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Structural analysis of proteins in Thailand: Identification of abnormal hemoglobins

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Abstract Many proteins and enzymes have been purified in Thailand, but little sequence analysis has been performed, except for the abnormal hemoglobins.. This paper reviews the hemoglobins discovered in Thailand, and presents experimental data on the abnormal hemoglobins found in five individuals, namely: Hb Anantharaj [α11 (Lys→Gln)], Hb Lepore-Washington-Boston [δ87-β116]/ Hb E [β26 (Glu→Lys)], Hb J (Bangkok) [β56 (Gly→Asp)]/ Hb E [β26 (Glu→Lys)], Hb Queens [α34(Leu→Arg)], and Hb C [β6 (Glu→Lys)] .

INTRODUCTION

Several proteins have been isolated in the purified state in Thailand, e.g. human protamines (ref. 1), rat testis-specific histones (ref. 2), and human seminal plasma progastricsin (ref. 3), structural studies on these proteins have been limited in nature, due partly to the small amounts of material available and partly to the limited facilities for protein sequence analysis. So the most extensive protein sequence data available in Thailand are on the abnormal hemoglobins, several of which have now been found in Thailand (ref. 4-21), as shown in Table 1. Initially structural analysis was carried by Dr. Sagna Pootrakul and others at the Faculty of Medicine, Siriraj Hospital, Mahidol University, either independently or in collaboration with overseas groups. However, upon his death in 1977, work on structural aspects lapsed until our group reinitiated such studies, using hemoglobin as a model to assist in the development of facilities for protein sequencing. This paper reviews the characterisation of hemoglobin variants found in five individuals.

Hb ANANTHARAJ

Hb Anantharaj (ref. 5), was discovered in a 58 year old male of pure Thai extraction, with three males out of the five offspring also having the abnormal hemoglobin which was characterised in 1975 as being due to the mutation 11 Lys-Glu. We have reexamined the abnormal hemoglobin in the eldest son ("S.K.") of the family, who is now 30 years old. The abnormal hemoglobin was isolated by DEAE-cellulose chromatography (ref. 22) and the abnormal chain was purified by CM-cellulose chromatography in 8 M urea (ref. 22). Tryptic peptide maps (ref. 21) showed that the normal α^A chain contained peptides T2 and T3 absent from the abnormal α-chain, which showed a new peptide T2-3 instead.

Peptides distinguishing the normal and abnormal α-chains were hydrolysed with mercaptoethane sulphonic acid to preserve tryptophan. The amino acid compositions of peptides T2 (Asx 1.08, Thr 0.97, Val 1.17, Lys 1.01) and T3 (Gly 1.00, Ala 1.96, Trp 1.04, Lys 1.00) corresponded to residues 8-11 and 12-16 of normal α^A chain respectively. Peptide T2-3 (Asx 0.84, Thr 1.16, Glx 0.95, Gly 0.99, Ala 1.99, Val 0.90, Trp 0.92, Lys 1.01) from the α chain of Hb Anantharaj indicated that this peptide originated from residues 8-16 by a replacement of Lys by Glx. However, peptide T2-3 of Hb

Anantharaj had a net positive charge at pH 6.5 ($m_{6.5}$ = - 0.25, relative to aspartic acid), indicating that the Glx residue must be glutamine, and cannot be glutamic acid as earlier reported. The earlier assignment of glutamic acid, based on amino peptidase digestion and amino acid analysis, is probably unreliable since the analysis procedure was unlikely to have been able to separate glutamine from glutamic acid. Thus, it is now concluded that Hb Anantharaj is due to the mutation α11 (A9) Lys-Gln, identical to Hb J-Wenchang-Wuming (ref. 23).

Table 1: HEMOGLOBIN VARIANTS FOUND IN THAILAND

HEMOGLOBIN	%	MUTATION		POSITION	HEMATOLOGY	REFS*
Hb Anantharaj	rare	α11	Lys-Gln	external	Normal	5,6
Hb Siam (Hb Ottawa)	rare	α15	Gly-Arg	external	Normal	7
Hb Queens (Hb Ogi)	rare	α34	Leu-Arg	$\alpha_1\beta_1$/ext	Normal	8
Hb Thailand	rare	α56	Lys-Thr	external	Normal	9
Hb Mahidol (Hb G Taichung)	rare	α74	Asp-His	external	Normal	10
Hb Suan Dok	rare	α109	Leu-Arg	internal	Mild α-thal	11
Hb Constant Spring	4%	αC.t.	elongation		HbH/α-thal	12
Hb C	rare	ß6	Glu-Lys	external	Target cell	13
Hb Siriraj	rare	ß7	Glu-Lys	external	No anemia	14
Hb E	13%	ß26	Glu-Lys	external	Microcytosis	15
Hb J Bangkok (J Meinung)	rare	ß56	Gly-Asp	external	Normal	16
Hb New York (Kaoshiung)	rare	ß113	Val-Glu	internal	Normal	17
Hb D Punjab (D Los Angeles)	rare	ß121	Glu-Gln	external	Normal alone	18
Hb Dhonburi	rare	ß126	Val-Gly	surface crevice	ß+-thal-like	19
Hb Tak	rare	ßC.t.	elongation		ß+-thal-like	20
Hb Lepore-Washington-Boston	rare	δ87-ß116	fusion		ß+-thal-like	21

*References shown are to publications on the discovery of each variant in Thailand and are listed in the Reference section.

Hb LEPORE-WASHINGTON-BOSTON/Hb E

The proposita ("B.Y.") was a Thai female (ref. 21) showing signs of mild anemia (Hb: 11.4 g/dl; RBC: 4.91 x 10^6/mm^3; reticulocytes: 2.4%; MCV: 70 fl; MCHC 23.3 g/dl). Red cells showed decreased osmotic fragility, moderate hyperchromia, slight aniso-poikilocytosis, and rare target cells. The 2,3 DPG level was 12.86 μmoles/g Hb, which is on the high side of normal. Hb F by alkali denaturation was 23.3%. Hemoglobins were isolated by DEAE-cellulose chromatography (ref. 22) in the following relative amounts: Hb E + Hb A_2 = 53%; Hb F_0 =30%; Hb ß-Lepore = 12.7%; Hb F_1 = 4.3%. Family studies suggested that the Hb E trait was inherited from the mother, and the Hb δß-Lepore was inherited from the father. Globin chains were prepared by CM-cellulose chromatography (ref. 22).

The tryptic peptide maps (ref. 21) of the $ß^E$ and the δß-Lepore chains were compared to those of the normal $ß^A$ chain. The $ß^E$ chain lacked peptide $ß^A$T3 and instead showed two new spots $ß^E$T3a (Asx 1.99, Glx 1.06, Gly 2.10, Val 3.03 and Lys 0.92) and $ß^E$T3b (Gly 1.02, Ala 1.24, Leu 0.90 and Arg 0.78), confirming the identification of the abnormal hemoglobin as Hb E [ß26 Glu- Lys]. Several differences were observed between the peptide maps of the δß-Lepore and the normal $ß^A$ chains (ref. 21). Amino acid analyses (Table 2) indicate that in the abnormal chain, peptides δßT2, δßT3, δßT5 and δßT10 were from the δ chain, while peptides δßT12b and δßT13 were from the $ß^A$ chain, indicating that the abnormal hemoglobin was Hb Lepore-Washington-Boston, where the non-α chain has a sequence like the δ chain in residues 1-87 and like the $ß^A$ chain in residues 116-146. The presence of the hybrid ß Lepore gene was further confirmed by Pst I digestion of the propositus DNA.

The association of Hb Lepore-Washington-Boston with Hb E produces mild clinical symptoms, in contrast to the heterozygous state of Hb E, which has no clinical effects. The level of Hb Lepore observed here is similar to other reports in association with ß chain variants. However, the level of Hb F (23-34%) is substantially higher than the 9-12% observed in most cases of association, e.g. with Hb S or Hb C, while the level of Hb E is rather lower than the content of other variants in association with Hb Lepore, due to abnormalities of $ß^E$ mRNA processing (ref. 24). These suggest that there is increased expression of the gamma chain genes to compensate for the lower levels of $ß^E$ and δß Lepore mRNA present.

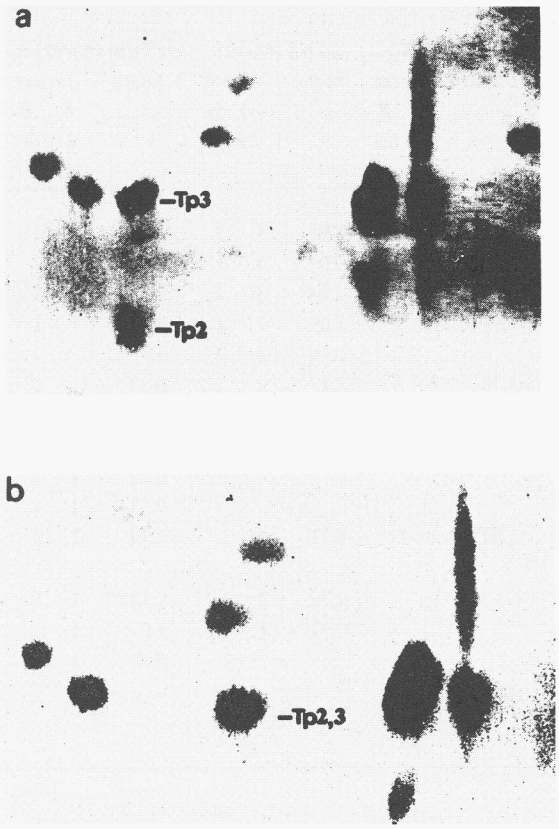

Fig. 1. Tryptic peptide map comparisons of *a:* normal α^A chains and b: abnormal chains from Hb Anantharaj. Peptides Tp2, Tp3 and Tp2-3 distinguished the two maps.

Hb J (BANGKOK)/Hb E

The proposita ("C.S.") was 22 year-old Thai female with mild pale conjunctiva, having a normal hematological profile (Hb: 14.8 g/dl; RBC: 4.81 x 10^6/mm^3; reticulocytes 0.4%; MCV 95 fl). Cellulose acetate electrophoresis indicated two abnormal hemoglobins Hb E, in the same position as HbA_2, and Hb J (Bangkok), moving faster than Hb A. The abnormal hemoglobins were isolated by DEAE-cellulose chromatography, and the abnormal ß chains were purified by CM-cellulose chromatography (ref. 22). The $ß^E$ and $ß^J$ chains were identified by peptide mapping on paper in comparison to the $ß^A$ chain. The $ß^E$ chain was confirmed by the characteristic replacement of peptide $ß^A$T3 by peptides $ß^E$T3a and $ß^E$T3b (above), which had the compositions expected.

The $ß^J$ chain showed replacement of the normal $ß^A$T5 by peptide $ß^J$T5 (Asp 4.06, $MetSO_2$ 0.90, Thr 1.00, Ser 1.93, Pro 1.97, Glx 1.09, Gly 1.06, Ala 1.05, Val 0.83, Leu 1.04, Phe 2.98, Lys 0.99) corresponding to positions 41-59 of the $ß^A$ chain, except for the replacement of one Gly by Asx. However, since there are two glycines in this sequence, peptide $ß^J$T5 was further cleaved by

cyanogen bromide producing two peptides β^J5a (Asp 2.09, Thr 0.98, Ser 1.90, Glx 1.10, Hsr 0.77, Pro 1.07, Gly 1.08, Ala 1.04, Val 1.00, Leu 1.04, Phe 2.94) and β^JT5b (Asx 2.06, Pro 0.72, Lys 0.97). Peptide β^JT5a had the composition expected of residues 41-55 of the β^A chain. However, peptide β^JT5b corresponded to residues 56-59 of the β chain, but had one extra Asx and one less Gly, and was also neutral, indicating that the abnormal hemoglobin was Hb J (Bangkok) [β56Gly-Asp]. The association of Hb E and Hb J (Bangkok) does not appear to cause any clinical manifestations.

Table 2: Amino Acid Composition (residues/mol) of Peptides in β-Chain of Propositus "B.Y."

Amino Acid	Tp 2 (9-17) found	expect δ T2	expect β T2	Tp 3 (18-30) found	expect δ T3	expect β T3	Tp 5 (41-59) found	expect δ T5	expect β T5	Tp10 (83-95) found	expect δ T10	expect β T10	Tp12b (113-120) found	expect δ T12b	expect β T12b	Tp 13 (121-132) found	expect δ T14	expect β T13
Asx	0.92	1	0	1.92	2	2	2.97	3	3	1.17	1	1		1	0			
Thr	0.86	1	1				0.09	0	1	1.12	1	2				1.07	1	1
Ser		0	1	0.13	0	0	2.91	3	2	2.27	2	1						
Glx				1.03	1	2	1.09	1	1	2.27	2	1				2.87	4	3
Pro							2.16	2	2							2.13	1	2
Gly	1.02	1	1	3.10	3	3	2.17	2	2	0.82	1	1	1.19	1	1			
Ala	2.09	2	2	2.08	2	1	1.12	1	1	0.12	0	1	1.12	1	1	2.24	2	2
Val	1.05	1	1	2.75	3	3	1.13	1	1				0.90	1	1	1.21	0	1
Met										0.92	1	1				0.00	1	0
Cys										0.53	1	1						
Leu	0.99	1	1	1.07	1	1	1.11	1	1	2.13	2	2	0.99	1	1			
Tyr																0.83	1	1
Phe							2.76	3	3	1.13	1	1	0.99	1	1	1.03	1	1
Lys	1.00	1	1				0.97	1	1	1.07	1	1	0.99	1	1	1.00	1	1
His										0.92	1	1	1.72	0	2			
Arg				1.01	1	1								1	0			
Trp	+	1	1															

Hb QUEENS (Hb OGI)

The propositus ("S.B.") was a 24 year-old male of Chinese ancestry (ref. 8) with normal hematological profile (Hb 13.8 g/dl, RBC 4.42 x 10^6 cells/mm^3; Hct 38.2; MCV 86 fl; MCH 31.3 pg; MCHC 36.0 g/dl). Cellulose acetate electrophoresis showed Hb A 85.3%, Hb Queens 11.2%, Hb A_2 2.2% and Hb F 0.8%. Hb Queens was identified by isolation of the abnormal hemoglobin by DEAE- cellulose chromatography and separation of chains by CM- cellulose chromatography (ref. 22), followed by tryptic peptide mapping. The α^{Queens} chain lacked peptide α^AT5 and instead showed two new peptides α^{Queens}T5a (Met 0.63, Phe 1.07, Arg 0.99) and α^{Queens}T5b (Thr 2.03, Ser 0.63, Pro 1.07, Phe 1.10, Lys 0.92). These data identify the abnormal hemoglobin as Hb Queens (Hb Ogi) [α34Leu-Arg].

Since the level of Hb Queens was rather low, we investigated the heat stability of the purified abnormal hemoglobin by heating at 60°C at various times. The results (ref. 8) indicate that Hb Queens precipitates sooner than normal Hb A, suggesting that the abnormal hemoglobin may be less heat stable *in vitro,* possibly due to substitution of a polar residue in the $\alpha_1\beta_1$ contact. If such instability also occurred *in vivo,* this may explain the lower concentration of Hb Queens observed. However, the α^{Queens} mutation maps at the α1 locus, which appears to be expressed at a 2-3 fold lower rate than the α2 locus (ref. 25), and this could contribute to the lower concentrations observed in the heterozygote.

Hb C

The propositus ("B.D.") was a 29 year-old male of Thai origin, with the following hematological profile: Hb 15.2 g/dl; RBC 5.16 x 10^6 cells/mm^3; PCV 44.8 %; MCV 77 fl; MCH 29 g/dl; MCHC 33 g/dl; % Hb F 0.48. Of these values, the MCV is rather lower than that found in normal subjects (92.5 ± 3.7 fl): in addition, red cell morphology showed few target cells. Blood typing suggested that the propositus was heterozygous for Hb A and Hb E, and that the abnormal hemoglobin was inherited from the father. However, the content of Hb E (42%) was substantially higher than that normally found (25-30%) in Hb E/Hb A heterozygotes. Accordingly further studies were carried out to verify the abnormal hemoglobin present.

The abnormal hemoglobin was purified from the propositus' hemolysate by DEAE-cellulose chromatography (ref. 22) and the abnormal β^C chain was purified by CM-cellulose chromatography in 8 M urea (ref. 22). Purified normal β^A-chain and abnormal β^C-chain were desalted by h.p.l.c. on a C_{18} column using H_2O: 2-propanol and separately subjected to sequence analysis on an Applied Biosystems protein sequencer (ABI Model 473A). The results showed the expected sequence for the normal β^A chain, but the abnormal β^C chain showed lysine instead of glutamic acid at residue 6:

	1	2	3	4	5	6	7	8
β^A	Val	His	Leu	Thr	Pro	Glu	Glu	Lys
β^C	Val	His	Leu	Thr	Pro	*Lys*	Glu	Lys

Preliminary data on DNA sequence analysis confirm this sequence, indicating that the abnormal hemoglobin of the propositus has a replacement of glutamic acid by lysine at position 6 of the ß-chain, showing that the abnormal hemoglobin is Hb C. This is the first report of Hb C in Thailand. The amino acid substitution Glu-Lys, found in Hb C, is exactly the same as that found in Hb E, except that in Hb C it occurs at position 6, while in Hb E it occurs at position 26, so it is not surprising that the two abnormal hemoglobins show the same electrophoretic behaviour. Hb C is common in black populations (ref. 26), while Hb E is common in Southeast Asian populations (ref. 1). In particular, in Thailand, Hb E is the most common abnormal hemoglobin, so that it is possible that some cases previously diagnosed as Hb E are actually Hb C.

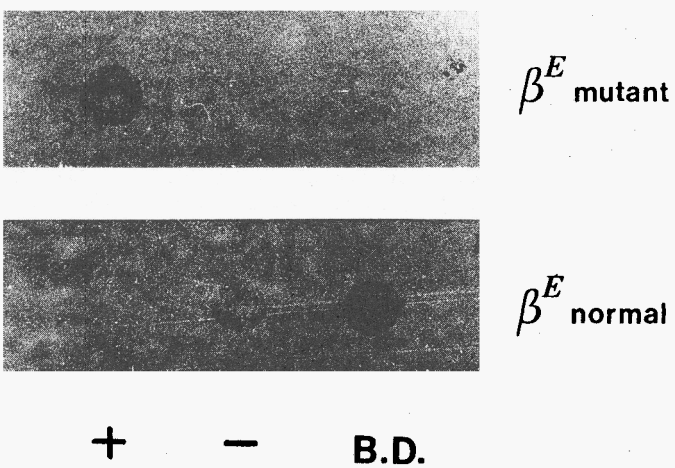

Fig. 2 Hybridisation of DNA from HbE/HbE heterozygote (+), normal HbA/HbA individual (-), and propositus (B.D.) with probe for HbE ($ß^E$ mutant) and probe for HbA ($ß^E$ normal).

Accordingly, we are exploring techniques for rapid differential diagnosis of Hb E and Hb C. One approach involves using specific DNA probes; so far we have a specific probe ($ß^E$ mutant) to recognise the $ß^E$ DNA and a specific probe ($ß^E$ normal) from the same region of sequence which recognises normal $ß^A$ DNA. Results using these probes indicate that the $ß^E$ mutant probe does not

recognise DNA from a HbA/Hb A normal individual (-) but recognises DNA from the homozygous Hb E/Hb E individual (+). Conversely, the β^E normal probe recognises DNA from the HbA/HbA individual (+) but not DNA from the homozygous HbE/HbE individual (-). DNA from the propositus (B.D.) is recognised by the β^E normal probe, but not by the β^E mutant probe, consistent with being derived from a HbA/HbC heterozygote. In addition, citrate-agar electrophoresis has been found to separate Hb C from Hb E, Hb A and Hb A_2, which run at the same position (data not shown). These approaches will be used to screen cases of Hb E with unusual hematology or unusually high levels of Hb E to see whether any are, in fact, Hb C.

CONCLUSIONS

This paper demonstrates the structural characterisation of several abnormal hemoglobins found in Thailand over the last five years. Although all of them have been reported before, some have not previously been discovered in Thailand. Moreover, some have been found in association with Hb E, the commonest variant in Thailand. All the variants described here were characterised locally without the need for collaboration with overseas laboratories. This undoubtedly indicates a greater capability for structural analysis of proteins developed in the last few years, using the readily hemoglobin variants as a model. Now that Thailand has its first protein sequencer at the Chulabhorn Research Institute, it is hoped that more extensive structural studies on proteins will be carried out locally, but this will also require training of personnel overseas.

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