HAEMOGLOBIN VOLGA, β27, (B9) Ala → Asp, A NEW HIGHLY UNSTABLE HAEMOGLOBIN WITH A SUPPRESSED CHARGE

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1. Introduction

A severe chronic haemolytic anaemia was found to be due to a new highly unstable haemoglobin in which alanine β27 (B9) is replaced by aspartic acid. The variant, which has been named Hb Volga, cannot be separated from Hb A by routine electrophoretic methods, suggesting that the charge on the aspartic acid side-chain is suppressed by being 'buried' in a hydrophobic region of the molecule.

2. Methods

2.1. *Routine investigations*

Haemolysates were subjected to electrophoresis on paper [1], starch gel (pH 7 and pH 8.6) [2], agar gel [3] and cellulose acetate [4], the latter being used to quantitate Hb A_2. Starch gel electrophoresis of ferricyanide treated haemolysate was carried out at pH 7 and pH 8.6. Isoelectric focusing on polyacrylamide gel [5] was performed over the pH ranges 3.5–9.5 and 6–9. Isopropanol stability tests were carried out according to Carrell and Kay [6] and Hb F was estimated by alkali denaturation [7]. Globin electrophoresis was carried out in 6 M urea [8]. Other haematological studies followed standard methods [9].

**Abbreviation*: TPCK, 1-chloro-4-phenyl-3-L-tosyl-amidobutan-2-one.

2.2. *Structural studies*

Globin was prepared from the isopropanol precipitate and separated into its component chains [10]. After gel filtration on a column (100 × 2.5 cm) of Sephadex G-25 (coarse) in 0.5% (v/v) aqueous acetic acid, the α- and β-chains were recovered by freeze drying. The β-chains were aminoethylated [11] and again subjected to gel filtration and freeze drying. Preparative 'fingerprints' of tryptic digests of the unmodified α-chain and aminoethylated (AE) β-chains were prepared at pH 6.4 and stained with fluorescamine, as recently described [12]. (Trypsin (Worthington TRL 33J786) was purified on CM-cellulose [13] and treated with TPCK* [14] before use). The abnormal tryptic peptide was eluted with 0.5 N NH_4OH; a portion was analysed and the remainder hydrolysed with thermolysin, followed by 'fingerprints' of the resultant peptide mixture, all as described previously [15]. L-aspartic acid, L-lysine.-HCl, N^{ϵ}-dinitro phenol-L-lysine·HCl and cyanol FF were used as electrophoresis markers. The net charges of peptides at pH 6.4 were calculated according to Offord [16].

3. Results

3.1. *Haematology and haemoglobin electrophoresis*

The patient was a 16 year old male from the Volga region. He had suffered from severe, chronic haemolytic anaemia from the first year of life and was

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Table 1
Haematological data for the Hb Volga heterozygote

	Hb g/dl	RBC $\times 10^{-6}/\mu l$	WBC $\times 10^{-3}/\mu l$	Reticulocytes %	Platelets $\times 10^{-3}/\mu l$
Before splenectomy	9.1	3.04	3.75	20–30	85
After splenectomy	10–13	3.85	9.70	20–40	280

splenectomised at the age of five. Following splenectomy, numerous Heinz bodies were noted in the erythrocytes. The Hb F and Hb A_2 levels were raised to 3.2% and 4.8% respectively; the isopropanol stability test produced a copious red precipitate after only three min. incubation. On starch gel electrophoresis a raised Hb A_2 was noted and a small band in the region where free α-chains would be expected to move; otherwise no abnormality was noticed on electrophoresis or on isofocusing of the oxy- or met-haemoglobin derivatives. Globin electrophoresis in 6 M urea, however, indicated the presence of fast-moving (β^J) chains which represented about 15–20% of the total β-chains. The relevant pre- and post-splenectomy haematological data for the patient are shown in table 1. The patient's parents and his sister did not have the unstable haemoglobin, which is therefore probably the result of a new mutation.

3.2. *Structural studies*

The CM-cellulose elution pattern of the globin from the isopropanol precipitate showed the latter to consist almost entirely of abnormal β-chains. The 'fingerprint' of the abnormal AEβ chain tryptic peptides showed that β^A Tp III, which consists of residues 18–30 of the β chain, (a in fig.1) was replaced by a new, more negatively-charged peptide (b in fig.1). The amino acid composition of the new peptide (table 2) differed from normal by the absence of alanine and the presence of an extra residue of aspartic acid. Since there is only one residue of alanine in β^A Tp III, at position β27 (B9) (see fig.2), the mutation in the unstable Hb is β27 (B9) Ala → Asx. It is not possible to decide from the amino acid composition alone whether Asx is Asp or Asn, since Asn is converted to Asp during the acid hydrolysis preceding amino acid analy-

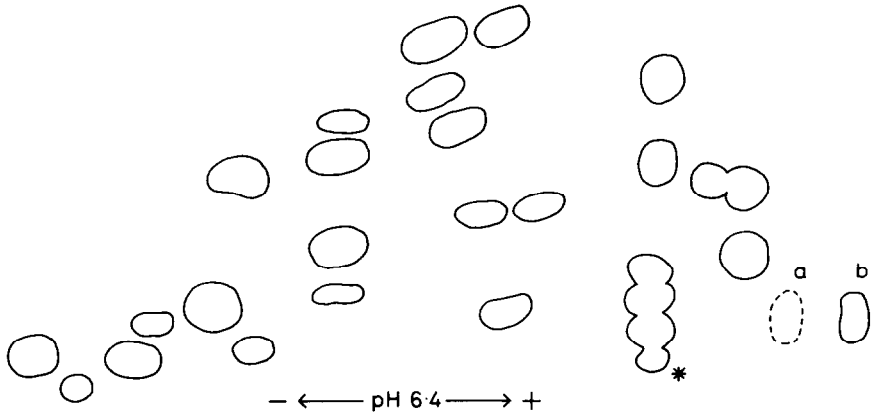

Fig.1. 'Fingerprint' of the tryptic peptides of the AEβ chain from Hb Volga. Electrophoresis at pH 6.4 (60 V/cm, 1 h); ascending chromatography in the upper phase of pyridine–isoamyl alcohol–water (6:6:7, by vol.) for 22 h. Peptides were located by staining with fluorescamine as described in the methods section. (*) Origin; (a) Position of β^A Tp III; (b) β^{Volga} Tp III.

Table 2
Haemoglobin Volga
amino acid composition of βTp III (β18–30)
from the isopropanol precipitate

Amino acid	Molar ratio	Expected for β^A Tp III
Asp	2.9	2
Glu	2.0	2
Gly	2.8	3
Ala	0	1
Val	3.3	3
Leu	1.0	1
Arg	1.0	1

sis. In view, however, of the increased negative charge of the abnormal haemoglobin chains in concentrated urea at pH 8.6 and pH 6.7, and of the increased anodal mobility of the variant βTp III at pH 6.4, it seemed likely that the substitution was Ala → Asp. This was confirmed by calculating the net charge on the abnormal thermolysin peptide β23-27 (see fig.3); it is −1.64 at pH 6.4, whereas the net charge of β^A 23–27 is −0.81 at pH 6.4. It follows that the substitution at position β27 (B9) is Ala → Asp. There is no reason to suppose that the substitution is Ala → Asn and that the resultant Asn–Leu (β27–28) sequence has undergone subsequent deamidation (as is often observed with Asn–Gly sequences [17]). No haemoglobin with the substitution β27 Ala → Asp has been described before, and in view of the patient's origin it was decided to name the new variant Hb Volga.

4. Discussion

The clinical and haematological picture of the patient is typical of a severe unstable haemoglobin haemolytic anaemia. The severity can be directly related to the instability of Hb Volga, which was observed to precipitate spontaneously from a haemolysate at

Residue No.	18	19	20	21	22	23	24	25	26	27	28	29	30
Helical No.	A15	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
Residues													
Hb A	Val	Asn↓Val		Asp	Glu↓Val		Gly	Gly	Glu	Ala↓Leu		Gly	Arg
Hb Volga	Val	Asn↓Val		Asp	Glu↓Val		Gly	Gly	Glu	Asp↓Leu		Gly	Arg

The arrows indicate the positions of hydrolysis by thermolysin.

Fig.2. Amino acid sequence of the tryptic peptide III of the β-chain of normal haemoglobin and Hb Volga.

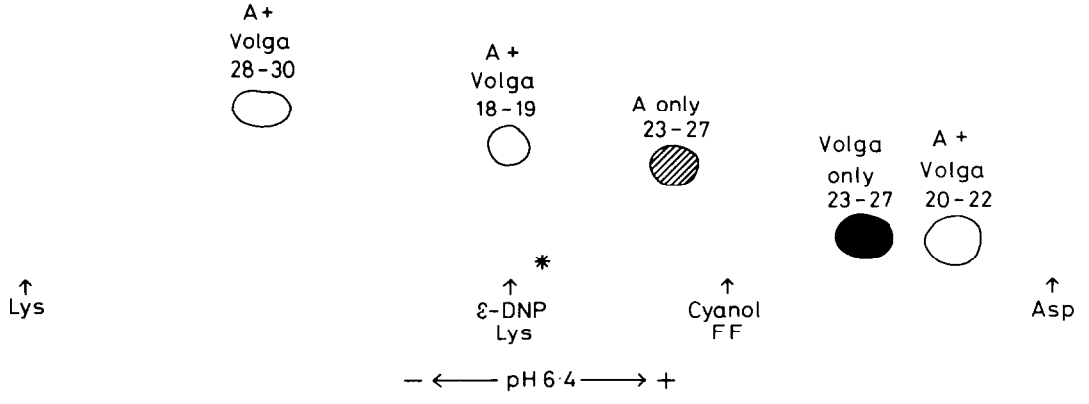

Fig.3. 'Fingerprint' of the thermolysin peptides of β^A Tp III and β^{Volga} Tp III prepared as described previously [15]. (*) Origin. The positions occupied by the electrophoresis markers are indicated at the bottom; the mobility of the blue cyanol FF dye is 0.38 of that of aspartic acid at pH 6.4 [19].

4°C. Since both this precipitate and the isopropanol precipitate were red, it seems unlikely that Hb Volga undergoes a significant loss of haem groups from the abnormal chains, particularly as the isopropanol precipitate consisted almost exclusively of the abnormal β-chains. It appears that Hb Volga is the result of a spontaneous mutation, a finding which is becoming increasingly common to the highly unstable haemoglobins.

Hb Volga represents the first known mutation at position B9 in any human globin chain. B9 is occupied by alanine in most of the known haemoglobin chains, although it can be occupied by threonine (as, for example, in the human γ-chain [18]; it is occupied by valine or isoleucine in all known vertebrate myoglobins. Dr M. F. Perutz showed us on his model of the haemoglobin molecule that the methyl group side-chain of Ala β(B9) normally points away from the β-haem group towards a very hydrophobic internal region comprising Val B5, Leu E12, Leu G12, Val G15 and Leu G16 of same β-chain. Since the variant does not separate from Hb A, it seems likely that the charge on the aspartyl side-chain in Hb Volga is suppressed whilst it is in position within the tetramer. However, the introduction of a polar group into such a hydrophobic region cannot be accommodated easily, and as there is no way in which the new side-chain can be extruded to the surface of the molecule without causing gross molecular disturbance, the substitution must give rise to a highly unstable haemoglobin.

Although unstable haemoglobin variants are known in which a newly-introduced charge is internally compensated (as in Hb Wien [20] and Hb Tacoma [21]), Hb Volga is the first instance of an unstable haemoglobin in which the ionisation of a new charge is suppressed by virtue of its intramolecular environment.

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