Pyruvate Kinase Greensboro. A Four-Generation Study of a High $K_{0.5s}$ (Phosphoenolpyruvate) Variant

By William N. Valentine, William B. Herring, Donald E. Paglia, Mary Christine Steuterman, Richard A. Brockway, and Misae Nakatani

The proband with lifelong hemolytic anemia has a high $K_{0.5s}$ phosphoenolpyruvate (PEP) erythrocyte pyruvate kinase (PK) variant substantially but incompletely normalized by the allosteric modifier fructose-1,6-diphosphate (F-1,6-P$_2$) with conversion of sigmoidal to hyperbolic kinetics. Heterozygotes in four generations express qualitatively identical but less severely abnormal kinetics and lack overt hemolysis. Kinetic abnormalities are closely mimicked by sulfhydryl modification of normal PK. Three distinct clinical and metabolic phenotypes characterize the proband and two sisters: variant PK and hemolytic anemia, variant PK without clinical manifestations or hemolysis, and complete normality. Their mother, whose red cell PK is entirely normal except for a questionably slightly low $V_{max}$, is postulated to express the gene products of nonidentical alleles, one encoding a product with mildly less favorable catalytic characteristics. At low PEP concentrations, the proband and heterozygotes for the PK mutant express only a very small fraction of normal PK activity despite apparent inheritance of one normal allele in the latter. Evidence suggests that this disproportionately lowered PK activity may be a property of a heterotetrameric PK. Illusory abnormalities in nucleotide specificity are artifacts of diminished substrate affinity characterizing the mutant PK.

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MATERIALS AND METHODS

Red cells and hemolysates. Blood was freed of leukocytes by passage over a column of microcellulose.4 Lysates were prepared as recommended by the International Committee for Standardization in Haematology (ICSH).5 Some hemolysates were largely freed of hemoglobin (Hb) after precipitation by ZnSO$_4$ at pH 8.0 as previously described.$^6$

Enzyme assays. Our PK assay differs somewhat from that recommended by the ICSH. It contained the following: 8.5 mmol/L triethanolamine-saline, pH 7.5, 0.8 mmol/L EDTA, 75 mmol/L KCl, 8 mmol/L MgSO$_4$, 2 mmol/L ADP, 0.3 mmol/L NADH, 14 IU lactic dehydrogenase (LDH), 3.0 or 0.4 mmol/L PEP, and 0.1 mmol/L F-1,6-P$_2$ (when present). Assays were performed at 37°C and were monitored continuously with a Gilford Multi-Sample Recorder (Gilford Instrument Laboratories, Oberlin, OH). All other enzymes were assayed, sometimes with minor modifications, as recommended by ICSH.$^7$

Perchloric acid extracts. Freshly drawn venous blood was delivered into 2 vol of 0.6 N perchloric acid. Neutralized supernatant was assayed for total adenine nucleotides and ATP, ADP, and adenosine monophosphate (AMP).$^5$ The concentration of 2,3-diphosphoglycerate (2,3-DPG) was determined as described in Technical Bulletin 35-UV, Sigma Chemical Co, St Louis.

Apparent $K_{0.5s}$ [PEP] and $K_{0.5s}$ [ADP]. The final reaction mixture, pH 7.8, contained 10 mmol/L Tris-HCl, 0.5 mmol/L EDTA, 100 mmol/L KCl, 10 mmol/L MgCl$_2$, 0.2 mmol/L NADH, and 14 IU LDH. In ADP kinetic studies, PEP was 1.0 mmol/L. When $K_{0.5s}$ [PEP] was determined, ADP was 1.5 mmol/L, and at an acid pH, Tris-maleate buffer, 100 mmol/L, pH 6.0 to 6.5 was substituted.
Nucleoside diphosphate specificity and ATP inhibition. In the former, ADP, guanosine diphosphate (GDP), uridine diphosphate (UDP) and cytosine diphosphate concentrations were 1.5 mmol/L and PEP, 1.0 mmol/L, in assay systems identical to those used in ADP kinetic studies. In the latter, PEP was 2.5 mmol/L and ADP, 1.5 mmol/L.

pH optimum. Assays contained 50 mmol/L Tris-maleate-glycine, 100 mmol/L KCl, 10 mmol/L MgCl2, 0.2 mmol/L NADH, 14 IU LDH, 1.5 mmol/L ADP, and 1.5 mmol/L PEP. The pH was adjusted with KOH or HCl.

Thermostability. In a slight modification of the method of Blume et al, packed red cells were diluted 1:6 with 10 mmol/L Tris-HCl, pH 7.5, 100 mmol/L KCl, 2 mmol/L β-mercaptoethanol (MCE), 10 mmol/L L-α-aminocaproic acid, 10 mmol/L EDTA, 0.1% bovine serum albumin, and 0.5% saponin. Aliquots of 0.2 mL were incubated at 53°C and activity for each incubation time expressed as a percentage of the present initially.

Case report. The proband, III-3, aged 35, has had lifelong hemolytic anemia for which she was splenectomized at the age of 3. In 1977 and 1979 there were episodes of marked worsening of anemia, the latter associated with an infectious ailment, the former with no identifiable illness. Transfusions unassociated with a febrile illness were administered. A transfusion unassociated with a febrile illness was administered. Transfusions unassociated with a febrile illness were administered. Transfusions unassociated with a febrile illness were administered.

The patient is white and has no Mediterranean or Jewish ancestry. Her husband, both parents, three children, two sisters (III-2 and III-7), and two nephews (IV-1 and -2) had normal findings on examination. The paternal grandmother is aged 88, and a single systolic cardiac murmur. Jaundice was minimal, though her skull show prominent frontal bossing and widened diploe.

RESULTS

Hematology. Hemograms obtained on parents of the proband and her husband, three children, paternal grand-

mother, two sisters, and two nephews were normal except for mild anemia in the paternal grandmother who is in ill health. For reasons unexplained, minimally elevated reticulocyte counts (2.0% to 2.5%) were observed in several kindred members, but there was no correlation with the presence or absence of abnormalities in red cell PK. The serum bilirubin level was normal in all.

Routine assay of PK with [PEP], 0.4 and 3.0 mmol/L. Family members segregate into two groups. Group I members (proband, I-1, II-1, II-2, III-6, IV-3, -4 and -5) exhibit erythrocyte PK activity with 0.4 mmol/L [PEP] 2 or more SD below our normal mean (2.6 ± 0.9 IU/1010 RBCs). There was a qualitatively similar but less markedly low activity with 3.0 mmol/L [PEP] and partial but incomplete normalization by F-1,6-P2. In contrast, group II (II-1, III-2, III-4, IV-1, and -2) had PK activities at both [PEP] approximating or within 1 SD of our normal mean when F-1,6-P2 was present or absent. There was no overlap in the two groups. PK activity in II-1 and III-4 was in the low normal range.

Other erythrocyte enzyme activities. In the proband, her paternal grandmother’s (I-1), her sisters’ (III-2 and III-6), and her nephews’ (IV-1 and IV-2) erythrocyte glutathione concentrations were measured as well as the activities of all enzymes of anaerobic glycolysis, the two dehydrogenases of the pentose phosphate shunt, glutathione peroxidase and reductase, AMP and adenosine deaminase, pyrimidine-5’-nucleotidase, glutamic-oxalacetic transaminase, malic dehydrogenase, and acetyl cholinesterase. In the proband, all age-dependent enzyme activities are elevated (that of hexokinase was five times the normal mean) as expected for reticulocyte-rich blood. No abnormalities other than those of PK activity were encountered. In all other subjects, enzyme activities were normal, with the exception of slightly elevated hexokinase levels in the paternal grandmother. In other kindred members only the age-dependent activities of hexokinase, phosphofructokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and glutamic-oxalacetic transaminase were assayed. All were entirely normal and gave no confirmation of a hemolytic component.

Adenine nucleotides and 2,3-DPG. In nine family members other than the proband, red cell 2,3-DPG concentrations varied from 13.2 to 18.4 μmol/g Hb (normal males, 12.8 ± 2.3; females, 13.6 ± 2.6). While a few were suggestively elevated, only that of the proband was dramatically and unequivocally so (28.1 μmol/g Hb). Total adenine nucleotides levels were reduced in proband erythrocytes to 3.75 μmol/g Hb. In contrast, these were normal in her husband (5.06 μmol), her mother (4.5 μmol), her sister III-2 (4.4 μmol), and her two nephews (5.6 and 4.5 μmol).

PK kinetics with PEP. All kindred members in group I share a kinetic abnormality with substrate PEP (Table 1, Figs 2 and 3). Despite the well-known increased PK activity of reticulocyte-rich blood, at [PEP] <5.0 mmol/L the proband’s PK, with or without F-1,6-P2 additions, is less active than that of any kindred member, and the K0.5 [PEP] is the highest by a wide margin. Kinetics in the red cells of her father, one sister (III-6), and her three children are qualitatively less markedly abnormal, are virtual carbon copies, and
Table 1. Kindred D. Erythrocyte $K_{m}$ [PEP] and Hill Coefficient n

<table>
<thead>
<tr>
<th>Subject</th>
<th>$K_{m}$ [PEP]</th>
<th>n</th>
<th>$K_{m}$ [PEP]</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.8</td>
<td></td>
<td>pH 6.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- FDP*</td>
<td>+ FDP</td>
<td>- FDP</td>
<td>+ FDP</td>
</tr>
<tr>
<td>I Proband, III-3</td>
<td>4.00</td>
<td>0.92</td>
<td>1.36</td>
<td>1.01</td>
</tr>
<tr>
<td>Sister, III-6</td>
<td>1.50</td>
<td>0.48</td>
<td>1.76</td>
<td>0.90</td>
</tr>
<tr>
<td>Father, II-2</td>
<td>1.70</td>
<td>0.40</td>
<td>1.67</td>
<td>0.97</td>
</tr>
<tr>
<td>Paternal grandmother, I-1</td>
<td>1.24</td>
<td>0.40</td>
<td>1.30</td>
<td>1.10</td>
</tr>
<tr>
<td>Daughter, IV-3</td>
<td>2.40</td>
<td>0.47</td>
<td>1.35</td>
<td>1.00</td>
</tr>
<tr>
<td>Son, IV-4</td>
<td>2.20</td>
<td>0.48</td>
<td>1.32</td>
<td>0.92</td>
</tr>
<tr>
<td>Son, IV-5</td>
<td>2.80</td>
<td>0.46</td>
<td>1.24</td>
<td>0.94</td>
</tr>
<tr>
<td>II Husband, III-4</td>
<td>0.60</td>
<td>0.15</td>
<td>1.36</td>
<td>1.01</td>
</tr>
<tr>
<td>Mother, II-1</td>
<td>0.60</td>
<td>0.17</td>
<td>1.35</td>
<td>1.05</td>
</tr>
<tr>
<td>Sister, III-2</td>
<td>0.75</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nephew, IV-1</td>
<td>0.50</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nephew, IV-2</td>
<td>0.60</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*FDP, fructose-1, 6-diphosphate (F-1,6-P2)*.  
*ADP = 1.5 mmol/L FOP (when present) = 0.1 mmol/L. Kindred members in group I express peptide Greensboro.

confirm the inheritance of peptide Greensboro in all. The paternal grandmother, I-1, appears to share the same variant peptide, albeit with modestly less abnormal kinetics. In contrast, the $K_{m}$ is normal in all members in group II (Table 1). The Hill coefficient “n” values document sigmoidal PK activity curves in both groups, and F-1,6-P2 restores hyperbolic kinetics with n values approximating 1.0 in all.

The proband and her two sisters are three different metabolic phenotypes. Proband red cells exhibit the most aberrant kinetics. Her asymptomatic sister, III-6, has also inherited the allele encoding kinetically abnormal peptide Greensboro from their father. Her red cell PK kinetic pattern is virtually identical with his. A second sister, III-2, presents as an entirely normal clinical and metabolic phenotype with erythrocyte PK activity at our normal mean and normal by all the usual criteria.

PK kinetics with PEP at acid pH. At pH 6.1 to 6.5, kinetic abnormalities attributable to peptide Greensboro continue to be expressed, although with significant differences (Table 1). As with normal red cell PK, an acid pH induces conformation changes characterized by abolition of cooperativity as evidenced by hyperbolic reaction kinetics and the Hill coefficient n values documented in Table 1. In distinction to its effect on normal erythrocyte PK, F-1,6-P2 markedly lowers the $K_{m}$ [PEP]. Additions of F-1,6-P2 induce abnormally large changes in reaction velocity at low to moderate concentrations of PEP. When the activity of PK partially purified by Zn2+ precipitation of Hb as described in Materials and Methods was compared at pH 6.5 with 3.0 mmol/L [PEP], the following were noted. F-1,6-P2 increased reaction velocity more than 350% in the case of the proband, over 200% in the proband’s three children and father who express peptide Greensboro, and less than 40% in the proband’s mother and a normal control.

PK kinetics with ADP. In all, the $K_{m}$ [ADP] was essentially normal, ranging from 0.075 to 0.19 mmol/L ADP. The Hill coefficient n was 1.0 or below, thus indicating that the kinetics were hyperbolic.

pH optimum. No abnormality of pH optimum with PEP was apparent in any members of kindred D.
Thermostability. Red cell PK was not thermostable in any kindred member. Without F-1,6-P2 additions, after 60 minutes at 35°C, activity was still above 70% of that of "time 0" in all but two whose residual activity approximated 50%. With F-1,6-P2 in the assay, all PK activities were >90% of that before incubation.

Activation by F-1,6-P2. Normal 50% activation of PK was noted at 0.5 to 1.0 mmol/L F-1,6-P2 in the red cells of the proband, I-1, III-2, -4, -6, II-1, and IV-1 and -2. This value was slightly higher in II-2 and IV-3, 4, and 5.

Nucleotide specificity. Illusory abnormalities in PK Greensboro. In the ICSH-recommended protocol, nucleotide specificity is assessed with 1 mmol/L PEP.5 Table 2 compares the effects of UDP ⋅ ADP in the proband and her sister, III-6, who express peptide Greensboro, with those of the proband's husband, III-4, and mother, II-1, who do not. Red cell PK activity of III-3 and III-6 in the absence of F-1,6-P2 additions is anomalously greater with UDP than with ADP, but normal ratios are fully restored by F-1,6-P2. When PK activity with ADP and F-1,6-P2 additions is expressed as a percentage of that with ADP alone, that of the proband and her sister increase nearly 400% to 500%. In contrast, that of II-1 and III-4 increase, but only 10% to 55%. Reaction curves for the proband and her sister III-6 reveal increased PK activity with UDP until [PEP] is 1.25 and 1.0 mmol/L, respectively. In their normal sister, III-2, PK activity with ADP exceeded that with UDP at [PEP] >0.5 mmol/L (data not shown).

High K0.5 [PEP] mutants and normal PK are not saturated to the same degree when [PEP] is 1.0 mmol/L. The anomalous nucleotide specificities evident in III-3 and III-6 in Table 2 may thus be artifacts secondary to diminished saturation of the mutant enzyme in the assay system.

Inhibition by negative effector ATP. Entirely consistent results were not obtained when ATP inhibition was assessed with PK partially purified by Hb precipitation with Zn2+ and unmodified hemolysates. In the latter, inhibition by ATP was substantially more marked in the case of the proband as compared with that in her mother, II-1, husband, III-4, and normal sister, III-2 (data not shown). Unlike the findings with normal PK, additions of F-1,6-P2 failed to fully reverse the inhibition. However, with high K0.5 [PEP] variants, inhibition by ATP is assessed at a diminished saturation with substrate as compared with that of normal PK at the usually used [PEP] of 2.5 mmol/L, and an abnormal reaction may thus be an epiphenomenon.

Table 2. Representative Data Comparing Hemolysate PK Activity (1.0 mmol/L [PEP] and 1.5 mmol/L [ADP] and [UDP]) in Four Members of Kindred D

<table>
<thead>
<tr>
<th>Subject</th>
<th>Activity (ADP) (%)</th>
<th>Activity + F-1,6-P2 (ADP) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proband, III-3*</td>
<td>205</td>
<td>520</td>
</tr>
<tr>
<td>Sister, III-6*</td>
<td>129</td>
<td>379</td>
</tr>
<tr>
<td>Mother, II-1</td>
<td>70</td>
<td>155</td>
</tr>
<tr>
<td>Husband, III-4</td>
<td>53</td>
<td>110</td>
</tr>
</tbody>
</table>

*Possess peptide Greensboro; F-1,6-P2 (when present), 0.1 mmol/L.

DISCUSSION

The proband's father, II-2, one sister, III-6, and three children, IV-3, -4, and -5, are judged to be heterozygous for the allele encoding peptide Greensboro. In II-2, the companion allele is normal as evidenced by its transmittal to his daughter III-2, whose erythrocyte PK activity equals our normal mean and is normal by all criteria. The characteristics of red cell PK in III-6 are almost identical with that of her father, II-2. The companion allele, inherited from her mother, II-1, thus appears to be normal. The PK of II-1 has activity in the low normal range and has a normal metabolic phenotype, and a normal allele has been transmitted to another daughter, III-2. The erythrocyte PK kinetics of all three of the proband's children IV-3, -4, and -5, faithfully reproduce nearly identically the pattern of their grandfather, II-2, and their aunt, III-6. That of their father, III-4, is normal by all criteria.

The question remains as to why the proband alone has overt hemolysis and, in all respects, substantially more severe PK kinetic abnormalities than any other member of the kindred. In our view, the most likely explanation is that her mother, despite the apparent normality of her erythrocyte PK, actually harbors a normal and variant allele. The unfavorable characteristics of the gene product of the latter presumably are not severe but sufficient in conjunction with peptide Greensboro to shift the balance to one resulting in hemolytic anemia. Heterozygosity for mildly abnormal variant alleles is inherently difficult or impossible to detect, being masked by their association with a normal allele and by the variation in red cell PK activity in entirely normal subjects.

Table 3 documents another phenomenon requiring explanation. At PEP concentrations <2.0 mmol/L, the proband and heterozygotes for the allele encoding peptide Greensboro in three generations invariably fail to express more than a small fraction of parental or normal PK activity at the same substrate concentrations. In normal subjects, about 50% of the activity of each parental PK is expressed; in the kindred members documented in Table 3, as little as 9% to 12% normal activity is noted at low substrate concentrations.

Normal red cell PK is a homotetramer. In simple heterozygosity, unlike subunits are normal and variant; in compound

<table>
<thead>
<tr>
<th>Subject</th>
<th>Parent*</th>
<th>0.25</th>
<th>0.50</th>
<th>0.75</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proband III-3</td>
<td>II-1</td>
<td>11.0†</td>
<td>8.0</td>
<td>9.0</td>
<td>12.0</td>
<td>19.0</td>
<td>27.0</td>
</tr>
<tr>
<td>Sister III-6</td>
<td>II-1</td>
<td>9.0</td>
<td>16.0</td>
<td>25.0</td>
<td>30.0</td>
<td>35.0</td>
<td>39.0</td>
</tr>
<tr>
<td>Daughter IV-3</td>
<td>III-4</td>
<td>8.0</td>
<td>13.0</td>
<td>16.0</td>
<td>21.0</td>
<td>24.0</td>
<td>22.0</td>
</tr>
<tr>
<td>Son IV-4</td>
<td>III-4</td>
<td>12.0</td>
<td>9.0</td>
<td>14.0</td>
<td>16.0</td>
<td>19.0</td>
<td>22.0</td>
</tr>
<tr>
<td>Son IV-5</td>
<td>III-4</td>
<td>12.0</td>
<td>11.0</td>
<td>13.0</td>
<td>16.0</td>
<td>19.0</td>
<td>18.0</td>
</tr>
<tr>
<td>Father II-2</td>
<td>‡</td>
<td>13.0</td>
<td>11.0</td>
<td>17.0</td>
<td>19.0</td>
<td>22.0</td>
<td>24.0</td>
</tr>
</tbody>
</table>

*Parent lacking peptide Greensboro.
†Values are percentages of PK activity of the subject divided by the PK activity of the parent.
‡Parent deceased. PK activity is expressed as a percentage of that of a normal son-in-law, III-4.
heterozygozy, neither unlike subunit is normal. Given equal subunit expression and random association, PK in simple or compound heterozygotes is a spectrum of heterotetrameric isoenzymes (A_1A_2B_2, A_1B_2, A_2B_1, A_2B_2, B_1B_2) in a theoretical ratio of 1:4:6:4:1. If B is a variant and A is normal, some 94% of all PK contains one or more mutant subunits. While unconfirmed by subunit analysis, the very low PK activity in subjects expressing peptide Greensboro at low molalities of isozymes (A_4, A_3B, A_2B_2, B_3A, B_4) heterozygosity, neither unlike subunit is normal. Given equal and heterotetramers. This would imply that (a) hybridization occurs and (b) the hybrid enzymes have appreciably less favorable catalytic properties than if the selfsame subunits were assembled into normal and mutant homotetramers. Depending on subunit characteristics, the affinity of unlike subunits for themselves and for each other in the tetramer assembly process, and subunit interaction affecting cooperativity and catalytic properties of hybrid enzymes, metabolic phenotypes of many species are possible.

With normal erythrocyte PK, diminished affinity for PEP, partially normalized by F-1,6-P_2, results from exposure to reagents and conditions having a high specificity for modification of cysteinyl residues. Further, modification by methylmethanethiosulfonate, for example, is completely prevented by the prior presence of small amounts of PEP and Mg_2^+ which strongly suggests that modification occurred at or near the PEP binding site. The same kinetic alterations in "L-type" PK have been reported under conditions expected to alter thiol residues. These included the effects of low concentrations of Cu^{2+}, prolonged incubation with oxidized glutathione, and autoxidation of partially purified PK. Additional reports suggest that, (a) in certain acquired PK deficiencies activity can be partially restored by thiol reductors and (b) the aberrant kinetics with PEP can be normalized in certain mutants by B-mercaptoethanol. It has been further claimed that temporary restoration of normal PK kinetics was achieved in two subjects with PK variant enzymes following intravenous injection of 2-mercaptopropionylglycine. Such circumstantial evidence does not prove involvement of thiol residues in the molecular lesion at or near the PEP binding site in this and similar PK mutants but is compatible with this possibility.

In kindred D, apparent abnormalities in nucleotide specificity and less dramatic increases in ATP inhibition of PK appear to be epiphenomena and, hence, illusory. With normal red cell PK, the V_max is much higher with ADP than with UDP or GDP as a cofactor. However, the K_m for ADP is appreciably higher than that for UDP (GDP). Consequently, while PK activity is highest with ADP at the usual 1.0 mmol/L PEP concentration used in the test, below 0.5 mmol/L PEP, the activity becomes progressively higher with UDP (GDP) than with ADP. With high K_m [PEP] variants, substrate saturation at 1.0 mmol/L PEP is much less than with normal PK. Higher activities with UDP (GDP) may thus be expected as an artifact of diminished affinity for PEP. ATP inhibition is also influenced by effective PEP saturation, which is complete with normal PK at the 2.5 mmol/L PEP concentration used in the test system. With K_m [PEP] variants this may not be the case.

Although PK kinetics are grossly abnormal, heterozygozy for peptide Greensboro is not accompanied by overt hemolysis. However, mitigation by F-1,6-P_2 provides a partial physiologic buffer against ill effects of the mutant peptide in vivo. There are no hard data dealing with the fluctuating availability of F-1,6-P_2 in erythrocytes during fasting, variable glycolytic conditions, and sojourns in regional areas of the circulation such as the spleen. The proband clearly cannot compensate for the kinetic abnormalities; although compensation may be marginal, other members of kindred D expressing peptide Greensboro escape significant hemolysis.

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REFERENCES

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