ENZYMATIC BASIS FOR THE COEXISTENCE OF MYOPATHY AND HEMOLYTIC DISEASE IN INHERITED MUSCLE PHOSPHOFRUCTOKINASE DEFICIENCY

Seiichiro Tarui, Norio Kono, Terushi Nasu and Mitsuo Nishikawa

Central Laboratory for Clinical Investigation, and
Second Department of Internal Medicine
Osaka University Medical School, Osaka, Japan

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Observation on a specific enzymatic defect has often led to recognition of the characteristics of the enzyme concerned. Phosphofructokinase (PFK, ATP:d-fructose-6-phosphate 1-phosphotransferase, EC 2, 7, 1, 11) is considered a key enzyme of glycolysis because of its low activity, allosteric property and one-way reaction. There have been many reports on the complicated kinetic properties of this enzyme. However, an extensive survey of patients lacking active PFK could throw further light on the basic properties, the genetic control and the physiological role of PFK.

Muscle PFK deficiency is a newly discovered type of metabolic myopathy, originally demonstrated in this laboratory (Tarui et al., 1965), and has been designated as Type VII glycogen-storage disease by Brown and Brown (1968). More clinical and immunochemical features of this disease were reported recently (Tarui, 1967; Layzer et al., 1967). Our previous report indicated that not only muscle PFK but also erythrocyte PFK was affected in this disease. The inherited deficiency of a specific enzyme of the glycolytic pathway in erythrocytes is known to be associated with reduced survival of erythrocytes. In patients with muscle PFK deficiency the clinical features for non-spherocytic hemolytic disease, including a reduced life span of ^{51}Cr-labelled erythrocytes of 13 to 16 days, erythroid hyperplasia of the bone marrow and increased reticulocyte counts ranging from 3.8 to 6.5%, were demonstrated in this laboratory besides the manifestations of muscle glycogenosis.

Of interest is the fact that erythrocyte PFK activity in the patients is found to be about half that of the normal controls in contrast to the almost complete absence of muscle PFK activity in this disease (Tarui et al., 1965). These peculiar enzymatic abnormalities of the disease were completely confirmed in a report from the United States on a second family with muscle PFK deficiency (Layzer et al., 1967). The experiments reported in this paper were undertaken to determine whether the difference in the degrees of enzyme dificiency in the muscles and erythrocytes could be elucidated by characterization of erythrocyte PFK.

Materials and Methods

The patients with muscle PFK deficiency examined in the present work were the same to those described previously (Tarui et al., 1965). PFK activity was measured in a system coupled with aldolase, triose-phosphate isomerase and glycerophosphate dehydrogenase. One unit of PFK activity was defined as before, i.e. the amount of enzyme which catalyzes the formation of 1 μmole of fructose-1,6-diP per min. Erythrocyte PFK was extracted from hemolyzates with DEAE-cellulose by the method of Hennessey et al. (1962) ; the enzyme protein fraction was desorbed from cellulose with 0.5M Tris phosphate, pH 8.0. Human skeletal muscle was obtained during surgical procedures. The muscles were freed from connective tissue and fat, stored at -20°, and then used for PFK purification.

Results and Discussion

Purification of human muscle PFK: Muscles, weighing 800g, were passed through a chilled grinder and homogenized for 20 mins., in a Waring Blendor with 1.5 volume of 0.04M EDTA, pH 8.0, containing 0.03M KF, 0.005M mercaptoethanol, 10^{-4}M ATP, 2×10^{-5}M fructose-1,6-diP. A high concentration of EDTA was required to prevent a marked drop in pH and to obtain a good yield on extraction. The homogenate was centrifuged at 0°C for 20 mins. at 10,000×g. The supernatant, filtered through glass wool, was heated and precipitated with isopropyl alchohol by the method of Ling et al. (1965). The precipitate was dialyzed against 0.05M Tris-phosphate, pH 8.0, containing stabilizing substrates, 0.005M mercaptoethanol, 2×10^{-4}M EDTA, 10^{-4}M ATP, and 2×10^{-5}M fructose-1,6-diP. PFK was then precipitated from the supernatant with between 35 and 60% satu-

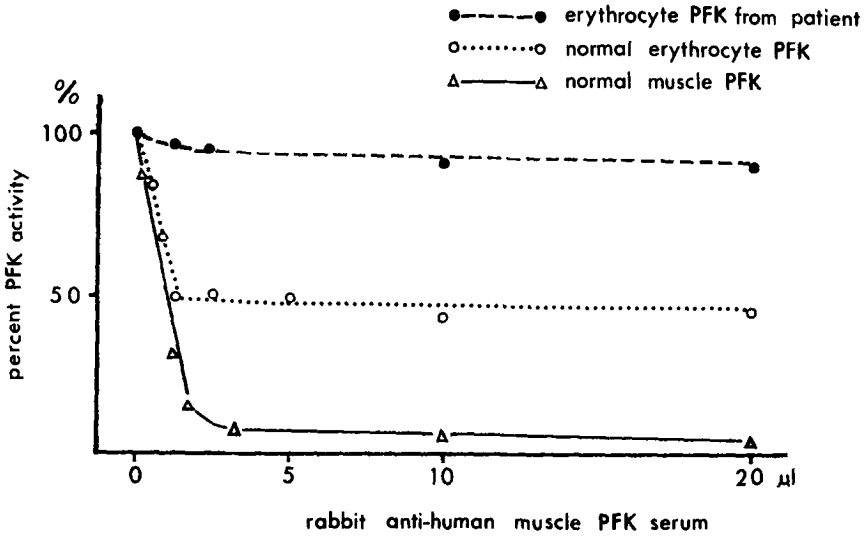

Fig. 1. Neutralization of muscle and erythrocyte PFK using anti-muscle PFK serum. In the tests a mixture of antiserum and enzyme was incubated for 30 mins. at 20°C before addition of the assay solution. Enzyme preparations were adjusted to the same initial activity. Assays were performed in the presence of dispersed antigen-antibody complex. The extent of enzyme inhibition was calculated from control experiments in which antiserum was replaced by normal rabbit serum.

Table I. Purification of human skeletal muscle PFK

Fraction	Volume	Total units	Protein	Specific activity	Yield
	ml	units	mg/ml	units/mg	%
Initial extract from 800g of muscle	700	4270	23.0	0.265	(100)
Heat treated and precipitated with 2-propanol	102	3010	24.5	1.21	70
Precipitated with 0.35 - 0.60 saturation of $(NH_4)_2SO_4$	44.0	2670	7.38	8.22	63
Fractionated with DEAE-cellulose and $(NH_4)_2SO_4$	3.0	1300	6.15	70.5	30

ration of $(NH_4)_2SO_4$, pH 6.0. This fraction was dialyzed against 0.05M Tris-phosphate buffer containing the above stabilizers and applied to a column of DEAE-cellulose. The protein was eluted with a linear gradient from 0.05M to 0.4M Tris-phosphate buffer, pH 8.0. PFK was found in the eluates with between 0.1M and 0.25M Tris-phosphate, and was collected by $(NH_4)_2SO_4$ fractionation. Results of a typical preparation are summarized in Table I.

Preparation of rabbit anti-enzyme serum: A solution of purified muscle PFK (5mg/ml) was completely emulsified with the same volume of Freund's complete adjuvant. Then 1.5ml of the emulsion was injected into the foot pads of rabbits two or three times at two week intervals. Immune serum was obtained from clotted blood, generally drawn two weeks after the last injection. Antibodies were partially purified by $(NH_4)_2SO_4$ fractionation.

Enzyme neutralization tests with anti-human muscle PFK serum: Fig. 1 summarizes the results of enzyme neutralization tests using various amounts of anti-human muscle PFK serum. Purified human muscle PFK was neutralized more than 90% with excess antibody. The PFK activity of normal erythrocytes was neutralized by anti-muscle PFK serum approximately 50% and this percentage of neutralization was not increased by further addition of antiserum. Generally immunochemical analysis does not provide conclusive criteria for identifying the enzyme protein. In these studies, however, materials from patients with the genetic defect of muscle PFK were available. When hemolyzates from the patients, in which PFK-activity was half the normal value, were employed for the tests, there was little inhibition of PFK by anti-muscle PFK serum. These data are interpreted as indicating that normal erythrocyte PFK is heterogeneous, and that 50% of the enzyme activity is due to "muscle-type" PFK protein since it not only possesses the same antigenicity as muscle PFK but is under the same genetic control as muscle PFK. Hence the fact that erythrocyte PFK decreases to about half the normal level when muscle PFK is completely deficient is considered to be due to the genetic heterogeneity of erythrocyte PFK protein.

DEAE-cellulose chromatography of muscle and erythrocyte PFK: As shown in Fig. 2, human muscle PFK and erythrocyte PFK were separated by DEAE-cellulose chromatography ; the former was eluted with between 0.15M and 0.2M Tris-phosphate while enzymatic activity of the latter was mostly eluted with between 0.2M and

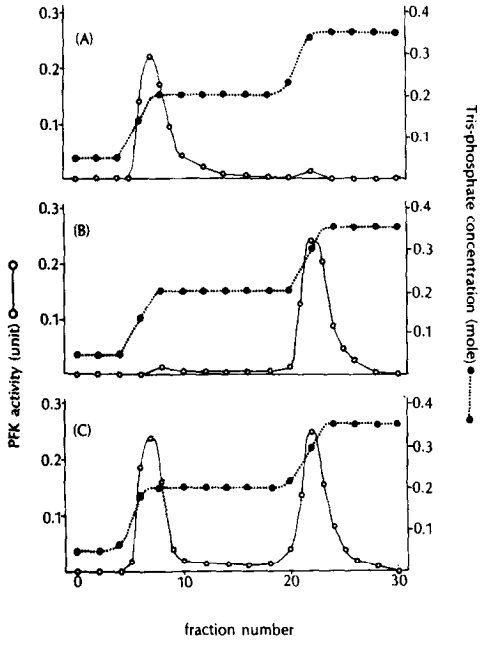

Fig. 2. Elution pattern of human muscle and erythrocyte PFK from DEAE-cellulose column. A, purified human muscle PFK ; B, PFK extracted from human hemolyzates ; C, a mixture of A and B. A column was prepared from DEAE-cellulose (1.5cm^2 × 12cm), equilibrated with 0.05M Tris-phosphate, pH 8.0, and the enzyme preparations were applied to the top of the column. The column was washed with 0.05M Tris-phosphate, pH 8.0, and eluted stepwise with from 0.2M to 0.35M Tris-phosphate at pH 8.0. All manipulations were performed at 4°C in a cold room and the eluants contained the stabilizing substrates described under "purification of human muscle PFK".

0.35M Tris-phosphate. Several fractions of the major peak in an elution pattern of erythrocyte PFK during DEAE-cellulose chromatography were examined for percentage neutralization of the enzyme activity by anti-muscle PFK serum ; 47 to 52% neutralization was observed for every fraction. These chromatograms, together with the results of immunochemical studies, indicate that the great portion of PFK in erythrocytes contains "muscle-type" PFK protein as a molecular subunit. It is also noteworthy that the curve of the neutralization of erythrocyte PFK by anti-muscle PFK serum (Fig. 1) is quite similar to the graph presented by Markert and Appella (1963) for neutralization using anti LDH-5 serum with LDH-3, A^2B^2 type molecules.

Summary and Comments

Antiserum to purified human muscle PFK was prepared in rabbits. Immunochemical studies on erythrocyte PFK of normal controls and muscle PFK deficient subjects with this antiserum revealed that normal erythrocyte PFK contains two or more components and 50% of the enzyme activity is attributable to "muscle-type" PFK protein. Therefore, it is quite natural that the genetic defect of muscle PFK results in subnormal activity of erythrocyte PFK. Hence it is better to refer to muscle PFK deficiency as "muscle-type" PFK deficiency.

PFK is known to play a major role in the control of glycolysis in erythrocytes (Minakami et al., 1964) ; the decreased activity of the enzyme is regarded as the cause of decreased erythrocyte survival. However, hemolytic jaundice is not remarkable in this disease, possibly because erythrocyte PFK is only partially affected. Neither anemia nor splenomegaly was observed in our three cases. Only one of the three cases had an elevated serum bilirubin of 2.9mg% total with 2.2mg% indirect reacting bilirubin ; the serum indirect bilirubin levels of the other two cases were at the upper limit of normal. Erythrocyte PFK deficiency coexistent with Type VII glycogenosis is a rare entity of hereditary hemolytic disease which shows about 50% defect of an enzymatic step in the homozygous form. It seems unlikely that hemolytic anemia would occur due to complete absence of erythrocyte PFK, since erythrocyte PFK consists of genetically heterogenous enzyme protein.

PFK of erythrocytes and PFK of skeletal muscle were found to be chromatographically separable. These data have led to the concept that the great portion of PFK in erythrocytes contains "muscle-type" PFK protein as a molecular subunit. Work is under progress on the chemical structure and enzymatic properties of erythrocyte PFK.

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