Hb ALTDORF $\alpha_2\beta_2$ 135 (H13) Ala → Pro: A NEW ELECTROPHORETICALLY SILENT UNSTABLE HAEMOGLOBIN VARIANT FROM SWITZERLAND

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1. Introduction

An Italian family living at Altdorf in Central Switzerland was investigated because of the presence of a hereditary haemolytic anaemia. The anaemia was of a mild to moderate type with haemoglobin concentrations of 9.1–12.1 g per 100 ml and reticulocyte counts of 2–17%. It was detected in three generations, viz. in the father of the family (born in 1923), in his daughter (born in 1957), and in a grandchild (born in 1971) of his brother. Other relatives of the family living in Italy were also reported to suffer from haemolytic anaemia but have not yet been investigated by us. A heat-unstable haemoglobin fraction was found in the blood of all three carriers although no abnormality could be detected by electrophoresis. A detailed investigation of the blood of the daughter led to the identification of the new unstable haemoglobin, Hb Altdorf $\alpha_2\beta_2$ (H13) Ala → Pro.

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2. Materials and methods

2.1. *Identification of the haemoglobin*

Haemolysates prepared from the patient's blood using CCl_4 were analysed by electrophoresis on paper or starch gel using Tris–EDTA–borate buffer pH 8.9 (50.4:5.0:3.8 g per litre) [1,2]. The dissociated globin chains were analysed by electrophoresis on cellulose acetate in 6 M urea–0.02 M phosphate pH 6.8 [3]. Instability tests were performed by incubation in 0.05 M Tris pH 7.4 [4,5] or in isopropanol–0.1 M Tris pH 7.4 (17:83 v/v) [6]. The 'isopropanol precipitate' was washed with 0.1 M Tris-HCl pH 7.4, redissolved in 0.1 N HCl and converted into globin by precipitation with 1.5% (v/v) conc. HCl in acetone. The dried globin (57 mg) was dissolved in a mixture of 1 M Tris (6 ml) and urea (3 g) adjusted to pH 9.6 with HCl, and β-mercaptoethanol (3 ml) was added. After incubation for 2 h the solution was gel-filtered on a column of Sephadex G-25 (coarse) in 0.5% (v/v) acetic acid and the pooled fractions containing the protein were freeze-dried. This treatment removed most of the dark brown material associated with the isopropanol precipitate. The recovered protein was separated into α- and β-chains by chromatography on CM-cellulose in 8 M urea with a linear gradient from 0.05 M to

0.3 M Na_2HPO_4 pH 6.7 [7] and the isolated β-chains were amino-ethylated. Fingerprints of tryptic digests of the α- and AE β-chains were prepared by standard techniques and stained with specific reagents for histidine, tyrosine, arginine, tryptophan and sulphur-containing amino acids [2]. Peptides were isolated for amino acid analysis by preparative finger-printing [8] and hydrolysed with 6 N HCl in sealed evacuated tubes at 108°C for 24 or 48 h. Sequencing of the abnormal peptide (β Tp XIV) was performed by combined Edman degradation plus 'dansylation' [9], the dansyl amino acids were identified by two-dimensional chromatography on polyamide layers [10].

2.2. *Oxygen affinity studies on the haemolysate*

Haemolysates were prepared both from the daughter's blood and from a normal control by lysis of the cells with 5 vol. 0.01 M phosphate buffer pH 6.8 at 4°C for 1 h, followed by centrifugation at 35 000 *g* for 20 min to remove the stroma. The use of CCl_4 was avoided to prevent possible precipitation of the unstable haemoglobin. Oxygen dissociation curves were measured by the method of Imai [11] using solutions containing approximately 0.1% haemoglobin in 0.2 M potassium phosphate pH 7.4 at 25°C.

3. Results

Starch gel electrophoresis of the haemolysates revealed the presence of a major component with the mobility of Hb A, Hb A_2, and a trace of material with the mobility of free α-chains. Hb F was also detected by alkali denaturation (2). In the daughter's blood the proportions of Hb A_2 and Hb F were 2.7% and 0.6% respectively. Although no haemoglobin component with an abnormal electrophoretic mobility was detected, incubation of the haemolysates in 0.05 M Tris-HCl pH 7.4 at 50°C or in isopropanol–Tris at 37°C produced red precipitates of unstable haemoglobin. Analysis of the globin from the precipitate by electrophoresis in 6 M urea–0.02 M phosphate pH 6.8 revealed α- and β-chains of normal electrophoretic mobilities, however, the β band was considerably more intense than the α band.

Fingerprints of tryptic digests of the α- and AE-β-chains isolated from the 'isopropanol precipitate' did not differ significantly in appearance from normal controls. Amino acid analysis revealed that all the tryptic peptides of the AE-β-chain had normal compositions except for β Tp XIV (residue 133–144) which contained less alanine than normal and a residue of proline which is normally absent from this peptide (table 1). These findings indicated that the haemolysate contained an unstable haemoglobin which differed from Hb A by the presence of an abnormal β Tp XIV with an Ala → Pro substitution at one of the positions β135, 138, 140 or 142 (fig.1). The substitution was located at position β135 by dansyl-Edman degradation.

Table 1
Amino acid composition of the abnormal β Tp XIV of Hb Altdorf

Amino acid	Yield (nmoles)	Molar ratios
Asp	27.8	1.00 (1)
Pro	21.4	0.77 (0)
Gly	29.3	1.05 (1)
Ala	92.0	3.29 (4)
Val	84.8	3.04 (3)
Leu	27.7	1.00 (1)
His	24.3	0.87 (1)
Lys	27.9	1.00 (1)

Expected molar ratios for normal β Tp XIV in parentheses

The Ala → Pro substitution is electrically neutral and causes no changes in the electrophoretic mobility of the abnormal haemoglobin or of its isolated β-chain. Furthermore, the normal and abnormal forms of peptide β Tp XIV have the same chromatographic mobility in the solvent system employed (pyridine–amyl alcohol–water 6:6:7 by vol), and consequently the fingerprint of the abnormal β-chain appeared to be normal. Amino acid analysis of β Tp XIV of the mixed β-chains isolated from a whole haemolysate indicated that the abnormal form containing proline comprised about 35% of the total β-chains.

Val–Val–Pro–Gly–Val–Ala–Asn–Ala–Leu–Ala–His–Lys
133 134 135 136 137 138 139 140 141 142 143 144

Fig.1. Amino acid sequence of β Tp XIV of Hb Altdorf

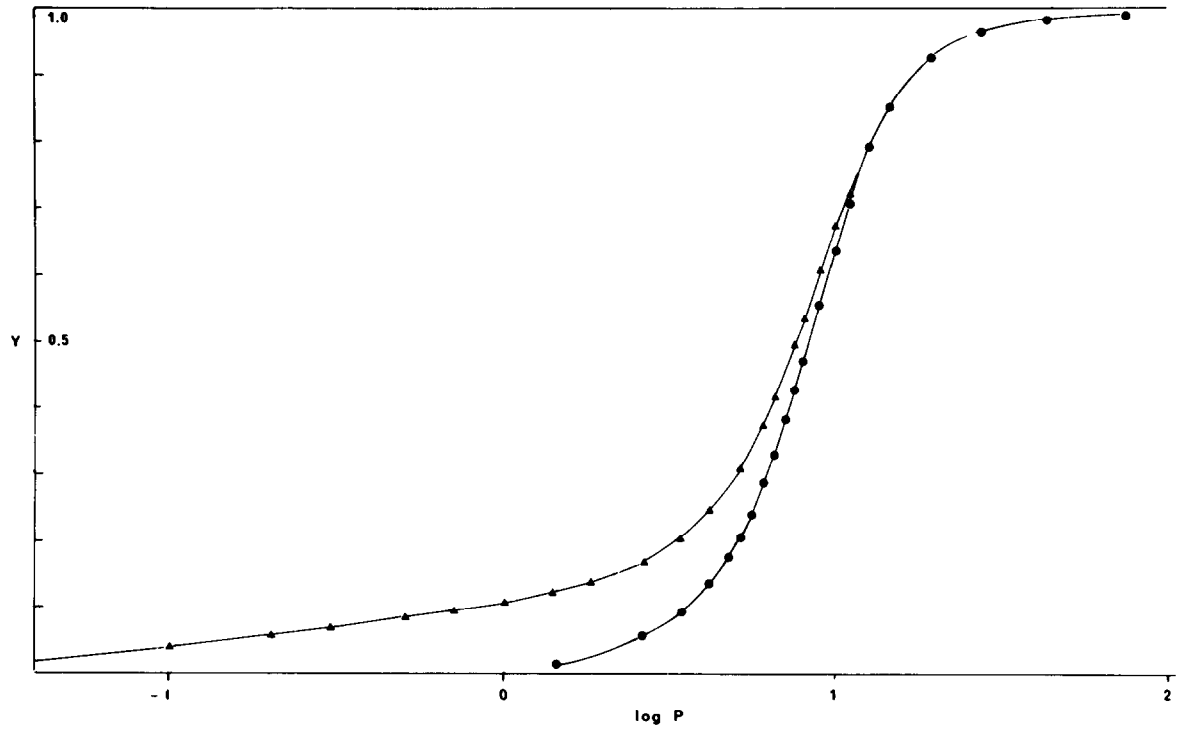

Fig.2. Oxygen dissociation curves of haemolysates from the patient (▲) and a normal control (●) in 0.2 M potassium phosphate buffer pH 7.4 at 25°C.

Fig.2 shows the oxygen dissociation curves of haemolysates from the daughter and a normal control in 0.2 M potassium phosphate pH 7.4. The curves were almost superimposable above about 80% oxygenation but below this the abnormal haemolysate showed a higher oxygen affinity. Numerical analysis of the data indicated that the abnormal curve could be best represented by a mixture of two non-interacting components: 11.3% of the abnormal haemoglobin (Hb Altdorf) with $n = 1.0$ and $\log P_{50} = 0.71$ ($P_{50} = 0.19$ mm Hg) and the remainder normal haemoglobin with n = 2.9 and $\log P_{50} = 0.9$ ($P_{50} = 7.94$ mm Hg). Hb Altdorf thus behaves as a non-cooperative haemoglobin with a high oxygen affinity comparable to that of myoglobin or monomeric chains.

4. Discussion

Hb Altdorf is the third example of an unstable haemoglobin with an Ala → Pro substitution, the others being Hb Duarte [12] and Hb Madrid [13]. As a general rule, proline can only be incorporated into an α-helix at the first or last three positions. Introduction of proline into internal positions causes disruption of the helix and leads to instability of the haemoglobin. In Hb Altdorf the substitution occurs approximately two thirds of the way along the H helix. This is likely to weaken the haem contacts of residues β137 (H15) Val and β14 (H19) Leu [14] thus conceivably causing loss of haem groups from the β-chains, as has been observed with other unstable haemoglobins such as Hb Köln [15–18] and Hb Santa Ana [17]. There is some experimental evidence that Hb Altdorf is deficient in haem. Thus the proportion of abnormal globin, estimated by amino acid analysis, is 35% while the proportion of abnormal haemoglobin, estimated from the oxygen affinity data, is only about 11%. This indicates that Hb Altdorf may be more than 50% deficient in haem.

In Hb Köln and Hb Santa Ana the loss of haem groups is associated with a reduction of the

electrophoretic mobility of the variants relative to Hb A though the amino acid substitutions are electrically neutral [19]. Moreover, the mobility is restored to normal in the presence of added haem [15]. This does not seem to be the case for Hb Altdorf which does not separate from Hb A on electrophoresis at pH 8.6. The loss of the haem groups from the β-chains could account for the high oxygen affinity of Hb Altdorf which would then behave as an intermediary compound [20] c.f. Hb Köln [16,18]. Other factors, in addition to the loss of the haem groups, may also inhibit the formation of a stable quaternary deoxy structure (T-state). For example, reorientation of the side chains of all the amino acids beyond position β135 could prevent both the proper movement of the penultimate tyrosine β145 residues into their internal pockets, and the formation of the salt bridges at the C-terminus of the β-chains which help to stabilize the deoxy-structure [21].

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