A New Unstable Hemoglobin Iwata, F8 (α87) His→Arg

Its Structure and Unique Properties

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ABSTRACT

An unstable hemoglobin, Hb Iwata, F8 (α87) His → Arg, was discovered during a screening program for hemoglobin anomalies in the newborns. Studies of the family members of the proband revealed the father and the paternal grandmother to also carry the abnormal hemoglobin. None of the carriers of the abnormal hemoglobin manifested clinical symptoms, probably because Hb Iwata comprised only a few percent of the total hemoglobin. Hb Iwata migrated between Hb A and A_2 in starch gel electrophoresis (pH 8.6), and was isolated by DE-52 column chromatography. Analysis of the amino acid composition of tryptic peptides from the α chain of Hb Iwata demonstrated the substitution of the proximal histidine by arginine. Results of the isopropanol, heat denaturation and Heinz body formation tests indicated instability of Hb Iwata. Hb Iwata rapidly auto-oxidized into semihemichrome and subsequently precipitated. The oxidation also accelerated heme loss, and the heme-depleted Hb Iwata tended to dissociate and precipitate. The unique properties of Hb Iwata were interpreted in reference to stereochemical properties in other abnormal hemoglobins with substitution of the proximal or distal histidine.

Key word: unstable hemoglobin

INTRODUCTION

Human hemoglobin is a tetrameric molecule composed of α and β subunits. Each subunit consists of a polypeptide (globin) and a heme. The globin is rich in α-helical structure. The helical regions are designated as A,B, ---, G and H in sequence from the N-terminal of the

peptide which are interrupted by non-helical regions NA, AB, ---, GH and HC. The polypeptide chain is folded at the non-helical regions to provide a globular cage-like conformation called "myoglobin-fold". Each amino acid residue is numbered by the position from the N-terminal of each helical or non-helical region, e.g., F8 designates the 8th residue from the start of the helical region F. Alternatively, amino acid residues are sequentially numbered from the N-terminal of the whole polypeptide of globin and, accordingly, the residue F8 is also identified as position 87 in the α chian (α87) or position 92 in the β chain (β92). The conformation of the globin provides a hydrophobic crevas to accomodate the heme (called "heme pocket"). There is a histidyl residue on each side of the heme plane in the heme pocket, the proximal histidine F8 on one side and the distal histidine E7 on the other side. The heme moiety ,in which an iron atom occupies the center of the porphyrin ring and shares electron with the four pyrrole nitrogens, is linked with the globin by sharing the fifth coordinate of the heme iron with imidazole nitrogen Nε of the proximal histidine F8. Thus, the proximal histidine F8 (α87 or β92) plays a crucial part in heme-globin linkage and proper function ing of the hemoglobin molecule. The sixth coordinate of the heme iron is free, having a space between the distal histidine E7. An oxygen molecule as well as other exogenous ligands, enters the space between the iron and the distal histidine to be reversibly bound by the sixth coordinate of the iron[1-3].

Studies of hemoglobin variants, most of which represent single amino acid substitution in the α or β chain, have made a considerable contribution to the understanding of the role of each amino acid residue in the hemoglobin molecule[4]. Of almost 300 hemoglobin variants so far discovered, the only example of substitution of the proximal histidine in the α chain has been Hb M Iwate, α87 (F8) His $\rightarrow$ Tyr[5]. Detailed analyses of Hb M Iwate, as well as other variants with substitution in the heme pocket of the α or the β chain, have confirmed the crucial role played by the proximal histidine in heme-globin bining and proper functioning of hemoglobin[6,7]. This paper deals with a new variant, Hb Iwata, with substitution of the proximal histidine in the α chain.

MATERIALS AND METHODS

The propositus, a male, was found to carry an abnormal hemoglobin during a survey of a general newborn population for abnormal hemoglobins. He was reexamined at 11 months of age, together with seven other members of the family (Fig. 1). The thin layer starch gel electrophoresis

method of Yamaoka et al[8], with the gel stained by o-toluidine, was used for the screening. The father (II-5) and the paternal grandmother (I-5) also carried the abnormal hemoglobin. The abnormal hemoglobin comprised 2.1% (propositus), 5.8% (father) and 4.6% (grandmother) of total hemoglobin. The three affected members shared hematological features of borderline anemia (11.6-13.6 g/dl), slight reticulocytosis (1.8-2.0%), decreased levels of haptoglobin (33.2-60.3 mg/dl) and hemopexin (33.5-48.2 mg/dl) and slight instability of hemoglobin which was revealed by isopropanol test (positive)[9], heat denaturation test (accelerated precipitate formation at 60°C)[10] and Heinz body formation test (39.6-5 58.5%, while normal control was 32.8-37.6%)[11]. Hemolysate from the father was used for further characterization of the abnormal hemoglobin.

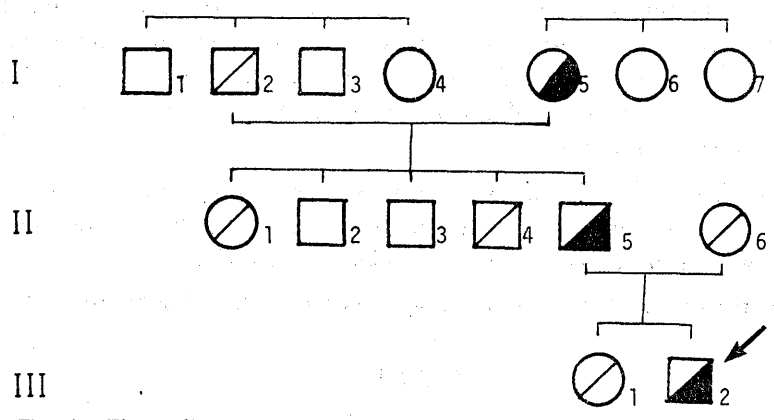

Fig. 1. The pedigree

□, ⊘: examined, normal

■, ◪: carrier of the abnormal hemoglobin

Hemolysate was fractionated at appr. 6°C by DE-52 column chromatography using a linear gradient of NaCl in 0.2 M glycine (pH 7.8, adjusted by NaOH)[12]. Fractions were concentrated at 4°C by ultrafiltration in 8/32 Visking Tubing. Globin was prepared either by acid methylethylketone method[13] or by acid acetone method[14]. Electrophoretic mobility of the globin chains was studied by polyacrylamide gel electrophoresis (0.4 M tris-glycine buffer, pH 9.8) in 6.4 M urea[15]. The α, β and δ chains were separated by CM-23 column chromatography in 8 M urea[16]. The normal and anormal α chains were compared by fingerprinting procedure[17]. The relevant tryptic peptides were eluted from fingerprints with constant boiling HCl, hydrolysed at 105°C for 20 h, and was then subjected to amino acid analysis by Hitachi Amino

Acid Analyzer 835.

Absorption spectra of whole hemolysate and separated hemoglobins were recorded by Hitachi 634 Spectrophotometer with nominal band width of 1 nm. The excess ferricyanide was removed by Sephadex G-25 gel filtration immediately before measurement. The ratio of oxyhemoglobin, methemoglobin and hemichrome was calculated from the absorption spectrum according to the method of Carrell et al[18].

Subunit dissociation was studied by comparative Sephadex G-75 gel filtration in 0.01 M phosphate-0.09 M NaCl (pH 7.0); the Sephadex column (1.0×60 cm) was first developed with Hb A (appr. 5 μM) as a reference until the column was equilibrated, and then the developer was switched to the sample solution of the same concentration. The absorbance of the effluent either at Soret band (absorption peak by porphyrin ring; 415 nm for oxyhemoglobin and 421 nm for cyanmethemoglobin) or at 280 nm was continuously recorded by a spectrophotometer. An increased dissociation of the sample, relative to the foregoing Hb A, is reflected by a deflection of absorbance at the leading boundary where the both hemoglobins meet.

The effect of addition of hemin was tested by incubation of 1 mM hemoglobin with up to 3.1 mM hemin cyanide in the presence of excess cyanide and subsequent starch gel electrophoresis.

Precipitation of hemoglobin was assessed by absorbance at 700 nm.

RESULTS

I Identification of the Abnormal Hemoglobin

Starch gel electrophoresis of the hemolysate of blood samples from the three affected members revealed an abnormal band midway between those for Hb's A and A_2 (Fig. 2). Also present in each of the three carriers was a band, tentatively called "A_2 minor", situated behind Hb A_2, near the origin. The major abnormal band, with an approximate mobility of Hb G, was separable by cellulose acetate membrane. It comprised 2.1-5.8% of the total hemoglobin, when assessed by extracting the band on the membrane with Drabkin's solution. The "A_2 minor" band was invisible on cellulose acetate membrane, presumably because the sensitivity of Ponceau staining of the membrane is much lower than that of o-toluidine staining of starch gel.

DE-52 column chromatography revealed four hemoglobin peaks, corresponding to those in starch gel electrophoretogram. The "A_2 minor" peak emerged before Hb A_2, and the peak for the major abnormal

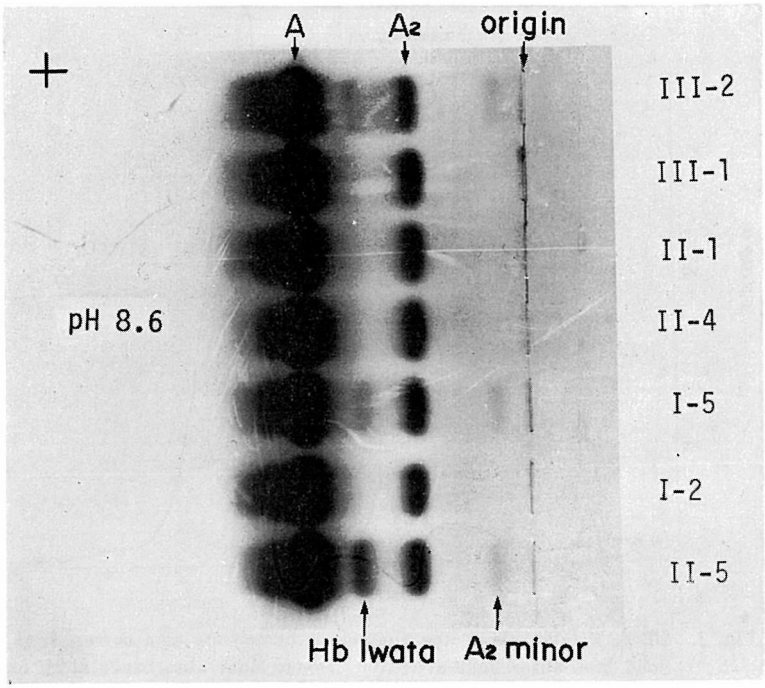

Fig. 2. Starch gel electrophoresis of hemolysate from family members. Ⅲ-2 (propositus), Ⅰ-5 (grandmother) and Ⅱ-5 (father) are carriers of the abnormal hemoglobin showing the same migration as Hb G which is known to migrate midway between Hb 's A and A_2.

hemoglobin appeared before Hb A (Fig. 3). In addition, a trace of poorly defined, hemoglobin-like pigment was observed between the peaks for Hb A_2 and the major abnormal hemoglobin. The amount of the major abnormal hemoglobin apparently decreased with aging of the hemolysate (e.g., 1 week at 4°C), accompanied by concomitant increase of Hb A_2 fraction with a broader peak on DE-52 (not shown). The undefined hemoglobin, apparently formed during aging of the hemolysate and eluted at the Hb A_2 position, is tentatively called "Hb X" in this paper.

Polyacrylamide gel electrophoresis (pH 9.8) of the major abnormal hemoglobin showed the abnormal α chain with a slower electrophoretic mobility than the normal α chain, and the normal β chain. "A_2 minor" consisted of the abnormal α and normal δ chains. The Hb A_2 fraction from the aged (1 week at 4°C) hemolysate, namely [Hb A_2+Hb X], contained the abnormal α chain in addition to the normal α, β and δ chains (Fig. 4). This indicates that the Hb A_2 fraction was a mixture of normal Hb A_2 and the major abnormal hemoglobin.

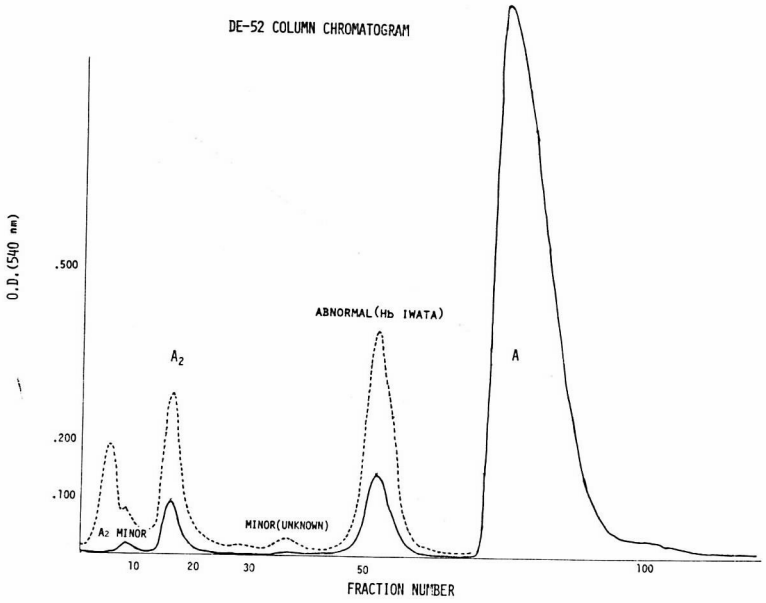

Fig. 3. DE-52 column chromatography of the hemolysate of a carrer(Ⅱ-5)
Solid line: absorbance at 540 nm Dotted line: absorbance at 280 nm

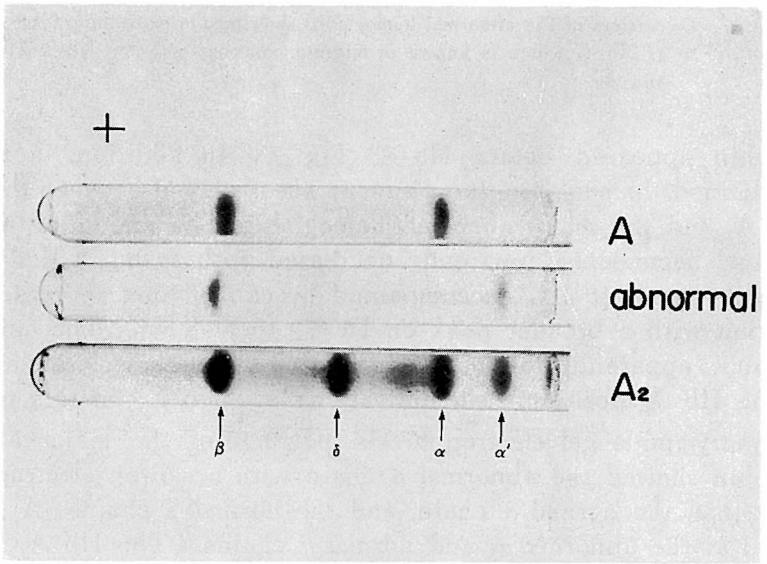

Fig. 4. Polyacrylamide gel disc electrophoresis (pH9.8) in 6.4M urea. A, and abnormal: the A and abnormal fractions in Fig. 3., respectively. A_2 : the A_2 fraction from an aged hemolysate of Ⅱ-5. α : α chain, α' : abnormal α chain, β : β chain, δ : δ chain

The aforementioned results suggested that 1) the abnormal α chain is lacking approximately one net negative charge compared to normal α chain, 2) the major abnormal hemoglobin consists of two abnormal α and two normal β chains (loss of two net negative charges per tetramer), while "A_2 minor" is composed of two abnormal α and two normal δ chains, and 3) a portion of the major abnormal hemoglobin loses during aging another pair of net negative charges, the resulting product (Hb X) being cochromatographed with Hb A_2.

Tryptic fingerprinting of the abnormal α chain revealed an extra spot (Spot E) between those for α Tp's 7 and 8 (Fig. 5); its location surmised the tripeptide α88-90 which is known to be produced by thermolysin digestion of αTp 9[19]. Also, there appeared four extra spots (Spots A, B, C and D) above the spot of αTp 4. The spots corresponding to αTp's 9 and 8-9 and their sulfoxide derivatives seemed to be

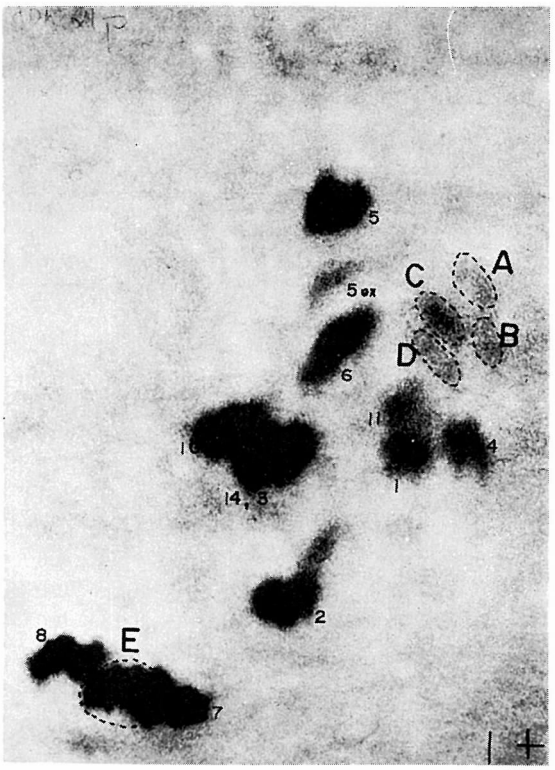

Fig. 5. Fingerprint of the abnormal α chain after tryptic hydrolysis. The abnormal spots are encircled by dotted line. Numerals denote the α chain tryptic peptide numbers.

missing. The same feature was confirmed by fingerprinting of the whole globin of the major abnormal hemoglobin; there was the same abnormal spot (E) between α and β Tp 7 complex and $\alpha\beta$Tp 8 (Lys), and the other four abnormal spots (A to D) between those for βTp 5 and its sulfoxide derivative above αTp 4. The absence of αTp 9 and the related peptides at their usual position was demonstrated by specific staining for histidine[20].

The composition of Spot A agreed with the N-terminal twenty-five residues of αTp 9, or α62-86, plus an arginine, while that of Spot E was identical with the tripeptide from the C-terminal of αTp 9, namely the α88-90 mentioned above (Table I). The composition of Spots B, C and D was consistent with α62-87 (methionine sulfoxide derivative), α61-87 and α61-87 (methionine sulfoxide derivative), respectively, all of which with an arginine substitution for a histidine. It was concluded, therefore, that arginine substituted histidine at position 87 in the α chain and the extra spots A through E were derived from the abnormal αTp 9 which was hydrolyzed at the carboxyl side of the newly introduced argininyl residue. We would like to name the new hemoglobin as Hb Iwata, after the town it was discovered.

Table I Amino acid composition (molar ratio) of abnormal spots.

	Spot A (α62-87)		Spot E (α88-90)		A+E	normal αTp9 (α62-90)	
	actua	integer	actual	integer		actual	integer
Asp	5.9	6	0.0	0	6	6.3	6
Thr	1.0	1	0.0	0	1	1.0	1
Ser	2.0	2	0.0	0	2	1.7	2
Glu	0.2	0	0.0	0	0	0.0	0
Gly	0.2	0	0.1	0	0	0.0	0
Ala	6.0	6	0.6*	1	7	7.0	7
Val	3.0	3	0.0	0	3	3.1	3
Met	1.0	1	0.0	0	1	1.0	1
Leu	4.0	4	0.0	0	4	4.3	4
Tyr	0.0	0	0.0	0	0	0.0	0
Phe	0.0	0	0.0	0	0	0.0	0
Lys	0.0	0	1.1	1	1	1.0	1
His	1.0	1	1.1	1	2	3.0	3
Arg	1.2	1	0.0	0	1	0.0	0
Pro	1.2	1	0.0	0	1	0.8	1
Trp	–	-	–	–	–	–	–

*Alanine is the N-terminal residue (α88) of the peptide for Spot E. N-terminal residue is occasionally partially destroyed by ninhydrin staining of fingerprint.

II Properties of Hb Iwata

Absorption spectrum The absorption spectrum of oxygenated whole hemolysate containing Hb Iwata, did not differ from that of a normal control. When oxygenated Hb Iwata was purified by DE-52 column chromatography, however, it came out of the column as partially oxidized form with decreased α (577 nm) and β (540 nm) peaks and increased absorbance at 630 nm (Fig. 6). A portion of Hb Iwata seemed to have auto-oxidized during its isolation by the column.

Oxidation of Hb Iwata with excess potassium ferricyanide resulted in partial loss of normal absorption peaks at 630 and 500 nm and the appearence of a new peak at 525 nm (Fig. 7). The absorption spectrum

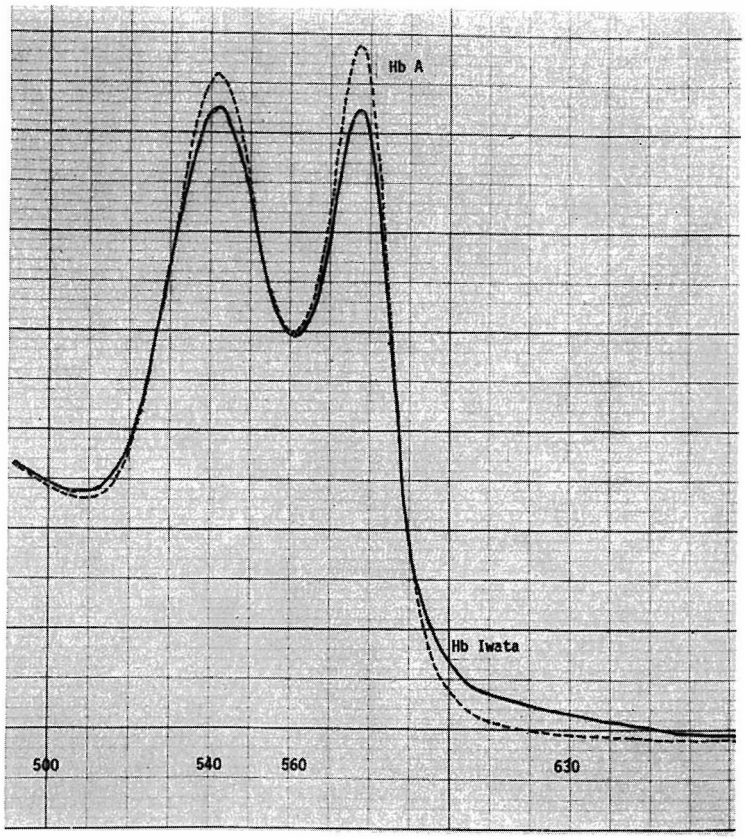

Fig. 6. Absorption spectra of Hb's Iwata (solid line) and A (dotted line) in oxy form. 0.2M glycine-NaOH buffer, pH 7.8. Spectra were taken immediately after separation of both hemoglobins by DE-52 column chromatography. Absorbance at 280 nm was adjusted to 0.600.

of the abnormal α subunit in methemoglobin Iwata was obtained by subtracting one half of the absorbance of normal methemoglobin A from that of methemoglobin Iwata. The α and β subunits in normal methemoglobin A are spectrophotometrically equivalent to one another[21]. Therefore, it was assumed that the absorbance by the β subunit in methemoglobin Iwata is a half that of normal methemoglobin A. The calculated spectrum by the α subunit featured: 1) an indentation at around 500 nm, 2) a prominent peak at 535 nm with a shoulder at 565 nm (Fig. 7). These features conform to those of hemichrome[22]. Methemoglobin Iwata, therefore, is a semihemichrome. The α subunit, upon reduction with sodium dithionite, showed absorption peaks at 559 and 435 nm, indicating its conversion to hemochrome. Cyanmethemoglobin Iwata was spectrophotometrically normal. These results indicated that Hb Iwata autooxidized rapidly and became a reversible semihemichrome. Hemichrome comprized 18% of Hb Iwata, when it was isolated hy chromatography from whole hemolysate. Hemichrome formation further proceeded to reach 45% after two days' incubation at room temperature. There was a somewhat smaller, parallel increase of methemoglobin (Fig. 8). Hemichrome formation was negligible in a control.

The absorption spectrum of A_2 minor ($\alpha_2^{Iwata}\delta_2$) was essentially the same as Hb Iwata. The undefined pigment that was eluted between Hb A_2 and Hb Iwata showed a curious absorption spectrum with a major peak at 670 nm and additional peaks at 577 and 542 nm (Fig. 9). Absorption spectrum of [Hb A_2+Hb X] fraction was much closer to

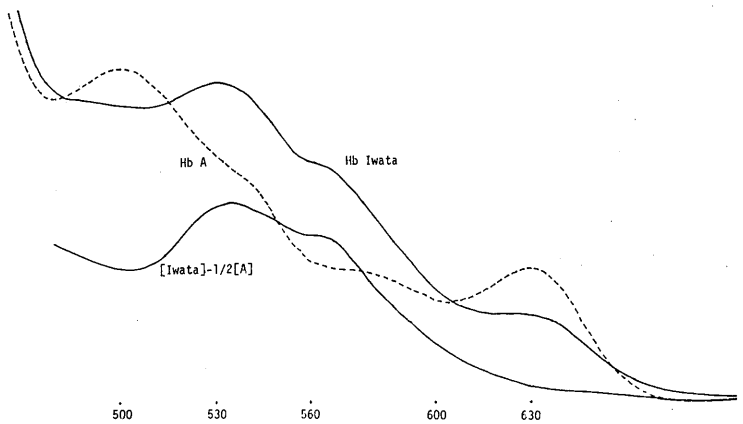

Fig. 7. Absorption spectra of Hb's Iwata (solid line) and A (dotted line) after complete oxidation by pottasium ferricyanide. [Iwata]-1/2 [A] : Difference between the absorbance of methemoglobin Iwata and a half of that of Hb A. 1/15 M phosphate buffer, pH 6.6.

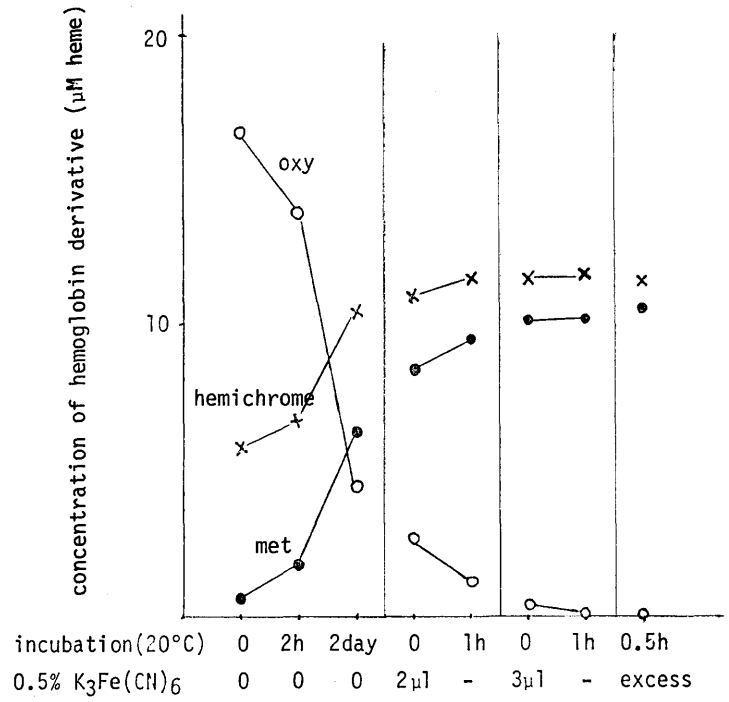

Fig. 8. Observation of oxidation process of Hb Iwata. Hb Iwata (appr. 22 μM) were first incubated for two days at room temperature, and then increasing amounts of potassium ferricyanide were added, during which absorption spectra were taken. Concentrations of oxy-, met- and hemichrome-derivatives were calculated from the absorption spectra.
○ : oxy form, • : met form, × : hemichrome

that of normal Hb A than Hb Iwata (not shown).

The heme to globin ratio in Hb Iwata was calculated using the absorbance ratio A_{540} (cyanmethemoglobin)/A_{280} (oxyhemoglobin). This would be justified in view of the fact that the absorption spectrum of cyanmethemoglobin Iwata was normal. The ratio was the same for both Hb Iwata and Hb A, while that for the [Hb A_2+Hb X] fraction was lower than that for the Hb A fraction. The latter result suggested that the Hb X corresponds to a partially heme-depleted molecule of Hb Iwata (Table Ⅱ).

Heme loss Heme loss from Hb Iwata was further exemplified by DE-52 column rechromatography of the purified Hb Iwata (Fig. 10), where approximately one half of it appeared in a fraction corresponding to the Hb X with a reduced visible/ultraviolet absorption ratio (Table Ⅱ). DE-52, with its affinity for heme higher than that of Hb Iwata, would

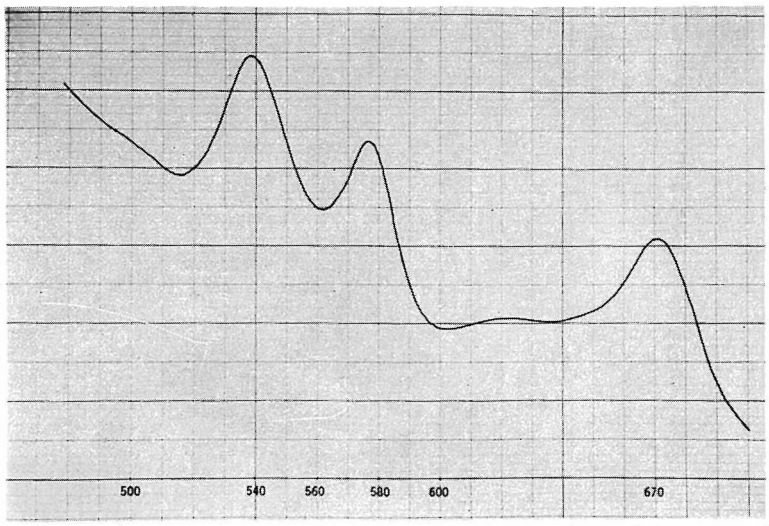

Fig. 9. Absorption spectrum of the Hb-like pigment obtained between Hb A_2 and Hb Iwata.

Table Ⅱ Spectrophotometrical estimation of heme content of a hemoglobin molecule in the DE-52 fractions

fraction	A_{280} (oxy)	A_{540} (cyanmet)	A_{540}/A_{280}	heme/hemogobin
A_0	.401	.164	0.410	4
Hb Iwata	.385	.158	0.410	4
A_2	.399	.157	0.393	3.8
Heme-depleted Hb Iwata*	.388	.106	0.274	2.7

*Heme-depleted Hb Iwata fraction in rechromatography of purified Hb Iwata (refer to Fig. 10).

compete with and deplete the latter. Sephadex G-25 gel filtration, on the other hand, was ineffective in removing heme.

Starch gel electrophoresis of the partially heme-depleted fraction of Hb Iwata revealed three bands at equal intervals (Fig. 11). The fastest band had the mobility of the intact Hb Iwata with all four hemes. The middle one with an approximate mobility of Hb A_2, comprised a very small fraction. The slowest one, slightly faster than the "A_2 minor", appeared in a broad band. The mobilities of the three bands agreed with those expected for the three forms of Hb Iwata, each with four, three and two hemes per tetramer, respectively. An addition of increasing amounts of hemin cyanide to the heme-depleted Hb Iwata fraction,

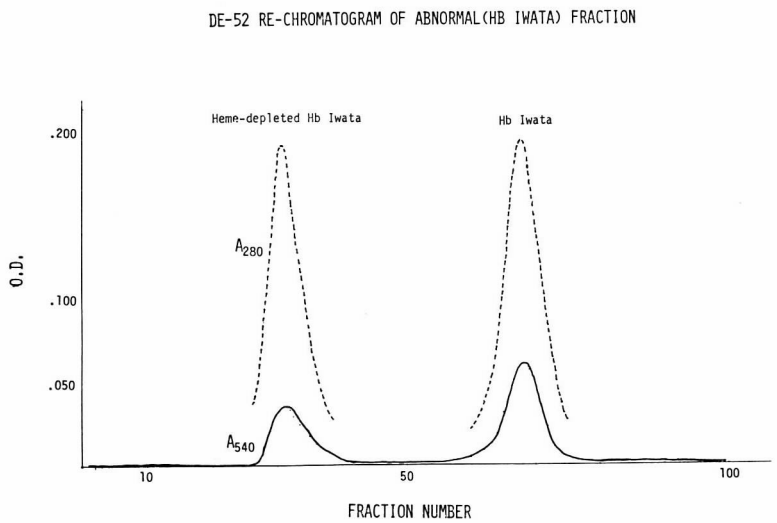

Fig. 10. DE-52 column rechromatography of the purified Hb Iwata. Loss of heme during chromatography produced a heme-depleted fraction corresponding to Hb X which was cochromatographed with Hb A_2 in Fig. 3. Solid line shows absorbance at 540 nm and dotted line at 280 nm. Note that the absorbance ratio A_{540}/A_{280} of Hb X is lower than that of the intact Hb Iwata.

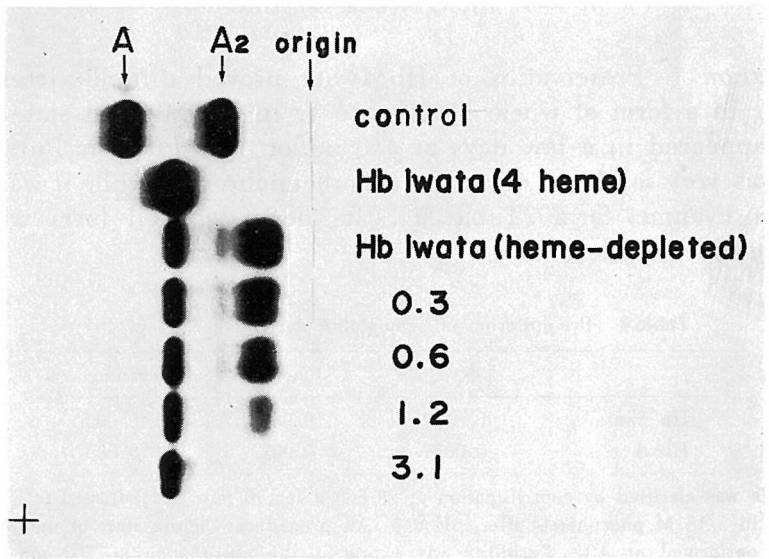

Fig. 11. Starch gel electrophoresis of heme-depleted fraction of Hb Iwata before and after addition of hemin. The numbers are concentrations of hemin cyanide (mM). Hb Iwata (1 mM) with all four hemes was completely restored by an addition of 3.1 mM hemin cyanide.

resulted in a progressive increase in intact Hb Iwata, eventually reaching 100% restoration (Fig. 11, lower half).

Subunit dissociation The oxygenated form of Hb Iwata (actually a mixture of oxy, met and hemichrome forms) in an extreme dilution showed an increased tendency to subunit dissociation. This tendency was less pronounced in cyanmet form. Dissociation was less than normal in met form (actually semihemichrome). The heme-depleted Hb Iwata (cyanmet form) showed a marked increase of subunit dissociation (Fig. 12).

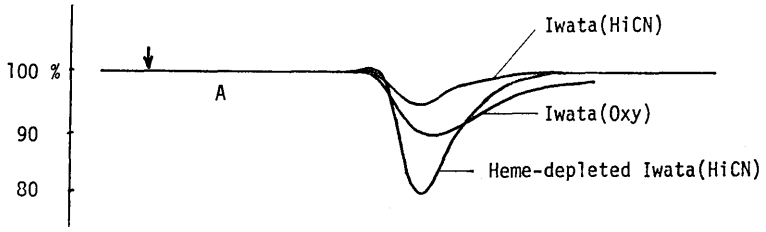

Fig. 12. Subunit dissociation of Hb Iwata as observed by gel filtration (Sephadex G-75 column chromatography). The column was equilibrated with Hb A (5 μM), and then eluted with the same concentration of Hb Iwata. The arrow indicates the point where the developer was switched from Hb A to Hb Iwata. HiCN: cyanmet derivative.

Precipitation Preservation of Hb Iwata proved difficult irrespective of either in a form of whole hemolysate or in the purified state; precipitates appeared in a few days at 4°C under CO saturation. Purified Hb Iwata was very labile in met form (semihemichrome), while it was rather stable in cyanmet form (Table Ⅲ). Its heme-depleted form was even more unstable (Table Ⅳ).

Table Ⅲ Precipitation of hemoglobin derivatives (37°C, 4h)

	oxy	met	cyanmet
Hb Iwata	0.017	0.081	0.000
Hb A	0.003	0.002	0.000

Sample was clarified by centrifugation at 28,680 g for 10 min and adjusted to 50 μM heme in 1/15 M phosphate buffer, pH 6.6. All procedures before start of incubation were performed at 4°C. Turbidity was expressed by absorbance at 700 nm after incubation at 37°C for 4 h. Actual composition (%) of the derivatives was as follows:

Hb Iwata oxy: oxy 62.6, hemichrome 30.0, met 7.4
Hb Iwata met: hemichrome 50, met 50
Hb A oxy: oxy 93.4, met 6.6
Hb A met: met 100

Table Ⅳ Stability of the hemoglobins obtained from DE-52 fractions

	A_{280}	A_{700}	20° C, 3h →	A_{700}
Hb A	.730	.000		.000
Hb Iwata	.736	.008		.010
Heme-deplet ed Hb Iwata.	.736	.004		.024

Turbidity was assessed by absorbance at 700 nm after incubation of appr. 30 μM (as globin chain) hemoglobin at 20°C for 3 h in 1/15 M phosphate buffer, pH 6.6.

The precipitates that formed during storage of purified Hb Iwata (oxy form) at 4°C for two weeks was studied by the urea-polyacrylamide gel electrophoresis; they consisted of approximately equal amounts of the abnormal α and normal β chains. No excess β chain (Hb H) was detected in the supernatant. The precipitates from the heme-depleted Hb Iwata showed the same chain composition as the native Hb Iwata, but its color was lighter than the precipitates of the native Hb Iwata. These observations suggested that Hb Iwata precipitated without complete dissociation into the α and β subunits, as was the case with other unstable hemoglobins, and that although heme loss much accelerated the precipitation it was not a prerequisite for precipitation of Hb Iwata.

DISCUSSION

Hemoglobin Iwata, as found in our pedigree, is the first example in which the proximal histidine of the α chain (α87) was replaced by arginine. Abnormal hemoglobins so far discovered with substitution of either the proximal or distal histidine are listed in Table Ⅴ. In Hb M

Table Ⅴ Amino acid substitution of the proximal or distal histidine

	α chain	β chain
F8 (proximal histidine)	α87 Hb M Iwate (His⟶Tyr) Hb Iwate (His⟶Arg)	β92 Hb M Hyde Park (His⟶Tyr) Hb St. Etienne (His⟶ Gln) Hb J Altgeld Gardens (His⟶Asp) Hb Newcastle (His⟶Pro)
E7 (distal histidine)	α58 Hb M Boston (His⟶Tyr)	β63 Hb M Saskatoon (His⟶Tyr) Hb Zürich (His⟶Arg) Hb Bicêtre (His⟶Pro)

Iwate, the proximal histidine F8 (α87) of the α chain is replaced by tyrosine[5]. Its X-ray crystallographic analysis disclosed that the heme iron is bound to the distal histidine E7 (α58) and that it is stabilized in ferric state by being internally liganded to the new tyrosine F8[23].

This situation does not extend to Hb M Hyde Park in which the proximal histidine of the β chain is replaced by tyrosine[24]. Here, the heme iron of the abnormal β chain is not bound to the distal histidine E7 (β63), probably because of steric hindrance of the binding by valyl residue E11 (β67). The helix F and non-helical corner FG of the β chain must recede from the heme to make room for the new tyrosyl residue F8 which is internally liganded to the ferric heme. This minor difference in the structure between the α and β heme pockets is the reason why Hb M Hyde Park is unstable with tendency to lose heme while Hb M Iwate is stable. The abnormal β chain of Hb St. Etienne does not bind heme at all because of substitution of the proximal histidine by glutamine which cannot be internally liganded to heme iron[25]. The distal histidine in the β chain of human hemoglobin helps to maintain the tension or restraint at the heme in the T structure. Therefore, it is essential for the maintenance of the low oxygen affinity. The distal histidine opposes acid-catalyzed oxidation of iron by its low pK and the basic nature of its Nε. Hb Zürich, with arginine substitution for the distal histidine E7 (β63) of the β chian, has the arginyl side chain swinging out of the distal site leaving there a gap. Its guanido nitrogen engages in a salt bridge with propionate side chain Ⅳ of the heme[26]. Hb Zürich is noted for its susceptibility to oxidizing agents.

Although our study of Hb Iwata determined neither the orientation of the arginyl residue F8 (α87) nor the exact position of the heme in the heme pocket of its α subunit, the heme in Hb Iwata, by analogy to Hb M Iwate, is expected to be held in place by the distal histidine E7 (α58). As has been demonstrated in Hb Zürich, the arginyl residue in Hb Iwata may also swing out. The gap formed there will facilitate accelerated oxidation with hemichrome formation and weak heme-globin contacts. More precise stereochemical interpretation of this abnormal hemoglobin should depend on crystallographic analysis.

According to the preliminary data of Hayashi et al (personal communication), the oxygen equilibrium curve of Hb Iwata is similar in shape to that of Hb M Iwate because both have lost cooperative subunit interation (n of Hill's equation $\fallingdotseq$ 1). However, Hb Iwata shows oxygen affinity higher than normal, especially at low oxygen saturation and shows normal Bohr effect; this is contrary to Hb M Iwate whose oxygen affinity is lower than normal and Bohr effect decreases[27]. The low oxy-

gen affinity of Hb M Iwate is due to tight T conformation in its semi-ferrihemoglobin form. The inappropriate tension that the tyrosyl residue exerts on the heme does not allow T to R conformational transition which otherwise should occur coincident with oxygenation as well as oxidation. The high oxygen affinity and diminished subunit cooperation in Hb Iwata, on the other hand, may result from the absence of the normal tension at the heme necessary for stabilization of the deoxy T structure. With the absence of the heme-linked histidyl residue, Hb Iwata may always remain in the R conformation even in deoxy state.

The sequence of events leading to the precipitation of Hb Iwata *in vito* is summarized in Fig. 13. This scheme essentially conforms to what has been proposed by Winterbourn and Carrell[28] for the explanation of the exaggerated hemolysis in unstable hemoglobin hemolytic diseases. The oxidation of heme iron, to form methemoglobin, is the first essential step in hemoglobin denaturation. Methemoglobin then gradually transforms into reversible hemichrome, irreversible hemichrome and precipitates. The ferric form of the abnormal α subunit of Hb Iwata may exist only in a low spin form, i.e., hemichrome, as against to the usual methemoglobin, a high spin ferric hemoglobin[29]. Its high spin

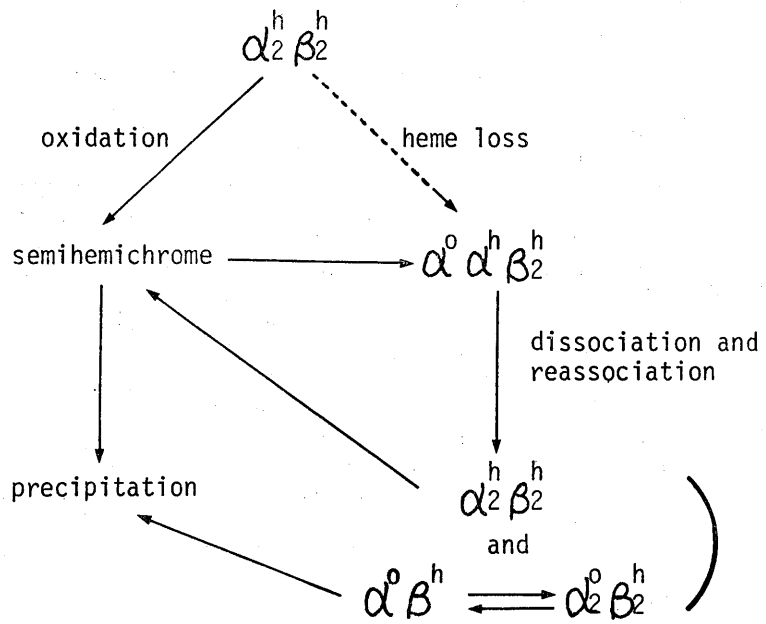

Fig. 13. The sequential events leading to precipitation of Hb Iwata. Superscripts h and o represent the presence and absence of heme in the subunit, respectively.

ferrihemoglobin appears to be extremely unstable and transforms into low spin hemichrome as soon as the heme iron is oxidized.

The results of electrophoresis and comparative Sephadex G-75 gel filtration of the heme-depleted form of Hb Iwata suggest that Hb Iwata with three hemes per tetramer tends to dissociate into two kinds of $\alpha\beta$ dimers, i.e., α^{h} β^{h} and α° β^{h} (Fig. 13). Their reassociation generates Hb Iwata with all four hemes (α_2^{h} β_2^{h}), leaving the heme-depleted counterparts (α° β^{h}, α_2° β_2^{h}) in a dimer-tetramer equilibrium. The broadness of the slow-moving band in Fig. 11 suggests the existence of such an equilibrium.

While several heme-depleted β chain variants[30-34] have been isolated, Hb Iwata is the first heme-depleted α chain variant isolated. The heme-depleted forms of Hb Iwata rapidly denature because they lack the stability provided by the heme which serves as a hydrophobic center of the molecule[4]. These findings illustrate that a single substitution of the proximal histidine in the α chain drastically affects the physical properties and function of the mutant hemoglobin. Hb Iwata deserves further investigation since it offers an opportunity to check the mutual correlation between the heme and globin and to define the structural and functional differences between the α and β chains.

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