# Amino Acid Sequences of the α and β chains of Adult Hemoglobin of the Patas Monkey, *Erythrocebus Patas*[1)]

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**Summary:** Globin prepared from hemoglobin of the patas monkey (*Erythrocebus patas*) was separated into α and β polypeptide chains by chromatography on a CM 52 column. After S-aminoethylation, both chains were digested with trypsin and the amino acid sequences of the tryptic peptides obtained were analyzed. Further, the order of these tryptic peptides in each chain was deduced from their homology with the primary structures of α and β chains of human adult hemoglobin. Comparing the primary structures of the α and β chains of adult hemoglobin of the patas monkey thus obtained with those of human hemoglobin, 5 amino acid substitutions in the α chains and 7 in the β chains were recognized.

As analysis of the primary structure of hemoglobins has progressed, the rate and mechanisms of their molecular evolution have been discussed in detail. We have been analyzing the primary structures of primate hemoglobins.[1-10] We report here the primary structures of the α and β chains of adult hemoglobin of the patas monkey (*Erythrocebus patas*), which belongs to *Cercopithecoidae*.

## Materials and Methods

### *Preparation of α and β Polypeptide Chains*

Hemoglobin solution was prepared from the blood of adult patas monkey (*Erythrocebus patas*) by the method of Drabkin[11] and the heme was removed from the globin by the method of Anson and Mirskey[12]. Globin was separated into α and β polypeptide chains by CM 52 column chromatography[13]. The polypeptide chains were reduced with 2-mercaptoethanol and S-aminoethylated with ethyleneimine by the method of Jones[14].

### *Enzymatic Digestion*

The aminoethylated (AE-) polypeptide chain, 170 mg was suspended in 20 ml of water and the pH of the suspension was adjusted to 8.5 by adding 0.2 N NaOH. Three mg of trypsin [EC 3. 4. 21. 4] (TPCK-treated, Worthington Biochemical Co.) was added and digestion was carried out at pH 8.5 and 37°C under nitrogen.

The procedures for digestion with pepsin [EC 3. 4. 23. 1], chymotrypsin [EC 3. 4. 21. 1], thermolysin [EC 3. 4. 24. 4], subtilisin [EC 3. 4. 21. 4], and carboxypeptidase A and B [EC 3. 4. 12. 2 and EC 3. 4. 12. 3] were described in previous papers[2,15] and the conditions in each case are described in Results and Discussion.

### *Chromatography, Gel Filtration, Electrophoresis*

Ion exchange chromatographies using Chromo Beads P (Technicon Co.) and AG 50 wx4 (Bio. Rad Lab.) were carried out in the same ways as in the previous paper[2] with the following buffer system. System I. chamber 1 — 120 ml of buffer A (0.1 M pyridine acetate, pH 3.1), chamber 2~3 — 120 ml of buffer B (0.2 M pyridine acetate, pH 3.1), chamber 4 — 60 ml of buffer B and 60 ml of buffer C (2.0 M Pyridine acetate, pH 5.0), chamber 5~6 — 120 ml of buffer C. System II; chamber 1 — 150 ml of buffer A, chamber 2~3 — 150 ml of buffer B, chamber 4~5 — 100 ml of buffer B and 50 ml of buffer C, chamber 6~7 — 150 ml of buffer C.

Chromatography on a AG 1x2 (Bio. Rad Lab.) column, paper chromatography, gel filtration on a Sephadex G-50 column and paper electrophoresis at pH 6.4 were carried out as described eleshwere[2,15]. Amino acid analysis, subtractive Edman degradation[16] and dansylation combined with Edman degradation[17] were also carried out as described in the previous papers[2,15].

## Results and Discussion

The globin from patas monkey was separated into

1. Biochemical Studies on nemoglobins and Myoglobins XVII
2. To whom all correspondence should be addressed. Abbreviation: AE- S-aminoethylated

α and β polypeptide chains by chromatography on a CM 52 column as shown in Fig. 1. The fractions corresponding to each of these chains were collected separately, subjected to a Sephadex G-25 column (3.6x60 cm) tion and the supernatant was subjected to gel filtration on a Sephadex G-50 column. Four fractions were obtained as shown in Fig. 2A. Further, peptides in each fraction were separated by column chromatography

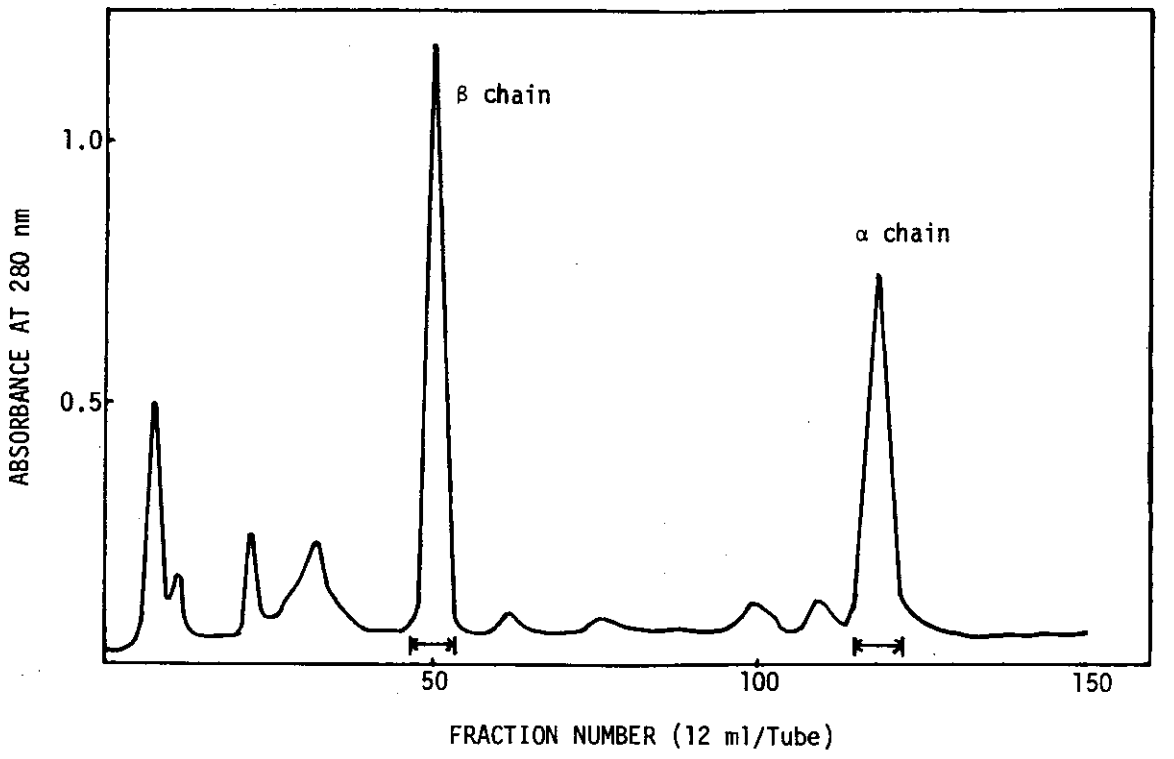


Fig. 1. Elution pattern of the α and β polypeptide chains of patas monkey hemoglobin on CM 52 column chromatography.

About 700 mg of the globin was applied to a column (3.6 x 15 cm) previously equilibrated with the first buffer; 0.02 M (in $Na^+$) phosphate buffer containing 8 M urea and 0.05 M 2-mercaptoethanol, pH 6.86. The polypeptide chains on the on the column were eluted with a linear gradient of the first buffer (900 ml) to the second buffer; 0.08 M (in $Na^+$) phosphate buffer containing 8 M urea and 0.05 M 2-mercaptoethanol, pH 6.86.

previously equilibrated with 0.5 % acetic acid to remove urea and salt, and finally lyophilized. The yield of the α and β chains were 29 % and 32 %, respectively, of the amounts of protein added.

*Tryptic Petides of the AE-α Polypeptide Chain*

AE-α polypetide chain was digested with trypsin for 5 h (see Materials and Methods). Insoluble materials in the digest were removed by centrifugausing AG 1x2 (Fraction I and II) or AG 50 wx4 (Fraction III and IV). The elution patterns of the peptides on the chromatographies are shown in Fig. 2B to 2E. The purity of the peptide in each peak in the figures was examined by paper chromatography and paper electrophoresis. Peaks containing more than one peptide were further subjected to preparative paper chromatography or electrophoresis to separate them or to purify the major component.

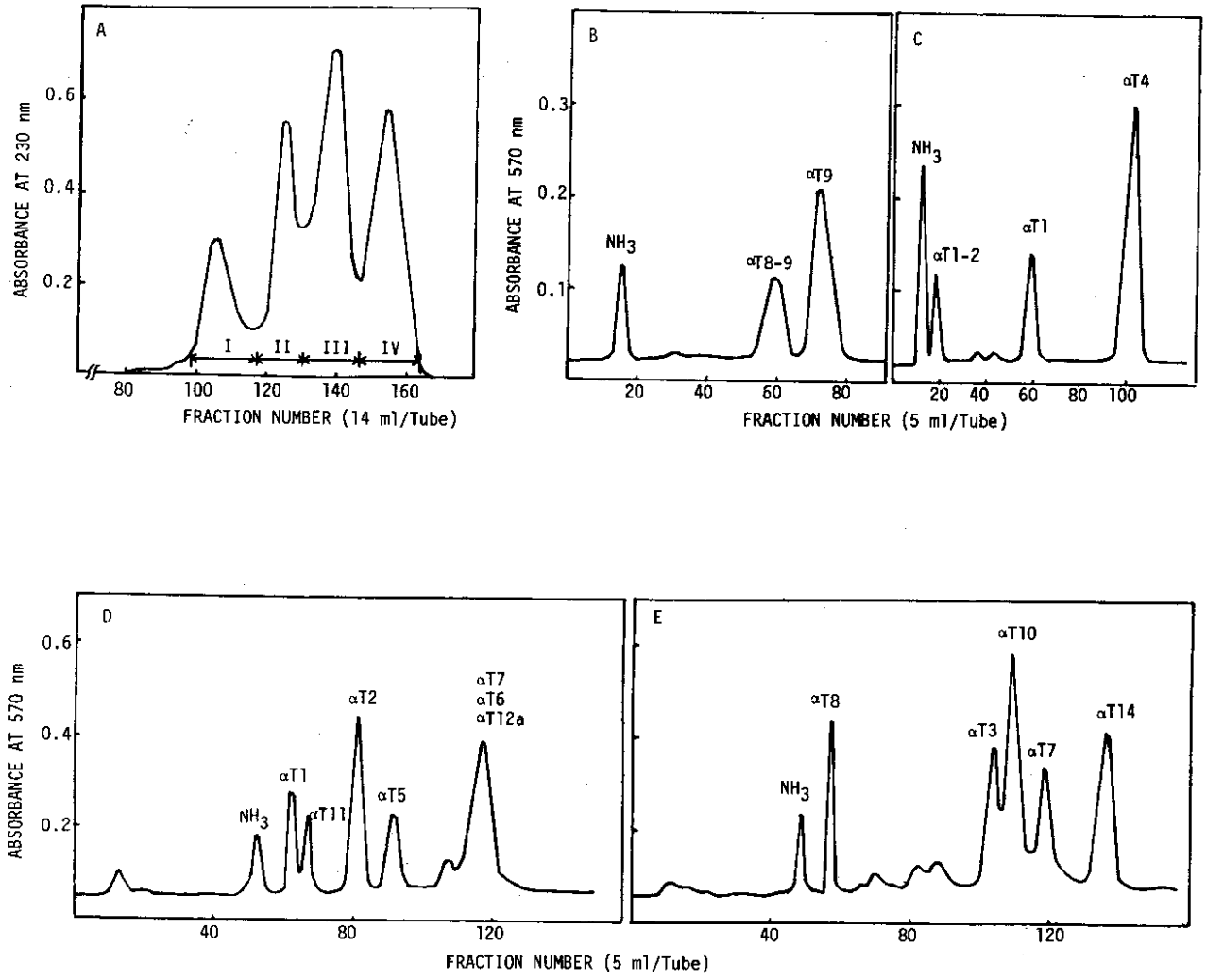


Fig. 2. Fractionation of the soluble tryptic peptides from the AE-α polypeptide chain.

A: Elution pattern of the soluble tryptic peptides on gel filtration on a Sephadex G-50 column. Peptides in the supernatant of a digest were applied to a column (5.0 × 140 cm), and eluted with 0.05 M $NH_4HCO_3$. B: Elution pattern of the peptides in Fraction I in Fig. 2A on AG 1×2 column chromatography. The lyophilized sample was dissolved in 3 ml of the starting buffer; 2 % pyridine 1% collidine acetate buffer, pH 8.5 and applied to a column (0.9×20 cm). The peptides were eluted at 40°C, with the starting buffer (fraction number 1 to 23) and with a exponential gradient of the buffer (400 ml) to successive 0.1 M acetic acid (fraction number 24 to 80) and 1.0 M acetic acid. Peptides were detected by means of the ninhydrin reaction using an automatic peptide analyzer (Technicon Co. P-I type). C: Elution pattern of the peptides in Fraction II in Fig. 2A on AG 1×2 column chromatography. The chromatography was carried out as described above. D: Elution pattern of the peptides in Fraction III in Fig. 2A on AG 50 w×4 column chromatography. The sample was dissolved in 2 ml of 0.05 M pyridine acetate buffer, pH 3.1 and the solution was applied to a column (0.9×20 cm). The peptides were eluted at 55°C with elution buffer system I described in "MATERIALS AND METHODS". E: Elution pattern of the peptides in Fraction IV in Fig. 2A on AG 50 w×4 column chromatography. The conditions were the same as in Fig. 2D.

The insoluble material of the tryptic digest was dissolved in 5 % acetic acid and the solution was subjected to gel filtration on a Sephadex G-50 column (2.0 x 150 cm) previously equilibrated with 3 % acetic acid. One major peptide (αT12b — 13) was eluted from the column with 3 % acetic acid.

The amino acid compositions, electrophoretic behaviors, purification procedures and yields of the peptides thus obtained are shown in Table I. The tryptic peptides are numbered according to their alignment in

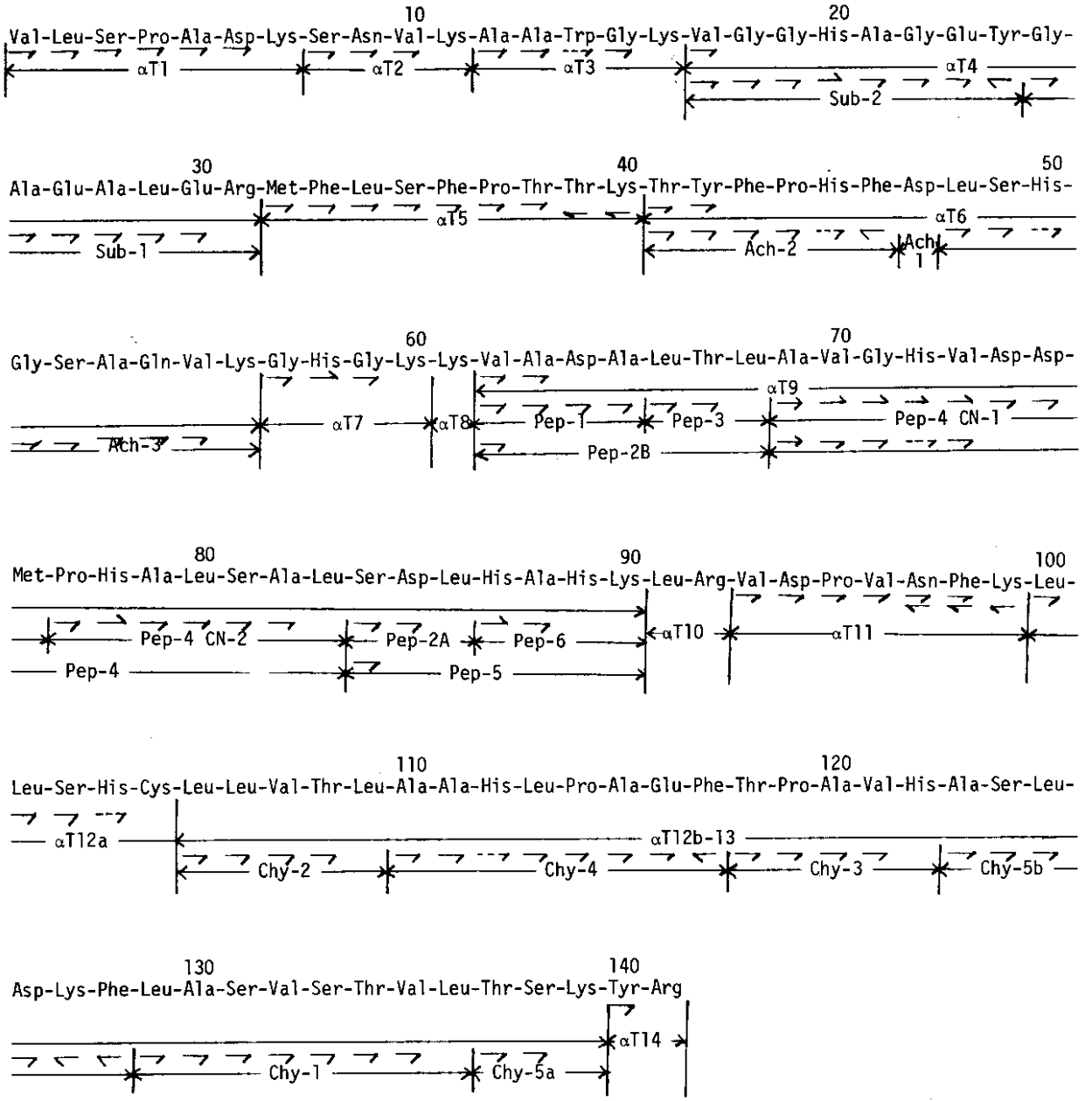


Fig. 3 The amino acid sequence of the α polypeptide chain of patas monkey hemoglobin.

αT1, αT2, . . . ., αT14 represent the tryptic peptides from the AE-α polypeptide chain. The hydrolytic peptides obtained with pepsin (Pep), chymotrypsin (Chy), subtilisin (Sub) and dilute acetic acid (Ach) from the tryptic peptides are shown by the abbreviations. The amino acid sequences determined by the dansyl Edman method (⇢), the substractive Edman method (⇢) and by carboxypeptidase digestion (←) are indicated by the arrows. In sequences marked ⇢ dansyl amino acid was found by the dansyl Edman method and the sequences were deduced from the amino acid compositions of the peptides and the homology of the amino acid sequences of the peptides with those of human hemoglobin.

the α polypeptide chain deduced from the homology of their amino acid sequences with that of human adult hemoglobin[18]. The amino acid sequences of smaller peptides were mainly determined by the dansyl Edman method, and the assignment of the amide residues was deduced from the electrophoretic mobilities at pH 6.4 and the results of carboxypeptidase digestion. Longer peptides were further digested with chymotrypsin, pepsin or subtilisin, or hydrolyzed with 0.25 M acetic acid, and the amino acid sequences of the resulting short peptides were determined as described above. The amino acid sequences of all the tryptic peptides determined are shown in Fig. 3.

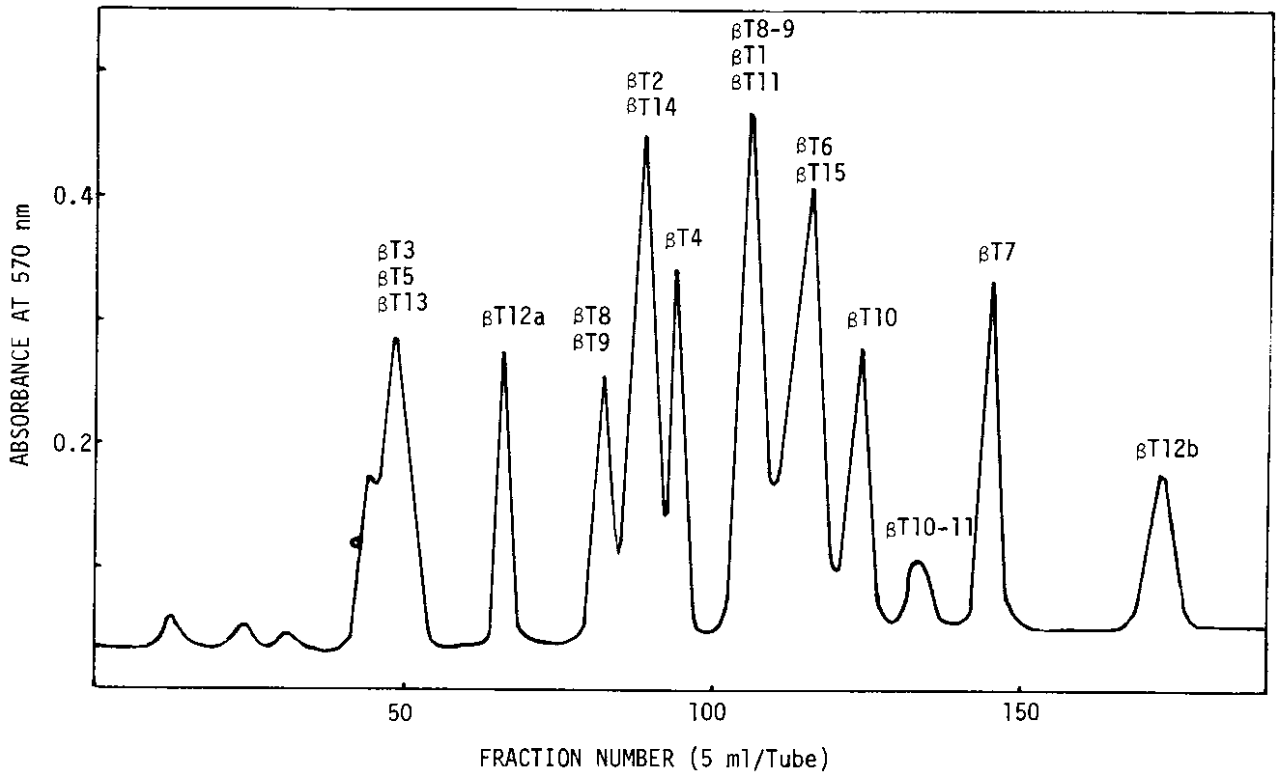


Fig. 4 Elution pattern of the tryptic peptides from the AE-β polypeptide chain on Chromo Beads P column chromatography.

The tryptic peptides (about 10 μmol) were applied to a column (0.9 × 20 cm), and eluted with elution buffer system II described in "MATERIALS AND METHODS".

Given below are some remarks on sequence analyses for a limited number of peptides among those shown in Fig. 3.

*αT4 (17-31):* The peptide (3.2 μmol) was digested with subtilisin (0.3 mg) at pH 8.5 and 30°C for 2 h and the resulting peptides were separated by chromatography on a AG 50 wx4 column under the same conditions as those in Fig. 2D. One acidic (Sub-1) and one neutral (Sub-2) peptides were obtained and their amino acid sequences were determined.

*αT6 (41-56):* The peptide (2.4 μmol) was hydrolyzed with 0.25 M acetic acid at 108°C for 16 h. Two basic peptides (Ach-2 and Ach-3) and aspartic acid (Ach-1) were isolated from the hydrolysate by paper electrophoresis. Ach-3 possessed two positive net charges as calculated from its relative electrophoretic mobility at pH 6.4 according to Offord[19]. αT6 must have an acidic residue because it possessed two positive net charges inspite of having three basic residues. Thus, the isolated aspartic acid (Ach-1) was considered not to have been produced from an asparagine residue during the hydrolysis.

*αT9 (62-90):* The peptide (4.2 μmol) was digested with pepsin (0.3 mg) at pH 2.0 and 30°C for 2 h and the resulting peptides were isolated by successive chromatography on a Chromo Beads P column (Elution buffer System I) and paper electrophoresis. Three acidic (Pep-1, Pep-2A and Pep 2B), two neutral (Pep-3 and Pep-4) and two basic (Pep-5 and Pep-6) peptides were obtained. To establish the amino acid sequence of Pep-4 the peptide (2.6 μmol) was left with cyanogen bromide in 70 % formic acid at 25 °C for 16 h and the resulting peptides were separated by paper electrophoresis. Pep-4 CN-1 was acidic, and Pep-4 CN-2 was basic. The alignment of the peptic peptides in αT9 was deduced from remarks described as follows: (1) The sum of the total residues in Pep-1, Pep-3, Pep-4, Pep-2A and Pep-6 coincided with the composition of αT9. (2) Pep-1 had N-terminal valylalanine and Pep-6 had C-terminal lysine. (3) The linkage of Pep-1 and Pep-3 and that of Pep-2A and Pep-6 was deduced from the compositions and terminal sequences of Pep-2B and Pep-5, respectively. Thus, the amino acid sequence of αT9 was deduced to be as shown in Fig. 3.

*αT12b-13 (105-139):* The large peptide was insoluble at pH 8.5. The peptide (3.2 μmol) was suspended in 0.05 M $NH_4HCO_3$, pH 8.5 and digested with chymotrypsin (0.4 mg) at 35°C for 16 h. Four neutral (Chy-1, Chy-2, Chy-4 and Chy-5b) and two basic (Chy-3 and Chy-5a) peptides were isolated from the digest by successive chromatography on a AG wx4 column and paper chromatography, and their amino acid sequences were determined. The alignment of the chymotryptic peptides in αT12b-13 was deduced from the homology of their amino acid sequences with that of human hemoglobin.

### *Tryptic Peptides of the AE-β Polypeptide Chain*

The AE-β polypeptide chain was digested with trypsin for 4 h, and no insoluble material was found in the digest. The tryptic peptides were separated by chromatography on a Chromo Beads P column as shown in Fig. 4. The peptide in each peak was further purified

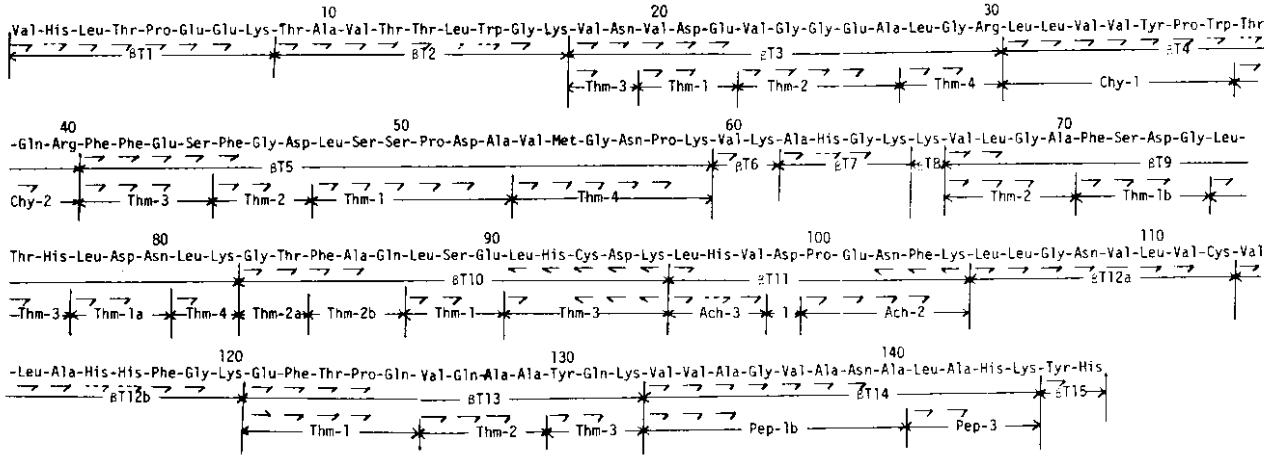


Fig. 5 The amino acid sequence of the β polypeptide chain of patas monkey hemoglobin.
βT1, βT2, . . . . , βT15 represent tryptic peptides from the AE-β polypeptide chain. Thm shows the hydrolytic peptides obtained with thermolysin from the tryptic peptides. Other symbols are the same as in Fig. 3.

by AG 1x2 column chromatography, paper chromatography or paper electrophoresis. The amino acid compositions of the isolated peptides are shown in Table II, and their amino acid sequences are shown in Fig. 5. The tryptic peptides are numbered according to their alignment in the $\beta$ polypeptide chain deduced in the same way as the $\alpha$ chain. Given below are some remarks on their sequence analysis for a limited number of peptides among those shown in Fig. 5.

*$\beta$T3 (18-30):* The peptide must have three acidic residues because it possessed two negative net charges. Four peptides (Thm-1~Thm-4) were obtained from a digest of $\beta$T3 (2.8 $\mu$mol) with thermolysin (0.3 mg) for 16 h by paper electrophoresis. Thm-1 had two negative net charges, Thm-2 was acidic, and Thm-3 was neutral.

*$\beta$T4 (31-40):* To establish the amino acid sequence of $\beta$T4, a digestion with chymotrypsin for 2 h was carried out. A basic peptide (Chy-2) was obtained from the digest by paper electrophoresis.

*$\beta$T5 (41-59):* The peptide (3.2 $\mu$mol) was digested with thermolysin (0.4 mg) at pH 8.5 and 40°C for 4 h and resulting peptides were isolated by chromatography on a Chromo Beads P column (Elution buffer System I). Thm-1, Thm-2 and Thm-3 were acidic, and Thm-4 was basic.

| | αT1 | αT2 | αT3 | αT4 | αT5 | αT6 | αT7 | αT8 | αT9 | αT10 | αT11 | αT12a | αT12b-13 | αT14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lysine | 1.08 | 0.96 | 1.00 | | 1.12 | 1.07 | 1.05 | 1.00 | 1.13 | | 1.13 | | 2.08 | |
| AE-cysteine | | | | | | | | | | | | 0.73 | | |
| Histidine | | | | 0.93 | | 1.91 | 0.91 | | 3.72 | | | 1.04 | 1.86 | |
| Arginine | | | | 0.90 | | | | | | 0.92 | | | | 1.12 |
| Aspartic acid | 1.11 | 1.00 | | | | 0.98 | | | 3.99 | | 2.04 | | 1.21 | |
| Threonine | | | | | 1.83 | 1.04 | | | 1.21 | | | | 3.69 | |
| Serine | 0.88 | 1.02 | | | 1.07 | 1.95 | | | 1.91 | | | 0.90 | 3.88 | |
| Glutamic acid | | | | 3.21 | | 1.11 | | | | | | | 1.23 | |
| Proline | 0.93 | | | | 1.11 | 0.90 | | | 1.06 | | 0.89 | | 2.07 | |
| Glycine | | | 1.14 | 3.96 | | 1.09 | 1.12 | | 1.08 | | | | | |
| Alanine | 1.12 | | 1.86 | 2.87 | | 0.97 | 0.93 | | 5.79 | | | | 5.78 | |
| Valine | 0.94 | 1.03 | | 1.09 | | 0.98 | | | 2.93 | | 1.94 | | 4.06 | |
| Methionine | | | | | 0.68 | | | | 0.78 | | | | | |
| Isoleucine | | | | | | | | | | | | | | |
| Leucine | 0.95 | | | 1.16 | 1.01 | 1.01 | | | 5.07 | 1.08 | | 2.00 | 7.11 | |
| Tyrosine | | | | 0.88 | | 0.90 | | | | | | | | 0.88 |
| Phenylalanine | | | | | 1.86 | 2.19 | | | | | 1.00 | | 2.03 | |
| Tryptophan | | | 1 | | | | | | | | | | | |
| Number of residues | 7 | 4 | 5 | 15 | 9 | 16 | 4 | 1 | 29 | 2 | 7 | 5 | 35 | 2 |
| Electrophoresis at pH 6.4 (a) | N | B 0.46 | B 0.35 | A 0.20 | B 0.25 | B 0.20 | B 0.85 | B 1.00 | S | B 0.62 | N | B 0.64 | S | B 0.56 |
| Purification (b) | PC | PE | PC | | PE | PE | PE | | | PC | PC | PE | G-50 | |
| Yield (%) | 44 | 52 | 58 | 63 | 56 | 62 | 56 | 36 | 46 | 60 | 48 | 42 | 38 | 60 |

Table I. Amino acid compositions, electrophoretic behaviors and purification procedures for the tryptic peptides from the AE-α polypeptide chain.

(a): Acidic (A), neutral (N), basic (B) peptides are shown by these abbreviations. S shows a peptide that remained at the origin on a electrophoregram. The value are mobilities relative to aspartic acid (acidic peptides) or lysin (basic peptides).

(b): Peptides were purified by paper chromatography (PC) or paper electrophoresis (PE) from the fractions shown in Fig. 2. αT12b-13 was isolated by Sephadex G 50 column chromatography from insoluble fraction of the digest at pH 8.5.

*βT9 (67-82):* Two acidic (Thm-1a and Thm-1b), one neutral (Thm-2) and two basic (Thm-3 and Thm-4) peptides were isolated from a digest of βT9 (1.8 μmol) with thermolysin for 4 h by successive paper electrophoresis and paper chromatography. Thm-1a had one negative net charge, and when two residues from N-terminal of this tripeptide were removed by two steps of Edman degradation, the resulting residue was neutral.

*βT10 (83-95):* The peptide (2.1 μmol) was digested with thermolysin (0.3 mg) for 4 h and the resulting peptides were isolated by successive paper electrophoresis and paper chromatography. Thm-2b was neutral and Thm-1 acidic.

*βT13 (121-132):* The peptide must have one glutamic acid and three glutamine residues because it was neutral. An acidic (Thm-1), a neutral (Thm-2) and a basic (Thm-3) peptides were isolated from a digest of βT13 with thermolysin for 4 h by paper electrophoresis. When N-terminal residue of Thm-1 was removed by Edman degradation, the resulting peptide was neutral.

*βT14 (133-144):* From a digest of the peptide with pepsin for 16 h, two major fragments (Pep-1b and Pep-3) of which amino acid sequences are shown in Fig. 5 and three minor fragments were isolated by successive paper electrophoresis and paper chromatography. Pep-1b was neutral. Amino acid compositions of the minor frag-

| | βT1 | βT2 | βT3 | βT4 | βT5 | βT6 | βT7 | βT8 | βT9 | βT10 | βT11 | βT12a | βT12b | βT13 | βT14 | βT15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lysine | 0.93 | 1.04 | | | 1.13 | 1.04 | 1.21 | 1.00 | 0.97 | 1.04 | 0.96 | | 1.12 | 1.07 | 0.99 | |
| AE-cysteine | | | | | | | | | | 0.63 | | 0.72 | | | | |
| Histidine | 1.07 | | | | | | 0.93 | | 0.89 | 1.02 | 1.03 | | 1.90 | | 1.00 | 1.13 |
| Arginine | | | 0.92 | 1.07 | | | | | | | | | | | | |
| Aspartic acid | | | 2.14 | | 2.93 | | | | 3.03 | 1.13 | 1.92 | 1.16 | | | 1.12 | |
| Threonine | 0.88 | 2.74 | | 1.15 | | | | | 1.02 | 0.99 | | | | 1.12 | | |
| Serine | | | | | 2.89 | | | | 1.00 | 1.06 | | | | | | |
| Glutamic acid | 2.11 | | 2.08 | 1.00 | 1.05 | | | | | 2.10 | 1.11 | | | 3.82 | | |
| Proline | 1.01 | | | 1.12 | 2.07 | | | | | | 1.02 | | | 0.96 | | |
| Glycine | | 1.13 | 2.90 | | 2.12 | | 0.94 | | 2.09 | 1.01 | | 1.03 | 0.90 | | 1.16 | |
| Alanine | | 1.16 | 1.11 | | 0.94 | | 0.92 | | 1.11 | 0.94 | | | 1.01 | 2.01 | 3.98 | |
| Valine | 0.93 | 0.91 | 2.78 | 1.64 | 0.95 | 0.96 | | | 1.10 | | 0.88 | 1.89 | 0.98 | 1.03 | 2.53 | |
| Methionine | | | | | 0.62 | | | | | | | | | | | |
| Isoleucine | | | | | | | | | | | | | | | | |
| Leucine | 1.06 | 0.92 | 1.08 | 2.21 | 1.01 | | | | 3.79 | 2.20 | 1.13 | 2.93 | 1.00 | | 1.21 | |
| Tyrosine | | | | 0.88 | | | | | | | | | | 0.89 | | 0.87 |
| Phenylalanine | | | | | 2.93 | | | | 0.99 | 1.05 | 0.98 | | 1.09 | 0.90 | | |
| Tryptophan | | 1 | | 1 | | | | | | | | | | | | |
| Number of residues | 8 | 9 | 13 | 10 | 19 | 2 | 4 | 1 | 16 | 13 | 9 | 8 | 8 | 12 | 12 | 2 |
| Electrophoresis at pH 6.4 | N | B 0.32 | A 0.33 | B 0.24 | A 0.31 | B 0.66 | B 0.82 | B 1.00 | N | B 0.20 | N | B 0.30 | B 0.71 | N | B 0.44 | B 0.44 |
| Purification(a) | PC | PE | AG-1 | PC | AG-1 | PE | PC | PC | PC | PE | PC | | | AG-1 | PE | PE |
| Yield (%) | 52 | 60 | 66 | 55 | 50 | 68 | 60 | 28 | 48 | 42 | 52 | 60 | 54 | 62 | 60 | 58 |

Table II. Amino acid composition, electrophoretic behaviors and purification procedures for tryptic peptides from the AE-β polypeptide chain.

(a): Three peptides (βT3, βT5 and βT13) were isolated from the first peak in Fig. 4 by chromatography on a AG 1×4 column under the same conditions as those in Fig. 2D. Other symbols are the same as in Fig. 1.

ments were as follows: Pep-la (Asx, $Ala_2$, Val), Pep-lc (Gly,$Ala_2$,$Val_3$), Pep-2 (Lys, His, Asx, $Ala_2$, Leu).

*The Primary Structures of the α and β Polypeptide Chains of Patas Monkey Hemoglobin*

The complete amino acid sequences of α and β polypeptide chains of adult hemoglobin from patas monkey were deduced to be as shown in Fig. 3 and Fig. 5, respectively, basing on the results of sequence analysis of all the tryptic peptides as described above. A comparison between the α polypeptide chain of adult hemoglobin from human and that from the patas monkey indicates 5 amino acid substitutions in total; Thr (human) ⟷ Ser (patas monkey) at the 8th, Ala ⟷ Gly at the 19th, Asn ⟷ Leu at the 68th, Ala ⟷ Gly at the 71st and Asn ⟷ His at the 78th positions from the N-terminus. Comparison of the β chains indicates 7 amino acid substitutions in total; Ser ⟷ Thr at the 9th, Ala ⟷ Thr at the 13th, Thr ⟷ Ser at the 50th, Ala ⟷ Thr at the 76th, Thr ⟷ Gln at the 87th, Arg ⟷ Lys at the 104th and Pro ⟷ Gln at the 125th positions from the N-terminus.

On the other hand, comparing hemoglobin of patas monkey with that of savannah monkey (*Cercopithecus aethiops*)[5], both belong to *Cercopithecoidae*, but are classified in different genus, the α chains are quite identical and in the β chains only one amino acid substitution (Ala ⟷ Thr) are recognized at the 76th position from the N-terminal. This fact would elucidate that the both have diverged in comparatively recently.

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