Structural study of hemoglobin Knossos, β27 (B9) Ala→Ser

A new abnormal hemoglobin present as a silent β-thalassemia

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Received 6 September 1982

A new electrophoretically silent hemoglobin variant is described that produces the classical phenotype of β thalassemic intermedia in association with β° thalassemia trait. This variant has the expression of a silent β thalassemia trait. The abnormal hemoglobin was detected by acid–urea–Triton–acrylamide electrophoresis and further demonstrated by isoelectric focusing. The amount of the variant in carrier is ~30% of the total hemoglobin. No instability was found. Absence of hemoglobin A in the propositus blood facilitated structural studies. Peptides maps were normal but analysis of individual peptide spots showed an Ala→Ser substitution in the βT3. This variant has been previously called Hb Knossos (β27 (B9) Ala→Ser).

Urea–Triton–acrylamide electrophoresis *β Thalassemia intermedia* *Neutral substitution* *Silent β thallasemia trait*

1. INTRODUCTION

This paper describes the structure of Hb Knossos, β27 (B9) Ala→Ser. This new abnormal hemoglobin was detected in a large Greek family in which 3 members, belonging to 2 branches of the third generation, had a syndrome of thalassemia intermedia [1]. In each branch, one parent is a typical high-A_2 β-thalassemia carrier while the other was considered as a silent carrier since the only detectable feature was a reduced β/α ratio among the specific criteria of β-thalassemia. The presence of the abnormal hemoglobin in the intermediates and in the silent carriers remained unknown for a long time since its behaviour was that of Hb A on the standard electrophoretic methods and because it was not unstable. Unpredictably, the abnormal hemoglobin was detected upon Triton/urea chain-electrophoresis performed to determine the $^{A}\gamma$, $^{G}\gamma$ ratio in Hb F. By this method an abnormal band migrating between the α and β chains was present. This result prompted a re-examination of the case.

2. MATERIALS AND METHODS

Electrophoretic studies were performed on cellulose acetate strips (Sebia, France) with Tris, EDTA, borate buffer (pH 8.6) and on agar gel with citrate buffer (pH 6.2). Isoelectric focusing (IEF) was done on thin-layer polyacrylamide gel in a pH 6–9 gradient as in [2]. Globin chain separation on Triton/urea acrylamide gel was done as in [3].

After desheminization by acid acetone precipitation of the hemolysate from one intermediate proband, the chains were separated according to [4] and freed of urea on a Biogel P_2 (Bio-Rad) column. Two methods were used for the separation of the peptides. In the first, the tryptic peptides of the β-aminoethylated chain were separated by fingerprint on silica-gel, thin-layer plates [5], eluted and submitted to hydrochloric acid hydrolysis. In the second, the tryptic peptides were isolated by column chromatography on Aminex A_5 as in [6]. The abnormal βT3 was purified on the same column

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Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

but using a modified gradient (buffer 1 = pyridine acetic, 0.05 M, pH 2.5; buffer 2 = 0.2 M, pH 3.1). The amino acid composition was determined on a Biotronik 6 000 1 E. (Biotronik, Munich).

3. RESULTS

3.1. *Case report*

The results of the clinical, hematological and genetical data have been presented in [1]. The hemoglobin patterns exhibited by the different members of this family were those normally found in β-thalassemia: modifications of the Hb A_2 and Hb F levels and absence of abnormal hemoglobin detectable by cellulose acetate and agar electrophoresis or by stability test. The abnormal chain detected by Triton/urea electrophoresis [1] was slightly more cathodal than β^A and amounted to 75% and 33% of the non-α-chains in the proband and in the atypical parent globins, respectively.

3.2. *Structural studies*

Isoelectric focusing of the hemolysate from the proband revealed (fig.1), in addition to 10% of Hb F the presence of an abnormal band more cathodal but very close to the Hb A position. This abnormal hemoglobin showed a pI higher than those of some controls migrating on IEF close to Hb A, some of them being electrophoretically silent variants: Hb Pitié Salpêtrière, Hb Spanish Town and Hb M Saskatoon (fig.1). The pI of Hb Knossos is very similar to that of Hb Saki and of heminized Hb Köln. No band of Hb A was distinguishable in the samples of the probands, contrasting with the presence on Triton/urea gel of a small band migrating as β^A chain [1]. Though the presence of Hb Knossos was clearly detectable upon IEF in the sample of the carrier parents, the overlapping between the bands of Hb Knossos and Hb A did not allow a precise estimation of their respective amounts by gel densitometry.

The structural study was performed on the globin of one of the probands without prior Hb purification, since no traces of Hb A were detected by IEF. The profile of the chain pattern obtained on CMC urea column chromatography exhibited in addition to the expected peaks of γ, δ and α chains, a peak of a chain which eluted with the same volume of buffer as a peak of β^A. On Triton/urea electrophoresis, this β-like chain was confirmed to be the abnormal component detected in the hemolysate. The fingerprint patterns of the tryptic peptides of this aminoethylated chain were identical to those of control aminoethylated β^A chain after ninhydrin staining and specific coloration for histidine, tyrosine, tryptophan, arginine and methionine. The amino acid composition of all the peptides was normal except for the βT3 where the single alanine residue was missing and a serine was present. Since peptides eluted from silica-gel thin layers may be occasionally contaminated by serine, we performed a new separation of βT3 by an another method. By chromatog-

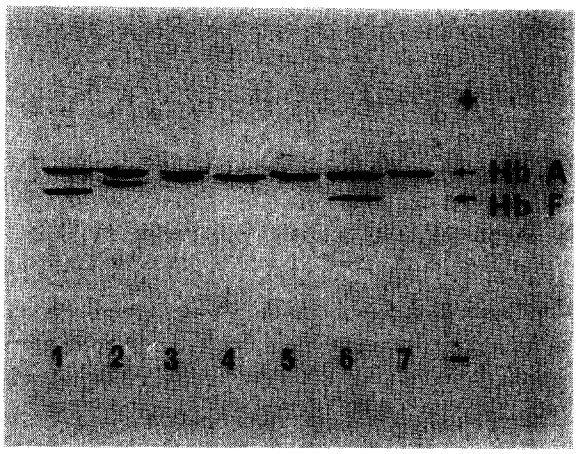

Fig.1. Isoelectric focusing in a pH 6–9 gradient: (1) hemolysate Pitié Salpêtrière; (2) hemolysate Spanish Town; (3) hemolysate M Saskatoon; (4) Hb Saki; (5) Hb Köln; (6) hemolysate Knossos; (7) control Hb A.

Table 1

Amino acid composition of peptide βT3 from Hb Knossos compared to that of normal βT3

Residue	βT3 Knossos (Molar ratio)	βT3 normal (Molar ratio)
Ac. Asp	2.04	2
Ser	0.96	–
Ac. Glu	2.10	2
Gly	3.15	3
Ala	0	1
Val	2.80	3
Leu	0.98	1
Arg	0.95	1

raphy on a column of Aminex A5 β^{Kn}T3 was eluted as a β^{A}T3 in the first peak which also contains βT13 and βT5. $\beta T^{Kn}3$ was repurified on the same column by using a more acidic gradient. Amino acid analysis of the peptide (table 1) showed it to have the same composition as that of $\beta^{A}3$ except for the replacement of the alanine residue by serine. Since peptide $\beta^{A}3$ contains only one alanine residue in position 27 the structure of Hb Knossos is $\alpha_2{}^{A}\beta$ 27 Ala→Ser (B9).

4. DISCUSSION

The alanine β27 is also replaced by an aspartic residue in Hb Volga [7], an unstable hemoglobin. In Hb St Louis $\alpha_2\beta_2$ 28 Leu→Gln the substitution of the residue B10 produces instability and hemichrome [8]. The Hbs with a mutation in β26 (Hb E $\alpha_2{}^{A}\beta_2$ Glu→Lys and Hb Henri Mondor $\alpha_2{}^{A}\beta_2$ Glu→Val [9]) produce mild hemolysis. It will be necessary to reinvestigate more carefully the physicochemical properties of Hb Knossos to define the exact role of the replacement of Ala 27 by a serine in this critical part of the B helix.

This report presents a new example of the value of using IEF for the detection of the silent variants, several cases of which, associated with a high oxygen affinity, have already been detected in our laboratory [10]. These findings suggest that this technique may also be useful for the detection of silent variants associated with β-thalassemia intermedia. In [1] β^{A} was thought to be present because a faint band was detected with the same mobility as β^{A} on Triton/urea globin-electrophoresis. The results obtained here by IEF of the intermedia hemolysate and by the amino acid analysis of β^{Kn}T3 indicate that Hb A is not present at a detectable level. The β^{A}-like band visible on Triton/urea electrophoresis could be due to a splitting of the β Knossos chain. Such an artefact is not exceptional with this method. Triton/urea electrophoresis will probably be also useful for the detection of silent variants and more specifically in cases of silent β-thalassemia. It is still difficult to explain the separation obtained with this method between β^{A} and β Knossos chains. Because replacement of an alanine in the $^{G}\gamma$ produces a difference of mobility of the same magnitude to that exhibited by β^{A} and β Knossos, the role of alanine could be hypothesized and it will be interesting to study the variants in which an alanine is replaced by another neutral amino acid residue.

The β Knossos mutation is presumably GGC to GGA at codon 27. Such a possibility, if confirmed, would permit a prenatal diagnosis using the restriction enzyme *Alu*I. This enzyme produces with the β^{A} gene the sequence 228–390 which should be absent from the hydrolysate of the Knossos DNA according to the specificity of this enzyme.

Several characteristics of Hb Knossos are similar to those present in Hb E where the substitution occurs on a nearby position. These characteristics are: reduced rate of β chain synthesis and presence of a β-thalassemia intermedia syndrome when the hemoglobin is associated with a high-A_2 β-thalassemia gene. It will be interesting to investigate at the DNA level the possible mechanisms of the thalassemic syndrome associated with Hb Knossos.

ACKNOWLEDGEMENTS

We are grateful to Mme Crofts for her help in the revision of the manuscript and to Mme Segear for typing the manuscript. This work was supported by a grant from INSERM (PRC 124 035), CNRS (ERA 914) and Ministère de la Recherche (PVD 81 L 0528).

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