Two new mutations of the P5'N-1 gene found in Italian patients with hereditary hemolytic anemia: the molecular basis of the red cell enzyme disorder

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Inherited pyrimidine 5'-nucleotidase type-1 (P5'N-1) deficiency is the most frequent abnormality of red cell nucleotide metabolism causing non-spherocytic hemolytic anemia. We describe two novel mutations in two Italian patients affected by P5'N-1 deficiency. One mutation is a two base deletion that occurs at the splice site junction between intron 7 and exon 8 (c.396-397del AG); the second is an in-frame deletion of three adjacent bases (c.427-429del CAA), leading to deletion of glutamine 143. The kinetic properties of Q143del variant were not grossly altered, but the variant was very heat unstable even at physiological temperatures.

Key words: P5'N-1 deficiency, hemolytic anemia, mutations, recombinant mutant.

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Hereditary pyrimidine 5'-nucleotidase type-1 (P5'N-1) deficiency is the most frequent disorder of nucleotide metabolism causing hereditary non-spherocytic hemolytic anemia.[1,2] It is an autosomal recessive disease and is characterized by mild-to moderate hemolytic anemia, reticulocytosis and hyperbilirubinemia. The hallmark of this red cell enzyme disorder is the presence of pronounced basophilic stippling in red blood cells (RBC) on a peripheral blood smear, associated with accumulation of pyrimidine nucleotides within the erythrocytes.[3,4]

P5'N-1 (EC 3.1.3.5) also known as uridine monophosphate hydrolase type-1 (UMPH-1) catalyzes the dephosphorylation of pyrimidine 5'-ribose monophosphates, especially UMP and CMP, allowing them to diffuse out of the cell as nucleosides. The enzyme has no activity on purine nucleotides.[3,5] Its major role is in the catabolism of the pyrimidine nucleotides, mainly resulting from ribosomal RNA degradation during final erythroid maturation.[6] The enzyme also transfers phosphate groups from pyrimidine nucleotides to pyrimidine nucleosides, suggesting that it has an additional role in nucleotide metabolism.[5] The P5'N-1 activity is dependent on magnesium ions (Mg^{2+}) and is readily inhibited by heavy metal ions, and metal chelating and thiol reactive reagents. P5'N-1 is a 286 amino acid-long monomer with an apparent molecular mass of approximately 34 kDa.[7] The gene encoding P5'N-1 (*NT5C3, UMPH-1*) has been mapped to chromosome 7p15-p14.[8] It consists of 11 exons leading to the production of three alternatively spliced mRNA.[9] Presently, only the 286 amino acid protein has been isolated from RBC.[1,2]

Eighteen different mutations of P5'N-1 gene have been so far described in 30 unrelated families.[6,8-13] Recently, four pathological variants of the P5'N-1 were expressed in an *E. coli* system and characterized. The correlation between the molecular defects and the residual enzyme activity of the patients' blood samples suggested that *in vivo* deficiency of P5'N-1 is at least in part compensated by other nucleotidases.[7]

In this study we investigated two unrelated Italian patients affected by hereditary hemolytic anemia associated with P5'N-1 deficiency. We found two novel mutations, a two bp deletion at the splice site junction between intron 7 and exon 8 (c.396-397del AG) and an in-frame deletion of one codon (c.427-429del CAA) corresponding to glutamine 143. The pathological variant resulting from the deletion of glutamine 143 (Q143del) was produced in a recombinant form, purified, and characterized.

Design and Methods

Patients

Case 1. The propositus was a 37-year old woman originating from southern Italy who had been affected by moderate chronic hemolytic anemia since childhood. She also had jaundice, hepatomegaly and gallstones,

but no splenomegaly. The patient needed one blood transfusion during a pregnancy.

Case 2. The propositus, a 37-year old woman originating from Northern Italy, had a history of chronic hemolytic anemia since childhood with jaundice, splenomegaly, and hepatomegaly. She was found to be a double heterozygote for HbD Punjab and Hb Zurich, which did not, however, justify the degree of hemolysis. Bone marrow morphology showed mild dyserythropoiesis; ferrokinetic studies revealed a slightly increased iron turnover associated with increased peripheral hemolysis. At the age of 14 years she had undergone cholecystectomy and splenectomy, without any significant increase in hemoglobin values. Before splenectomy she had had low serum iron levels (55 μg/dL). Thereafter, progressive rises in serum ferritin and transferrin saturation were observed associated at the age of 22 with marked hepatic siderosis at liver biopsy and increased cardiac iron burden detected by magnetic resonance imaging. The patient was treated with desferrioxamine. At the age of 34 blood transfusions (4U) were required because of exacerbation of anemia (Hb 4.7g/dL) during parvovirus B19 infection.

Hematologic assays, enzyme detection and molecular studies

Hematologic investigations were carried out as reported elsewhere.[12] The ratio of purine/pyrimidine nucleotides was calculated as described by Beutler *et al*.[4] P5'N-1 activity was determined by micellar electrokinetic chromatography.[14] After obtaining informed consent, genomic DNA was extracted from leukocytes. The entire coding region and intronic flanking regions of the P5'N-1 gene were analyzed as described previously.[12] The nucleotide numbering adopted is referred to cDNA reported in EMBL (coding sequence) accession no. AAF36153, whereas amino acid numbering is according to Swiss-Prot accession no. Q9H0P0-2.

Construction of pEA9 coding for the Q143del mutant form.

The DNA sequence encoding the mutant enzyme Q143del was obtained by a two-step total gene synthesis method.[15] pEA1, a derivative of pET23b(+) containing the P5'N-1 cDNA, was used as the template.[7] 5'-GAGAATTTCTTTGATAAGCTCCAACATAGCATCCCCGTGTTC-3' and 5'-GAACACGGGGATGCTATGTTGGAGCTTATCAAAGAAATTCT-3', as sense and antisense mutagenic oligonucleotides, respectively, and T7 Reverse and T7 Forward as outer primers, were used. The construct, pEA9, was checked by sequencing.

Expression, purification and characterization of the Q143del enzyme

The mutant protein was expressed in *E. coli* BL21(DE3)pLysS transformed with pEA9 by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (21°C, 12 hours) and purified using the method previously reported.[7] Kinetic analyses, thermal stability assays and far-UV circular dichroism (far-UV CD) measurements were performed as described in the same report.[7]

Table 1. Hematologic data of the two P5'N deficient patients at the time of the study.

	Case 1	*Case 2*	*Reference range*
Hb (g/dL)	8.4	9.3	11.8-14.7
MCV (fL)	106	124	82-99
Reticulocytes (10^9/L)	300	752	24-84
Unconjugated bilirubin (UI/L)	54.4	226	<12.8
Lactate dehydrogenase (U/L)	1218	440	230 - 460
Serum ferritin (μg/L)	36	1980	19-238
Transferrin saturation (%)	45	100	16-54
Osmotic fragility	normal	normal	

Results and Discussion

P5'N-1 deficiency (OMIM 606224) has been suggested to be the third most common hereditary red cell enzyme disorder causing hemolysis after glucose 6-phosphate dehydrogenase and pyruvate kinase deficiencies.[8] It can also be acquired as a result of lead poisoning or oxidative threat.[6,10] In this study, we investigated two Italian patients affected by non-spherocytic hemolytic anemia who were found to have two new mutations in the gene encoding P5'N-1.

The hematologic data at the time of the study are reported in Table 1. In case 1 the molecular analysis of the P5'N-1 gene showed the presence of a new two bp homozygous deletion (c.396-397del AG); given the DNA sequence (cttacagAGAAGGAT), it is not possible to determine whether the deletion affects the last bases of intron 7 or the first bases of exon 8. In any case, the deletion may affect the splice site resulting in a splicing alteration and in the absence of a functional protein in RBC. Moreover, if splicing should occur, the two bp deletion would generate a frameshift and a premature stop codon at position 135. A functional protein is not expected in this situation either. In fact, P5'N-1 activity could not be detected in the patient's RBC, despite using a very sensitive detection method such as capillary electrophoresis.[14] Intriguingly, the degree of anemia was moderate, suggesting that there is a mechanism of compensation, as there is in other enzyme deficiencies.[16-17]

In case 2 complete sequencing of the P5'N-1 gene showed that the patient was a compound heterozygote. One allele had the frameshift mutation c.710-711insGG, already described in Italian and Turkish patients,[11,12] while the second allele showed a new in-frame deletion of three base pairs (c.427-429del CAA), leading to dele-

Table 2. The main features of recombinant Q143del mutant P5'N-1.

	kinetics						thermal stabilty		
	CMP			UMP					
enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	$t_{1/2}$ 37°C (min)	$t_{1/2}$ 46°C (min)	T_{50} (°C)
wild type	26.1±0.7	0.18±0.02	145	11.9±0.3	0.056±0.006	213	>120	>120	51.5
Q143del	19.8±0.7	0.16±0.01	124	14.7±0.5	0.170±0.010	86	38	3	43.5

tion of Q143. The c.710-711insGG mutation is predicted to lead to a truncated enzyme lacking 42 residues at the C-terminus. Thus, conceivably, the gene product is completely unable to display any activity. To evaluate the effect of Q143del on the activity of P5'N-1, the mutant enzyme was produced in *E. coli* as a recombinant form, purified to homogeneity and characterized. Overall, the Q143del mutant enzyme behaved as the wild-type enzyme, exhibiting similar kinetic parameters towards UMP and CMP (Table 2). Only a small decrease (2.5 fold) of the specificity constant (k_{cat}/K_m) for UMP was observed (86 vs 213 $s^{-1}mM^{-1}$), due to an increase of the Km value. Thus, the Q143 deletion turned out to have only marginal effects on the catalytic properties of the enzyme, strongly suggesting that it is not involved in the catalytic site, as supported by structural data.[18]

To unravel the cause of the lowered enzyme activity, the stability of the mutant form was investigated by thermal inactivation assays and circular dichroism analysis. Treated at 46°C, the enzyme showed high thermal instability with the loss of 50% of its initial activity within the first 3 minutes of incubation (Table 2). Moreover, even at 37°C the mutant enzyme was very unstable, with its activity halved within 38 minutes of incubation (Table 2). The evaluation of the behavior of the Q143del mutant in a wider range of temperatures (25°C-60°C) led to a better appreciation of the reduced stability of the mutant with respect to the wild-type enzyme (Figure 1A). The T_{50} of the mutant enzyme was 8°C lower than that of the wild-type protein (Table 2).The marked instability of the Q143del mutant enzyme is also supported by the findings of the far-UV circular dichroism analysis carried out at 25°C and 37°C (Figure 1B). Compared to the wild type enzyme, Q143del displayed altered α-helical and β-sheet content (15%±2% and 24%±1% vs 37%±2% and 26%±1% for the wild type) even at 25°C. A substantial drop of the α-helical content was observed when the temperature was increased to 37°C (3.2%±0.2% and 22%±1% for α-helices and β-sheets, respectively). Therefore, the deletion of Q143 destabilizes the secondary structure of the enzyme which leads to premature intracellular degradation *in vivo*. The mutation is similar to the pathological variant L131P, which is also func-

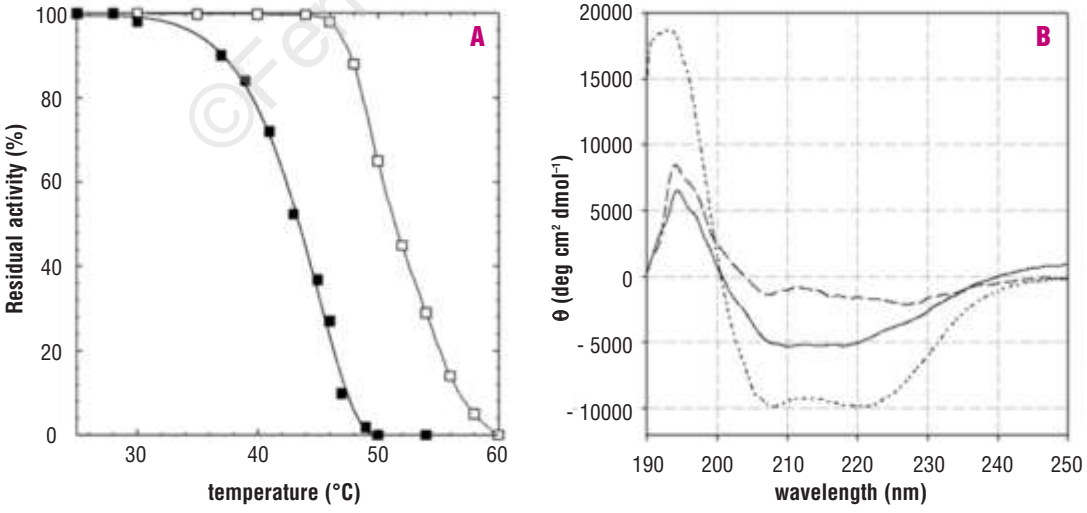

Figure 1. Thermal stability of recombinant wild-type and Q143del mutant P5'N-1. **A.** Plot of the residual activities at 5 minutes versus temperatures. Wild type (open symbol) and Q143del mutant enzyme (filled symbol) were subjected to heat inactivation in a given range of temperatures. Residual activity is expressed as a percentage of initial activity. **B.** Far-UV circular dichroism spectra of wild-type (dotted line, 25°C and 37°C) and Q143del mutant enzyme (solid line, 25°C; dashed line, 37°C). Observed ellipticities were converted to mean residue ellipticity (θ=deg cm^2 $dmol^{-1}$).

tional, but highly unstable ($t_{1/2}$ at 37°C about 5 min) and susceptible to proteolysis.[7,9] In the present case, the residual activity was rather high (40%). This may be due, at least in part, to the elevated number of reticulocytes that, in normal conditions, are known to display a much higher enzyme activities than older cells. The mutated enzyme, although unstable, is catalytically efficient and is therefore likely to be fully active in younger cells, but then very rapidly degraded during RBC maturation. However, the presence of compensatory mechanisms such as up-regulation of other nucleotidases and/or alternative pathways for nucleotide metabolism cannot be excluded.[7] It is worth noting that the P5'N deficiency in propositus 2 was found in association with HbD Punjab and Hb Zurich. The presence of associated hemoglobin variants could have exacerbated the clinical pattern in this patient, as reported for HbE.[19] Moreover, this patient had overt iron overload requiring iron chelation. Molecular analysis of the HFE gene showed that she was also heterozygous for the H63D mutation; this mutation, which does not affect iron homeostasis in normal conditions, may result in iron overload in co-operation with other genetic or non-genetic factors.[12,20]

In conclusion, as already observed,[7] the severity of the phenotype is not directly related to the enzyme activity detected in RBC and this in turn is not a simple and direct consequence of the genotype. In fact, the clinical manifestations of a genetic disease reflect the interactions of a variety of physiological and environmental factors and do not solely depend on the molecular properties of the altered molecule. Caution should be exercised in drawing prognostic conclusions only from genotypic information.

AZ recruited the patients and contributed to the data interpretation. DCC provided clinical data. EF and PB analyzed hematologic data and performed the molecular studies. PA carried out the site-directed mutagenesis analysis. LRC carried out the biochemical experiments. GV and AG contributed to the study design, data interpretation and wrote the manuscript. All authors critically revised the manuscript and approved the final version to be published. None of the authors has any potential conflict of interest.

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