The Molecular Mechanism of the Inherited Phosphofructokinase Deficiency Associated With Hemolysis and Myopathy

By Shobhana Vora, Laurence Corash, W. King Engel, Susan Durham, Carol Seaman, and Sergio Piomelli

Normal human erythrocyte phosphofructokinase (ATP: d-fructose-6-P-1-phosphotransferase, EC 2.7.1.11; PFK) has recently been shown to consist of a heterogeneous mixture of five tetrameric isozymes: M₄, M₃L, M₂L₂, ML₃, and L₄ (M, muscle type; L, liver type). In the light of these findings, we have investigated the molecular basis of the inherited erythrocyte PFK deficiency associated with myopathy and hemolysis (Tarui disease). The propositus, a 31-yr-old male, suffered from muscle weakness and myoglobinuria on exertion. He showed mild erythrocytosis despite laboratory evidence of hemolysis. In his erythrocytes a metabolic crossover point was found at the level of PFK; 2,3-diphosphoglycerate (2,3-DPG) was also significantly reduced. The PFK from the patient’s erythrocytes consisted exclusively of the L₄ isozyme, and there was a complete absence of the other four. The leukocyte and platelet PFKs from the patient showed normal activities, chromatographic profiles, and precipitation with anti-M₄ antibody. These studies provide direct evidence that in Tarui disease the M₄-type subunits are absent; but the liver- and platelet-type subunits of PFK are unaffected. The paradox of mild erythrocytosis despite hemolysis reflects the decreased production of 2,3-DPG.

In the erythrocytes, inherited deficiencies have been described for almost all the glycolytic enzymes. Since the glycolytic pathway is universally present, an enzymatic defect of the erythrocytes should reflect a generalised defect in all the tissues. Although most glycolytic defects of the erythrocytes are associated with a hemolytic syndrome, concomitant dysfunction of other organ systems is not always observed. Neurologic syndromes occur with triose-P-isomerase, phosphoglycerate kinase, and aldolase deficiencies, while the deficiency of phosphofructokinase (ATP: d-fructose-6,P-1-phosphotransferase, EC.2.7.1.11; PFK) is often associated with severe generalized muscle dysfunction. An inherited deficiency of PFK (complete lack in the muscles, but partial deficiency in the erythrocytes) was first reported by Tarui et al. in 1965. As a moderate deposition of glycogen occurred in the muscles, Brown and Brown designated this syndrome as glycogen storage disease VII. Since the original description, eight additional cases of inherited PFK deficiency have been reported. Of these, only one manifested the same clinical features of Tarui disease, i.e., myopathy and hemolysis. The other cases exhibited either only myopathy or hemolysis or no clinical symptoms at all. A relative deficiency of erythrocyte PFK has been described in normal newborns that has been shown to be due to increased in vivo lability of the enzyme. Like other allosteric enzymes, PFK is an oligomeric protein, the smallest active oligomer being a tetramer. The existence of two distinct subunits in human erythrocyte PFK (one of which is identical to the sole subunit present in muscle PFK) had been suggested initially by the immunologic studies of the residual erythrocyte PFK from the patients with Tarui disease and demonstrated more recently by SDS-polyacrylamide gel electrophoresis of normal erythrocyte PFK. Recently, we have elucidated the molecular structure of normal human erythrocyte PFK. We have shown it to consist of a heterogeneous mixture of five isozymes resulting from all the possible combinations of the M (muscle-type) and L (liver-type) subunits to form various tetramers, i.e., M₄, M₃L, M₂L₂, ML₃, and L₄. In this article, we report an additional patient with Tarui disease and observations on his residual erythrocyte PFK, in the light of the recent knowledge. A preliminary report of these studies has been presented.

CASE REPORT

The propositus, an apparently healthy and bright 31-yr-old male, was referred to the Medical Neurology Branch of the National Institute of Neurological and Communicative Disorders and Stroke in 1974. He complained of easy fatigability, muscle weakness, and myoglobinuria following vigorous exercise since his early childhood. On one occasion myoglobinuria led to acute renal failure, which resolved uneventfully. His physical examination was essentially normal except for slight scleral icterus (total bilirubin: 2.4 mg/dl; direct: 0.3 mg/dl), secondary to a well compensated hemolytic process (hemoglobin 14.7 g/dl; reticulocytes, 6.3%; ⁵¹Cr survival, 12.5 days; haptoglobin, 12.0 mg/dl). There was modest macrocytosis (mean cell volume [MCV]: 100 cu μ). Other normal studies included autohemolysis, direct and indirect Coombs tests, hemoglobin A₂ and F levels, hemoglobin electrophoresis, renal and liver function tests, intravenous pyelogram (IVP), chest X-ray, electro-
cardiogram (EKG), serum GOT, glutamate pyruvate transaminase (GPT), lactate dehydrogenase (LDH), aldolase, and creatine phosphokinase (CPK). Nerve conduction and electromyographic studies were completely normal. During several ischmic exercise tests, the patient failed to increase the levels of venous lactate and pyruvate. A diagnosis of muscle PFK deficiency was then established by direct biochemical analysis of the patient’s muscle biopsy specimen. The simultaneously measured erythrocyte PFK was found to be approximately 50% of normal. These findings suggested that his defect was the same as in the families reported by Tarui et al.2 and Layzer et al.7

The patient is of Jewish ancestry and his parents are nonconsanguineous. The erythrocyte PFK in both the parents was also found to be half-normal. However, both of them and a female sibling (not studied) are completely asymptomatic. Further biochemical and immunologic studies carried out on the patient in 1978 form the basis of this report.

MATERIALS AND METHODS

Adenosine 5'-mono-, di-, and triphosphates, nicotinamide adenine dinucleotide/phosphate (NAD/NADP) and their reduced forms, hexose mono- and diphosphates, diithiothreitol, glycylglycine, and the sodium salt of β-glycerol-P were purchased from Sigma Chemical Co., St. Louis, Mo. Aldolase, α-glycerol-P-dehydrogenase, triose-P-isomerase, hexokinase, glucose-6-phosphate dehydrogenase (G6PD), LDH, and other enzymes were purchased from Boehringer Mannheim. Enzyme-grade ammonium sulfate was obtained from Schwarz/Mann. Phosphofructokinase and phosphoglycerases were obtained from Calbiochem. Freund’s complete and incomplete Freund’s adjuvants came from Difco. DEAE-Cellulose (DE-52) was obtained from Whatman, Inc., St. Louis, Mo. Agarose from Calab Labs, and Nonidet (NP-40) from Particle Data Inc. All other chemicals were of reagent grade.

Cell suspensions were prepared from blood samples stored at 4°C immediately after collection. Simultaneously collected blood samples from normal individuals served as controls. The removal of leukocytes and platelets was achieved by filtration of whole blood on dextran (Particle Data Inc.). To assay glycolytic intermediates, whole blood was immediately added to an equal volume of cold perchloric acid buffer (pH 7.4) containing 1 mM EDTA and 1 mM β-mercaptoethanol. Leukocytes and platelets were suspended in 10 mM K$_2$HPO$_4$ buffer, pH 8.0 containing 0.2 mM EDTA, 0.2 mM AMP and 0.7 mM diithiothreitol and disrupted by two freeze-thaws and sonication, respectively. The resulting suspensions were centrifuged at 12,000 g for 10 min and the supernatants were assayed.

Ischemic exercise test was performed according to the method of McArdle.27

Muscle specimens were obtained by biopsy of the left quadriceps. Another biopsy was obtained from the left forearm muscles after an ischemic exercise test. PFK was assayed in the supernatant of the muscle homogenate prepared according to the method described by Tarui et al.2 Phosphorylase, acid maltase, and neutral maltase were assayed according to the methods described by Layzer et al.7

Immunologic studies consisted of double diffusion and enzyme precipitation studies and were performed using an anti-human muscle PFK rabbit antiserum as previously described.2

Partial purifications of erythrocyte and liver PFKs were carried out according to the methods of Hennessy et al.28 and Brock,29 respectively. Muscle PFK was purified according to Kemp et al.90

Metabolic studies included glycolytic intermediates, glucose consumption, and lactate production, as well as PK, HK, and G6PD activities, and were determined according to Beutler.26

Chromatographic separation of PFK isozymes was obtained on DEAE-Sephadex A-25, equilibrated with 0.1 M Tris-P04 buffer (pH 8) containing 0.2 mM EDTA, 0.2 mM AMP, and 1 mM dithiothreitol. A concave gradient of NaCl was used for elution. Details of this technique have been previously described.21

Enzyme stability studies were performed to assess the in vivo stability of the enzyme; the biological half-life (t½) of the erythrocyte PFK was determined according to the method of Corash et al.31 In vitro stability was determined by allowing hemolysates of similar enzyme concentrations to stand at various temperatures with periodic assays over the next 24 hr.

Protein determinations in the solutions were performed according to Lowry et al.32 using bovine serum albumin as a standard.

RESULTS

Hematologic and enzyme studies. Table 1 summarizes the representative hematologic values and erythrocyte, leukocyte, and platelet enzyme activities of the patient and his parents. From September 1974 through March 1977, the propositus demonstrated normal hemoglobin values (14.7–16 g/dl) with hematocrits ranging between 45% and 47%. At the time of the study in August 1978, he exhibited a hemoglobin level of 18.0 g/dl with a hematocrit of 53%. He showed a persistent reticulocytosis of approximately 7% (range 4%-11%). The hemoglobin levels and reti-

<p>| Table 1. Hematologic Values and Enzyme Activity in the Patient and his Parents |
|-----------------|--------|--------|--------|--------|--------|--------|--------|--------|</p>
<table>
<thead>
<tr>
<th>Hemoglobin (g/dl)</th>
<th>Hematocrit (%)</th>
<th>Reticulocytes (%)</th>
<th>Erythrocyte</th>
<th>Leukocyte</th>
<th>Platelet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propositus</td>
<td>14.7–18†</td>
<td>53</td>
<td>7.0</td>
<td>7.22</td>
<td>0.95</td>
</tr>
<tr>
<td>Father</td>
<td>15.5</td>
<td>44</td>
<td>0.9</td>
<td>7.80</td>
<td>0.76</td>
</tr>
<tr>
<td>Mother</td>
<td>13.1</td>
<td>39</td>
<td>1.5</td>
<td>6.35</td>
<td>0.85</td>
</tr>
<tr>
<td>Control</td>
<td>15.0</td>
<td>45</td>
<td>1.0</td>
<td>14.35</td>
<td>0.51</td>
</tr>
</tbody>
</table>

*In μmole/min/g hemoglobin at 37°C.
†In μmole/min/10⁹ cells at 37°C.
‡Range of measurements, see text.
culocyte counts of the parents were within the normal range. The PFK activity of the patient’s erythrocytes was 48% of normal and those of the father and mother were 54% and 44%, respectively. The activities of hexokinase, pyruvate kinase, and G6PD were moderately elevated in the patient’s erythrocytes. Similar but milder increases were noticed in the erythrocytes of the parents. In all three, PFK activities of leukocytes and platelets were completely normal.

**Metabolic studies of the erythrocytes.** Figure 1 illustrates the metabolic crossover in the patient’s erythrocytes at the level of the PFK step. The levels of glycolytic intermediates prior to the block, i.e., glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P), were elevated and those subsequent to it, i.e., dihydroxyacetone phosphate (DHAP) and fructose-1,6-diphosphate (FDP) were depressed; 2,3-diphosphoglycerate (2,3-DPG) was only 35% of normal. The glucose consumption by the patient’s erythrocytes was increased (8 μM/hr/g Hb versus a control of 5 μM/hr/g Hb) to a degree commensurate with a young mean cell age. However, lactate production was identical to that of the control (10 μM/hr/g Hb).

**Ischemic exercise tests.** In several ischemic exercise tests, the patient showed only minimal elevations in the levels of lactate and pyruvate. The patient’s mother also failed to increase her levels, whereas the father exhibited normal increases.

**Muscle biopsy.** As shown in Table 2, the activity of PFK in the patient’s muscle was absent, while those of phosphorylase and acid maltase were normal, and that of neutral maltase was increased. No specific histologic abnormalities were detected in muscle fibers, connective tissue, vessels, or nerves. The histochemical reactions of ATPase and alkaline phosphatase were normal, those for acid phosphatase and esterase showed a mild increase, whereas PAS staining was markedly increased in the region of the sarcolemmal membrane in many fibers, suggesting the presence of glycogen. The crystal violet reaction for amyloid was negative. The muscle specimen obtained after ischemic exercise showed acute necrosis of muscle fibers with subsarcolemmal vacuolization.

**Characterization of the erythrocyte PFK isozymes.** On chromatography, the erythrocyte PFK from the patient was eluted as a single peak at the position of the liver isozyme (Fig. 2B). There was a complete absence of the four isozymes containing the M-subunits usually found in normal erythrocyte PFK (Fig. 2A). The patient’s platelet PFK was shown to consist of three isozymes, eluting in the general positions of the hybrid isozymes of erythrocyte PFK, a finding similar to that observed with normal platelet PFK. The patient’s leukocyte PFK consisted predominantly of the L4 isozyme, as observed in the leukocytes of normal individuals.

**Immunologic studies.** Immunodiffusion experiments utilizing a monospecific anti-human muscle PFK antibody were carried out against partially purified normal muscle, liver, and erythrocyte preparations. Normal muscle and erythrocyte PFKs each yielded a single precipitin line with a reaction of identity; however, the liver isozyme did not exhibit any cross-reactivity. Partially purified erythrocyte PFK from the patient exhibited a similarly complete lack of cross-reactivity. Unfortunately, the muscle biopsy specimens from the patient were not available for immunologic studies.

Figure 3 illustrates the results of the enzyme precipitation tests: an excess of anti-muscle PFK antibody precipitated more than 90% of muscle PFK and approximately 50% of normal erythrocyte PFK, but both liver PFK and the residual erythrocyte PFK from the patient were almost completely resistant to precipitation. The isozymes observed in platelets and leukocytes showed a metabolic crossover at the PFK step.

**Table 2. Muscle Enzyme Activities in the Propositus**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Patient (per g muscle)</th>
<th>Control (Mean/g muscle)</th>
<th>Control (Range/g muscle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphofructokinase</td>
<td>0.13*</td>
<td>17.4 (8)</td>
<td>14–22.6</td>
</tr>
<tr>
<td>Phosphorylase</td>
<td>8.39*</td>
<td>9.6 (13)</td>
<td>5.3–17.2</td>
</tr>
<tr>
<td>Acid maltase</td>
<td>97.20†</td>
<td>99.0 (17)</td>
<td>52.0–145</td>
</tr>
<tr>
<td>Neutral maltase</td>
<td>70.90†</td>
<td>34.2 (14)</td>
<td>19.0–51.2</td>
</tr>
</tbody>
</table>

Number in parentheses is number of normal individuals studied.

*μM of NADH or NADP oxidized or reduced/min/g of muscle.
†μM maltose split/min/g of muscle.

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**Fig. 1.** Glycolytic intermediates in the patient’s erythrocytes showing a metabolic crossover at the PFK step.
cytes, from the patient as well as normal individuals, were resistant to immunoneutralization.

**Enzyme stability studies.** The biologic half-life of the erythrocyte PFK from the patient was essentially identical to that of liver-type isozyme from erythrocytes of normal individuals, indicating a normal rate of in vivo decay. The chromatographic profile of the erythrocyte PFK from the patient was investigated in cell populations of increasing mean age. It was found to consist exclusively of the L4 type, even in the youngest red cells, thus virtually excluding a mutant M-type subunit with increased instability. The in vitro stability of the erythrocyte PFK from the patient under optimum conditions of storage was essentially similar to that of normal erythrocyte PFK.

**DISCUSSION**

Enzymopathies involving erythrocyte glycolysis generally produce nonspherocytic hemolytic anemia, which in some cases is associated with dysfunction of other organ systems. A generalized enzymopathy may be selectively expressed in mature erythrocytes, as these are unable to replenish an unstable mutant enzyme and to utilize mitochondrial respiration as an alternate route for energy generation. On the other hand, the defect may only involve an erythrocyte-specific isozyme under separate genetic control, such that generalized enzymopathy does not occur.

In 1967, the absence of muscle-type subunit(s) in erythrocyte PFK from patients with Tarui disease was demonstrated by Tarui et al. and Layzer et al., utilizing immunoneutralization studies and Ouchterlony analysis, respectively. The clinical findings of myopathy and hemolysis with total and partial deficiencies of muscle and erythrocyte PFKs, respectively, together with these immunologic studies, were interpreted as resulting from the lack of a subunit common to muscle and erythrocyte PFKs and the persistence of the non-muscle-type subunits in erythrocyte PFK.

Layzer et al. further observed in their patient that a cross-reacting PFK-like material was completely absent from his muscles and that the leukocyte PFK was normal, suggesting a separate genetic control. Additional biochemical and immunologic studies by both groups of investigators confirmed the original findings.

In 1977, both groups have shown the existence of two nonidentical subunits in erythrocyte PFK, one of which is identical to the sole subunit present in muscle, utilizing SDS-polyacrylamide gel electrophoresis. Until recently, lack of a sensitive technique for the resolution of PFK isozymes has prevented the clarification of the question whether erythrocyte PFK is a single hybrid isozyme or a family of five isozymes,
comprised of both muscle and non-muscle types of subunits. Using a high-resolution chromatographic technique, we have recently demonstrated that erythrocyte PFK is indeed a mixture of five isozymes resulting from all the possible combinations of two distinct subunits, M (muscle-type) and L (liver-type), to form various tetramers. These findings have been corroborated by the fact that an identical set of five isozymes is produced when purified muscle and liver PFKs are hybridized in vitro.21 The similarity between the non-muscle subunit of erythrocyte PFK and the subunit from liver PFK is supported by the identical chromatographic elution patterns of the respective homotetramers, nonreactivity with anti-M4 antibody, and by the fact that the kinetic properties of erythrocyte PFK are intermediate between those of muscle and liver isozymes.21 The recent observation by Kahn et al. that normal erythrocyte PFK is also precipitated by anti-liver PFK antibody further corroborates our interpretation that the non-muscle subunit of erythrocyte PFK is of liver-type.36

The patient reported in this study exhibits clinical and biochemical characteristics essentially identical to those reported by Tarui et al.2 and Layzer et al.7 the findings in his parents are also identical to those already reported (partial deficiency of erythrocyte PFK and normal hematologic values), except that his mother showed an abnormal response to ischemic exercise test. The results of the muscle biopsy are also in agreement with those reported by other workers.8,9 The observation that in our patient acute necrosis of muscle fibers with subsarcolemmal vacuolization occurred after ischemic exercise provides an explanation of the mechanism of the exertional myopathy, at times resulting in myoglobinuria.

Our metabolic studies indicate that the deficiency in the erythrocytes, although partial, nevertheless results in a significant block in glycolysis. A metabolic crossover was found at the level of the PFK reaction. The increased glucose consumption without a concomitant rise in lactate production attests to the block and the probable shunting of glycolytic flux via the hexose monophosphatase shunt. These metabolic alterations most probably account for the significant shortening of the erythrocyte lifespan. The block in glycolysis also accounts for the observed marked decrease in the 2,3-DPG level in the patient’s erythrocytes. This result in increased oxygen affinity, which in turn produces a compensatory erythrocytosis. The combination of these factors appears to be responsible for the paradoxical finding of shortened erythrocyte lifespan, accompanied by erythrocytosis, instead of anemia.

The stability of the L4 from patient’s erythrocytes was assessed, since hemolysis was present despite 50% residual PFK activity. Both in vivo t½ and in vitro stability on storage were normal, indicating that the cause of hemolysis must be sought elsewhere. It is known that under existing intraintracellular conditions, the activity of PFK is scaled down to 0.5% of its potential capacity and is commensurate with the observed rate of erythrocyte glycolysis.37 It is possible that even a 50% reduction in erythrocyte PFK becomes critically rate-limiting for glycolysis and energy generation, which results in premature cell death. Alternatively, the turnover number of liver-type isozyme or its apparent km and ki could be of such magnitude that glycolysis in the erythrocyte is severely impaired despite adequate in vitro enzymatic activity.

Our chromatographic studies provide direct molecular evidence that the PFK deficiency results from the absence of the M-type subunits as originally proposed by Tarui et al.8 and Layzer et al.7 The chromatographic profiles of PFKs from the normal and deficient erythrocytes were strikingly different. The normal PFK was resolved into five peaks, whereas that from the patient was eluted as a solitary peak in the same position as the final peak of the normal enzyme. Immunologically, the residual PFK from our patient failed to react with anti-muscle PFK antibody, as previously demonstrated by Tarui et al.8 and Layzer et al.7 We have additionally shown that purified human liver PFK, which chromatographically is eluted in the same position as the patient’s erythrocyte PFK, also fails to react with the anti-muscle PFK antibody.

The presence of an unstable M-subunit instead of its total absence in our patient was virtually excluded by the failure to detect any M-containing isozymes in the reticulocytes and youngest erythrocytes separated by gradient density centrifugation. Thus, the most likely reason for undetectable M-subunits in our patient would seem to be absent or extremely reduced synthesis.

The existence of multiple isozymes of PFK in different organs had been suggested initially by Layzer et al.38,39 on the basis of the differing immunologic reactivity of PFK from various tissues to anti-muscle PFK antibody. Based on immunologic studies utilizing three different antisera, Kahn et al.40 have recently suggested the existence of three types of PFK subunits, i.e., muscle, liver, and fibroblast type. Using molecular hybridization, chromatographic and immunologic techniques, we have recently shown that in leukocytes L4 predominates and that in platelets there are three isozymes (P4, P1L, P2L2) consisting of a unique platelet-type or P-subunit (probably the same
as Kahn's fibroblast type) and liver-type or L subunit.

These findings are further corroborated by the observations that in our patient, leukocyte and platelet PFKs were found to be normal, both chromatographically and immunologically.

So far, 12 cases of inherited PFK deficiency occurring in 10 unrelated families (including the present report) have been described. Table 3 lists the clinical and biochemical profiles of these cases. In the light of the present findings, one can speculate on the molecular basis of PFK deficiency in these cases. The cases reported by Serratrice et al.8 and Tobin et al.9 both showed a total lack of muscle PFK and partial reduction of erythrocyte PFK. The hemolysis was (probably unjustifiably) presumed to be absent only because the patients were found not to be anemic; however, laboratory evidence of hemolysis (i.e., reticulocyte count or erythrocyte survival) was not sought. It appears most likely that these cases were essentially similar to those mentioned above, i.e., homozygous deficient for M subunits. In contrast, the patients reported by Waterbury et al.10 and Miwa et al.11 presented with hemolysis but no myopathic symptoms and had normal lactate production after exercise. Their molecular defect could be due either to the absence or the extreme instability of L-type subunits or to the instability of the M-subunits. The case described by Oda et al.6 showed partial reduction in erythrocyte PFK with a compensated hemolytic syndrome. Despite the absence of myopathic symptoms, involvement of the muscle was implicated because of an increase in the plasma level of pyruvate kinase of the muscle type. In this case, the molecular defect could be due to an unstable M-type PFK subunit, which is compensated in the muscle cells because of active protein synthesis. Boulard et al.12 described a healthy individual, with partial reduction of erythrocyte PFK, discovered during a large-scale survey; he could be a heterozygote for the deficiency of M- or L-subunit. Kahn et al.5 reported another individual with normal muscle function and PFK level, despite a half-normal erythrocyte PFK level; the muscle PFK was unstable and had fast electrophoretic mobility. These findings were interpreted as due to the presence of a mutant M-subunit.

From the foregoing discussion, it is obvious that a variety of clinical syndromes associated with erythrocyte PFK deficiency may stem from different defects of either of the two subunits. Some syndromes may even result from the inheritance of a combination of different genetic lesions. Our studies provide direct and conclusive evidence that the most common form of PFK deficiency (myopathy with compensated hemolysis—Tarui disease) stems from the exclusive deficiency of the M-type subunit of PFK. The precise characterization of the other forms of this enzymatic defect will demand detailed studies along similar lines.

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REFERENCES


ERYTHROCYTE PFK DEFICIENCY


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