

Posttranslational Deamidation of Proteins: The Case of Hemoglobin J Sardegna [α 50(CD8)His \rightarrow Asn \rightarrow Asp]

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Hemoglobin J Sardegna [α 50(CD8)His \rightarrow Asn \rightarrow Asp] is a human Hb variant in which a posttranslational deamidation process takes place, transforming an Asn to an Asp residue. This variant, particularly widespread in northern Sardinia, has for the first time been characterized at the DNA level (codon 50 C \rightarrow A) on the selectively amplified α_2 -globin gene. We determined the protein and DNA sequences and performed cellulose acetate electrophoresis, isoelectric focusing, globin chain separation, stability tests with isopropanol and heat precipitation, and oxygen affinity analyses on whole blood to fully characterize the variant. A comprehensive review of the deamidation processes involving Asn and Gln residues in mutant proteins is reported, together with a discussion of the molecular mechanisms of such deamidations. Finally, examples of other proteins of clinical importance in which Asn or Gln residues have been implicated by DNA analysis alone are presented. These findings point out the importance of the complete characterization of variant proteins by use of both DNA and protein analyses.

Hemoglobin (Hb) J Sardegna is a well-known Hb variant, being one of the most common α -globin structural mutants. Hb J Sardegna was first reported in 1968 in members of a family from the island of Sardinia, Italy (1). At that time, the variant was described as a fast-moving Hb and characterized by the classic techniques of protein

chemistry, which demonstrated the substitution of a His with an Asp residue at position 50 of the α chain. Since then, large screening programs performed by means of isoelectric focusing and reverse-phase HPLC have produced detailed information on the prevalence of this variant and its association with α - and β -thalassemia.

Researchers have discovered that Hb J Sardegna has a relatively high frequency in Sardinia, where it occurs in \sim 0.09% of the population. The geographic distribution is not uniform: some variations of incidence are found in different areas of the island. A higher incidence has been reported in the north, where it reaches 0.25% (2, 3).

Because thalassemic diseases are also widespread in the Sardinian population, the association of Hb J Sardegna with α - or β -thalassemia is not rare (4, 5). Studies that correlate the Hb pattern with the α - and β -globin genotype have shown that in double heterozygotes for Hb J Sardegna and α -thalassemia, the quantity of the abnormal Hb is substantially higher than in heterozygous carriers of the Hb variant alone (6). On the other hand, the concomitant presence of β -thalassemia leads to a slight decrease in the amount of the variant, probably because of a lower affinity of the abnormal α chain for the β chain. Hb J Sardegna has also been described in association with Hb G Philadelphia, another α -chain variant that occurs rather frequently in the Sardinian population (2, 5).

The presence of Hb J Sardegna does not seem to produce any particular clinical manifestation. Appreciable clinical complaints have never been reported in heterozygous carriers of this abnormal Hb, even if it occurs in combination with α - or β -thalassemia. To date, no data are available on the DNA molecular defect associated with Hb J Sardegna; information on the stability of this variant and its functional properties is also not available.

In the past few years, methods based on molecular biology techniques have become reliable and easy tools for identifying Hb variants. Recently, we used a DNA sequencing approach for additional characterization of

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Hb J Sardegna. Surprisingly, the results obtained by DNA sequencing analysis appeared to disagree with the results of protein analysis reported originally (1). The mutation found at the genomic level indicated a His→Asn substitution, whereas protein structural analysis demonstrated that it was His→Asp. This enigmatic finding prompted us to reinvestigate this abnormal Hb. We report here on the characterization of Hb J Sardegna performed at both the DNA and protein levels.

Materials and Methods

BLOOD SAMPLES

Blood samples were obtained from different heterozygous carriers of Hb J Sardegna, all coming from Cagliari on the island of Sardinia, Italy. None of them were affected with any clinical symptoms or hematological abnormalities. For Hb studies, blood samples were collected in EDTA, shipped by fast air mail, and analyzed within 2 days. Twenty-six different carriers of Hb J Sardegna were studied by DNA sequencing as well as by HPLC analysis.

DNA ANALYSIS

DNA was obtained from white blood cells by standard methods. Gene mapping of the α -globin genes was carried out by PCR (7, 8). The α_2 -globin gene was selectively amplified by choosing the upstream primer in the 5' promoter region of both α -globin genes and the downstream primer in the 3' noncoding α_2 -specific region. Sequencing reactions were performed on amplified single-stranded DNA by the dideoxy chain termination method of Sanger et al. (9). The sequencing reverse primer was located at positions 355 to 375 upstream from the Cap site (5'-ACGTGCGCCACGGCGTTGGT-3'). The detection of codon mutation by use of restriction enzymes was performed with primers to amplify the 127-bp sequence encompassing codon 50 of the α_2 -globin and α_1 -globin genes. The forward and reverse primers (5'-CCCTCACTCTGCTTCTCCCC-3' and 5'-GGGCGTCGGCCACCTTCTT-3', respectively) are located at positions 226 and 353 upstream from the Cap site of the α -globin genes. The PCR conditions were as follows: denaturation at 94 °C for 1 min, annealing at 65 °C for 2 min for 30 cycles after an initial incubation at 95 °C for 5 min. The product of amplification was digested by restriction enzyme *DsaI*, which recognizes the sequence C↓C(A or G)(T or C)GG.

RNA ANALYSIS

RNA was extracted from lymphocytes by the acid guanidinium thiocyanate-phenol-chloroform method (10). The cDNA fragment, obtained by reverse transcription-PCR, was sequenced with the same primer used for DNA sequencing, as described above.

Hb CHARACTERIZATION

Hemolysate was prepared from washed erythrocytes by the conventional CCl₄-water method. Hb stability was assessed by isopropanol and heat denaturation

tests (11). Methemoglobin and carboxyhemoglobin were measured by standard spectrophotometric techniques (12). Oxygen affinity was studied on fresh blood at pH 7.1 and 37 °C by a continuous recording method, using a Hemox Analyzer (TCS) as described previously (13). The measured venous P_{50} was corrected to standard values for the most important factors that affect oxygen affinity, i.e., pH, P_{CO_2} , 2,3-diphosphoglycerate, carboxyhemoglobin, and methemoglobin (14). Red cell 2,3-diphosphoglycerate was measured with an enzymatic test (Boehringer Mannheim), according to the manufacturer's instructions.

Electrophoresis of the hemolysate was performed at pH 8.4 in Tris-EDTA-borate buffer on cellulose acetate plates. Isoelectric focusing was performed on 0.5 mm thick polyacrylamide gel containing 20 mL/L ampholine, pH 6–8. The matrix concentration was 6% T (6 g monomers/100 mL) and 4% C (4 g cross-linker/100 g monomers). Hemolysate samples were applied in a 10- μ L volume corresponding to 100 μ g of total Hb loaded per track. Running conditions included a prerun at 400 V for 15 min and, after sample application, separation at 750 V for 2 h. Focusing was performed at 10 °C.

Additional characterization of Hb fractions was carried out by HPLC analysis using a Kontron 400 system (Kontron Instruments) composed of two pumps (model 422), an autosampler (model 360) with a 10- μ L injector and a ultraviolet-visible detector set at 415 nm. The system was equipped with a cation-exchange polyCAT A column (0.46 × 20 cm, 5 μ m particle size, 300 Å pore size; PolyLC). The flow rate was 2 mL/min. Two buffers of the following composition were used: 35 mmol/L bis-Tris, 3.0 mmol/L ammonium acetate, 0.1 g/L KCN, pH 6.47 (buffer A); and 35 mmol/L bis-Tris, 16.9 mmol/L ammonium acetate, 150 mmol/L sodium acetate, 1 g/L KCN, pH 7.0 (buffer B). Samples (10 μ L, containing 10 μ g of Hb) were applied to the column. The chromatographic separation was achieved by the following step gradient: 20–70% buffer B (20-min step), 70–100% buffer B (1-min step), and 100% buffer B (3-min step). The original conditions were then rapidly restored, and the column was equilibrated with 20% buffer B:80% buffer A for 3 min. Integration of the peaks was performed with the Kontron 450 Data System, Ver. 3.4.

Globin chain analysis was performed by reverse-phase HPLC using a Vydac 218TP54, C₁₈ column (0.46 × 25 cm, 5 μ m particle size). The hemolysate was diluted with water to deliver an Hb concentration of 5 g/L, and 10- μ L samples were injected in the column. Buffer A was acetonitrile-methanol-155 mmol/L sodium chloride, pH 2.7 (50:20:30, by volume); and buffer B was acetonitrile-methanol-155 mmol/L sodium chloride, pH 2.7 (25:40:30, by volume). The elution was performed at a flow rate of 1.4 mL/min for 65 min, with the following gradient: 50–20% buffer B (55-min step) and 20% buffer B (5-min step). The detector was set at 215 nm.

STRUCTURAL STUDIES

For preparative purposes, the heme was removed from the globins by precipitation of the total hemolysate in cool acetone (-20°C) containing 0.3 mol/L HCl. Precipitated globins were washed with cold acetone, dried at room temperature, and then redissolved in 8 mol/L urea buffer. Globin chain separation was obtained by preparative chromatography on carboxymethyl cellulose (Whatman CM-52) in the presence of urea and dithiothreitol (15). After extensive dialysis to remove all traces of urea and salts, the abnormal α chains were digested with trypsin (*N*-methyl-tosyl-L-phenylalanine chloromethyl ketone-treated) in 50 mmol/L ammonium acetate, pH 8.3, using 20 mg of enzyme per 1 g of substrate. The reaction was performed at 37°C for 5 h and stopped by acidification.

Tryptic peptide separation was performed by reverse-phase HPLC essentially as described (16). Briefly, 1 mL of the tryptic digest from native and mutant α chains (1 mg of starting protein) was centrifuged, and the supernatant was applied to an Aquapore RP-300 C_8 column (25×0.46 cm; Applied Biosystems), equilibrated in 0.01 mol/L ammonium acetate, pH 5.7. Peptides were eluted with a 125-min linear gradient (0–30%) of 0.01 mol/L ammonium acetate, pH 5.7, in 500 mL/L acetonitrile at a flow rate of 1 mL/min. Approximately 60 mL of eluted native and mutated $\alpha\text{T-6}$ peptides were N-terminal sequenced on a 477/A protein sequenator (Applied Biosystems) according to the manufacturer's instructions.

BFU-e STUDIES

Mononuclear cells were isolated from peripheral blood on a gradient of Histopaque 1077 (Sigma) and seeded at a density of 5×10^9 cells/L in α minimum essential medium supplemented with 100 g/L fetal calf serum (both from Life Technologies), 1 mL/L cyclosporin A (Sandoz), and 100 mL/L conditioned medium collected from cultures of the 5637 bladder carcinoma cell line (17). The cultures were incubated at 37°C in an atmosphere of 5% CO_2 . After a 5-day incubation period in this phase I culture, the nonadherent cells were harvested, washed, and recultured for 10–15 days in a fresh medium composed of α medium, 300 mL/L fetal calf serum, 10 g/L deionized bovine serum albumin, 10 $\mu\text{mol/L}$ β -mercaptoethanol, 1.5 mmol/L L-glutamine, 1 $\mu\text{mol/L}$ dexamethasone, and 1 kilounit/L human recombinant erythropoietin.

The Hb composition was studied by ion-exchange HPLC as described for hemolysate obtained from mature red blood cells. Globin chain biosynthesis was determined in mature erythroid colonies incubated with ^3H -leucine (TRK 754; Amersham). Separation was carried out on a Beckman HPLC System Gold, and the total area under the chromatogram was measured. The percentage of globin synthesis was calculated according to the following equation: $[\alpha\text{-globin}/(\alpha\text{-globin} + \beta\text{-globin})] \times 100$ (18).

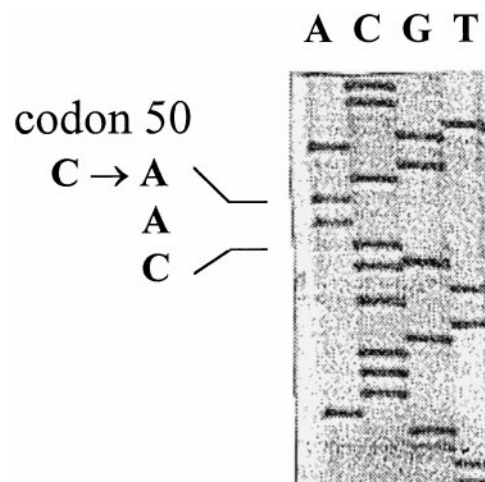


Fig. 1. Sequence analysis of the α_2 -globin gene in a carrier of Hb J Sardegna and $-\alpha/\alpha$ genotype.

Results

Direct sequencing of DNA and cDNA from patients with Hb J Sardegna detects a single nucleotide substitution $\text{C} \rightarrow \text{A}$ in the first position of codon 50 (Fig. 1). The result was the same for all 26 subjects studied. The $\text{CAC} \rightarrow \text{AAC}$ mutation is expected to produce a change of His to Asn. In the patient with Hb J and $-\alpha/\alpha$ (-3.7) genotypes the nucleotide usually at the first position of codon 50 is not evident because only one α gene (i.e., the α_2 gene) carrying the $\text{C} \rightarrow \text{A}$ mutation is present. The $\text{C} \rightarrow \text{A}$ mutation was also confirmed by use of a restriction enzyme (Fig. 2). *DsaI* digestion of amplified DNA (127 bp) from a healthy subject produces three DNA fragments of 78, 24, and 25 bp (Fig. 2, lane 1). The codon 50 mutation ($\text{C} \rightarrow \text{A}$) removes a recognition site for this enzyme, generating a novel DNA fragment of 102 bp (Fig. 2, lane 2). The faint band at the position corresponding to a 102-bp fragment

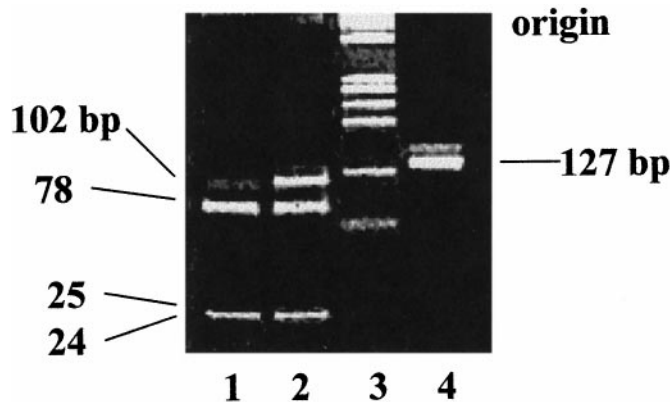


Fig. 2. Identification of the mutation by *DsaI* digestion of the amplified DNA.

The 127-bp sequence encompasses the codon 50 of the α_2 and α_1 genes. The forward and reverse primers are located at positions 226 and 353 upstream from the Cap site of the α genes. Lane 1, control DNA from a healthy subject; lane 2, DNA from an Hb J Sardegna carrier; lane 3, size markers; lane 4, amplified undigested DNA.

in the DNA from a healthy subject represents a small amount of amplified partially digested DNA.

The results of both isopropanol and heat precipitation tests for Hb stability were within reference values. Methemoglobin and carboxyhemoglobin were within reference values (0.5% and 2.5%, respectively). The whole blood of the Hb J Sardegna carrier displayed an oxygen equilibrium curve of standard sigmoidal shape and shifted towards the left side of the health-related reference range. After standardization, the P_{50} value was found to be 24.9 mmHg (reference range, 24.6–28.9 mmHg). The red cell 2,3-diphosphoglycerate concentration was slightly decreased (0.68 mol/mol Hb; reference range, 0.77–1.07 mol/mol Hb).

Cellulose acetate electrophoresis revealed the presence of an abnormal band on the anode side of Hb A₀ and migrating in a position consistent with a difference of two negative charges compared with normal Hb. Isoelectric focusing (Fig. 3) demonstrated the presence of two abnormal Hb components. The first component, amounting to 23% of the total Hb, displayed a much more acid isoelectric point than Hb A₀; the second component focused slightly to the anode side of Hb A₀ and was 5% of the total Hb.

The separation of Hb by cation-exchange HPLC is shown in Fig. 4. The elution profile showed an anomalous Hb fraction that eluted just after the Hb A_{1c} peak, in the Hb F position (indicated as X₁ in Fig. 4). It amounted to ~25% of the total Hb. A second difference was a distinct increase in the proportion of the Hb A_{1d} peak, probably suggesting the presence of an additional Hb component that eluted in the same position (indicated as X₂ in Fig. 4). This peak was ~9% of the total Hb, whereas in fresh hemolysates from healthy individuals it never exceeded 3%. The Hb A₂ concentration was slightly reduced (1.8%), whereas Hb F was not detectable because it was over-

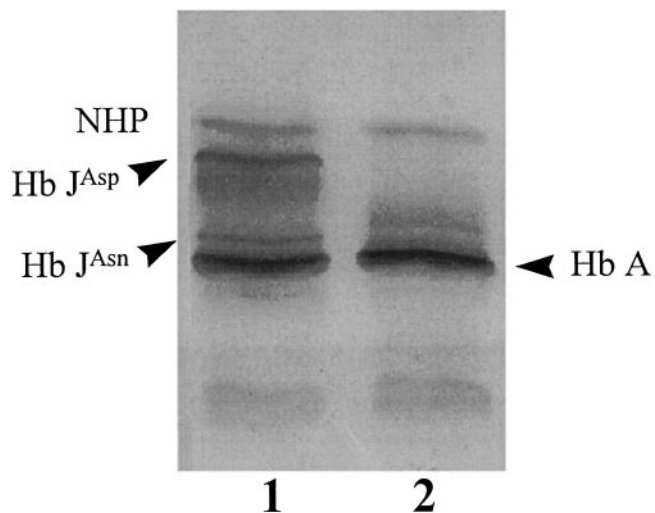


Fig. 3. Isoelectric focusing pattern at pH 6–8.

The anode is at the top. Lane 1, hemolysate from an Hb J Sardegna carrier; lane 2, hemolysate from healthy control; NHP, non-hemoglobin proteins.

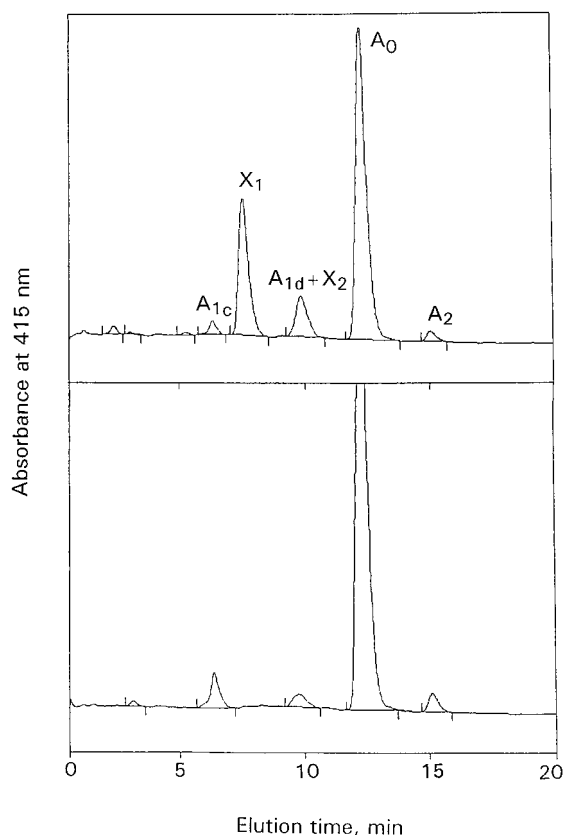


Fig. 4. Cation-exchange HPLC separation of whole blood hemolysates prepared from an Hb J Sardegna carrier (top) and from a healthy subject (bottom).

X₁, Hb J^{ASP}; X₂, Hb J^{ASN}.

lapped by the variant peak. This elution pattern was confirmed in all Hb J Sardegna carriers examined. HPLC analysis of the hemolysate from one Hb J Sardegna carrier was periodically repeated during a 20-day storage at 4 °C. The elution profiles showed no evident differences in the relative proportion of the different Hb fractions. HPLC analysis of Hb was also performed in culture-grown erythroid cells obtained from an Hb J Sardegna carrier. The results (not reported in detail here) showed that Hb composition of BFU-e-derived erythroblasts was similar to that observed in the hemolysate prepared from peripheral blood.

The separation of globin chains from whole hemolysate obtained by analytical reverse-phase HPLC is shown in Fig. 5. Three main peaks were present, corresponding to β chains, native α chains, and more hydrophilic variant α chains, which accounted for 24% of the total α subunits. Globin chain biosynthesis was analyzed in BFU-e-derived erythroblasts and indicated an α -chain/non- α -chain ratio of 1.02, suggesting a balanced rate of synthesis of the globin chains. Furthermore, the HPLC elution pattern presented no different peaks with respect to those detected in the separation performed on peripheral blood.

For structural studies, the variant α chains were sepa-

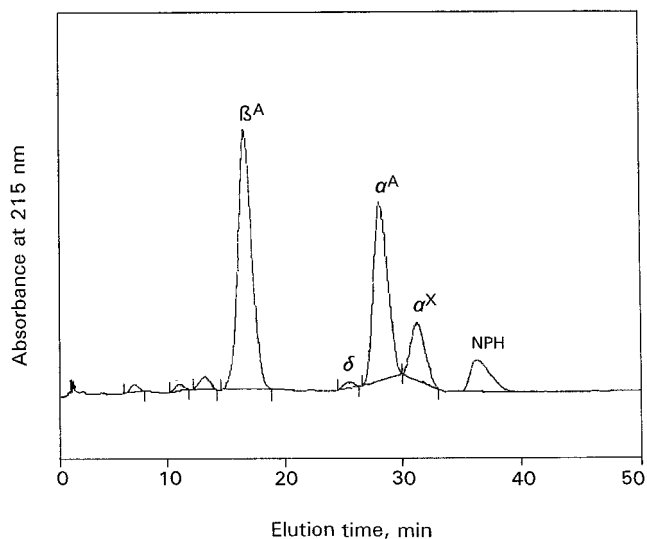


Fig. 5. Reverse-phase HPLC analysis of globin chains from an Hb J Sardegna carrier.

β^A , normal β chain; δ , normal δ chain; α^A , normal α chain; α^X , variant α chain; NPH, non-hemoglobin proteins.

rated by preparative ion-exchange chromatography and digested with trypsin. The separation of tryptic peptides from native and variant α chains by reverse-phase HPLC is shown in Fig. 6. From the comparison of the tryptic digests, it was noted that one peptide peak was missing, whereas a new, more hydrophilic peak was present. N-terminal sequence analysis revealed this new peptide as $\alpha 41-56$, which corresponds to an $\alpha T-6$ peptide in which the His at position 50 was replaced by Asp. During the N-terminal sequencing of tryptic peptide 6, the expected amount of L-aspartic acid was recovered. This finding should exclude the presence of isoaspartic acid, because it probably would not be released efficiently during the Edman degradation. Moreover, no other additional peaks,

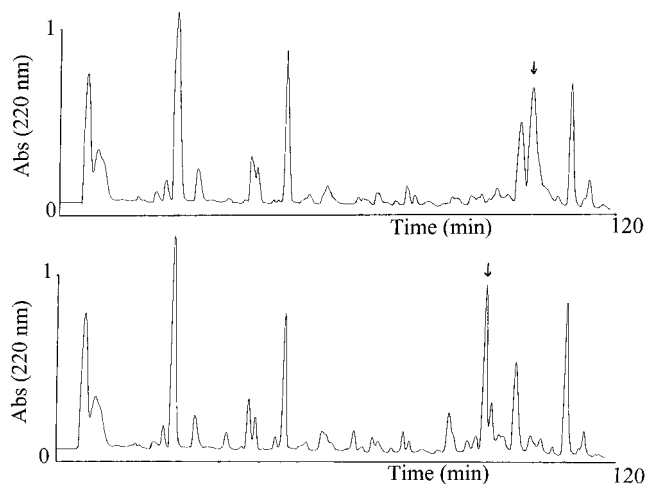


Fig. 6. Elution profile obtained by reverse-phase HPLC separation of tryptic digests of normal (top) and mutant (bottom) α chains.

The arrows indicate the corresponding T-6 peptides.

with the exception of a small quantity of Ala, were detected. The small amount of Ala was probably derived from cross-contamination of the chromatographic peak with $\beta T-9$ peptide, which carry Ala in position 10.

Discussion

We have reported the characterization of Hb J Sardegna by both DNA and protein analyses. The peculiar feature that emerged from our data was an evident disagreement between nucleotide sequence and protein analysis, the first showing a His \rightarrow Asn mutation, the latter showing a His \rightarrow Asp substitution in the same position. Moreover, we have proven that the mutation occurs in the α_2 gene because the subjects heterozygous for Hb J Sardegna and α -thalassemia (deletion of one of the two α_2 genes) possess an α_2 gene sequence with no trace of the normal sequence of the α^A -globin. Furthermore, the abnormal Hb seemed to be present as two fractions. One possible explanation for these findings is that a C \rightarrow A mutation occurs in codon 50 of the α -globin gene, which causes the substitution of His for Asn, and that the new $\alpha 50$ Asn residue subsequently undergoes a posttranslational partial deamidation to Asp.

Indeed, two forms of Hb J Sardegna were found. Hb J Sardegna-Asp was easily seen in the ion-exchange HPLC chromatogram, as well as in the electrophoretic and isoelectrophoretic pattern, because it is more negatively charged than Hb A₀. The presence of Asp at position $\alpha 50$ was confirmed by the Edman degradation. The Hb fraction that coeluted with Hb A_{1d} in an intermediate position between Hb J Sardegna-Asp and Hb A₀ is probably Hb J Sardegna-Asn, in agreement with a difference of charge attributable to the replacement of the slightly protonated His by a neutral amino acid. No evidence for two abnormal α chains was seen, however, in reverse-phase HPLC separation of globin chains where only variant α -Asp chains could be detected. The α -Asn chains have polar properties similar to normal α chains and would be expected to coelute with them. Therefore, we were not able to purify α -Asn chains, which are present in very small amounts, to prove the presence of Asn in position 50 by Edman degradation. However, the presence of α -Asn chains can be inferred from the results of the isoelectric focusing.

In previous reports on Hb J Sardegna, the fraction carrying Asn had never been observed, probably because its detection requires high resolution techniques. Manca and Masala (3), in their paper on the identification of Hb J Sardegna by means of isoelectric focusing, reported the presence of an additional band focusing closer to the anode than Hb A₀ and accounting for 3.5–11.6% of the total Hb; however, they did not identify it. It is reasonable to suppose that this unknown band corresponds to Hb J Sardegna-Asn.

In an attempt to verify where the conversion of Hb J Sardegna-Asn to Hb J Sardegna-Asp occurs, we have analyzed the Hb composition of erythroid progenitor

cells. The fraction containing Asp that was found in BFU-e-derived erythroblasts suggests that the conversion of Asn to Asp takes place in a very early phase of red cell maturation. Afterward, the proportions of the two Hb fractions do not seem to change in peripheral blood or in vitro during sample storage. On the other hand, in one study of Hb Redondo, deamidation reportedly occurred rapidly in vitro, and after a few days of storage at 4 °C the relative proportions of the two fractions changed (19).

In addition to Hb J Sardegna, six other rare Hb variants have been reported in which deamidation of an Asn residue to an Asp occurs as a spontaneous posttranslational modification. A list of these variants, including Hb J Sardegna, is given in Table 1. The mutation, the amino acid sequence surrounding the Asn undergoing deamidation, and the relative percentage of the two protein forms containing, respectively, Asn or Asp detected in blood have been reported for each variant. In most of these variants, including Hb J Sardegna, the deamidation reaction involves the mutant Asn itself, whereas in Hb La Roche-sur-Yon (20) and Hb J Singapore (21) the variant amino acids, a Gly and a His, respectively, induce the deamidation of the adjacent Asn residue. To support the possibility of the existence of particular tridimensional structures and sequences favoring deamidation reaction, it may be observed that in three of the seven Hb variants—Hb J Singapore, Hb La Roche-sur-Yon, and Hb Providence (22)—the deamidation is localized within the EF corner, and in Hb Osler (23) it occurs in the C-terminal region, which exhibits a flexible local conformation. Moreover, the sequences flanking the Asn site often include amino acid residues known to be potentially catalytic in the deamination reaction (see below). In particular, in Hb J Sardegna the α 50 Asn residue is located near a serine residue at position α 49 and a Gly residue at position α 51. In all reported cases, the Asn residue is only partially deamidated, as proven by the finding that both of the Hb forms, containing either Asn or Asp, are present in peripheral blood. Hb Redondo occurs in rather low proportion to Hb A₀ because it is a highly unstable variant (19), whereas the unusually low percentage of Hb Wayne is probably the result of an increased rate of destruction

because of a relevant structural abnormality of the 3' terminal region (24).

Indeed, the molecular mechanism proposed for deamidation reactions of Asn residues involves the formation of a cyclic succinimidyl intermediate from whose opening aspartyl and isoaspartyl residues are formed (25). The reaction is influenced by different factors, including both primary and tertiary structural characteristics of the protein and general properties of the media surrounding the protein (26). From the comparison of amino acid sequences in model peptides, it has been noted that the Asn residue undergoing deamidation is frequently found nearby a residue with a small, nonbulky side chain, such as Gly, Ser, or Ala, which can be contiguous in sequence or brought close to the amide residue by the tertiary structure of the protein (27). Other studies point out that Asn is more susceptible to deamidation when it has a basic or acid residue on one side and a threonine, serine, or cysteine residue on the other side (28). In addition to the nature of neighboring residues, the polypeptide conformation seems to be equally important. It has been shown, in fact, that a high degree of flexibility of the polypeptide chain and the proximity of a His residue are required for the deamidation reaction (20, 29). Moreover, the Gln residues also may undergo posttranslational deamidation (27), and it has been postulated that these deamidations may serve as molecular timers that control protein turnover and aging (28). In addition, in the case of the deamidation of Gln residues, the polypeptide conformation and sequence, especially in the surroundings of Gln residues, has been proven to be important determinants for the occurrence of these spontaneous deamidations (27, 28, 30).

Apart from this proposed deamidation mechanism, there are other possible explanations, including errors in codon reading because of unusual mRNA structure or mischarging of tRNA (31). We did not test such hypotheses, but such alternative mechanisms do appear to take place rather rarely.

From a more general viewpoint, with regard to the eventual consequences of the deamidation of an Asn residue, it is important to remember that Asn residues are

Table 1. Hb variants exhibiting posttranslational deamidation of an Asn residue.

Variant Hb	Mutation	Sequence	Asn, %	Asp, %
J Sardegna	α 50(CD8)His→Asn→Asp	–Ser–Asn–Gly–	6	25
J Singapore	α 79(EF7)Ala→Gly α 78(EF7)Asn→Asp	–Pro–Asn–Gly–	ND ^a	ND
La Roche-sur-Yon	β 81(EF5)Leu→His β 80(EF5)Asn→Asp	–Ala–Asn–His–	15	12
Osler	β 145(HC2)Tyr→Asn→Asp	–Lys–Asn–His–	ND	28
Providence	β 82(EF6)Lys→Asn→Asp	–Leu–Asn–Gly–	19	32
Redondo	β 92(F8)His→Asn→Asp	–Leu–Asn–Cys–	10	6
Wayne	α 139(H17)Lys→Asn→Asp elongated chain	–Ser–Asn–Thr–	3	3

^a ND, not determined.

important determinants of posttranslational self-splicing (32), and that there is relatively extensive literature about deamidation of Asn residues, especially concerning well-characterized proteins of pharmaceutical interest (26, 33). Therefore, we strongly recommend that, in the case of mutant proteins, both DNA and protein structure analysis should be undertaken to fully characterize the mutation, especially when Asn or Gln residues have been introduced in the mutant protein.

In conclusion, in consideration of the widespread use of DNA techniques for the molecular characterization of mutant proteins, we would like to note that protein structural studies are sometimes not performed or are performed as a second step. We report here some cases in which, to our knowledge, only DNA analysis has been exploited to characterize the mutations: (a) factor V Leiden (1696G→A, 506Arg→Gln), a mutant of factor V associated with resistance to activated protein C known to be a genetic risk factor for venous thrombosis (34); (b) the first discovered apolipoprotein B-100 mutant (10708G→A, 3500Arg→Gln), which causes familial defective apolipoprotein B-100, an autosomal disorder associated with hypercholesterolemia (35); (c) a recently discovered polymorphism in human orosomucoid (*ORM1*F1* 60CAA→CAG) (36); and (d) a variant of red cell pyruvate kinase (1529G→A, 509Arg→Gln), which causes severe nonspherocytic hemolytic anemia (37). We hope that this missing information (i.e., protein structure analysis) will soon be available to define these abnormal proteins completely.

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References

1. Tangheroni W, Zorcolo G, Gallo E, Lehmann H. Haemoglobin J Sardegna: $\alpha 50(\text{CD}8)\text{Histidine}\rightarrow\text{Aspartic Acid}$. *Nature* 1968;218:470-1.
2. Masala B. Hemoglobinopathies in Sardegna. *Hemoglobin* 1992;16:331-51.
3. Manca L, Masala B. Identification of Hb J-Sardegna [$\alpha 50(\text{CE}8)\text{His}\rightarrow\text{Asp}$] by HPLC and its incidence in northern Sardinia. *Hemoglobin* 1989;13:33-44.
4. Gallo E, Pugliatti L, Ricco G, Pich PG, Pinna G, Mazza U. A case of haemoglobin J Sardegna- α -thalassaemia double heterozygosis. *Acta Haematol* 1972;47:311-20.
5. Meloni T, Pilo G, Camardella L, Concedda F, Lania A, Pepe G, Luzzato L. Coexistence of three hemoglobins with different α -chains in two unrelated children (with family studies indicating polymorphism in the number of α -globin genes in the Sardinian population). *Blood* 1980;55:1025-32.
6. Maccioni L, Galanello R, Melis MA, Cao A. Hemoglobin constitution of double heterozygotes for α or β -thalassaemia and Hb J Sardegna. *Hemoglobin* 1984;8:497-507.
7. Dode C, Krishnamoorthy R, Lamb J, Rochette J. Rapid analysis of α -3.7 thalassaemia and alpha alpha alpha anti 3.7 triplication by enzymatic amplification analysis. *Br J Haematol* 1993;83:105-11.
8. Bowden DK, Vickers MA, Higgs DR. A PCR-based strategy to detect the common severe determinants of alpha thalassaemia. *Br J Haematol* 1992;81:104-8.
9. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain termination inhibitors. *Proc Natl Acad Sci U S A* 1977;74:5463-7.
10. Chomeczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-9.
11. US Department of Health and Human Services, Public Health Service. Laboratory methods for detecting hemoglobinopathies. Atlanta, GA: Centers for Disease Control, 1984:45-58.
12. Fairbanks VF, Klee GG. Biochemical aspects of hematology. In: Tietz NW, ed. *Fundamentals of clinical chemistry*, 3rd ed. Philadelphia, PA: WB Saunders, 1987:803-6.
13. Mosca A, Paleari R, Rubino FM, Zecca L, De Bellis G, Debernardi S, et al. Hb Abruzzo [$\beta 143(\text{H}21)\text{His}\rightarrow\text{Arg}$] identified by mass spectrometry and DNA analysis. *Hemoglobin* 1993;17:261-8.
14. Wimberley PD, Burnett RW, Covington AK, Fogh-Andersen N, Maas AHJ, Muller-Plathe O, et al. Guidelines for routine measurement of blood hemoglobin oxygen affinity. *J Int Fed Clin Chem* 1991;3:81-6.
15. Clegg JB, Naughton MA, Weatherall JD. Abnormal human haemoglobins, separation and characterization of the α and β chains by chromatography, and the determination of two new variants, Hb Chesapeake and Hb J (Bangkok). *J Mol Biol* 1966;19:91-108.
16. Wilson JB, Lam H, Pravatmuang P, Huisman THJ. Separation of tryptic peptides of normal and abnormal α , β , and δ hemoglobin chains by high-performance liquid chromatography. *J Chromatogr* 1979;179:271-90.
17. Fibach E, Manor D, Oppenheim A, Rachmilewitz EA. Proliferation and maturation of human erythroid progenitors in liquid culture. *Blood* 1989;73:100-3.
18. Leone L, Monteleone M, Gabutti V, Amione C. Reversed-phase high-performance liquid chromatography of human haemoglobin chains. *J Chromatogr* 1985;321:407-19.
19. Wajcman H, Vasseur C, Blouquit Y, Esperito Santo D, Peres MJ, Martins MC, et al. Hemoglobin Redondo [$\beta 92(\text{F}8)\text{His}\rightarrow\text{Asn}$]: an unstable hemoglobin variant associated with heme loss which occurs in two forms. *Am J Hematol* 1991;38:194-200.
20. Wajcman H, Kister J, Vasseur C, Blouquit Y, Trastour JC, Cotenceau D, Galacteros F. Structure of the EF corner favors deamidation of asparaginyl residues in hemoglobin: the example of Hb La Roche-sur-Yon [$\beta 81(\text{EF}5)\text{Leu}\rightarrow\text{His}$]. *Biochim Biophys Acta* 1992;1138:127-32.
21. Blackwell RQ, Hock Boon W, Liu CS, Weng MI. Hemoglobin J Singapore: $\alpha 78\text{Asn}\rightarrow\text{Asp}$; $\alpha 79\text{Ala}\rightarrow\text{Gly}$. *Biochim Biophys Acta* 1972;278:482-90.
22. Moo-Penn WF, Jue DL, Bechtel KC, Johnson MH, Schmidt RM, McCurdy PR, et al. Hemoglobin Providence. A human hemoglobin variant occurring in two forms in vivo. *J Biol Chem* 1976;251:7557-62.
23. Kattamis AC, Kelly KM, Ohene-Frempong K, Reilly MP, Keller M, Cubeddu R, et al. Hb Osler [$\beta 145(\text{HC}2)\text{Tyr}\rightarrow\text{Asp}$] results from posttranslational modification. *Hemoglobin* 1997;21:109-20.
24. Seid-Akhavan M, Winter WP, Abramson RK, Rucknagel DL. Hemoglobin Wayne: a frameshift mutation detected in human hemoglobin alpha chains. *Proc Natl Acad Sci U S A* 1976;73:882-6.

25. Galletti P, Ingrosso D, Manna C, Clemente G, Zappia V. Protein damage and methylation-mediated repair in the erythrocyte. *Biochem J* 1995;306:313–25.
26. Wright HT. Nonenzymatic deamidation of asparaginyl and glutaminyl residues in proteins. *Crit Rev Biochem Mol Biol* 1991;26:1–52.
27. Wright HT. Sequence and structure determinants of the nonenzymatic deamidation of asparagine and glutamine residues in proteins. *Protein Eng* 1991;4:283–94.
28. Robinson AB. Evolution and the distribution of glutamyl and asparaginyl residues in proteins. *Proc Natl Acad Sci U S A* 1974;71:885–8.
29. Clarke S. Propensity for spontaneous succinimide formation from aspartyl and asparaginyl residues in cellular proteins. *Int J Peptide Protein Res* 1987;808–21.
30. Ingrosso D, Clarke S. Human erythrocyte D-aspartyl/L-isoaspartyl methyltransferases: enzymes that recognize age-damaged proteins. *Adv Exp Med Biol* 1991;307:263–75.
31. Momand JA, Clarke S. The fidelity of protein synthesis: can mischarging by aspartyl-tRNA (Asp) synthetase lead to the formation of isoaspartyl residues in proteins? *Biochim Biophys Acta* 1990;1040:153–8.
32. Clarke ND. A proposed mechanism for the self-splicing of proteins. *Proc Natl Acad Sci U S A* 1994;91:11084–8.
33. Bischoff R, Kolbe HV. Deamidation of asparagine and glutamine residues in proteins and peptides: structural determinants and analytical methodology. *J Chromatogr B* 1994;662:261–78.
34. Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994;369:64–7.
35. Soria LF, Ludwig EH, Clarke HRG, Vega GL, Grundy SM, McCarthy BJ. Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. *Proc Natl Acad Sci U S A* 1989;86:587–91.
36. Yuasa I, Umetsu K, Vogt U, Nakamura H, Nanba E, Tamaki N, Irizawa Y. Human orosomucoid polymorphism: molecular basis of the three common *ORM1* alleles, *ORM1*F1*, *ORM1*F2*, and *ORM1*S*. *Hum Genet* 1997;99:393–8.
37. Rouger H, Valentin C, Craescu CT, Galacteros F, Cohen-Solal M. Five unknown mutations in the LR pyruvate kinase gene associated with severe hereditary nonspherocytic haemolytic anemia in France. *Br J Haematol* 1996;92:825–30.