HEMOGLOBIN Ty GARD ($\alpha_2^A\beta_2$ 124 (H_2) Pro → Gln)

A stable high O_2 affinity variant at the $\alpha_1\beta_1$ contact

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1. Introduction

In normal HbA the $\alpha_1\beta_1$ contact involves 32 amino acid residues equally shared by the α and the β chains [1]. Among these contacts only 5 or 6 include a hydrogen bond, the remaining bonds being due to van der Waals forces [1]. The similarity of the structure of the $\alpha_1\beta_1$ contact in oxy and deoxy hemoglobin has been reported from X ray crystallography [2], it is in contrast to the wide differences observed at the $\alpha_1\beta_2$ contact between these two quaternary structures. Fourteen abnormal hemoglobins have so far been described with various substitutions at the $\alpha_1\beta_1$ interface [3]. Most of these variants were discovered in clinically symptom-free patients. In several cases signs of hemolytic anemia were present [5–8], due to instability of these abnormal hemoglobins. Only a few functional studies of these abnormal hemoglobins have been reported. The aim of this article is to report structural and some functional studies of a new high O_2 affinity mutant at the $\alpha_1\beta_1$ contact: Hb Ty Gard (Hb^{TG}): $\alpha_2^A\beta_2$ 124 (H_2) Pro → Gln, discovered in a polycythemic patient. It was silent by routine electrophoresis and isolated only by isofocusing electrophoresis.

2. Material and methods

Hematological studies were obtained by routine Coulter counter analysis.

Abbreviation: PMB, *p*-Hydroxymercuribenzoate Na salt (Sigma, St. Louis, MO)

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2.1. *Structural studies*

The structural abnormality was determined using the following techniques: globin preparation; chain separation and characterization; amino ethylation and tryptic hydrolysis; analytical and preparative finger prints; isolation and purification of large amounts of tryptic peptides by ion exchange column chromatography (Beckman M 72, Bio Rad AG 50 X 4 and AG 1 X 2 resins); amino acid composition of the resulting peptides. The sequence of the abnormal peptide was determined after manual Edman degradation. The methods are in [9]. PMB reacted chains were obtained as in [10].

2.2. *Functional studies*

Oxygen dissociation curves of washed fresh erythrocytes were obtained in isotonic phosphate buffer in physiological conditions of pH, PCO_2 at 37°C as in [11]. Functional studies of the purified component were done after its separation from HbA and other minor components by preparative isoelectrofocusing (LKB multiphor, Stockholm) of freshly prepared hemolysate. The abnormal Hb was further stripped on ion exchange resin to remove the ampholytes and other low molecular weight contaminants [12] and concentrated under nitrogen pressure (Aminco system – PM 10 membrane). O_2 binding curves were obtained on 0.2% Hb_4 (tetramer) solutions by the spectrophotometric equilibrium method in [13] in Tris or bis-Tris buffer at varying pH values and chloride concentrations at 25°C. Methemoglobin content of the purified component never exceeded 4% [14]. Heat denaturation and isopropanol stability tests were done as in [15].

Purified native HbA stored in liquid nitrogen was used as control. 2,3-Diphosphoglycerate (DPG) concentrations in the fresh red cells were measured as in [16].

3. Results

The propositus, a 45 year-old man, from the west centre of France reported to the laboratory because of the recent discovery of a high red cell count from routine blood examination (table 1). He was symptom free. White cell, platelet and reticulocyte counts were normal. No Heinz bodies could be detected in fresh erythrocytes. Physical examination did not reveal any cardio-pulmonary abnormality. The spleen was not enlarged. Blood uric acid was normal. Heat denaturation and isopropyl alcohol stability tests gave identical results relative to purified HbA. Electrophoresis on cellulose acetate strips at pH 8.6 of fresh stripped hemolysates was identical to that of normal adult hemolysates. However, oxygen dissociation curves of fresh erythrocytes of the propositus, which are part of our screening tests, showed a shift to the left of the curve: P_{50} pH 7.40, PCO_2 = 40 mm Hg, 37°C = 20.8 mm Hg compared to the normal adult value of 26.5 ± 0.6 mm Hg. The slope of the Hill plot was comparable to that of normal erythrocytes and did not indicate the presence of two components (fig.1, left panel). [DPG] of the propositus fresh erythrocytes was 0.875 mol/mol Hb_4 (Normal controls = 0.930 ± 0.070). Isofocusing electrophoresis in polyacrylamide gels containing 5% ampholytes (LKB, Stockholm) revealed two distinct major bands, the abnormal one migrating slightly ahead of HbA (pH_i 6.90 compared to 6.95 for HbA). Only one band of HbA_2 was observed indicating that the abnormal component was due to a β chain variant (fig.2).

3.1. *Structural characterization of the abnormal component*

The abnormal β PMB chains could be isolated from the α^A and β^A chains by starch block electrophoresis of the PMB-reacted whole hemolysate. βSH globin chains were obtained after adding a sufficient amount of β-mercaptoethanol to the usual acid–acetone solution. Finger prints of the tryptic peptides from the amino ethylated (AE) β chains were found identical to those obtained from AE β^A chains. Amino acid compositions of all tryptic peptides of the abnormal chains were identical to those of normal β^A chains except for βT 13. Amino acid content of this peptide (table 2) suggested that β 124 Pro or β 125 Pro were substituted by a Gl_x. Sequential analysis of βT 13 was done to assess the exact nature of the mutation. Sufficient amount of this peptide was prepared after successive ion exchange chromatographies. From the M 72 column the abnormal β T 13 eluted with the same volume of buffer as the β^A T 13. By chromatography on an AG X 2 column β^x T 13 was eluted after β^A T 13 and free of βT 5 and βT 3 contamination (fig.3). The results of the sequential analysis performed according to Edman indicated that the actual structure of the abnormal β chain was β_2 (H_2) 124 Pro → Gln.

3.2. *Functional studies of purified Hb Ty Gard*

The right panel of fig.1 shows that purified Hb^{TG} retained the high O_2 affinity as observed with the erythrocytes and normal cooperativity. Stripped

Table 1
Hematological data of the propositus and members of his family

Generation	Age (years)	Hb (g/dl)	RBC (× 10^{-12}/l)	PCV ratio	MCV (fl)	MCH (pg)	MCHC (g/dl)
I.1 (m)[a]	45	19.2	6.0	54.3	88	31.3	35.8
I.2 (f)	42	14.1	4.6	40.0	87	30.1	35.3
II.2 (f)	17	14.5	4.9	39.8	81	29.0	36.4
II.2 (f)[a]	11	14.7	4.8	43.0	89	30.3	34.0
II.3 (m)	6	13.5	5.0	38.0	75	26.3	35.9

[a] Carriers of Hb Ty Gard

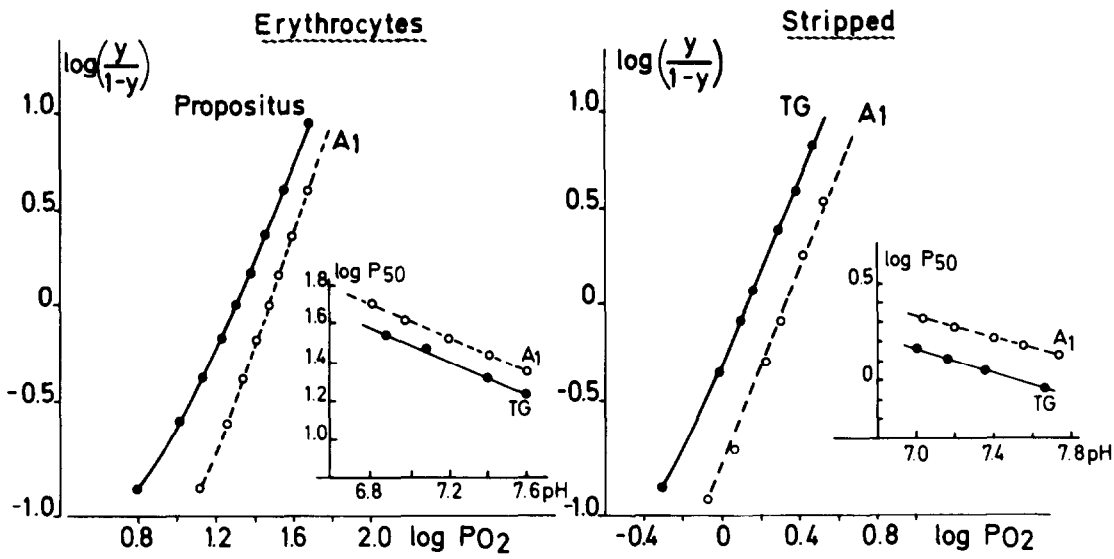

Fig.1: Hill plots of O_2 dissociation curves. Left panel: on fresh erythrocytes, in isotonic phosphate bicarbonate buffer at pH 7.40, PCO_2 = 40 mm Hg and 37°C. Right panel: on purified Hb solutions. Conditions: bis-Tris 0.02 M; [NaCl] 0.005 m; [EDTA] 10^{-6} M; pH 7.0; T 25°C; [Hb_4] 3×10^{-5} M. The inserts show the pH dependence of $\log P_{50}$ in the two conditions.

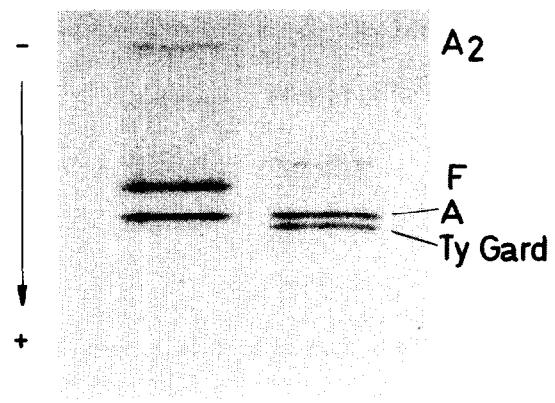

Fig.2. Isofocusing electrophoresis in polyacrylamide gels. Left: from top to bottom HbA_2, HbF_{II} and HbA. Right: hemolysate of the propositus with Hb Ty Gard.

Table 2
Amino acid composition of peptide β T 13 in Hb^{TG} and HbA

Residue	Hb^{TG} (Found)	HbA (Exp.)
Lys	0.80	1.0
Thr	0.91	1.0
Glu	3.6	3.0
Pro	1.16	2.0
Ala	2.17	2.0
Val	0.80	1.0
Tyr	1.05	1.0
Phe	1.00	~1.0

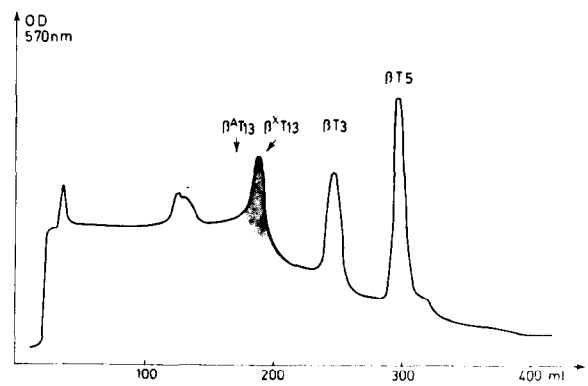

Fig.3. Elution profile of a chromatogram of the β Ty Gard AE chain tryptic peptides on an AG 1 X 2 resin column. The sample was the first peak eluted from a M 72 resin Beckman column chromatogram.

Table 3
Oxygen binding parameters measured on purified Hb^{TG} and HbA

Additions[a]	Hb Ty Gard		HbA_1		
	$\log P_{50}$	n	$\log P_{50}$	n	$\Delta (\log P_{50}A_1 - \log P_{50}HbTG)$
(1) Stripped	0.178	2.50	0.337	2.80	0.159
(2) + NaCl 0.15 M	0.782	2.70	0.969	2.90	0.187
(3) + DPG	1.176	2.81	1.34	2.90	0.164
(4) + NaCl 0.15 M + DPG	1.042	2.70	1.305	2.87	0.263

[a] Conditions: (1) bis-Tris 0.02 M, [NaCl] 0.005 M; (2) bis-Tris 0.05 M, [NaCl] 0.150 M; (3) as in (1) plus [DPG Na_2] 5 mol/mol Hb_4; (4) as in (2) plus [DPG] 5 mol/mol Hb_4. In all instances [EDTA] 10^{-6} M, [Hb_4] 3×10^{-5} M (tetramer), pH 7.0, T 25°C

hemoglobins A and TG had identical alkaline Bohr effects ($\Delta \log P_{50}/\Delta pH = -0.28$). Table 3 shows comparative values for $\log P_{50}$, Hill coefficient n, of both hemoglobins and their interactions with anions, at pH 7.0. The $\Delta \log P_{50}$ column indicates that Hb^{TG} retained its high O_2 affinity relative to HbA whether in the presence or absence of chloride or DPG. This demonstrates also normal interactions of Hb^{TG} with anions. The larger $\Delta \log P_{50}$ observed in the presence of both DPG and chloride may indicate however a lower DPG binding to Hb^{TG} in these conditions.

4. Discussion

Hb^{TG} was discovered in a symptom free patients and in one of his daughters, the second member of the family carrying the abnormal Hb. The amount of the abnormal component in the erythrocytes was estimated to approx. 40%. This is a new case of an electrophoretically silent Hb variant uncovered by both recording the O_2 binding curve and isoelectrofocusing. Its main interest comes from both the location of the abnormality and from its functional properties. The substitution of Gln for Pro at the β 124 (H_2) is interesting as this residue is invariant in all the human α and non-α chains which have been sequenced except in embryonic ζ chains where it is replaced by Ile [17]. Only one other mutation has been described for this residue, Hb Khartoum or $\alpha_2\beta_2$ 124 (H_2) Pro → Asp but unfortunately without functional studies [5]. Despite the substitution of a Pro at the $\alpha_1\beta_1$ contact Hb^{TG} is not unstable and it is noticeable that its physiological consequence is an increase of the erythrocyte mass which is at variance with two other $\alpha_1\beta_1$ Hb variants, Hb Tarrant [18] and Hb Philly [4] where the increased O_2 affinity is accompanied by hemolytic anemia. Hb^{TG} is also unique among abnormal Hb at the $\alpha_1\beta_1$ contact in that it has a moderately increased O_2 affinity (P_{50} Hb^{TG}/P_{50} HbA = 0.7), normal cooperativity, normal alkaline Bohr effect and almost normal interactions with anionic effectors. Such features are not usually described in Hb variants at the $\alpha_1\beta_1$ contact but have been reported in a large variety of Hb variants either α mutants such as Hb Broussais [19], Fort de France [20], Sawara [21] or β mutants such as Hb Brigham [22], Little Rock [23], Hsi-Tsou or Szuhu [24]. Any molecular explanation for the high O_2 affinity of stripped Hb^{TG} will demand further studies. One may postulate that due to a change in polarity of Gln relative to Pro and/or of its charge, the substitution may weaken the contact between the H helix and the opposing β helix of the α chain, thus favouring a more R-like structure by narrowing the central cavity between the two β chains. This is suggested by the difference in DPG binding to Hb^{TG} in the presence of chloride.

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