Phosphofructokinase Deficiency Associated with Congenital Nonspherocytic Hemolytic Anemia and Mild Myopathy: Biochemical and Morphological Studies on the Muscle

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Department of Internal Medicine, Institute of Medical Science, University of Tokyo, Tokyo 108, *Department of Medical Research, Institute for Nuclear Medicine and Biology, University of Hiroshima, Hiroshima 734 and †Department of Anatomy, Faculty of Medicine, University of Tokyo, Tokyo 113

TANI, K., FUJII, H., MIWA, S., IMANAKA, F., KURAMOTO, A, and ISHIKAWA, H. Phosphofructokinase Deficiency Associated with Congenital Nonspherocytic Hemolytic Anemia and Mild Myopathy: Biochemical and Morphological Studies on the Muscle. Tohoku J. exp. Med., 1983, 141 (3), 287-293 — Enzymatic and electron microscopical studies were performed on the muscle of a proband with phosphofructokinase deficiency. Enzymatic studies showed that muscle phosphofructokinase activity of the proband was decreased to about a half of normal. This enzyme was quite thermodabile and had low affinity for fructose 6-phosphate. Electron microscopical studies showed the accumulation of glycogen granules beneath the sarcolemma and between the myofibrils in spite of a lack of accumulation of the intermediates before the step of phosphofructokinase. The proband's clinical symptoms, i.e., hemolytic anemia and myopathy, were considered to be due to the unstable, mutant, muscle-type phosphofructokinase in the red blood cells and muscle. — phosphofructokinase deficiency; myopathy; congenital nonspherocytic hemolytic anemia

Phosphofructokinase (PFK) deficiency associated with myopathy and congenital nonspherocytic hemolytic anemia was first reported by Tarui et al. (1965). Several investigators showed that PFK in human tissues is a tetramer made up of three types of subunits; a muscle-type subunit, a liver-type subunit and a fibroblast (platelet)-type subunit (Tarui et al. 1969; Kahn et al. 1979; Vora 1981). Tarui et al. (1969) and Layzer and Rasmussen (1974) demonstrated that the muscle-type subunit was defective in the probands' red cell PFK. Since the first discovery of congenital PFK deficiency, twenty-one cases of PFK deficiency occurring in seventeen unrelated families have been reported. In addition to these cases, we recently found two additional Japanese kindreds with this deficiency (Tani et al. 1983). One of our probands exhibited well-compensated hemolytic anemia and mild myopathy. The proband's red cell enzyme studies

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showed that red cell PFK activity was reduced to about a half of normal level. No demonstrable muscle-type PFK was shown in the proband’s red cell PFK either by starch gel electrophoresis or by DEAE-Sephadex A-25 chromatographic studies. Based on the proband’s mild myopathy, mutant, not completely defective, muscle-type PFK was considered to be a pathogenic cause of our case (Tani et al. 1983).

Recently, the permission for muscle biopsy was obtained from the proband. In this report we analyzed the muscle specimen to elucidate the precise mechanism of hereditary nonspherocytic hemolytic anemia and mild myopathy.

**Materials and Methods**

A case report of the proband, a 35 year-old Japanese male, was done previously (Tani et al. 1983).

Muscle specimens were taken from the proband and control subjects by surgical biopsy in accordance with the Declaration of Helsinki and after informed consent was obtained. Tissue from a proband’s skeletal muscle (red portion of the quadriceps) and control subjects’ skeletal muscle (red portion of the rectus abdominis), were frozen in dry ice and transported to our laboratory for biochemical studies.

For morphological studies, a strip of the biopsied muscle specimen was fixed at a stretched state in 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.3, overnight at room temperature. After being washed with 10% sucrose in the phosphate buffer, small pieces of the tissue were postfixed in 1% OsO₄ in the same buffer, dehydrated, and then embedded in Epon 812. Thin sections were cut, doubly stained with uranyl acetate and lead citrate, and examined with a Hitachi HU-12 electron microscope.

For the determinations of muscle glycolytic enzyme activities, 450 mg of frozen muscle was homogenized in 4 ml of 0.05 M Tris-chloride, pH 7.5, containing 5 mM ethylenediaminetetraacetic acid in a glass-Teflon, motor driven homogenizer at 0°C for 5 min. After centrifugation at 26,800 x g for 15 min, the supernatant was assayed according to the methods of Beutler et al. (Beutler 1975; Beutler et al. 1977).

For the determinations of muscle glycolytic intermediates and adenine nucleotides, 450 mg of frozen muscle was homogenized with 1 ml of 3 M HClO₄ in a glass-Teflon, motor driven homogenizer cooled with an ice-alcohol bath. After vigorous stirring for 3 min, 2 ml of ice-cold water was added and the mixture was homogenized for 3 more min. After centrifugation at 26,800 x g for 15 min, the supernatant was neutralized to pH 7.0 with 10 M KOH and the final volume was adjusted to 10 ml with ice-cold water. The precipitated potassium perchloride was removed by centrifugation and the clear extract was used for assay according to the methods of Minakami et al. (1965).

Thermal and urea stability tests were performed by the method of Kahn et al. (1975) with minor modifications of the temperature and time course where the thermal stability test was performed at 59°C and the residual activities were measured after 10, 20, 40 and 60 min incubations. Muscle enzyme preparations for determining Michaelis constants for fructose 6-phosphate (Km F6P) and adenine triphosphate (Km ATP) were prepared as described by Kahn et al. (1975) except that 0.1 M Tris-phosphate, pH 8.0 containing 0.2 mM ethylenediaminetetraacetic acid, 0.7 mM dithiothreitol and 1% bovine serum albumin were used as the homogenizing buffer. Km for F6P and Km for ATP were determined as previously described (Tani et al. 1983).

The preparations for anti-muscle antibody and immunoneutralization tests were done as previously described (Vora et al. 1980) using partially purified antibody (Harboe and Inglid 1973).

All substrates and nucleotides were purchased from Boehringer Mannheim. All other reagents were of analytical grade. The enzyme reaction was read with a Gilford recording spectrophotometer at 37°C.
RESULTS AND DISCUSSION

Muscle PFK activity, muscle glycolytic intermediates, Km for ATP and Km for F6P of the proband and normal control subjects are shown in Table 1. The proband’s muscle PFK was unstable during heating at 59°C as shown in Fig. 1 and slightly unstable in the presence of 4 M urea (data not shown).

| Table 1. Muscle PFK activity, muscle content of glycolytic intermediates, Km ATP and Km F6P of proband and normal control subjects |
|-----------------|-----------------|-----------------|
|                 | Patient         | Control means (range) |
| PFK activity (units/g.w.w.) | 12.7 | 22.1 (16.7–28.7) (n=3)* |
| Glucose 6-phosphate (G6P) | 315 | 430 (191–848) (n=3)* |
| Fructose 6-phosphate (F6P) (nmol/g.w.w.) | 49.4 | 110 (61–187) (n=3)* |
| Km ATP (mM) | 0.111 | 0.093 (0.091–0.095) (n=2)* |
| Km F6P (mM) | 0.870 | 0.295 (0.22–0.37) (n=2)* |

* The numbers of control subjects examined.

Fig. 1. Thermal-stability test of the proband’s muscle PFK (○—○) and normal controls’ muscle PFKs (●—●) at 59°C.

Immunoneutralization tests using human muscle PFK antibody showed that no apparent differences existed between the proband’s muscle PFK and control muscle PFK (Fig. 2).

Electron microscopy showed that most muscle fibers contained a moderately increased amount of glycogen. Glycogen particles were deposited in the sarcoplasm beneath the sarcolemma and between the myofibrils (Fig. 3). Large accumulations of glycogen particles were occasionally found in the subsarcolemmal region. Also prominent in many fibers was the deposit of glycogen particles within the myofibrils preferentially at the I band (Fig. 4).

Our studies showed that the proband’s muscle PFK activity was decreased to about a half of normal. The enzyme was thermolabile and had a decreased
affinity for F6P. There was no increase of G6P or F6P in the muscle compared with normal controls. The accumulation of glycogen granules beneath the sarcolemma and between the myofibrils was demonstrated with the electron microscope.

Since PFK deficiency associated with myopathy and congenital nonspherocytic hemolytic anemia was first described by Tarui et al. (1965), twenty-four cases of congenital PFK deficiency occurring in 19 unrelated families have been reported (Tani et al. 1983). In these cases, 12 cases had apparent myopathy and hemolytic anemia, six cases had only myopathy and four cases had only anemia. Muscle PFK activity was measured in 15 cases. PFK activity was decreased to between zero and a few percent in 14 cases. These cases were all accompanied with myopathy. In one case reported by Kahn et al. (1975), PFK activity was not decreased at all, and hemolytic anemia was predominant. Their studies demonstrated that proband’s muscle PFK was an unstable and electrophoretically fast variant.

The proband did not attempt to do vigorous exercise because exercise has induced abdominal pain and fever since his early childhood. So actually it was difficult to determine clinically whether he had real myopathy or not. However, the muscle exercise test showed no increase of lactate in the venous blood of the proband (Tani et al. 1983). This suggested that clinical myopathy would appear if he did vigorous exercise. Examination by electron microscopy showed an accumulation of glycogen granules in the sarcolemma and between myofibrils, which was commonly seen in PFK deficiency with myopathy (Serratrice et al. 1969; Tobin et al. 1973; Guibaud et al. 1978; Agammanolis et al. 1980; Hays et al. 1981; Dannon et al. 1981; Zanella et al. 1982). The accumulation was considered to be due to the increased level of G6P which accelerates glycogen synthesis (Layzer et al. 1967). In one case (Tobin et al. 1973), muscle G6P level was remarkably increased with the accumulation of glycogen in the muscle. In our case, glycogen accumulation was demonstrated electron microscopically in the

![Graph of immunoneutralization test](image-url)
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The increase of G6P in the muscle might be demonstrated if the biopsy was performed after the exercise.

Our data demonstrated that the muscle-type PFK was very unstable and had a low affinity for F6P. This mutant muscle-type PFK was considered to be a pathogenic factor that leads to hemolysis and mild myopathy in this proband. In spite of the muscle PFK activity being decreased to about a half of normal, the findings in our proband were very similar to the report by Kahn et al. (1975). Recently, Agammanolis et al. (1980) and Zanella et al. (1982) have reported the PFK deficiency due to the synthesis of a structurally abnormal and catalytically inactive enzyme protein. Exact mechanisms still remain unclear without the knowledge of the primary structure of the enzyme protein (Fujii and Yoshida 1980). But there can be various clinical pictures based on the structure of the mutant protein, e.g. our case and the case reported by Kahn et al. were accompanied with mild or no myopathy because of the reduced enzyme catalysis, whereas the cases reported by Agammanolis et al. and Zanella et al. were accompanied with severe myopathy because of catalytically inactive muscle-type PFK. As muscle tissue might actively synthesize this mutant muscle-type PFK, a very mild myopathy and no increase of G6P or F6P in the muscle were noted in our proband. On the other hand, a serious case of hemolytic anemia was noted because of the loss of protein synthesis in red blood cells.

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Fig. 3 (upper). Electron micrograph of transversely cut muscle fibers. Note a subsarcolemmal accumulation of glycogen particles (arrowheads).

Fig. 4 (lower). Electron micrograph of a transverse section of muscle fibers. In the red fiber with the subsarcolemmal aggregates of mitochondria (M) and many lipid droplets (L), numerous glycogen particles are deposited within the I band of myofibrils (arrowheads) as well as in the sarcoplasm between the myofibrils.
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