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Identification of Abnormal Hemoglobin IX. HbA_2 Yokoshima δ25(B7) Gly→Asp Found in Homozygous State in Two Japanese Families

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Abstract A variant of HbA_2 was found in two patients during a random survey by isoelectric focusing. Chemical characterization of the abnormal δ chain was facilitated by reverse phase high performance liquid chromatography and automated gas phase amino acid sequencing. The same amino acid substitution, from glycine to aspartic acid at position 25 (B7) of the δ chain, was demonstrated for both cases. This is the second example of identification of δ chain anomaly and includes the second and third examples of homozygosity for an abnormal hemoglobin among Japanese.

Key Words : Abnormal hemoglobin, Hemoglobin A_2

Introduction

$HbA_2(\alpha_2\delta_2)$ usually accounts for only 2.5% of the total hemoglobin throughout human life after 6 months of age. Alteration in its percentage provides a clue to the differential diagnosis of thalassemia syndrome[1]. Qualitative abnormality of HbA_2 is considered to be of no clinical importance, however, because of its normally low concentration, and a relatively small number of δ chain variants have been identified by amino acid sequence analysis[2]. Also the heavy work load and requirement for a large amount of blood sample used to discourage detailed characterization of variants of HbA_2. Recent development of technology, especially of high performance liquid chrmatography (HPLC) and of automated amino acid sequencing, has scaled down the amount of sample to be used in determination of amino acid substitution by one-tenth.

Availability of new instruments has recently enabled us to determine the amino acid substitution in the abnormal δ chain of HbA_2 Yokoshima. Soon after publication of a preliminary report on this material[3], a homozygote for an abnormal HbA_2 of the same isoelectric point was found. Its structural abnormality was studied side by side with HbA_2 Yokoshima and the same amino acid substitution was demonstrated. This paper

presents technical data in the chemical characterization of the δ chain variant which have largely been omitted in the previous report[3].

Materials and Methods

Red cell lysates were prepared by a conventional water and toluene method, dialyzed successively against 0.1 mM sodium EDTA (pH 7.4) and deionized water. They were stored about a year at 4℃ under carbonmonoxide without any sign of deterioration.

Thin layer polyacrylamide gel isoelectric focusing[4] was used for screening of hemoglobin variants. Normal and abnormal HbA_2 were prepared by DEAE-cellulose (DE-52, Whatman) column chromatography[5]. Globin was prepared by a HCl acetone method and δ chains were purified by urea CM-cellulose (CM-52, Whatman) column chromatography[6].

The δ chains were digested with trypsin and the soluble tryptic peptides were subjected to peptide mapping by reverse phase HPLC on a 3.9mm X 25cm column of μBondapak C_{18} (Waters)[7]. For preparation of relevant tryptic peptides, the column was loaded with 2mg digest (120 nmol) and developed by the trimethylamine-acetic acid/acetonitrile system (see legend for Fig. 4b). Effluent fractions were manually collected. Approximately 30-100nmol of each peptide was digested with 20% HCl at 105℃ for 24h and amino acid composition determined by Model 835 Amino Acid Analyzer (Hitachi). Approximately 5nmol abnormal δT3 was subjected to automated gas phase Edman degradation by Model 470 Gas Phase Protein Sequencer (Applied Biosystems). A standard program provided by the manufacturer was used. One-fifth of the product of each step (PTH-amino acid) was loaded on a Novapak C_{18} column (Waters), which was developed by a convex gradient of acetonitrile in 50mM ammonium acetate (pH 6.8)-10% methanol. Alternatively, a convex gradient between 25mM sodium acetate (pH 5.15)-methanol (9/1 by vol) and isopropanol-water (1/1)[8]. was used. Since our HPLC system was not equipped with an integrator, no attempt was made to quantitate PTH-amino acids. This caused no difficulty in identification of the major peaks and interpretation of the results.

Results

The minor variant was detected together with a decreased amount of normal HbA_2 for the first time in a 3-year-old boy. His patternal grandparents, both from Yokoshima, a tiny island in Setonaikai (the Inland Sea), carried the same variant, and one of their children was a homozygote (Fig. 1). Isoelectric focusing pattern of the red cell lysates has been published[3].

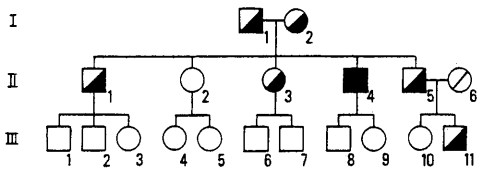

Fig. 1 Pedigree of the first family segregating HbA_2 Yokoshima. Square and circle: male and female, respectively. Oblique line: examined normal. Half filled: heterozygote. Filled: homozygote.

The second case was a 68-year-old man from another island near Yokoshima. His blood lacked normal HbA_2 and had an abnormal band, which comprised a similar amount and focused at the mid point between HbA_2 and HbA in isoelectric focusing. No family member was available for study. Marrital relation to the first family was unknown.

The variant hemoglobin emerged from the DEAE-cellulose column behind normal HbA_2 and in front of HbA_0 (Fig. 2). Up to 1g hemoglobin could be applied without overlapping of the leading edge of HbA_0 over the abnormal peak, and about 10mg of the variant was purified from a heterozygote (considerablly more from a homozygote). It consisted of the normal α chain and the abnormal δ chain whose elution from the urea CM-cellulose column was almost as fast as the normal β chain and faster than the normal δ chain (Fig. 3).

One tryptic peptide was missing and there

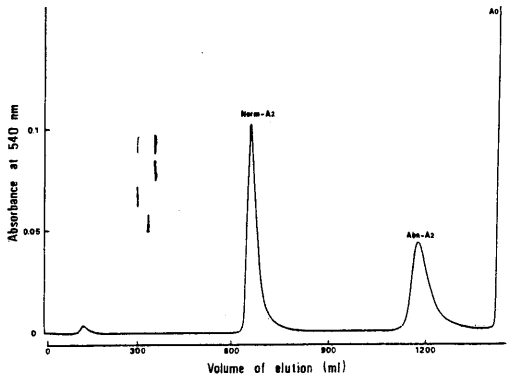

Fig. 2 DEAE-cellulose column chromatography of red cell lysate from a heterozygote (Fig. 1, II-5). A 2.6 X 25 cm column of DE-52 was loaded with approximately lg hemoglobin and developed by a linear gradient of NaCl, from 5mM to 30mM, in 200mM glycine-NaOH (pH 7.8). The total volume of gradient was 4 l at a flow rate of lml/min at 6℃. Abnormal hemoglobin comprised 42.2 % of the total HbA_2. The inserted picture shows isoelectric focusing of each fraction. From top to bottom : normal red cell lysate, HbA_0, normal HbA_2 and the variant. The anode is to the right.

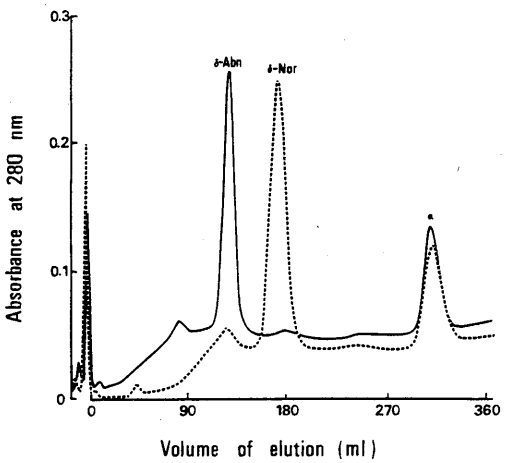

Fig. 3 Urea CM-cellulose column chromatography of the globin from HbA_2. A 1.6 X 15 cm column of CM-52 was developed by a linear gradient of sodium phosphate buffer (final pH about 6.8), from 5 to 40 mM of Na_2HPO_4 in 8M urea and about 2mM dithiothreitol, total volume 500ml and flow rate 45ml/h at room temperature. Solid line : the variant. Broken line : normal HbA_2. Sample size : each about 20mg.

was one abnormal peak in peptide maps of the abnormal δ chain. The abnormal peak emerged slightly slower than the missing peptide in the trifluoroacetc acid/acetonitrile developer system (Fig. 4a) and slightly faster in the trimethylamine-acetic acid/acetontrile system (Fig. 4b). Its composition indicated a substitution from glycine to aspartic acid (or asparagine) in δT3 (Table 1). Composition of the whole δ chain was consistent with the substitution and suggested no other abnormality.

Automated gas phese Edman degradation on the abnormal δT3 from the second case identified the sequence of 12 residues from the N-terminal. The 8th residue was aspartic acid rather than glycine. This result established the substitution from glycine to aspartic acid (rather than asparagine) at position 25 of the δ chain (Fig. 5). No PTH-amino acid was recovered for the 13th residue (the C-terminal arginine of δT3). Essentially the same results were obtained for the first case[3].

Discussion

Purification of peptides by reverse phase HPLC and subsequent sequencing by an automated gas phase Edman degradation proved to be a very efficient system of the chemical identification of variant hemoglobin. A few mg of the abnormal δ chain was enough, and there remained no ambiguity as to the amino acid substitution in HbA_2 Yokoshima. The C-terminal arginine of the δT3 served as an anchor in this particular case during extraction of the amino acid derivatives and excess reagents ; it remained on the sample disc even after derivation at the final step (data not shown).

Hemoglobin variants are infrequent among the Japanese according to the results of surveys of the total number of some 500,000 individuals[9]. Report of only one homozygote [Hb Takamatsu β120(GH3)Lys→

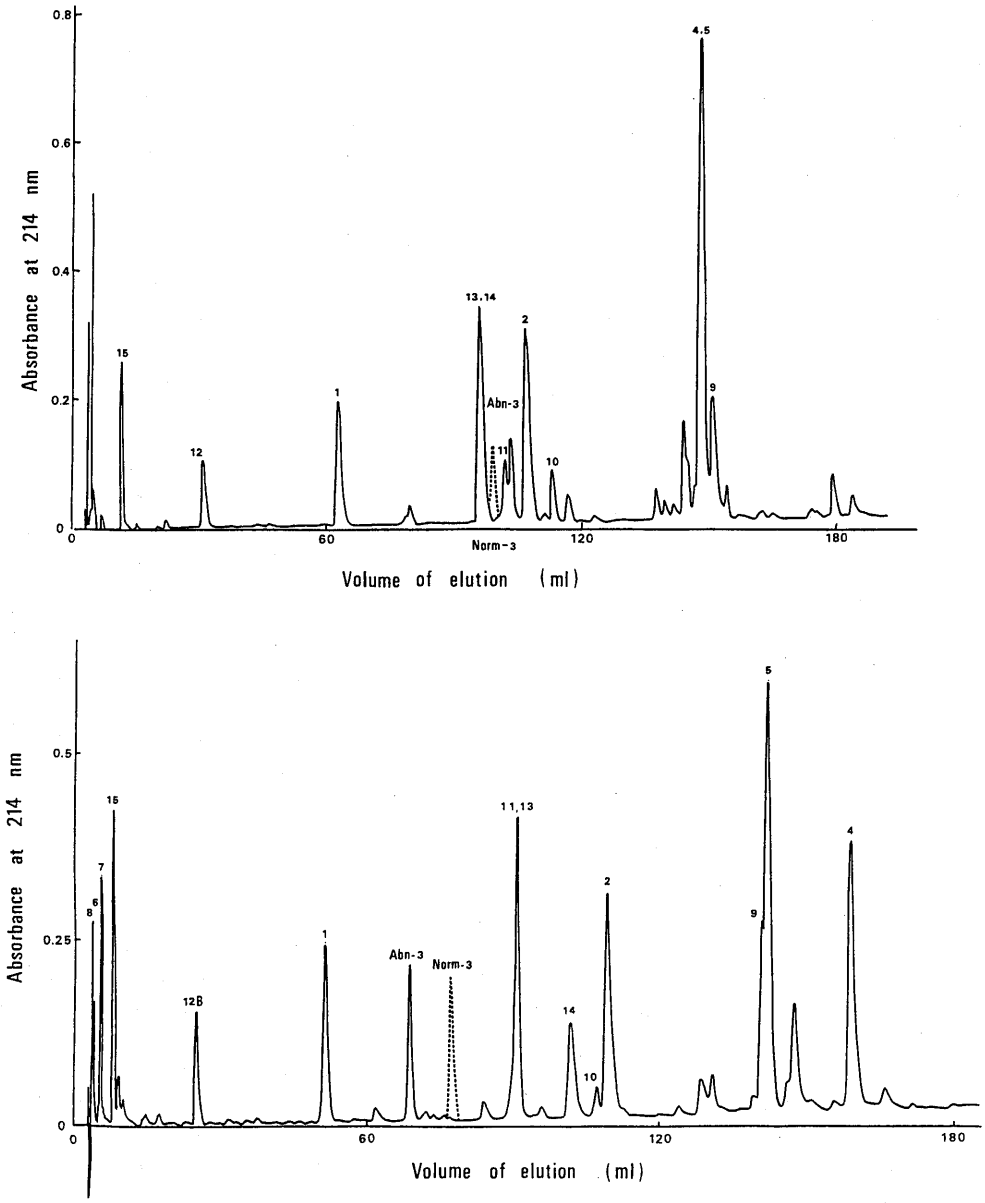

Fig. 4 Peptide mapping of tryptic digest. The sample was dissolved to 2% (w/v) in the starting developer and 10 μl of the supernatant was applied to a 3.9mm X 25 cm column of μBondapak C_{18}, which was developed by a linear gradient of acetonitrile, from 0 to 32% in 180ml, in (a) 0.05% trifluoroacetic acid or (b) 9mM-trimethylamine-10mM acetic acid (pH 5.6), at a flow rate of 1.5ml/min at room temperature. The peak for normal δT3, which is missing in the abnormal δ chain, is shown by a broken line. Numerals are tryptic peptide numbers.

18 19 20 21 22 23 24 25 26 27 28 29 30
Val-Asn-Val-Asp-Ala-Val-Gly-*Asp*-Glu-Ala-Leu-Gly-Arg
→ → → → → → → → → → → →
1 2 3 4 5 6 7 8 9 10 11 12

Fig. 5 Amino acid sequence of abnormal δT3. Numbers above amino acid are residue numbers from the N-terminal of the δ chain. Arrows indicate sequenced residues. Residue at position 25 is Gly in normal δT3.

Table 1 Results of Amino Acid Analysis

Amino acid	Whole δ chain Case 1 abnormal	Whole δ chain normal	Expected normal	δT3 Case 2 abnormal	δT3 Case 1 abnormal	δT3 normal	Expected normal
Asp *	15.94	15.15	15	3.04	2.86	1.87	2
Thr	4.98	4.93	5	—	—	—	0
Ser	5.50	5.60	6	—	—	—	0
Glu *	12.38	12.43	12	0.93	1.15	1.21	1
Pro	6.55	6.52	6	—	—	—	0
Gly	12.31	13.24	13	2.03	2.04	2.99	3
Ala	15.14	15.11	15	2.01	2.06	2.08	2
Val	15.27	15.07	17	2.75	2.86	2.83	3
Cys	0.63	0.67	2	—	—	—	0
Met	2.07	2.01	2	—	—	—	0
Ile	—	—	0	—	—	—	0
Leu	18.70	18.65	18	1.01	1.07	1.04	1
Tyr	3.11	3.07	3	—	—	—	0
Phe	8.12	8.19	8	—	—	—	0
Trp	(+)	(+)	2	—	—	—	0
Lys	11.78	11.84	11	—	—	—	0
His	7.16	7.02	7	—	—	—	0
Arg	4.31	4.41	4	1.07	0.96	0.96	1

Expressed by molar ratios. * Asparagine and glutamine have been hydrolyzed to aspartic acid and glutamic acid, respectively.
Underlined are abnormal values.

Gln] [10] reflects this situation. Although about 100 variants of the α or β chain have been reported, only 2 δ chain variants have been identified including HbA_2 Yokoshima. The two patients we have presented here constitute the only examples with a well documented δ chain variant detected during a survey of 22,000 non-repetitive blood samples by isoelectric focusing (unpublished). The discovery of 2 homozygotes will not reflect a high frequency of this variant among the Japanese. Like Hb Takamatsu, HbA_2 Yokoshima is presumed to be a local hemoglobin prevalent in a number of families from a small geographical area.

The latest version of variant list from International Hemoglobin Information Center[2] includes 15 variants of the δ chain, to which the newly identified Japanese variants, HbA_2 Honai δ90(F6)Glu→Val[11] and HbA_2 Yokoshima are to be added (Table 2). In an excellent review of the subject published in 1977, Vella[12] collected 10 δ chain variants including all relatively common types. HbA_2'(also called HbB_2) is by far the most frequent. Its incidence in some Negro Populations exceeds 1 : 100. Double heterozygosity with HbS or HbC has occasionally been reported, but the gene locus for δ-A_2' chain has always been in trans to that for β^S or β^C chain. The δ-A_2' locus is in cis to the β-thalassemic locus in some families while it

Table 2 The Variants of the δ and β Chains at the Homologous Sites

δ chain Substitution	Name	Properties	β chain Substitution	Name	Properties
2(Na2) His→Arg	A_2Sphakia		His→Arg	Deer Lodge	high
			His→Gln	Okayama	high
12(A9) Asn→Lys	A_2NYU		Thr		
16(A13) Gly→Arg	A_2'(B_2)		Gly→Arg	D-Bushman	
			Gly→Asp	J-Boltimore	
20(B2) Val→Glu	A_2Roosevelt		Val→Met	Olympia	high*
22(B4) Ala→Glu	A_2Flatbush		Glu→Ala	G-Coushatta	normal
			Glu→Gln	D-Iran	normal
			Glu→Gly	G-Taipei	
			Glu→Lys	E-Saskatoon	unstable
24(B6) Gly→Asp	A_2Victoria		Gly→Asp	Moscva	unstable*, low
			Gly→Val	Savannah	unstable*
			Gly→Arg	Riverdale-Bronx	unstable*, high
25(B7) Gly→Asp	A_2Yokoshima		Gly→Arg	G-Taiwan Ami	
43(CD2) Glu→Lys	A_2Melborne		Glu→Gln	Hoshida	normal
			Glu→Ala	G-Galveston	
51(D2) Pro→Arg	A_2Adria		Pro→Arg	Willamette	unstable, high
69(E13) Gly→Arg	A_2Indonesia		Gly→Arg	Kenitra	
			Gly→Asp	J-Cambridge	
			Gly→Ser	City of Hope	
90(F6) Glu→Val	A_2Honai		Glu→Lys	Agenogi	low
99(G1) Asp→Asn	A_2Canada	high	Asp→Asn	Kempsy	high*
			Asp→Gly	Hotel-Dieu	high*
			Asp→Ala	Radcliffe	high*
			Asp→Val	Chemilly	high*
			Asp→Tyr	Ypsilanti	high*
			Asp→His	Yakima	high*
116(G18) Arg→His	A_2Coburg		His		
121(GH4) Glu→Val	A_2Manzanares	unstable	Glu→Val	Beograd	
			Glu→Gln	D-Los Angeles	high*
			Glu→Lys	O-Arab	normal
125(H3) Gln→Glu	A_2Zagreb		Pro		
136(H14) Gly→Asp	A_2Babinga		Gly→Asp	Hope	unstable, low
142(H20) Ala→Asp	A_2Fitzroy		Ala→Asp	Ohio	high*
			Ala→Pro	Toyoake	unstable*, high

High, normal, low : oxygen affinity. *Cause of clinical manifestation.
See reference 2 for references of individual hemoglobin variant.

is in trans in others in double heterozygosity for HbA_2' and β-thalassemia. Levels of the normal and abnormal HbA_2 in these cases have indicated an increased production of the δ chain both in cis and in trans to the β-thalassemia determinant.

Other δ chain variants more or less popular in some populations include HbA_2Flatbush and HbA_2Babinga (Negro), HbA_2Sphakia (Canadian Amerindian, but not South American Indian), HbA_2Indonesia (Indonesian/Malay), and HbA_2NYU (Eastern European). Homozygotes have been found for HbA_2Sphakia, HbA_2', HbA_2Flatbush and HbA_2Yokoshima.

None of the hetrozygous or homozygous carriers of one of the δ chain variants has shown anemia, erythrocytosis, jaundice or nigremia which is ascribable to the hemoglobin. This is due to the low level of HbA_2

rather than the physical and functional properties of the vatiant. It is expected from analogy to the β chain variants that some of the δ chain variants will exhibit molecular instability and/or functional abnormality if they are specifically tested. For example, HbA_2Canada showed a high oxygen affinity just like its β chain homologue Hb Kempsy, a high affinity hemoglobin causing erythrocytosis in its heterozygous carriers. Purification of an amount of a variant of HbA_2 that is enough for such studies requires a few hundred ml of blood from a single donor, although chemical identification per se demands only 10-20ml.

Structural and synthetic variations of the δ chain have served as a genetic marker, have opened the door into some insight on the thalassemia syndromes, and, it is hoped, may provide useful informations for the elucidation of the mechanism of gene activity.

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