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Red cell pyruvate kinase deficiency: 17 new mutations of the *PK-LR* gene

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Summary

The *PK-LR* gene was studied in 23 patients with congenital haemolytic anaemia associated with erythrocyte pyruvate kinase (PK) deficiency. Twenty-seven different mutations were detected among the 42 mutated alleles identified: 19 missense mutations, four splice site mutations and one nonsense, one single base deletion and two large deletions. Seventeen of them (107G, 278T, 403T, 409A, 661A, 859C, 958A, 1094T, 1190T, 1209A, 1232C, 1369G, 507A, IVS9 −1c, IVS9 +43t, del C224, del 5006bp IVS3 → nt 1431) were new. Although all the exons, the flanking regions and the promoter were sequenced in all cases, we failed to detect the second expected mutation in four subjects. To correlate genotype to phenotype, the molecular results were related to the biochemical properties of the mutant enzymes by an analysis of the three-dimensional structure of erythrocyte PK. The new mutant 409A, found in association with the large deletion of 5006 bp in a newborn baby who died soon after birth, was functionally characterized by mutagenesis and *in vitro* expression of the protein to investigate its contribution in the severity of the clinical pattern. However, the biochemical data obtained for the mutant enzyme cannot explain the severe anaemia found in the PK-deficient patient hemizygous for this mutation.

Keywords: pyruvate kinase deficiency, *PK-LR* gene, chronic haemolytic anaemia, erythrocyte metabolism, mutations.

Pyruvate kinase (PK; ATP: pyruvate 2-*o*-phosphotransferase, EC 2.7.1.40) catalyses the final step of glycolysis, converting phosphoenolpyruvate (PEP) to pyruvate with the concomitant generation of ATP.

The PK deficiency, transmitted as an autosomal recessive trait, is the most frequent enzyme abnormality of glycolysis, with over 400 cases described (Zanella & Bianchi, 2000) since its detection in 1961 (Valentine *et al*, 1961). Clinical symptoms, ranging from neonatal jaundice requiring exchange transfusions to a fully compensated haemolytic anaemia (Mentzer & Glader, 1989), occur only in homozygotes and in compound heterozygotes for two mutant alleles. Heterozygotes are hematologically normal, with rare exceptions (Sachs *et al*, 1968; Kahn *et al*, 1976).

The *PK-LR* gene, specific for liver and red blood cell PK, is located on chromosome 1q21 (Satoh *et al*, 1988), where it directs tissue-specific transcription for both the liver-specific isozyme PK-L and the red blood cell-specific isozyme PK-R by the use of alternate promoters. The cDNA of the *R*-type is 2060 bp long and codes for 574 amino acids. The codifying region is split into 12 exons, 10 of which are common to the two isoforms, while exons 1 and 2 are specific for the erythrocytic and the hepatic isoenzyme, respectively (Tani *et al*, 1988; Kanno *et al*, 1991).

So far, more than 130 different mutations have been identified in PK deficiency (Bianchi & Zanella, 2000; Zarza *et al*, 2000; Zanella *et al*, 2001; van Wijk *et al*, 2003; Sedano *et al*, 2004; Costa *et al*, 2005). The majority of these mutations consist of single-base substitutions, resulting in amino acid changes of conserved residues in structurally and functionally important domains of PK. In contrast, mutations that affect *PK-LR* transcription (van Solinge *et al*, 1997; Kugler *et al*, 1999; Manco *et al*, 2000; van Wijk *et al*, 2003), or processing of pre-mRNA (Lenzner *et al*, 1994; Kanno *et al*, 1997; Zanella *et al*, 1997, 2001; Manco *et al*, 2002; van Wijk *et al*, 2004) are less frequent. Two large deletions have been described in PK deficiency, PK 'Gypsy' (Baronciani & Beutler, 1995) and PK 'Viet' (Costa *et al*, 2005).

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Here, we report the results of a molecular study of 23 patients affected by PK deficiency. Twenty-seven different mutations were detected, 17 of which were novel. Correlation was made between mutations, biochemical characteristics of the enzyme and clinical phenotypes by the analysis of the three-dimensional structure of erythrocyte PK. Moreover, the new mutant 409A, found in association with the large deletion of 5006 bp in a newborn baby who died soon after birth, was functionally characterized by mutagenesis and *in vitro* expression of the protein.

Materials and methods

Patients

Twenty-three patients (from 21 unrelated families) affected by congenital non-spherocytic haemolytic anaemia associated with PK deficiency were investigated. Fifteen were Italian, three from northern Europe, one Gypsy, one Australian, one from Guinea, one from Pakistan and one from Oman.

The most important clinical and haematological data at the time of the study are summarized in Table I. Most patients were newly diagnosed. With regard to Patient 1 (Zanella *et al*, 2001), the molecular diagnosis was completed in this work.

The patients underwent the following haematological investigations: complete blood count, reticulocyte count, serum bilirubin, iron status parameters, screening for abnormal or unstable haemoglobins (Hb), red cell osmotic fragility, autohaemolysis, direct antiglobulin test, Ham and sucrose haemolysis test, and assay of the most important erythrocyte enzyme activities of the glycolytic pathway and nucleotide metabolism (hexokinase, glucose phosphate isomerase, phosphofructokinase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, pyruvate kinase, triose phosphate isomerase, lactate dehydrogenase, enolase, aldolase, diphosphoglycerate mutase, monophosphoglycerate mutase, adenylate kinase) and hexose monophosphate shunt (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione reductase, glutathione peroxidase). The diagnosis of PK deficiency was made by exclusion of the most common causes of haemolytic anaemia and by demonstrating a reduced PK activity. The activities of the other investigated red cell enzymes were normal or increased in relation to reticulocytosis in all patients.

Table I. Clinical and haematological data of 23 PK-deficient patients grouped according to clinical phenotype.

Patient	Sex	Origin	Age at diagnosis (years)	Neonatal jaundice	ExTx	Tx (units)	Splenectomy	Hb (g/dl)	Retics (10^9/l)	Unconj bil (IU/l)	SF (μg/l)
Severe											
1	M	Northern Italy	3 months	Yes	No	>100	Yes	6·1	249	1·9	828
2	F	Central Italy	10 months	No	No	10	No	5·3	116	42·7	ND
3	F	Gypsy	7	Yes	Yes	>50	Yes	8·4	1347	ND	ND
4	M	Belgium	11	ND	ND	>50	No	8·2	305	128·2	25
5	F	Northern Italy	2	Yes	Yes	5	No	4·3	138	37·6	ND
6	M	Australia	Birth	Yes	Yes	0	No	8·9	ND	46·2	>4000
7	F	Central Italy	40	No	No	0	Yes	10·5	87	22·2	3530
8	M	Central Italy	33	Yes	Yes	>50	Yes	9	1166	130	2290
9	M	Central Italy	35	Yes	No	>50	Yes	9	990	71·8	3210
10	F	Central Italy	65	ND	ND	ND	Yes	12	139	12·5	540
11	F	England	2 weeks	Yes	No	8	No	2·2	101	39	ND
12	M	Oman	Birth	No	No	>30	No	6·7	36	35	2310
13	F	Pakistan	Birth	No	No	15	No	8	112	28	ND
Mild-to-moderate											
14	F	Southern Italy	8	Yes	Yes	2	No	10·3	146	39·3	ND
15	M	Northern Italy	16	Yes	Yes	0	No	12·4	54	27·4	109
16	F	Southern Italy	19	No	No	3	No	8·3	203	112·9	212
17	F	Belgium	44	No	No	0	No	8	200	47·9	600
18	M	Northern Italy	10 months	No	No	0	No	9·5	122	34·2	107
19	M	Central Italy	18	No	No	0	No	15·1	139	59·8	143
20	F	Southern Italy	14	Yes	Yes	2	No	10·1	187	104·3	146
21	F	Southern Italy	9	No	No	3	No	9·5	377	63·3	192
22	F	Northern Italy	9	No	No	0	No	13·2	140	39·3	33
23	F	Guinea	1 month	Yes	No	0	No	14·4	ND	312·9	ND
Reference values								12·2–16·7	24–84	<12	19–238

ExTx, exchange transfusion; Tx, transfusions; Unconj bil, unconjugated bilirubin; retics, reticulocyte count; SF, serum ferritin; ND, not determined; PK, pyruvate kinase; Hb, haemoglobin.

Ten patients displayed a moderate-to-mild phenotype (defined as Hb >8 g/dl, fewer than five transfusions, not splenectomized), and 13 a severe phenotype (presplenectomy Hb levels lower than 8 g/dl, and/or transfusion dependence, and/or splenectomized).

Haematological assays and enzymes studies

Routine haematological investigations were carried out according to Dacie and Lewis (2001). The assay of glycolytic and pentose phosphate pathway enzyme activities was performed as reported elsewhere (Zanella *et al*, 1976), following the methods of Beutler (1984). Serum ferritin (SF) was measured by an enzyme immune assay procedure (IMX System, Abbott Laboratories, Abbott Park, IL, USA).

Molecular analysis of the PK-LR gene

Blood samples were collected from patients and 50 normal controls from various Italian regions, after obtaining informed consent. Leucocytes were isolated and the genomic DNA was extracted using standard manual methods (Sambrook *et al*, 1989). The entire coding region and promoter of the *PK-LR* gene were amplified by polymerase chain reaction (PCR) (Zanella *et al*, 1997) and automatically sequenced (ABI-PRISM 310 Genetic Analyzer, Applied Biosystems, Warrington, UK) using the Big Dye Terminator Cycle Sequencing Kit.

The variant PK 'Gypsy' was identified by amplifying the genomic DNA with primers Gypsy-F (5′-GTT GGC AGG ATT AAC AGG AG-3′) and Gypsy-R (5′-GGA TGT GAC CAC TGC AGT A-3′), which give a deleted PCR product of 451 bp in patients carrying the mutation, in comparison with the 1600 bp of the normal allele.

The DNA of normal controls was investigated for the presence of missense mutations.

Reticulocyte mRNA extraction and cDNA analysis

The polysomal RNA was prepared from reticulocytes, by selectively lysing the red cells with ammonium chloride (Boyer *et al*, 1976); the ribonucleoproteins contained in the red cell lysate were precipitated at pH 5 and the RNA was extracted with phenol, chloroform and isoamyl alcohol (Goossens & Kan, 1981) and subsequently reverse transcribed (AMV reverse transcriptase, Roche, Mannheim, Germany) to cDNA using random primers.

The complete *PK-LR* cDNA was amplified by PCR (94°C for 30 s, 68°C for 30 s and 72°C for 60 s, five cycles; 94°C for 30 s, 65°C for 30 s and 72°C for 110 s, 30 cycles) using primers designed according to the cDNA sequence reported by Kanno *et al* (1991). The forward primer was 5′-ATT GGA GCT CAA GGA GGC TGA AAG CAT GTC GAT CCA GGA GA-3′ and the reverse primer was 5′-CCG GAA TTC AGG GGG GAG GGG CGT CTC AGG A-3′.

Identification of del 5006bp

To identify the deletion in Patient 6, the entire *PK-LR* cDNA obtained from father's reticulocytes was amplified by PCR and sequenced.

The deletion was confirmed in the baby's DNA using primers 3173F (5′-GAG ATC GAT ACC ATC CTG GC-3′), located in intron 3, and I9145B (5′-GCC TCC AGC TGT CAT TGC TG-3′) located in intron 10.

Production of mutant enzyme Ala137Thr (409A)

To obtain the mutant enzyme Ala137Thr, the recombinant vector pLC1, a derivative of pTrcHisB with an insert encoding *PK-R* (Valentini *et al*, 2002), was subjected to site-directed mutagenesis with Quick Change® XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The sense and antisense mutagenic oligonucleotides used were: 5′-CGC CAA CGT CCG GGA GAC GGT GGA GAG CTT TGC-3′ and 5′-GCA AAG CTC TCC ACC GTC TCC CGG ACG TTG GCG-3′. The underlined letters indicate the mutated bases. PCR was performed as follows: 95°C for 50 s, 60°C for 50 s, 68°C for 12 min, 18 cycles. The appropriate substitution and the absence of unwanted mutations were confirmed by sequencing the insert.

Expression and purification of the mutant protein were performed according to the method previously reported (Valentini *et al*, 2002). Enzyme activities were measured at 37°C by the assay recommended by the International Committee for Standardization in Haematology (Miwa *et al*, 1979).

The kinetic parameters (Wang *et al*, 2001) were determined with the Enzyme Kinetic Module™ 1.1 [Statistical Package for the Social Sciences (SPSS) Science Software Gmb]. The thermal stability was measured by incubating the enzyme (100–200 μg/ml) at 53°C in a solution consisting of 20 mmol/l potassium phosphate, pH 6·5, 100 mmol/l KCl and 1 mmol/l ethylenediaminetetraacetic acid (EDTA), in the absence and in the presence of 1 mmol/l fructose 1,6-bisphosphate (FBP).

Results

Molecular studies

Table II reports the molecular data of 23 PK-deficient patients from 21 unrelated families. Three patients were found to be homozygous. Twenty-seven different mutations were detected among the 42 mutated alleles identified: 19 missense mutations (107G, 278T, 403T, 409A, 661A, 859C, 958A, 994A, 1094T, 1178G, 1190T, 1209A, 1232C, 1369G, 1456T, 1484T, 1492T, 1529A, 1706A), four splice site mutations (507A, IVS9 −1c, 1269A, IVS9 +43t), one nonsense mutation (721T), one single base deletion (del C224) and two large deletions (del 1149bp, Gypsy; del 5006bp IVS3 → nt 1431). Seventeen of the mutations were new.

Table II. Molecular data of 23 PK-deficient patients grouped according to clinical phenotype (mutations reported in bold are novel).

Case	PK activity (IU/g Hb)	Mutation	Effect
Severe			
1	9·5	721T/**507A**	Glu241End/**splicing**
2	8·2	1456T/**278T**	Arg486Trp/**Thr93Ile**
3	5·4	Del 1149bp 'Gypsy'/Del 1149bp 'Gypsy'	Del ex11/Del ex11
4	2·0	1484T/**IVS9 −*1c***	Ala495Val/**splicing**
5	12·1	994A/994A	Gly332Ser/Gly332Ser
6	nd	**409A/del 5006bp (IVS3 → nt 1431)**	**Ala137Thr/del ex 4-11**
7	10·7	1706A/?	Arg569Gln/?
8	5·3	**661A/1209A**	**Asp221Asn/Met403Ile**
9	5·5	**661A/1209A**	**Asp221Asn/Met403Ile**
10	5·5	1456T/**1209A**	Arg486Trp/**Met403Ile**
11	6·4	**1369G**/?	**Ile457Val**/?
12	5·3	**DelC244/DelC244**	**Frameshift/frameshift**
13	3·4	**403T/1190T**	**Arg135Trp/Asp397Val**
Mild-to-moderate			
14	5·9	1465T/**1232C**	Arg486Trp/**Gly411Ala**
15	3·2	1456T/721T	Arg486Trp/Glu241End
16	5·6	1529A/**859C**	Arg510Gln/**Phe287Leu**
17	2·4	1456T/1178G	Arg486Trp/Asn393Ser
18	13·6	1456T/**958A**	Arg486Trp/**Val320Met**
19	10·6	**107G**/?	**Ala36Gly**/?
20	3·9	1465T/994A	Arg486Trp/Gly332Ser
21	3·4	994A/1492T	Gly332Ser/Arg498Cys
22	8	**1094T**/?	**Lys365Met**/?
23	4·3	1269A/**IVS9 +43t**	Splicing/**splicing**
Reference values	11·1–15·5		

PK, pyruvate kinase; Hb, haemoglobin.

The large deletion of 5006 nucleotides was identified in a baby (Patient 6) who had died during exchange transfusion soon after birth; he was born to an Australian family with recurrent abortions within the first trimester of gestation. The direct sequencing of the *PK-LR* gene performed on the propositus led to the detection of a new mutation G409A (Ala137Thr) apparently at the homozygous level. The mutation was present at the heterozygous level in the mother but not in the father (Fig 1A). The study of the father's reticulocytes mRNA revealed a large cDNA deletion encompassing exons 4–11. The DNA analysis of father and baby showed the presence of a large deletion extending from intron 3 to the last nucleotides of exon 10 (Fig 1B).

Of the three new splicing mutations identified, 507A and IVS9 −1c were localized in the donor/acceptor sites; in mutation IVS9 +43t the effect on normal splicing was not clear, being localized in the internal portion of intron 9. The cDNA analysis from Patient 23 (1269A/IVS9 +43t) failed to detect any *PK-LR* transcript, suggesting that mutations 1269A and IVS9 +43t generated an unstable mRNA that was rapidly degraded. The cloning of the patient's DNA showed that the two mutations were localized on different alleles.

The frameshift mutation del C244 determined the creation of a stop codon at nucleotide 320.

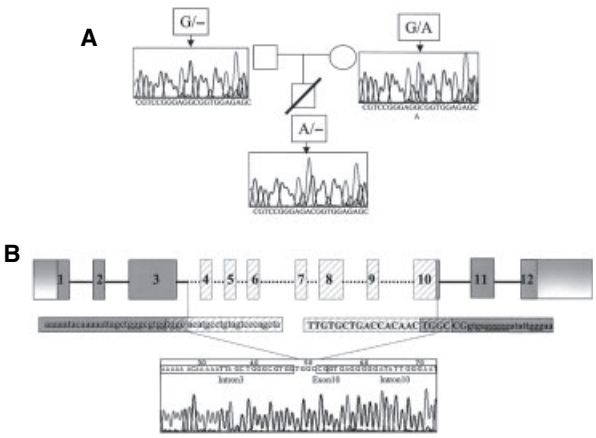

Fig 1. (A) Direct sequencing of exon 5 of *PK-LR* gene in Patient 6 and his parents. The patient appeared to be homozygous for mutation 409A, the mother heterozygous and the father normal. (B) Molecular characterization of 5006 bp deletion in Patient 6, extending from intron 3 to the last bases of exon 10 (hatched square, deleted region).

Missense mutation 1706A was found in a patient carrying the polymorphism 1705C at the homozygous level; the mutation, in association with the polymorphism, determined the amino acid substitution Arg596Gln.

All the new missense mutations described were not detected among the DNA of 50 normal controls studied.

Biochemical characterization of Ala137Thr mutant enzyme

To investigate the contribution of this variant in the severity of the clinical pattern of Patient 6 we functionally characterized the mutant 409A by mutagenesis and *in vitro* expression of the protein.

Ala137Thr mutant enzyme, purified to homogeneity, exhibited a specific activity of 320 U/mg. The oligomeric state of the mutant protein was identical (data not shown) to that of the wild-type enzyme (Wang *et al*, 2001). Similarly, the kinetic parameters and the thermostability properties (Table III) appeared to be substantially unaffected by the Ala137Thr replacement. Furthermore, the enzyme was less sensitive to ATP inhibition (IC_{50}: 2·3 mmol/l vs. 0·53 mmol/l for the wild-type enzyme). Therefore, the biochemical data obtained for the mutant enzyme could not explain the severe anaemia found in the PK-deficient patient hemizygous for this mutation.

Discussion

This paper describes 17 novel mutations of the *PK-LR* gene, including a deletion of 5006 bp which is the largest so far reported. Two large deletions were previously described, PK 'Gypsy' (del 1149bp) (Baronciani & Beutler, 1995) and PK 'Viet' (del 4-10) (Costa *et al*, 2005).

The 5006 bp deletion might originate from a misalignment of the two alleles during crossing over in meiosis, as hypothesized for PK 'Gypsy' (Baronciani & Beutler, 1995) and in other inherited red cell defects (Nicholls *et al*, 1987; Bianchi *et al*, 1997). At the extremity of the deleted fragment we found two 4 bp identical sequences (tggc), in intron 3 and in exon 10 respectively that might have caused this unequal recombination event. The effect of this deletion is more severe at the cDNA level, resulting in the loss of exons 4–11; the skipping of exon 11 suggests that its efficient inclusion in wild-type *PK-LR* mRNA could depend on the presence of splicing-enhancer elements in exon 10 in the primary transcript, as already hypothesized for exons 5 and 6 by van Wijk *et al* (2004).

The deletion affects the same gene portion of PK 'Viet' (del 4-10) recently detected in a Vietnamese child with severe transfusion-dependent anaemia, but not extensively characterized (Costa *et al*, 2005).

The del 5006bp was found associated with the new missense mutation 409A in a severely affected Australian newborn baby who had died soon after birth. Hydrops fetalis and death in the neonatal period are unusual clinical manifestations in PK deficiency, with only five cases having been reported so far (Hennekam *et al*, 1990; Gilsanz *et al*, 1993; Afriat *et al*, 1995; Ferreira *et al*, 2000; Sedano *et al*, 2004). Among them, the molecular defect was investigated only in the large family

Table III. Kinetic parameters of the mutant Ala137Thr compared with wild-type enzyme.

	PEP													
		FBP (−)				FBP 1 mmol/l				ADP			$t_{1/2}$ at 53°C	
Enzyme	k_{cat} (/s)	$S_{0·5}$ (mmol/l)	$k_{cat}/S_{0·5}$ (/s/mmol/l)	n_H	k_{cat} (/s)	$S_{0·5}$ (mmol/l)	$k_{cat}/S_{0·5}$ (/s/mmol/l)	n_H	k_{cat}	K_m	k_{cat}/K_m	FBP (−) (min)	FBP 1 mmol/l (min)
A137T	302 ± 10	0·71 ± 0·02	425	1·55 ± 0·10	288 ± 9	0·15 ± 0·01	1920	1·00 ± 0·06	330 ± 12	0·32 ± 0·01	1031	40	>120
Wt	355 ± 12	1·10 ± 0·04	323	1·60 ± 0·16	355 ± 11	0·18 ± 0·02	1972	1·05 ± 0·07	355 ± 13	0·17 ± 0·02	2088	80	>120

PEP, phosphoenolpyruvate; FBP, fructose 1,6-bisphosphate; ADP, adenosine diphosphate.

described by Sedano *et al* (2004), in which 12 patients, five of whom died within 2 d of birth, carried the homozygous nonsense mutation 1318T; although the authors were not able to establish directly the 'null' expression of this variant, it was assumed that the mutation could result in the absence of a functional protein product.

Considering the very severe clinical picture of Patient 6, with del 5006bp/409A, we hypothesized that mutation 409A could generate a non-functional protein, and consequently a 'null' phenotype. To confirm this hypothesis we functionally characterized the 409A mutant obtained by the mutagenesis and *in vitro* expression of the protein. The lack of critical abnormalities of the kinetic parameters suggests that, in the hemizygous state, this mutant should not *per se* result in serious clinical consequences. However, we cannot exclude that this substitution could have some effect at the mRNA level; alternatively, additional defects other than PK deficiency could exacerbate the clinical pattern, as already reported in some cases (Baughan *et al*, 1968; Tanphaichitr *et al*, 1997; Cohen-Solal *et al*, 1998).

Three new splicing mutations were identified. Whereas mutations 507A and IVS9 −1c altered the consensus sequences of the donor/acceptor sites, the effect on the splicing of mutation IVS9 +43t is not clear because it is localized in the internal portion of intron 9. The mutation IVS9 +43t was heterozygously found with the already described splicing mutation 1269A. Amplification of *PK-LR* cDNA from 1269A/IVS9 +43t patient failed to reveal any transcript, suggesting that the mRNA generated by these mutations is unstable and rapidly degraded. The absence of *PK-LR* mRNA as a consequence of mutation 1269A was expected, because a similar effect was reported for substitution 1269C (Zanella *et al*, 1997). In contrast, the effect of mutation IVS9 +43t on the mRNA splicing cannot be directly demonstrated due to the lack of *PK-LR* mRNA. However, we believe that IVS9 +43t is likely to be involved in PK deficiency as it was not detected in the normal population, it possibly introduces a new acceptor splice site as suggested by computerized splicing prediction, and no other abnormalities were detected in the patient's DNA. Moreover, mutations in internal portions of exons or introns, which result in aberrant splicing, have been described in several disease-related genes (Cartegni *et al*, 2002; Coutinho *et al*, 2005).

With regard to the new missense mutations found in this study, the comparison of the amino acid sequences among several species (Munoz & Ponce, 2003) showed that all replacements target strictly conserved amino acids with the exception of Ala137Thr, Val320Met and Ile457Val.

We looked into the available three-dimensional structure of erythrocyte PK (Valentini *et al*, 2002), crystallized in the active state (R-state), and that of the *Escherichia coli* enzyme, crystallized in the inactive state (T-state) (Mattevi *et al*, 1995), to advance hypotheses on how and to what extent the new point mutations alter the enzyme's molecular properties, and to correlate genotype to phenotype.

Among the nine mutations localized in the A domain, 278T and 1209A were found in two severely affected patients in heterozygosity with 1456T, which is known to have only a limited effect on the molecular properties of the enzyme (Valentini *et al*, 2002). Both 278T and 1209A mutations are likely to be critical as they target amino acid residues located in the proximity of the PEP-binding site, and introduce into the binding site pocket a bulky and hydrophobic side chain pointing towards the substrate. These findings suggest that mutations Thr93Ile and Met403Ile greatly impair the catalytic efficiency of the enzyme, providing an explanation for the severity of the clinical manifestations.

Mutation 661A results in amino acid substitution Asp221Asn in domain B. Asp221 is a highly conserved amino acid that plays an important functional role in the allosteric transition, as observed by comparing the structures of the PK enzyme in T- and R-state (Mattevi *et al*, 1995). It has been stated that, during the allosteric transition, the subunit rotations drive a pivotal Arg of domain A (Arg292 in *E. coli* PK) to move towards Asp221 (Asp127 in *E. coli* PK), engaging it in a salt bridge and allowing the catalytic site to gain the correct set-up. The replacement of this charged residue with the uncharged Asn hampers the salt bridge formation, probably preventing the catalytic site from becoming properly conformed. Actually, the corresponding *E. coli* mutant enzyme obtained as recombinant form was 10-fold less active than the wild-type enzyme and highly unstable ($t\frac{1}{2}$ at 55°C 2 min vs. >1 h; data not shown).

Mutations 1209A and 661A were identified in a family with an apparently dominant transmission of PK deficiency; both the mother (Patient 10) and two siblings (Patients 8 and 9) being affected. It is worth noting that the clinical pattern observed in this family can be fully explained on the basis of the detected mutations; both siblings, compound heterozygous for the two detrimental mutations 1209A and 661A, showed critical clinical conditions (need of regular transfusions until splenectomy, postsplenectomy Hb levels of 9 g/dl), whereas the mother, compound heterozygous for 1209A and the mild mutation 1456T, was less severely affected, with postsplenectomy Hb of 12 g/dl.

It is known that, in most cases of erythroenzymopathy, the enzyme deficiency is due to a low intracellular level of enzyme as a consequence of decreased stability. We think that this is the case with mutations 403T (Arg135Trp) and 1190T (Asp397Val). In the wild-type PK-R, Arg135 is located in helix α-2 of the A domain, with its side chain fully solvent-accessible, whereas Asp397 is placed in loop connecting helix α-7 to strand β-8 of A domain, with its side chain most likely engaged in a salt bridge with Lys354. The substitution of charged residues with hydrophobic amino acids results in the energetically unfavourable exposure of the hydrophobic Trp135 residue on the protein surface and in the removal of the bridge that stabilises Lys354. The association of these two mutations in Patient 13 would make the enzyme unable to maintain proper folding *in vivo*, increasing the susceptibility to

degradation by the ubiquitin-proteasome pathway, as suggested for other very unstable erythrocyte enzymes (Kanno *et al*, 2004).

Although all the exons, flanking regions and the promoter were sequenced in all cases, we failed to detect the second mutation in four subjects. This is likely to be due to technical reasons; in fact some variants, for example, large deletions, mutations in the regulative regions of the gene, or mutations that may activate a cryptic splice site in an intron, are often difficult to identify and may not be detected by the normal panel of primers used for the PCR amplification of the *PK-LR* gene. The presence in our series of two of these 'unusual' mutations (the large deletion of 5006 bp and the intronic splicing mutation IVS9 +43c) proves the difficulty of the genetic analysis in some cases, and underlines the importance of family studies in the molecular diagnosis of PK deficiency.

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