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<td>著者</td>
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An Abnormal Human Hemoglobin (Hb Rampa) 
Found in Nagasaki

Bunji WATANABE,1 Tomoyuki MAEKAWA,1 Naoko FUJIWARA2 and Genji MATSUDA3

Abstract An abnormal human hemoglobin (Hb Rampa) was found in a 
Japanese adult male living in Nagasaki. The hybridization test of this hemo-
globin showed that the abnormality existed in its \( \alpha \) chain, but the difference 
between the fingerprints of soluble tryptic peptides of this hemoglobin and 
normal human hemoglobin could not be observed. The purification of the ab-
normal \( \alpha \) chain was performed by CM-cellulose column chromatography after S-
carboxymethylation of this chain. The tryptic peptides from the S-carbo-
xymethylated abnormal \( \alpha \) chain were purified by high performance liquid 
chromatography. From the amino acid analysis of the tryptic peptides, it was 
concluded that the amino acid substitution of this abnormal hemoglobin was \( \alpha \) 
95 (Pro→Ser) and this hemoglobin was identified as Hb Rampa.


Key words : Human abnormal hemoglobin, amino acid sequence, HPLC

Introduction

Since Pauling et al. described hemoglobin S (Hb S)1, a number of 
abnormal hemoglobin have been reported in the world with respect to the 
function of hemoglobin and the genetic investigation.2 The Hb Rampa, found 
in 1970, is a relatively stable hemoglobin variant in which the proline residue 
at position 95 of the \( \alpha \) chain is replaced by a serine residue3. Three other 
hemoglobin which have substitutions at \( \alpha \)95 have been described. They are 
Hb G Georgia4 \( \alpha \)95 Pro→Leu, Hb Denmark Hill5 \( \alpha \)95 Pro→Ala and Hb St.

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Lukes$^6$ $\alpha 95$ Pro$\rightarrow$ Arg.

In Nagasaki, three abnormal hemoglobins, Hb Nagasaki$^7$ $\beta 27$ Glu$\rightarrow$Lys, Hb Atago$^8$ $\alpha 85$ Asp$\rightarrow$Tyr and Hb E$^9$ $\beta 26$ Glu$\rightarrow$Lys have been reported. In this paper, the authors describe the fourth abnormal hemoglobin, Hb Rampa, found in Nagasaki.

**Materials and Methods**

**Materials**

Hemoglobin solution was prepared from a Japanese adult male who showed no significant clinical symptoms by the method of Drabkin$^{10}$.  

*Starch gel electrophoresis*

Thin-layer starch gel electrophoresis of hemoglobin was carried out according to a modification of the method of Smithies$^7,^{11}$. Hemoglobin was stained with amido black or O-dianisidine.

*Column chromatography of hemoglobin*

The CM-cellulose (Whatman Biochemical Co.) column chromatography for the separation of normal and abnormal hemoglobin was performed according to the method previously described$^7$.

*Hybridization test*

Hybridization of the separated abnormal hemoglobin (Hb Aô) and normal human hemoglobin (Hb Ao) with canine hemoglobin was performed by the method of Gammack et al.$^{12}$.

*Fingerprint of hemoglobin*

Fingerprints of the separated abnormal hemoglobin and the normal hemoglobin were carried out by the method of Ingram$^{13}$.

*Separation of the $\alpha$ and $\beta$ chains of globin*

The heme was removed from the globin by the method of Anson and Mirsky$^{14}$. The globin was separated into $\alpha$ and $\beta$ chains by CM-52 column chromatography by the method reported previously$^{15}$.

*S-carboxymethylation*

S-carboxymethylation of the $\alpha$ chain was carried out by the method of Crestfield et al.$^{16}$.

*Cellogel electrophoresis of S-carboxymethylated $\alpha$ chain*

The purity and the mobility of the CM $\alpha$ chain were analyzed by Cellogel (Chemetron Co., Italy) electrophoresis with 30 mM phosphate buffer (pH 8.6) or 20 mM phosphate buffer (pH 6.8) containing 8 M urea and 20 mM 2-mercaptoethanol.

*Purification of the abnormal CM $\alpha$ chain*

The CM $\alpha$ chain was purified on a column of CM-52 as described
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previously\(^\text{17}\).

**Fragmentation of the CM \(\alpha\) chain**

The purified S-carboxymethylated abnormal \(\alpha\) chain was digested with TPCK-trypsin (Worthington Biochemical Co.) at pH 8.6 and 37° C for 6 h.

**Isolation of the tryptic peptides**

The tryptic peptides were isolated by high performance liquid chromatography on a reversed phase column (0.4 × 15 cm, \(\mu\) Bondasphere, Waters Co.). Peptides were eluted with a linear gradient of acetonitrile in 0.1% trifluoro acetic acid.

**Amino acid analysis**

The amino acid compositions of the purified peptides were determined using a JEOL JLC 300 amino acid analyzer.

**Results**

On the starch gel electrophoresis of the hemoglobin solution from the propositus the major abnormal hemoglobin (Hb \(A_6^\prime\)) migrated more slowly than Hb \(A_6\) and a minor abnormal hemoglobin (Hb \(A_2^\prime\)) migrated relatively slower than Hb \(A_2\) and similar to non hemoglobin protein zone (NHP) were observed (Fig. 1).

![Fig. 1. Thin-layer starch gel electrophoresis of hemoglobins in tris-EDTA-borate buffer, pH 8.6.](image)

- I : Normal human hemolysate
- II : Hemolysate of the propositus
- III : Hemolysate of an infant
- NHP : non hemoglobin protein
The existence of Hb A₂ component suggested that the abnormality of this abnormal hemoglobin existed in its α chain. The elution pattern on CM-52 column of the hemoglobin from the propositus is shown in Fig. 2. The abnormal hemoglobin (Hb A₆) was eluted between the normal Hb A₀ and Hb A₂ under the conditions described in Fig. 2.

![Figure 2](image)

**Fig. 2.** Separation of the abnormal Hb on a column of CM-52 (2.5×50cm) equilibrated with 0.01 M phosphate buffer, pH 6.8, and eluted with a exponential gradient of pH (pH 6.8-8.5). The effluent was pooled as indicated by the bars (pools 1-2). 8 ml fractions were collected.

The electrophoretic pattern of hemoglobins after hybridization is presented in Fig. 3. The hybrid molecule of α₂^{HbA₀} β₂^{canine} showed the different mobility from the hybrid molecule of α₂^{HbA₀} β₂^{canine}. On the other hand, the hybrid molecule of α₂^{canine} β₂^{HbA₀} showed the same mobility as the hybrid molecule of α₂^{canine} β₂^{HbA₀}. From above results, it was concluded that the Hb A₀ had its abnormality in its α chain. The difference between the fingerprints of soluble tryptic digests of Hb A₀ and of Hb A₀ could not be observed.

Chromatographic separation of the globin from whole hemolysate of the propositus on CM-52 or DE-52 in 8 M urea could not obtained in the expected separation of the normal and abnormal α chains. Therefore, the abnormal hemoglobin was separated by CM-52 column chromatography under the condition described in Mayerials and Methods. After the removal
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Fig. 3. Hybridization test of hemoglobins.

I : Mixture of normal human Hb (Hb A₀) and canine Hb
II : Hybrid molecules between normal human Hb and canine Hb
III : Hybrid molecules between abnormal Hb (Hb A₁₀) and canine Hb
IV : Mixture of abnormal Hb (Hb A₁₀) and canine Hb
(1) α₂HbA₀ β₂canine (2) α₂HbA₁₀ β₂canine (3) canine Hb
(4) HbA₀' (5) HbA₀ (6) α₂canine β₂HbA₀

of the heme from the separated abnormal hemoglobin, the globin was applied to the CM-52 column to separate into the abnormal α chain and the β chain (Fig. 4). However the result of Cellogel electrophoresis of the α chain fraction (peak 2 in Fig. 4) after S-carboxymethylation suggested that this fraction was still contaminated with a fairly amount of normal CM α chain. Therefore the further purification of the abnormal CM α chain was performed on a column of CM-52. The abnormal CM α chain was eluted in the peak 3 in Fig. 5 and identified as pure by Cellogel electrophoresis.

The purified abnormal CM α chain was digested with TPCK trypsin. The pH of the digestion mixture was adjusted to 6.4 and the resulting precipitate was separated by centrifugation. The peptides in the supernatant were isolated by the reversed phase HPLC on a column of μBondasphere as shown in Fig. 6. Amino acid compositions of the purified tryptic peptides are summarized in Table 1. The peptides were designated αT1 to αT14 based on the nomenclature of the tryptic peptides from the normal human α chain. Except for αT11, all peptides show compositions which were not at variance with those of the corresponding normal α chain peptides. The composition of αT11 which occupies positions 93 to 99 is Asp (2), Ser (1), Val (2), Phe (1) and Lys (1). The abnormal αT11 apparently lacked the proline residue normally present.

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Fig. 4. Separation of the α and β chain of the abnormal hemoglobin by CM-52 column (2 × 15 cm) equilibrated with 0.07 M Na₂HPO₄, pH 6.7 containing 8 M urea and 0.05 M 2-mercaptoethanol, and eluted with a gradient of Na₂HPO₄ (0.007-0.03 M, 500-500 ml). 6 ml fractions were collected. The effluent was pooled as indicated by the bars (pools 1-2).

Fig. 5. Further purification of the α chain of Hb A₀ on a column of CM-52 (2 × 15 cm).
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Fig. 6. Separation of tryptic peptides of the abnormal CM α chain by reversed phase HPLC on a column of µBondasphere C18 (0.4×15 cm). The elution was performed with a linear gradient of acetonitrile in 0.1% trifluoro acetic acid from 0 to 45% in 60 min at a flow rate of 0.7 ml/min.

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

<table>
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<tr>
<th></th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
<th>T7</th>
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<tr>
<td>Asp</td>
<td>0.97(1)</td>
<td>0.98(1)</td>
<td>1.04(1)</td>
<td></td>
<td></td>
<td></td>
<td>1.04(1)</td>
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<tr>
<td>Thr</td>
<td>0.93(1)</td>
<td>0.97(1)</td>
<td></td>
<td>1.00(2)</td>
<td>1.05(1)</td>
<td></td>
<td></td>
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<tr>
<td>Ser</td>
<td></td>
<td></td>
<td>2.95(3)</td>
<td>1.22(1)</td>
<td>1.05(1)</td>
<td></td>
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<tr>
<td>Glu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.14(1)</td>
</tr>
<tr>
<td>Pro</td>
<td>1.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Gly</td>
<td></td>
<td>2.86</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ala</td>
<td></td>
<td></td>
<td></td>
<td>2.09</td>
<td>4.03(4)</td>
<td>1.01(1)</td>
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<tr>
<td>Val</td>
<td>0.93</td>
<td>1.12(1)</td>
<td></td>
<td></td>
<td></td>
<td>1.08(1)</td>
<td>0.92(1)</td>
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<tr>
<td>Met</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.89(1)</td>
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<tr>
<td>Leu</td>
<td>1.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.10(1)</td>
<td>1.13(1)</td>
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<tr>
<td>Tyr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.01(1)</td>
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<tr>
<td>Phe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.94(2)</td>
</tr>
<tr>
<td>His</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.90(1)</td>
</tr>
<tr>
<td>Lys</td>
<td>0.96</td>
<td>1.05(1)</td>
<td></td>
<td>1.10(1)</td>
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<td>0.98(1)</td>
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<tr>
<td>Trp</td>
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<td>0.77(1)</td>
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<tr>
<td>Arg</td>
<td></td>
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Total Res. 7 4 5 15 9 16 4

αT12 was not analysed. Amino acids of αT8 and αT10 were analysed after purification by paper chromatography.
in position 95 of the α chain, and contained instead a serine residue. From the above results, it is concluded that in this abnormal hemoglobin, α95 pro in the normal hemoglobin was substituted into Ser. And thus this abnormal hemoglobin was determined to be Hb Rampa.

**Discussion**

Hemoglobin Rampa was found in a Japanese adult male who lived in Nagasaki and was clinically and hematologically normal. This paper describes the first confirmed evidence of the Hb Rampa discovered in Japan. Hb Rampa was found first in India and described by W. W. W. DE Jong et al.\(^ {3} \). All carriers of Hb Rampa are clinically and hematologically normal\(^ {3} \). This hemoglobin variant migrates more slowly towards the anode than Hb A\(_ {0} \) on electrophoresis at pH 8.6 despite having a neutral mutation, α95 pro→Ser. Hb Denmark Hill and Hb Georgia having the same neutral mutations at α95, and also show slow electrophoretic mobilities\(^ {4,5} \). The conformational changes caused by the substitution at α95 may alter surface charge of the abnormal hemoglobin molecule. And proline residue in position α95 is involved in the α\(_ {1} \) β\(_ {2} \) contact which is of great importance for the physiological properties of the hemoglobin molecule.\(^ {18} \) Actually, all abnormal hemoglobins which have substitutions at α95 show altered oxygen affinities, having decreased heme-heme interaction, increased oxygen affinities and reduced Bohr effect\(^ {3,4,5} \).

In the course of evolution, the hemoglobin molecule has undergone extensive amino acid substitutions. Recently, Nagai et al.\(^ {19} \) and Perutz et al.\(^ {20} \) described the method to distinguish functionally important substitut-
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ions from those which might be neutral in evolution\textsuperscript{21} by using authentic $\beta$-globins produced in Esherichia coli. The studies of the human mutant hemoglobins also contribute greatly to developing our understanding of the molecular basis of the function and the evolution of human hemoglobin.

In this study, the separation of CM globin on CM-52 was a fairly useful method for the abnormal hemoglobin. For the isolation of tryptic peptides of hemoglobin, various approaches, such as ion exchange chromatography, gel filtration and paper chromatography have been employed. We have had great success with RP HPLC for the isolation of peptides. With the HPLC methodology it is now possible to rapidly perform the process of peptide isolation of abnormal hemoglobin, and handle microquantities of material. Such a miniaturization may lead to new strategies both in the isolation and in the analysis of the abnormal hemoglobin component.

References


(1988年12月13日受理)
長崎で発見されたヒト異常ヘモグロビン・ヘモグロビン・ランパ（Hb Rampa）の一次構造

渡辺 文治 1 前川 知之 1 藤原 直子 2 松田 源治 3

1 長崎大学医療技術短期大学部一般教育
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3 長崎大学医学部生化学教室

要旨 この報告はヒト異常ヘモグロビン Hb Rampa についての我国最初の詳細な記載である。Pauling が HbS を報告して以来、Hb 分子の機能や遺伝の面から多くの種類のヒト異常ヘモグロビンが報告されている。我々は長崎在住の男性の Hb α鎖の異常を電気泳動で見出し、部分精製した α鎖のトリプシン消化物をフィンガープリン
ト法で検索したが、異常を発見できなかった。このため Hb を化学修飾後に CM−セ
ルロース・カラムクロマトを行なう方法で正常と異常 α鎖を分離し得ることを見出し
た。分離精製した異常 α鎖のトリプシン分解を行ない生じたペプチド断片は高速液体
クロマトグラフィー（HPLC）で精製した。精製ペプチドのアミノ酸組成分析の結果、
α鎖の95番目のプロリンがセリンに置換されていることが明らかとなり、この Hb を
Hb Rampa と確認した。中性アミノ酸の変化にもかかわらず電気泳動で発見される例は他にも報告があり、蛋白のコンフォーメーションの変化に基づくものと考えられ
る。またこの α95 の部位のアミノ酸置換例は他にも報告があり、Hb 分子の機能との
関連、進化の中立説など最近の知見も混えて考察した。HPLC を用いた異常ヘモグロ
ビンの分離同定は今後重要な方法となり得るものと考えられた。

長大医短紀要 2:1−11, 1988