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<td>Author(s)</td>
<td>Watanabe, Bunji; Maekawa, Tomoyuki; Fujiwara, Naoko; Matsuda, Genji</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 hemoglobin showed that the abnormality existed in its α 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 abnormal α chain was performed by CM-cellulose column chromatography after S-carboxymethylation of this chain. The tryptic peptides from the S-carboxymethylated abnormal α 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 α95(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 α chain is replaced by a serine residue3. Three other hemoglobin which have substitutions at α95 have been described. They are Hb G Georgia4 α95 Pro→Leu, Hb Denmark Hill5 α95 Pro→Ala and Hb St.

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In Nagasaki, three abnormal hemoglobins, Hb Nagasaki$^7$ $\beta17$ Glu$\rightarrow$ Lys, Hb Atago$^8$ $\alpha85$ Asp$\rightarrow$ Tyr and Hb E$^9$ $\beta26$ 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 A) 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\textsuperscript{17}.

\textit{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.

\textit{Isolation of the tryptic peptides}

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

\textit{Amino acid analysis}

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

\section*{Results}

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

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{Thin-layer starch gel electrophoresis of hemoglobins in tris-EDTA-borate buffer, pH 8.6. \(I\) : Normal human hemolysate \(II\) : Hemolysate of the propositus \(III\) : Hemolysate of an infant \(NHP\) : non hemoglobin protein}
\end{figure}
The existence of Hb A′ 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 A′) was eluted between the normal Hb A0 and Hb A2 under the conditions described in Fig. 2.

![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 an 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.](image)

The electrophoretic pattern of hemoglobins after hybridization is presented in Fig. 3. The hybrid molecule of α2⁢HbA0 β2⁢canine showed 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 A′ had its abnormality in its α chain. The difference between the fingerprints of soluble tryptic digests of Hb A′ 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 Materials and Methods. After the removal
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Fig. 3. Hybridization test of hemoglobins.

I: Mixture of normal human Hb (Hb A\textsubscript{0}) and canine Hb
II: Hybrid molecules between normal human Hb and canine Hb
III: Hybrid molecules between abnormal Hb (Hb A\textsubscript{0}) and canine Hb
IV: Mixture of abnormal Hb (Hb A\textsubscript{0}) and canine Hb

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

The purified abnormal CM \(\alpha\) 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 \(\mu\)Bondasphere as shown in Fig. 6. Amino acid compositions of the purified tryptic peptides are summarized in Table 1. The peptides were designated \(\alpha\text{T1}\) to \(\alpha\text{T14}\) based on the nomenclature of the tryptic peptides from the normal human \(\alpha\) chain. Except for \(\alpha\text{T11}\), all peptides show compositions which were not at variance with those of the corresponding normal \(\alpha\) chain peptides. The composition of \(\alpha\text{T11}\) which occupies positions 93 to 99 is Asp (2), Ser (1), Val (2), Phe (1) and Lys (1). The abnormal \(\alpha\text{T11}\) apparently lacked the proline residue normally present.
Fig. 4. Separation of the $\alpha$ and $\beta$ chain of the abnormal hemoglobin by CM-52 column ($2 \times 15$ cm) equilibrated with 0.07 M $\text{Na}_2\text{HPO}_4$, pH 6.7 containing 8 M urea and 0.05 M 2-mercaptoethanol, and eluted with a gradient of $\text{Na}_2\text{HPO}_4$ (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 $\alpha$ chain of Hb A$_0$ on a column of CM-52 ($2 \times 15$ 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 (0.4×15cm). 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>
<thead>
<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>1.04(1)</td>
<td></td>
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<tr>
<td>Thr</td>
<td></td>
<td>0.97(1)</td>
<td></td>
<td>1.80(2)</td>
<td>1.05(1)</td>
<td></td>
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</tr>
<tr>
<td>Ser</td>
<td>0.92(1)</td>
<td></td>
<td></td>
<td>1.00(1)</td>
<td>1.79(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td></td>
<td></td>
<td>2.95(3)</td>
<td></td>
<td>1.05(1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>1.11(1)</td>
<td></td>
<td></td>
<td>1.22(1)</td>
<td>1.14(1)</td>
<td></td>
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</tr>
<tr>
<td>Gly</td>
<td></td>
<td></td>
<td>2.88(3)</td>
<td></td>
<td>1.04(1)</td>
<td>1.97(2)</td>
<td></td>
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<tr>
<td>Ala</td>
<td>1.10(1)</td>
<td></td>
<td>2.09(2)</td>
<td>4.03(4)</td>
<td>1.01(1)</td>
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<tr>
<td>Val</td>
<td>0.93(1)</td>
<td>1.12(1)</td>
<td></td>
<td>1.08(1)</td>
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<td>0.92(1)</td>
<td></td>
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<tr>
<td>Met</td>
<td></td>
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<td></td>
<td>0.89(1)</td>
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</tr>
<tr>
<td>Leu</td>
<td>1.02(1)</td>
<td></td>
<td></td>
<td>1.10(1)</td>
<td>1.13(1)</td>
<td>1.12(1)</td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td></td>
<td></td>
<td></td>
<td>1.01(1)</td>
<td></td>
<td>0.92(1)</td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td></td>
<td></td>
<td></td>
<td>1.94(2)</td>
<td></td>
<td>2.08(2)</td>
<td></td>
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<tr>
<td>His</td>
<td></td>
<td></td>
<td>0.90(1)</td>
<td></td>
<td>1.84(2)</td>
<td>0.97(1)</td>
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<tr>
<td>Lys</td>
<td>0.96(1)</td>
<td>1.05(1)</td>
<td>1.10(1)</td>
<td></td>
<td>0.98(1)</td>
<td>0.98(1)</td>
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<td>Trp</td>
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<td>0.77(1)</td>
<td></td>
<td></td>
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<tr>
<td>Arg</td>
<td></td>
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<td></td>
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Total Res. 7 4 5 15 9 16 4

aT12 was not analysed. Amino acids of aT8 and aT10 were analysed after purification by paper chromatography.

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in position 95 of the $\alpha$ chain, and contained instead a serine residue. From the above results, it is concluded that in this abnormal hemoglobin, $\alpha_{95}$ 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 $\alpha_0$ on electrophoresis at pH 8.6 despite having a neutral mutation, $\alpha_{95}$ pro$\rightarrow$ Ser. Hb Denmark Hill and Hb Georgia having the same neutral mutations at $\alpha_{95}$, and also show slow electrophoretic mobilities\(^4,5\). The conformational changes caused by the substitution at $\alpha_{95}$ may alter surface charge of the abnormal hemoglobin molecule. And proline residue in position $\alpha_{95}$ is involved in the $\alpha_1 \beta_2$ contact which is of great importance for the physiological properties of the hemoglobin molecule.\(^6\) Actually, all abnormal hemoglobins which have substitutions at $\alpha_{95}$ 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 evolution2 by using authentic β-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


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

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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－セロース・カラムクロマトを行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ぜて考察した。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