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ORIGINAL ARTICLE

Hb Cardarelli [β86(F2)Ala→Pro]: A New Unstable and Hyperaffine Variant in Association with β^+-Thalassemia

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ABSTRACT

Hb Cardarelli [β86(F2)Ala → Pro] is a new unstable and high oxygen affinity variant found in several members of a family from Naples, Southern Italy. A detailed structural and functional characterization of the variant was performed on two subjects, at both the protein and DNA level. The first patient exhibited 43% of the variant hemoglobin (Hb) without major hematological problems. The proband showed 82% of the abnormal Hb in association with β^+-thalassemia (thal) that caused relevant erythrocytosis requiring frequent phlebotomies. Structural investigation of the Hb variant by mass spectrometric methodologies identified the amino acid replacement as Ala → Pro at β86. The corresponding DNA mutation *G*CC → *C*CC at codon 86 of the β-globin gene was assessed by both DNA sequencing and amplification refractory mutation system (ARMS) techniques. Functional studies carried out on whole blood

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and diluted hemolysates from both patients demonstrated increased oxygen affinity, decreased Bohr effect, reduced heme–heme interaction and nearly halved 2,3-diphosphoglycerate (2,3-DPG) and chloride effects.

Key Words: Variant hemoglobin (Hb); Erythrocytosis; High affinity; Mass spectrometry.

INTRODUCTION

The term erythrocytosis refers to a variety of clinical and pathological conditions induced by various factors, including the presence of a hemoglobin (Hb) variant. A number of anomalous hyperaffine Hbs have been described in which the increased oxygen affinity is responsible for a compensatory erythrocytosis. The degree of erythrocytosis depends primarily upon the molecular defect of the abnormal Hb and is roughly proportional to the increase in whole blood oxygen affinity. Besides a shift to the left of the oxygen dissociation curve, these variants usually show decreased heme–heme interactions and Bohr effect, and diminished affinity for 2,3-bisphosphoglycerate (2,3-DPG).

Most of the hyperaffine Hbs described so far are characterized by amino acid substitutions either occurring at or affecting the $\alpha 1\beta 2$ interface. This region is crucial to Hb function as it is involved in the T→R conformational transition. However, a few high oxygen mutations have been found in helix F of the β chain that contains the proximal His(F8), and is close to the FG corner belonging to the $\alpha 1\beta 2$ interface. Only two variants carrying substitutions at position β86(F2)Ala have been reported (1,2).

This paper reports structural and functional studies of Hb Cardarelli, a new high affinity variant carrying an Ala→Pro mutation at position β86(F2). This variant was in association with β^+-thalassemia (thal) and was responsible for a rather high degree of erythrocytosis. The amino acid replacement was identified by mass spectrometric procedures, and the corresponding base mutation confirmed by DNA sequencing and amplification refractory mutation systems (ARMS) techniques. Besides the increased oxygen affinity, functional studies revealed that the Bohr effect and the heme–heme interactions were reduced, and the 2,3-DPG and chloride effects were decreased to nearly half of their normal values.

MATERIALS AND METHODS

Routine Examinations

Unless otherwise specified, blood was collected with EDTA as anticoagulant. Hematological data were obtained by standard procedures using a Coulter Counter MAXM (Coulter Electronics, Inc., Miami Lakes, FL, USA). Red cell lysates were analyzed by electrophoretic techniques at alkaline pH on cellulose acetate and acidic pH on agar citrate. The different Hb components were separated and measured by cation exchange high performance liquid chromatography (HPLC) using the VARIANT

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I™ system and the elution program suggested by the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA). The heat stability and isopropanol precipitation tests were carried out as indicated by Huisman and Jonxis (3).

Structural Characterization of Variant Hemoglobins

Individual globin chains were separated either by reversed phase HPLC (HP 1100 system; Agilent Technologies, Palo Alto, CA, USA) or analyzed by liquid chromatography-mass spectrometry (LC/MS) on a DECA LC-MS system (Thermo-Finnigan Corp., San Josè, CA, USA) as described by Carbone et al. (4). The variant globin was carboxyamidomethylated with iodoacetamide, digested with trypsin and the resulting peptide mixture was directly analyzed by matrix assisted laser desorption ionization (MALDI) mass spectrometry (MS) as reported (5) using a Voyager DE linear instrument (Applied BioSystems, Boston, MA, USA). Manual Edman degradation steps were performed on the unfractionated peptide mixtures using 5% phenylisothiocyanate in pyridine as coupling agent, as described previously (6).

DNA Analysis

DNA was obtained from peripheral blood leukocytes by phenol-chloroform extraction. The mutation of Hb Cardarelli was assessed by DNA sequencing and polymerase chain reaction (PCR)-ARMS. Amplification of the β-globin gene DNA for sequence analysis was accomplished by using the following primers: 5′-CTG ACT CTC TCT GCC TAT TG-3′ (nucleotides 62369–62399, as listed in GenBank) and 5′-ACA CTG ATG CAA TCA TTC GTC-3′ (nucleotides 62738–62759, as listed in GenBank) which produced a 390 bp fragment. Sequence analysis of the amplified fragment was carried out on a gel purified sample (Quiagen Gel Purification Kit; Applied BioSystems, Norwalk, CT, USA) by an Applied BioSystems 373 automated sequencer using the PRISM™ dye terminator cycle sequencing kit (Applied BioSystems). The allele specific amplification analysis was accomplished by PCR amplification of the β-globin gene using the following primers: 5′-CTT GTC ACA GTG CAG CTC ACT CAG TGT CGG-3′ and 5′-ACC TCA CCC TGT GGA GCC AC-3′ according to Old et al. (7). The β-globin haplotype was constructed by using various polymorphic restriction enzyme sites within the β-globin gene cluster according to Orkin et al. (8).

Functional Studies

The whole blood oxygen parameters were determined by tonometry at 37°C of heparinized samples using an RNA Medical Equilibrator (Acton, MA, USA). Since Hb Cardarelli could not be separated from Hb A, the oxygen binding parameters were determined on diluted hemolysate samples from the two patients, previously stripped to remove all the 2,3-DPG. Dilution was performed to a final Hb concentration of 0.1 mM in heme, and the samples were analyzed by tonometry at 25°C using an Oximeter-3000 WTW (WTW, Weilheim, Germany), coupled to a double-beam spectrophotometer (9).

Oxygen equilibrium curves were represented according to Hill's equation [log (y/(1−y) vs. log pO2], and functional parameters were calculated following standard procedures (10,11). Chloride and 2,3-DPG binding was examined at pH 7.3, the former as $\Delta\log P_{50}$ between 600 and 100 mM NaCl, and the latter as $\Delta\log P_{50}$ at 100 mM NaCl, in the presence or the absence of 1 mM 2,3-DPG (12,13). When necessary, cytochrome C-reductase, NADH and methylene blue were added to diluted Hb samples to minimize oxidation of Hb to metHb (14).

RESULTS

Case Report

Hb Cardarelli was identified in several members of a large family from Naples, Southern Italy; their pedigree is shown in Fig. 1. The proband, (II-9), was a 28-year-old male who was referred to the Thalassemia Unit of the Cardarelli Hospital because of headaches and weakness. Physical examination showed sclerae jaundice, inflamed conjunctiva, skin redness without cyanosis and moderate enlargement of the spleen (lower tip 4 cm below the costal margin) and liver (lower margin 4 cm below the costal margin). The patient was affected by erythrocytosis in association with β^+-thal and practiced therapeutic phlebotomies at the age of 20.

A bone marrow biopsy showed an erythroblastic increase series, and cytogenetic studies revealed a normal karyotype. Hematological findings of the proband and other member of his family are reported in Table 1. The abnormal Hb was inherited through

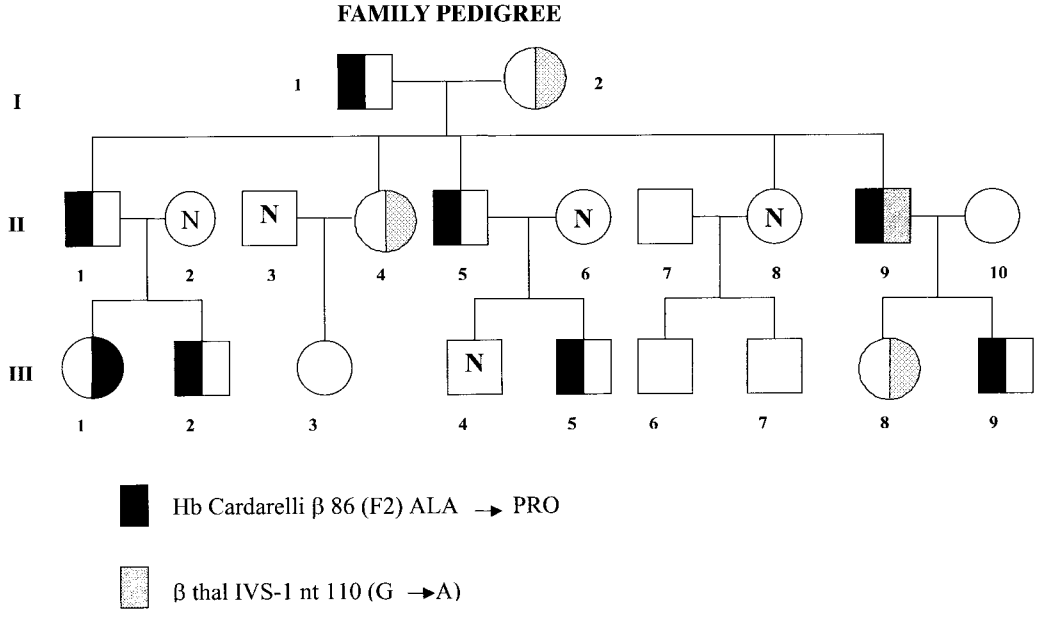

Figure 1. Pedigree of the Italian family with Hb Cardarelli.

Table 1. Hematological findings of the proband (II-9) and other members of his family.

Subjects	I-1	I-2	II-1	II-4	II-5	II-8	II-9[a]	III-1	III-2	III-5	III-8	III-9
Sex-age	M-74	F-69	M-40	F-37	M-35	F-33	M-30	F-13	M-12	M-3	F-8	M-6
β-Thal mutation		IVS-I-110		IVS-I-110			IVS-I-110				IVS-I-110	
RBC (10^{12}/L)	5.2	4.9	5.3	4.9	5.0	4.2	7.5	4.9	4.7	5.4	5.7	4.7
Hb (g/dL)	16.8	12.2	16.9	11.7	16.8	13.8	18.6	14.8	13.9	14.8	11.6	13.3
PCV (L/L)	0.48	0.34	0.49	0.35	0.49	0.38	0.58	0.42	0.40	0.44	0.38	0.39
MCV (fL)	91.0	69.0	92.0	70.0	98.0	95.0	77.0	85.0	84.0	83.0	64.0	84.4
MCH (pg)	31.6	24.0	32.0	24.0	33.0	32.0	24.0	29.0	28.0	27.0	19.0	28.3
Reticulocytes (%)	1.0	0.8	0.9	1.0	1.0	0.9	5.0	1.0	0.8	0.9	1.0	1.0
Hb A_2 (%)	3.3	4.4	3.2	4.8	3.1	2.7	5.8	3.2	3.3	3.4	5.1	3.0
Hb F (%)	1.0	3.5	1.0	3.0	0.9	0.9	6.0	1.0	0.9	1.8	2.0	1.0
Hb X (%)	45.0		48.0		50.0		78.0	47.0	48.0	50.0		46.0
Isopropanol	[+]		[+]		[+]		[+]	[+]	[+]	[+]		[+]
Serum iron (μg/dL)	80.0	83.0	70.0	76.0	86.0	60.0	76.0	98.0	80.0	80.0	57.0	101.0
Ferritin (ng/mL)	350.0	97.0	514.0	35.0	316.0	34.0	215.0	30.0	28.0	26.0	52.0	52.0

[a]Proband.

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the paternal line (I-1), whereas the thalassemia trait was of maternal origin (I-2). The proband showed erythrocytosis (RBC 7.5×10^{12}/L), increased Hb concentration (18.6 g/dL), and a Hb A_2 level of 5.8%, with a high reticulocyte count (5.0%). Both the isopropanol precipitation and heat stability tests showed a relevant molecular instability of the variant Hb. A second carrier of the abnormal Hb (II-1) was found to be heterozygous for Hb Cardarelli. However this subject showed only mild erythrocytosis and a modest instability of Hb and had a normal life. Both cellulose acetate (pH 8.6) and agar citrate (pH 6.0) electrophoreses showed only normal Hb components. The cation exchange HPLC analysis on the VARIANT I™ instrument (Bio-Rad Laboratories) was also normal.

Abnormal Hemoglobin Analysis

Red cells from the proband (II-9) were hemolysed and the individual globins directly analyzed on an LC/MS system. The total ion current (TIC) profile of the LC/MS analysis, i.e., the current associated to the ionization of each individual protein component in the electrospray (ES) source, revealed the presence of an abnormal peak eluting before the normal β-globin, together with a large decrease in the amount of the β chain. The anomalous peak exhibited a mass value of 15894.4 ± 0.8 Da, confirming

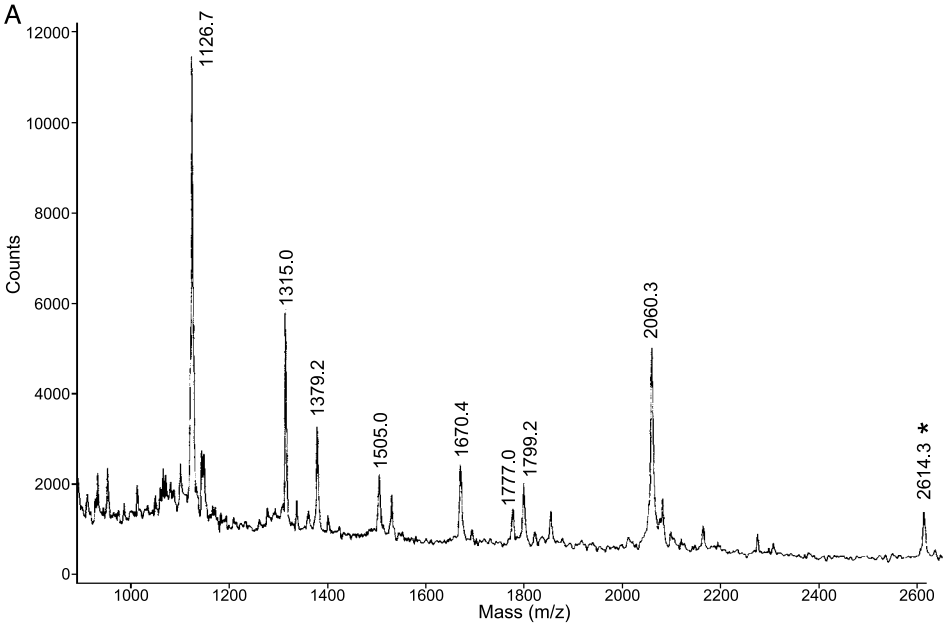

Figure 2. A) The MALDI/MS analysis of the tryptic digest of the variant β chain. The mass signals were associated with the expected tryptic peptides within the β-globin sequence. The signal at m/z 2614.3 representing the varied peptide is marked with an asterisk (*). B) Partial MALDI/MS spectra of the truncated peptide mixture following one (a), two (b), three (c) and four (d) steps of manual Edman degradation.

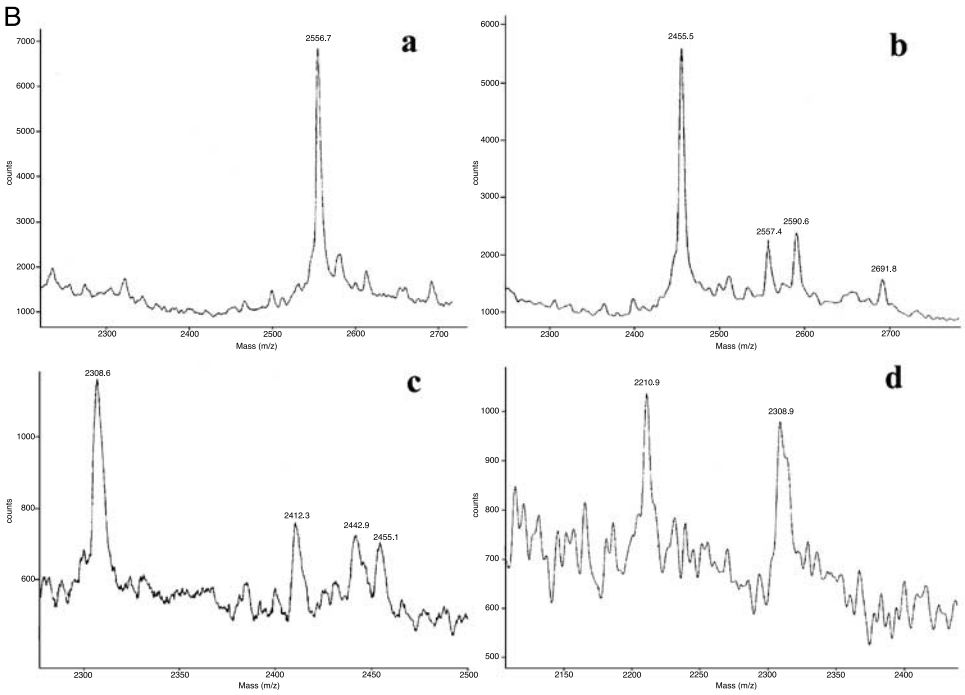

Figure 2. Continued.

the occurrence of a β variant. The relative abundance of the various globin chains was estimated from the ES mass spectral data, from which the percentage of the different Hb components was extrapolated as follows: Hb X 82.0%; Hb A 4.6%; Hb F 7.7% and Hb A_2 5.7%. A similar analysis was performed on the hemolysate from the second carrier of the variant Hb (II-1), and a Hb X value of 43.0% was estimated.

The measured molecular mass of the variant globin was about 26 Da higher than the normal β chain. On the basis of a DNA single point mutation, this difference could only be accounted for by four amino acid substitutions, i.e., His→Tyr, Ala→Pro, Ser→Leu or Ser→Ile (15). The variant chain was purified by preparative HPLC, carboxyamidomethylated and digested with trypsin as previously described (5). The resulting peptide mixture was directly analyzed by MALDI/MS, producing the spectrum shown in Fig. 2(A). All the mass signals recorded in the spectrum could be assigned to the predicted tryptic peptides within the β-globin sequence with the exception of the peak at m/z 2586.9. This mass value occurred 26 Da higher than the peptide 83–104 (βT-10,11, expected mass value 2560.7 Da), thus confining the structural variation within this region. The amino acid replacement was eventually defined by submitting the whole peptide mixture to four manual Edman degradation steps and re-analyzing the truncated peptide mixture generated after each cycle by MALDI/MS [Fig. 2(B; a–d)]. Following three reaction steps, the anomalous mass signals moved back to m/z 2529.8, 2428.6 and 2281.4, due to the removal of Gly83, Thr84, and Phe85, respectively. Finally, the fourth Edman step shifted the mass signal back by 97 Da, to m/z 2184.3, thus demonstrating the presence of a proline residue replacing the normally occurring alanine at position 86. The

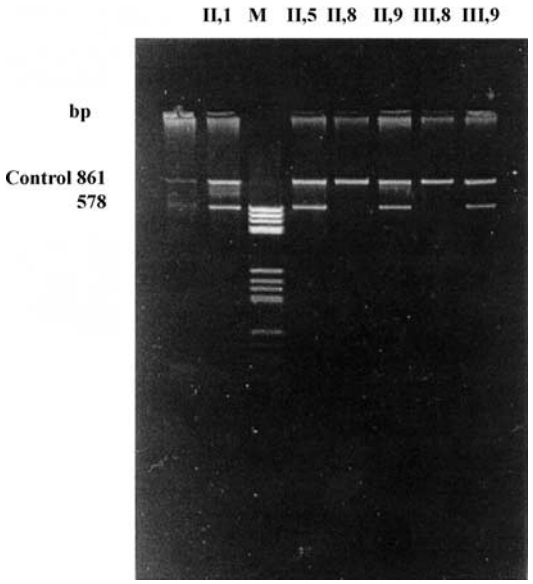

Figure 3. Amplification refractory mutation system for Hb Cardarelli in various members of the family. Lane 2: II-1, heterozygous for Hb Cardarelli; lane 3: markers; lane 4: II-5, heterozygous for Hb Cardarelli; lane 5: II-8, normal; lane 6: II-9, proband; lane 7: III-8, heterozygous for the thalassemia mutation; III-9, heterozygous for Hb Cardarelli. The 578 bp fragment detected in lanes 2, 4, 6 and 8 indicates the presence of Hb Cardarelli. The 861 bp fragment is the amplification control.

abnormal variant was named Hb Cardarelli [β86(F2)Ala→Pro] after the hospital where it was first identified.

DNA Analysis

The DNA mutation corresponding to Hb Cardarelli was assessed both by direct sequencing and by allele specific amplification analysis. For the DNA sequence, the

Table 2. Oxygen binding parameters of the whole blood from the proband (II-9) and his brother (II-1).

Patient	pH	P_{50}	Log P_{50}	Hill coefficient	MetHb (%)
A) Hill's plot					
II-1	7.33 ± 0.02	17.1 (26.2)	1.22 (1.41)	1.58 (2.66)	2.67 ± 0.12
II-9	7.31 ± 0.03	13.8 (26.2)	1.14 (1.41)	1.80 (2.66)	1.90 ± 0.13
Patient		pH range		Bohr effect	
B) Bohr effect					
II-1		6.98–7.70		−0.58 (−0.44)	
II-9		6.74–7.73		−0.57 (−0.44)	

Comparative values for normal whole blood are in parentheses.

β-globin genes from the proband's DNA were amplified by PCR using suitable oligonucleotide probes, yielding 700 bp products that were gel purified. Both strands were sequenced showing heterozygosity for a G→C change at the first position of codon 86, from *G*CC to *C*CC. This mutation was confirmed by the ARMS technique using an oligonucleotide probe synthesized on the basis of mass spectral data. The DNA from the proband and other member of his family was analyzed and the corresponding electrophoretic results are shown in Fig. 3. The occurrence of an abnormal 578 bp band, specific for Hb Cardarelli and absent in the control, was detected in the proband (II-9) and three members of his family (II-1, II-5 and III-9).

Haplotype analysis of DNA polymorphisms on the β-globin gene cluster was performed by amplification of the seven DNA segments containing the common polymorphic restriction sites occurring within the β-globin gene cluster, and using different sets of oligonucleotide primers. The restriction sites included the three *Hin*cII sites located 5′ to the ε-globin gene, within and 3′ to the ψβ-globin gene, the two

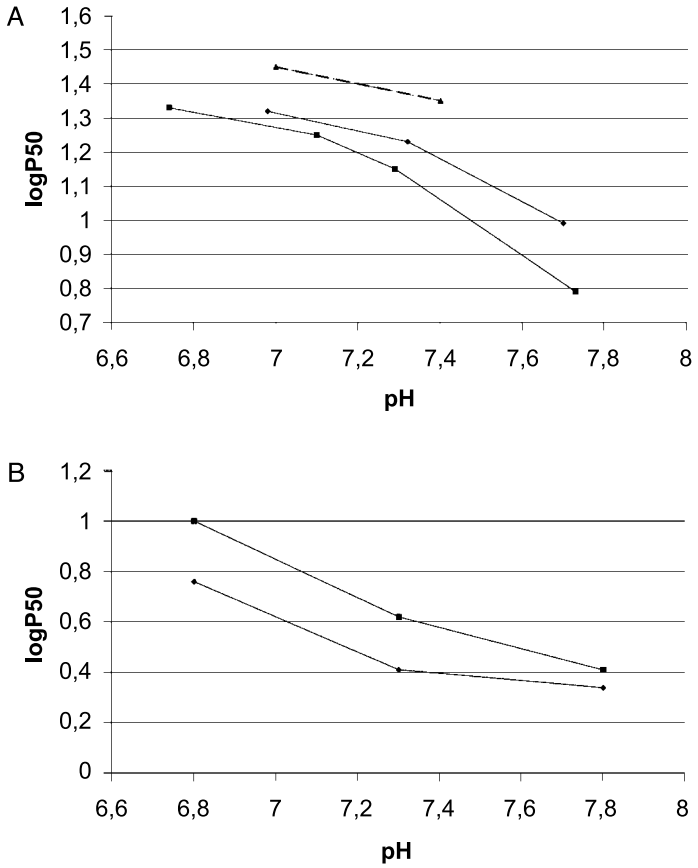

Figure 4. A) Plot of log P_{50} vs. pH measured in the red blood cells of the proband (II-9), his brother (II-1) and the control: proband (II-9) (■); subject (II-1) (◆); control (▲). B) Plot of log P_{50} vs. pH measured on dilute hemolysate samples from the proband (II-9) (◆); control (■).

*Hin*dIII sites within the $^{G}\gamma$- and $^{A}\gamma$-globin genes, the *Ava*II site in the β-globin gene and the *Bam*HI site located 3′ to the β-globin gene. This analysis showed that the β-globin gene from Hb Cardarelli was associated with haplotype II and the (β^{+}) IVS-I-110 (G→A) mutation was associated with haplotype I, according to Orkin et al. (8).

Functional Studies

The oxygen binding parameters measured in the red blood cells of the proband (II-9) and his brother (II-1) are summarized in Table 2; normal values are given in parentheses for comparison. The affinity for oxygen of the abnormal red cells in standard conditions (pH 7.3, 37°C) was increased and the Hill coefficient (*n*) decreased in both cases. In particular, the sample of patient II-9, containing 82% of Hb Cardarelli, showed an almost two-fold increase in oxygen affinity as compared to the control. The P_{50} ratio between the normal and proband red cells was measured as 1.9. Figure 4(A) shows the plot of log P_{50} vs. pH for the two subjects and the control; an increased Bohr effect was determined for both abnormal samples from these data. Moreover, a biphasic aspect of the two anomalous curves was observed, indicating the presence of two Hb species with widely different oxygen affinities. This behavior was clearly detected in the heterozygous sample containing 43.0% of Hb Cardarelli, whereas it could only be observed at low pH values in the proband's sample, as it contains only 20.0% of non Hb Cardarelli components.

Since the variant Hb could not be separated from the other Hb components by a number of chromatographic techniques, and because of its molecular instability and auto-oxidation rate, the oxygenation measurements in solution were performed on dilute hemolysate samples from the proband. The final concentration of Hb was maintained at

Table 3. Oxygen binding parameters and allosteric properties of dilute hemolysate samples from the proband (II-9).

Effectors	pH	Hill coefficient	P_{50}	Log P_{50}	MetHb (%)
No effectors (Tris 50 mM + Cl^{-} mM)	7.3	1.8 (2.8)	2.4 (2.5)	0.4 (0.40)	13.2
+ NaCl 100 mM	6.8	2.1 (2.66)	5.8 (10.1)	0.8 (1.0)	10.9
+ NaCl 100 mM	7.3	2.3 (2.6)	2.5 (4.2)	0.4 (0.6)	8.6
+ NaCl 100 mM	7.8	2.2 (2.7)	2.2 (2.6)	0.3 (0.4)	2.4
+ NaCl 600 mM	7.3	1.7 (2.7)	4.0 (10.5)	0.6 (1.0)	12.0
+ NaCl 100 mM + 2,3-DPG	7.3	2.1 (2.7)	3.8 (11.0)	0.6 (1.0)	4.5
Allosteric measurements					

Effector	pH Range	Log P_{50}	Bohr effect
NaCl 100 mM	6.8–7.8		−0.42 (−0.59)
Chloride	7.3	0.20 (0.40)	
2,3-DPG	7.3	0.18 (0.42)	

Samples were added with the enzyme reductase mixture to minimize oxidation of Hb Cardarelli to metHb. Comparative values are in parentheses.

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0.1 mM of heme (9). When necessary, samples were treated with the enzyme reductase mixtures to minimize the oxidation reaction (14). The observed oxygen binding parameters of Hb Cardarelli are reported in Table 3. The variant showed an almost two-fold increase in oxygen affinity, with decreased heme–heme interactions with respect to Hb A. As illustrated in Fig. 4(B) and Table 3, the alkaline Bohr effect was diminished to about 71.0% of the Hb A values. The addition of 2,3-DPG did not cause any major change in either the oxygen affinity or heme–heme interactions. The effects of 2,3-DPG and heterotropic cofactor Cl^- were measured at half their normal values, suggesting that some structural rearrangements had occurred that affected both the entry and/or the global internal charge of the central cavity. Under all the experimental conditions used, Hb Cardarelli retained a two-fold increase of oxygen affinity as compared to Hb A.

DISCUSSION

The occurrence of an abnormal Hb was detected in a patient affected by erythrocytosis in association with β^+-thal. A detailed structural analysis of the variant Hb was carried out at both protein and DNA levels, eventually demonstrating the presence of a new Hb variant never before described, that was named Hb Cardarelli or β86(F2)Ala→Pro. The amino acid replacement was assessed by mass spectrometric methodologies, and the corresponding DNA mutation was established as G→C at the first position of codon 86 (*G*CC→*C*CC) by both direct DNA sequencing and PCR-ARMS experiments, using a synthetic oligonucleotide probe designed on the basis of the MS results. Functional studies showed that Hb Cardarelli belongs to the high affinity group of Hb variants, with reduced Bohr effect and reduced heme–heme interactions.

Two other mutations at position β86(F2)Ala have been described so far: Hb Olomuc (→Asp) found in a Czechoslovakian family, was the first reported abnormal Hb with a mutation in the F2 residue (1), and the same amino acid replacement was later discovered in an unrelated family in Japan (16). All patients were affected by erythrocytosis, but functional studies were only carried out on the European subjects. Increased oxygen affinity was found in all cases with normal stability tests. A second F2 mutation occurred in the doubly substituted, unstable and hyperaffine Hb Poissy, β56(D7)Gly→Arg;β86(F2)Ala→Pro, one of the very few Hb variants carrying two amino acid variations (2).

Hb Poissy showed mild molecular instability, increased oxygen affinity, low *n* factor and diminished Bohr effect. These functional abnormalities were attributed to the single β86(F2)Ala→Pro mutation, the same as Hb Cardarelli, since the second substitution [β56(D7)Gly→Arg] was considered unable to determine functional alteration (2). The presence of proline at position β86 causes a displacement of the F helix closer to the heme plane, a distortion of the FG segment and a large increase of the dynamic fluctuation of the tertiary structure of Hb at the proximal side of the affected hemes. As a consequence, these hemes and $\alpha_1\beta_2$ contacts are functionally altered (2). Accordingly, Hb Cardarelli and Hb Poissy display similar Hill's plots, and Hb Cardarelli showed a two-fold increase in oxygen affinity, with decreased heme–heme interactions (n_{50}) and a diminished alkaline Bohr effect.

However, a number of differences in the functional behavior of the two variants was observed. In particular, the addition of 2,3-DPG did not restore the functional

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parameters of Hb Cardarelli, as occurred in Hb Poissy. The new variant retained both the high oxygen affinity and the low *n* value in the presence of the cofactor. Moreover, the sensitivity of Hb Cardarelli to heterotropic cofactors was quite dissimilar to that of Hb Poissy, these effects having been measured at half their normal values. These discrepancies might be related to the different methods employed for oxygen equilibrium curve recordings or to the different percentage of metHb occurring in the hemolysates. However, a synergic effect of the two mutations on the functional behavior of the doubly mutated Hb Poissy cannot be ruled out.

The association of mutated β-globin genes with the corresponding haplotype was defined by restriction fragment length polymorphisms (RFLPs) through family linkage studies. The (β^+) IVS-I-110 (G→A) mutation was associated with haplotype I, according to Orkin et al. (8), whereas the β variant gene giving rise to Hb Cardarelli was associated with haplotype II. This result reflects the frequency of association of the IVS-1-110 mutation with haplotype I in Campania and the settlement of the β-Cardarelli gene on the most frequent genetic background in our region, haplotype II. Haplotype frequency analysis, in fact, had shown that haplotypes I and II account for more than 50.0% of both β^A and β-thal chromosomes (17).

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