An Abnormal Human Hemoglobin (Hb Rampa) Found in Nagasaki

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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 purifild 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 α 95 (Pro→Ser) and this hemoglobin was identified as Hb Rampa.

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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 residue[3]. Three other hemoglobin which have substitutions at α95 have been described. They are Hb G Georgia[4] α95 Pro→Leu, Hb Denmark Hill[5] α95 Pro→Ala and Hb St.

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Lukes[6] α95 Pro→ Arg.

In Nagasaki, three abnormal hemoglobins, Hb Nagasaki[7] β17 Glu→Lys, Hb Atago[8] α85 Asp→Tyr and Hb E[9] β26 Glu→Lys have been reported. In this paper, the authers 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 symptome 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 Aó) 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 α and β chains of globin

The heme was removed from the globin by the method of Anson and Mirsky [14]. The globin was separated into α and β chains by CM-52 column chromatography by the method reported previously[15].

S-carboxymethylation

S-carboxymethylation of the α chain was carried out by the method of Crestfield *et al.*[16].

Cellogel electrophoresis of S-carboxymethylated α chain

The purity and the mobility of the CM α 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 α chain

The CM α chain was purified on a column of CM-52 as described

previously[17].

Fragmentation of the CM α chain

The purified S-carboxymethylated abnormal α chain was digested with TPCK-trypsin (Worthington Biochemical Co.) at pH 8.6 and 37℃ 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㎝, μ 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_0') migrated more slowly than Hb A_0 and a minor abnormal hemoglobin (Hb A_2') migrated relatively slower than Hb A_2 and similar to non hemoglobin protein zone (NHP) were observed (Fig. 1).

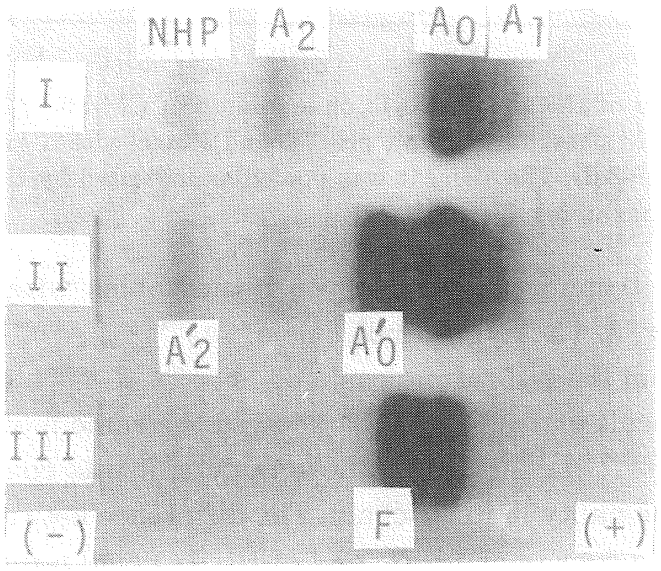

Fig. 1. Thin-layer starch gel electrophoresis of hemoglobins in tris-EDTA-borate buffer, pH 8.6.
I : Normal human hemolysate
II : Hemolysate of the propositus
III : Hemolysate of an infant
NHP : non hemoglobin protein

The existence of Hb A_2' 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 hemoglobim (Hb A_0') was eluted between the normal Hb A_0 and Hb A_2 under the conditions described in Fig. 2.

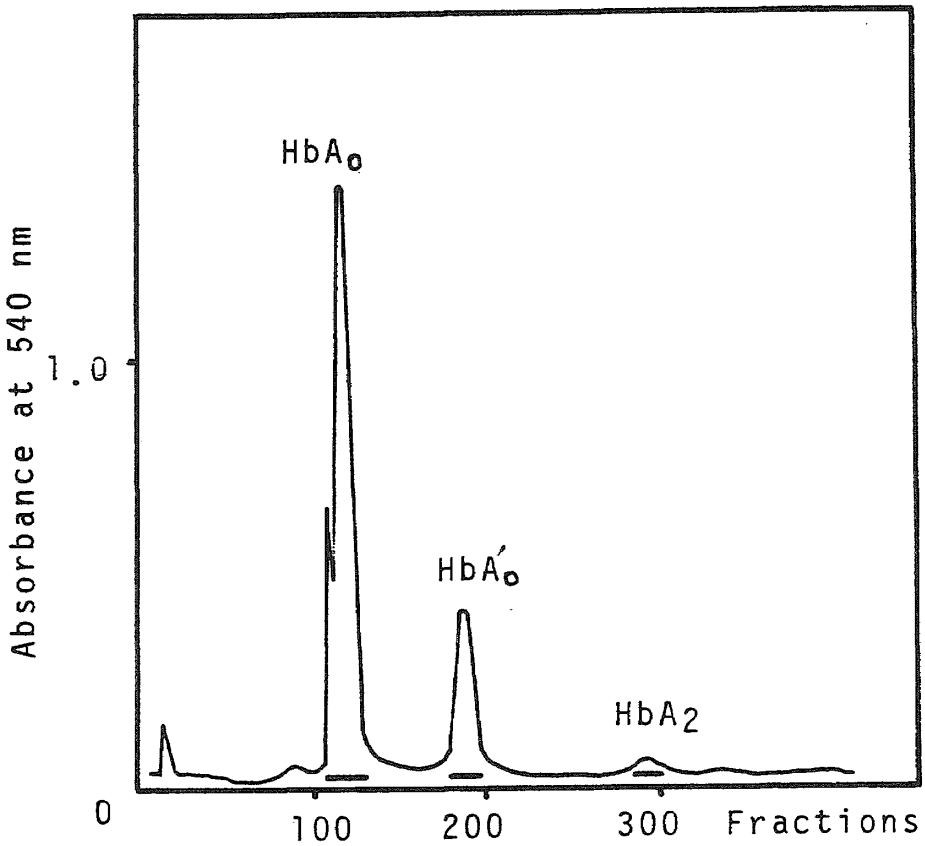

Fig. 2. Separation of the abnormal Hb on a column of CM-52 (2.5×50cm) equilibrated with 0.01 M phosphate buffer, pH 6.8, and eluted with a exponential gradient of pH (pH 6.8-8.5). The effluent was pooled as indicated by the bars (pools 1-2). 8 ml fractions were collected.

The electrophoretic pattern of hemoglobins after hybridization is presented in Fig. 3. The hybrid molecule of $\alpha_2^{HbA_0'}\ \beta_2^{canine}$ showed the different mobility from the hybrid molecule of $\alpha_2^{HbA_0}\ \beta_2^{canine}$. On the other hand, the hybrid molecule of $\alpha_2^{canine}\ \beta_2^{HbA_0'}$ showed the same mobility as the hybrid molecule of $\alpha_2^{canine}\ \beta_2^{HbA_0}$. From above results, it was concluded that the Hb A_0' had its abnormality in its α chain. The difference between the fingerprints of soluble tryptic digests of Hb A_0' and of Hb A_0 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 *Metarials and Methods*. After the removal

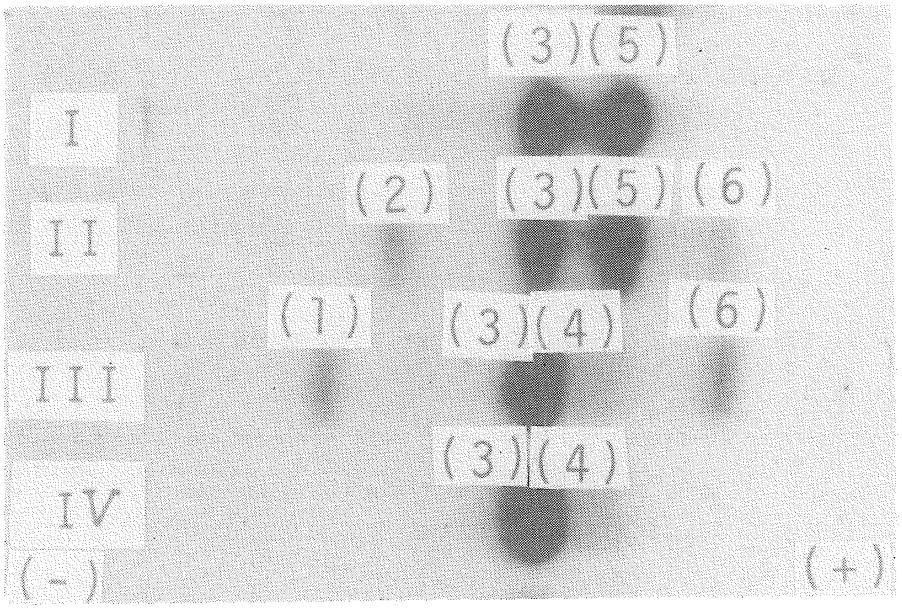

Fig. 3. Hybridization test of hemoglobins.
I : Mixture of normal human Hb (Hb A_0) and canine Hb
II : Hybrid molecules between normal human Hb and canine Hb
III : Hybrid molecules between abnormal Hb (Hb A_0') and canine Hb
IV : Mixture of abnormal Hb (Hb A_0') and canine Hb
(1) $\alpha_2^{HbA_0'}\beta_2^{canine}$ (2) $\alpha_2^{HbA_0}\beta_2^{canine}$ (3) canine Hb
(4) HbA_0' (5) HbA_0 (6) $\alpha_2^{canine}\beta_2^{HbA_0}$

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

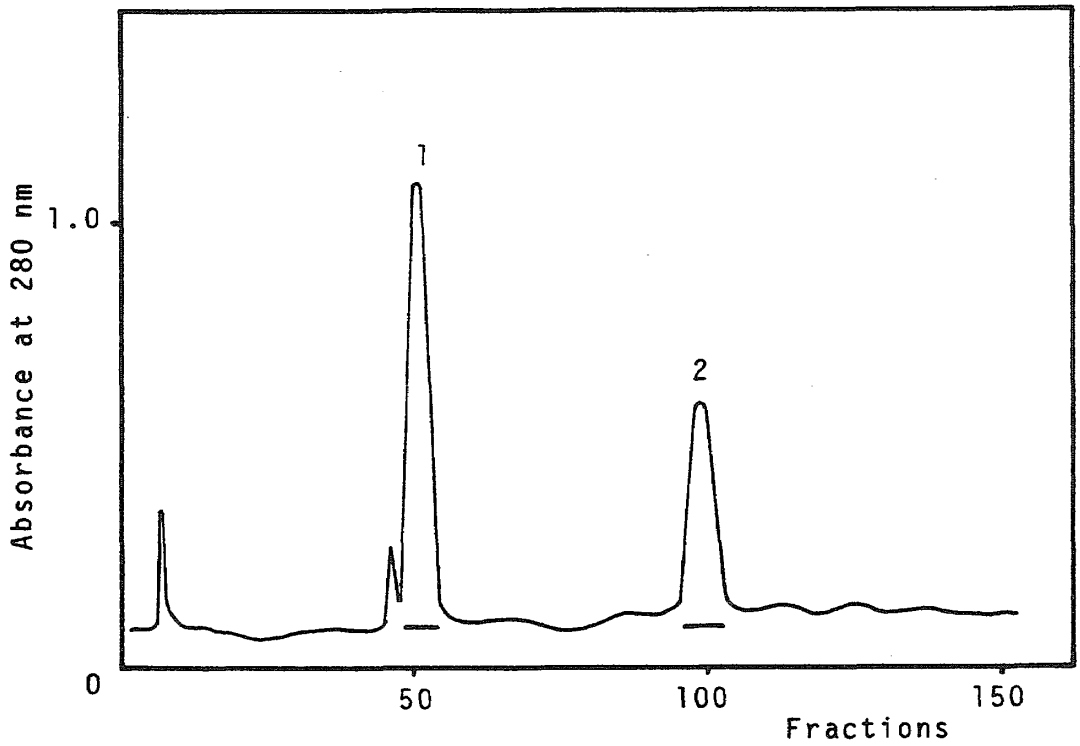

Fig. 4. Separation of the α and β chain of the abnormal hemoglobin by CM-52 column (2×15cm) equilibrated with 0.07 M Na_2HPO_4, pH 6.7 containing 8 M urea and 0.05 M 2-mercaptoethanol, and eluted with a gradient of Na_2HPO_4 (0.007-0.03 M, 500+500 ml). 6 ml fractions were collected. The effluent was pooled as indicated by the bars (pools 1-2).

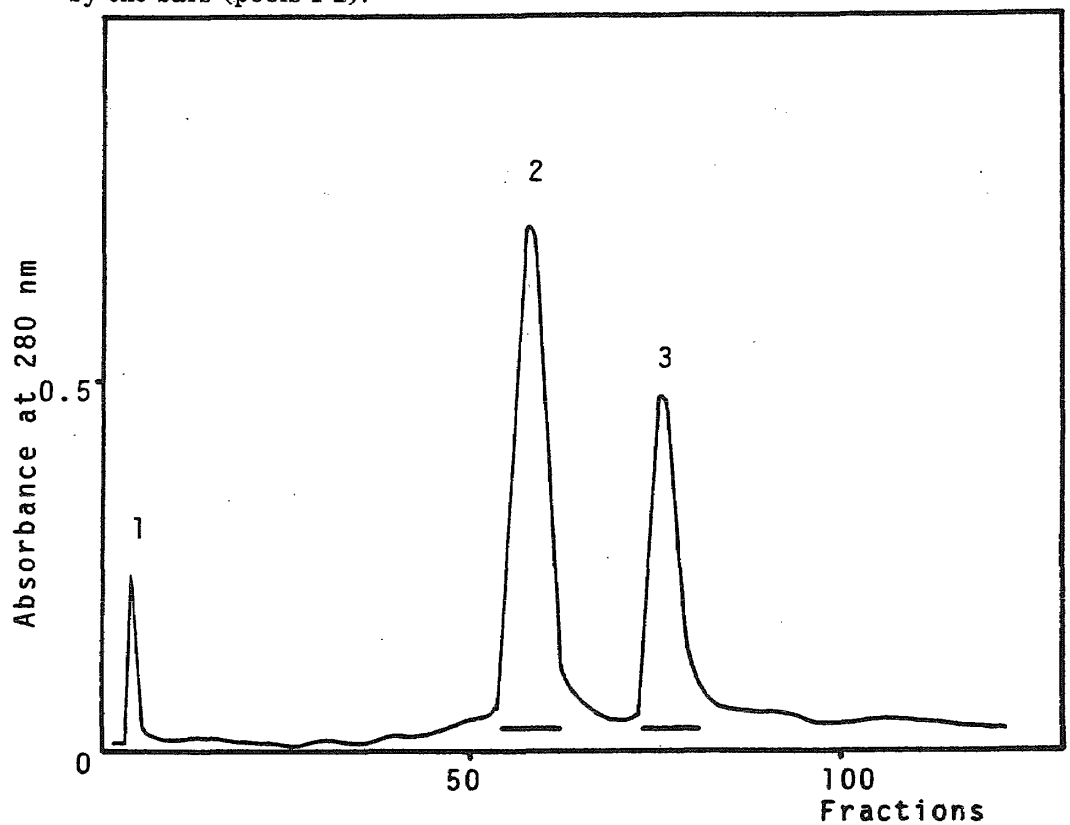

Fig. 5. Further purification of the α chain of Hb A_0' on a column of CM-52 (2×15cm).

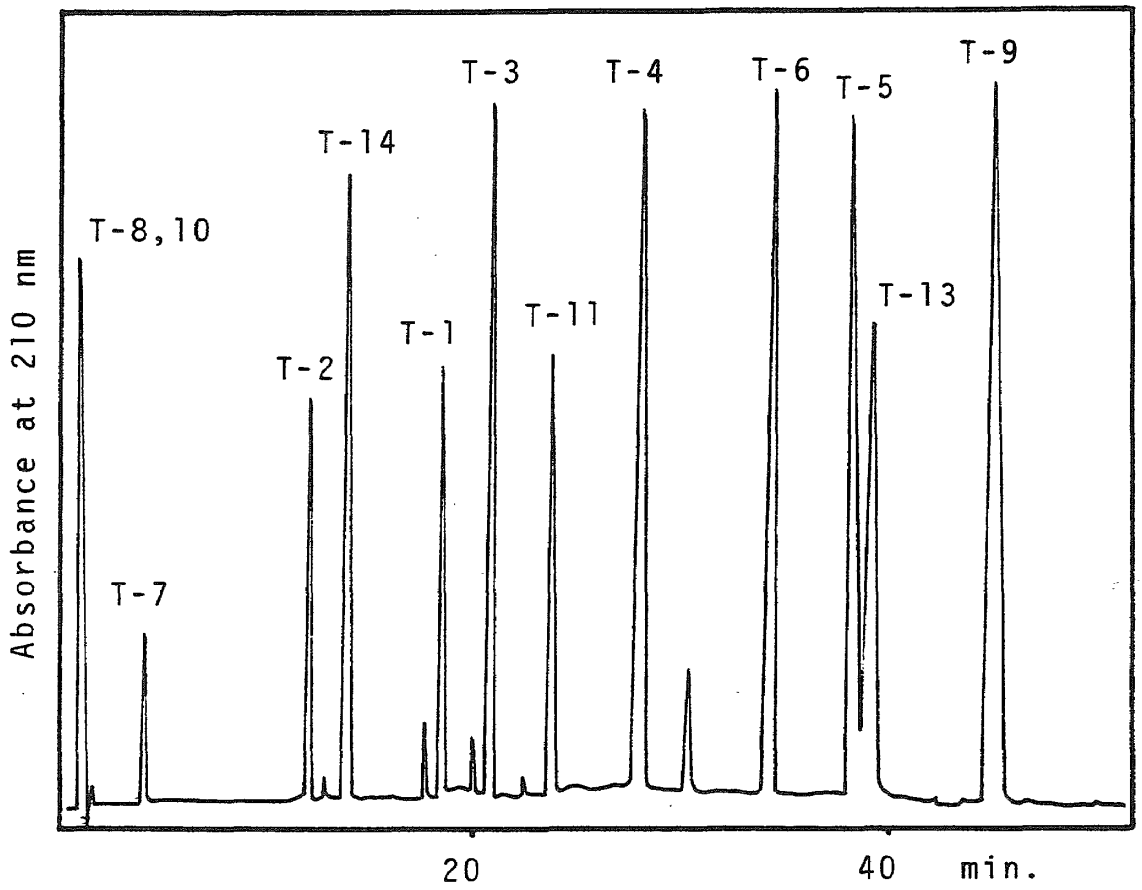

Fig. 6. Separation of tryptic peptides of the abnormal CM α chain by reversed phase HPLC on a column of μBondasphere C_{18} (0.4×15cm). The elution was performed with a linear gradient of acetonitrile in 0.1% trifluoro acetic acid from 0 to 45% in 60 min at a flow rate of 0.7 ml/min.

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

	T1	T2	T3	T4	T5	T6	T7
Asp	0.97(1)	0.98(1)	1.04(1)			1.04(1)	
Thr		0.97(1)			1.80(2)	1.05(1)	
Ser	0.92(1)				1.00(1)	1.79(2)	
Glu				2.95(3)		1.05(1)	
Pro	1.11(1)				1.22(1)	1.14(1)	
Gly				2.88(3)		1.04(1)	1.97(2)
Ala	1.10(1)		2.09(2)	4.03(4)		1.01(1)	
Val	0.93(1)	1.12(1)		1.08(1)		0.92(1)	
Met					0.89(1)		
Leu	1.02(1)			1.10(1)	1.13(1)	1.12(1)	
Tyr				1.01(1)		0.92(1)	
Phe					1.94(2)	2.08(2)	
His				0.90(1)		1.84(2)	0.97(1)
Lys	0.96(1)	1.05(1)	1.10(1)		0.98(1)	0.98(1)	1.06(1)
Trp			0.77(1)				
Arg				1.03(1)			
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.

Table 1B.

	T8	T9	T10	T11	T13	T14
Asp		6.13(6)		2.06(2)		
Thr		0.96(1)			2.00(2)	
Ser		1.83(2)		0.93(1)	2.73(3)	
Pro		1.00(1)				
Ala		7.29(7)			1.18(1)	
Val		3.06(3)		1.94(2)	1.91(2)	
Met		0.88(1)				
Leu		4.14(4)	1.02(1)		2.10(2)	
Tyr						0.75(1)
Phe				1.05(1)	1.06(1)	
His		2.68(3)				
Lys	1.00(1)	1.04(1)		1.02(1)	1.01(1)	
Arg			0.98(1)			1.25(1)
Total Res.	1	29	2	7	12	2

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 $\alpha_1\beta_2$ 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-

ions from those which might be neutral in evolution[21] 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 fitration 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

1. Pauling L, Itano H, Singer S. J, Wells Z. C : Sickle cell anemia, a molecular disease. Science **100** : 543-548, 1949.
2. Bunn H F, Forget B G : Hemoglobin : molecular, genetic and clinical aspects. Saunders, Philadelphia, 1986.
3. De Jong W W W, Bernini L F, Khan P M : Hemoglobin Rampa : α95 Pro → Ser. Biochim. Biophys. Acta **236** : 197-200, 1971.
4. Huisman T H J, Adams H R, Wilson J b, Efremov G D, Reynolds C A, Wrightstone R N : Hemoglobin G Georgia or $\alpha_2^{95\,\mathrm{Leu\,(G\text{-}2)}}\beta_2$. Biochim. Biophys. Acta **200** : 578-580, 1970.
5. Wiltshire B G, Clark K G A, Lorkin P A, Lehmann H : Hemoglobin Denmark Hill $\alpha^{95\,(\mathrm{G\,2})\,\mathrm{Pro}\rightarrow\mathrm{Ala}}$, A variant with usual electrophoretic and oxygen-binding properties. Biochim. Biophys. Acta **278** : 459-464, 1972.
6. Bannister W H, Grech J L, Plese C F, Smith L L, Barton B P, Wilson J B, Reynolds C A, Huisman T H J : Hemoglobin St Luke's or $\alpha_2^{95\,\mathrm{Arg(G2)}}\beta_2$. Eur. J. Biochem. **29** : 301 -307, 1972.
7. Maekawa M, Maekawa T, Fujiwara N, Tabara K, Matsuda G:Hemoglobin Nagasaki ($\alpha_2^{\mathrm{A}}\ \beta_2^{17\mathrm{Glu}}$) A new abnormal human hemoglobin found in one family in Nagasaki. Int. J. Protein Reserch II(3) : 147-156, 1970.
8. Fujiwara N, Maekawa T, Matsuda G : Hemoglobin Atago ($\alpha_2^{85\mathrm{Tyr}}\ \beta_2$) A new abnormal human hemoglobin found in Nagasaki. Int. J. Protein Reserch III (1) 35-39, 1971.
9. Hunt J A, Ingram V M : Abnormal human hemoglobin. The clinical difference between hemoglobin A and E. Biochem. Biophys. Acta **49** : 520-536, 1961.

10. Drabkin D L : Spectrophotometric studies XIV. The crystallographic and optical properties of the hemoglobin of man in composition with those of other species. J. Biol. Chem. **156** : 703-723, 1946.
11. Smithies O : Zone electrophoresis in starch gels.: Group variations in the serum proteins of normal human adults. Biochem. J. **61** : 629-641, 1955.
12. Gammack D B, Huehns E R, Lehmann H, Shooter E M : The abnormal polypeptide chains in a number of haemoglobin variants. Acta Genet. **11** : 1-16, 1961.
13. Ingram V M : Abnormal human haemoglobins. I. The comparison of normal human and sickle-cell haemoglobins by fingerprinting. Biochim. Biophys. Acta **28** : 539-545, 1958.
14. Anson M L, Mirsky A F : Protein coagulation and it's reversal. The preparation of insoluble globin, soluble globin and heme. J. Gen. Physiol. **13** : 469-476, 1930.
15. Watanabe B, Fujii T, Nakashima Y, Maita T, Matsuda G : Amino-acid sequences of the α and β chains of adult hemoglobins of the Grand Galago, *Galago crassicaudatus*. Biol. Chem. Hoppe-Seyer **366** : 265-269, 1985.
16. Crestfield A M, Moore S, Stein W H : The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated protein. J. Biol. Chem. **238** : 622-627,1963.
17. Watanabe B, Maita T, Matsuda G, Goodman M, Johnson M L : Amino acid sequence of the α and β chains of adult hemoglobin of the Harbor Seal, *Phoca Vitulina*. Biol. Chem. Hoppe-Seyer **367** : 1251-1258, 1986.
18. Matsukawa S, Mawatari K, Yoneyama Y, Shimokawa Y : Close correlation between Monad-Wyman-Changeux parameters, L, and C, and its implication for the stereochemical mechanism of hemoglobin allostery. J. Mol. Biol. **150** : 615-621, 1981.
19. Nagai K, Thogersen H C : Generation of β-globin by sequence specific proteolysis of a hybrid protein produced in Esherichia coli. Nature (London) **309** : 810-812, 1984.
20. Luisi B F, Nagai K, Perutz M F : X-ray crystallographic and functional studies of human hemoglobin mutants produced in Escherichia coli. Hemoglobin Proc. Int. Meeting, London, 1986, Acta Hemat. **78** : 85-89, 1987.
21. Kimura M : The neutral theory of molecular evolution. Sci. Am. **241** : 94-104, 1979.

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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

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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主要な方法となり得るものと考えられた．

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