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Hereditary Hemolytic Anemia with Erythrocyte Phosphofructokinase Deficiency

Studies of Some Properties of Erythrocyte and Muscle Enzyme

J. Etiemble*, A. Kahn**, P. Boivin, J. F. Bernard, and M. Goudemand

Centre de Recherches sur les Enzymopathies, Unité 24 de l'INSERM, E.R.A. 573 du C.N.R.S. et Université Paris, Hôpital Beaujon, Clichy, France

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Summary. A case of hereditary nonspherocytic hemolytic anemia associated with partial erythrocyte PFK deficiency without muscular symptoms is reported: erythrocyte enzyme activity in the propositus was 60% of normal. Kinetic studies of erythrocyte PFK revealed increased sensitivity to ATP inhibition and decreased sensitivity to citrate inhibition.

Muscle PFK from the patient had a normal enzymatic activity, but was highly unstable to heat, dilution without stabilizer and urea; furthermore its starch gel electrophoretic mobility was markedly faster than the one of a normal control. The results suggested that a muscle type's subunit was deficient in the erythrocyte PFK.

The authors hypothesize that there was no PFK deficiency in the patient's muscle because of the active synthesis of proteins by this tissue. In contrast, the deficiency of PFK would be easily detected in erythrocytes, because of the absence of protein synthesis.

In human erythrocytes the rate limiting enzymes of glycolysis are hexokinase, phosphofructokinase, pyruvate kinase. The importance of phosphofructokinase in erythrocyte metabolism is also suggested by the deficiency of erythrocyte phosphofructokinase as a cause of hereditary non spherocytic hemolytic anemia.

This deficiency has been observed in two different circumstances.

Severe PFK deficiency in skeletal muscle was first demonstrated by Tarui *et al.* (1965) in 3 Japanese siblings in 1965; a similar case was described by Layzer *et al.* (1967). These cases were characterized by severe myopathy associated with a mild hemolytic process, transmitted as an autosomal recessive trait. The erythrocytes showed about half normal PFK activity. This disorder has been referred to as type VII glycogen storage disease. An antihuman muscle PFK antiserum was able to inhibit normal erythrocyte enzyme by approximately 40% although there was no inhibition of erythrocyte enzyme of affected patients (Tarui *et al.*, 1970).

Hereditary non spherocytic hemolysis associated with abnormal erythrocyte PFK activity without muscular symptoms was later demonstrated by Waterbury and Frenkel (1972). Enzyme activity in the propositus (his mother and maternal

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* Attachée de recherche à l'INSERM; present address: Hôpital Beaujon, Pavillon Abrami, F-92110 Clichy, France (for reprint requests).

** Chargé de recherche à l'INSERM.

grandmother) was approximately 60% of normal controls. The propositus enzyme activity was not inhibited by antihuman muscle PFK antiserum.

The absence of muscle disease differentiated this case from those with type VII glycogen storage disease. In 1972 Miwa *et al.* (1972) described a similar case with an erythrocyte PFK activity of only 8% of the normal. In none of these patients was muscle phosphofructokinase activity assayed.

We report a new case of congenital non spherocytic hemolytic anemia with partial erythrocyte PFK deficiency without muscular symptoms but in which an abnormal muscle PFK was present.

Immunological results on this observation have been briefly reported (Kahn *et al.*, 1975).

Case Report

The propositus, a 22-year-old French woman had normal growth and development until she was 5 years old; at this age, icterus and anemia were noted for the first time and hereditary spherocytosis was first diagnosed.

At age 17, splenomegaly was found, and laboratory studies revealed an erythrocyte count of 3×10^{12}/l with 5.6% reticulocytes, a bilirubin 6.2 mg/dl (unconjugated bilirubin: 5 mg/dl). A ^{51}Cr-labelled red cell survival study utilizing the patient's own cell revealed a T ½ of 17 days, with splenic sequestration.

Splenectomy was performed 1 year later. Anemia and icterus, however, remained unchanged.

Three years after the splenectomy the patient was admitted to the department of Hematology of the "Centre Hospitalo-Universitaire" of Lille. At this period hemogram showed: hemoglobin 9.8 g/dl; hematocrit 28.8%; red blood cells 2.8×10^{12}/l; reticulocytes 1.2%; normal white blood cells count; thrombocytes 630×10^{9}/l; erythroblastes: 190 per 100 leukocytes.

Blood smears showed anisopoïkilocytosis, target cells, Howell-Jolly bodies and 24% basophilic stippling erythrocytes.

Bilirubin was 5.1 mg/dl, with 4.3 mg/dl of unconjugated bilirubin.

The enzymatic studies of red blood cells allowed to find a partial PFK erythrocyte deficiency contrasting with the high activities of the other red blood cell enzymes (Table 1).

Table 1. Phosphofructokinase activity in various tissues

	Patient	Control	Father	Mother
Red blood cells (IU/g Hb)	8.1	13 ± 2	13.2	10.8
Blood platelets (IU/mg of proteins)	0.0175	0.015 ± 0.05		
White blood cells (IU/mg of proteins)	0.12	0.095 ± 0.02		
Muscle (IU/mg of proteins)	0.5	0.48 ± 0.13		

Material and Methods

The substrates of the enzymatic reactions were provided by Boehringer Mannheim. The Cleland reagent (dithiothreitol) were obtained from Calbiochem. The reagents used to make buffers were provided by Merck and Calbiochem. Starch hydrolyzed for gel electrophoresis was

furnished by Connaught. Phenazine methosulfate and nitroblue tetrazolium were obtained from Sigma.

Preparation of Muscle Samples. Muscle specimens were obtained by biopsies of the quadricep femoris under local anesthesia. The samples were minced with scissors and placed in a motor driven potter with two volumes of a cold extraction medium consisting of Tris chloride buffer 0.05 M pH 8.0, 60 mM NaF, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1 mM AMP. The mixture was homogenized twice for 30 sec each, centrifuged at 30000 g for 10 min at 4°C and the adipose layer was removed.

Separation of the Platelets and the Leukocytes. Platelets were purified by means of a differential centrifugation at 4°C (30 min at 300 g then three times 2 min at 600 g in order to eliminate red and white cells completely). Total leukocytes were isolated by means of sedimentation in a macromolecular medium (plasmagel) and separated from the remaining erythrocytes by an incubation of 15 min at 20°C with 0.85% ammonium chloride (W/V).

Partial Purification of PFK from Erythrocytes. The enzyme from erythrocytes was purified 150 fold as previously reported (Mandereau and Boivin, 1973) with DEAE cellulose batch and ammonium sulfate precipitation. The specific activity of the enzyme at 25°C was 0.3 IU/mg proteins.

Assay. The activity was measured at 25°C in a coupled system by following the oxidation of NADH in a Zeiss PMQII spectrophotometer according to the method of Ling *et al.* (1955) with minor modifications. The ammonium sulfate was removed from the auxiliary enzymes by centrifugation, dilution and dialysis of the precipitate enzymes in Tris chloride buffer pH 7.5. The composition of the assay medium was previously described (Mandereau and Boivin, 1973).

Electrophoresis. Horizontal starch gel electrophoresis was performed according to Niessner *et al.* (1974) in Tris phosphate buffer pH 7.75. Enzymatic activity was revealed according to Layzer *et al.* (1969) with the following solution: 50 mM tris chloride buffer pH 8.0, 0.5 mM ATP, 5 mM $MgCl_2$, 1 mM F6P, 1 mM NAD^+, 10 mM $NaASIO_4$, 10 IU/ml glyceraldehyde 3 phosphate dehydrogenase, 1 IU/ml aldolase, 0.024 mg/ml phenazine methosulfate, 0.4 mg/ml p nitroblue tetrazolium.

Immunoprecipitations by anti-muscle PFK antiserum and antileukocyte PFK antiserum were studied by methods previously published (Kahn *et al.*, 1975).

Kinetic Studies. A stabilized enzyme preparation was used for the kinetic studies and enzyme stability. The kinetic studies were performed at 20 or 30°C in the presence of 2 mg/ml protein with the same ionic strength buffer and without NH_4^+, the ratio Mg:ATP was equal to 10.

The kinetics with respect to the substrates: F6P, ATP, GTP, ITP, the inhibition by ATP, citrate, 2—3 DPG were performed in 100 mM tris chloride buffer pH 7.5, in the absence of NH_4^+ according to previously reported methods (Mandereau and Boivin, 1973).

The effects of inhibitors such as: ATP and citrate were studied in various conditions:

1. In 100 mM tris chloride pH 7.5 containing 2 mM F6P for ATP inhibition and 0.4 mM F6P for citrate inhibition.

2. The inhibition by ATP was performed at a pH and in the presence of various salts at a concentration close of the intracellular conditions consisting by 10 mM potassium phosphate buffer pH 7.25, 100 mM KCl, 0.44 mM Mg^{2+}, 7.5 mM 2—3 DPG, 7.5 mM glucose, 0.33 mM ADP, 0.1 mM G 1—6 diphosphate.

3. Effects of high concentrations of ATP were studied after precipitation of normal hemolysate by anti muscle PFK serum and anti human leukocyte PFK serum.

Cations Concentrations. Erythrocytes Na^+, K^+ concentrations were determined by methods recently published (Afifi *et al.*, 1974). Ca^{2+} and Mg^{2+} concentrations were measured by atomic absorption spectrophotometry.

Results

1. *Cations Concentrations* in the erythrocytes are given in Table 2.

2. *Total Activity in Different Cells.* The PFK activities of erythrocytes, leukocytes, platelets, muscle from normal subject, patient and parents are shown in Table 1.

Table 2. Cation concentrations in the erythrocytes from normal human and patient

Cations: per kilogram of erythrocytes	Propositus	Control
Mg^{2+}	2.51 mM	2.97 mM ± 0.46
Ca^{2+}	40 μM	9.75 μM ± 3.25
Na^{+}	7.41 meq	6 ± 2
K^{+}	92 meq	89 ± 6

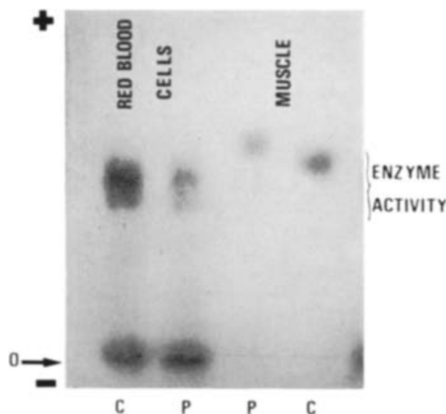

Fig. 1. Starch gel electrophoresis of erythrocyte and muscle PFK of propositus and control. *C* control. *P* patient

3. *Starch Gel Electrophoresis.* The isozyme patterns of erythrocyte PFK from the propositus, the mother, and the father did not differ from the normal (Fig. 1), exhibiting 2 active bands. Normal muscle enzyme fielded a single band, slightly faster than the erythrocyte PFK bands. The muscle enzyme from the patient had faster mobility than the one of a control.

4. *Immunoprecipitation Studies,* previously reported, showed that the precipitation by antimuscle PFK antiserum was more pronounced for normal erythrocyte enzyme than for deficient erythrocyte enzyme. In contrast, inhibition by anti leukocyte phosphofructokinase antiserum was more pronounced for deficient erythrocyte enzyme than for normal erythrocyte enzyme.

5. *Kinetic Studies.* The kinetic characteristics of the patient's erythrocyte and muscle enzymes in respect to ATP substrate, F6P, ITP, GTP, the inhibition by 2—3 DPG and the optimum pH were similar to those of normal enzymes.

Inhibition by ATP and Citrate

The patient's erythrocyte PFK was inhibited by ATP at lower concentrations than those required to inhibit the control PFK (Fig. 2). Inhibition by citrate, in contrast, was more apparent with normal PFK (Fig. 3). In "physiological conditions" (material and methods) the concentration of ATP required to give half maximum inhibition was higher for patient's erythrocytes by a factor of two.

Fig. 4 shows the inhibition of normal hemolysate after precipitation by anti muscle PFK antiserum. This figure reproduces the same pattern as the patient's erythrocyte PFK.

Enzyme Stability

The erythrocyte enzyme of propositus was slightly more unstable to heat and to 4 M urea than control PFK. Control and propositus erythrocytes enzymes were identically stable to storage and dialysis.

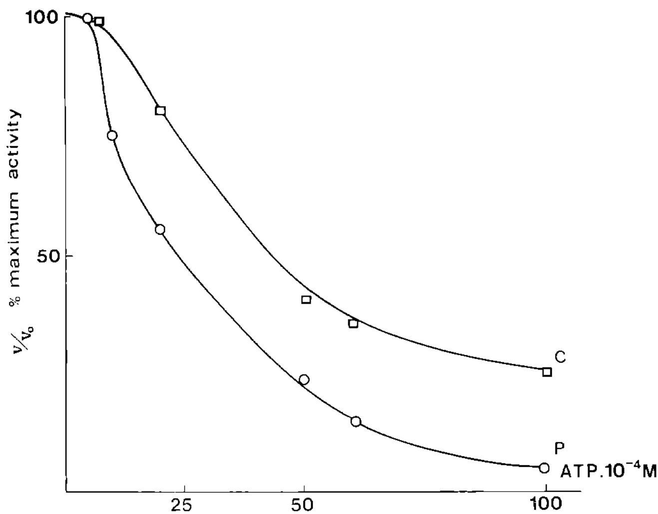

Fig. 2. Effect of ATP concentration on PFK activity of erythrocyte from patient (*P*) and control (*C*). F6P concentration was constant at 2 mM in Tris chloride buffer (100 mM) at pH 7.5. *Vo* velocity at 0.5 mM ATP (V max)

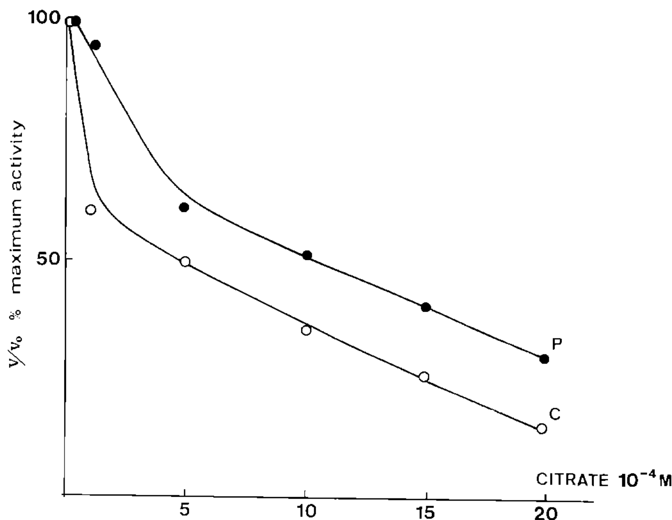

Fig. 3. Effect of citrate concentration on PFK activity of erythrocyte from patient (*P*) and control (*C*) with F6P at 0.4 mM in 100 mM Tris chloride buffer pH 7.5. *Vo* velocity without citrate

Fig. 5 shows first order plots of the time dependency of activity loss at 56° C for the muscular PFK of propositus and control. After 45 min at 56° C, the enzyme from patient was almost completely inactivated while less than 55% of the initial activity of control enzyme was lost in the same conditions.

After a 10 fold dilution in Tris chloride buffer pH 7.6 without stabilizers the activity of propositus muscle enzyme was more reduced than that of a control enzyme (Fig. 6).

The effect of specific antibodies on the stability of the muscle enzyme from normal and pathological subjects was tested (Figs. 5 and 6). After 45 min at 56° C the residual activity

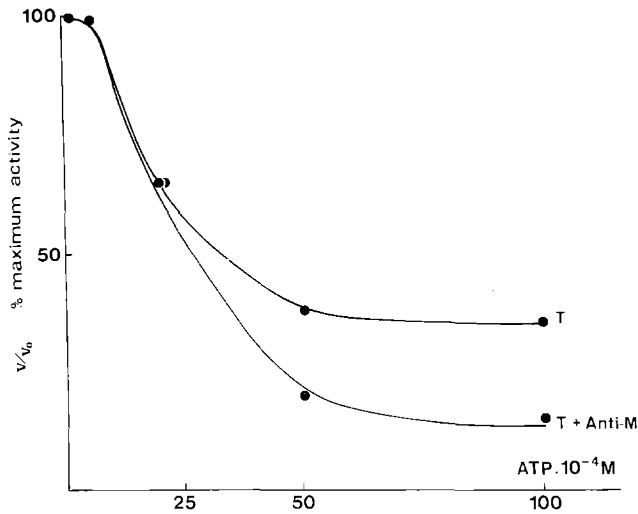

Fig. 4. ATP inhibition in normal hemolysate (*T*) and in normal hemolysate after incubation with antimuscle PFK serum (*T* + *Anti M*). The hemolysates were incubated in the conditions previously reported (Kahn *et al.*, 1975) with antiserum and normal serum. Antiserum concentration was choosen for inactivating PFK activity by half. After centrifugation, the inhibition by ATP was studied in the supernatants dialyzed against the Tris chloride buffer pH 7.6

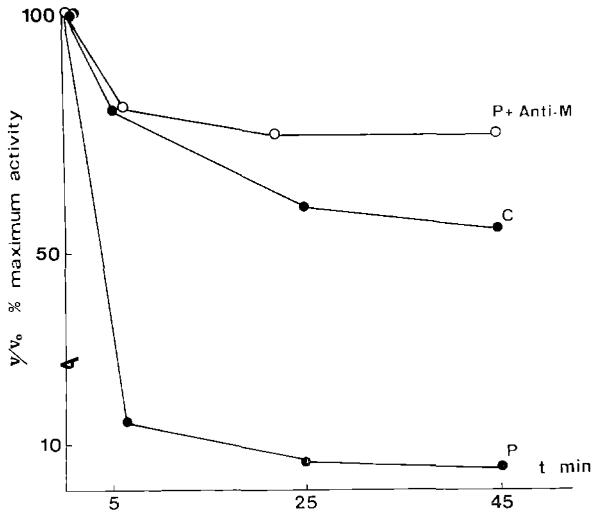

Fig. 5. Thermal stability at 56°C of muscle PFK from patient (*P*) and control (*C*). The residual activity was measured after preincubation at 56°C for periods of 5, 10, 15, 20, 30 and 45 min in Tris phosphate buffer pH 7.7 containing 1 mM EDTA, 1 mM εaminocaproic acid, 1 mM 5′AMP, 5 mM dithiothreitol, 30 mM kalium fluoride, 2 mg/ml serum albumine. The stability was studied after incubation of muscular PFK with antimuscle PFK serum (*P* + *anti M*) in the optimal proportions of precipitation (*i.e.* no activity was detected after centrifugation)

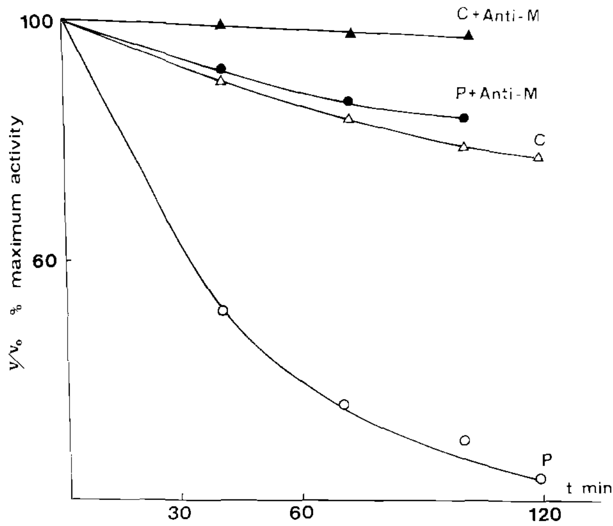

Fig. 6. Stability to dilution (10 folds) without stabilizers of muscle PFK from patient (*P*) and control (*C*) in 100 mM Tris chloride buffer pH 7.5 at laboratory temperature. The stability was studied after binding with anti muscle PFK serum (*P + anti M*)

was still 75% for the patient's enzyme in the presence of antimuscle PFK antiserum and nul without antimuscle PFK antiserum. In the same way, the deleterious effect of dilution on the pathological enzyme was strikingly decreased after its linkage with antimuscle PFK anti serum. This phenomenon has already been described for other altered enzymes.

Discussion

Our purpose was to investigate erythrocyte and muscle PFK in a patient with erythrocyte PFK partial deficiency and congenital non spherocytic hemolytic anemia. Such association has been previously reported by various authors (Miwa *et al.*, 1972; Waterbury and Frenkel, 1972). In the majority of cases, there was either complete muscle and partial erythrocyte deficiency (Layzer *et al.*, 1969; Tarui *et al.*, 1965) or immunological evidences for the absence of muscle type PFK in the deficient erythrocytes (Tarui *et al.*, 1970). These data have led several authors (Layzer *et al.*, 1967; Layzer and Conway, 1970; Miwa *et al.*, 1972; Tarui *et al.*, 1970; Waterbury and Frenkel, 1972) to suggest that there are at least 2 PFK isoenzymes and that erythrocyte PFK is a composite enzyme, with muscle type's subunits. It would be quite possible that there were also common subunits between erythrocyte and leukocyte PFK (Staal *et al.*, 1973).

Muscle and erythrocyte PFK, although immunologically related, are not identical. In starch electrophoresis we have separated muscle and erythrocyte PFK. Hemolysate from normal controls showed at least one extra band of erythrocyte enzyme activity as reported before by Niessner and Beutler (1974) and Layzer *et al.* (1969). The red cell enzyme is characterized by its high sensitivity to the inhibitory action of ATP, compared with the muscle enzyme: moreover the muscle PFK is more inhibited by citrate than erythrocyte PFK (Layzer *et al.*, 1969).

In the patient, the antimuscle PFK antibody precipitation studies (Kahn *et al.*, 1975) resemble to those described by Tarui *et al.* (1970), Layzer *et al.* (1967) and Waterbury and Frenkel (1972). These findings seem to indicate that an enzyme subunit of muscle type is deficient in erythrocyte and that an hypothetical common subunit between erythrocyte and leukocyte enzyme is not involved in the deficiency. Kinetic experiments, inhibition of erythrocyte PFK by ATP and citrate confirm the assumption of the deficiency in patient's erythrocyte of muscle type PFK; the residual activity being due to the "non muscle" PFK subunit. The normal erythrocyte enzyme after precipitation of the muscle subunits by anti-muscle PFK serum gives the same results as the pathological enzyme.

In spite of the absence of muscle symptoms, we have demonstrated that the deficiency in the patient's erythrocyte of muscle subunits is related with the instability and the abnormal electrophoretic migration of the patient's muscle PFK. This was proposed but not demonstrated by Waterbury and Frenkel (1972) and Miwa *et al.* (1972). There was no PFK deficiency in the patient's muscle probably because the tissue actively synthesize proteins. In contrast, because of the absence of protein synthesis, the deficiency is easily detected in the erythrocytes.

The relationships between the erythrocyte PFK deficiency and hemolysis could be explained by the increased sensitivity of the patient's erythrocyte enzyme to ATP inhibition. In this assumption only muscle type PFK, the least inhibited by ATP would be efficient for glycolysis in the metabolic conditions of intra erythrocytic medium (*e.g.* high ATP concentration). These data would suggest that an enzyme deficiency to 60% of normal but involving the muscle type subunit would lead to a severe metabolic disturbance and to hemolysis.

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Dr. M. Goudemand
Centre regional de transfusion sanguine
de Lille
19—21 rue Camille Guérin
59012 Lille RP Cedex

Dr. J. Etiemble
attachée de Recherche à l'INSERM
Hôpital Beaujon, Pavillon Abrami
F-92110 Clichy, France