Molecular Abnormality of Erythrocyte Pyruvate Kinase Deficiency in the Amish

By Hitoshi Kanno, Samir K. Ballas, Shiro Miwa, Hisaichi Fujii, and Herbert S. Bowman

We describe the cellular and molecular biologic studies of the erythrocyte pyruvate kinase (PK) deficiency of the Amish deme in Pennsylvania. Nucleotide sequencing of the patient's PK gene showed a point mutation, CAC to CAC, corresponding to no. 1436 from the translational initiation site of the R-type PK (R-PK) mRNA, and it caused a single amino acid substitution from Arg to His at the 479th amino acid residue of the R-PK. The substituted Arg residue is located in the C domain of PK subunit, that is essential for both the intersubunit contact and the allosteric regulation. Because this enzyme shows the catalytic activity only as a dimer or tetramer, it is rational that the structural alteration would result in severe PK deficiency. To elucidate the effect of the PK deficiency on red blood cell (RBC) membrane, we performed the cellular studies of the patients' RBCs. Ouabain-insensitive K+ efflux was increased to 142% to 145% of normal controls and not inhibited by furosemide, as previously observed in HbSC disease RBCs.

PYRUVATE KINASE (PK) deficiency is the most common hereditary nonspherocytic hemolytic anemia caused by a glycolytic enzyme defect. The residual erythrocyte PK activity is not usually related to the severity of anemia, whereas the enzymatic characteristics such as a decreased substrate affinity, thermal instability, or impaired response to the allosteric activator correspond well to the phenotype. We have analyzed the R-PK cDNA of six Japanese PK-deficient families, including five true homozygotes and a compound heterozygote, and identified four distinct missense mutations. Another group found point mutations in the Turkish and Lebanese PK-deficient individuals, and one mutation found in the Lebanese was identical with the mutation identified in two Japanese families. It was noteworthy that these mutations may cause the structural changes near the potassium-binding sites; consequently, the variant PKs showed low affinity with the substrate, phosphoenolpyruvate (PEP). We have also shown that the variant PK synthesized in Escherichia coli had a thermal instability. Although the reticulocyte mRNA is the appropriate source for cloning and sequencing the R-PK cDNA, the genomic DNA is more convenient for handling. Recently, we have clarified the structure of the human L-type PK (L-PK) gene, and it becomes possible to search the mutation in the genomic DNA analysis.

In 1963, five PK-deficient kindred found in the Mifflin County Amish deme in Pennsylvania were reported. Previous studies showed that all the PK variants descended from a common ancestor, and consanguineous marriage was often noted in the family. Therefore, the PK-deficient subjects in the population are homozygous with an identical mutation. The clinical manifestation of the Amish PK variant was reported to be severe, and sometimes death occurred in the neonatal or infantile period unless splenectomy was performed. To clarify the molecular abnormality of the Amish PK variant might be useful not only for the understanding of the relationship between the structure and the function of PK but also for the screening of the variant gene in the population. In this report, we present the molecular abnormality identified in an Amish family, and discuss the structure-function relationship of PK and the cellular characteristics of PK-deficient erythrocytes.

MATERIALS AND METHODS

Clinical features of the Amish PK-deficient subjects. AY and JY are siblings, being brother and sister and members of the STY, Sr. family (Fig 1). STY, Sr, is a distant consanguineous relative of SIJ and AB who are believed to be the ancestral pair of the Mifflin County Pennsylvania Amish red blood cell (RBC) PK-deficient gene. Family history showed JY to be one of 10 Old Order Amish siblings of the STY, Sr, pedigree. Two sisters had been affected with the Amish RBC PK-deficient hemolysis, one of whom died. A subsequent sister born later, AY, likewise was found to have this RBC disorder. JY was initially seen at age 1 month, having been icteric at 48 hours of life with deepening jaundice during the subsequent 5 days. As the icterus gradually faded, he evidenced increasing pallor and was referred to the Hematology Center, Harrisburg Hospital. He was transfused with packed RBCs sufficient to maintain the hematocrit at 26%, and such transfusions were maintained every eighth week. The pretransfusion hematologic values were: RBCs, 1.56 x 10^12/L; hemoglobin (Hb), 5.1 g/dL; hematocrit (Het), 18.5%; mean cell volume (MCV), 112 FL; reticulocytes, 15.4%. Blood film showed 58 nucleated RBCs/100 white blood cells (WBCs), macrocytosis, polychromasia, and marked anisocytosis. Total bilirubin was 2.6 mg/dL, and lactate dehydrogenase (LDH) 1,250 U. RBC PK activity was 0.1 U/10^11 RBCs. At 6 months of age, his spleen became palpable 5 cm below the left costal margin. Transfusion therapy was continued until 17 months of age, when splenectomy was performed. Splenic red pulp preparations showed reticuloendothelial cell, RBCs, and reticulocyte phagocytosis and increased reticulocytes. Subsequent examinations have shown normal findings and no transfusion has been required. Current examinations at age 31 years have shown.

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Submitted June 18, 1993; accepted December 8, 1993.

Supported in part by a Scientific Research Grant from the Ministry of Education, Science, and Culture, and by a Research Grant for Specific Diseases from the Ministry of Health and Welfare, Japan, and in part by the National Institutes of Health Comprehensive Sickle Cell Grant No. HL 38632.

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RBCs 2.55 × 10⁶/μL, Hb 10 g/dL, Hct 29.6%, MCV 116 fl, and reticulocytes 26%. In the blood film, macrocytosis, polychromasia, and echinocytosis were noted. LDH was 278 U.

**Materials.** Blood samples from the probands were obtained under informed consent. High molecular weight genomic DNAs were purified by the standard protocol.12 Restriction endonucleases and modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (Beverly, MA). Taq DNA polymerase (AmpliTaq) was obtained from Perkin Elmer Cetus (Norwalk, CT). DNA was sequenced using a DNA sequencing system (Model 373A; Applied Biosystems, Foster City, CA). The computer software, DNASIS (Hitachi Software Engineering, Yokohama, Japan), was used to analyze the hydrophobicity properties of the variant protein based on the theory of Hopp and Woods.15

**Hematologic data and cellular studies about PK-deficient RBCs.** Routine hematologic methods were used to determine the Hb level, Hct, RBC indices, and reticulocyte count. Whole RBC deformability and RBC sodium (Na⁺) and potassium (K⁺) concentrations were determined as previously described.13 The ouabain-insensitive component of K⁺-efflux was measured as described previously.15

**Amplification and sequencing of the human L-PK gene.** Oligonucleotides used for the amplification of the exons of human L-PK gene are listed in Table 1. L1 (5'-CGCGGGAGGCAGAGGGCCGCAGT-3') and L2 (5'-CTGACCGCAGCTGGCTCATG-3') were used for amplifying the 3' end sequences of exon 10. Polymerase chain reaction (PCR) was performed in a total volume of 100 μL containing 1 μg of the template DNA, deoxynucleotides at 200 mmol/L each, a pair of primers, 10 mmol/L Tris/HCl (pH 8.3), 50 mmol/L KCl, 1.5 to 3 mmol/L MgCl₂, 0.001% gelatin, and 5 U of Taq DNA polymerase. The amplified DNA fragments were subcloned into pBluescript (Stratagene, La Jolla, CA) by the restriction endonuclease digestion method with fluorescent primers.

**RESULTS**

**Cellular studies of the PK-deficient RBCs.** Hematologic characteristics of the subjects studied and the rheologic properties of PK-deficient RBCs are listed in Table 2. Because both JY and AY had been splenectomized, the anemia was not severe on the examination. The MCV in PK deficiency was increased, and it seemed to be reflected on the high reticulocyte counts, which were usually observed in PK deficiency after splenectomy. It should be noted that the PK-deficient RBCs showed increased maximum deformability as well as increased deformability at isotonic conditions.

To elucidate further the hydration status and surface area-volume ratio of PK-deficient RBCs, we measured the density distribution and cation content of these cells (Fig 2, Table 3). There is a significant shift to lower density in PK-deficient RBC populations as seen in Fig 2. Table 3 shows that the sodium content of PK-deficient cells is similar to that of controls, whereas the potassium and total cation contents are significantly increased to 117% to 121% of normal controls.

To investigate the nature of the abnormal cation regulation in PK-deficient RBCs further, we determined the ouabain-insensitive K⁺ efflux in the presence or absence of 1.0 mmol/L furosemide, an inhibitor of Cl⁻ transport and chloride-dependent K⁺ flux. Table 4 shows that PK-deficient cells had a markedly increased K⁺ efflux. The first-order rate constant for K⁺ efflux of the PK-deficient cells was 0.034 to 0.035 h⁻¹, compared with 0.024 for control cells (142% to 145% increase). The addition of furosemide reduced the rate constant for K⁺ efflux of normal cells, but did not decrease that of the PK-deficient cells.

**Identification of a point mutation in the L-PK gene of the Amish PK variant.** Sequencing of the L-PK gene from the Amish PK variant showed that the variant had a single nucleotide change at the 3'-end nucleotide of exon 10 of human L-PK gene, corresponding to no. 1436 of human R-PK cDNA, 1436 CGC → CAC. This missense mutation caused a single amino acid substitution; 479 Arg → His (R479H), which subsequently decreased the hydrophobicity properties near the mutated site (Fig 3). The predicted secondary structure by the theory of Chou and Fasman showed that the core of α-helix might be formed by this amino acid change (data not shown). This Arg residue is well conserved during evolution, including chicken M1-, cat M1-, rat R-, and human M2-type PK (Fig 4).16-19

**Demonstration of the point mutation by the Hpa II digestion of the genomic PCR products.** To confirm this nucleotide change in genomic DNAs of the probands, the 3' end

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**Table 1. Oligonucleotides for PCR of Genomic PK**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Product (bp)</th>
<th>Restriction Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5'-CTAAAGCTTCCTGGGACAGGTTGCC-3'</td>
<td>5'-GAGGCTCTGAGAAGCTACG-3'</td>
<td>374</td>
<td>HindIII/Sty I</td>
</tr>
<tr>
<td>III</td>
<td>5'-GTGACATGGCAGTCCCTGAAGCCGCTCC-3'</td>
<td>5'-ATACCAATAGGCGCTTGTGGGC-3'</td>
<td>260</td>
<td>Sac I/Pat I</td>
</tr>
<tr>
<td>IV-V</td>
<td>5'-GGCTGGTTGAGAATGTTTAGTGG-3'</td>
<td>5'-TCCACTTTCGAGCCTGGACC-3'</td>
<td>559</td>
<td>Eco RI/Apa I</td>
</tr>
<tr>
<td>VI</td>
<td>5'-GACTCCGGAGCTCAGAATCTA-3'</td>
<td>5'-CAGCGCAGGATGTCAATCGG-3'</td>
<td>272</td>
<td>Sac I/Bcl I</td>
</tr>
<tr>
<td>VII-VIII</td>
<td>5'-GTACCGCGAGCTGGGCTTTATCGT-3'</td>
<td>5'-CCTCTGGCTGTTGGATTCACCA-3'</td>
<td>626</td>
<td>Pvu II/Pst I</td>
</tr>
<tr>
<td>IX-X</td>
<td>5'-GGTTGCTCAGAAGAGTACCTTGCGGC-3'</td>
<td>5'-CAAAAGCTCCATCTGGACATT-3'</td>
<td>531</td>
<td>Kpn I/Bam HI</td>
</tr>
<tr>
<td>XI</td>
<td>5'-GAAATCACCATCCATCTGG TGCGC-3'</td>
<td>5'-CTGCCGATGCAATATGAGAG-3'</td>
<td>281</td>
<td>Eco RI/Bam HI</td>
</tr>
<tr>
<td>XII</td>
<td>5'-CCACAGCTGCTCAATGATTG-3'</td>
<td>5'-AAGGATCTTGAGGCGCTGAGAG-3'</td>
<td>468</td>
<td>Pvu II/Pst I</td>
</tr>
</tbody>
</table>

The primers used in sequencing the entire coding region for the R-type PK are shown. To create appropriate restriction sites, the underlined nucleotides are added or substituted to the primers.
Table 2. Hematologic and Rheologic Data on the Patients Studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age/</th>
<th>Hb (g/dL)</th>
<th>Hct (%)</th>
<th>MCV (fL)</th>
<th>Reticulocytes (%)</th>
<th>Max Dl</th>
<th>DI 290</th>
<th>% of Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY</td>
<td>F</td>
<td>29</td>
<td>11.0</td>
<td>37.3</td>
<td>116.1</td>
<td>18.5</td>
<td>111</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>JY</td>
<td>M</td>
<td>31</td>
<td>9.3</td>
<td>27.3</td>
<td>115.8</td>
<td>25.4</td>
<td>120</td>
<td>120</td>
<td></td>
</tr>
</tbody>
</table>

Maximum deformability index (Max Dl) was attained by osmotic gradient ektacytometry. DI 290 is the deformability index of RBCs suspended in a buffer the osmolarity of which is 290 mOsm/kg.

Table 3. Cation Content of PK-Deficient RBCs

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patient</th>
<th>Control</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺, mEq/L RBCs</td>
<td>AY</td>
<td>JY</td>
<td>11.9</td>
</tr>
<tr>
<td>K⁺, mEq/L RBCs</td>
<td>84.6</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Na⁺ + K⁺, mEq/L RBCs</td>
<td>96.4</td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Effect of Furosemide on Ouabain-Insensitive K⁺ Efflux of Normal and PK-Deficient RBCs

<table>
<thead>
<tr>
<th>Additive</th>
<th>Normal RBCs</th>
<th>PK-Deficient RBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.024</td>
<td>0.034</td>
</tr>
<tr>
<td>0.0 mmol/L furosemide</td>
<td>0.014</td>
<td>0.037</td>
</tr>
</tbody>
</table>

Cells were incubated for 2 hours at 37°C in 10 mmol/L HEPES (pH 7.5), 5 mmol/L glucose, 0.2 mmol/L ouabain, and NaCl to bring the final osmolarity to 290 mOsm/kg, in the presence or absence of the indicated additive. Values shown are the negative Δ in of the calculated intracellular K⁺ concentration as a function of time.

DISCUSSION

Here we report a molecular abnormality in the Amish PK deficiency. The probands had suffered from severe hemolytic anemia, and required transfusion every 8 weeks. After splenectomy the anemia was compensated, with a Hb level of about 9 to 10 g/dL. Cellular studies of the PK-deficient RBCs showed increased K⁺ content and a markedly expanded ouabain-insensitive K⁺ efflux. The pathophysiology of PK-deficient erythrocytes has been studied. Keitt has shown that PK-deficient erythrocytes were almost entirely dependent on mitochondrial oxidative phosphorylation rather than on glycolysis to maintain adenosine triphosphate (ATP) levels. A rapid decrease of ATP levels because of decreased oxidative phosphorylation during maturation of erythrocytes allows massive losses of potassium and water, resulting in the formation of spiculated erythrocytes, i.e., echinocytes. These cells usually have decreased deformability, and are easily sequestered by the reticuloendothelial system. Spleenectomy may prevent reticulocytes and young erythrocytes from being trapped by the reticuloendothelial system, resulting in improvement of the anemia in most cases. The hematologic data of the Amish PK deficiency showed postsplenectomy reticulocytosis, which was usually observed in PK deficiency, and the large, lower density cells with increased deformability may reflect mainly the increased number of reticulocytes. Despite the increased K⁺ content, these cells are prone to lose K⁺ and water rapidly because of impaired ATP availability, and become dehydrated and en...
Fig 3. Hydrophobicity profiles near the mutated site. The dotted line indicates the hydrophobicity properties of normal R-PK from no. 471 to no. 490 amino acid residues, and the solid line shows the altered hydrophobicity properties of the Amish PK variant. The arrows show the position of the 479th amino acid residue, which is substituted from Arg to His, and the shift of the hydrophobicity profile caused by the amino acid substitution.

trapped in the splenic environment. This hypothesis is supported by the finding of entrapped reticulocytes in the splenic red pulp of the patient’s spleen.11 It should be noted that the potassium efflux of the Amish PK-deficient erythrocytes was increased up to 142% to 145% of normal controls. Activation of Na+/Cl− cotransport seemed unlikely in PK deficiency, because the addition of 1.0 mmol/L furosemide (an inhibitor of KCl cotransport) to the medium did not reduce the rate constant of K+ efflux. The K+ efflux may be increased by a mechanism similar to that observed in HbSC RBCs.12,13 The reported enzymatic characteristics22 of the identical variant PK were a low Michaelis constant (Km) for PEP, low Km for adenosine diphosphate, narrow nucleotide specificity, less ATP inhibition than normal, more fructose-1,6-diphosphate (FDP) required for 50% activation, thermal instability, normal optimum pH, and normal migration in polyacrylamide gel electrophoresis. It should be noted that lower response to the allosteric effector, FDP, and thermal instability were unfavorable characteristics of this variant, which may be responsible for the phenotype.

There are four mammalian PK isozymes: type-M1, M2, L, and R. The M-type PK (M-PK) gene encodes both M1 and M2 isozymes.3,25 In contrast, L and R isozymes are encoded by the L-PK gene.5,18 Both the M- and L-PK gene of rats and humans have 12 exons.5,18,22,26 Alternative splicing produces two distinct mRNAs, which contain either exon 9 (M1) or 10 (M2) of the M-PK gene. Exons 1 and 2 of the L-PK gene are specifically transcribed to the R- and L-PK mRNA by the tissue-specific promoter. It is of interest that among four isoenzymes only M1-type is not allosterically regulated.23 Because M1 and M2 isozymes differ only in the exon 9-10 region, exon 10 should encode the amino acid residues responsible for the allosteric property. Comparison of exon 10 between the M- and L-PK gene disclosed that there was an extensive homology, indicating that the exon 10-encoding region of L-PK gene also contained the allosteric structure (Fig 4). It seemed to be rational that the variant showed lower response to the allosteric effector, because the missense mutation identified in exon 10 would cause the drastic change of hydrophobicity and secondary structure.

The identification of the molecular lesion of erythrocyte PK deficiency in the Mifflin County Pennsylvania Amish enables us to detect the mutation by a simple procedure, PCR and restriction endonuclease digestion. Thus, it can be applied to genetic counseling or the prenatal diagnosis of the Amish PK-deficient hemolytic anemia, which has been known as a most severe form of PK deficiency. As only the Geauga County Ohio Amish are related to the Mifflin County Pennsylvania Amish deme by an ancestral genetic member (ie, AB),22 no other demes of Amish within the United States are believed to exist sharing this missense mutation or the erythrocyte PK deficiency hemolytic anemia.
Fig 5. *Hpa* II digestion of the PCR products flanking the 3' end of exon 10 from the probands and a normal control. Because there are three *Hpa* II recognition sites in the amplified DNA, the PCR product of normal control is digested into four fragments: 122, 39, 28, and 2 bp. When the 1436 G to A mutation is present, the 201-bp products are digested into three fragments: 171, 28, and 2 bp. M, pBR322 digested with *Msp* I; 1, normal control; 2, the Amish PK-deficient subject JY; 3, the Amish PK-deficient subject JY.

ACKNOWLEDGMENT

We are indebted to Y. Okamura, A. Kawaguchi, and M. Watanabe for their technical assistance.

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