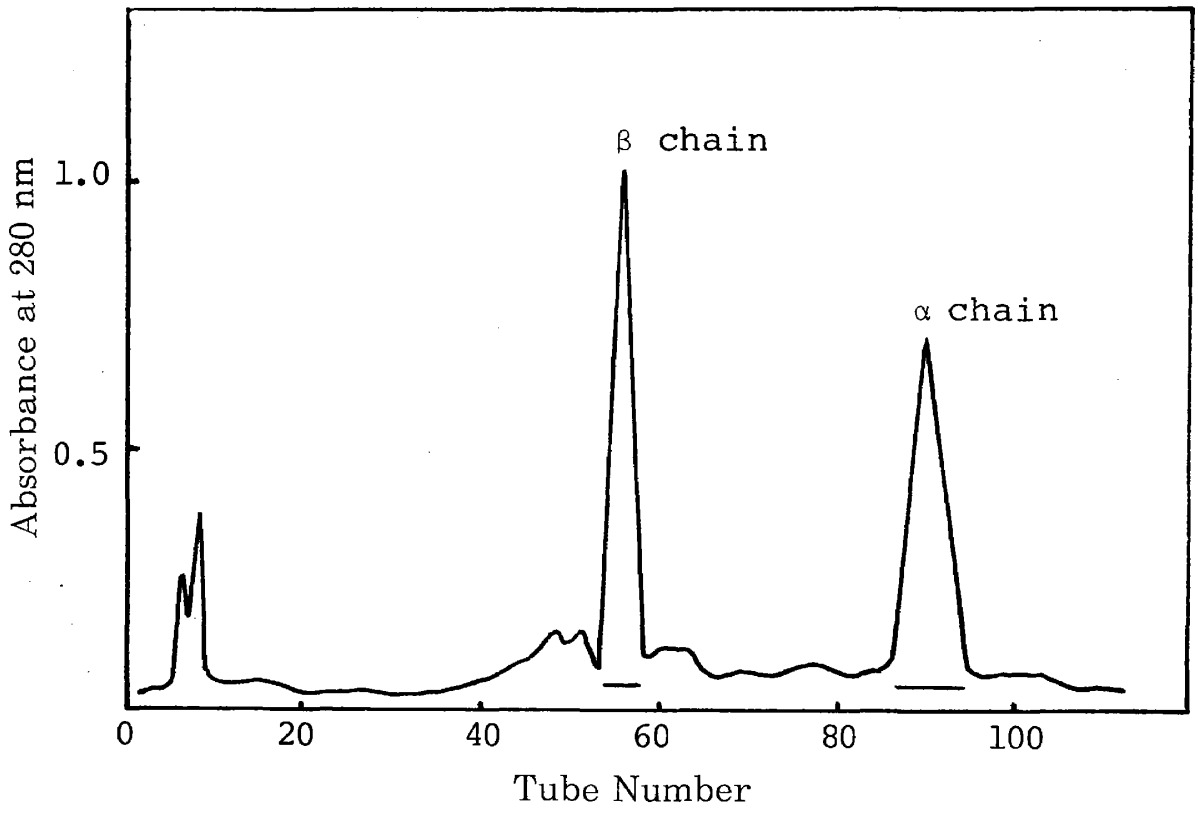

Fig. 2. Isolation of the α and β chains of Hb 3.

About 400 mg of the globin from Hb 3 was dissolved in 5 ml of the first buffer : 0.02 M (in Na^+) phosphate buffer (pH 6.8) containing 8 M urea and 50 mM 2-mercaptoethanol, and applied to a CM-52 column (3.2 x 18 cm) equilibrated with the first buffer. Elution was carried out with a linear gradient of the first buffer (900 ml) to the second buffer (900 ml) : 0.08 M (in Na^+) phosphate buffer (pH 6.8) containing 8 M urea and 50 mM 2-mercaptoethanol. Fraction volume : 12 ml.

separated each other.

Amino acid sequences of the tryptic peptides of the α chain from Hb 3

About 120 mg of the S-carboxymethylated α chain was digested with trypsin (2 mg) at pH 8.5 and 37℃ for 4h. The pH of the digestion mixture was adjusted to 6.4 by addition of 0.1 M acetic acid, and the resulting insoluble materials were collected by centrifugation. Peptides in the supernatant were separated by Chromo Beads P column chromatography as shown in Fig. 3. Peptides in each peak were further purified by paper chromatography or electrophoresis. The precipitate of the tryptic digest at pH 6.4 contained a large peptide, αT12, which was purified on a Sephadex G-50 column with 0.05 M NH_4HCO_3 (pH 8.5).

The amino acid compositions of the isolated peptides are shown in Table 1. The peptides are numbered according to their alignment in the α chain deduced from the homology of their amino acid sequences with that of human

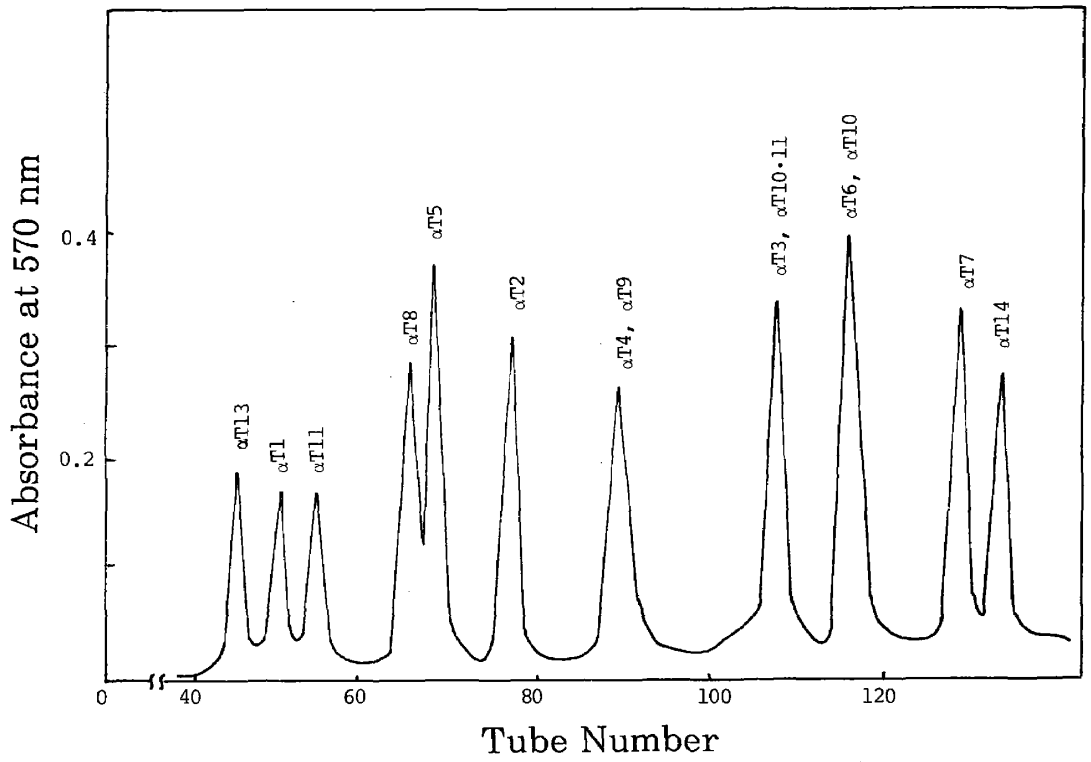

Fig. 3. Elution pattern of soluble tryptic peptides from the α chain of Hb 3 on Chromo Beads P column chromatography.

The soluble tryptic peptides of the α chain were applied to a column (0.9 x 15 cm). Elution was carried out with pyridine acetate buffer, system I described in ref.[15] at 55℃. Peptides were detected by means of the ninhydrin reaction using an automatic peptide analyser (Technicon Co.). Fraction volume : 5 ml.

Table 1. Amino acid compositions of the tryptic peptides of the α chain.

	αT 1	αT 2	αT 3	αT 4	αT 5	αT 6	αT 7
CmCys							
Asp	0.96(1)	1.03(1)				1.03(1)	
Thr		1.02(1)			1.96(2)	0.97(1)	
Ser	0.92(1)				0.99(1)	1.93(2)	
Glu				2.85(3)		1.06(1)	
Pro	0.88(1)				0.94(1)	1.02(1)	
Gly			1.02(1)	3.88(4)		1.04(1)	2.00(2)
Ala	0.99(1)		1.85(2)	3.10(3)		1.09(1)	
Val	0.96(1)	0.92(1)		1.06(1)		0.93(1)	
Met					0.89(1)		
Leu	0.96(1)			1.06(1)	0.99(1)	1.02(1)	
Tyr				0.93(1)		0.67(1)	
Phe					2.10(2)	2.04(2)	
His				1.07(1)		2.15(2)	0.94(1)
Lys	1.11(1)	1.02(1)	1.13(1)		1.12(1)	1.04(1)	1.06(1)
Trp			(+)				
Arg				1.06(1)			
Total	7	4	5	15	9	16	4

Table 1. continued

	αT 8	αT 9	αT 10	αT 11	αT 12	αT 13	αT 14
CmCys					0.89(1)		
Asp		4.06(4)		1.96(2)	0.96(1)		
Thr		1.02(1)			1.79(2)	1.79(2)	
Ser		1.93(2)			1.95(2)	2.86(3)	
Glu					1.00(1)		
Pro		1.00(1)		1.00(1)	2.09(2)		
Gly		1.06(1)					
Ala		6.02(6)			5.19(5)	1.13(1)	
Val		2.93(3)		1.89(2)	1.89(2)	1.99(2)	
Met		0.97(1)					
Leu		5.21(5)	0.95(1)		7.18(7)	2.04(2)	
Tyr							0.95(1)
Phe				1.03(1)	1.10(1)	0.98(1)	
His		3.64(4)			2.91(3)		
Lys	1.00(1)	1.16(1)		1.11(1)	1.03(1)	1.20(1)	
Trp							
Arg			1.05(1)				1.05(1)
Total	1	29	2	7	28	12	2

adult hemoglobin.[1] The sequence studies of the α chain are summarized in Fig. 4. All peptides except for αT 9 and αT 12 were sequenced completely by manual Edman degradation and digestion with carboxypeptidase A plus B.

αT 9 was sequenced up to 16 residues by the manual Edman degradation. In order to complete the sequence, αT 9 (2.0 μmol) was fragmented with cyanogen bromide (15 mg) in 3 ml of 70% formic acid for 22h, and two peptide, CN-1 and CN-2 were isolated by paper electrophoresis and sequenced (see Fig. 4). Sequence of α T 12 was not completely determined by the Edman method. Then, this 28-residue peptide (2.3 μmol) was further digested with chymotrypsin (1 mg) at pH 8.5 and 37℃ for 5h. The resulting peptides were isolated by Chromo Beads P column chromatography (the chromatogram is not shown) and paper electrophoresis. Five peptides, CH-1 to CH-5 were obtained, and sequenced so that the sequence of αT 12 was established.

Amino acid sequences of the tryptic peptides of the β chain from Hb 3

About 150 mg of the S-carboxymethylated β chain was digested with trypsin (3 mg) for 5h, and the resulting peptides were separated by Chromo Beads P column chromatography as shown in Fig. 5. The peptides were further purified by chromatography on an AG 1 x 2 column (βT 5, βT 3), Sephadex G-50 column (βT 1, βT 8・9) and on paper (βT 2, βT 9, βT 14), and by paper electrophoresis (βT 12, βT 15). The amino acid compositions and the results of sequence studies of the isolated peptides are summarized in Table 2

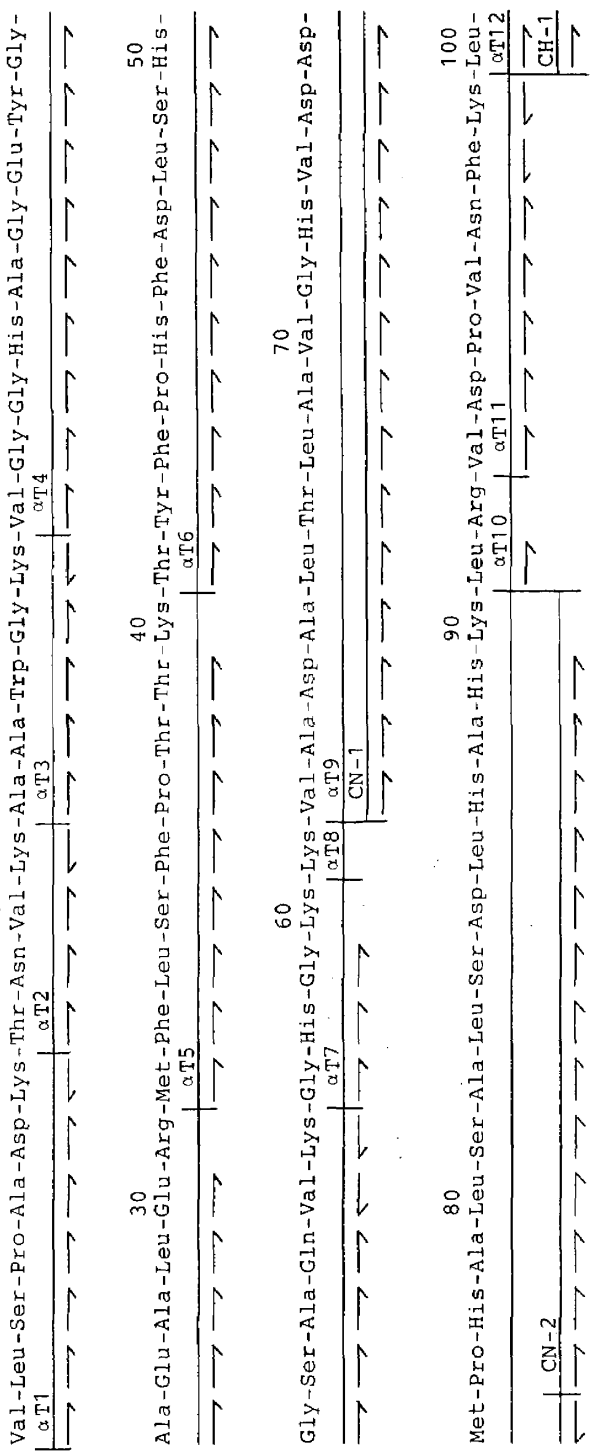

Fig. 4. Summary of sequence studies of the α chain of Hb 3 from the bonnet monkey.
αT 1 to αT 14 represent the tryptic peptides of the α chain. CN and CH are peptides fragments obtained from αT 9 with cyanogen bromide and from αT 12 with chymotrypsin, respectively. The sequence was determined by manual Edman degradation (⟶) and carboxypeptidase A plus B digestion (⟵).

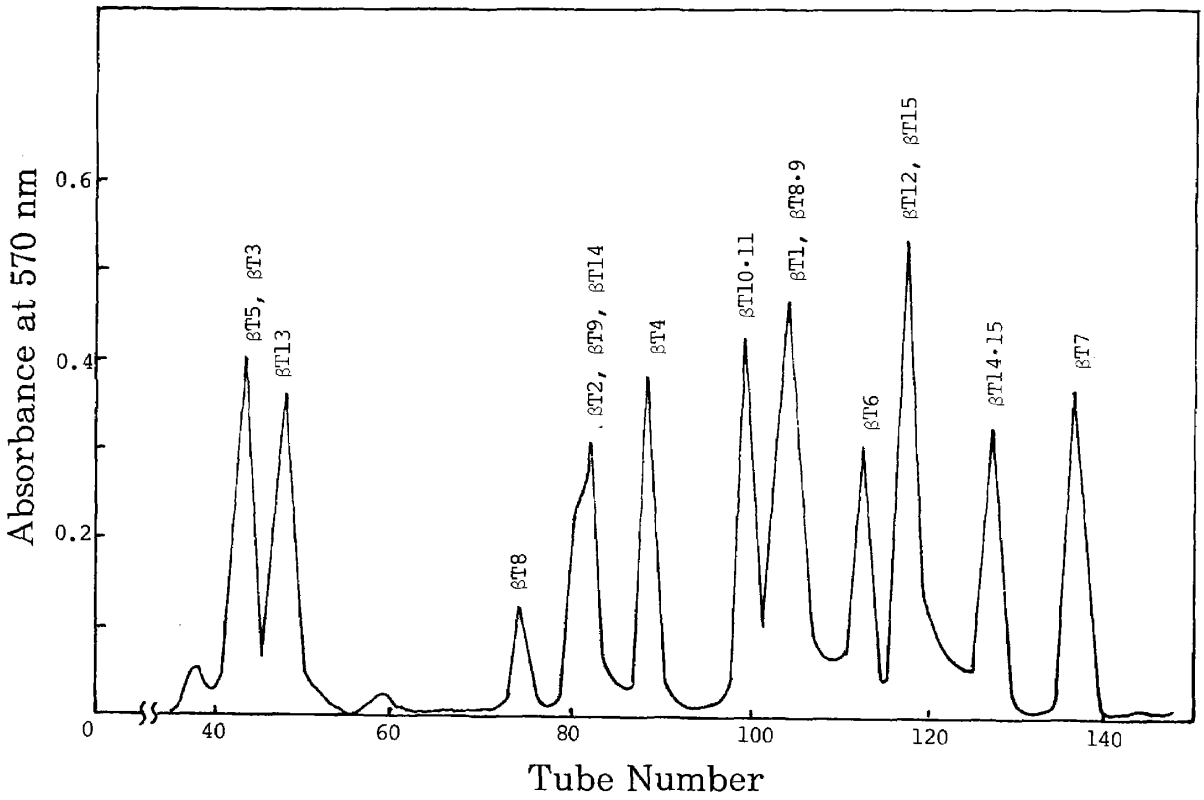

Fig. 5. Elution pattern of the tryptic peptides from the β chain of Hb 3 on Chromo Beads P column chromatography.
Conditions were the same as in Fig. 3.

and Fig. 6, respectively. βT 5 (2.3 μmol) was further digested with thermolysin (0.7 mg) at pH 8.5 and 45℃ for 5h, and βT 10 • 11 (2.5 μmol) was hydrolyzed with 0.25 M acetic acid at 110℃ in evacuated sealed tube for 16h. The resulting peptides were isolated by Chromo Beads P column chromatography and sequenced, respectively.

Discussion

The bonnet monkey had three kinds of major hemoglobins, Hb 1 to Hb 3. The amino acid sequences of all the tryptic peptides of the α and β chains from Hb 3 were determined. The alignment of the tryptic peptides in each chain was deduced from the homology of their sequences with those of human adult hemoglobin. So that, the sequences of the α and β chains from Hb 3 were determined to be as shown in Fig. 4 and Fig. 6, respectively. Comparing the primary structures of Hb 3 and human adult hemoglobins, only three amino acid substitutions are recognized in the α chain ; at 68 Leu ↔ Asn, at 71 Gly ↔ Ala, and at 78 His ↔ Asn, and 7 substitutions, in the β chain ; at 9 Asn ↔ Ser, at 13 Thr ↔ Ala, at 50 Ser ↔ Thr, at 76 Asn ↔ Ala, at 87 Gln ↔ Thr, at

Table 2. Amino acid compositions of the tryptic peptides of the β chain.

	βT 1	βT 2	βT 3	βT 4	βT 5	βT 6	βT 7
CmCys							
Asp		0.97(1)	2.05(2)		2.99(3)		
Thr	1.02(1)	1.92(2)		1.05(1)			
Ser					2.50(3)		
Glu	2.05(2)		1.83(2)	1.02(1)	1.14(1)		
Pro	0.99(1)			1.03(1)	2.03(2)		
Gly		1.04(1)	3.00(3)		2.20(2)		0.96(1)
Ala		1.04(1)	1.07(1)		1.11(1)		0.97(1)
Val	0.80(1)	0.94(1)	2.82(3)	1.60(2)	1.19(1)	0.94(1)	
Met					0.65(1)		
Leu	1.02(1)	1.02(1)	1.08(1)	2.09(2)	1.14(1)		
Tyr				1.08(1)			
Phe					2.95(3)		
His	0.95(1)						0.92(1)
Lys	1.18(1)	1.07(1)	1.15(1)		1.10(1)	1.06(1)	1.15(1)
Trp		(+)		(+)			
Arg				1.15(1)			
Total	8	9	13	10	19	2	4

Table 2. continued

	βT 8	βT 9	βT10 • 11	βT 12	βT 13	βT 14	βT 15
CmCys			0.96(1)	0.92(1)			
Asp		3.75(4)	3.01(3)	1.09(1)		1.05(1)	
Thr			1.00(1)		1.01(1)		
Ser		0.88(1)	0.96(1)				
Glu			2.77(3)		3.48(4)		
Pro			0.96(1)		1.06(1)		
Gly		2.09(2)	1.03(1)	2.28(2)		1.05(1)	
Ala		1.15(1)	1.10(1)	1.09(1)	2.14(2)	4.14(4)	
Val		1.08(1)	0.96(1)	2.80(3)	1.06(1)	2.64(3)	
Met							
Leu		3.99(4)	3.08(3)	3.77(4)		1.00(1)	
Tyr					1.00(1)		0.98(1)
Phe		0.95(1)	2.00(2)	1.02(1)	1.03(1)		
His		0.90(1)	1.91(2)	1.81(2)		0.99(1)	1.02(1)
Lys	1.00(1)	1.19(1)	2.22(2)	1.13(1)	1.21(1)	1.14(1)	
Trp							
Arg							
Total	1	16	22	16	12	12	2

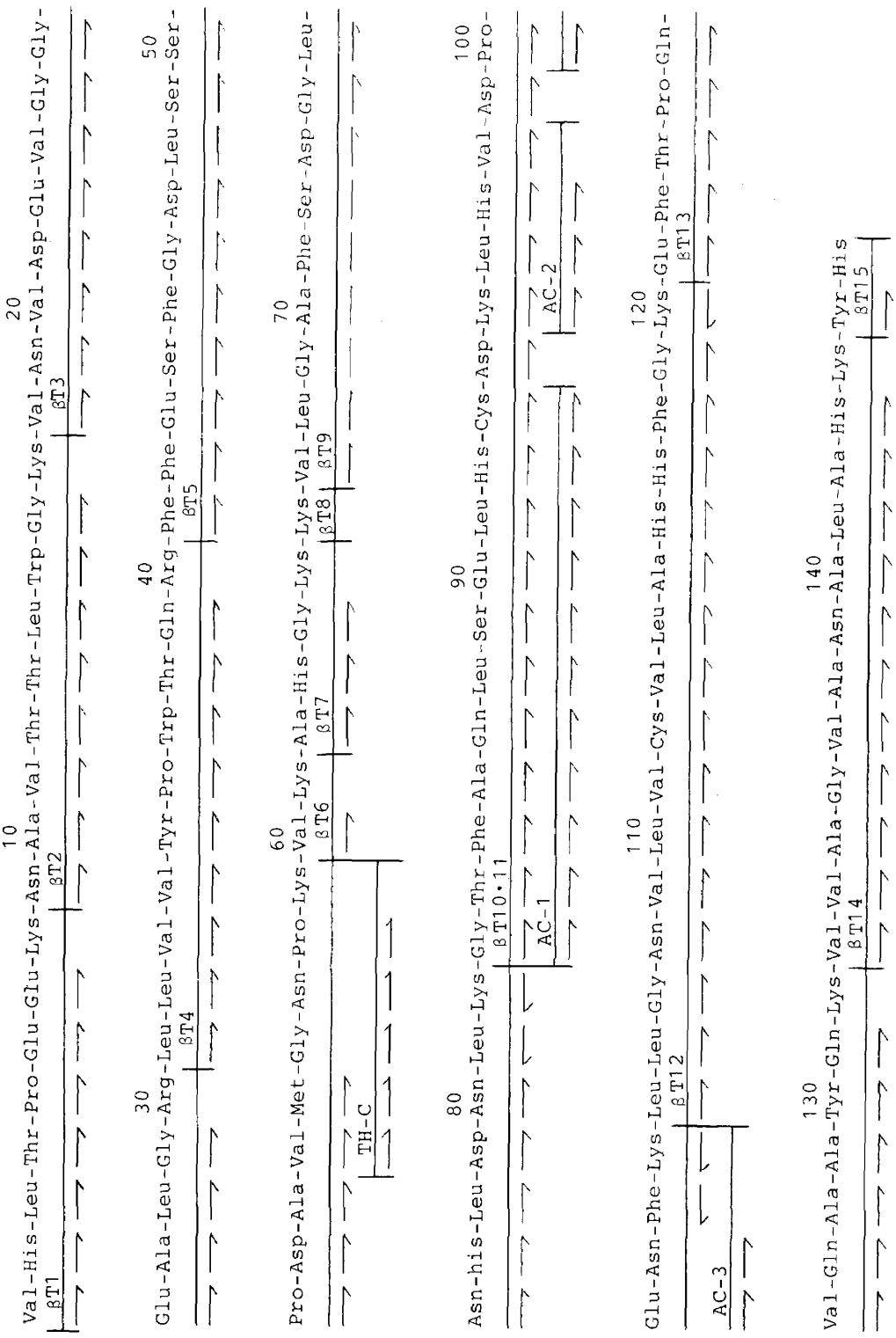

Fig. 6. Summary of sequence studies of the β chain of Hb 3 from the bonnet monkey.
TH-C shows C-terminal thermolysin peptide of β T 5. AC stand for peptides obtained from β T 10 • 11 by partial hydrolysis with 0.25 M acetic acid. Other symbols are the same as in Fig. 4.

104 Lys ↔ Arg, and at 125 Gln ↔ Pro, respectively. Amino acid residues at these positions, except for β-125, are considered not to be related to function of hemoglobin. The residue at 125 in β chain is considered to be one of the α 1-β 1 contact residues.[21]

The bonnet monkey is a kind of macaque which belongs to Cercopithecoidea ; Old World monkey. It is known that macaques generally have more than two major hemoglobin components, for instance the stump-tailed macaque has two, Hb 1 and Hb 2,[10] the pig-tailed macaque has three, Hb I, Hb II and Hb III,[9] the Assamese macaque has three, Hb 1 to Hb 3,[11] and the crab-eating macaque has four, Hb A, Hb Q, Hb R and Hb T,[22] respectively. Further, it is reported that the β chains of Hb 1 and Hb 2 from the stump-tailed macaque are identical, and those of Hb 1 to Hb 3 from the Assamese macaque are also identical.[10, 11] Electrophoretic mobilities of the β chains of the three hemoglobins from the bonnet macaque on Cellogel were almost equal (data is not shown). Therefore, we speculate that Hb 1 and also Hb 2 contain the identical β chain with that of Hb 3 reported in this paper.

Table 3 shows amino acid substitutions recognized in the α chains from macaque hemoglobins. The sequence of the α chain of bonnet Hb 3 was the same as those of stump-tailed Hb 2 and Assamese Hb 3. This may be a variable event to speculate the divergence of macaques. It is considered that macaques have two α globin genes because some individuals have three kinds of α chains.[9-11] Further, the α 1 chain (α chain of Hb 1) of the Assamese macaque was assumed to be an allele of the α 3 chain, and the α1 and α2 chains are thought to be duplicated.[11] Recently, Sawada and Schmid reported duplicated α globin genes in the family of α globin gene from primates.[23] In the case of the bonnet monkey, the alleles and duplication of the α chain may be revealed

Table 3. Amino acid substitutions in the α chains of macaque hemoglobins.

Residue No. in α chain		8	15	71	78	133
Bonnet macaque	Hb 3	Thr	Gly	Gly	His	Ser
Assamese macaque[11]	Hb 1	Thr	Asp	Gly	His	Ser
	Hb 2	Ser	Gly	Gly	Gln	Ser
	Hb 3	Thr	Gly	Gly	His	Ser
Pig-tailed macaque[9]	Hb I	Thr	Gly	Asp	Gln	Gly
	Hb II	Thr	Gly	Asp	His	Gly
	Hb III	Thr	Gly	Gly	Gln	Gly
Stump-tailed macaque[10]	Hb 1	Thr	Asp	Gly	His	Ser
	Hb 2	Thr	Gly	Gly	His	Ser
Japanese macaque[8]		Ser	Gly	Gly	Asn	Ser
Rhesus macaque[7]		Ser	Gly	Gly	Asn	Ser

by the sequence analysis of the α chains from Hb 1 and Hb 2.

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ボンネットモンキー（*Macaca radiata*）の主要ヘモグロビンHb 3の一次構造

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要　旨　分子進化を知る目的で，多くのヘモグロビンの一次構造が報告されている．今回著者らは旧世界猿のマカク属に分類されるボンネットモンキーの主要ヘモグロビンHb 1～Hb 3の中，Hb 3の α 鎖および β 鎖の一次構造を決定し，ヒトならびに同属のヘモグロビンと比較した．ヒトとの比較では α 鎖で3箇所68 Leu⟷Asn，71 Gly⟷Ala，78 His⟷Asn，β 鎖で7箇所9 Asn⟷Ser，13 Thr⟷Ala，50 Ser⟷Thr，76 Asn⟷Ala，87 Gln⟷Thr，104 Lys⟷Arg，125 Gln⟷Proのアミノ酸置換が認められた．また同属の他のヘモグロビンとの比較検討も行った．さらにボンネットモンキーを含めたマカク属では一般に複数個のヘモグロビンが認められるが，この原因となる α グロビンの対立遺伝子あるいは遺伝子重複の点についても比較考察を加えた．

長大医短紀要 1：43－56，1987