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Further Examples of Hemoglobin Hirose: Study of Molecular Stability

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Abstract Two new cases of Hb Hirose, β37 (C3) Trp→Ser, are reported. Hemoglobin Hirose exhibits unusual electric properties, a high oxygen affinity and slightly increased rates of autooxidation, heat denaturation and precipitation on mechanical shaking. These unique properties of Hb Hirose seem to depend on the abnormal manner of association of the aberrant β subunit with the α subunit and on the enhanced tendency to dissociate into subunits. In spite of the abnormal functional properties and the molecular instability of the hemoglobin, none of the carriers of Hb Hirose reported so far (only four unrelated Japanese in the world) manifested erythrocytosis or a hemolytic disorder, presumably because the dissociation into subunit is prevented *in vivo* by the very high hemoglobin concentration in the red blood cell.

Key Word: Abnormal hemoglobin

Introduction

Human hemoglobin is a tetramer consisting of two α and two β subunits. The subunits are non-covalently bound chiefly by van der Waals forces and hydrogen bonds between complementary surfaces and by a small number of salt bidges. Each subunit has two complementary phases against the unlike subunit, one extensive and the other rather limited, and it combines firmly with one unlike subunit (α_1-β_1, or α_2-β_2 interface) and loosely with another (α_1-β_2, or α_2-β_1 interface) in constructing a tetramer. This tetrameric structure is the major source of the stability and reasonably low oxygen affity of the hemoglobin molecule.

Hemoglobin Hirose or β37 (C3) Trp→Ser is one of several abnormal hemoglobins with a marked tendency to dissociate into $\alpha\beta$ dimers. This unique hemoglobin was first identified by Yamaoka a decade ago[1]. Fujita[2] characterized it as a high oxygen affinity variant, and detailed physico-chemical studies by Sasaki *et al*[3] disclosed that its unusual functional properties depended entirely, or almost entirely, on the markedly enhanced rate of dissociation into subunits.

In spite of the very high oxygen affinity *in vitro*, decreased subunit cooperativity and alkaline Bohr effet, carriers of the hemoglobin did not manifest erythrocytosis. This apparent paradox was explained by the fact that the oxygen affinity of Hb Hirose depended much more on hemoglobin concentration than that of Hb A. Recent clinical investigation on the physiological implication of this hemoglobin by Imamura *et al*[4] demonstrated its efficient oxygen transport *in vivo*.

Only two unrelated Japanese carriers of Hb Hirose have been reported in the world. We add here two apparently unrelated cases of heterozygosity for Hb Hirose. Stability of the hemoglobin is studied from various aspects.

Materials and Methods

The abnormal hemoglobin was discovered during random surveys of patients either by agar gel electrophoresis (pH 8.6 and 7.0)[5] or by starch gel electrophoresis (pH 8.6)[6]. Clinical observations of the first and the second cases were performed in Shimane Prefecture Central Hospital and Yamaguchi University Hospital, respectively, according to their routine methods.

Hemoglobin fractions were quantitated by cellulose acetate membrane electrophoresis (pH 8.6)[7]. Level of Hb F was determined by alkali denaturation of cyanmethemoglobin[8]. The hemolysates were studied by following stability tests: isopropanol test[9], qualitative heat precipitation test at 50°C[10], quantitative observation of heat denaturation at 50 and 60°C[11] and p-chloromercuribenzoic acid (PCMB) treatment[12]. Mechanical instability was studied by means of Hemoshake (TCS)[13] on the purified hemoglobins. Heinz body formation test was by the method of Beutler *et al*[14]. Subunit dissociation was studied by equilibrium gel filtration on Sephadex G-75[15]. The abnormal hemoglobin was purified either by DEAE-cellulose column chromatography[16] or by the cellulose acetate membrane electrophoresis.

Chain anomaly was studied by PCMB method[17] and by hybridization with canine hemoglobin[18]. Globin chains were isolated by urea CM-cellulose column chromatography[19]. The β chains were digested with trypsin and studied by filterpaper fingerprinting[20]. Tryptophan was detected by specific staining of fingerprints with Ehrlich's aldehyde reagent. For a large scale preparation of βT4, the soluble tryptic peptides from the β chain were fractionated by Sephadex G-25 gel filtration[21] and the relevant fractions were further subjected to fingerprinting. Beta T4 was digested with constant boiling HCl for 20 h at 105°C, and the amino acid composition was determined by an automated amino acid analyzer.

Results

Case Report

The first case was discovered in September 1970, during a survey by agar gel electrophoresis at Shimane Prefecture Central Hospital, in a 51-year-old Japanese woman who was suffering from a psychiatric disease. She had no known relatives. The second was a 64-year-old Japanese woman (discovered in a survey by starch gel electrophoresis at Yamaguchi University Hospital in October 1979) who underwent total hysterectomy for the treatment of *prolapsus uteri* with *cystocele* and *rectocele*. Although she had

Table 1 Hematological Data of the Propositi and the Normal Daughter of Case 2.

		Case 1	Case 2	Daughter
RBC	$\times 10^{12}$/l	4.32	4.55	4.65
VPRC	l/l	0.420	0.386	0.399
Hb	g/dl	13.5	12.7	13.4
MCV	fl	89.6	87.9	85.8
MCH	pg	nd	28.5	28.8
MCHC	g/dl	31.7	32.7	33.6
Reticulocyte	%	nd	1.8	0.6
WBC	$\times 10^{9}$/l	8.2	9.5	5.8
Thrombocyte	/10 OIF	78	288	196
Total bilirubin	mg/dl	normal*	0.5	normal*

Each value is the median of several determinations.
OIF: oil immersion field. nd: not determined.
* Icteric index, 3 to 4.

had five children and one still birth, only one daughter was alive and she did not inherit the variant hemoglobin. The two propositi had never had any hematologic problems. They were neither anemic nor erythremic (Table 1), and were free from clinical and laboratory evidence of accelerated hemolysis. Both showed a slight anisopoikilocytosis with mild ovalocytosis. Results of routine chemical analyses of serum were also within normal ranges. An abnormal, slow-moving hemoglobin comprised 37.7 and 37.8% of total hemoglobin in the first and the second cases, respectively; Hb A_2, 2.6 and 2.8%; Hb F, 1.1 and 0.6%.

Electric Property

Starch gel electrophoresis (pH 8.6) of the hemolysates revealed a major abnormal band migrating intermediate between Hb A and Hb G (Fig. 1). A minor abnormal band, which is usually accompanied by an α chain abnormal hemoglobin, was absent. The abnormal band separated also in agar gel and cellulose acetate membrane electrophoresis at pH 8.6. The abnormal hemoglobin was retarded like Hb S in agar gel electrophoresis at pH 7.0. The abnormal hemoglobin eluted from the DEAE-cellulose column just in front of Hb A.

An attempt to establish the chain anomaly by starch gel electrophoresis of the PCMB treated hemoglobin was unsuccessful because both α and β subunits showed normal mobilities. Hybridization of the abnormal hemo-

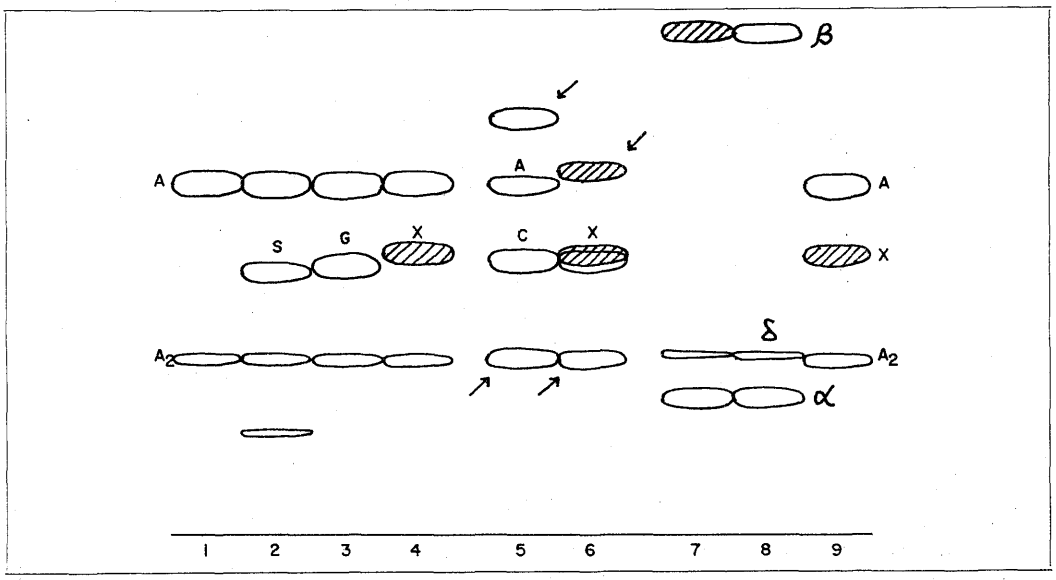

Fig. 1 Schematic drawing of the starch gel electrophoretograms (pH 8.6). The samples were applied on the line at the bottom and run towards the anode (top).

Columns 1–4 and 9: hemolysates. 1. Normal adult. A: Hb A. A_2: Hb A_2. (Hb A_1, or Hb A_3, is omitted.). 2. Carrier of Hb Shimonoseki (α54 Gln→Arg). There is a minor abnormal band behind Hb A_2. 3. Carrier of Hb G Coushatta (β22 Glu→Ala). 4. and 9. Carrier of the abnormal hemoglobin in question.

Columns 5 and 6: hybrids between human and canine hemoglobins. 5. Hb A (A) vis canine Hb (C). 6. Purified abnormal hemoglobin (X) vis canine Hb. ↙: $\alpha_2^{canine}\beta_2^{human}$. ↗: $\alpha_2^{human}\beta_2^{canine}$.

Columns 7 and 8: PCMB treated hemoglobins. 7. Purified abnormal hemoglobin. 8. Hb A. α, β and δ: α, β, and δ subunit, respectively, with p-mercuribenzoic acid attached to all cysteinyl residues. The bands for the molecules containing the abnormal subunit (β^X) are indicated by hatched lines.

globin with canine hemoglobin revealed, however, that the hybrid consisting of the canine α subunits and the β subunits of the abnormal hemoglobin ($\alpha_2^{can}\beta_2^{X}$) was slower than the one consisting of the canine α and the normal human β subunits ($\alpha_2^{can}\beta_2^{A}$) in the starch gel electrophoresis (Fig. 1). It was therefore concluded that the abnormal β subunit (its homotetramer) carried the same net electric charge as the normal β subunit, but it rendered a slightly decreased net negative charge to the heterotetramer molecule because it combined with α chains in an abnormal manner.

Absorption Spectrum

The purified abnormal hemoglobin had a normal visible absorption spectrum in the oxygenated form, while it appeared to autooxidize into methemoglobin slightly faster than normal. It had unusually low ultraviolet absorption arround 275 nm and at 290 nm (the tryptophan notch).

Subunit Dissociation

The abnormal hemoglobin was eluted slower than Hb A from the Sephadex G-75

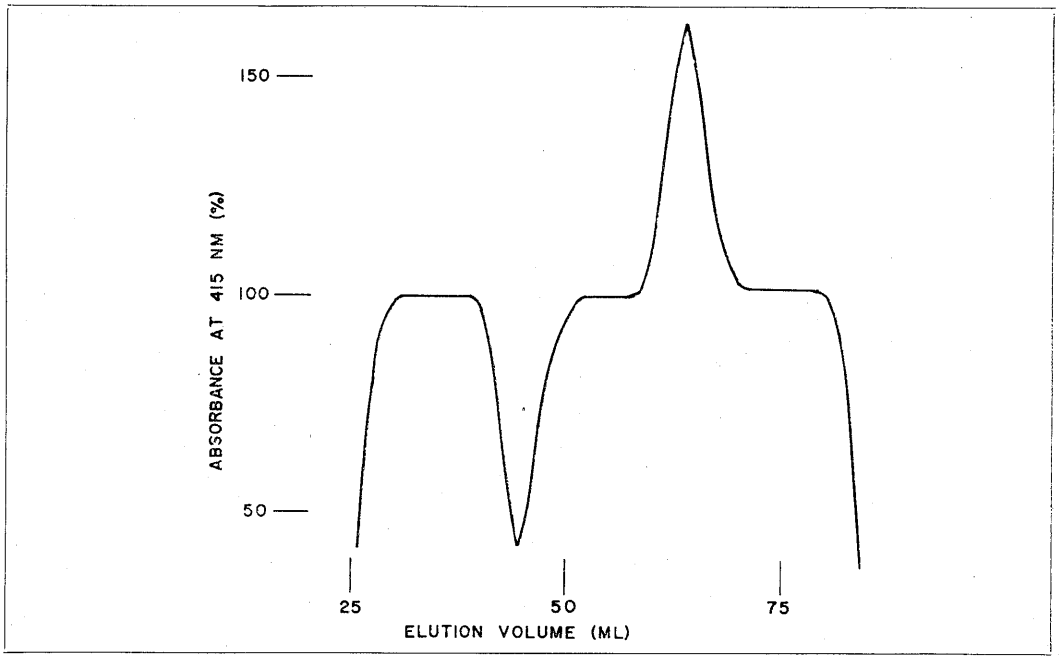

Fig. 2 Equilibrium gel filtration on Sephadex G-75. The column (1×60 cm) was eluted by successive changes of the eluent at a flow rate of 15 ml/h, and the effluent absorbance at 415 nm was continuously recorded. When the eluent was switched from a 5 μM solution of oxyhemoglobin A in an isotonic, phosphate buffered saline containing 0.02 M KCN at pH 7.2 (A) to a solution of the abnormal hemoglobin of the same concentration (X), solution A equilibrated the column (the first plateau of absorbance at 100%) before solution X emerged. Since the average molecular size of the abnormal hemoglobin was smaller than that of Hb A because of dissociation into subunits, the abnormal hemoglobin was eluted slower than Hb A and a gap was formed between the preceeding Hb A and the following abnormal hemoglobin (a deep, V-shaped, negative deflection of absorbance) before the column was equilibrated with solution X (second plateau). When the order of the eluents was reveresed, i.e. switched from solution X to solution A, the leading front of Hb A ran over the tail of the abnormal hemoglobin, forming an upward deflection.

column, causing a deep downward deflection of absorbance at the interface from Hb A to the abnormal hemoglobin and a high upward deflection at the interface from the abnormal to Hb A (Fig. 2). The retention volume of the abnormal hemoglobin (relative to myoglobin as a reference) was less concentration dependent than that of Hb A in hemoglobin concentrations from 1 to 50 μM (as heme), suggesting that the abnormal hemoglobin had already dissociated to near maximum at 50 μM, while Hb A progressively dissociated as the concentration decreased.

Stability

Stability of the hemoglobin was tested in various ways because it appeared to autooxidize somewhat faster than normal, although there was no evidence for increased hemolysis *in vivo*. The erythrocytes gave negative results in the Heinz-body formation test. The hemolysate gave negative results (or equivocal at most) in the routine isopropanol and qualitative heat precipitation tests. Quantitative observations of heat denaturation, however, uncovered a slightly increased lability of the abnormal hemoglobin (Fig. 3). There was no difference between the abnormal and normal hemolysates in the denaturation rate at 50°C until 45-min incubation, when autooxidation started to proceed slightly faster than normal. Precipitation started after 2-h incubation, and approached 2 % of total hemoglobin after 4 h, while it was negligible in the control. Denaturation of the sample was slightly faster than normal from the start of incubation at 60°C. The shape of the precipitation curve of the abnormal hemolysate was closer to normal, however, than expected for an unstable hemoglobin.

Treatment of the hemolysate (1 g/dl, pH 6.0) with 12-fold molar excess of PCMB overnight under CO at 4°C produced a trace amount of precipitate, which contained approximately equal amounts of α and β chains of normal mobility in urea polyacrylamide gel electrophoresis at pH 9.8 and at pH 4.6

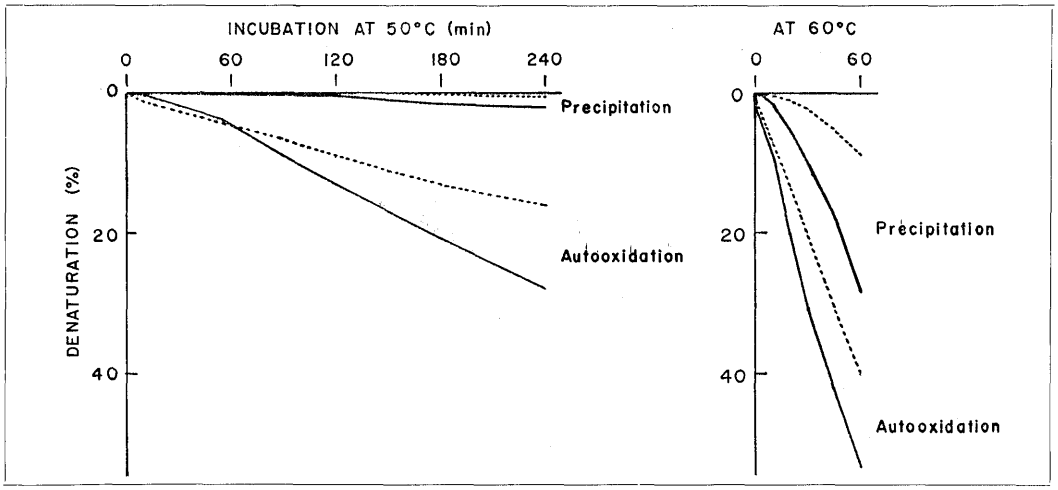

Fig. 3 Heat denaturation of hemolysaye (1 mM heme-equivalent in 0.025 M Tris-HCl, pH 7.40). The amount of precipitation was determined by subtracting the hemoglobin concentration of the supernatant (measured by a cyanmethemoglobin method) from the initial value. Autooxidation was expressed by the sum of the amount of precipitate and that of methemoglobin in the supernatant. Solid line: abnormal hemolysate. Broken line: control.

The selective precipitation of the abnormal subunit which is usual in unstable hemoglobins was not revealed in this slightly unstable hemoglobin.

Mechanical shaking of the purified abnormal hemoglobin (20–100 μM cyanmethemoglobin in 0.01 M tris buffer, pH 8.0) gave a value for an apparent precipitation rate constant, 1×10^{-2}/min (Hb A, 0.5×10^{-2}).

Structure

Tryptic fingerprinting of the β chain from the purified abnormal hemoglobin revealed the feature reported by Yamaoka[1]: the abnormal spot for βT4 with a smaller chromatographic Rf value and with negative reaction for tryptophan (Fig. 4).

The abnormal β chain did not separate from the normal counterpart in the urea CM-cellulose column chromatography of total globin from the unfractionated hemolysate. Sephadex G-25 gel filtration of the soluble tryptic peptides from the mixture of the two β chains was characterized by reduction of the absorption peak of βT4 by almost a half (Fig. 5). Fingerprinting of the relevant fractions disclosed that the abnormal βT4, which no longer had the high ultraviolet absorption, had emerged together with βT13, i.e. much faster than normal. Elution of βT4 is retarded because of the high affinity of its tryptophanyl residue to the gel matrix. This would not be the case in the abnormal βT4 because it had lost the tryptophanyl residue.

The amino acid composition of the abnormal βT4 after acid hydrolysis (Table 2) agreed with that expected for βT4 except for the presence of a serine which was absent in the normal control. Since tryptoph-

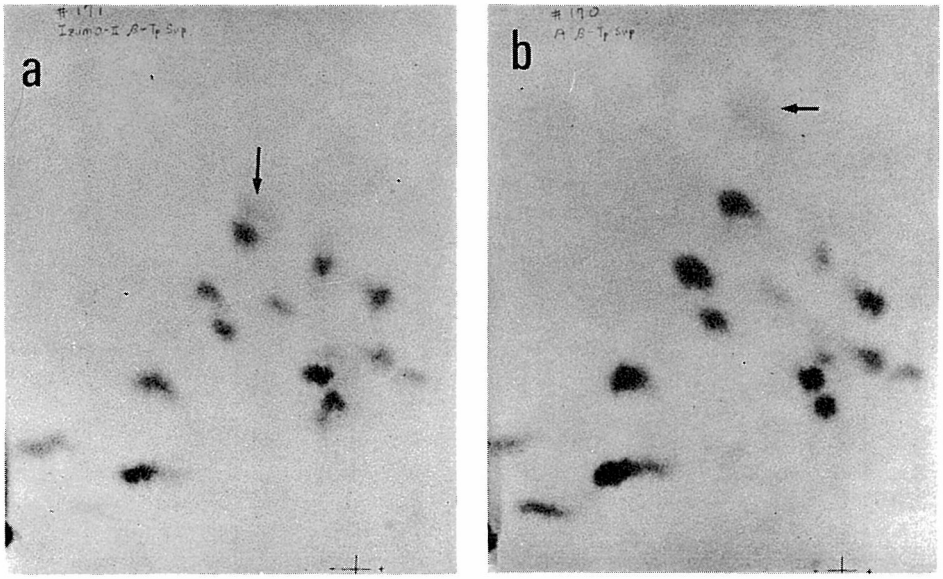

Fig. 4 Tryptic fingerprint of the abnormal (a) and normal (b) β chain. A vertical arrow (a) points to the abnormal βT4 and it also indicates the direction of movement from the normal location. A horizontal arrow (b) points to the normal βT4 which is faintly stained.

Electrophoresis: pyridine-acetic acid-water (500/20/4, 480, v/v, pH 6.4), appr. 45 volts/cm, 90 min. The anode is to the right.

Chromatography: n-butanol-pyridine-acetic acid-water (90/60/18/72, v/v), ascending development from botom to top for 36 h.

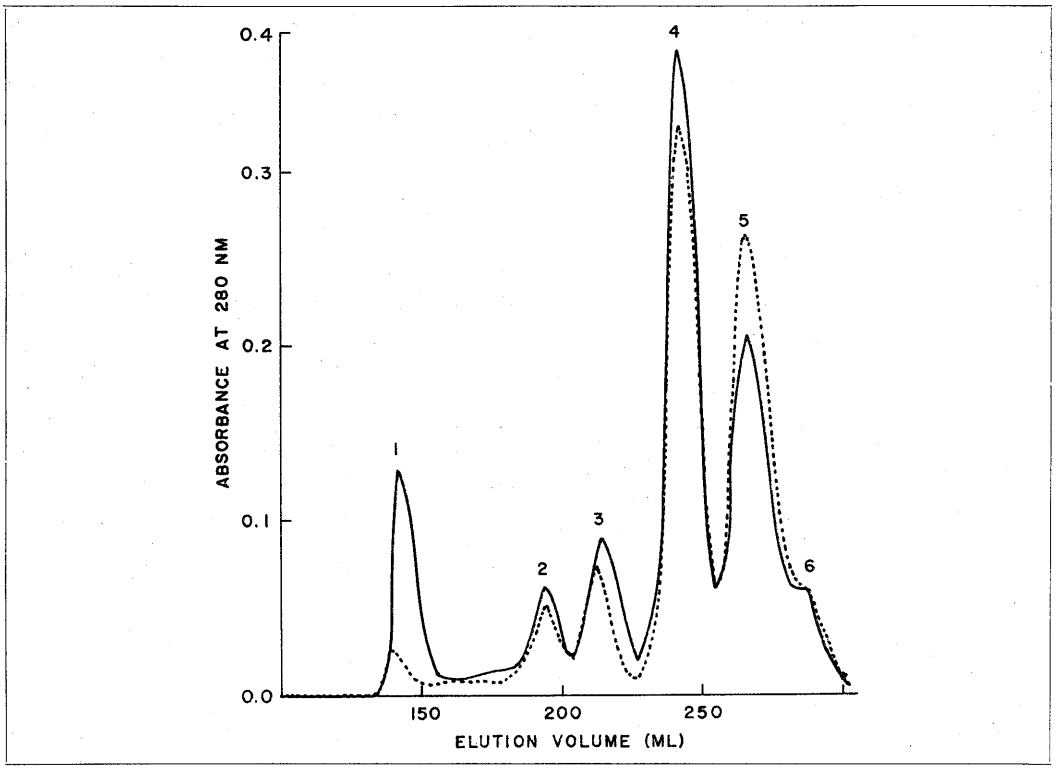

Fig. 5 Sephadex G-25 gel filtration of soluble tryptic peptides from the β chain.

The pH 6.4 supernatant of tryptic digest from 15-20 mg β chain was loaded on an 1.8×145 cm column of Sephadex G-25 fine beads equilibrated with 0.2 M acetic acid. Flow rate, 30 ml/h.

Solid line: the total β chain containing appr. 38% abnormal globin.

Broken line: normal β chain.

Light absorption at 280 nm in peaks 3, 4, 5 and 6 is due to a tyrosyl residue of βT13, a tryptophanyl of βT2, a tryptophanyl (and a tyrosyl) of βT4, and a tyrosyl in βT15, respectively.

an was not detectable by the specific staining and its absence was indicated by the spectroscopic property and by the Sephadex G-25 chromatogram, it was concluded that the only tryptophan in βT4, namely at position 37 of the β chain, had been substituted by serine. This abnormal hemoglobin is therefore identical with Hb Hirose or β37 (C3) Trp→Ser.

Discussion

Functional properties of Hb Hirose have been studied in detail and correlated with its enhanced tendency to dissociate into subunits[1-4]. Oxygen equilibrium studies on our cases by Hayashi and Kidoguchi and by Iuchi and his associates (personal communications) confirmed the previous results. An oxygen equilibrium curve (OEC) of the erythrocyte suspension (pH 7.4, 37°C) revealed a slightly increased oxygen affinity with a P_{50} value (the oxygen tension at 50% saturation) of 23.0 mmHg (control, 25.1) and subnormal intersubunit cooperation (decreased sigmoidity). OECs of the purified Hb Hirose (60 μM heme in 0.05 M bis-

Table 2 Amino Acid Composition (molar ratios) of βT4

	Case 1 Normal	Expected Normal	Case 1 Abnormal	Case 2 Abnormal
Thr	1.0	1	1.0	1.1
Ser	—	0	1.0	1.0
Glu	1.2	1	1.1	1.2
Pro	0.6	1	0.4	1.1
Val*	1.9	2	1.9	1.6
Leu	2.2	2	2.3	1.9
Tyr	0.9	1	0.8	1.0
Trp#	(+)	1	(−)	(−)
Arg	0.7	1	0.8	1.1

Partial loss of Pro in case 1 is due to ninhydrin staining of the fingerprints. Fingerprints were stained by fluorescamine in case 2.

* Contained in a Val-Val sequence which is subject to incomplete hydrolysis.

\# By staining of a fingerprint with Ehrlich's reagent.

tris-0.1 M NaCl) gave P_{50} values of 4.42, 3.43 and 3.05 mmHg (Hb A: 19.19, 14.09 and 10.79) at pH 6.5, 6.8 and 7.0, respectively, i.e. an oxygen affinity approximately 3.5–4.4 times higher than normal. The discrepancy between the result on the erythrocytes and that on the purified hemoglobin is a characteristic feature of Hb Hirose because the very high oxygen affinity of this hemoglobin *in vitro* depends largely on its dissociation into $\alpha\beta$ dimers in a highly diluted solution (from 9,000 μM in erythrocytes to 60 μM in the purified hemoglobin solution). Hb A remains mostly in $\alpha_2\beta_2$ tetramers in such a diluted solution.

The results of stability tests characterize Hb Hirose also as a slightly unstable hemoglobin. Its mechanical lability is comparable to that of Hb Hofu, a clinically insignificant, slightly unstable hemoglobin[11]. Iuchi (personal communication) estimated, from his results of stopped flow experiments, the activation energy for oxidation of Hb Hirose with ferricyanide as 8.59 Kcal/mol (Hb A, 13.44), i.e. Hb Hirose was oxidized slightly more easily than Hb A. He also showed that the precipitation curves of methemoglobin Hirose at 45, 50 and 55°C were comparable to, or slightly steeper than, those of methemoglobin A at 5°C higher temeratures, respectively.

The PCMB precipitation is selective to the abnormal subunit in all unstable hemoglobins we have studied so far. This selectivity is realized even in clinically insignificant, very slightly unstable hemoglobins, e.g. Hb Kokura[22], Hb Miyashiro[23] and Hb Hofu[11]. Hb Hirose is an exception in this regard. Presumably, the instability of Hb Hirose resides in the abnormal manner of association of $\alpha\beta$ dimers in constructing the tetramer molecule. Hb Hirose would render a less pronounced stabilizing effect than normal Hb A.

Hb Kansas[24] is the only abnormal hemoglobin in which we have observed an unselective PCMB precipitation similar to Hb Hirose. This is another variant with a markedly enhanced subunit dissociation comparable to Hb Hirose, but its oxygen affinity is so low as to cause a marked cyanosis to the carriers under the atomospheric oxygen tension of 120 mmHg. The preliminary results of stability tests (unpublished) are also similar to those we have decribed in Hb Hirose. It is interesting to note that both of the two abnormal hemoglobins exhibited a markedly enhanced tendency to dissociate into subunits and a slight instability in a similar manner, even though the abnormality of oxygen affinity is to the opposite directions.

Oxidation, heme loss and subunit dissociation are claimed to be the three major factors which trigger irreversible denaturation of hemoglobin[25]. Hb Hirose exemplifies, however, that subunit dissociation is not by itself an important factor in denaturation of hemoglobin *in vivo* because it is suppressed by the high hemoglobin concentration in the red blood cells.

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