

Heterogeneity of the Molecular Lesions in Inherited Phosphofructokinase Deficiency

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ABSTRACT Human phosphofructokinase (PFK; EC 2.7.1.11) exists in tetrameric isozymic forms. Muscle and liver contain the homotetramers M₄ and L₄, whereas erythrocytes contain five isozymes composed of M (muscle) and L (liver) subunits, i.e., M₄, M₃L, M₂L₂, ML₃, and L₄. Inherited defects of erythrocyte PFK are usually partial and are described in association with heterogeneous clinical syndromes. To define the molecular basis and pathogenesis of this enzymopathy, we investigated four unrelated individuals manifesting myopathy and hemolysis (glycogenosis type VII), isolated hemolysis, or no symptoms at all.

The three symptomatic patients showed high-normal hemoglobin levels, despite hemolysis and early-onset hyperuricemia. They showed total lack of muscle-type PFK and suffered from exertional myopathy of varying severity. In the erythrocytes, a metabolic crossover was evident at the PFK step: the levels of hexose monophosphates were elevated and those of 2,3-diphosphoglycerate (2,3-DPG) were depressed, causing strikingly increased hemoglobin-oxygen affinity. In all cases,

the residual erythrocyte PFK consisted exclusively of L₄ isozyme, indicating homozygosity for the deficiency of the catalytically active M subunit. However, presence of immunoreactive M subunit was shown in cultured fibroblasts by indirect immunofluorescence with monoclonal anti-M antibody. The fourth individual was completely asymptomatic, had normal erythrocyte metabolism, and had no evidence of hemolysis. His residual erythrocyte PFK showed a striking decrease of the L₄, ML₃, and M₂L₂ isozymes, secondary to a mutant unstable L subunit. Identical alterations of erythrocyte PFK were found in his asymptomatic son, indicating heterozygosity for the mutant unstable L subunit in this kindred.

These studies show that, except for the varying severity of the myopathic symptoms, glycogenosis type VII has highly uniform clinical and biochemical features and results from homozygosity for mutant inactive M subunit(s). The absence of anemia despite hemolysis may be explained by the low 2,3-DPG levels. The hyperuricemia may result from hyperactivity of the hexose monophosphate shunt. In contrast, the clinically silent carrier state results from heterozygosity for mutant M or L subunit. Of the two, the M subunit appears to be more critical for adequate glycolytic flux in the erythrocyte, since its absence is correlated with hemolysis.

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INTRODUCTION

Mature human erythrocytes generate energy exclusively via the glycolytic pathway. It is not, therefore, surprising that glycolytic enzymopathies generally cause nonspherocytic hemolytic anemia, which in some cases is associated with dysfunction of other organ systems

(1). Inherited deficiency of phosphofructokinase (PFK;¹ ATP: D-fructose-6-phosphate [Fru-6-P], 1-phosphotransferase, EC 2.7.1.11), a key rate-limiting enzyme of glycolysis, is generally partial in the erythrocyte and is associated with a group of clinically heterogeneous syndromes (2). The most common syndrome, glycogenosis type VII (Tarui disease), is characterized by partial reduction of erythrocyte PFK and total lack of muscle PFK. The clinical effects of the enzymatic defect are intolerance to intense exercise with cramps and myoglobinuria and compensated hemolysis. Other syndromes associated with partial deficiency of erythrocyte PFK include isolated hemolytic trait or asymptomatic state. Two myopathic syndromes have also been described in association with the isolated deficiency of muscle PFK (2). The clinical heterogeneity of these syndromes suggests that the underlying enzymatic defects must be heterogeneous at the molecular level.

We and others have previously demonstrated the existence of a multilocus isozyme system for human PFK (3-6). Three structural loci code for muscle (M), liver (L), and platelet (P; same as fibroblast) subunits, which are variably expressed in different tissues. Random tetramerization of these subunits produces various isozymes, which are distinguishable from one another by ion-exchange chromatography (3, 4) and subunit-specific antibodies (3-6). The molecular basis of inherited erythrocyte PFK deficiency has been investigated only in a few cases (7-13). In two unrelated patients with glycogenosis type VII, the enzymatic defect was conclusively shown to result from a total deficiency of the M subunit (7, 8). In contrast, in three unrelated patients with isolated hemolytic syndrome (9-13) and in one asymptomatic kindred (14), the presence of an unstable M subunit was postulated.

In the present article, we describe clinical features, hematological data, and biochemical studies (including PFK isozyme patterns) in four unrelated patients with diverse clinical syndromes. Two of them have been previously reported to suffer from isolated hemolysis (11-13). Our results demonstrate that glycogenosis type VII (Tarui disease) results from homozygosity for catalytically inactive M subunit(s), whereas a clinically silent state (carrier state) results from heterozygosity for mutant M or L subunit. In addition, the metabolic studies in glycogenosis type VII offer some clues about

the physiological role and significance of PFK isozymes in the control of glycolysis at the cellular level.

METHODS

Case reports

D.S., a 31-yr-old white male presented with life-long history of intolerance to strenuous exercise, with bouts of weakness and pigmenturia after exertion; the symptoms had progressively worsened for the past 2-3 yr. At the age of 27 yr, he developed symptomatic gout, for which he was placed on allopurinol. Physical examination was normal, except for slight scleral icterus. Blood counts revealed the presence of a well-compensated hemolytic process (Table I). Other positive findings included increased levels of serum creatine kinase: 35-4,990 U/dl (normal, 1-145), aldolase: 5.6-30 U/dl (normal, 1.8-7.8), lactate dehydrogenase: 321-342 U/dl (normal, 1-40), and uric acid 7.2-12.3 mg/dl (normal, 4-9). Since venous lactate and pyruvate failed to increase during a forearm ischemic exercise test, myophosphorylase deficiency (McArdle disease) or PFK deficiency (Tarui disease) were suggested. The diagnosis of muscle PFK deficiency was then established by direct biochemical analysis of the patient's erythrocytes and muscle biopsy. Since the patient is adopted, family history is not available. He is married, but has no children.

C.C. (37 yr), and B.R. (59 yr), two unrelated white males, are known cases of inherited erythrocyte PFK deficiency who have been reported previously (11-13). Both patients presented with mild icterus secondary to well-compensated hemolytic process (Table I). Since there was no clear history of muscle disease, and muscle biopsy was not obtained, they were considered to suffer from an isolated hemolytic trait, probably secondary to an unstable muscle-type isozyme. The following laboratory data were abnormal in one or both patients: high-normal hemoglobin levels; increased hemoglobin-oxygen affinity (P_{50} : 19.5 mmHg; normal, 27.0); decreased erythrocyte ATP and 2,3-diphosphoglycerate (2,3-DPG) levels; and increased ATP inhibition and decreased neutralization with anti-M antibody of residual erythrocyte PFK; normal leukocyte PFK.

However, more detailed clinical histories revealed that both patients had little tolerance for exercise, noticing weakness and pigmenturia after vigorous exercise. One of the patients has abolished these symptoms simply by avoiding vigorous activities, while the other appears to tolerate bouts of moderately vigorous exercise reasonably well with premature fatigue as his only complaint. Both patients suffer from early-onset hyperuricemia, but only one has symptomatic gout and he has undergone several surgical procedures for the removal of gouty tophi; his serum uric acid ranges from 11 to 13.6 mg% (normal, 2.5-8.0).

Both patients are of Jewish (Russian) ancestry and are born of nonconsanguineous marriages. Their parents and children, who are known to be heterozygote deficient, are asymptomatic. In the present study, muscle and skin biopsy specimens were investigated in addition to erythrocytes from B.R., whereas only the erythrocytes were investigated in C.C.

P.S., an asymptomatic 47-yr-old white male was discovered to have partial erythrocyte PFK deficiency in 1977, when he volunteered as a normal control for PFK assays in our laboratory. His erythrocytes have consistently shown 60-65% of normal activity, but blood counts and erythrocyte ^{51}Cr $t_{1/2}$ have been completely normal (Table I; ^{51}Cr survival 32 d, normal, 28-32 d). Both physical examination and routine

¹ Abbreviations used in this paper: CRM, cross-reactive material; 2,3-DPG, 2,3-diphosphoglycerate; Fru-6-P, fructose-6-phosphate; Fru-1,6-P₂, fructose-1,6-diphosphate; Glu-6-P, glucose-6-phosphate; L, M, and P, liver, muscle, and platelet subunits, respectively, of PFK; P_{50} , O₂ partial pressure at which 50% of hemoglobin is oxygenated; PFK, phosphofructokinase; PRPP, 5-phosphoribosyl pyrophosphate.

blood chemistries have been normal. He is of Italian ancestry and his parents are nonconsanguineous; his two children show similar degree of erythrocyte PFK deficiency, whereas his wife has normal enzyme activity.

Procedures

Ischemic exercise tolerance test. The ischemic exercise tolerance test was performed according to the method of McArdle (15). Venous blood specimens were collected immediately and at timed intervals of 2, 4, 6, and 10 min for the measurement of lactate and pyruvate levels.

Muscle specimens. Open biopsies of the left deltoid muscles were obtained from D.S. and B.R. after informed consent. Glycogen content, PFK, phosphorylase, and acid and neutral maltases were assayed by described procedures (16). In addition to routine histochemical stains for glycogen (periodic acid-Schiff), the presence of abnormal polysaccharide was investigated in both biopsies by biochemical and ultrastructural methods (16).

Metabolic studies. For the assay of erythrocyte glycolytic intermediates, fresh whole blood was added immediately from the syringe to an equal volume of ice-cold 2 N perchloric acid, and the neutralized supernatant was stored at -20°C . The levels of glucose-6-phosphate (Glu-6-P), fructose-6-phosphate (Fru-6-P), fructose-1,6-diphosphate (Fru-1,6-P₂), and triose phosphates were measured by spectrofluorometric methods, and 2,3-DPG by spectrophotometry within the next 4–6 d, as described previously (17).

Preparation of pure cell suspensions. Fresh venous blood samples (heparinized and defibrinated) were obtained simultaneously from the PFK-deficient individuals and healthy volunteers serving as controls. Pure erythrocyte (18), leukocyte (19), and platelet (20) suspensions were obtained by published procedures.

PFK assays. PFK assays were performed on a Gilford model 250 spectrophotometer (Gilford Instrument Laboratories, Inc., Corning Glass Works, Oberlin, OH) at 26°C , as described previously (3). 1 unit is defined as that amount of enzyme that converts 1 μmol of Fru-6-P to Fru-1,6-P₂ in 1 min. The enzyme activity was expressed as units per gram of hemoglobin or units per 10^{10} cells. Hemolysates and cell extracts were prepared immediately before assay or other experimental procedures, as described previously (4).

Isozymic complements of various cell types. Chromatographic separation of PFK isozymes was carried out at 4°C with DEAE-Sephadex A-25 equilibrated with 0.1 M Tris-PO₄ buffer (pH 8.0) containing 0.2 mM EDTA, 0.2 mM AMP, and 1 mM dithiothreitol. A concave gradient of NaCl (0.025–0.525 M) was used for elution. Details of this technique have been previously described (3).

Production and characterization of subunit-specific antibodies. Subunit-specific monoclonal antibodies against the M and L subunits were produced by the secretory lymphocyte hybridoma technique. The details of the immunization protocol, technique of hybridization, screening of the secretory hybrids, production of antibody-rich mouse ascites fluids and characterization of the secreted antibodies are described elsewhere (21). The production and characterization of a P subunit-specific BALB/c antiserum has been described in detail elsewhere (22). The five monoclonal antibodies (four anti-L and one anti-M) and the P subunit-specific antiserum react strictly with the respective subunit only (4, 21, 22). At the dilutions used, these antibodies retained full precipitating efficacy and were always in excess of the amount of PFK used for immunoprecipitation (see below). An excess of each

of these antibodies precipitates completely not only the respective homotetramer but also heterotetramers containing one or more of the specific subunits. For instance, anti-M antibody precipitates completely not only M₄ homotetramer, but also the M₃L, M₂L₂, and MP₂L heterotetramers (4).

Enzyme immunoprecipitation assays. Cell extracts were prepared just before the precipitation studies with a stabilizing buffer as described previously (4). The extracts were diluted to a final concentration of 0.06 U/ml with the extraction buffer. 50- μl samples of a given extract were mixed in duplicate, with 50 μl of diluted mouse ascites fluid (1:200 in phosphate-buffered saline) containing either the anti-M (V96-26) or anti-L (V 65-06) monoclonal antibody or with 50 μl of diluted anti-P antiserum (1:64). The mixtures were incubated for 30 min at 37°C , when 100 μl of freshly washed (23), 10% wt/vol suspension of staphylococci-bearing protein A (IgG-sorb) was added. The mixtures were incubated at 4°C for 30 min with continuous shaking, centrifuged at 8,000 g for 10 min, and assayed for residual enzyme activity. The precipitated enzyme activities were expressed as percentages of the concurrent controls; only the precipitation values $>7\%$ were considered significant (24). Each extract was tested in duplicate with duplicate controls.

In vivo stability of the residual erythrocyte PFK. In vivo stability of the residual erythrocyte enzyme was assessed by determining the biological half-life ($t_{1/2}$), according to the method of Corash et al. (25). Briefly, the technique uses discontinuous gradients of arabinogalactane (Stractan) to separate erythrocytes into populations of differing mean cell age. Total PFK contents and isozymic profiles of these erythrocyte populations were determined as described above, and the $t_{1/2}$ of the total PFK was calculated by probit analysis.

Cell lines and cell cultures. Long-term lymphoblastoid cell lines were established from D.S. and B.R. through the courtesy of Dr. A. Bloom (Department of Human Genetics, College of Physicians & Surgeons of Columbia University, New York). Primary fibroblast and muscle cultures were also established from the same patients as described previously (26). Viable cells were counted by the dye exclusion technique by the standard manual method before biochemical analysis. The enzyme activity was expressed in units per 10^{10} viable cells.

Hemoglobin-oxygen affinity of intact erythrocytes. The oxygen-affinity curves of intact erythrocytes from PFK-deficient individuals and normal controls were determined with Hemox-Analyzer (TCS-Medical Products Co., Southhampton, PA) at 37°C , CO₂ 5%, and pH 7.4. Measurements were performed in 50- μl aliquots of freshly collected anticoagulated blood. The P₅₀ values were expressed in millimeters of mercury at which hemoglobin was half saturated with O₂.

Demonstration of immunoreactive M subunit. Coverslip preparations of primary fibroblasts from D.S. and B.R. and normal individuals were used to demonstrate the presence of M-PFK antigen by indirect immunofluorescence. Mouse monoclonal anti-M antibody (V96-26) and a fluorescein-labeled goat anti-mouse antibody were used as the primary and secondary antibody, respectively. In control smears, similar dilutions of nonimmune mouse ascites fluid (1:50) were used instead of monoclonal anti-M antibody. Details of these techniques have been described previously (27).

RESULTS

Hematologic studies and PFK assays. Table I summarizes the ranges of hematologic values and PFK ac-

TABLE I
Hematologic Values and PFK Activity Levels in Various Cell Types of the PFK-deficient Individuals

Propositi	Hemoglobin	Hematocrit	Reticulocyte	PFK activity				
				Erythrocytes	Leukocytes	Platelets	Lymphoblastoid cell line	Fibroblast cell line
	g/dl	%				units/10 ⁶ cells		
Symptomatic								
DS*	14.1-17.2	44-51	2.9-4.7	1.08±0.08	18.7±3.6	1.19±0.14	81.7±19.5	267.5±86.2
BR*	16.8-18.0	49-54	6.0-11.0	1.40±0.08	21.2±4.3	1.05±0.07	77.2±23.8	280±78.5
CC*	16.0-16.9	48-52	4.0-9.0	1.42±0.05	ND†	ND	ND	ND
Asymptomatic								
PS*	14.9-15.5	46-48	0.8-1.2	1.43-0.05	13.8±0.6	0.86±0.03	98.0±11.1	ND
AS*	14.3-15.3	43-46	1.0-1.1	1.44-0.04	ND	ND	ND	ND
Controls§ (n = 6)	13.9-17.3	42-52	0.8-1.2	2.11±0.12	19.4±3.4	1.29±0.15	179.6±53.3	244.4±73.3

ND, not done.

* At least two to six observations were made on each single cell type or cell line.

† Normal activity was found previously (13).

§ At least six separate donors or cell lines were investigated; data are mean±1 SD.

tivity levels (mean±1 SD) in various cells from the four patients and from A.S., the PFK-deficient son of P.S. The three symptomatic patients (D.S., B.R., and C.C.) showed high-normal hemoglobin levels, despite persistent reticulocytosis. Their erythrocyte PFK activity levels ranged from 50 to 65% of normal. This degree of deficiency was also observed in the hematologically normal parents and in several children of B.R. and C.C. (data not shown). The PFK activity levels were normal in leukocytes, platelets, and cultured fibroblasts from D.S. and B.R., but were only 50% of normal in lymphoblastoid cell lines. Despite partial PFK deficiency in erythrocytes, P.S. and his son, A.S., had normal hematological values. In P.S., the enzyme activity was approximately half-normal in all other cell types studied.

Ischemic exercise test. Repeated ischemic exercise tests in D.S. and B.R. caused only minimal increases of venous lactate and pyruvate; the test was not done on the other two propositi. The lactate levels rose minimally from the resting values of 9.8 and 8.5 mg% (normal, 12-14 mg%) to 13.5 and 14 mg% at 2 min, 14.3 and 14 mg% at 4 min, 19 and 13 mg% at 6 min, and 15.3 and 12 mg% at 10 min in D.S. and B.R., respectively; (normal values, 50-65 mg%). Similarly, the pyruvate levels showed minimal increments in the patients. The resting values of 0.5 and 0.8 mg% (normal, 1.0 mg%) rose to 0.8 and 1 mg% at 2 min, 0.9 and 0.6 mg% at 4 min, 0.8 and 1.0 mg% at 6 min, and 0.65 and 0.8 mg% at 10 min in D.S. and B.R., respectively (normal values, 1.2-1.6 mg%).

Enzyme assays in muscle biopsies. PFK activity was almost absent in muscle biopsies from D.S. and B.R. (0 and 0.4 U/g protein; normal, 11-18.8 U/g), whereas activities of phosphorylase and acid and neutral maltase were normal. Glycogen concentration was modestly increased (2.1 and 1.98 g%; normal, <1.5 g%). Histochemical stains for ATPase, alkaline phosphatase, and acid phosphatase showed normal reactions. Electron microscopy showed abnormal accumulation of normal-looking glycogen β -particles, but failed to show deposits of structurally abnormal glycogen.

Metabolic studies of the erythrocytes. The pattern of glycolytic intermediates in the erythrocytes from D.S. and B.R. showed a clear metabolic block at the level of PFK (Fig. 1); the levels of Glu-6-P and Fru-6-P were increased and those of dihydroxyacetone phosphate and Fru-1,6-P₂ were decreased; the content of 2,3-DPG was strikingly decreased in D.S. and B.R., (36 and 46% of normal). The glycolytic intermediates were normal in the erythrocytes from P.S.

PFK isozymes in various blood cells. In contrast to the five-membered isozyme pattern (M₄, M₃L, M₂L₂, ML₃, and L₄) usually found in erythrocytes (Fig. 2 A), the residual erythrocyte PFK in the three symptomatic patients eluted as a single peak in the position of the liver isozyme (Fig. 2 B). PFK isozymic profiles were normal in platelets and leukocytes. In both patients, platelet PFK consisted of three isozymes, P₄, P₃L, and P₂L₂, eluting in the general positions of the hybrid isozymes of M and L subunits (Fig. 2 C). However, these isozymes differed from the erythrocyte hybrids,

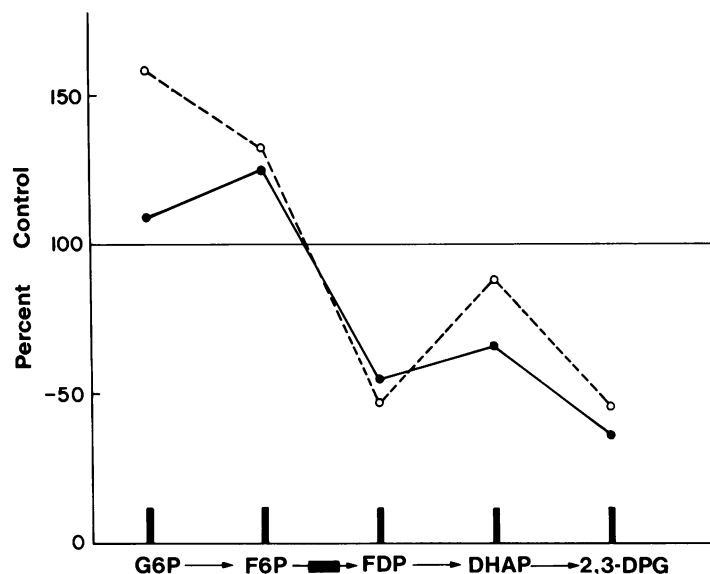


FIGURE 1 Profile of glycolytic intermediates in the erythrocytes of the symptomatic propositi, D.S. (—) and B.R. (---). The results are expressed as percentage of normal values. A distinct metabolic crossover was evident at the PFK step in the erythrocytes; the levels of 2,3-DPG were only 36 and 46% of normal, respectively.

because they were not precipitated by anti-M antibody. Leukocyte PFK consisted predominantly of L_4 type isozyme, but hybrid species of the P and L subunits were also observed (Fig. 2 D; see below). As shown in

Fig. 3 A, erythrocyte PFK from the asymptomatic propositus and his son showed an almost total lack of L_4 isozyme, and a marked decrease in the relative proportions of M_2L_2 and ML_3 isozymes; the chromatograms

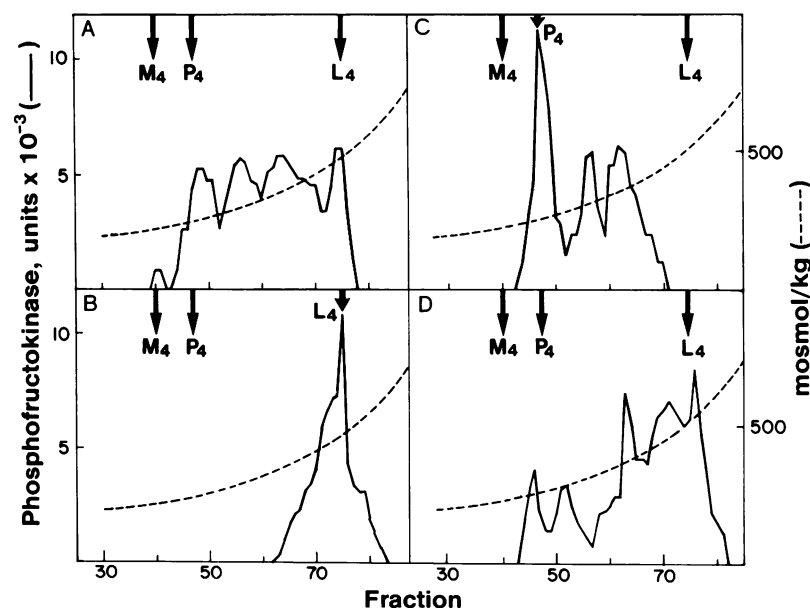


FIGURE 2 Isozymic profiles of PFK from various cell types of the symptomatic propositi. (A) Normal erythrocyte PFK showing the presence of M_4 , M_3L , M_2L_2 , ML_3 , and L_4 isozymes; (B) the residual erythrocyte PFK from the patients showing the exclusive presence of L_4 isozyme; (C) platelet PFK showing the presence of P_4 , P_3L , P_2L_2 isozymes; and (D) leukocyte PFK showing the presence of P_4 , P_3L , P_2L_2 , PL_3 , and L_4 isozymes.

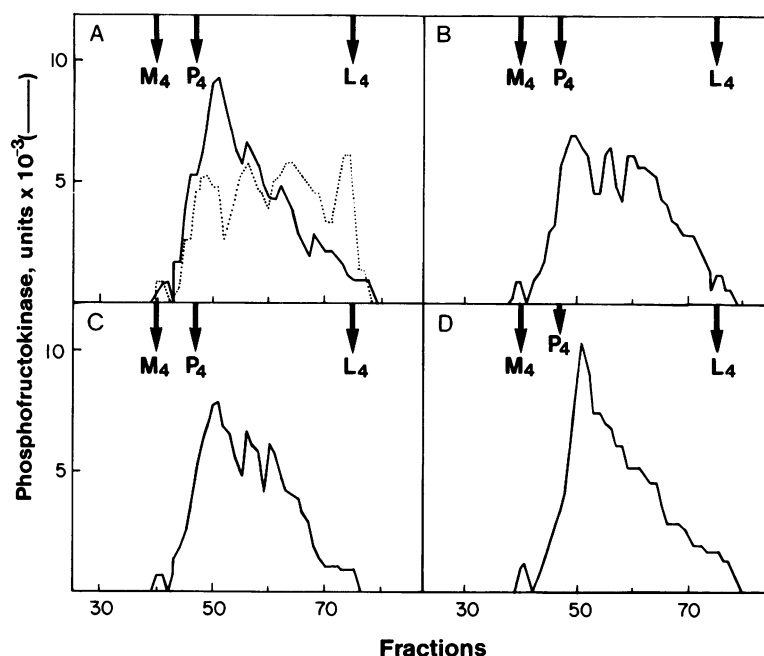


FIGURE 3 Isozymic profiles of PFK from age-fractionated erythrocytes from the asymptomatic propositus, PS (—), and from normal individuals (---). (A) Unfractionated erythrocytes showing the absence of L_4 and diminution of ML_3 and M_2L_2 species in P.S., and representative profile in controls. (B) The youngest cells showing barely visible L_4 peak and nearly normal ML_3 and M_2L_2 species. (C) Middle-aged cells showing a profile identical to (A) and (D), the oldest cells showing total lack of L_4 , ML_3 , and M_2L_2 species.

from these individuals showed a predominance of the M_3L isozyme. Leukocyte and platelet PFK from propositus P.S. showed normal isozymic profiles (data not shown).

Immunochemical studies. In Table II, the percent precipitation values of PFK from various blood cells and cultured cell lines in the two symptomatic patients are compared with those from control cells. Precipi-

TABLE II
Immunoprecipitation Values of PFK from Various Cell Types of the Propositi

Cell type	Percentage of precipitation*					
	Anti-M antibody		Anti-L antibody		Anti-P antibody	
	Controls (6)† and asymptomatic propositus§	Symptomatic propositi (2)	Controls (6) and asymptomatic propositus	Symptomatic propositi (2)	Controls (6) and asymptomatic propositus	Symptomatic propositi (2)
Erythrocytes	74.9±6.1	1.1±1.4	88.0±4.1	92.4±1.9	1±2.1	1.8±1.4
Leukocytes	18.5±4.0	1.0±1.4	79.7±7.0	88.9±1.6	56.1±5.6	60.0±7.1
Platelets	1±2.3	0.5±0.7	67±8.0	67.3±3.4	85±6.4	80.4±3.39
Fibroblasts¶	24.3±10.4	2.2±1.7	72±4.4	71.7±8.4	88.2±11.9	93.5±5.8
Lymphoblastoid cells	38.6±13.4	1.9±0.9	68.9±9.5	74.0±10.0	76.2±5.5	74.4±9.5

* Values given are percentage of enzyme activity precipitated by the respective antibody compared with that of the concurrent control assay from which antibody is omitted. Each value represents the average of four to eight determinations; values <7.2% are considered not significant (24).

† Numbers of separate donors investigated are in parentheses.

§ Values of the asymptomatic propositus are included with those of the controls since these were very similar.

|| Data are mean±1 SD.

¶ Since fibroblast cell line was not established from the asymptomatic propositus, these data are derived from normal individuals only.

tation values in the asymptomatic propositus were normal and are therefore included with those of control subjects for clarity of presentation. PFK activity in all cells from the two patients was completely resistant to precipitation by anti-M antibody, indicating that the deficiency of the M subunit is universally expressed. In contrast, PFK activity was precipitated by the anti-P and anti-L antibodies, indicating normal expression of these subunits.

In vivo stability of residual erythrocyte PFK. Total PFK activity in erythrocyte populations of differing mean cell age from each of the three patients were similar to that of unfractionated erythrocytes. The PFK activity ratios of the youngest ($\sim 15\%$ of the total) to the oldest erythrocytes ($\sim 15\%$ of the total) were normal, i.e., 1.1–1.2, indicating that the residual L_4 isozyme is not excessively unstable in vivo. In contrast, the PFK activity ratio of the youngest to the oldest erythrocytes in the asymptomatic propositus was 1.5, indicating increased in vivo instability of the enzyme. The rates of decline of PFK activity in erythrocytes from P.S. and from normal individuals are compared in Fig. 4. $T_{1/2}$

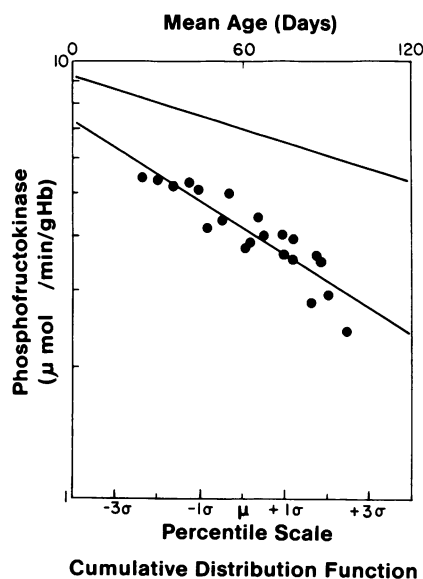


FIGURE 4 Rate of decline of total PFK activity with erythrocyte aging. The upper line representing the average rate of decline was derived from measurements of 10 normal individuals. The lower line, representing the average rate of decline of the asymptomatic propositus (PS), was derived from four separate experiments. The $t_{1/2}$ of the propositus' PFK is ~ 76 d as compared with 155 d for the controls (range, 100–200 d). Erythrocytes were separated according to age by density gradient ultracentrifugation on arabinogalactane. The two slopes were obtained by weighted regression of the logarithm of the enzyme activity on the probit-transformed midpoint cumulative percentile of each age-separated fraction.

value of his PFK was 76 d (average of four experiments), as compared with 155 d (range 100–200 d) in normal controls (average of 10 experiments).

Isozymic profiles of age-fractionated erythrocytes. Isolated L_4 type isozyme was present in erythrocyte populations of all ages from all three symptomatic patients; no M_4 - or M -containing species were eluted even when the columns were overloaded with enzyme from the youngest erythrocytes (1 U, instead of 0.1 U). These data excluded the presence of an unstable M subunit in these patients. In contrast, chromatograms of PFK from young (layer 1 and 2), intermediate (layer 3), and old (layer 5) erythrocytes from P.S. showed a dramatic progressive decline in the relative proportions of M_2L_2 , ML_3 , and L_4 species with cell aging (Fig. 3). Although L_4 is not detectable in unfractionated erythrocytes (Fig. 3 A), it is found in small amounts in the youngest fraction (Fig. 3 B), which shows near-normal proportions of ML_3 and M_2L_2 . These L-containing isozymes decline rapidly with cell aging (Fig. 3 C), so that the oldest fraction shows mainly the L_3M species (Fig. 3 D). Aging of normal erythrocytes is accompanied by decline and, occasionally, complete disappearance of the L_4 isozyme from the oldest fraction (Vora, S., unpublished observations). The striking decrease of L_4 isozyme in the youngest fraction and the total absence of L_4 , ML_3 , and M_2L_2 in old erythrocytes from P.S. are distinctly abnormal.

Hemoglobin-oxygen affinity values. The oxyhemoglobin dissociation curves in the three symptomatic patients (D.S., B.R., and C.C.), were shifted to the left (Fig. 5), with mean P_{50} values of 15, 17, and 19.5 mmHg (normal, 27 ± 0.5). In contrast, the oxyhemoglobin dissociation curve was normal in the asymptomatic propositus.

Demonstration of immunoreactive M subunit. Both cultured fibroblasts and cultured muscle cells from D.S. and B.R. showed the presence of immunoreactive M subunit (Fig. 6). The results of immunocytochemical studies in muscle cultures have been reported elsewhere (27). As expected, immunofluorescence with the anti-L antibody was present in both fibroblast and muscle cultures.

DISCUSSION

Since the original reports by Tarui et al. (28) and Layzer et al. (29), 21 additional cases of PFK deficiency from 18 unrelated families have been described (for details, see reference 2). These cases can be divided into five major groups, according to clinical symptoms and laboratory findings: group I, patients with the "classic" syndrome, i.e., simultaneous presence of myopathy and hemolysis (7, 16, 30–32); group II, patients with isolated myopathy (33–35); group III, individuals with hemolysis

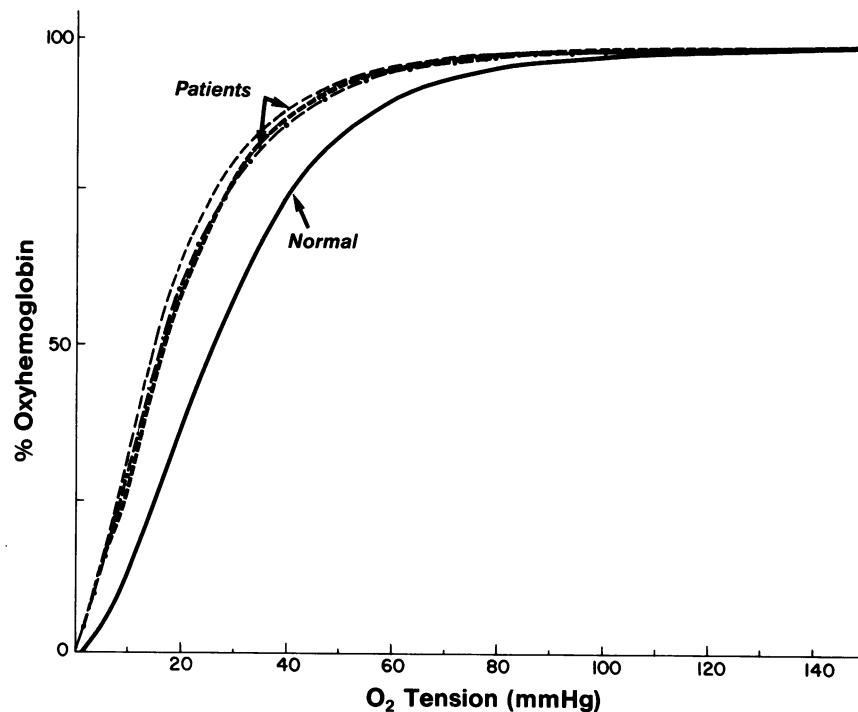


FIGURE 5 Oxygen-hemoglobin dissociation curves of the symptomatic probands. Note the remarkable shift of the patients' curves to the left with a mean P_{50} value of 15, 17, and 19.5 mmHg, respectively (normal value, 27 ± 0.5 mmHg). The oxyhemoglobin dissociation curve was normal in the asymptomatic proband.

only (9, 11, 13, 36, 37); group IV, individuals who are completely asymptomatic despite partial deficiency of erythrocyte PFK (14, 38); group V, patients with progressive, fatal myopathy associated with other atypical features (39, 40). Since only a few cases have been investigated in detail, the clinical classification of some of these syndromes remains uncertain and the nature of the underlying enzymatic defects unknown. To clarify these problems, we investigated in detail four unrelated individuals with different clinical syndromes.

D.S. presented with the classical syndrome and P.S. was asymptomatic, while B.R. and C.C. had been considered to suffer from an isolated hemolytic syndrome secondary to an unstable M subunit (11–13). The absence of muscle symptoms in patients with unstable M subunit was attributed to constant replenishment of the unstable mutant enzyme by the active protein synthesis of muscle. In fact, detailed clinical histories showed that both patients suffered from exertional myopathy. The apparent lack of myopathy was probably due to the mild and infrequent nature of muscle symptoms in one patient (C.C.), and, in the other (B.R.), to his shift from an active to a sedentary life-style. In D.S. and B.R., the diagnosis was established by biochemical analysis of muscle biopsies. In C.C., analysis of eryth-

rocyte PFK and other studies proved sufficient for the diagnosis, and muscle biopsy was not considered necessary.

Muscle biopsy studies in two of these patients were in agreement with those reported by other workers (7, 16, 31, 32). The increase of glycogen was initially attributed not only to the block of glycolysis but also to increased activity of glycogen synthetase (41). Subsequent studies showed that the increased activity of glycogen synthetase was secondary to the increased content of Glu-6-P (see below), an activator of the enzyme (29, 42). Recently a fraction of the accumulated glycogen was found to be structurally abnormal, with long peripheral chains, resembling that found in glycogenosis type IV (brancher enzyme deficiency) (16, 31). However, abnormal polysaccharide was not seen in the muscle biopsies of our patients by biochemical or ultrastructural criteria.

The three patients had almost identical hematological findings and PFK activity levels in various cell types (Table I). There was high-normal hemoglobin level, despite moderately severe hemolysis, and PFK activity was approximately half-normal in erythrocytes and cultured lymphoblastoid cell lines, but normal in leukocytes, platelets, and cultured fibroblasts. Although

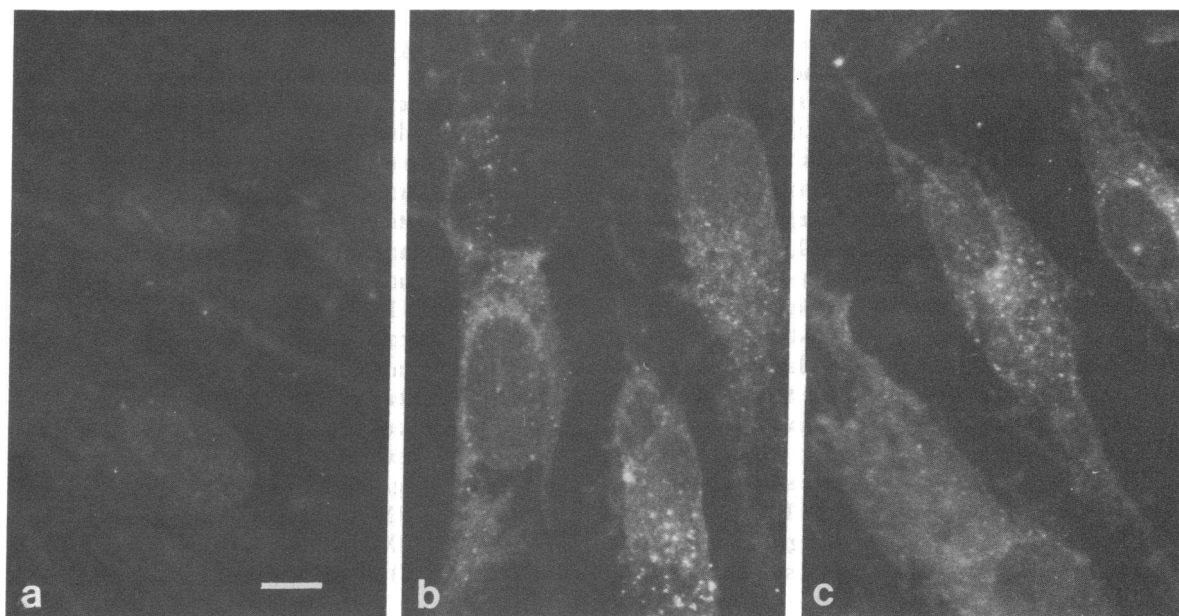


FIGURE 6 Indirect immunocytochemical staining of cultured human skin fibroblasts with monoclonal anti-M antibody (1:50), followed by fluorescein-tagged goat anti-mouse IgG antibodies. (a) Normal cells stained with nonimmune mouse peritoneal fluid show only faint nonspecific fluorescence, mostly nuclear. (b) Normal cells stained with anti-M, show distinct cytoplasmic fluorescence (the punctate staining of some cells may represent glycogen-bound enzyme). (c) Cells from D.S. also show similar staining, indicating the presence of immunoreactive M-type PFK antigen. (Bar is 10 μ m).

erythrocyte PFK activity was decreased to a similar extent, propositus P.S. and his children, and parents and children of B.R. and C.C. showed no hemolysis. These findings are consistent with heterozygosity for a recessively inherited deficiency state.

Metabolic studies of the patients' erythrocytes offered an explanation for the paradoxical presence of erythrocytosis. As previously demonstrated (7), there was a metabolic block in erythrocytes at the level of PFK: glycolytic intermediates above the block, such as hexose monophosphates, were increased, whereas those below the block, such as Fru-1,6-P₂ and triose phosphates, were decreased (Fig. 1). The consistently decreased concentration of 2,3-DPG was associated with markedly increased hemoglobin-oxygen affinity (Fig. 5), indicating that the high normal hemoglobin in these patients is compensatory in nature (7, 11, 43).

Two of our three patients and several other patients reported previously from the United States (7, 16, 29) were of Jewish (Russian-Jewish) ancestry, suggesting that PFK deficiency may be more prevalent in this ethnic group. Recently, Agamanolis et al. (16) reported the development of hyperuricemia and gout in a 23-yr-old patient, a feature characteristically present in glycogenosis type I (glucose-6-phosphatase deficiency).

Our studies confirm the association of muscle PFK deficiency with early-onset hyperuricemia. Moreover, there may be a direct correlation between (a) age at onset and severity of hyperuricemia and (b) frequency and severity of muscle disease. D.S. and B.R., who had severe and moderately severe muscle symptoms, developed symptomatic gout at age 27 and 33 yr, while C.C., who had better tolerance for moderately vigorous exercise, showed only mildly elevated uric acid levels.

Early-onset hyperuricemia and gout are known to be associated with the following three inherited enzymopathies: glucose-6-phosphatase deficiency (glycogenosis type I), hypoxanthine guanine phosphoribosyl transferase deficiency (Lesch Nyhan syndrome), and hyperactive variants of 5-phosphoribosyl pyrophosphate (PRPP) synthetase. The enzyme defects are considered to result in an excess of PRPP, the key substrate for *de novo* purine and pyrimidine biosynthesis and therefore overproduction of uric acid (44). In glycogenosis type I, an increased shunting of accumulated Fru-6-P via hexose monophosphate shunt is proposed to result in increased production of PRPP (45, 46). A similar mechanism may be operative in inherited muscle PFK deficiency, which is associated with increased hexose monophosphates concentrations.

In all three patients, the residual erythrocyte PFK consisted exclusively of the L₄-type isozyme, suggesting homozygosity for the deficiency of the M subunit. This interpretation was supported by the fact that in parents and several children of B.R. and C.C. a partial PFK deficiency (50–65% of normal) was observed in the erythrocytes. In addition, the autosomal recessive mode of inheritance of PFK deficiency is apparent from the previously reported family studies (7, 16, 29). Immunoprecipitation studies indicated that catalytically active M subunit was lacking in all cell types studied. However, whether this was reflected in a significant decrease of total PFK activity depended on the relative contribution of the M subunit to the total enzyme activity in normal cells. Only long-term lymphoblastoid cell lines and erythrocytes showed consistently decreased PFK activity (Table I), since M-subunit represents a major proportion of total PFK (Table II) in these cells. In contrast, lymphocytes, granulocytes, and cultured fibroblasts normally express small amounts of the M subunit (12–25% M-containing species), and, therefore, deficiency of the M subunit, although demonstrable immunologically, was not detectable by enzyme assays (Tables I and II).

Considering the organ distribution of the M-type subunit and the preferred mode of carbohydrate metabolism utilized by various organs, it is not surprising that only the erythrocytes and muscle sustain injury in the M-deficient patients; the dysfunction of other organ systems is conspicuously absent. Only the muscle exclusively contains M-type subunits; all other organs contain P and/or L subunits as well. For instance, the erythrocytes and platelets contain M plus L, and P plus L subunits, respectively. During steady state, the muscle utilizes oxidative phosphorylation for energy generation, but it utilizes glycolysis during ischemic exercise, owing to anaerobiosis. The inability of the M-deficient muscle to utilize glycolysis results in muscle weakness and rhabdomyolysis during vigorous exercise. In contrast, the erythrocytes depend exclusively on glycolysis for energy generation and hence undergo chronic premature cell death. Thus, the complete and universal deficiency of the M subunit in glycogenosis type VII is not fatal and results in a theoretically expected disease complex.

The deficiency of the M subunit may be caused either by total synthetic failure or by synthesis of a structurally or functionally abnormal gene product. The mutant M subunit may be extremely unstable or it may be catalytically nonfunctional. Presence of an unstable M subunit was not supported in all our patients by the failure to detect any M-containing isozymes in reticulocytes and young erythrocytes separated by density-gradient centrifugation. In addition, the ratios of young to old erythrocyte PFK activities were normal in all patients, indicating that the L₄ isozyme was not unstable

within the existing intraerythrocytic conditions. Initial studies had suggested that unstable M subunit was expressed in the erythrocytes of patient B.R., because young erythrocytes showed a normal five-membered isozyme set, whereas old erythrocytes expressed only the L₄ isozyme (12). However, this finding could have been due to platelet contamination of the reticulocyte-rich erythrocyte population obtained by density-gradient fractionation. Since platelet isozymes (P + L) elute in the same positions as the hybrid erythrocyte isozymes (M + L), this contamination can result in an apparently normal isozyme profile of young erythrocytes that contain only the L₄-species.

Immunologically cross-reactive material (CRM) was present in the cultured fibroblasts from D.S. and B.R., indicating the existence of a catalytically dysfunctional M subunit (Fig. 6). Conflicting data exist about the presence of CRM in glycogenosis type VII (7, 16, 29, 31, 32). No CRM was demonstrated in muscle (29) or erythrocytes (7) of some patients, but was present in muscle of other patients (6, 31, 32). CRM may be demonstrable only in nucleated cells, because of their ability for continued protein synthesis, or there may be heterogeneity of the molecular lesion, (i.e., CRM+ and CRM- patients), despite the similar phenotypic expression.

In contrast to the three M-deficient patients who showed similar clinical problems, the fourth propositus (P.S.) and his son are completely asymptomatic. The absence of hemolysis in this kindred is puzzling because the degree of PFK deficiency in erythrocytes is similar (60–65% of normal) to that of M-deficient patients. As anticipated, glycolytic intermediates, including 2,3-DPG, and whole blood P₅₀ were normal in the erythrocytes of P.S.

The erythrocyte PFK from P.S. and his son showed a marked deficiency of L₄, ML₃, and M₂L₂ species, indicating a deficiency of the L subunit (Fig. 3). Heterozygosity for the deficiency was inferred in both, since identical quantitative and qualitative alterations of erythrocyte PFK were present in father and son. The molecular mechanism of the enzyme deficiency in the P.S. kindred was due to instability of the mutant L subunit, as shown by the markedly shortened $t_{1/2}$ value of total erythrocyte PFK. The isozymic profiles of density-gradient fractionated erythrocytes from P.S. clearly showed an age-dependent loss of predominantly L-containing species, i.e., L₄, ML₃, and M₂L₂, thus confirming the presence of an unstable mutant L subunit (Fig. 3).

The approximately half-normal PFK activity of leukocytes, platelets, and lymphoblastoid cell line of P.S. is an expected finding, since the L subunit constitutes a major portion of their total PFK and indicates that the deficiency of the L subunit is universally expressed. The fact that nucleated cells also exhibit the deficiency

casts doubt on the generally accepted belief that an unstable mutant enzyme protein is rapidly replenished by nucleated cells because of their synthetic ability. Although attractive, there is no serious experimental proof for this hypothesis. In fact, there appears to be no reason why an unstable mutant enzyme should be replenished to normal level, since in vivo most enzyme activities are present in excess of the physiological needs. Only protein turnover studies can conclusively demonstrate whether the rate of synthesis of an unstable enzyme is increased *pari passu* with its rate of degradation.

These data taken together with those available in the literature permit a critical reevaluation of the existence and pathogenesis of the various syndromes associated with inherited PFK deficiency (2). The classic syndrome emerges as a consistent and well-defined disease entity (group I). It is characterized not only by myopathy and hemolysis, but also by high-normal hemoglobin and early-onset hyperuricemia. The isolated myopathic syndrome (group II) appears to be a doubtful nosologic entity, probably resulting from incomplete investigation of the patients' erythrocytes; the available data suggest that they suffer from the classic disease. Of the four reported patients with isolated hemolytic syndrome, two have been reinvestigated in the present study and found to suffer from the classic disease. However, available data on the other two patients indicate that an isolated hemolytic syndrome (group III) may exist in some cases (10, 36). One of the two asymptomatic kindreds with PFK deficiency (group IV) was reported to express an unstable M subunit (14). In contrast, the P.S. kindred reported in this article expresses an unstable L subunit. Lastly, the association of congenital fatal myopathy with PFK deficiency (group V) is unclear, since the presence of a superimposed defect or a major primary disease unrelated to PFK deficiency was not excluded.

These data also shed some light upon the physiological roles of different PFK isozymes. The M subunit appears to be essential for adequate glycolytic flux in erythrocytes since its absence is correlated with hemolysis. This is probably due to the fact that M₄ isozyme is less sensitive to inhibition by ATP and 2,3-DPG than the L₄ isozyme (47). This may also explain why M-deficient patients show moderately severe hemolysis despite residual erythrocyte PFK activity of 50–65% of normal. In contrast, M- and L-deficient heterozygotes with similar degree of enzyme deficiency are asymptomatic, because the residual M subunits protect against a metabolic block in glycolysis.

In summary, the studies reported in this article have defined the disease entities and the nature of the enzymatic lesions associated with inherited PFK deficiency. Of the five clinical syndromes previously reported (2), only two commonly exist as distinct bio-

chemical entities. Homozygosity for a mutant inactive M subunit results in glycogen storage disease type VII, the most common syndrome. Heterozygous deficiency of M or L subunit results in an asymptomatic carrier state. The M subunit confers upon the erythrocyte PFK regulatory advantages most suited for their metabolic regulation.

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