Structural study of hemoglobin Hazebrouck, β38(C4)Thr⟶Pro

A new abnormal hemoglobin with instability and low oxygen affinity

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A new β-variant has been detected and structurally defined in a French male, with a life-long history of hemolytic anemia. This variant is moderately unstable and has a low oxygen affinity. The abnormal hemoglobin was not detected by standard electrophoretic procedures. It moved slightly slower than Hb A during isoelectric focusing (IEF). Two minor fractions were also seen; the first migrated just cathodal to Hb F, as did partially oxidized Hb A or hemichrome derivatives of some unstable hemoglobins; the second in the position of free α-chains. The abnormal β-chain was readily separated from both β_A- and α_A-chains by acid–urea–Triton globin chain electrophoresis. Structural study was conducted simultaneously by fingerprinting and high-performance liquid chromatography (HPLC) of tryptic peptides. A new mutation β38(C4)Thr⟶Pro was found, which was named Hb Hazebrouck.

Hemoglobinopathy *β-variant* *Molecular instability* *Low oxygen affinity* *HPLC* *Structural analysis*

1. INTRODUCTION

Molecular instability in the different variants of human adult hemoglobin has been documented in about 65 cases. Among these, about 42 involved the β-chain. The perturbation of oxygen affinity in these variants is frequent, but a low affinity has seldom been described. The most studied of these variants are Hb Hammersmith β42(CD1)Phe⟶Ser [1], Hb Louisville β42(CD1)Phe⟶Leu (2) and Hb Niteroi β43–45 deleted [3]. The modified residues alter both the $\alpha_1\beta_2$ contacts and the heme pocket. In such cases, anemia is determined both by hemolysis and low oxygen affinity. Here we report, for the first time a β-variant, Hb Hazebrouck $\alpha_2\beta_2$ 38(C4)Thr⟶Pro.

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2. MATERIALS AND METHODS

Standard hematological procedures were followed. Heinz body staining was performed with Cresyl brilliant blue (1%) after 3 h sterile incubation at 37°C. Freshly prepared hemolysates were subjected to the 4 electrophoretic systems as proposed in [4]. Electrophoresis at alkaline pH (Tris–EDTA–borate buffer) was performed on cellulose acetate strips (Helena Titan III H), citrate agar electrophoresis (pH 6.2) on agar from Helena (Titan IV), and globin chain electrophoresis in 6 M urea, 1% β-mercaptoethanol (pH 6 and 9) on cellulose acetate strips (Sepraphore X Gelman). Isoelectric focusing (IEF) was carried out on a thin-layer polyacrylamide gel (pH 6–9) as in [5]. Densitometry of IEF slabs was done with a Cellosystem Sebia densitometer (France). Globin chain analysis on acid–urea–Triton acrylamide gel was performed as in [6]. Quantification of Hb F

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was as in [7] and the Hb A_2 level was measured chromatographically as in [8]. Glycosylated Hb (Hb A_{1c}) was estimated by an automatic apparatus using Biorex 70 chromatography as in [9]. Stability was tested by the isopropanol test as in [10]. The methemoglobin fraction in the fresh hemolysate was spectrophotometrically evaluated with a Cary 118 C instrument. The oxygen affinity of the whole blood was determined with an Aminco Hemoscan (Silver Springs, USA) and the 2–3 DPG assay was performed as in [11]. The abnormal β-chain was precipitated during 30-min incubation with *p*-mercuribenzoate (pMB) as in [12]. Purity of the abnormal Hb after precipitation was controlled by acid–urea–Triton electrophoresis and then subjected to desheminisation by acid–acetone precipitation, separated by CM–cellulose chromatography [13], aminoethylated, and stripped of urea on a Biogel P_2 (Bio-Rad) column. Two methods were used for the analysis of tryptic peptides: (i) fingerprinting on silica gel thin-layer plates [14] and specific staining for histidine, tyrosine, tryptophan, arginine and methionine [15]; (ii) HPLC using a Beckman model 343 with an Altex wavelength detector set at 214 nm. The column system was a Waters μBondapack C-18 (10 μm, ID 3.9 mm, 30 cm). The solvent system was based on [16]: solvent A, 0.02 M ammonium acetate (pH 5.7); solvent B, 0.01 M ammonium acetate (pH 5.7) mixed 50/50 with acetonitrile (Baker, v/v). The gradient was linear from 0 to 60% in 50 min and from 60 to 100% in 10 min. Amino acid composition was determined on a Biotronik 6000 1E (Biotronik, Munich) after 40 h hydrochloric acid hydrolysis of separated peptides.

3. RESULTS

3.1. *Case report*

The patient was a 30-year-old French male whose family comes from the north of France near Hazebrouck. Other carriers of the trait were detected in the family. The propositus had well tolerated hemolytic anemia with slight cyanosis. Typical red cell indices were: Hb, 10.8 g/dl; RBC, 3.56 10^{12}/l; PCV, 29.5%; MCV, 83; reticulocytes, 300 $\times$ 10^9/l. Numerous Heinz bodies were seen after 3 h incubation with Cresyl brilliant blue. The abnormal hemoglobin was undetectable by the 4 standard electrophoreses. IEF showed 3 abnormal

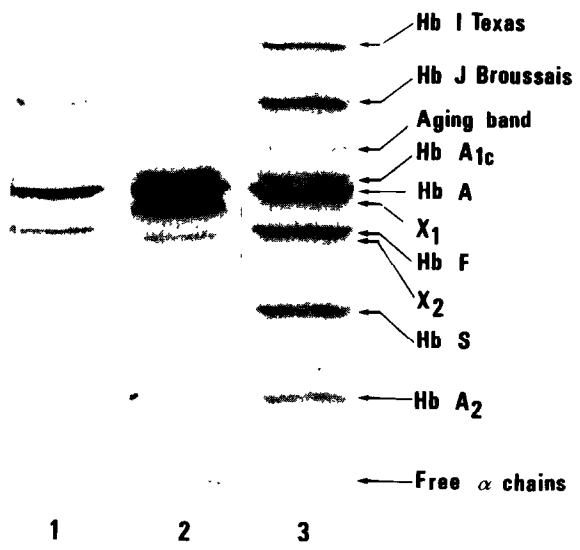

Fig.1. IEF in a pH 6–9 gradient of the propositus hemolysate (2). The main fraction is X(1) and the minor one X(2), X, abnormal hemoglobin Hazebrouck. 1 and 3, mixed controls.

fractions migrating cathodically to Hb A (fig.1). The main band was very close to Hb A, amounting to 31% as evaluated by densitometry. The second was slightly slower than Hb F and amounted to 2.5–5% on successive IEF. A faint hemoglobin fraction in the position of α-chains was regularly seen. An abnormal β-chain was visualized by acid–urea–Triton electrophoresis (fig.2). Hb A_2 was 3.64% (N = 2.1 $\longrightarrow$ 3.1) and Hb F 0.42% ($N \leq 1$). The elution pattern from Biorex 70 chromatography had no distinctive feature and Hb

Fig.2. Acid–urea–Triton electrophoresis pattern of Hb Hazebrouck whole hemolysate (2) and of the precipitated total Hazebrouck. Globin (1) compared to an Hb A control (3).

Table 1

Amino acid composition of the abnormal BT_4 obtained by HPLC

	$\beta^A T_4$ expected	Hazebrouck βT4 HPLC
Thr	1	–
Glu	1	1.1
Pro	1	1.8
Val	1	1.1
Leu	2	2.1
Tyr	1	0.9
Arg	1	1.0

A_{1c} was lower than normal: 2.8% (N = 4.6 ⟶ 6.2). The isopropanol test was positive after 3 min. The whole blood P_{50} was 36 mmHg (N = 27 ⟶ 29) and 2–3 DPG 18.6 μM/g of total Hb (N = 13 ⟶ 17). The level of ferrihemoglobin in the fresh hemolysate was below 1%.

3.2. *Structural studies*

Fingerprints of the tryptic hydrolysate of the isolated abnormal β-chain prepared from pCMB-precipitated total globin showed normal spots. Specific staining for histidine, tyrosine, tryptophan, arginine and methionine was normal. Then, each individual spot was analysed for its amino acid composition. All had the expected composition, except βT4 were the threonine normally present was absent and replaced by an additional proline.

An HPLC trace of the same tryptic hydrolysate showed a normal pattern. All the isolated peptides had the expected amino acid composition, except the peak in the place of β^AT4 where the threonine residue was missing, being replaced by one proline (table 1).

4. DISCUSSION

Hb Hazebrouck is the first occurrence of Hb mutation with the Thr⟶Pro substitution. This substitution is allowed by the genetic coda and the published sequence of the human β-gene [17] Thr-β38:ACC⟶Pro:CCC. The substitution β38 Thr⟶Pro was demonstrated by amino acid analysis of the abnormal βT4 and this new variant was named Hb Hazebrouck according to the origin. This unstable hemoglobin produces discrete, compensated hemolytic anemia due to the effect of the low oxygen affinity. In our patient, the P_{50} of whole blood is higher than expected for the observed level of 2–3 DPG.

The presence of this abnormal unstable hemoglobin was easily detectable by the standard methods of detection of unstable Hbs: presence of Heinz bodies, stability test, globin chain electrophoresis in the presence of urea–Triton and by IEF of the hemolysates. This method was able to separate two minor components: the first with a pI close to that of Hb F and different from the hemichrome observed in Hb St Louis which migrated close to the valency hybrid IB_{II} [18]; the second with the IEF pattern of free β-chains frequently associated with unstable β-variants.

Further studies are in progress to investigate the molecular mechanisms of instability and low oxygen affinity determined by this point mutation introducing a proline in the C helix in contact with the heme pocket. One must consider that it is the first example of substitution in Thr (C4) which is an invariant residue, in contact with heme and located between residues β37(C3)Trp and β40(C6)Arg, which are greatly involved in $\alpha_1\beta_2$ contacts. Considering the model of deoxyhemoglobin described in [19], the substitution β38 Thr⟶Pro occurs at the $\alpha_1\beta_2$ contact, which forms the two-way switch between the T and R quaternary structures. Pro in position 38 fixes the switch in the T structure. Subunit dissociation will be studied to compare what will occur with the close variants Hb Rotschild β37 Trp⟶Arg [20], Hb Hirose β37 Trp⟶Ser [21] and Hb Philly β35 Tyr⟶Phe [22].

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