The Amino Acid Sequence of the Insoluble Tryptic Peptide, $\beta\text{T10, 11, 12}$ from the $\beta$-Polypeptide Chain in Macaca mulatta Monkey Hemoglobin

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Globin prepared from macaca mulatta monkey hemoglobin was separated into $\alpha$ and $\beta$-polypeptide chains by countercurrent distribution method. The $\beta$-polypeptide chain was digested with trypsin. The insoluble tryptic peptide (so-called $\beta$-core), the precipitate of the digest at pH 6.4, was obtained by centrifugation. This insoluble tryptic peptide after oxidized with performic acid was digested with pepsin or redigested with trypsin, and the amino acid composition was examined. Next, the amino acid sequence of the $\beta$-core containing 38 amino acid residues was determined by the DNP method, the PTC method, and the carboxypeptidase method. The result was compared with the known sequence of the $\beta$-core of human hemoglobin. Between these two hemoglobins, two amino acid substitutions were found; that is, the 5th and 22nd residues from the N-terminus are threonine and arginine in case of human hemoglobin, but glutamine and lysine, respectively in case of macaca mulatta monkey hemoglobin.

INTRODUCTION

Since DARWIN, classification and evolution of living organisms have been studied by comparative investigations on their shapes and habits. ANFINSEN, however, proposed to deduce the evolulational process of genes which control protein synthesis, by comparing structures of proteins on molecular level, which play an important role in constiuutions and functions of living organisms. Since then a new branch called chemical paleogenetics has been noted. For elucidation of this branch, the sequences of the subunits of DNA which are codes in genes are necessary to be determined. At this stage that the determination of basic sequences of DNA is difficult, however, it is significant to get information on the primary structures of proteins which are the primary products of DNA, and to deduce the structure of DNA by the information obtained.

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For the structural study of proteins, hemoglobin is one of suitable materials because of its presence in most animals and easy isolation. It is also available because a living organism has several kinds of resembling hemoglobins so that it enables to deduce how they have evolved from the common origin. The primary structures of the \( \alpha^{15} \) and \( \beta \)-polypeptide\( ^{24} \) chains of human hemoglobin and those of the \( \alpha^{17} \) and \( \beta \)-polypeptide\( ^{27} \) chains of horse hemoglobin have already been determined. Besides them, pig\( ^{5} \), rabbit\( ^{8} \), and sheep\( ^{9} \) hemoglobins are being studied. The differentiating time of living organisms can be assumed by comparing these known primary structures of hemoglobins. It is said that it takes \( 1 \times 10^{7} \) years to substitute one amino acid residue to another.\( ^{32} \)

The structural differences in abnormal hemoglobins are interesting problems of genetics especially because they are due to mutation. Since Hb M was discovered by HÖRLEIN et al.\( ^{13} \) many kinds of abnormal hemoglobins such as Hb S\( ^{22} \), Hb E, and so forth have been discovered and their primary structures determined. ZUCKERKANDL and PAULING\( ^{34} \) took up abnormal hemoglobins as proteins during the molecular evolution or molecular disease. BEAL and LEHMAN\( ^{2} \) studied on the correlation between abnormal hemoglobins and genetic code and presented the schema of one-step mutation of amino acids. They reported that most abnormal hemoglobins were due to one-step mutation although some could not be explained without double-step mutation. Recently, however, with the advance of studies on code, it is said that abnormal hemoglobins are almost due to single base change.

From comparative biochemical interest in heridity and evolution, the present author chose, as the experimental material, hemoglobin from macaca mulatta monkey which evolutionally resembles man pretty well.

It was reported by ZUCKERKANDL and SCHROEDER\( ^{36} \) that the amino acid composition of gorilla hemoglobin differed from human hemoglobin at one point in the \( \alpha \) and \( \beta \) polypeptide chains, respectively. BUETTNER-JANUSH and HILL\( ^{6} \) demonstrated a pretty large difference in the amino acid composition of the \( \beta \)-polypeptide chain between lemur fulvus and human hemoglobins. Concerning macaca mulatta monkey hemoglobin, since SCHAPIRA and KRUCH\( ^{25} \) reported its immunological resemblance to human hemoglobin, CABANNES and SEARAIN,\( ^{7} \) RODNAN and EBAUGH\( ^{23} \) showed the difference between these two hemoglobins by paper chromatography. FINE et al.\( ^{12} \) observed the similarity by agar gel electrophoresis, but ZUCKERKANDL et al.\( ^{34} \) found a slight difference by fingerprinting method. According to MATSUDA and MAITA,\( ^{18} \) the N-terminal structures of both the \( \alpha \) and \( \beta \)-polypeptide chains of macaca mulatta monkey hemoglobin were respectively Val-Leu and Val-His-Leu, which are exactly the same as those of human hemoglobin. SHIKAYA\( ^{26} \) clearly
demonstrated the difference between *macaca mulatta* monkey and human hemoglobins by using such techniques as agar gel electrophoresis, cellulose acetate membrane electrophoresis, CM cellulose column chromatography, alkaline denaturation, and finger-printing method. Maita investigated the amino acid compositions of the tryptic peptides soluble at pH 6.4 of the α-polypeptide chain in *macaca mulatta* monkey hemoglobin and deduced that it differed from human hemoglobin at five points among 101 amino acid residues. Similarly, Takei discovered six differences among 108 amino acid residues of the β polypeptide chain. Successively, Igawa, by the sequence determination of 60 amino acid residues of the soluble tryptic peptides of the α-polypeptide chain in *macaca mulatta* monkey hemoglobin, found two differences between these two hemoglobins. Matsuda et al. determined the whole amino acid sequence of the soluble tryptic peptides of the β-polypeptide chain and discovered six amino acid substitutions between human and *macaca mulatta* monkey hemoglobins. Their findings agree with Takei's report on the amino acid composition. The present author, by using various kinds of techniques, determined the amino acid composition and sequence of the insoluble tryptic peptide (so-called core) of the β-polypeptide chain in *macaca mulatta* monkey hemoglobin.

**MATERIALS AND METHODS**

1) Preparation of the β-Polypeptide Chain from *Macaca mulatta* Monkey Hemoglobin

Blood was obtained from abdominal aorta of *macaca mulatta* monkey. According to Drabkin's method, hemoglobin was prepared. It was removed the heme by Anson and Mirsky's method. Globin thus prepared was separated into α and β-polypeptide chains by counter-current distribution method with the system of sec-butanol containing 0.86% trichloroacetic acid, propionic acid, and water (8.7:1.5:11.0, v/v). The solution containing the β-polypeptide chain was transferred in a visking tube 24/32, dialyzed against deionized water, and lyophilized after the organic solvent in the solution was fully removed.

2) Preparation of the Insoluble Tryptic Peptide from the β-polypeptide Chain

The β-polypeptide chain (2g) dissolved in 100ml of 8M urea was denatured by stirring at 60°C for 45 min. It was dialyzed against more than 20 changes of 3l of deionized water overnight for complete removal of urea. The urea-denatured β-polypeptide chain, after adjusted to pH 8.5 with 0.1 N NaOH, was digested at 37°C for 4 hours with 20mg of trypsin (Worthington Biochemical Corporation, twice crystallized). The pH of the digest maintained at 8.5 with 0.1N NaOH. After the
digestion ceased, the solution stirred and adjusted to pH 6.4 by adding 0.1 N acetic acid was allowed to stand overnight at 0°C in a low temperature room, and then the pH was measured and readjusted to pH 6.4 precisely. The insoluble tryptic peptide (so-called core), which was precipitated at pH 6.4, was isolated by cold centrifugation (9000 r.p.m. x 45 min.).

3) Oxidation of the Insoluble Tryptic Peptide with Performic Acid.

The insoluble tryptic peptide (Ca 400mg) was oxidized with performic acid. Performic acid used was prepared by mixing 18 ml of 99% formic acid and 2ml of 30% hydrogen peroxide in a round-bottomed flask with an interchangeable ground glass joint, which was sealed perfectly and allowed to stand at room temperature for 2 hours. Later, the performic acid solution thus prepared was cooled to be 0°C. Subsequently, the insoluble tryptic peptide dissolved in 5ml of formic acid cooled at 0°C was oxidized at 0°C for 2 hours with 20ml of performic acid. The reaction was stopped by adding 100ml of deionized water. The performic acid-oxidized insoluble tryptic peptide was dried in a rotary evaporator below 37°C for removal of performic acid.

4) Digestion of the Oxidized Insoluble Tryptic Peptide with Pepsin

The insoluble tryptic peptide (β-core) dissolved in 50ml of deionzied water and adjusted to pH 2.0 with 1N HCl was digested with 4mg of pepsin dissolved in 1ml of 1/16N HCl. Pepsin, three time crystallized, was obtained from Sigma Chemical Company. The digestion was stopped by adjusting the solution to pH 9.0 with 0.1N NaOH.

5) Column Chromatography of the Peptic Peptides from Insoluble Tryptic Peptide

The resin (Dowex 1×2, Dow Chemical Company, 200–400 mesh) was washed with 1N NH₄OH, deionized water, glacial acetic acid, and deionized water, successively, suspended in the starting buffer, and thoroughly evacuated. It was packed in a column (2×150cm), which was fully equilibrated by pouring the starting buffer (Ca 4). To this column was applied 30ml of the peptic digest solution which was adjusted to pH 9.5 by adding 1N NaOH. Acetate buffer (the starting buffer), pH 8.5, containing 1% pyridine, 1% picoline, and 1% collidine was used as the first developer. During the development, the column was warmed at 37°C and the flow rate was 160ml per hour. The first 11 fractions were developed with the starting buffer and thereafter the gradient elution was employed, that is, the development between Fractions No. 12–150 was carried out by supplying 0.1N acetic acid in the upper chamber to 1 liter of the starting buffer in the mixing cham-
ber, and from Fraction No. 241 to 270, the content of the upper chamber was changed into 1N acetic acid. The column was finally washed with glacial acetic acid. All the organic bases used had been purified by distillation and deionized water decarbonized and thoroughly evacuated. A eluate was collected in 10 ml-fractions by a fraction collector. The 0.5ml portion of individual fractions was subjected to ninhydrin reaction according to Yemm and Cocking. The ninhydrin-positive fractions were combined respectively, dried under reduced pressure below 37°C, redissolved in 5ml of deionized water, and finally lyophilized.

6) Redigestion of the Insoluble Tryptic Peptide with Trypsin in 1M NH₄OH

The performic acid-oxidized insoluble tryptic peptide (β-core), Ca 300mg, was dissolved in 3ml of 1M NH₄OH at 0°C, stirred, and adjusted to pH 9.0 with 1N acetic acid. Redigestion was carried out at 0°C for 7 hours with 3mg of trypsin (Worthington Biochemical Corporation, Twice crystallized). The digest was dried in a rotary evaporator for removal of ammonia. All the operations described above were carried out in a low temperature room (0°C). The pH of the solution was not adjusted during digestion, but was 8.7 after 7 hour digestion.

7) Column Chromatography of Tryptic Peptides from the Oxidized Insoluble Tryptic Peptide

Peptides produced by the tryptic redigestion of the insoluble tryptic peptide (β-core) were isolated by column chromatography. Resin used was Dowex 1 × 2 and a column was 1 × 60 cm. Tryptic peptides, after dissolved in 10 ml of deionized water and adjusted to pH 9.5 with 1N NaOH, were applied to the column. Starting buffer was acetate buffer, pH 8.5, containing 4% pyridine and 1% picoline. Development was carried out at the flow rate of 60–70 ml per hour, the column being kept at 37°C. The first 10 fractions were developed with the starting buffer and thereafter gradient elution was employed; that is, from Fraction No. 11 to 67, acetate buffer, pH 5.0, containing 4% pyridine and 1% picoline in the upper chamber was fed into 500 ml of the starting buffer in the mixing chamber, and from Fraction No. 68 to 100, the content in the upper chamber was 1N acetic acid. 10 ml-fractions were collected by a fraction collector and treated in the same way as the peptic peptides described previously.

8) Paper Chromatography of Various Peptides from the Insoluble Tryptic Peptide
The peptides isolated by column chromatography were again isolated and purified by paper chromatography. Descending method was employed on a sheet of Toyo Filter Paper No. 51 with the upper phase of the mixture of n-butanol, glacial acetic acid, and deionized water (4:1:5). The peptides were located by spraying with 2% ninhydrin-n-butanol solution and heating to color with an iron. Besides, location of the peptides which contained histidine was performed by Pauli's reaction. The peptides were eluted from paper with 5% acetic acid.

9) Digestion of the Peptic Peptides with Chymotrypsin and Isolation of Chymotryptic Peptides by Paper Chromatography

Ca 2.0 μmoles of individual peptides, after dissolved in 5 ml of deionized water and adjusted to pH 8.0 was digested at 37°C for 22 hours with 2 mg of chymotrypsin (Sigma Chemical Co., 3 time crystallized). During the beginning 8 hours, the pH was maintained at 8.0 with 0.1N NH₄OH, but was not adjusted after that. The digest was dried under reduced pressure and then dissolved in deionized water. The chymotryptic peptides were isolated and purified by descending paper chromatography with the mixture of n-butanol, glacial acetic acid, deionized water (4:1:5). Elution was performed with 5% acetic acid.

10) Paper Electrophoresis of the Peptides

Electric charge of individual peptides was examined by high voltage paper electrophoresis in order to distinguish between aspartic acid and asparagine, and between glutamic acid and glutamine.

A sample was applied on near the center of a sheet of Toyo Filter Paper No. 51 (10×60cm). Electrophoresis was carried out at 2 KV for 2 hours in pyridine acetate buffer, pH 6.4 (pyridine: glacial acetic acid: deionized water=100:4:900) by hanging the paper in an electrophoretic apparatus (Ishidai type). Detection of the peptides was performed by ninhydrin reaction as in case of paper chromatography.

11) The N-Terminal Amino Acid Analyses of the Peptides by the DNP Method

Dinitrophenylation of the peptides was performed in 1% NaHCO₃ solution. A peptide (0.2–0.5 μmole) was dissolved in 3 ml of 1% NaHCO₃, and to this was added 0.05 ml of 2, 4-dinitro-1-fluorobenzene (DNFB). The reaction was carried out at 40°C for 2 hours. After the reaction was stopped, the excess of DNFB was extracted. The aqueous phase, after dried and redissolved in 4 ml of constant boiling point HCl, was hydrolyzed at 105°C for 16 hours in a sealed tube. The N-terminal
DNP-amino acid was extracted with three 10ml-portions of ether from the hydrolysate. The ether phase transferred in a heart-shaped flask, a modification of Mills' apparatus, was dried under reduced pressure. The flask was then fixed to a cold finger and evacuated by a vacuum pump in order to remove as much dinitrophenol as possible. Two dimensional paper chromatography was employed for identification of the DNP-amino acids. On a sheet of Toyo Filter Paper, ascending method was performed by using n-butanol saturated with 1N NH₄OH for the first dimension and 0.5M phosphate buffer for the second dimension.

12) The PTC Method for Amino Acid Sequence Analyses of the Peptides

Phenylisothiocyanation of the peptides was carried out according to a modification of Edman original method.¹⁰,¹¹ For ring formation, trifluoroacetic acid was used.²⁹ Analyses were performed by so-called elimination method, that is, by examining amino acid composition of the remaining peptide after each stage of the PTC method.

Peptide solution (0.5—2.0 µmole), after dried under reduced pressure in a heart-shaped flask of Mills' apparatus, was dissolved in 2.5ml of 66% pyridine and to this was added 0.05ml of phenylisothiocyanate (PTC) obtained from Hayashi Pure Chemical Industry Ltd. In case the pH of this mixture was below 7.5, it was brought to pH 7.5 with 0.1N NH₄OH. The mixture was incubated at 37°C for 3 hours. After reaction stopped, the reaction mixture was dried under reduced pressure below 37°C and evacuated for 30 min. by a vacuum pump, the flask being fixed to Mills' apparatus. After that, in order to perform ring formation, the mixture was added 1ml of trifluoroacetic acid and allowed to stand at room temperature for 4 hours. After reaction stopped, trifluoroacetic acid was removed under reduced pressure. The residue dissolved in 3ml of deionized water was extracted with four 3ml-portions of benzene. By this treatment, the resultant PTH-amino acids were extracted in the benzene phase and the peptide lacking one N-terminal residue of the original peptide remained in the aqueous phase. A portion of the aqueous phase was dried and hydrolyzed with constant boiling point HCl for 24 hours. The amino acid composition was examined and compared with that of the peptide before subjected to the PTC method. The remainder of the aqueous phase after dried was used in the next stage of the PTC method.

13) The C-Terminal Amino Acid Analyses of the Peptides by Carboxypeptidase A Method

In 0.5 ml of 10% LiCl solution, 0.01ml of carboxypeptidase A (Sigma Chemical Co., 50 mg/ml toluol water suspension) was dissolved.
A sample (0.5–1.0 μmole) in a heart-shaped flask was dissolved in 2 ml of borate buffer, pH 8.1, and to this was added 0.5 ml of carboxypeptidase A solution prepared as above. Digestion was performed at 37°C for the appropriate time and then in order to stop enzymatic reaction, the reaction mixture was added several drops of glacial acetic acid. The digest dried under reduced pressure below 37°C was subjected to amino acid analysis.

Borate buffer was prepared as follows; in 100 ml of 1N NaOH, 12.404g of H₃BO₃ was dissolved and to this was added deionized water so that the volume was 1 liter. This solution was further adjusted to pH 8.1 with 0.1N HCl. It was diluted ten times when used.

14) Amino Acid Analyses of the Peptides

Amino acid compositions of the purified peptides and the remaining peptides after the PTC method were determined after they were completely hydrolyzed with HCl.

Peptides dissolved in 4ml of constant boiling point HCl were hydrolyzed at 105°C for 24 hours in a sealed tube. Dried hydrolysates, after redissolved in a small portion of deionized water and again dried for removal of HCl, were subjected to an amino acid analyzer, Hitachi KLA 2 for the amino acid analyses.

RESULTS AND DISCUSSION

1) Determination of the N-Terminal Amino Acid of the Insoluble Tryptic Peptide by the DNP Method

The N-terminal amino acid of the insoluble tryptic peptide was known to be glycine by the DNP method. It was also confirmed by the comparison with that of human hemoglobin. Hydrolysis was therefore carried out only for 10 hours for keeping out destruction of DNP-glycine as minimal as possible.

2) Amino Acid Sequences of the Peptic Peptides from the Insoluble Tryptid Peptide, βT10, 11, 12 of the β-chain in Macaca mulatta Monkey Hemoglobin

The insoluble tryptic peptide after oxidized with performic acid was digested with pepsin at pH 2.0 for 8 hours at 37°C. The digest was isolated by Dowex 1×2 column chromatography and purified by paper chromatography. Fig. 1 and Fig. 2 show the column and paper chromatograms. As shown in Fig. 1, eight main peptides and some other peptides whose amounts of yield were very small. Amino acid compositions of these eight main peptides were examined and the results are given in Table I.
Table I Amino acid compositions of peptic peptides from the insoluble tryptic peptide, βT10, 11, 12 of the β-chain in *macaca mulatta* monkey hemoglobin

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Table I Amino acid compositions of peptic peptides from the insoluble tryptic peptide βT10, 11, 12 of the β-chain in *macaca mulatta* monkey hemoglobin

Subsequently, the amino acid sequences of individual peptides were determined by the DNP, PTC, and carboxypeptidase methods.

a) PI-a eluted at pH 8.4 Rf Leu 1.00

Three stages of the PTC method were performed.

Stage 1 Phe 0.00 Lys 1.00 Leu 3.11 Asp 0.91 Gly 1.07 Val 0.88
Stage 2 Phe 0.00 Lys 0.00 Leu 3.00 Asp 1.06 Gly 1.00 Val 0.90
Stage 3 Phe 0.00 Lys 0.00 Leu 2.11 Asp 1.06 Gly 1.00 Val 0.90

This peptide was known basic by high voltage paper electrophoresis. This indicates that the residue presented as Asp in the analysis is asparagine. The sequence of this peptide was, therefore, Phe-Lys-Leu [Leu 2, Asp (NH₂) 1, Gly 1, Val, 1]. As shown in Table I, the composition of the C-terminal region of this fragment including the third residue from its N-terminus accords with that of P IV-a, which therefore the subject of the next analysis.

b) PIV-a eluted at pH 6.3 Rf Leu 1.15

The N-terminal residue was leucine by the DNP method. This peptide was neutral, confirming the presence of asparagine. Therefore, PIV-a was deduced to be a peptide positioned next to lysine of PI-a at the C-terminal side. The yield of PI-a was lower than other peptide, whereas that of PIV-a was higher. This fact enabled to confirm that PIV-a overlapped PI-a as mentioned above. The peptide containing one residue each of phenylalanine and lysine must have been discovered but such a peptide could not be detected from any part of paper chromatogram. Four stages of the PTC method were carried on on PIV-a.

Stage 1 Leu 2.04 Gly 0.96 Asp 0.94 Val 1.03
Stage 2 Leu 1.20 Gly 0.87 Asp 0.99 Val 0.92
Stage 3 Leu 1.16 Gly 0.22 Asp 0.98 Val 0.84
Stage 4 Leu 1.16 Gly 0.10 Asp 0.23 Val 0.83

PIV-a (0.5 µmole) was further subjected to carboxypeptidase method. By 30 minute digestion, 0.325 µmole of leucine and 0.16 µmole of valine were obtained. This indicates that the C-terminal residue of
this peptide was leucine. Therefore, the amino acid sequence of PIV-a was determined as follows:

\[ \text{Leu-Leu-Gly-Asp(NH\textsubscript{2})-Val-Leu} \]

By combining the above-mentioned results, the sequence of PI-a was determined as follows:

\[ \text{Val-Cys-Val-Leu} \]

\[ \text{Ala-His-His-Phe} \]

\[ \text{Gly-Lys} \]

c) PIII-a eluted at pH 6.4 Rf Leu 0.22

The N-terminal residue was known to be valine by the DNP method. This peptide was digested with chymotrypsin at pH 8.0 for 22 hours at 37°C. Three peptide fragments were isolated from the digest by paper chromatography. They were designated PIIIa-chy-1, PIIIa-chy-2, and PIIIa-chy-3 in the order of their Rf Leu values from the highest.

**PIIIa-chy-1** Rf Leu 1.00
Composition: Val 1.96, Cys 1.00, Leu 1.03
Three stages of the PTC method were carried out.
Stage 1 Val 1.01 Cys 1.00 Leu 0.98
Stage 2 Val 1.00 Cys 0.00 Leu 0.99
Stage 3 Val 0.20 Cys 0.00 Leu 1.00
The sequence was, therefore, Val-Cys-Val-Leu.

**PIIIa-chy-2** Rf Leu 0.30
Composition: Ala 1.16, His 1.93, Phe 0.90
Stage 1 Ala 0.16 His 2.06 Phe 0.93
Stage 2 Ala 0.00 His 1.00 Phe 1.00
Stage 3 Ala 0.00 His 0.00 Phe 1.00
From the above result, the sequence was determined to be Ala-His-His-Phe.

**PIIIa-chy-3** Rf Leu 0.13
Composition: Gly 1.21, Lys 0.78
Stage 1 Gly 0.30 Lys 1.00
The sequence was therefore, Gly-Lys.
Since the N-terminal residue of PIII-a had already been known to be valine, PIIIa-chy-1 appears to be the N-terminal peptide of P-IIIa. There could be found no peptic peptides in which lysine was positioned at the C-terminus except PIIIa-chy-3. Therefore, PIIIa-chy-3 was decided to be the C-terminal peptide not only of PIII-a but also of the insoluble tryptic peptide.

By putting the above-mentioned results together, the amino acid sequence of PIII-a was determined as follows;

\[
\text{Val - Cys - Val - Leu - Ala - His - His - Phe - Gly - Lys}
\]

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\text{D.P} \quad \text{P} \quad \text{P} \quad \text{P} \quad \text{P} \quad \text{P}
\]

\[\text{PIIIa-chy-1} \quad \text{PIIIa-chy-2} \quad \text{PIIIa-chy-3} \]

P III - a

d) PIII-b eluted at pH 6.4 Rf Leu 0.7
Two stages of the PTC method were carried out.
Stage 1 Ala 0.15 Glu 0.98 Leu 1.02
Stage 2 Ala 0.00 Glu 0.25 Leu 1.00
The result of high voltage paper electrophoresis showed this peptide was neutral, confirming the presence of glutamine. The sequence was, therefore, determined to be Ala-Glu (NH$_2$)-Leu.

e) PVI-a eluted at pH 5.9 Rf Leu 0.7
Two stages of the PTC method were performed.
Stage 1 Gly 0.23 Thr 1.03 Phe 0.97
Stage 2 Glp 0.00 Thr 0.11 Phe 1.00
Since there could be found no peptic peptides whose N-terminal residue was glycine except this peptide, it was decided to be the N-terminal peptide of the $\beta$-core.

f) PVIII-a eluted at pH 4.4 Rf Leu 0.25
By the DNP method, the N-terminal amino acid residue was known to be histidine. Further analysis was not performed because this peptide was known to be identical with PIXa-p-3 of PIX-a.

g) PIX-a eluted at pH 3.8 Rf Leu 0.31
The N-terminal residue was serine as a result of the DNP method. This peptide (30 $\mu$moles) was digested with 2mg of pepsin at pH 2.0 for 22 hours at 37°C. Three peptide fragments were isolated from the digest by paper chromatography. They were designated PIXa-p-1, PIXa-p-2, and PIXa-p-3 in the order of their Rf Leu values from the lowest. The amino acid sequence of each fragment was determined in
the following procedure.

**PIXa-p-1** Rf Leu 0.20  
Composition: His 0.91, Cys 1.00, Asp 1.09, Lys 0.85, Leu 1.13  
Three stages of the PTC method were carried out.  
Stage 1: His 0.00, Cys 1.00, Asp 1.06, Lys 1.00, Leu 0.93  
Stage 2: His 0.00, Cys 0.00, Asp 0.98, Lys 1.00, Leu 1.01  
Stage 3: His 0.00, Cys 0.00, Asp 0.25, Lys 1.00, Leu 1.00  
This peptide after Stage 2 was known to be neutral by high voltage paper electrophoresis. This indicates that the amino acid presented as Asp in the analysis was aspartic acid. The sequence was, therefore, His-Cys-Asp-(Lys, Leu).

**PIXa-p-2** Rf Leu 0.29  
Composition: Lys 1.00, His 1.00, Cys 1.00, Asp 0.94, Ser 0.70, Glu 1.04, Leu 2.30  
Since the composition of this peptide accorded with that of PIX-a, it seemed an indigested peptide. Carboxypeptidase method was carried out on this peptide and the C-terminal amino acid residue was known to be leucine, namely, it was digested at 37°C for 8 hours and subjected to amino acid analysis. Leucine was only recognized.

**PIXa-p-3** Rf Leu 0.87  
Composition: Ser 0.90, Glu 1.03, Leu 1.06  
Two stages of the PTC method were carried out.  
Stage 1: Ser 0.00, Glu 0.94, Leu 1.05  
Stage 2: Ser 0.00, Glu 0.18, Leu 1.00  
This peptide was acidic as a result of high voltage paper electrophoresis, indicating the presence of glutamic acid. The sequence was, therefore, Ser-Glu-Leu.

The sequence of PIX-a peptide was determined as follows by combining the above-mentioned results together.

| Ser - Glu - Leu - His - Cys - Asp - Lys - Leu |
|---|---|---|---|---|---|---|---|
| D.P | P | P | P | P | P | C |

h) **PIX-a** eluted at pH 3.7 Rf Leu 0.22  
The C-terminal amino acid residue of this peptide was known to be asparagine by the carboxypeptidase method. Namely, the amino acid analysis of the digest with carboxypeptidase at 37°C for 75 min. gave asparagine alone. Four stages of the PTC method were carried out.
Stage 1  His 0.00  Val 0.93  Asp 1.96  Pro 1.12  Glu 1.00
Stage 2  His 0.00  Val 0.15  Asp 2.00  Pro 1.08  Glu 0.90
Stage 3  His 0.00  Val 0.00  Asp 1.22  Pro 0.97  Glu 0.95
Stage 4  His 0.00  Val 0.00  Asp 1.04  Pro 0.00  Glu 0.95

According to the result obtained by high voltage paper electrophoresis, this peptide was acidic. It was again subjected to high voltage paper electrophoresis after Stage 2. Its mobility was -2. Therefore, among the two residues presented as Asp and one residue as Glu, two residues were deduced to be acidic amino acids. However, since the C-terminal residue had been known to be Asp (NH$_2$), the following sequence was determined.

His-Val-Asp-Pro-Glu-Asp (NH$_2$).

3) Amino Acid Compositions of Redigested Peptides with Trypsin from the Oxidized $\beta$-Core

In order to determine the whole amino acid sequence of the insoluble tryptic peptide, the performic acid-oxidized insoluble tryptic peptide...
was redigested with trypsin (0.01%) at 0°C for 7 hours in 1M NH₄OH solution. The digest was isolated by Dowex 1×2 column chromatography and purified by paper chromatography. The column chromatogram and the paper chromatogram are given in Fig. 3 and Fig. 4, respectively.

As shown in Fig. 4, two main peptides and several whose yields were very little were obtained. The amino acid compositions of these two peptides were analyzed and shown in Table II.

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<th></th>
<th>Lys</th>
<th>His</th>
<th>Arg</th>
<th>Cys</th>
<th>Asp</th>
<th>Thr</th>
<th>Ser</th>
<th>Glu</th>
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<th>Val</th>
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<th>Leu</th>
<th>Tyr</th>
<th>Phe</th>
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<th>Total</th>
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<td>TVIa-2</td>
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Table II  Amino acid compositions of the tryptic peptides from the oxidized β-core

a) TVI-a luted at pH 5.5  Rf Leu 0.66
The N-terminal was known to be glycine by the DNP method. This peptide was redigested with trypsin at 0°C for 5 hours in 1M NH₄OH solution and the digest was subjected to paper chromatography. As a result, two distinct ninhydrin-positive spots were observed. They were
designated TVIa-1 and TVIa-2, respectively in the order of the Rf value from the lowest.

TVIa-1

The amino acid composition of this peptide was Lys 1.28, His 0.71, Asp 1.98, Glu 1.27, Pro, 1.00 Val 0.96, Leu 1.14, and Phe 0.64. This peptide was first subjected to the DNP method. The N-terminal residue was known to be leucine.

TVIa-2

The composition of this peptide was Lys 1.01, His 0.81, Cys 1.00, Asp 0.96, Thr 0.96, Ser, 1.01, Glu 2.26: Gly 0.96, Ala 1.00, Leu 2.25, and Phe 0.81. According to the result of the DNP method, the N-terminal amino acid was glycine. This may indicate that this peptide is located at the N-terminus of the β-core.

In comparison of macaca mulatta monkey hemoglobin with human hemoglobin, this TVIa-2 appears to correspond to the peptide BRAUNITZER et al. called βT10+11, and there are two amino acid substitutions between these two peptides in their amino acid compositions.

b) TIV-a eluted at pH 5.8 Rf Leu 0.76

The N-terminal amino acid residue of this peptide was known to be leucine by the DNP method. Compared with human hemoglobin, this peptide had the same composition with βT12 of human hemoglobin.

4) Whole Amino Acid Sequence of the Insoluble Tryptic Peptide βT10, 11, 12 (β-Core)

By combining two series of the results, that is, the amino acid sequences of the peptic peptides (PI-a, PIII-a, PIII-b, PVI-a, PIX-a, and PX-a) and the amino acid compositions and the N-terminal analyses of the trypsin-redigested peptides (TIV-a, TVI-a, TVIa-1, TVIa-2),

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Fig. 5 Amino acid sequence of the insoluble tryptic peptide βT10,11,12 from the β-polypeptide chain of macaca mulatta monkey hemoglobin
the amino acid sequence of the $\beta$-core was determined as shown in Fig. 5.

This sequence was compared with the known sequence of the corresponding peptide of human hemoglobin. Two amino acid substitutions were found between them, that is, the 5th and the 22nd residues from the N-terminus are threonine and arginine in case of human hemoglobin, whereas they are glutamine and lysine, respectively in case of macaca mulatta monkey hemoglobin. The substitution at the 22nd residue is seen between gorilla and human hemoglobins\(^{43}\). Furthermore, on viewing these amino acid substitutions from the triplet code suggested by NIRENBERG and OCHOA, the results given in Table III were obtained.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Genetic code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monkey Lys</td>
<td>AAA AGA</td>
</tr>
<tr>
<td>Human Arg</td>
<td>AGA AGG CGA CGG CGC CGU</td>
</tr>
<tr>
<td>Monkey Glu (NH$_2$)</td>
<td>CAA CAG</td>
</tr>
<tr>
<td>Human Thr</td>
<td>ACA ACG ACC ACU</td>
</tr>
</tbody>
</table>

**Table III** The relation between the amino acid substitution and triplet code

As shown in Table III, the substitution, Arg$\rightarrow$Lys is due to single base change, however, Thr$\rightarrow$Glu (NH$_2$) is due to double base change. This fact seems very interesting when considered together with the fact that the abnormal hemoglobin so far discovered are all due to single base change.

**ACKNOWLEDGEMENTS**

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