

Red cell pyruvate kinase deficiency: molecular and clinical aspects

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Summary

Red cell pyruvate kinase (PK) deficiency is the most frequent enzyme abnormality of the glycolytic pathway causing hereditary non-spherocytic haemolytic anaemia. The degree of haemolysis varies widely, ranging from very mild or fully compensated forms, to life-threatening neonatal anaemia and jaundice necessitating exchange transfusions. Erythrocyte PK is synthesized under the control of the *PK-LR* gene located on chromosome 1. To date, more than 150 different mutations in the *PK-LR* gene have been associated with PK deficiency. First attempts to delineate the biochemical and clinical consequences of the molecular defect were mainly based on the observation of the few homozygous patients and on the analysis of the three-dimensional structure of the enzyme. More recently, the comparison of the recombinant mutants of human red cell PK with the wild-type enzyme has enabled the effects of amino acid replacements on the enzyme molecular properties to be determined and help to correlate genotype to clinical phenotype.

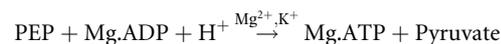
Keywords: pyruvate kinase deficiency, *PK-LR* gene, mutations, chronic haemolytic anaemia, mutagenesis.

Red cell pyruvate kinase (PK) deficiency, firstly identified in the early 1960s (Valentine *et al*, 1961), is the most frequent enzyme abnormality of the glycolytic pathway, and the most common cause of hereditary non-spherocytic haemolytic anaemia, together with class I glucose-6-phosphate dehydrogenase deficiency (Glader, 2004). The disease is transmitted as an autosomal recessive trait, clinical symptoms usually occur in compound heterozygotes for two mutant alleles and in homozygotes. PK deficiency has a world-wide geographical distribution. The prevalence of PK deficiency, as assessed by gene frequency studies, has been estimated to

be 1:20 000 in the general white population (Beutler & Gelbart, 2000).

Structure and function of PK

Pyruvate kinase (ATP: pyruvate 2-o-phosphotransferase, EC 2.7.1.40) is a key glycolytic enzyme that catalyses the transphosphorylation from phosphoenolpyruvate (PEP) to ADP, yielding pyruvate and ATP. The reaction is the last step of the glycolytic pathway and is essentially irreversible under physiological conditions. The enzyme requires K⁺ and Mg²⁺ (or Mn²⁺) for activity.



The reaction is critical for the control of the metabolic flux in the second part of glycolysis. Moreover, the substrate PEP and the product pyruvate are involved in a number of energetic and biosynthetic pathways and the tight regulation of PK activity has been shown to be of great importance not only for glycolysis itself, but also for the entire cellular metabolism. Thus, one of the main features of this enzyme is its allosteric response to a large number of effectors, whose precise chemical nature depends on the type of organism or tissue.

Pyruvate kinase is an homotetramer in almost all organisms (Fothergill-Gilmore & Michels, 1993), although it may exist in different forms, from monomer to decamer (Munoz & Ponce, 2003). A high degree of structural homology among PKs from different species has been reported. Crystal structures have been published for PKs from cat muscle (Muirhead *et al*, 1986), rabbit muscle (Larsen *et al*, 1994), *Escherichia coli* (Mattevi *et al*, 1995), yeast (Jurica *et al*, 1998), *Leishmania mexicana* (Rigden *et al*, 1999) and human erythrocyte (Valentini *et al*, 2002). These structures resemble each other in that each subunit is organized into three principal domains, an A domain with (β/α)₈ barrel topology; a β-stranded B domain, inserted between strand β₃ and helix α₃ of the A domain, and a C domain with an α + β topology (Fig 1). With the exception of prokaryotes (Valentini *et al*, 1991) a fourth small domain, corresponding to the N-terminus, is also present. Moreover, in *Bacillus* PKs an additional C-terminal domain is also observed (Munoz &

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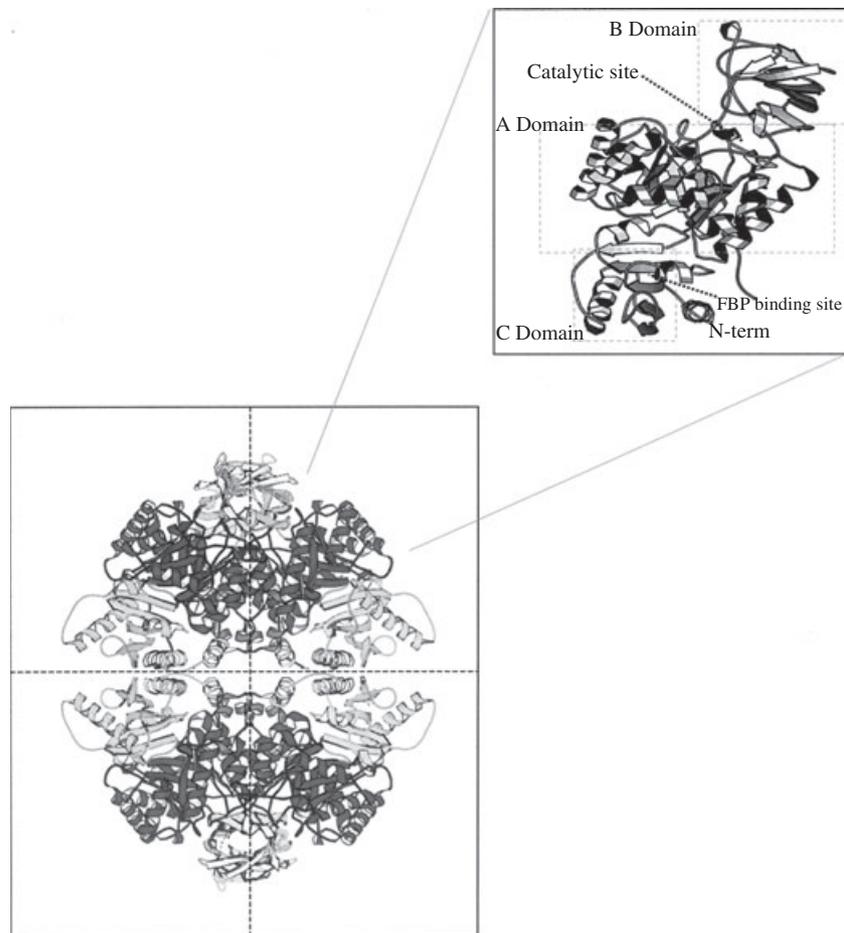


Fig 1. Three-dimensional crystal structure of RPK monomer (upper right) and tetramer (lower left). In this orientation, a molecular twofold axis is perpendicular to the plane of the paper, whereas the other two molecular twofold axes are vertical and horizontal to the plane of the paper (indicated with vertical and horizontal lines respectively).

Ponce, 2003). The crystal structure shows that the four subunits of the tetramer are assembled to form a D₂ symmetric oligomer (i.e. three, twofold rotation axes intersecting each other at right angles). The intersubunit interactions define two large contact areas; the A/A' interface involves the A domains of subunits related by the vertical twofold axis whereas the C/C' interface involves the C domains of subunits interacting along the horizontal axis.

The multidomain architecture of PK is instrumental to the regulation of the activity of this enzyme that can exist in different conformational states. Transition between these states can be triggered on binding one or more effectors (Kahn & Marie, 1982; Fothergill-Gilmore & Michels, 1993). The switching from a low-affinity tight (T) state (inactive state) to a high-affinity relaxed (R) state (active state) is thought to involve a combination of domain and subunit rotations coupled to conformational changes in the active site. Within this mechanism, the residues located at the domain and subunit interfaces play a crucial role in the communication between the activator-binding site and the catalytic centre, the

former being entirely located inside the C-domain and the latter between A and B domains respectively.

Four PK isoenzymes (M₁, M₂, L, and R) have been identified in mammals, expressed in a tissue-specific manner (Fothergill-Gilmore & Michels, 1993). Each isoenzyme exhibits different kinetic properties that reflect the particular metabolic requirements of the expressing tissues. In humans, two separate genes (*PK-M* and *PK-LR*) encode the four different PK isoenzymes. The *PK-LR* gene is located on chromosome 1 (1q21) (Satoh *et al*, 1988), and codes for both the L isoenzyme (liver) and the R isoenzyme (red blood cells, RBC) through the use of alternate promoters (Noguchi *et al*, 1987). The *PK-M* gene is located on chromosome 15 (15q22) (Tani *et al*, 1988a) and codes for M₁ and M₂ isoenzymes by alternative splicing of the same RNA (Noguchi *et al*, 1986). M₁ protein predominates in skeletal muscle, heart and brain, whereas M₂ isoenzyme is found primarily in the rapidly proliferating fetal tissues. Subsequently, M₂ is progressively replaced by the other tissue-specific isoforms, although it remains the principal form in kidney, leucocytes, platelets, lung, spleen and adipose tissue

(Takegawa *et al*, 1983). L-type can be found in liver, renal cortex and small intestine, whereas R-type is exclusively expressed in erythrocytes. M₂, L and R isoenzymes display sigmoidal reaction kinetics with respect to PEP and are allosterically activated by fructose 1,6-bisphosphate (FBP), whereas the M₁ displays no co-operative properties (Muirhead *et al*, 1986). N-terminal phosphorylation favouring the T-state (Kahn & Marie, 1982), partial activation through proteolysis (Kahn & Marie, 1982) as well as hormone-triggered dimerization for some forms of enzyme (Ashizawa *et al*, 1991) have also been reported.

Pyruvate kinase plays a central role in erythrocyte metabolism, because it catalyses one of the two major steps of ATP production in the cell. As mature RBCs lack mitochondria, these cells are absolutely dependent on glycolysis for maintaining cell integrity and function. Therefore, PK deficiency leads to ATP depletion, which ultimately affects the viability of the cell. Moreover, PK deficiency results in the accumulation of the glycolytic intermediates proximal to the metabolic block, particularly 2,3-diphosphoglycerate (2,3-DPG), which may increase up to threefold and further impair the glycolytic flux through the inhibition of hexokinase (Zanella & Bianchi, 2000).

Red cell PK (RPK), either isolated from mature erythrocytes (Kahn & Marie, 1982; Kilinc & Ozer, 1984) or obtained as a recombinant form by means of the *E. coli* system (Wang *et al*, 2001), is a heterotetramer build up by two subunits of 62 kDa and two of 58 kDa, the last ones resulting from a proteolytic cut at their N-terminus (Wang *et al*, 2001). It is conceivable that the shortening of the chains suits a different organization, within the tetramer, of the N-terminal segments of the four subunits. Unfortunately, the available RPK crystal structure is that of a truncated protein lacking the first 49 amino acids (Valentini *et al*, 2002). Thus, the actual structure and the functional role of the N-terminal extension of RPK are still puzzling.

Red cell PK is allosterically activated by FBP and inhibited by ATP (Kahn & Marie, 1982; Wang *et al*, 2001). The allosteric regulation can be described according to the sequential model of Monod *et al* (1965) with V_{\max} unchanged either in T- or R-state (350 U/mg). Thus, either in the presence or in the absence of effectors, the enzyme displays identical k_{cat} (turnover number) values (355/s). Conversely, the apparent affinity ($S_{0.5}$, a parameter used for sigmoidal kinetics and defined as K_m) and Hill coefficient (nH, an empirical parameter related to the degree of cooperativity) values are affected by the presence of effectors ($S_{0.5}$ and nH towards PEP, 1.1 mmol/l and 1.6 respectively in the absence of effectors; 0.18 mmol/l and 1.05 respectively in the presence of FBP). ATP inhibits RPK activity (IC_{50} at 0.1 mmol/l PEP, 0.53 mmol/l) (Kahn & Marie, 1982; Wang *et al*, 2001) but under physiological conditions the ATP inhibition is almost completely counteracted by FBP (Wang *et al*, 2001). Finally, RPK displays hyperbolic behaviour towards ADP with a K_m value of 0.17 mmol/l. Thus, the portrait emerging from these molecular properties of RPK is

that of a very complex protein that is finely regulated to fulfill the erythrocyte metabolic requirements. The flexibility and modularity of the protein are at the heart of regulatory mechanism.

Genetic characteristics of PK deficiency

The *PK-LR* gene (over 9.5 kb) is located on chromosome 1q21 (Sato *et al*, 1988) where it directs tissue-specific transcription for both the liver-specific and the red cell-specific (RPK) isoenzyme by the use of alternate promoters (Noguchi *et al*, 1987; Kanno *et al*, 1992a).

The coding region is split into 12 exons, 10 of which are shared by the two isoforms, while exons 1 and 2 are specific for the erythrocyte and the hepatic isoenzyme, respectively (Noguchi *et al*, 1987; Tani *et al*, 1988b; Kanno *et al*, 1991). The cDNA encoding RPK is 2060 bp long and codes for 574 amino acids (Kanno *et al*, 1991). In the R-type promoter region of the *PK-LR* gene, which is located in the 5' flanking region upstream from the first exon, two CAC boxes and four GATA motifs have been identified within 270-bp from the translational initiation codon. The proximal 120-bp region has a basal promoter activity and the region from -120 to -270 functions as a powerful enhancer in erythroid cells (Kanno *et al*, 1992a).

One hundred fifty-eight mutations associated with nonspherocytic haemolytic anaemia (Table I) and eight polymorphic sites (Table II) have been so far reported in the *PK-LR* gene. Mutations and polymorphisms are usually designated using the RPK cDNA sequence of the *PK-LR* gene, with the A of the initiation ATG being assigned number +1 (GenBank accession numbers D10326 and D90465); the GenBank accession numbers are U47654 and D13232 for the genomic DNA and the putative promoter region respectively.

Only two mutations, -72A → G and -83G → C, have been identified in the promoter region and functionally characterized: the former causes the disruption of the consensus binding motif for GATA-1 at nts -69 to -74 (Manco *et al*, 2000), and the latter alters a novel regulatory element (PKR-RE1) whose core CTCTG extends from nts -87 to -83 (van Wijk *et al*, 2003). The mutations identified are mostly missense (69%), splicing and stop codon (11% and 5%, respectively), whereas small deletions, insertions and frameshift mutations are rare (12%). Only a few large deletions have been reported: the 'Gypsy' deletion of 1149 bp, which results in the loss of exon 11 (Baronciani & Beutler, 1995), PK 'Viet' (del 4-10) (Costa *et al*, 2005), and a deletion of 5006 bp which results at the cDNA level in the loss of exons from 4 to 11 (Fermo *et al*, 2005). Most of the missense mutations involve highly conserved amino acids as assessed by homology studies (Munoz & Ponce, 2003).

Three of the most prevalent mutations in patients with PK deficiency are 1529A, 1456T and 1468T; these variants have been found to be distributed with a strong ethnic and regional background. In particular, the 1529A is the most common

Table I. Mutations in the *PK-LR* gene associated with congenital non-spherocytic haemolytic anaemia reported in the literature.

| cDNA nucleotide substitution | Effect | Exon(s) | Reference |
|---------------------------------|---------------------------|-----------|----------------------------------|
| -83g → c | | Promoter | van Wijk <i>et al</i> (2003) |
| -72a → g | | | Manco <i>et al</i> (2000) |
| IVS2(-1) g → a | Splice Site | IVS2 | Lenzner <i>et al</i> (1994a) |
| 107 C → G | 36 Ala → Gly | 3 | Fermo <i>et al</i> (2005) |
| 110 G → A | 37 Gly → Gln | 3 | Beutler <i>et al</i> (1997) |
| 183 16 bp 184 ins | Frameshift | 3 | Kugler <i>et al</i> (2000) |
| 227-231 TGGAC del | Frameshift | 3 | Zanella <i>et al</i> (1997) |
| 238 T → C | 80 Ser → Pro | 3 | Uenaka <i>et al</i> (1995) |
| 244C del | Frameshift | 3 | Fermo <i>et al</i> (2005) |
| 269 T → A | 90 Ile → Asn | 3 | van Solinge <i>et al</i> (1997b) |
| 278 C → T | 93 Thr → Ile | 3 | Fermo <i>et al</i> (2005) |
| 283 G → A | 95 Gly → Arg | 3 | van Solinge <i>et al</i> (1997b) |
| IVS3(-2) a → c | Splice Site | IVS3 | Zanella <i>et al</i> (1997) |
| IVS3(-2) a → t | Splice Site | IVS3 | Kanno <i>et al</i> (1997) |
| 5006 bp (IVS3 → nt 1431) del | ex 4-11 del | IVS3-ex10 | Fermo <i>et al</i> (2005) |
| ex 4-10 del | - | 4-10 | Costa <i>et al</i> (2005) |
| 307 C del | Frameshift | 4 | Baronciani and Beutler (1995) |
| 320 T → C | 107 Met → Thr | 4 | Baronciani <i>et al</i> (1995a) |
| 331 G → A | 111 Gly → Arg | 4 | van Solinge <i>et al</i> (1997b) |
| 343 G → C | 115 Ala → Pro | 4 | Rouger <i>et al</i> (1996b) |
| C 346-349del → C 346 insAACATTG | Arg Leu 116 → Gln His Cys | 4 | Pissard <i>et al</i> (1999) |
| 359 C → T | 120 Ser → Phe | 4 | Rouger <i>et al</i> (1996b) |
| 389 C → A | 130 Ser → Tyr | 5 | Cohen-Solal <i>et al</i> (1998) |
| 391-393 ATC del | 131 Ile del | 5 | Baronciani and Beutler (1993) |
| 401 T → A | 134 Val → Asp | 5 | Baronciani and Beutler (1993) |
| 403 C → T | 135 Arg → Trp | 5 | Fermo <i>et al</i> (2005) |
| 409 G → A | 137 Ala → Thr | 5 | Fermo <i>et al</i> (2005) |
| 434 C del | Frameshift | 5 | Kanno <i>et al</i> (1994c) |
| 458T → C | 153 Ile → Thr | 5 | Kugler <i>et al</i> (2000) |
| 464 T → C | 155 Leu → Pro | 5 | Baronciani and Beutler (1993) |
| 476 G → T | 159 Gly → Val | 5 | Demina <i>et al</i> (1998) |
| 487 C → T | 163 Arg → Cys | 5 | Neubauer <i>et al</i> (1991) |
| 507 G → A | Splice Site | 5 | Fermo <i>et al</i> (2005) |
| IVS5(+1) g → a | Splice Site | IVS5 | van Wijk <i>et al</i> (2004) |
| 514 G → C | 172 Glu → Gln | 6 | Zanella <i>et al</i> (1997) |
| 601 C → T | 201 Trp → Arg | 6 | Pissard <i>et al</i> (1999) |
| 603 G → A | 201 Trp → End | 6 | Baronciani <i>et al</i> (1995a) |
| 628-629 GT del | Frameshift | 6 | Lenzner <i>et al</i> (1997a) |
| 656 T → C | 219 Ile → Thr | 6 | Kugler <i>et al</i> (2000) |
| 661 G → A | 221 Asp → Asn | 6 | Fermo <i>et al</i> (2005) |
| 663 GAC 664 ins | 221 Asp 222 ins | 6 | Kanno <i>et al</i> (1994c) |
| 671 T → C | 224Ile → Thr | 6 | Pissard <i>et al</i> (1999) |
| IVS6(-2) a → t | Splice Site | IVS6 | Zanella <i>et al</i> (2001a) |
| 721 G → T | 241 Glu → End | 7 | Baronciani and Beutler (1993) |
| 757 A → G | 253 Asn → Asp | 7 | van Wijk <i>et al</i> (2001) |
| 787 G → A | 263 Gly → Arg | 7 | Lenzner <i>et al</i> (1997a) |
| 787 G → T | 263 Gly → Trp | 7 | Zanella <i>et al</i> (1997) |
| 808 C → T | 270 Arg → End | 7 | Baronciani and Beutler (1995) |
| 814 C → G | 272 Leu → Val | 7 | van Wijk <i>et al</i> (2001) |
| 823 G → C | 275 Gly → Arg | 7 | Baronciani <i>et al</i> (1995a) |
| 823 G → A | 275 Gly → Arg | 7 | Zanella <i>et al</i> (1997) |
| 841 G → A | 281 Asp → Asn | 7 | Kanno <i>et al</i> (1994c) |
| 859 T → G | 287 Phe → Val | 7 | Kanno <i>et al</i> (1994c) |
| 859 T → C | 287 Phe → Leu | 7 | Fermo <i>et al</i> (2005) |
| 862 G → T | 288Val → Leu | 7 | Aizawa <i>et al</i> (2003) |
| 877 G → A | 293Asp → Asn | 7 | Kugler <i>et al</i> (2000) |

Table I. Continued.

| cDNA nucleotide substitution | Effect | Exon(s) | Reference |
|------------------------------|-----------------|---------|----------------------------------|
| 884 C → T | 295 Ala → Val | 7 | Demina <i>et al</i> (1998) |
| 929 T → A | 310 Ile → Asn | 7 | van Solinge <i>et al</i> (1996) |
| 941 T → C | 314 Ile → Thr | 7 | Kanno <i>et al</i> (1994a) |
| 943 G → A | 315 Glu → Lys | 7 | Demina <i>et al</i> (1998) |
| 948 C → G | 316 Asn → Lys | 7 | Costa <i>et al</i> (2005) |
| 958 G → A | 320 Val → Met | 7 | Fermo <i>et al</i> (2005) |
| IVS7(+1) g → t | Splice Site | IVS7 | Kanno <i>et al</i> (1997) |
| 991 G → A | 331 Asp → Asn | 8 | Kugler <i>et al</i> (2000) |
| 993 C → A | 331 Asp → Glu | 8 | Baronciani and Beutler (1995) |
| 994 G → A | 332 Gly → Ser | 8 | Lenzner <i>et al</i> (1994a) |
| 1003 G → A | 335 Val → Met | 8 | Zanella <i>et al</i> (2001b) |
| 1006 G → T | 336 Ala → Ser | 8 | Lenzner <i>et al</i> (1994a) |
| 1010 G del | Frameshift | 8 | Cotton <i>et al</i> (2001) |
| 1010 G → C | 337 Arg → Pro | 8 | Pastore <i>et al</i> (1998) |
| 1010 G → A | 337 Arg → Gln | 8 | Lenzner <i>et al</i> (1997a) |
| 1015 G → C | 339 Asp → Gln | 8 | Zarza <i>et al</i> (1998) |
| 1022 G → C | 341 Gly → Ala | 8 | Baronciani and Beutler (1995) |
| 1022 G → A | 341 Gly → Asp | 8 | Demina <i>et al</i> (1998) |
| 1024 A → T | 342 Ile → Phe | 8 | Layton <i>et al</i> (1996) |
| 1042–1044 AAG del | 348 Lys del | 8 | Zanella <i>et al</i> (2001b) |
| 1044 G → T | 348 Lys → Asn | 8 | Kanno <i>et al</i> (1997) |
| 1055 C → A | 352 Ala → Asp | 8 | Kugler <i>et al</i> (2000) |
| 1060–1062 AAG del | 354 Lys del | 8 | Lenzner <i>et al</i> (1994a) |
| 1070 T → C | 357 Ile → Thr | 8 | Zarza <i>et al</i> (1998) |
| 1073 G → A | 358 Gly → Glu | 8 | van Wijk <i>et al</i> (2001) |
| 1075 C → T | 359 Arg → Cys | 8 | Kanno <i>et al</i> (1994c) |
| 1076 G → A | 359 Arg → His | 8 | Baronciani and Beutler (1993) |
| 1081 A → G | 361 Asn → Asp | 8 | Lenzner <i>et al</i> (1994a) |
| 1089 G 1090 ins | Frameshift | 8 | Baronciani and Beutler (1995) |
| 1091 G → A | 364 Gly → Asp | 8 | van Solinge <i>et al</i> (1997b) |
| 1094 A → T | 365 Lys → Met | 8 | Fermo <i>et al</i> (2005) |
| 1102 G → T | 368 Val → Phe | 8 | Kanno <i>et al</i> (1993a) |
| IVS8(+2) t → g | Splice site | IVS8 | Manco <i>et al</i> (1999) |
| 1121 T → C | 374 Leu → Pro | 9 | van Wijk <i>et al</i> (2001) |
| 1127 G → T | 376 Ser → Ile | 9 | Lenzner <i>et al</i> (1997a) |
| 1151 C → T | 384 Thr → Met | 9 | Neubauer <i>et al</i> (1991) |
| 1153 A → T | 385 Arg → Trp | 9 | Beutler and Gelbart (2000) |
| 1154 G → A | 385 Arg → Lys | 9 | van Wijk <i>et al</i> (2001) |
| 1160 A → G | 387 Glu → Gly | 9 | Zanella <i>et al</i> (2001b) |
| 1168 G → A | 390 Asp → Asn | 9 | Zanella <i>et al</i> (1997) |
| 1174 G → A | 392 Ala → Thr | 9 | Lenzner <i>et al</i> (1994a) |
| 1178 A → G | 393 Asn → Ser | 9 | Baronciani and Beutler (1995) |
| 1179 T → A | 393 Asn → Lys | 9 | Baronciani and Beutler (1995) |
| 1181 C → A | 394 Ala → Asp | 9 | Zanella <i>et al</i> (2001b) |
| 1181 C → T | 394 Ala → Val | 9 | Zanella <i>et al</i> (2001b) |
| 1190 A → T | 397 Asp → Val | 9 | Fermo <i>et al</i> (2005) |
| 1193 G → C | 398 Gly → Ala | 9 | Pissard <i>et al</i> (1999) |
| 1195 G del | Frameshift | 9 | Rouger <i>et al</i> (1996a) |
| 1203 AGC 1204 ins | 401 Ser 402 ins | 9 | Lenzner <i>et al</i> (1994a) |
| 1209 G → A | 403 Met → Ile | 9 | Fermo <i>et al</i> (2005) |
| 1223 C → T | 408 Thr → Ile | 9 | Zarza <i>et al</i> (1998) |
| 1228A → G | 410 Lys → Glu | 9 | Pissard <i>et al</i> (1999) |
| 1231G → A | 411 Gly → Ser | 9 | Park-Hah <i>et al</i> (2005) |
| 1232 G → C | 411 Gly → Ala | 9 | Fermo <i>et al</i> (2005) |
| 1261 C → A | 421 Gln → Lys | 9 | Kanno <i>et al</i> (1992b) |

Table I. Mutations in the *PK-LR* gene associated with congenital non-spherocytic haemolytic anaemia reported in the literature. Continued.

| cDNA nucleotide substitution | Effect | Exon(s) | Reference |
|------------------------------|-------------------------------|---------|----------------------------------|
| 1269 G → A | Splice Site | 9 | Kanno <i>et al</i> (1994c) |
| 1269 G → C | Splice Site | 9 | Zanella <i>et al</i> (1997) |
| Ivs9(+43)c → t | Splice site | IVS9 | Fermo <i>et al</i> (2005) |
| Ivs9(-1)g → c | Splice site | IVS9 | Fermo <i>et al</i> (2005) |
| 1276 C → T | 426 Arg → Trp | 10 | Kanno <i>et al</i> (1994c) |
| 1277 G → A | 426 Arg → Gln | 10 | Kanno <i>et al</i> (1993b) |
| 1281 G → T | 427 Glu → Asp | 10 | Lenzner <i>et al</i> (1997a) |
| 1291 G → A | 431 Ala → Thr | 10 | Zarza <i>et al</i> (1998) |
| 1318 G → T | 440 Glu → End | 10 | Sedano <i>et al</i> (2004) |
| 1369 A → G | 457 Ile → Val | 10 | Fermo <i>et al</i> (2005) |
| 1373 G → A | 458 Gly → Asp | 10 | Baronciani and Beutler (1995) |
| 1376 C → T | 459 Ala → Val | 10 | Baronciani <i>et al</i> (1995a) |
| 1378 G → A | 460 Val → Met | 10 | Baronciani and Beutler (1995) |
| 1403 C → T | 468 Ala → Val | 10 | Kanno <i>et al</i> (1994a) |
| 1409C → A | 470Ala → Asp | 10 | Pissard <i>et al</i> (1999) |
| 1436 G → A | 479 Arg → His and Splice site | 10 | Kanno <i>et al</i> (1994a) |
| Ivs10(+1) g → c | Splice site | IVS10 | Manco <i>et al</i> (1999) |
| 1437–1618 del | Frameshift | 11 | Baronciani and Beutler (1995) |
| 1454 C → T | 485 Ser → Phe | 11 | Lenzner <i>et al</i> (1997a) |
| 1456 C → T | 486 Arg → Trp | 11 | Baronciani and Beutler (1993) |
| 1462 C → T | 488 Arg → End | 11 | van Solinge <i>et al</i> (1997b) |
| 1468 C → T | 490 Arg → Trp | 11 | Kanno <i>et al</i> (1994c) |
| 1483 G → A | 495 Ala → Thr | 11 | Kugler <i>et al</i> (2000) |
| 1484 C → T | 495 Ala → Val | 11 | Baronciani and Beutler (1993) |
| 1488 C del | Frameshift | 11 | Rouger <i>et al</i> (1996b) |
| 1492 C → T | 498 Arg → Cys | 11 | van Solinge <i>et al</i> (1997b) |
| 1493 G → A | 498 Arg → His | 11 | Lenzner <i>et al</i> (1994a) |
| 1501 C → T | 501 Gln → End | 11 | Baronciani <i>et al</i> (1995a) |
| 1508 C → T | 503 Ala → Val | 11 | Zarza <i>et al</i> (1999) |
| 1511 G → T | 504 Arg → Leu | 11 | Demina <i>et al</i> (1998) |
| 1515–1518dupl | Frameshift | 11 | Zanella <i>et al</i> (2001b) |
| 1516 G → A | 506 Val → Ile | 11 | Zarza <i>et al</i> (2000) |
| 1523 T → G | 508 Leu → End | 11 | Pastore <i>et al</i> (1998) |
| 1528 C → T | 510 Arg → End | 11 | Demina <i>et al</i> (1998) |
| 1529 G → A | 510 Arg → Gln | 11 | Baronciani and Beutler (1993) |
| 1552 C → A | 518 Arg → Ser and Splice Site | 11 | Zanella <i>et al</i> (1997) |
| 1574 G 1575 ins | Frameshift | 11 | Baronciani <i>et al</i> (1995a) |
| 1594 C → T | 532 Arg → Trp | 11 | Lakomek <i>et al</i> (1994) |
| 1595 G → A | 532 Arg → Gln | 11 | Zarza <i>et al</i> (1998) |
| 1618 Ivs11(+1) g Del | Splice Site | IVS11 | van Wijk <i>et al</i> (2001) |
| Ivs11(-3) c → g | Splice Site | IVS11 | van Wijk <i>et al</i> (2001) |
| 1654 G → A | 552 Val → Met | 12 | Baronciani <i>et al</i> (1995a) |
| 1670 G → C | 557 Gly → Ala | 12 | Manco <i>et al</i> (1999) |
| 1675 C → G | 559 Arg → Gly | 12 | Baronciani <i>et al</i> (1995a) |
| 1675 C → T | 559 Arg → End | 12 | Zarza <i>et al</i> (1998) |
| 1698 C → A | 566 Asn → Lys | 12 | Kanno <i>et al</i> (1994c) |
| 1706 G → A | 569 Arg → Gln | 12 | van Wijk <i>et al</i> (2001) |

mutation in the USA (42%) (Baronciani & Beutler, 1995) and in Northern and Central Europe (41%) (Lenzner *et al*, 1997a). 1456T is most common in southern Europe (32% in Spain, 35% in Portugal and 29% in Italy), where, in contrast, mutation 1529A is rare (Demina *et al*, 1998; Pastore *et al*, 1998; Zarza *et al*, 1998; Manco *et al*, 2000; Zanella & Bianchi, 2000). 1468T occurs more frequently in Asia (9/16 unrelated

families) (Kanno *et al*, 1994a). Each of these mutations is found in the context of its own haplotype, arguing that each has a unique origin. Other mutations, in particular 721T and 994A, are present with a lower frequency in White people (Baronciani & Beutler, 1993; Zarza *et al*, 1998; Fermo *et al*, 2005). Only two mutations (1151T, 1436A) are common to Japanese and White populations.

Table II. Polymorphisms reported in the *PK-LR* gene.

| Polymorphic Site CDNA Number | Polymorphic site genomic number | Exon | Reference |
|------------------------------|---------------------------------|----------|---------------------------------|
| -342 T/A | | Promoter | van Wijk <i>et al</i> (2003) |
| -248T del | | | van Wijk <i>et al</i> (2003) |
| IVS5(+51)C/T | 2838 C/T | IVS5 | Baronciani <i>et al</i> (1995b) |
| T _{10/19} * | 5972-5981 (T ₁₀) | IVS10 | Lenzner <i>et al</i> (1997b) |
| Microsatellite ATT | 7181-7222 (14 ATT) | IVS11 | Lenzner <i>et al</i> (1994b) |
| 1705 A/C | 7619 A/C | 12 | Kanno <i>et al</i> (1992b) |
| 1738 C/T | 7652 C/T | 12 | Zanella <i>et al</i> (1997) |
| 1992 C/T | 7906 C/T | 12 | Lenzner <i>et al</i> (1994b) |

*T-stretch occurring in the two forms (T)₁₀ and (T)₁₉.

Clinical, haematological and diagnostic aspects of PK deficiency

Clinical features

Although abnormalities in *PK-LR* gene may result in alterations of both erythrocyte and liver enzyme, clinical symptoms are confined to red blood cells, the hepatic deficiency being usually compensated by the persistent enzyme synthesis in hepatocytes (Nakashima *et al*, 1977). Clinical manifestations of PK deficiency comprise the usual hallmarks of lifelong chronic haemolysis. The degree of anaemia varies widely, ranging from very mild or fully compensated anaemia to life-threatening neonatal anaemia and pronounced jaundice necessitating exchange transfusions and subsequent continuous transfusion therapy (Zanella & Bianchi, 2000). Hydrops foetalis and death in the neonatal period have also been reported in rare cases (Hennekam *et al*, 1990; Gilsanz *et al*, 1993; Afriat *et al*, 1995; Ferreira *et al*, 2000; Sedano *et al*, 2004; Fermo *et al*, 2005). In infants, the anaemia tends to improve with age, and may even disappear in some cases (Boivin & Ottenwaelter, 1982). The degree of anaemia is relatively constant in adulthood, although occasional exacerbation may occur during acute infections and pregnancy. Pregnancy is usually well tolerated in PK deficiency and associated with favourable perinatal outcome (Fanning & Hinkle, 1985; Esen & Olajide, 1998; Dolan *et al*, 2002); haemolysis can increase, requiring blood transfusions, before and after delivery (Amankwah *et al*, 1980; Dolan *et al*, 2002). It is worth noting that anaemia may be surprisingly well tolerated in PK-deficient patients (Osiki *et al*, 1971) because of the increased red cell 2,3-DPG content, which is responsible for a rightward shift in the oxygen dissociation curve of haemoglobin.

Figure 2 summarizes the main clinical features of PK deficiency, as assessed by the study of 61 cases from 54 families (44 of whom of Italian origin) referred to our Centre. The median age at the time of diagnosis was 16 years (range 1 day to 65 years). Eighteen were splenectomized (11 before diagnosis and seven thereafter). Twenty patients had long clinical and laboratory follow-ups (median 26 years, range 17-32 years). Anaemia was severe in 17 and moderate-to-mild

in 31 of unsplenectomized (or before splenectomy) patients. Six more cases were not anaemic, and the disease was detected in adult age by chance, or concomitant with pregnancy. Neonatal jaundice requiring exchange transfusion was common; one patient died during exchange transfusion soon after birth. The early onset of symptoms was usually associated with a severe clinical course: 16 of the 25 exchange-transfused newborns subsequently required multiple transfusions and/or splenectomy. Overall, 38/59 patients received blood transfusions (1 to >100, median 15), of whom 19 were transfusion-dependent in childhood or until splenectomy.

Gallstones are detected with increased frequency after the first decade of life, and may occur even after splenectomy. Rare complications include aplastic crisis following parvovirus infections, kernicterus, chronic leg ulcers, acute pancreatitis secondary to biliary tract disease, splenic abscess, spinal cord compression by extramedullary haematopoietic tissue and thromboembolic events (Tanaka & Zerez, 1990; Pincus *et al*, 2003).

Iron overload is a predictable complication in chronic transfusion therapy, but it may also occur in patients with limited or no history of transfusions (Zanella *et al*, 1993, 2001a). The pathogenesis of iron overload in patients with PK-deficient haemolytic anaemia is considered to be multifactorial. Chronic haemolysis alone, although resulting in

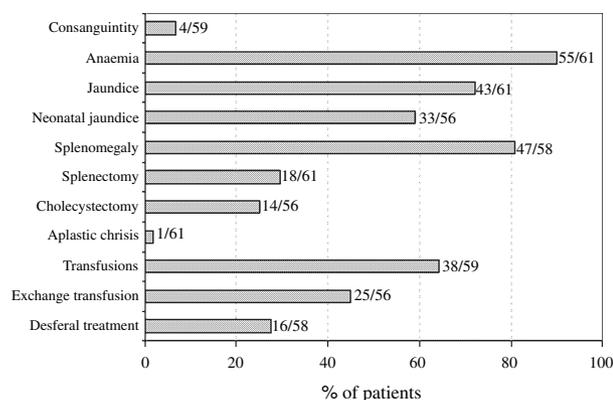


Fig 2. Clinical characteristics of 61 PK-deficient patients.

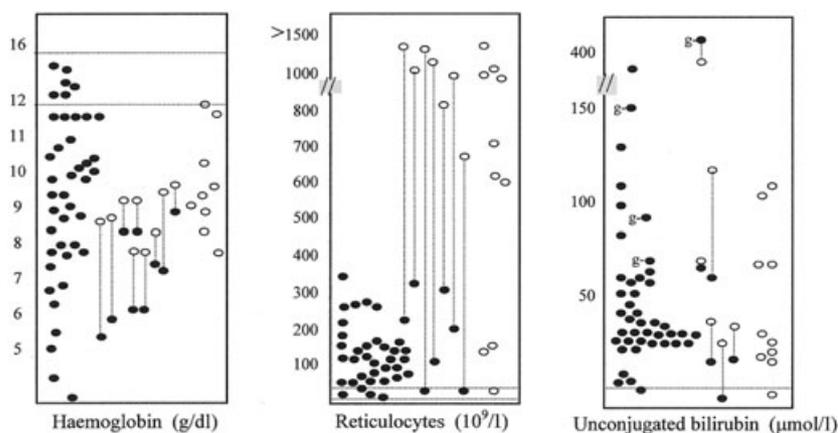


Fig 3. Routine haematological data of 61 PK-deficient patients (● not splenectomized; □ splenectomized). The horizontal dotted lines delimit the reference range for each parameter. Vertical bars indicate pre- and postsplenectomy values in single subjects. g, Gilbert's syndrome.

increased iron turnover, does not seem, *per se*, to be sufficient to cause iron accumulation in this disease (Zanella *et al*, 1993, 2001a). Splenectomy, which is a recognized risk factor for iron loading in untransfused haemolytic states (Pootrakul *et al*, 1981; Porter, 2001), and ineffective erythropoiesis have been regarded as possible cofactors in some cases (Zanella *et al*, 1993). Moreover, it has been hypothesized that the presence of hemochromatosis mutations C282Y and H63D may contribute to iron overload in PK patients in co-operation with other genetic or non-genetic factors (Piperno *et al*, 1998; Zanella *et al*, 2001a).

Haematological features

The haematological features of PK deficiency are common to other hereditary non-spherocytic haemolytic diseases. Figure 3 reports some routine laboratory findings in 61 PK-deficient patients, divided into splenectomized and unsplenectomized groups. Median haemoglobin concentration was 9.8 g/dl in unsplenectomized patients and 7.3 g/dl in candidates for splenectomy. Splenectomy usually resulted in stabilization of the haemoglobin at a slightly higher level (median Hb increase 1.8 g/dl, range 0.4–3.4).

The reticulocyte count in unsplenectomized patients is usually increased (median $166 \times 10^9/L$); however, reticulocytosis is not proportional to the severity of haemolysis, contrary to that observed in other haemolytic diseases, as younger PK defective erythrocytes are known to be selectively sequestered by the spleen (Mentzer *et al*, 1971; Matsumoto *et al*, 1972). Consequently, splenectomy results in a conspicuous rise of reticulocytes (median $796 \times 10^9/l$), even if the anaemia becomes less severe. This is a peculiar feature of PK deficiency and may be of some diagnostic value.

Unconjugated bilirubin concentration is very often increased, but usually $<85 \mu\text{mol/l}$, and may show a slight rise after splenectomy. In the presence of higher levels, a concomitant Gilbert's syndrome should be suspected (Sampietro *et al*, 2003).

Red cell morphology is commonly unremarkable, displaying some degree of anisocytosis, poikilocytosis and polychromatophilia; a variable proportion (3–30%) of contracted echinocytes, i.e. small, densely staining spiculated cells, is occasionally observed (15% of patients in our series), particularly after splenectomy. Although not specific, the presence of many shrunken echinocytes on a postsplenectomy blood smear has been considered to be suggestive of PK deficiency (Leblond *et al*, 1978).

Red cell osmotic fragility was normal in 75% of our patients and decreased in the remaining ones, in line with that observed by others (Dacie, 1985). Autohaemolysis was abnormal in only 21% of cases, confirming that this test is of no diagnostic value in this disease (Zanella & Bianchi, 2000).

Iron status parameters were increased in 33/49 patients: 18 had increased serum ferritin (SF) alone, 14 had increased SF and transferrin saturation (TS) and one had TS alone.

As the haematological features of PK deficiency are not distinctive, the diagnosis ultimately depends upon the demonstration of low enzyme activity, although it is known that some patients may display normal or even increased activity (Zanella & Bianchi, 2000). In our series, PK values were decreased (median 35% of normal) in all patients but four. PK activity is not related to the severity of haemolysis, as already reported (Tanaka & Paglia, 1971), or to the reticulocyte count (Fig 4).

Care must be taken in interpreting *in vitro* PK assays: contamination with normal donor red cells in recently transfused patients and incomplete leucocyte removal (leucocyte PK activity is 300 times higher than that of RPK) may result in a false normal red cell enzyme activity. Moreover, the M_2 isoenzyme may be expressed in mature red cells of some patients (Kanno *et al*, 1993a; Lenzner *et al*, 1994a), contributing to the measured activity. Finally, kinetically abnormal mutant PKs, although ineffective *in vivo*, may display normal or even higher catalytic activity under the optimal, artificial conditions of laboratory assays (Zanella & Bianchi, 2000); this possibility makes it advisable to determine the enzyme activity

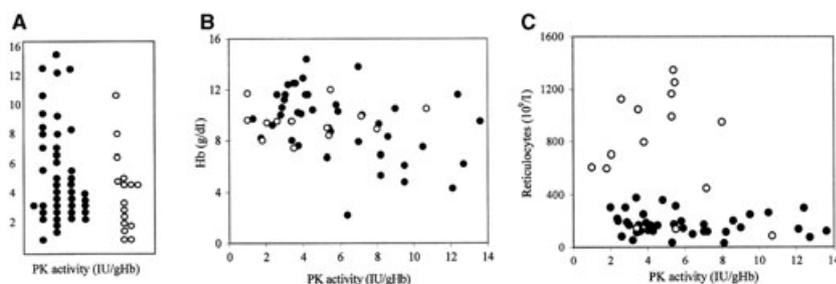


Fig 4. (A) PK activity in 61 PK-deficient patients (●, not splenectomized; ○, splenectomized) (B) Correlation between PK activity and haemoglobin levels. (C) Correlation between PK activity and reticulocytes.

at both high and low phosphoenol pyruvate concentration (Beutler, 1984). Moreover, it may be helpful to study parents and other family members for the presumed heterozygous state of the enzyme deficiency.

For many years, the adoption of standardized methods established by the International Committee for Standardisation in Haematology (Miwa *et al*, 1979) has enabled the identification of a number of PK variants characterized by single or multiple biochemical alterations. The biochemical characterization has been rapidly supplanted by DNA testing that, through the identification of the gene mutation, allows a more precise genotype/phenotype correlation (Miwa *et al*, 1993) and enables prenatal diagnosis to be performed in the more severe cases (Baronciani & Beutler, 1994; Rouger *et al*, 1996a).

Molecular diagnosis of PK deficiency is usually made by sequencing all the exons, flanking regions and erythroid promoter of the *PK-LR* gene. Some variants, for example large deletions, mutations in 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 polymerase chain reaction amplification. In addition, the presence of a large deletion in one allele can give a false result of homozygosity. The recent finding of 'unusual' mutations in the *PK-LR* gene (Costa *et al*, 2005; Fermo *et al*, 2005) confirms the difficulty of genetic analysis in some cases, and underlines the importance of familial studies in the molecular diagnosis of PK deficiency.

Relation between molecular defect and disease severity

The biochemical and clinical consequences of PK mutations have usually been deduced from the investigation of a few homozygous patients and, to a lesser extent, from the study of larger series of compound heterozygotes grouped according to their clinical phenotype (Zanella & Bianchi, 2000). More recently, the production and characterization of the recombinant mutant proteins of human RPK made it possible to define the effects of amino acid replacements on the stability and kinetic properties of PK and helped to correlate genotype to clinical phenotype (Wang *et al*, 2001; Valentini *et al*, 2002).

Clinical studies (Zanella & Bianchi, 2000) indicated that severe syndrome was commonly associated with some missense mutations (in particular 994A and 1529A) in the homozygous state, or with disruptive mutations, such as stop codon in the first part of the protein (for example 721T), frameshift and splicing mutations, or with missense mutations involving the last part of the protein. The rare patients with homozygous 'null' mutations (i.e. mutations resulting in the absence of a functional protein product) displayed intrauterine growth retardation, severe anaemia at birth, need of exchange blood transfusion and transfusion dependence until splenectomy and, in rare cases, intrauterine death or death in the first days of life (Rouger *et al*, 1996a; Kanno *et al*, 1997; Manco *et al*, 1999; Cotton *et al*, 2001; Sedano *et al*, 2004; Fermo *et al*, 2005).

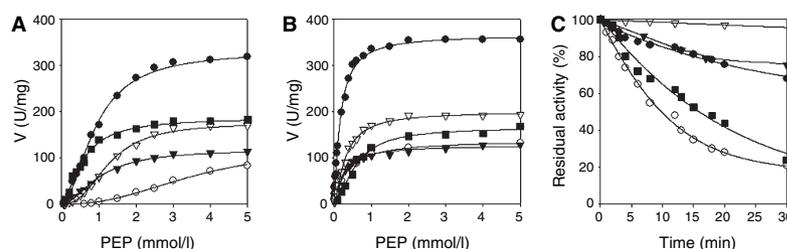


Fig 5. Characterization of wild-type RPK (●) and four types of mutants (▽, Arg486Trp, A/C interface; ▼, Thr384Met, A/A' interface; ○, Gly332Ser, hydrophobic core; ■, Arg532Trp, allosteric site). (A) Steady-state kinetics as a function of PEP in the absence of FBP; (B) steady-state kinetics as a function of PEP in the presence of FBP; (C) thermal stability (residual activity after incubation at 53°C expressed as a percentage of the initial activity).

A survey of the mutations associated with PK-deficient non-spherocytic haemolytic anaemia shows that most of the missense mutations cluster in specific regions of the protein three-dimensional structure: the interface between the A and C domains, the A/A' intersubunit interface, the hydrophobic core of the A domain, and the FBP-binding site (Mattevi *et al*, 1996; Valentini *et al*, 2002). We generated and functionally characterized nine RPK mutants (Gly332Ser, Gly364Asp, Thr384Met, Asp390Asn, Arg479His, Arg486Trp, Arg504Leu, Arg510Gln and Arg532Trp) targeting residues belonging to each of these regions of the protein (Wang *et al*, 2001; Valentini *et al*, 2002). In addition, for three of them (Thr384Met, Arg479His and Arg486Trp) we solved the crystal structure in complex with FBP, the allosteric activator, and phosphoglycolate, a substrate analogue (R-state). Almost all selected mutations have been found in homozygous patients. The kinetic, allosteric and thermostability parameters of all mutants were evaluated and related to the clinical pattern. Figure 5 shows, for each type of mutation (A/C and A/A' interface, hydrophobic core, allosteric site), an example of the effect on the thermal stability of the enzyme and on the steady-state kinetics as a function of PEP in the presence and in the absence of FBP.

Mutations at the A/C interface

1529A mutation (Arg510Gln) at the homozygous state results in very low residual PK activity (10%-25% of normal) associated with severe to moderate haemolytic anaemia, with haemoglobin levels ranging from 5.8 to 12.2 g/dl (Zanella & Bianchi, 2000). Actually, the recombinant mutant protein (Wang *et al*, 2001) shows a kinetic behaviour towards PEP and ADP very similar to that of the wild-type enzyme. Conversely, it exhibits an higher susceptibility to ATP inhibition and, most of all, a dramatically lowered thermal stability. Thus, PK deficiency associated with mutation 1529A appears to be primarily the result of a lowered intracellular level of RPK, rather than because of the altered kinetic and regulatory properties of the enzyme.

Mutation 1456T determines the amino acid substitution Arg486Trp, thus changing the local conformation of the protein and the local distribution of the charges. Arg486 is hydrogen-bonded to the carbonyl oxygen of Leu362 at the C terminus of the A domain helix 6. The functional and structural characterization of the recombinant mutant protein reveals that such a drastic amino acid replacement results in small effects on the molecular properties. The mutant three-dimensional structure shows that the Trp side chain is accommodated without any structural perturbation. Actually, this mutant is even more stable than the wild-type protein and properly responsive to effectors. The only significant perturbation is in the catalytic efficiency, which drops to 30% with respect to the wild-type RPK. However, the kinetic behaviour exhibited by the recombinant Arg486Trp is puzzling because Arg486 is >20Å away from the catalytic centre, which is left unperturbed by the mutation. Possibly, the Trp aromatic ring

introduced may restrict the overall ability of the enzyme to undergo the conformational changes occurring during catalysis, thereby perturbing the reaction kinetics. The moderate alterations of the kinetic parameters of Arg486Trp mutant correlate with the clinical symptoms because the few patients homozygous for 1456T generally exhibit a lifelong history of mild anaemia (haemoglobin 10–12 g/dl) (Zanella *et al*, 1997). The mild nature of the anaemia may mean that this mutation is underdiagnosed, and this could explain why 1456T is only rarely found in the homozygous state in spite of being one of the most frequent mutations (Beutler & Gelbart, 2000).

The other two investigated mutants targeting the A/C interface affect Gly364 and Arg504, which are part of a region of close association between the A and C domains.

As regards mutation 1091A (Gly364Asp), Gly364 allows a sharp turn of the polypeptide chain with a backbone conformation that is unfavourable for a non-glycine residue. The mutation leads to a drastic reduction of the enzyme stability, probably impairing the domain assembly. Thus the severe anaemia observed in patients homozygous for this mutation (van Solinge *et al*, 1997a) could be a consequence of a lowered intracellular level of the enzyme.

The mutation 1511T (Arg504Leu) affects Arg504, a C domain residue that is partly solvent-accessible and engaged in a salt bridge with the Asp281 of domain A. The mutation removes this interdomain interaction and introduces a hydrophobic Leu side chain in a solvent-exposed site close to a negatively charged Asp. Such amino acid replacement is clearly unfavourable, as it causes full inactivation of the protein, providing a reason for its extreme instability. This feature explains the severe anaemia found in PK-deficient patients homozygous for this mutation (Demina *et al*, 1998).

Mutations in the A domain hydrophobic core

Among mutations targeting the hydrophobic core of A domain, mutation 994A (Gly332Ser) is the most frequent and severe. It affects a residue that is strictly conserved among PK sequences. The replacement Gly332Ser leads to a significant decrease of the catalytic efficiency (1 order of magnitude in the absence of FBP and fivefold in the presence of FBP) and drastic reduction of stability, accounting for the very severe haemolytic anaemia (haemoglobin 4.2–7.4 g/dl, with transfusion dependence until splenectomy) displayed by the three homozygous patients so far reported (Zanella & Bianchi, 2000; Fermo *et al*, 2005). In one family of our series this defect was associated with intrauterine death.

Mutations at the A/A' interface

The mutation 1151T, found in Europe and Japan, leads to the amino acid substitution Thr384Met. Thr384 is located at the N terminus of helix 7 of the A domain (β/α)₈ barrel and, although not directly involved in intersubunit interactions, lies very close to the A/A' molecular twofold axis. The replacement

Thr384Met minimally impairs either stability or kinetic properties. Thr384 is not part of the binding sites for PEP and ADP. The crystal structure of the Thr384Met protein shows that active site is really well conformed and the Met side chain is easily accommodated, the only change being the removal of the helix-capping hydrogen bonds, normally engaged by the amino acid affected by this mutation. It is remarkable that homozygosity for the Thr384Met mutation is associated with severe anaemia (Zanella & Bianchi, 2000), implying that even moderate changes in the enzyme catalytic efficiency can have pathological effects.

Mutation 1168A (Zanella *et al*, 1997) produces the amino acidic substitution Asp390Asn. Asp390 is a solvent-inaccessible residue located in the A/A' interface, at the heart of a hydrogen bond network that involves Arg337 and Ser389, belonging to two different subunits. Based on crystallographic studies performed on *E. coli* PK, Asp390 is crucial for the allosteric transition by coupling changes in the quaternary structure with rearrangements of the active site (Mattevi *et al*, 1995; Valentini *et al*, 2000). The molecular analysis shows that the Asp390Asn amino acid replacement causes the almost complete inactivation of the protein, which, however, is as thermostable as the wild-type RPK. Thus Asp390Asn mutation is likely to lock the protein in an inactive conformation, impairing the transition to the R state.

Mutations in the allosteric site

The negative charges of FBP are compensated by the N terminus of helix 479–486 for the 1'-phosphate, and Arg532 side chain for the 6'-phosphate. Two mutations, 1436A and 1594T, found in patients affected by haemolytic anaemia target both elements involved in FBP binding. Mutation 1594T (Arg532Trp) has been found in compound heterozygosity with the nonsense mutation 721T. The clinical symptoms in this patient were severe (Lakomek *et al*, 1994). Arg532Trp recombinant protein is fully unresponsive to FBP, highlighting the pivotal role of Arg532 in activator binding. The lack of allosteric properties is associated with a decreased thermostability, possibly reflecting the energetically unfavourable exposure on the protein surface of the hydrophobic Trp residue.

The 1436A (Arg479His) mutation has been found in severely affected PK-deficient patients (Kanno *et al*, 1994a,b; Kugler *et al*, 2000). The side chain of Arg479 is located in the neighbourhood of FBP, although it does not directly interact with the activator. The crystal structure of Arg479His is identical to that of the wild-type protein, with the His side chain being fully solvent-exposed. Similarly, the kinetic parameters appear to be essentially unaffected by the mutation. These features are in contrast with the severe clinical symptoms. An explanation for this riddle is given by the observation that mutation 1436A, which is located on a splicing site at the 3'-end of exon 10, has recently been found to be associated with strongly reduced *PK-LR* gene transcript levels (van Wijk

et al, 2004). Thus, defects in the mRNA processing may be the actual cause of the RPK deficiency associated with this mutation.

In conclusion, the molecular characterization of RPK mutants highlights that mutations affect, to different extents, thermostability, catalytic efficiency and regulatory properties of the molecule. Mutations that target amino acids engaged in interdomain interactions at the A/C interface are generally harmful to the stability of the protein, highlighting the crucial role of these amino acids in maintaining the correct enzyme folding. Mutations that greatly impair thermostability, and/or activity, are associated with severe anaemia. However, mutations that cause moderate kinetic alterations may also give rise to mild to severe anaemia, underlining the essential role of RPK for the entire erythrocyte metabolic process.

The correlation between molecular and clinical parameters in PK deficiency suggests that biochemical characterization of mutant proteins may be a valuable tool to assist with diagnosis and genetic counselling. However, although there is in general correlation between the nature and location of the replaced amino acid and the type of molecular perturbation, caution is needed in predicting the consequence of a mutation by simply considering the target residue *per se*: 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. Actually, intrafamily variability of clinical pattern has been reported in some PK-deficient kindred (Lenzner *et al*, 1997a; Sedano *et al*, 2004). The variability of clinical expression has been related to possible individual differences in metabolic or proteolytic activity that may diversely modulate the basic effect of the mutation, or to the compensatory persistence of the M₂-type enzyme in some cases (Kanno *et al*, 1993a; Lenzner *et al*, 1994a, 1997a). Moreover, other factors, such as recurrent infections, or some degree of ineffective erythropoiesis, may interfere with the severity of anaemia. In addition, iron overload, particularly in splenectomized patients and/or concomitant with heterozygous hereditary haemochromatosis may greatly impair the clinical course of the disease (Zanella *et al*, 1993, 2001a).

Treatment

In spite of a variety of drugs and chemicals administered to improve *in vivo* activity (Zanella *et al*, 1976; Dacie, 1985; Mentzer & Glader, 1989), no specific therapy for PK deficiency is available, and the treatment of this disease is therefore based on supportive measures. Red cell transfusions may be required in severely anaemic cases, particularly in the first years of life; the haemoglobin then tends to stabilize in many cases at about 6–8 g/dl, and transfusions are no longer necessary unless the anaemia is exacerbated by intercurrent infections, pregnancy or other conditions (Tanaka & Paglia, 1971; Dacie, 1985). As the delivery of oxygen to tissues is highly efficient because of

the high 2,3-DPG content, the decision to transfuse a PK-deficient patient should be based on the clinical condition rather than the haemoglobin level.

Splenectomy does not arrest haemolysis, and usually results in an increase of 1–3 g/dl in haemoglobin; however, it often reduces or even eliminates the transfusion requirement in most transfusion-dependent cases (Dacie, 1985). The removal of the spleen should therefore be reserved to severely affected, young patients who need regular blood transfusions, and to patients who do not tolerate anaemia (Tanaka & Paglia, 1971). Splenectomy should also be considered in patients requiring cholecystectomy, because of the possibility of combining the two operations during the same laparoscopic surgical procedure (Watanabe *et al*, 2002). There is no way to predict the therapeutic efficacy of splenectomy, other than the response of other affected family members who may have undergone the operation. Aplastic or haemolytic crises may still occur after splenectomy.

Iron chelation may be required, as iron overload is rather common in PK deficiency, even in untransfused patients (Zanella *et al*, 1993, 2001a): repeated courses of desferrioxamine were needed in 16/58 patients of our series. Deferiprone has been employed in one case (Marshall *et al*, 2003). Erythropoietin has also been reported as an effective treatment of iron overload in one patient (Vukelja, 1994). Bone marrow transplantation has been successfully performed in one severely affected child (Tanphaichitr *et al*, 2000). Finally, gene transfer studies of the human RPK cDNA into haematopoietic stem cells of a lethally irradiated mouse (Tani *et al*, 1994) have shown the feasibility of gene therapy in this disease.

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