Comparative Biochemistry of Hemoglobins

IX. Amino Acid Sequences in So-called Soluble Tryptic Peptides from the α-Polypeptide Chain of *Macaca mulatta* Monkey Hemoglobin

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Globin was prepared from *Macaca mulatta* monkey hemoglobin. α-polypeptide chain in this globin was isolated and purified by countercurrent distribution method. Then, it was digested with trypsin. By column chromatography, seven so-called soluble tryptic peptides in the hydrolysate which were soluble at pH 6.4 were isolated and purified. The amino acid sequence of each peptide was determined by applying hydrolysates with various enzymes, DNP method, and PTC method. The sequences of 60 amino acids in α-polypeptide chain of *Macaca mulatta* monkey hemoglobin, which were determined by the present work, were compared with those of α-polypeptide chain of human hemoglobin. The exchange of amino acids was found at two points between the two hemoglobins.

INTRODUCTION

Hemoglobins are good materials for the studies of protein structure, not only because they are heme proteins which are widely distributed in nature, but also because its isolation and purification are comparatively easy. Though there are various hemoglobins, their difference depends not on their protoheme but on their globin part. The studies on human hemoglobin have been made with determination of the primary structure of α, β, and γ polypeptide chain of its globin part. The primary structures of horse and other hemoglobins are being elucidated. Since PAULING et al. discovered Hemoglobin S, various types of abnormal human hemoglobins have been found at parts of the world. Moreover, abnormality of the primary structures of many abnormal human hemoglobins have been confirmed.
Recently, ZUCKERKANDL and PAULING\textsuperscript{24,25} have been studying abnormal hemoglobins in view of the evolutionary aspects and considered their molecular diseases as one process of molecular evolution of protein. In connection with the evolutionary problem of protein moiety, it is interesting how primatian hemoglobins resemble human one.

The author et al. chose *Macaca mulatta* monkey hemoglobin as the experimental material. Human and monkey hemoglobin have been comparatively studied by several groups of workers in recent years. First, by SCHAPIRA and KRUCH's\textsuperscript{19} immunological studies, and next by the studies of CABANNES and SERAIN\textsuperscript{2}, RODNAN and EBAUCH\textsuperscript{17}, and FINE et al\textsuperscript{27}, close resemblance between the two hemoglobins was recognized. By fingerprinting method, ZUCKERKANDL et al.\textsuperscript{24} compared the soluble tryptic peptides of various hemoglobins. In their investigations, they recognized the difference on the primary structure between human and *Macaca mulatta* monkey hemoglobin. On the other hand, though MASIAR\textsuperscript{10} made the same investigation, he did not recognize the difference. MATSUDA and MAITA\textsuperscript{12} studied the N-terminal structure of *Macaca mulatta* monkey hemoglobin and reported that it consisted of the two *α*-polypeptide chains with Val-Leu N-terminus and the two *β*-polypeptide chains with Val-His-Leu N-terminus just as in case of human hemoglobin. Following that, SHIKAYA\textsuperscript{22} compared the two hemoglobins by agar gel electrophoresis, cellulose acetate membrane electrophoresis, CM-cellulose column chromatography, alkaline denaturation, and fingerprinting method. He concluded that there was a slight but clear difference on their structures though they resembled each other closely. Furthermore, MAITA\textsuperscript{9} digested *α*-chain of *Macaca mulatta* monkey hemoglobin with trypsin, isolated tryptic peptides soluble at pH 6.4, and investigated their amino acid compositions. As the results, he presumed there were at least five exchanges in 101 amino acids contained in these peptides. The present author chose seven so-called so-called soluble tryptic peptides from *α*-chain of *Macaca mulatta* monkey hemoglobin and determined the amino acid sequences in these peptides by using various methods.

**MATERIALS AND METHODS**

1) **Digestion of the *α*-Polypeptide Chain with Trypsin**

*Macaca mulatta* monkey hemoglobin, which was isolated and purified by DRABKIN's\textsuperscript{3} method, was dehemed to be globin by TEALE's\textsuperscript{22} methyl ethyl ketone method. Next, according to MATSUDA et al's\textsuperscript{14} method, this globin was separated into *α* - and *β* -polypeptide chain. *α*-polypeptide chain was digested with trypsin in the same way as that used by MAITA\textsuperscript{9}. In this hydrolysate, so-called "core" part precipitated at pH 6.4, and so it was removed by centrifugation. In this way, so-
called "soluble tryptic peptides" were obtained.

2) Isolation and Purification by Column Chromatography

The column (2.0×150cm) was prepared with the adsorbent, Dowex 1×2 (Dow Chemical Co., 200–400 mesh). The mixture of the soluble tryptic peptides was put on the column and eluted. The effluent was collected in 20 ml fractions by fraction collector. After 0.2 ml of each fraction was subjected to alkaline hydrolysis, ninhydrin reaction was performed by Yemm and Cocking's method. Each ninhydrin-positive peak was collected respectively and evaporated under reduced pressure below 30°C in a rotary evaporator.

3) Identification of Peptides by Paper Chromatography

By spotting a small portion of each concentrated peptide solution on filter paper (Toyo filter paper No. 50), paper chromatography was carried out by descending method at 24–25°C with the system of n-butanol, acetic acid, and water (4:1:5). Peptides on the paper were sprayed 0.2% ninhydrin-butanol solution and heated with iron to color. Besides, Ehrlich's reaction, Paul's reaction, α-nitrosonaphthol reaction, Sakaguchi's reaction, and the iodoplatinic acid reaction were done as the specific color reaction of amino acids.

4) Purification of Peptides by Paper Chromatography

Paper chromatography of the peptides mentioned above was also used for purification of the peptides. Namely, peptides on the paper were colored lightly with 0.2% ninhydrin-butanol solution. Next, the paper on which the peptides were spotted was cut out, washed with acetone to remove the color, and dried at room temperature. They were eluted with 5% acetic acid, and then evaporated under reduced pressure in a rotary evaporator.

5) Analyses of Amino Acid Compositions of the Peptides

The peptides isolated and purified were dissolved in 5 ml of constant boiling point HCl and hydrolyzed at 110°C for 24 hours in the sealed tubes.

These hydrolysates were concentrated to dryness under reduced pressure and dissolved in deionized water. Then, they were subjected to HITACHI-KLA-2 amino acid analyzer.

6) Partial Hydrolyses of Large Peptides into Small Peptide Fragments

a) Hydrolysis with HCl: The partial hydrolysis with HCl was used mainly when the residues of serine and threonine were contained in a peptide. About 2 p mole of the peptides was added 5 ml of constant boiling point HCl and hydrolyzed at 37°C for 2 or 3 days in a
circulating constant temperature bath. The peptide fragments in this hydrolysate were evaporated to dryness in a rotary evaporator. They were isolated and purified by paper chromatography and by high voltage paper electrophoresis.

b) Digestion with Pepsin: About 2 μ mole of the peptides was dissolved in 0.01 N HCl and added pepsin solution which was dissolved in 0.05 N HCl so that its concentration was 1%. After shaking, it was adjusted to pH 1.9 with 0.1 N HCl and digested at 37°C for 2 or 3 hours in a water bath. The peptide fragments were evaporated to dryness under reduced pressure, isolated, and purified in the same way with (a) method.

c) Digestion with Chymotrypsin: About 2 μ mole of the peptides was dissolved in 5 ml of deionized water and adjusted to pH 9 with 0.1 N NH₄OH. By addition of the adequate quantity of Chymotrypsin (Sigma Chemical Co., 3 time crystallized), it was digested in the circulating constant temperature bath at 37°C for 4 hours. During the digestion, the pH of the digestion mixture was measured every one hour and kept to 9.0. After digestion, it was readjusted to pH 6 with acetic acid, evaporated under reduced pressure, and then isolated and purified in the same way as in (a).

7) High Voltage Paper Electrophoresis

The electrophoretic apparatus was ISHIDAI Type (Fuji Iryo Kikai Seisakusho). The filter paper used here was Toyo filter paper No.51A (60 x 10 cm). Two systems of the buffer, pyridine, acetic acid, and water, (1: 10: 289, pH 3.7) and pyridine, acetic acid, and water (10: 0.4: 90, pH 6.5) were used. The sample was spotted near the center of the filter paper and dried in air. Directly after this filter paper was sprayed with the buffer, it was put in the electrophoretic tank. The time was 2 to 3 hours; the voltage was 1.5 to 3 KV.

8) DNP Method

a) Dinitrophenylation of the Peptides: To 3 ml of 1% trimethylamine solution, 0.2-0.5 μ mole of the purified peptide was dissolved. By addition of 0.6 ml of ethanol and 0.05 ml of 2,4-dinitro-1-fluorobenzene (DNFB), this mixture was shaken. After it was allowed to stand for 2 hours at room temperature, it was added 3 ml of deionized water and 0.1 ml of trimethylamine. Furthermore, in order to remove the excess DNFB, it was extracted three times with 4 ml of ether. The aqueous solution containing DNP peptides was evaporated to dryness.

b) Hydrolysis of DNP Peptides: Dissolved in 5 ml of constant
boiling point HCl (twice distilled), DNP peptides were hydrolyzed in the sealed tubes at 110°C for 17 hours. This hydrolysate was put in the separating funnel and extracted four times with 10 ml of ether. Furthermore, this ether extracts were washed three times with 5 ml of deionized water with addition of one drop 6 N HCl. The washings were returned to the aqueous phase mentioned previously. The ether extract containing DNP-amino acids was put into a heart-shaped flask, evaporated to dryness under reduced pressure. It was added a small portion of acetone and again evaporated to dryness on the inside surface of the flask. Into the cold finger condenser, dry ice was loaded. The pressure in the flask which was heated in a water bath at 50–60°C was reduced. In this way, sublimated dinitrophenol was attached on the surface of the drawn-up tip of the cold finger condenser. By repeating the above-mentioned procedure two or three times, almost all of dinitrophenol contained in this hydrolysate was removed.

c) Identification of DNP Amino Acids: After sublimation, the remained DNP amino acids were dissolved in a small portion of acetone and developed on Toyo filter paper No. 51 by two dimensional paper chromatography. As a developer, n-butanol saturated with aqueous ammonia and 1.5 M phosphate buffer were used.

9) PTC Method

The present author carried out PTC method modifying EDMAN's original one. At the same time, the amino acid sequence was assayed mainly by the degradation method of DU VIGNEAUD et al.

a) Preparation of PTC Peptides: Mills' apparatus applied in DNP method was used here. About 0.2 μ mole of the peptide solution, which was put in a heart-shaped flask, was evaporated to dryness under reduced pressure. To this, 2 ml of 66% pyridine solution and 0.05 ml of phenyl isothiocyanate (PTC) were added. After this mixture was shaken, it was adjusted to pH 7.2 with 0.1 N NH₄ OH solution, incubated at 37°C for three hours, and then evaporated under reduced pressure. It was put in Mills' apparatus in order to remove the excess PTC by sublimation method. In this way, PTC peptides were prepared.

b) Preparation and Identification of PTH: The sample containing PTC peptides was dissolved in 1 ml of trifluoroacetic acid and allowed to stand for four hours at room temperature. Then, N-terminal amino acid was isolated as the phenythiohydantoin (PTH) derivatives. After this solution containing these derivatives was evaporated to dryness under reduced pressure, it was added 3 ml of deionized water and
extracted three times with 3 ml of benzene. The aliquot of the aqueous phase which was evaporated to dryness under reduced pressure was hydrolyzed with 6 N HCl in the sealed tubes for 20 hours at 110°C. The amino acid composition was assayed by amino acid analyzer. The kind of the N-terminal amino acid of the peptide was determined by comparing this result with the amino acid composition of the primary peptide.

10) Carboxypeptidase Method

a) Preparation of Borate Buffer: After 12.04 g of H$_3$BO$_3$ was dissolved in 100 ml of 1 N NaOH, its volume was made to be 1 L by addition of decarbonated water. This solution was adjusted to pH 8.1 with 0.1 N HCl. In using, it was diluted 10 times.

b) Preparation of Carboxypeptidase Solution: Carboxypeptidase (Worthington Biochemical Co., 3 time crystallized, water suspension), 0.01 ml, was used by dissolving in 0.5 ml of 10% LiCl solution.

c) Digestion of Peptide: After about 1 μ mole of a peptide was dissolved in 2 ml of diluted borate buffer (pH 8.1), it was incubated at 37°C for the suitable time and digested. After digestion, in order to terminate the enzymatic reaction, it was added some drops of glacial acetic acid. Then, it was evaporated to dryness under reduced pressure and then assayed its amino acid composition.

RESULTS AND DISCUSSION

Maiz a$^{93}$ investigated the amino acid compositions of the peptides soluble at pH 6.4, i.e. of the twelve soluble tryptic peptides by the digestion of α-polypeptide chain. Subsequently, he reported it consisted of 101 amino acids. The present author chose α-T1, α-T2, α-T3, α-T4, α-T5, α-T6, and α-T7 among these soluble tryptic peptides and determined the amino acid sequences of the seven peptides. The results were compared with those of the corresponding peptides from α-chain of human hemoglobin. The procedures of the determination of each peptide were described hereafter.

(1) α-T1-Peptide

The amino acid composition of this peptide was as follows; Lys, 1.03; Asp, 1.03; Ser, 0.95; Pro, 0.99; Ala, 1.03; Val 0.95; Leu, 1.02. The N-terminal amino acid of this peptide was confirmed to be valine by DNP method. In order to digest this peptide, partial hydrolysis was performed with HCl at 37°C for 48 hours. The digest was submitted to descending paper chromatography. Consequently, three peptide fragments, α-T1-HI (Rf Leu = 0.24, Lys, 0.98; Aps, 1.02),
α-T1-HII (Rf Leu = 0.63, Ser, 1.06; Pro, 0.93; Ala, 1.01) and α-T1-HIII (Rf Leu = 1.19, Val, 1.05; Leu, 0.95) were obtained. α-T1-HI fragment is presumed to be in C-terminus of this tryptic peptide because of its lysine, and moreover its sequence is considered to be Asp-Lys. α-T1-HIII fragment contained valine, so according to the results obtained by DNP method, it is presumed to be in N-terminus of this peptide linking Val-Leu. The N-terminal amino acid of α-T1-HII fragment is confirmed to be serine by DNP method. Moreover, its sequence was determined to be Ser-Pro-Ala by PTC method (Stage 1: Ser, 0.32; Pro, 0.93; Ala, 1.07/Stage 2: Ser, 0.02; Pro, 0.26; Ala, 1.02).

From the above-mentioned facts, the amino acid sequence of α-T1 peptide is presumed as follows:

\[ \text{Val} \rightarrow \text{Leu} \rightarrow \text{Ser} \rightarrow \text{Pro} \rightarrow \text{Ala} \rightarrow \text{Asp} \rightarrow \text{Lys} \]

In this figure, D means DNP method; P, PTC method.

The amino acid sequence of α-T1 of Macaca mulatta monkey hemoglobin is quite the same as that of the corresponding peptide of human hemoglobin.

(2) α T2 peptide

The amino acid composition is as follows: Lys, 1.07; Asp, 1.01; Ser, 0.88; Val, 1.04.

The N-terminal amino acid of this peptide was recognized to be serine by DNP method. Serine in N-terminus and aspartic acid next to it were also confirmed by PTC method. The following results were obtained by PTC method. Stage 1: Ser, 0.09; Asp, 1.06; Val, 1.00; Lys, 0.94/Stage 2: Ser, 0.01; Asp, 0.16; Val, 1.04; Lys, 0.96. As α T2 peptide was confirmed to be a basic peptide by electrophoresis, aspartic acid, which was recognized by complete hydrolysis, is considered to be the one originated from asparagine. Accordingly, the amino acid sequence of α T2 peptide is presumed as follows:

\[ \text{Ser} \rightarrow \text{Asp(NH2)} \rightarrow \text{Val} \rightarrow \text{Lys} \]

In this figure, D means DNP method; P, PTC method.
Serine, the N-terminal amino acid of this peptide, is exchanged into threonine in case of human hemoglobin.

(3) $\alpha$ T3 peptide

The amino acid composition of this peptide is as follows; Lys, 0.99; Gly, 1.06; Ala, 1.95; and Try. Tryptophan was located on paper chromatography by Ehrlich's reaction. Alanine, the N-terminal amino acid was determined by DNP method. Moreover, about 3$\mu$ mole of $\alpha$T3 peptide was digested with pepsin for 16 hours (pH 1.8, 37°C). From this digest, three kinds of ninhydrin-positive spots were obtained. One of them was confirmed to be alanine. As to the amino acid compositions of other spots, $\alpha$T3-PI (Rf Leu=0.18) had Lys, 1.03; Gly, 0.99; Ala, 1.02; Try and $\alpha$T3-PII (Rf Leu=0.32) had Gly, 1.01; Ala, 1.02; Try. The N-terminal amino acid of $\alpha$T3-PII fragment was found to be alanine by DNP method. The C-terminal one was recognized to be glycine by Carboxypeptidase method. Therefore, the amino acid sequence of $\alpha$T3-PII fragment is presumed to be Ala-Try-Gly. On the other hand, the N-terminal amino acid of $\alpha$T3-PI fragment was recognized to be alanine by DNP method. The amino acid sequence of $\alpha$T3-PI is presumed to be Ala-Try-Gly-Lys. From the above-mentioned results, the amino acid sequence of $\alpha$ T3-peptide is presumed as follows:

\[
\begin{array}{cccc}
D & D & C \\
\text{Ala} & \text{Ala-Try-Gly-Lys} \\
\text{PI} & \text{PI} & \\
\end{array}
\]

C indicates that the amino acid sequence was determined by Carboxypeptidase method.

It is quite the same as that of human hemoglobin.

(4) $\alpha$ T4 Peptide

By DNP method, the N-terminal amino acid of this peptide was recognized to be valine. The amino acid composition was as follows; His, 0.97; Arg, 1.02; Glu, 3.07; Gly, 4.12; Ala, 3.01; Val, 0.87; Leu, 0.99; Tyr, 0.95. As this peptide was a pretty long one consisted of 15 amino acids, it was digested with pepsin for 16 hours (pH 1.8, 37°C). In order to isolated each peptide fragment in the digest, column chromatography performed in 0.9×60 cm column by using Dowex 1×2 as the adsorbent and 1% pyridine-1% picoline acetate buffer (pH 8.2) as the starting buffer. 5 ml fractions were collected. From Fraction No. 16, pH gradient was carried out by pouring 0.4 N acetic acid
into the mixing chamber which contained 500 ml of the starting buffer. The result of this column chromatography was given in Fig. 1.

As shown in Fig. 1, four peaks, PI, PII, PIII, and PIV were obtained. Fractions corresponding to these peaks were collected, evaporated to dryness under reduced pressure, and then subjected to paper chromatography. The result was shown in Fig. 2.

There were two kinds of peptide fragments, αT4-Pla and αT4-Plb, in PI fraction. Besides, from each fraction αT4-PII, αT4-PIII, and αT4-PIV were respectively purified. First, the N-terminal amino acid of αT4-Plb was found to be valine by DNP method. The amino acid composition was Gly, 1.94 and Val, 1.06. The amino acid sequence was considered to be Val-Gly-Gly. In αT4 peptide, only one mole of valine was contained. Therefore, this αT4-Plb fragment is considered to exist in N-terminus of αT4 peptide. The amino acid composition of αT4-Pla was as follows; Arg, 0.97; Glu, 1.00; Ala, 0.98; Leu, 1.05.

By PTC method, the N-terminal and the next amino acid were determined.
Stage 1: Ala, 0.22; Leu, 0.96; Glu, 1.03; Arg, 1.01
Stage 2: Ala, 0.03; Leu, 0.17; Glu, 1.03; Arg, 0.97

Furthermore, as $\alpha$-T4 peptide was a tryptic peptide, it is considered that arginine exists in C-terminus of this peptide and that this $\alpha$-T4-Pla fragment exists in C-terminus of $\alpha$T4 peptide. It is also considered the amino acid sequence is presumed to be Ala-Leu-Gly-Arg.

Next, the amino acid composition of $\alpha$T4-PII fragment was as follows; His, 0.97; Glu, 0.96; Gly, 3.04; Ala, 1.01; Val, 1.02.

After about 3$\mu$ mole of this fragment was partially hydrolyzed with HCl at 37°C for 48 hours, this hydrolysate was investigated by fingerprinting method (paper electrophoresis; pyridine acetate buffer, pH 6.5, 2 KV, 1.5 hour/paper chromatography; n-butanol-acetic acid-water 4:1:5). Fig.3 shows the result.

In this way, four kinds of the main fragments, PIla, PIIb, PIIC, and PIId were obtained. $\alpha$T4-PIIa had the composition (His, 0.93; Gly, 1.00; Ala, 1.07). It was submitted to PTC method.

Stage 1: Gly, 0.06; His, 0.94; Ala, 1.06
Stage 2: Gly, 0.00; His, 0.21; Ala, 1.00

Accordingly, the amino acid sequence of this fragment was Gly-His-Ala. Next, $\alpha$T4-PIIb fragment was a dipeptide of Gly-Glu since it
had the composition (Gly, 1.02; Glu, 0.98) confirming that glycine existed in N-terminus by DNP method. αT4-PIIc fragment was also a dipeptide of Ala-Gly since it had the composition (Gly, 1.01; Ala, 0.99) confirming that alanine was N-terminal by DNP method. αT4-PIId had the composition (Gly, 0.93; Val, 1.07) confirming that valine existed in N-terminus by DNP method. It is considered to be contained in αT4-PIIb fragment mentioned previously. From the results of above-mentioned analysis, the amino acid sequence of αT4-PII is considered to be Val-Gly-Gly-His-Ala-Gly-Glu.

The amino acid composition of αT4-PIII was as follows; His, 0.99; Glu, 0.93; Gly, 3.10; Ala, 1.02; Val, 0.96; Tyr, 1.00. This fragment is supposed to be the one added tyrosine to C-terminus of αT4-PII. αT4-PIV fragment had the composition (Glu, 0.90; Gly, 0.99; Ala, 1.03) and was subjected to PTC method.

Stage 1: Gly, 0.16; Ala, 0.98; Glu, 1.02
Stage 2: Gly, 0.04; Ala, 0.26; Glu, 1.00

The amino acid sequence of this fragment is considered to be Gly-Ala-Glu.

From the results of above-mentioned analyses, the amino acid sequence is presumed as follows:

This sequence was compared with the corresponding one of human hemoglobin to find that glycine, the third residue from the N-terminal amino acid of this peptide, was exchanged into alanine in case of human hemoglobin.
(5) αT5-Peptide

By DNP method, the N-terminal amino acid of this peptide was confirmed to be methionine. This peptide had the composition (Lys, 1.00; Thr, 1.90; Ser, 0.91; Pro, 1.09; Leu, 1.03; Phe, 2.07; and Met). It was digested with pepsin for 16 hours (pH 1.8, 37°C). From this digest, three kinds of peptide fragments, αT5-PI (Rf Leu=0.14), αT5-PII (Rf Leu=0.9), and αT5-PIII (Rf Leu=1.08) were isolated by descending paper chromatography.

αT5-PI fragment had the composition (Lys, 1.02; Thr, 2.07; Pro, 0.91) and was subjected to PTC method. Stage 1: Pro, 0.28; Thr, 1.96; Lys, 1.04. This fragment is considered to be in C-terminus of this peptide since it contained lysine. Its amino acid sequence is presumed to be Pro-Thr-Thr-Lys. αT5-PIII fragment had the composition (Ser, 0.95; Leu, 1.02; Phe, 1.03) confirming that leucine was N-terminal by DNP method. Moreover, it was subjected to PTC method to confirm the N-terminal to be leucine and the next residue to be serine. The result obtained by PTC method was as follows;

Stage 1: Leu, 0.31; Ser, 0.99; Phe, 1.01
Stage 2: Leu, 0.09; Ser, 0.18; Phe, 1.00

From the above-mentioned results, the amino acid sequence of this fragment is considered to be Leu-Ser-Phe. αT5-PII fragment had the composition (Phe, 1.96; Leu, 1.03; Ser, 1.01) confirming that the N-terminal amino acid was phenylalanine. The amino acid composition of αT5-PIII was compared with that of αT5-PII to find that αT5-PII had one mole more phenylalanine than αT5-PIII. Moreover, as mentioned previously, since the N-terminal amino acid of αT5-PII was phenylalanine, its sequence is presumed as follows;
This result was the same as that of human hemoglobin.

(6) αT6 Peptide

The amino acid composition of this peptide was as follows; Lys, 1.07; His, 1.99; Asp, 1.06; Thr, 0.91; Ser, 1.88; Glu, 1.00; Pro, 1.14; Gly, 0.98; Ala, 1.13; Val, 1.14; Leu, 0.92; Tyr, 0.79; Phe, 1.99.

The N-terminal amino acid was confirmed to be threonine by DNP method. This peptide was digested with pepsin for 3 hours (pH 1.8, 47°C). In order to isolate and purify various peptide fragments in this digest, column chromatography was performed by using Dowex 1×2 as the adsorbent and 1% Pyridine-1% lutidine-acetate buffer (pH 7.7) as the starting buffer. They were collected in 5 ml fractions. From Fraction No. 20, pH gradient elution were carried out by pouring 0.075 N acetic acid into the mixing chamber which contained 500 ml of the starting buffer. The result was given in Fig. 4.

As shown in Fig. 4, three main peaks, αT6-PI, αT6-PII, and αT6-PIII were obtained. The fractions of these peaks were respectively collected and evaporated to dryness under reduced pressure. It was used in the experiments hereafter. αT6-PI was purified by high voltage paper electrophoresis to find two main spots, αT6-PIa and αT6-PIb. They were analyzed their amino acid compositions.

αT6-PIb had the composition (Glu, 0.97; Val, 1.04; Lys, 0.99).
It must be the C-terminal tripeptide of \( \alpha \)T6 since this peptide had lysine. Moreover, by DNP method, the N-terminal amino acid was confirmed to be glutamic acid. As this peptide was known to be a basic peptide by high voltage paper electrophoresis, the amino acid sequence is \( \text{Glu (NH}_2\text{-)}\text{Val-Lys} \). \( \alpha \)T6-P1a will be discussed afterwards.

After \( \alpha \)T6-PII fragment was purified by descending method of paper chromatography, it was analyzed the composition (His, 1.02; Asp, 0.96; Ser, 2.08; Gly, 0.99; Ala, 1.00; Leu, 0.95). It was confirmed that its N-terminal amino acid was aspartic acid by DNP method. This peptide was partially hydrolyzed with HCl. The peptide fragments contained in this hydrolysate, \( \alpha \)T6-PII-HI (Rf Leu=0.21), \( \alpha \)T6-PII-HII (Rf Leu=0.40), and \( \alpha \)T6-PII-HIII (Rf Leu=0.94), were isolated and purified by descending method of paper chromatography. \( \alpha \)T6-PII-HI had the composition (His, 0.92; Ser, 1.00; Gly, 1.08), confirming that serine was the N-terminal residue by DNP method. \( \alpha \)T6-PII-HII had the composition (Ser, 1.04; Gly, 0.96), confirming that glycine was N-terminal. \( \alpha \)T6-PII-HIII had the composition (Asp, 1.01; Leu, 0.99). It is considered to be N-terminal peptide of \( \alpha \)T6-PII since \( \alpha \)T6-PII contained only one mole of aspartic acid and it was N-terminal amino acid.

From these results, the amino acid sequence of \( \alpha \)T6-PII is considered in the following two ways:

\[
\text{Asp-Leu-Ser-His-Gly-Ser-Ala}
\]
or
\[
\text{Asp-Leu-Ala-Ser-His-Gly-Ser}
\]

But, the amino acid composition of \( \alpha \)T6-P1a was as follows; Lys, 1.01; Ser, 0.98; Glu, 1.03; Gly, 1.01; Ala, 1.03; Val, 0.94. The N-terminal amino acid was glycine. Therefore, from the above-mentioned results on \( \alpha \)T6-PIb, \( \alpha \)T6-P1a, and T6-PII, the sequence must be the former.

After T6-PIII fragment was purified by descending method of paper chromatography, its amino acid composition was analyzed (His, 1.11; Thr, 1.00; Pro, 0.97; Tyr, 0.89; Phe, 2.03). Threonine was N-terminal of this peptide by DNP method. As \( \alpha \)T6 peptide contained only one mole of threonine, threonine in N-terminus of \( \alpha \)T6-PIII is threonine in N-terminus of \( \alpha \)T6 peptide. This fragment was digested with chymotrypsin for 4 hours, isolated and purified by descending method of paper chromatography. Consequently, two kinds of fragments, \( \alpha \)T6-PIII-CI (Rf Leu=0.79) and \( \alpha \)T6-PIII-CII (Rf Leu=0.93) were obtained. \( \alpha \)T6-PIII-CI had the composition (Thr, 1.09; Tyr, 0.91). Threonine must be N-terminal residue and tyrosine, the next to it. \( \alpha \)T6-PIII-CII had the composition (His, 1.05; Pro, 0.99; Phe, 1.96). The amino acid sequence of this fragment was investigated by PTC method

The results were as follows;
Stage 1: His, 1.08; Pro, 0.92; Phe, 1.12
Stage 2: His, 0.92; Pro, 0.28; Phe, 1.08
Stage 3: His, 0.16; Pro, 0.03; Phe, 1.00

The sequence of αT6-PIII-CII is considered to be Phe-Pro-His-Phe.
From the results of the above-mentioned analyses, the amino acid sequence of αT6 peptide was presumed as follows:

The sequence of 60 amino acids of α-chain of *Macaca mulatta* monkey hemoglobin was determined. The sequence of each peptide was compared with that of the corresponding peptide of human hemoglobin. Consequently, the exchange of amino acid was recognized in the eighth (Ser—Thr) and in the nineteenth (Gly—Ala) from N-terminus between the two hemoglobins.

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