HAEMOGLOBIN G NORFOLK α 85 (F6) Asp → Asn. STRUCTURAL CHARACTERIZATION BY SEQUENATOR ANALYSIS AND FUNCTIONAL PROPERTIES OF A NEW VARIANT WITH HIGH OXYGEN AFFINITY

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1. Introduction

This report describes the structural characterization and the functional properties of haemoglobin G Norfolk, an unfrequent variant of human haemoglobin found in a French family in association with an acute lymphoblastic leukemia. The substitution was determined by sequenator analysis as an α 85 (F6) Asp → Asn. This abnormal variant has previously been characterized [1], but a much more simple and faster method was followed in this laboratory. Functional studies demonstrated a high oxygen affinity which is difficult to interpret on a stereochemical basis.

2. Material and methods

Standard methods were followed for the electrophoresis of hemolysates and for the separation of chains by electrophoresis on cellulose acetate strips in 6 M urea [2]. The thermostability of haemoglobin was studied according to Rieder [3]. The abnormal variant was isolated by column chromatography [4]; the abnormal α chain was separated according to Clegg et al. [5], aminoethylated and hydrolysed by trypsin as described elsewhere [6]. Fingerprints were performed on silica gel thin layers according to a procedure developed in our laboratory [7], and were stained by ninhydrin and by specific reagents following the sequential technique of Easley [8]. Separation of tryptic peptides was performed by chromatography on a column of resin Aminex A5 (0.9 × 18 cm) using a linear gradient of pyridine acetate buffer (0.2 M, pH 3.1, to 2.0 M, pH 5) [9], and purifications performed on a column of AG 50 WX_2 0.9 × 50 cm. Amino acid compositions were determined using an automatic amino acid analyzer (Jeol JLC 5 AH).

Cyanogen bromide cleavage was performed in 70% formic acid at room temperature in the dark for 24 hr, using a molar ratio BrCN/methionine of 10/1 [10]. Peptides were then isolated on a column of Sephadex G50 (0.9 × 150 cm) eluted by 7% formic acid. Automatic Edman degradation was carried out in a Beckman 890 B updated Sequenator [11] using a dimethylbenzylamine (DMBA) buffer [12]. Thiazolinones were converted into phenylthiohydantoins (PTH) amino acids and identified by gas chromatography [13] and thin layer chromatography on miniature silica gel sheets 6.3 × 6.3 cm as described in [14]. Small peptides were treated with 3-isothiocyano, 1.5 naphtalene disulfonic acid disodium salt (Braunitzer reagent III) prior sequencing [15].

Oxygen affinity was studied on intact red blood cells and purified haemoglobin according to the technique of Benesch et al. [16] as modified by Bellingham and Huehns [17]. Alkaline Bohr effect was studied in 0.05 M bis–Tris and Tris–HCl buffers ranging from pH 6.45 to 9. In order to determine its effect, 2,3-DPG was added to purified stripped haemoglobin in a molar ratio DPG/Hb of 2.5/1 at pH 7.15. 2,3-DPG was assayed according to the enzymatic technique of Rose and Liebowitz [18].

3. Results

3.1. *Case report and haemoglobin data*

The abnormal haemoglobin was detected in a 68

years old patient suffering from a lymphoblastic acute leukemia. Electrophoresis of haemoglobin revealed 15% of an abnormal slow moving component migrating as Hb D; Hb A_2 was slightly decreased (1%), and neither Hb F nor methaemoglobin were detected. The abnormal Hb was thermo-stable.

This abnormal haemoglobin was presumably independent from the leukemia since the same elektrophoretic abnormality was found in two unaffected members of the family (brother and son). The later had a well compensed hyperhaemolysis (RBC = $4.7 \times 10^6/mm^3$, Hb = 14.9 g/100 ml, MCH = 42%, MCV = 89 μ3, reticulocytes = 273 000/mm^3 bilirubin = 13 mg/l), osmotic fragility and auto-haemolysis in vitro were normal. The structural and functional studies were performed on his haemoglobin.

3.2. *The abnormal haemoglobin was isolated by*

DEAE-Sephadex column chromatography at pH 8.05; it was free of Hb A_2 and Hb A_1 by electrophoresis control.

3.3. *Functional properties*

Dissociation curves of red blood cells showed similar results to those obtained from normal subjects in standard conditions: P_{50} = 30 mm of Hg (normal value = 30 ± 2). This value is in keeping with a normal 2,3-DPG value (11.5 μmol per g of Hb). Hill's '*n*' coefficient was normal (*n* = 2.6). Oxygenation curves of the isolated abnormal Hb showed a high oxygen affinity and a normal Hill's coefficient (fig.1): P_{50} at pH 7.15 = 14 mm of Hg (normal value = 20 ± 1.5), *n* = 2.6 (normal value = 2.8); the percentage of methaemoglobin after the experiments was lower than 4%. The Bohr effect is normal for all the pH values investigated (fig. 2). 2,3-DPG effect is also normal since the P_{50} ratio of haemoglobin with DPG and stripped haemoglobin is 1.5.

3.4. *Structural study*

The α chain was abnormal as indicated by its more cathodic migration with electrophoresis in 6 M urea.

Fingerprints on silica gel thin layers of the tryptic hydrolysate of the isolated aminoethylated α G chain showed the absence of two peptides αT–IX and αT–VIII–IX). These two peptides were replaced by two new peptides slightly more positively charged at pH 6.4 and fast moving in the chromatographic step.

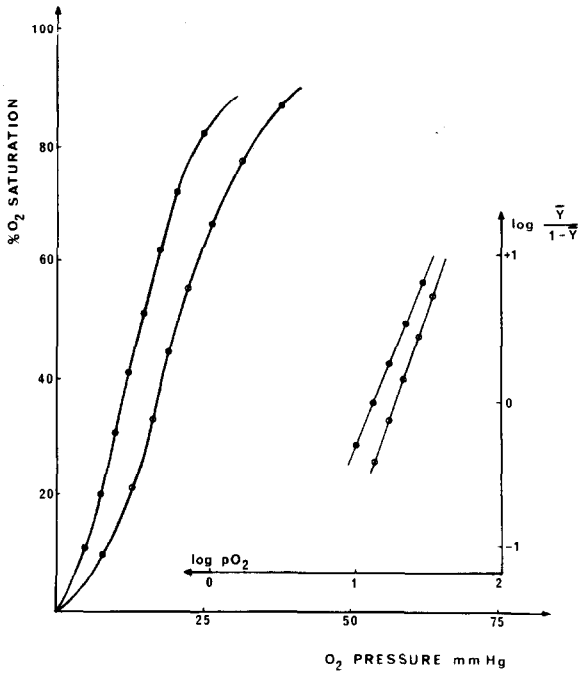


Fig. 1. Oxygen dissociation curves at pH 7.15 and Hill's plots of Hb A and pure stripped Hb G Norfolk. (o—o—o) Hb A. (•—•—•) Hb G Norfolk.

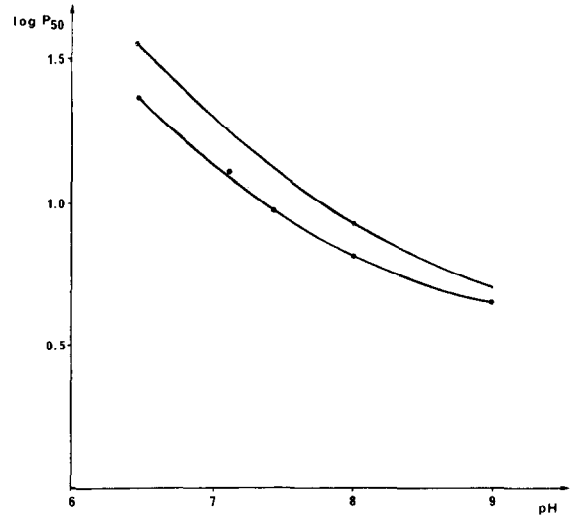


Fig. 2. Influence of pH on oxygen affinity of Hb G Norfolk. (o—o—o) Hb A. (•—•—•) Hb G Norfolk.

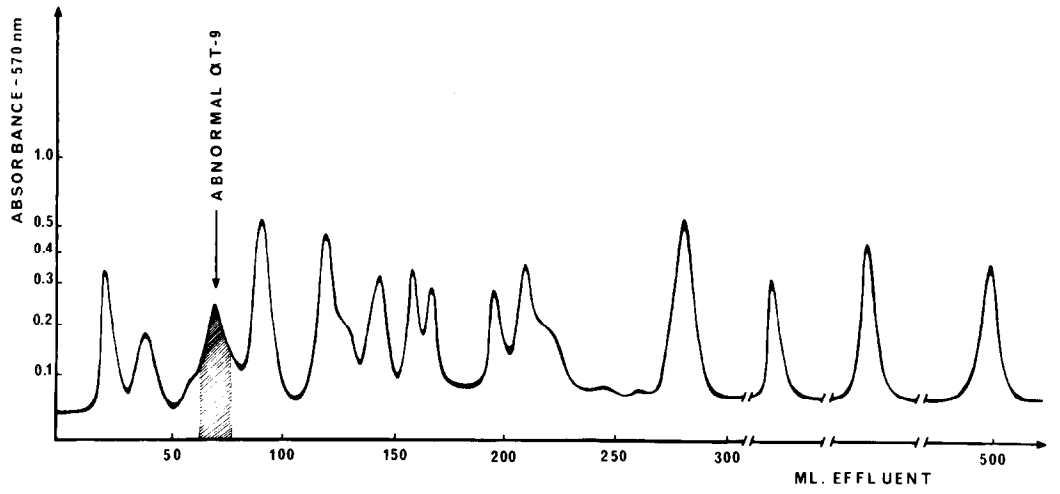


Fig. 3. Peptide elution pattern of tryptic peptides of α G aminoethylated chain. The separation was achieved on a column of Aminex A5 resin developed with a gradient of pyridin acetic buffer. The arrow indicates the zone where the abnormal αT–IX was found.

The tryptic peptide αT–IX was eluted from a Aminex A5 column with the same vol. as the normal peptide (fig.3), and purified by chromatography on AG 50 WX 2. Its amino acid composition was normal (table 1).

These results suggested a substitution of an aspartic residue by an asparagine in the αT–IX peptide. This residue is four times represented in the αT–IX. The localization of a substitution in such a large peptide containing 29 residues is very difficult and should have been achieved by indirect techniques [1]. The sequence determination by sequenator analysis seemed to be the more rapid and elegant method to confirm the substitution. By this method, it was possible to determine the sequence of the 23 first residues (62–84) of the peptide αT–IX showing that the Asp 64, 74 and 75 were unchanged. To reach the C-terminal segment of the αT–IX the cyanogen bromide product was used, allowing the determination of the 77 → 98 sequence. This sequence showed the substitution at 9th position of the α CB_3 (corresponding to the 85th residue in the intact α chain) of an Asn residue replacing the expected normal Asp (table 2). The αT–IX amino acid substitution was thus directly determined by two sequencing experiments as being α 85 (F6) Asp → Asn which is known to be Hb G Norfolk [1].

Table 1

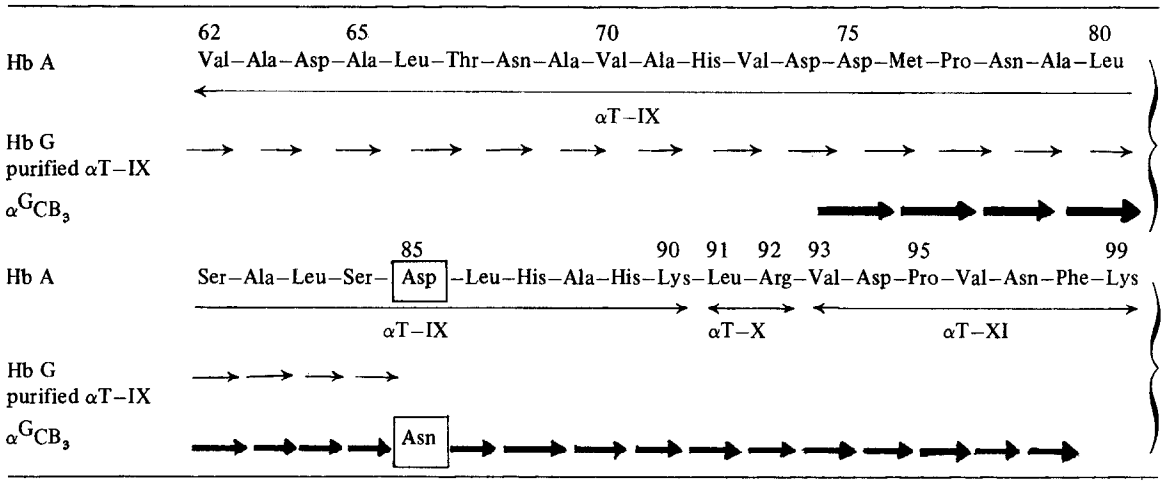
Amino acid composition of the tryptic αT–IX of aminoethylated α G Norfolk chain. αT–IX was isolated on a column of resin Aminex A5 and purified on a column of resin AG 50 WX 2.

Amino Acid	αT–IX theoretical	α^{G}T–IX found
Lys	1	1.05
His	3	2.90
Asp	6	5.90
Thr	1	1.00
Ser	2	1.75
Pro	1	1.10
Ala	7	7.15
Val	3	2.80
Met	1	0.70
Leu	4	4.00

4. Discussion

Three cases of haemoglobin G Norfolk have been until now described [1]. The discovery of that haemoglobinopathy in a subject suffering from acute leukemia has to be compared to the case reported by Lorkin et al. since an association was reported in one of the

Table 2
Sequence of the 23 first amino acids of the purified αT–IX (→→) and of the 22 first amino acids of the α CB 3 from α G Norfolk chain (↛↛), showing the substitution at 85th residue



three unrelated cases of Hb G Norfolk studied [1]. Between the four affected families a common ancestor could be suggested since our patient is native of Normandy and the three other patients of English origin.

A slight but controlled haematologic disorder (haemolysis) was observed in our patient in contrast to the three other observations of Lorkin et al. [1] where no haematological abnormality was reported.

The high oxygen affinity of Hb G Norfolk is not easy to explain. The 85th residue of the α chain (F6) occupies an external position which is not involved in a subunit contact or in a haem bond. The abnormal function suggests (Perutz, personal communication) the possibility of an interaction of Asp 85 with His 89 which lies just below it in the next turn of the helix. But such interaction cannot be seen on the electron density map. Nevertheless it must be observed that Asp 85 is constant in all the species except the horse [19]. This suggest that it plays an important role in the molecule.

The advantage of an automatic sequencing method to determine the substitution has to be emphasized. The αT–IX which is a very large peptide, contains four Asp residues and the substitution could only been investigated in the past by indirect methods such as enzymatic cleavages into very small peptides very difficultly isolated according to of their charge (several peptides are neutral) and their insolubility. In this case, sequenator analysis of the αT–IX and of the α CB_3 is the faster and the more simple method in order to confirm the assumed substitution.

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