Hb Marseille [$\alpha_2 \beta_2$ N methionyl – 2 (NA_2) His → Pro]: a new β chain variant having an extended N-terminus

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A new abnormal hemoglobin was found in a diabetic Maltese woman by citrate agar electrophoresis. This variant was undetectable by isoelectric focusing. No hematological abnormalities were observed. The structural analysis included isolation of the abnormal β chain, high pressure liquid chromatography of the corresponding tryptic peptides and then microsequencing of the abnormal T_1. These procedures revealed a double abnormality: the presence of a methionyl residue extending the NH_2 terminus and a histidine to proline substitution in position NA_2.

Hemoglobinopathy *β variant* *HPLC* *Hemoglobin Marseille* *Protein synthesis*

1. INTRODUCTION

An increasing number of hemoglobin variants are detected in the course of Hb A_{1c} evaluation in diabetic patients [1,2]. Here we detected a hemoglobin fraction behaving like Hb F or Hb A_{1c} by citrate agar electrophoresis (Corning Medical, USA) in a 64-year-old woman with mild diabetes. She was of Maltese origin but lived in Marseille. Structural studies allowed the observation of a very unusual modification, since, together with a His to Pro substitution at the NA_2 position, we have demonstrated the persistence of the initiating methionine. This illustrates that structural studies of hemoglobin variants can still provide some insight into molecular mechanisms.

2. MATERIALS AND METHODS

Standard hematological procedures were followed. Heinz bodies staining was performed with Cresyl brilliant blue (1%) after 3 h sterile incubation at 37°C. Freshly prepared hemolysates were subjected to the 4 classical electrophoretic systems as proposed in [3]. 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 plates 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 [4]. Globin chain analysis on acid–urea–Triton acrylamide gel was performed as in [5]. Densitometry of acid–urea–Triton polyacrylamide slabs was done with a Cellosystem Sebia densitometer (France). Quantification of Hb F was as in [6] and the A_2 level was measured chromatographically as in [7]. Stability was tested by the isopropanol test as in [8]. 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 [9]. The abnormal hemoglobin was separated by Biorex 70 chromatography according to [10] with some modifications [11] (fig.1). Purity of the abnormal

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hemoglobin was assessed by citrate agar electrophoresis and by acid–urea–Triton polyacrylamide gel electrophoresis (fig.1). The abnormal β chain after desheminisation by acid-acetone precipitation was prepared by CM-cellulose 8 M urea chromatography [12], aminoethylated and stripped of urea on a Biogel P2 (Biorad) column and hydrolysed with trypsin. Two methods were used for the characterization of tryptic peptides: (i) fingerprinting on silica gel thin-layer plates [13] and specific staining for histidine, tyrosine, tryptophan, arginine and methionine [14]; (ii) HPLC using a Beckman model 343 with an Altex wavelength detector set at 214 nm. The column system was a Waters μBondapack C18 (10 μm, ID 3.9 mm, 30 cm). The solvent system was based on [15]: 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 IE (Biotronik, Munich) after 40 h hydrochloric acid hydrolysis of separated peptides. The abnormal peptide (T_1) was in parallel subjected to the microsequencing procedure according to [16].

3. RESULTS

3.1. *Case report*

The propositus and one of her nieces were found to carry the β globin variant trait. Hematological data for both were in the normal range. The following data concern only the propositus. The abnormal Hb component constituted 44% of the total β chains as evaluated by densitometry of an acid–urea–Triton polyacrylamide gel electrophoresis (fig.2, lane 2). Hb A_2 was 2.6% (N = 2 ⟶ 3.1) and Hb F 0.45% (N < 1). No Heinz bodies were seen after 3 h incubation with Brilliant Cresyl blue. The isopropanol test was negative. The P_{50} of whole fresh blood was 27 mmHg (N = 25 ⟶ 29). 2,3-DPG was 14.55 μM/g Hb (N = 13 ⟶ 15). The level of ferrihemoglobin in the fresh hemolysate was below 1%. Electrophoretic mobilities according to [3] were, respectively: cellulose acetate electrophoresis (pH 8.7), 0.0; citrate agar (pH 6.2), −4.4; globin chain electrophoresis (pH 9.0 and 6.0), 20.0 and 21.7.

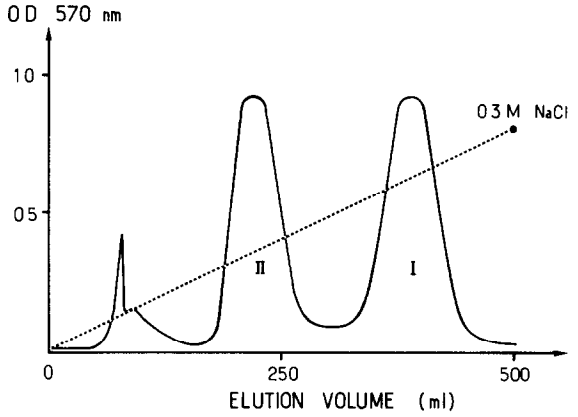

Fig.1. Biorex 70 chromatographic pattern of the propositus hemolysate. Peak I, Hb A_0; peak II, Hb Marseille; peak III, NHP (non-heme proteins), and minor fast hemoglobin derivatives. 300 mg hemolysates; column, ϕ 2.5 × 20 cm; Biorex 70 (200–400 mesh), pH 6.75; phosphate (NaH_2PO_4) buffer, 0.048 M; NaCl, linear gradient, 0 ⟶ 0.3 M.

Mobility in acid–urea–Triton polyacrylamide gel electrophoresis was 23.1; isoelectric focusing, no separation.

3.2. *Structural studies*

Fig.1 shows the elution profile of the abnormal hemoglobin after Biorex 70 chromatography and fig.2 the acid–urea–Triton polyacrylamide electrophoretic pattern of the successive peaks I and II. The data obtained indicated that peak II was composed of the abnormal Hb fraction and was

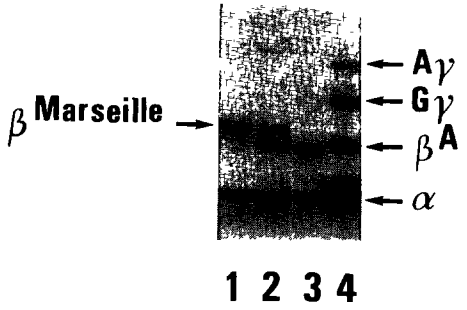

Fig.2. Acid–urea–Triton polyacrylamide gel electrophoretic analysis of the hemoglobin composition in the peaks (I and II) obtained by Biorex 70 chromatography compared to a control and to the whole hemolysate. Lanes: (1) peak II; (2) whole hemolysate; (3) Hb A_0; (4) cord blood sample (with a Hb α variant).

slightly (less than 10%) contaminated by the glycosylated form of tHb A_0 as shown in fig.2 (β A_{1c} has the same mobility as β A_0 in this electrophoretic system). Peak III was mainly composed of non-heme proteins (NHP) and some minor fast Hb derivatives. Peak I was Hb A_0.

Structural studies were conducted using the abnormal hemoglobin component present in peak II. Tryptic peptides of the β abnormal chain were first studied by fingerprinting. The T_1 is absent and a new spot of higher chromatographic mobility and more anodic was seen. Specific stainings revealed besides an otherwise normal pattern for the other spots, a negative histidine and a positive methionine coloration for the atypical spot.

Secondly, in the HPLC separation of the non-aminoethylated tryptic peptides, as shown in fig.3, βT_1 was absent and a peak with a longer retention time was present. Three successive HPLC steps permitted the accumulation of a sufficient amount of this peptide to perform amino acid analysis and a manual microsequence analysis. The amino acid molar ratios (table 1) showed a β T_1 with a histidine to proline substitution and an extra methionine, besides an otherwise normal amino acid composition. Five steps of microsequencing procedure performed on this abnormal peptide

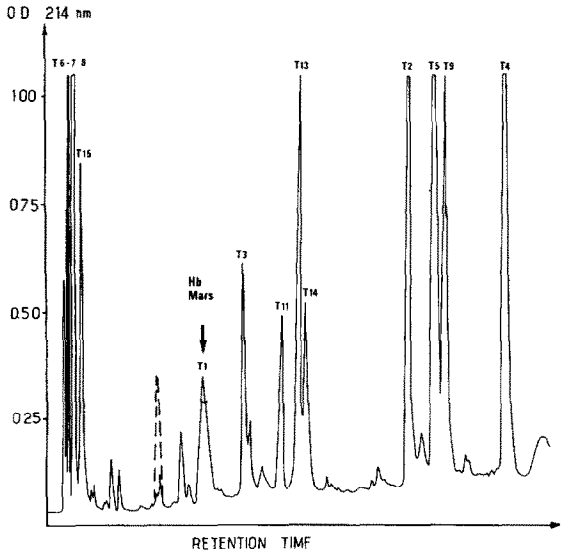

Fig.3. HPLC elution pattern of the non-aminoethylated tryptic peptides of the Hb Marseille β chain. Arrow, abnormal βT_1; dotted line, elution position of normal βT_1.

Table 1

Amino acid composition of βT_1 of Hb Marseille compared to normal βT_1

	βT_1 expected AA molar ratio	β Marseille T_1 observed AA molar ratio
Thr	1	0.90
Glu (Gln)	2	1.90
Pro	1	1.80
Val	1	1.00
Met	0	0.80
Leu	1	1.00
His	1	0.00
Lys	1	0.90

revealed the following sequence: Met ⟶ Val ⟶ Pro ⟶ Leu ⟶ Thr.

4. DISCUSSION

Hb Marseille has some unusual biochemical features. One of the structural abnormalities involves the substitution of the histidine NA_2 by a proline. This modification is homologous to that described in Hb Deer Lodge β (NA_2) His ⟶ Arg [17] and Okayama β (NA_2) His ⟶ Gln [18].

The electrophoretic behaviour of Hb Marseille deserves some comment. Citrate agar electrophoresis is known to explore, in part, the differences in the electropositivity of the central cavity of Hb variants [19,20]. In this system, like Hb A_{1c} and Hb Raleigh β (NA_1) Val ⟶ Ac Ala [21], Hb Marseille, in which the histidine NA_2 is substituted by a neutral residue, has a higher mobility. The separation of Hb Marseille, like Hb Okayama [18] in Biorex 70 chromatography, is sustained by a similar mechanism [10].

The substitution of His (NA_2) by Pro probably affects negatively the interaction between Hb and 2,3-DPG. In Hb Marseille the whole blood P_{50} was expected to be lowered as in Hb Deer Lodge [17] and Hb Okayama [18]. The finding of a normal P_{50} may indicate a low oxygen affinity of the Hb Marseille tetramers in the absence of allosteric effectors. Detailed Hb function studies are currently underway to provide a full description of the functional properties of Hb Marseille.

The finding of a methionine residue at the NH_2

terminus of the Hb Marseille β chain was firmly demonstrated by amino acid analysis, by stainings of the fingerprint, and by microsequencing data. Furthermore the achievement of the sequence analysis procedure showed that the NH_2 of the methionine was not blocked in, at least, a large fraction of Hb Marseille.

In the absence of data on the patient's DNA, and since we were unable to propose, starting from the DNA sequence of the Hb β chain [22], a unique DNA rearrangement that could produce such a modified β chain, we propose that the NH_2 terminal methionine present in Hb Marseille is the initiating methionine. In this hypothesis the occurrence of a proline in position NA_2 would constitute a steric impairment for the NH_2 methionyl peptidase normally eliminating rapidly the initiating methionine [23]. A very similar hypothesis has been stated for naturally occurring proteins with a met--X NH_2-terminal sequence, in which X is either a charged amino acid [24,25] or a proline [26,27].

Thus the NH_2 terminus of Hb Marseille β chain seems to be an inadequate substrate for both *N*-methionyl peptidase and NH_2 terminal acetyl transferase in contrast to Hb F γ chains [28] and Hb Raleigh β chains [21]. Finally, the amino acid analysis showed that NH_2 terminal methionine was not in its oxidized form, a result expected after the observed neutral electrophoretic behaviour in globin chain pH 9.0 electrophoresis.

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