HEMOGLOBIN C ZIGUINCHOR α_2^A β_2^6 (A3) Glu → Val β^{58} (E2) Pro → Arg: THE SECOND SICKLING VARIANT WITH AMINO ACID SUBSTITUTIONS IN 2 RESIDUES OF THE β POLYPEPTIDE CHAIN

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Received 28 July 1975

1. Introduction

Most human hemoglobin variants arise from a single amino acid substitution of either chain. Hemoglobin C Harlem was the first to be described which contains two amino acid substitutions in the β polypeptide chain, and which differs electrophoretically from Hb S and yet causes sickling [1]. An α chain variant, Hb J Singapore [2] exhibits changes at two adjoining positions in the α chain. The present report is concerned with a new hemoglobin variant, designated Hb C Ziguinchor, which is associated with erythrocyte sickling and which contains both the 'sickle' mutation β^6 Glu → Val, and a second amino acid substitution β^{58} Pro → Arg in the β polypeptide chain, as in hemoglobin Dhofar [3] or Yukahashi [4]. In this paper we give a description of the studies leading to this structural formulation.

2. Material and methods

The abnormal hemoglobin was detected in a 40 year old African negro man under investigation at the Hôpital Principal de Dakar.

The procedures used for the identification of the abnormal hemoglobin were electrophoresis in various buffers and media [5], and isoelectric focusing on polyacrylamide gel [6]. Heat stability tests and solubility determinations were carried out in either isopropanol [7] or concentrated phosphate [8] as previously described. Hb C Ziguinchor was isolated by DEAE cellulose chromatography [9] and globin prepared by the acid–acetone precipitation method [10]. Pure abnormal β chains were obtained by chromatography on carboxymethyl cellulose in urea according to Clegg [11], and globin chain electrophoresis was performed as described by R. G. Schneider [12]. The methods for analytical and preparative peptide mapping of tryptic digests of S. aminoethylated β chains either by fingerprinting [13] or by column chromatography [14] have been described. Amino acid analyses were carried out on a JEOL JLC 5 AH amino acid analyser. Peptides resulting from cyanogen bromide cleavage [15] were fractionated on 0.9 × 200 cm columns of Sephadex G-50 (fine) equilibrated and developed with 7% formic acid at a flow rate of 2 ml/h. Sequential analysis of amino acid residues was performed by the manual Edman degradation as previously described [16].

3. Results and discussion

3.1. *Case report*

The association of a hemoglobin C trait with erythrocyte sickling was observed in a 40 year old African negro man who originated from Ziguinchor (Casamance, Senegal) and in two of the eight children of the propositus, whose slight clinical and hematological disorders will be described in a separate report. Blood smears showed target cells and the hemolysate presented no abnormalities in either its heat stability or isopropanol solubility, but was insoluble when

reacted at 20°C with $Na_2S_2O_4$ in 2.24 M phosphate buffer, pH 6.8. Isolated Hb C Ziguinchor exhibited an oxygen equilibrium like that of Hb A, and in high concentration gelled on deoxygenation like Hb S, with subsequent liquefaction on cooling.

3.2. *Electrophoretic properties*

Electrophoresis of the proband's hemolysate in Tris–EDTA–borate buffer at pH 8.6 revealed two hemoglobin bands (fig.1a), corresponding to Hb A and to the new variant Hb C Ziguinchor, which migrated slightly cathodally to the position of Hb C. However, it was not possible to separate it from Hb A_2 under these conditions. Hb C Ziguinchor also travelled cathodally to Hb C during electrophoresis in phosphate buffer at pH 6.5 (fig.1b). Electrophoresis in agar gel in citrate buffer at pH 6.2 showed Hb C Ziguinchor to be indistinguishable from Hb S. When the erythrocyte hemolysate was electrophoresed on cellulose acetate in either acidic or alkaline buffer (pH 6.0 and 8.9), in the presence of urea and 2-mercaptoethanol, the β chains of Hb C Ziguinchor migrated at a characteristic rate, which differed under acidic and alkaline conditions and distinguished them from hemoglobins C and E. Fig.2 illustrates the electrophoretic properties of the different globin chains, and as previously described [12], β^E moves very slightly cathodally to β^C in acidic buffer and slightly anodally to it in alkaline buffer. β^C Ziguinchor moves more anodally than these two abnormal β chains both in alkaline and acidic media. These electrophoretic properties may provide a rapid means of characterising this new hemoglobin variant.

3.3 *Chromatographic data*

In preliminary experiments, microcolumns of different ion exchange resins with appropriate developers were used. We purified Hb C Ziguinchor on DEAE cellulose (DE–52) which had been equilibrated with a 0.05 M Tris–HCl buffer, pH 8.5. The abnormal hemoglobin was eluted after Hb A_2 at pH 8.3. This separation of Hb C Ziguinchor from Hb A_2 subsequently allowed its quantitative measurement. The relative amount of the different hemoglobins was: Hb A = 61.7% Hb A_2 = 3.3% and Hb C Ziguinchor = 35%. Complementary studies were performed in order to compare the specific mobilities of Hb C and Hb C Ziguinchor in CM-Sephadex [17] on a microcolumn, which was equilibrated and then developed with 0.05 M Tris – maleic acid buffer pH 6.8. When a mixture of different hemoglobins was tested, Hb F was eluted from the resin in the first ml of buffer at pH 6.8; Hb A and Hb A_2 were both eluted at pH 7.2 just before Hb S, and Hb C Ziguinchor was subsequently eluted at

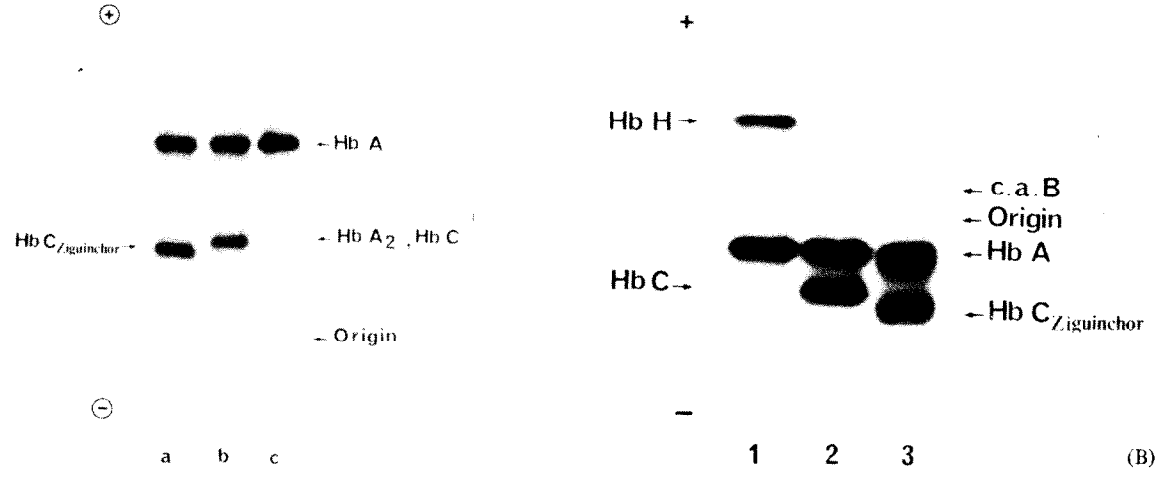

Fig.1. (A) Cellulose acetate strips electrophoresis (Tris–EDTA–borate buffer, pH 8.6, amino black stain) of hemolysates prepared from the following (left to right): (a) proband's blood, (b) hemoglobin C trait, and (c) normal adult. (B) Cellulose acetate strips electrophoresis (phosphate buffer system, pH 6.5, amino black stain) of the following, left to right (1) adult with hemoglobin H disease, (2) adult with hemoglobin C trait, and (3) the propositus. (c. a. B denotes carbonic anhydrase B).

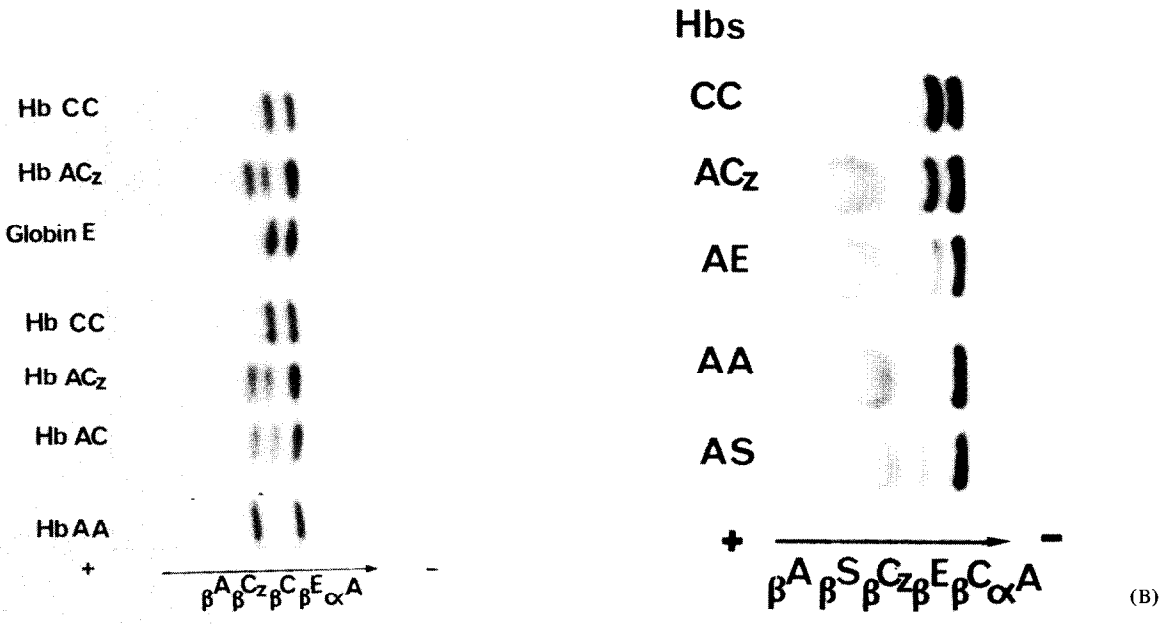

Fig.2. Globin-chain electrophoresis on cellulose acetate strips in urea–2-mercaptoethanol buffers of pH 6.0 (A) and 8.9 (B), of hemolysates containing various hemoglobins. (A) Tris–EDTA–citrate buffer, pH 6.0, 225 V, 2 h. (B) Tris–EDTA–borate buffer pH 8.9, 300 V, 2 h.

pH 7.4 Hb C is not normally eluted from the column under these conditions.

3.4. *Localisation of the substitution*

Comparison of peptide maps of tryptic digests of aminoethylated β C Ziguinchor with those prepared from normal β chains showed the following differences: (a) on the fingerprint at pH 6.4, β T1 was missing and a new peptide with a greater RF was present in a position resembling that of β T1 of Hb S. This spot gave a staining reaction for histidine. (b) The spot representing the peptide β T5 unexpectedly gave a staining reaction for arginine, and diminished staining with ninhydrin. (c) The β T6 spot stained with ninhydrin less intensely than normal whereas the free

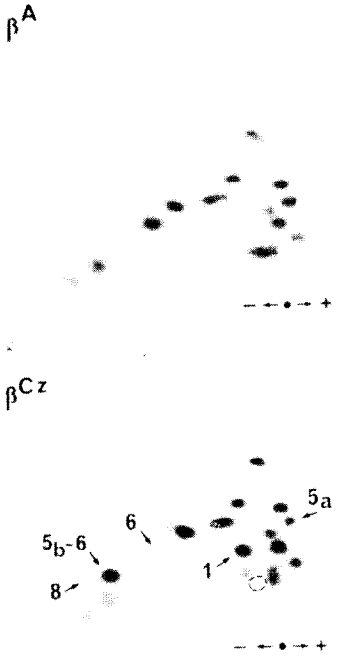

Fig.3. Fingerprints of the tryptic peptides of the β chain of Hb C Ziguinchor (below) and Hb A (above). Electrophoresis was carried out at pH 4.7 for 2 h and followed by ascending chromatography. The dotted circle indicates the normal position of peptide β T1. Numbers refer to tryptic peptides as designated in table I.

Table 1
Amino acid composition of tryptic peptides

Tryptic peptide	β T1		β $T5_a$		β $T5_b$-6		β T6	
Sequence position	1–8		41–58		59–61		60–61	
Lysine	1.11	(1)			1.98	(2)	1.01	(1)
Histidine	0.80	(1)						
Arginine			1.06					
Aspartic acid			3.19	(3)				
Threonine	0.95	(1)	0.90	(1)				
Serine			1.70	(2)				
Glutamic acid	1.14	(2)	1.05	(1)				
Proline	1.01	(1)	0.97	(2)				
Glycine			2.06	(2)				
Alanine			0.96	(1)				
Valine	1.78	(1)	1.03	(1)	1.02	(1)	0.98	(1)
Methionine			0.68	(1)				
Leucine	1.20	(1)	1.15	(1)				
Tyrosine								
Phenylalanine			2.82	(3)				

Values in parenthesis indicate the amino acid composition of the corresponding sequences in the isolated β^A chain.

lysine spot (β T8) showed an increased intensity of ninhydrin staining. When electrophoresis was run at pH 4.7 (fig.3) instead of pH 6.4, a supplementary peptide spot was seen, which did not stain for histidine and was situated between peptides β T8 and β T6. Analysis of this peptide showed that it had the composition Lys (2) Val (1) (Table 1). Fig.4 shows the pattern of tryptic peptides of abnormal β chains obtained by Aminex A5 (Bio Rad) chromatography. The pattern is the same as for a normal β chain except for the cross-hatched zones. Table 1 gives the amino acid composition of abnormal peptides. The relative amount of amino acids in the analysis of β T1 corresponds to that expected in β^S T1. Analysis of β T5 showed that the abnormal peptide differed in having one residue of proline instead of two, and a residue of arginine instead of lysine, with one less amino acid residue than normal (β T5a). Peptides β T5a (β 41–58) and β T5b (β 59 Lys) are the result of the tryptic hydrolysis of the peptide bond between β 58 Arg and β 59 Lys. Incomplete cleavage of the β 59–60 peptide bond (as a result of the adjacent Arg β 58) explains the presence of peptide β $T5_b$-6, where β 59 Lys remains attached to the following peptide. The penultimately eluted peptide from the cation–exchange resin (fig.4) corresponds to the abnormal spot found on the finger-

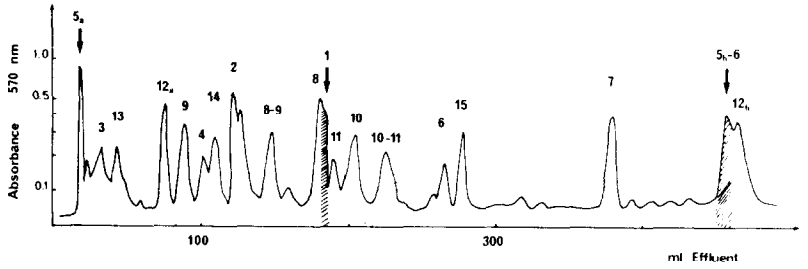

Fig.4. Peptide pattern of the tryptic hydrolysate of 30 mg of aminoethylated β chain from hemoglobin C Ziguinchor. Chromatographic separation was obtained on a column of Aminex A5 cation-exchange resin developed with a gradient of pyridine–acetic buffers from pH 3.1 to pH 5.0. The numbers above the zones identify the tryptic peptides found therein.

print performed at pH 4.7. Since there is no change in the overall electric charge of the new β T5, and since the removal of a proline residue from this moderately large peptide might have little effect on its chromatographic behaviour, no positional abnormality was found either on the chromatographic pattern or on the finger print maps. In order to confirm the two substitutions by manual Edman degradation, and to facilitate sequence analysis of the two regions in which the structural abnormalities were known to reside, the abnormal β chain of the proband was subjected to specific cleavage at methionyl residue (β 55) with cyanogen bromide. Gel filtration of the products separated the two major fragments. Edman degradation of each peptide gave the following sequences:

NH_2 terminal-

1 2 3 4 5 6 7 8 9

Val–His–Leu–Thr–Pro–Val–Glu–Lys–Ser. . . .

56 57 58 59 60 61

and N-Gly–Asn–Arg–Lys–Val–Lys. . . .

This demonstrates that the actual structure of Hb C Ziguinchor is β Glu (A3)6 → Val, β Pro (E2) 58 → Arg. The replacement β 58 Pro → Arg was previously described by Marengo-Rowe et al. [3] for hemoglobin Dhofar, and by Yanase et al. [4] for hemoglobin Yukuhashi.

4. Conclusions

The occurence of two substitutions in a single polypeptide chain of hemoglobin has been previously observed in the case of Hb C Harlem [1] in an American negro family of African descent, and in Hb J Singapore [2]. The new hemoglobin variant described here also contains two amino acid substitutions in the β chain with one, β^6 Val, responsible for sickling, as in Hb C Harlem. The second substitution, β 58 Pro → Arg, has been described for Hb Dhofar [3] in a South Arabian Veddoid from the Qara mountains of Dhofar, and for Hb Yukuhashi [4] in a Japanese person. These two substitutions, which are consistent with a one step mutation (a single base substitution) in the triplet code of messenger RNA, could have originated either by a second mutation in a β chain which already carried a single mutation for either the 6 or 58 residue, or could have resulted from homologous crossing over in an individual who was doubly heterozygote for Hb S and for Hb Dhofar ($\alpha_2^A \beta_2$ 58 Arg).

In connection with this latter suggestion it should be noted that Hb S occurs in 40 per cent of the population in certain areas of Africa and that 90 per cent of the population of Senegal are of the Muslim religion as are the people of Dhofar. The distance between determinants for β^6 and β^{58}, although smaller than those between β^6 and β^{73} of Hb C Harlem, nevertheless might favor crossing over between the two sites. Examination of the tertiary structure shows that the second substitution β^{58} Arg occurs at a corner and at the beginning of the E helix (E2). E2 is a non-polar residue in all chains of known hemoglobins, but its replacement by the polar arginine does not seem to be of particular importance in the structure of the variant β chain [18], and does not explain the low proportion (15%) of Hb Dhofar in the heterozygote [3], since Hb Yukuhashi surprisingly constituted 56 per cent of the total hemoglobin in the heterozygote. There is no significant decrease in the proportion of Hb C Ziguinchor (35%) as expected for a β chain variant. Another hypothesis which might explain the phenotype Dhofar arises from the possible association with a gene for α thalassemia. Hb C Ziguinchor retains many properties of Hb S (sickling, gelation and relative insolubility in its deoxy form), but in contrast to Hb C Harlem, a detailed comparison of these properties does not reveal differences resulting from the second substitution. A separate report will describe these studies, together with the hematological and clinical findings associated with this new hemoglobin variant.

Acknowledgements

This work was supported by l'Institut National de la Santé et de la Recherche Médicale (INSERM) grant n° 73-5-037-2, by la Délégation Générale à la Recherche Scientifique et Technique grant n° 72-7-028-2, and in part by l'Université de Paris-Val de Marne.

We thank Drs Robin and Oudart from l'Institut Pasteur de Dakar, and Dr Ducloux from l'Hôpital Principal de Dakar, Senegal, for help in investigating the patient. We are indebted to Dr M. J. Chapman for revising manuscript.

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