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MOUSE MUSCLE PHOSPHOFRUCTOKINASE IS PARTIALLY PHOSPHORYLATED

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SUMMARY: Phosphofructokinase isolated from mouse skeletal muscle 18 hours after intraperitoneal injection of $[^{32}P]$-PO_4^{3-} contained 0.12 to 0.15 moles of covalently bound phosphate per protomer on the basis of the specific activity of radiolabel in the γ-position of ATP. Under identical conditions, muscle pyruvate kinase and aldolase had no covalently bound phosphate.

In the past few years, several reports have appeared indicating that phosphofructokinase may be subject to regulation by a covalent phosphorylation mechanism. Brand and Soling (1,2) first reported a MgATP dependent activation of rat liver phosphofructokinase accompanied by the formation of covalently bound phosphate. In 1976, Hofer and Furst (3), suggested that the muscle isozyme of mouse phosphofructokinase is also phosphorylated on the basis of the immunoprecipitation by antibody to rabbit muscle phosphofructokinase of $[^{32}P]$ from muscle extracts after intraperitoneal injection of $[^{32}P]$-PO_4^{3-}. They concluded that 80-90% of the enzyme was phosphorylated by making the perhaps unjustified assumption of equilibration of the protein phosphate with the inorganic phosphate pool. Very recently, Hussey *et al.* (4) have reported that on the basis of alkali-labile phosphate content, rabbit muscle phosphofructokinase contains 0.15 to 0.24 moles of phosphate per protomer. These contrasting results, coupled with the known high affinity of phosphofructokinase for a variety of phosphorylated metabolites, suggested a thorough examination of the extent of phosphorylation of phosphofructokinase in mouse muscle.

EXPERIMENTAL PROCEDURE. Purification of Mouse Muscle Phosphofructokinase. Phosphofructokinase was prepared from the muscle of male ARS HA/ICR mice (Swiss Albino) by a procedure essentially similar to that of Ramadoss *et al.* (5). Mice were killed by cervical dislocation, decapitated, skinned, and eviscerated. The carcass, consisting of bone and skeletal muscle,was minced and subsequently homogenized in a Waring Blendor with

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3 ml/g tissue of 30 mM KF, 4 mM EDTA, adjusted to pH 7.5 (Buffer A). The homogenization and all following operations were performed at 4°. The homogenate was centrifuged at 27,000 x g for 30 min. The supernatant was removed, filtered through glass wool, and pumped through 1.5 x 10 cm column containing ATP-Sepharose 4B, prepared as described by Lindberg and Mosbach (6), that was previously equilibrated with Buffer A. The column was washed sequentially with three bed volumes of each of the following solutions at flow rates of 80 ml/hr: 50 mM Tris-PO_4 (pH 8.0), 0.2 mM EDTA, 1.0 mM dithiothreitol (Buffer B); Buffer B plus 0.1 mM fructose-6-P, Buffer B, Buffer B plus 0.1 mM ADP, Buffer B. Phosphofructokinase was eluted at a rate of 15 ml/hr with Buffer B plus 0.2 mM ADP and 0.2 mM fructose-6-P. Enzyme activity was assayed at pH 8.2 and 30° as described by Kemp (7).

Crystallization of Mouse Muscle Phosphofructokinase. The eluted fractions containing enzyme were pooled and precipitated by adding solid ammonium sulfate to 55% saturation. The precipitate was collected by centrifugation, resuspended in Buffer B plus 0.1 mM ATP to a final protein concentration greater than 10 mg/ml, and dialyzed overnight against the resuspension solution. The solution was then clarified by centrifugation. Solid ammonium sulfate (0.19 g/ml) was added with stirring to 0.32 saturation and the sediment that formed was removed by centrifugation. The supernatant was dialyzed against Buffer B plus 0.1 mM ATP and ammonium sulfate at 0.32 saturation. Crystals, usually as rods, began to appear after several days.

Gel Electrophoresis. Acrylamide gel electrophoresis in sodium dodecylsulfate was carried out on 7.5% acrylamide slab gels (3 mm x 10 mm) with buffers and solutions prepared according to Laemmli (8). Samples to be analyzed were diluted 1:1 in 120 mM Tris-PO_4 (pH 7.2) containing 50% glycerol, 2% mercaptoethanol, 2% sodium dodecylsulfate, and 0.004% bromophenol blue. The samples were heated at 100° for 3 min to assure complete denaturation. Electrophoresis in 5% acrylamide acid-urea gels was carried out in 0.6 cm diameter tubes with 6 M urea and 0.9 N acetic acid as described by Panyim and Chalkley (9). The protein was reduced with dithiothreitol prior to acidification and the gels were prerun for 5 hrs to remove persulfate before sample application.

Gels were fixed by shaking in 40% isopropanol/10% acetic acid for 30 min, stained for protein 1 hr with 0.1% Coomassie Blue R-250 in 10% isopropanol/10% acetic acid, and destained overnight against 10% isopropanol/10% acetic acid. The gels were sliced, digested with H_2O_2 (10), and counted in a scintillation counter.

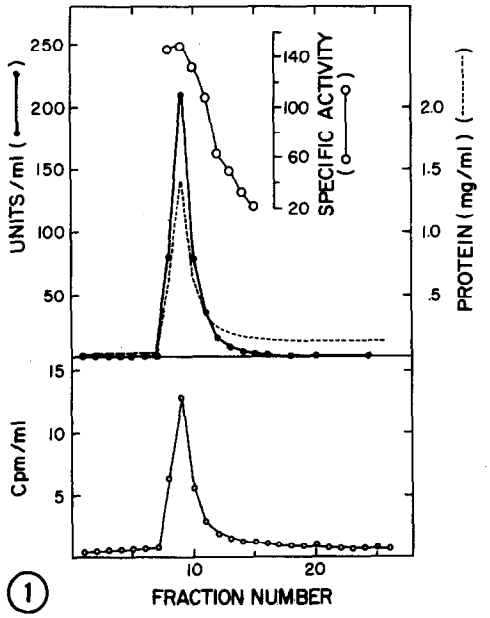
Electrophoresis on non-denaturing gels for pyruvate kinase was performed in 3 mm 10% acrylamide slabs as described by Davis (11), and the gels were stained for activity according to Cardenas and Dyson (12).

Specific Activity of ATP. The specific activity of the γ-phosphate of ATP was determined using a modification of the hexokinase technique of Mayer and Krebs (13). A small sample of the tissue that was to be homogenized for phosphofructokinase isolation was frozen on solid CO_2. The sample was then extracted with perchloric acid. Isolation procedures for ATP and for glucose-6-P following hexokinase treatment were carried out on DEAE-Sephadex A-25 in the bicarbonate form with a linear gradient of NH_4HCO_3 from 50 to 600 mM.

Protein was assayed by the method of Bradford (14) with rabbit muscle phosphofructokinase as a standard.

RESULTS AND DISCUSSION. Isolation of [^{32}P] Phosphofructokinase.

[^{32}P]-PO_4^{3-} in 0.3 ml of saline was injected into the peritoneal cavity of mice and, after 18 hrs, the phosphofructokinase was isolated as described in the Experimental Procedure. A typical elution profile is shown in Fig. 1,



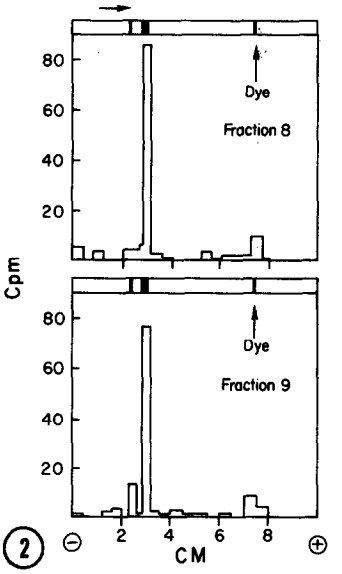


Fig. 1. Elution of Phosphofructokinase from ATP-Sepharose. To a 1.5 x 10 cm column was applied 2300 units of enzyme. Fractions of 2 ml were collected. Activity and acid-precipitable radioactivity were determined as described in the Experimental Procedure.

Fig. 2. SDS-Gel Electrophoresis of Phosphofructokinase. Electrophoresis was carried out 100 volts for 180 min with 24 and 22 µg of protein from fractions 8 and 9, respectively, from the elution described in Fig. 1.

The acid precipitable radioactivity was determined by the method of Bollum (15) and was found to be approximately 70 cpm/unit of phosphofructokinase in the peak tubes. In seven different experiments in which 3 to 9 mCi were injected, specific activity ratios varied from 25 to 90 cpm/enzyme unit. One mouse was injected 72 hrs prior to sacrifice with no significant difference noted in the specific activity ratio from the 18-hr treatment. In a second experiment an animal was anesthetized with Nembutal, then injected with 0.5 mg/g body weight epinephrine bitartrate 25 min prior to sacrifice. The specific radioactivity ratio obtained with this animal also fell within the above mentioned range.

The specific enzyme activity of the first several tubes from the affinity column (Fig.1) indicated a nearly homogeneous enzyme. That a trace contami-

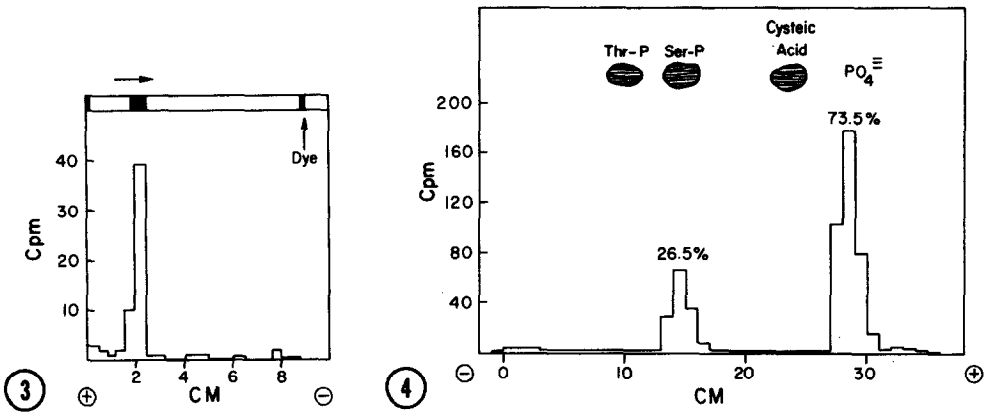


Fig. 3. Acid-Urea Gel Electrophoresis of Phosphofructokinase. Fourteen µg of protein of a pool of fractions 8 and 9 of Fig. 1 was applied. Electrophoresis was performed at 100 volts.

Fig. 4. Separation of Serine-P from Hydrolysate of [^{32}P]-Phosphofructokinase. Paper electrophoresis of acid hydrolyzed, carboxymethylated enzyme was carried out at 2750 volts for 120 min. Internal standards of threonine-P, serine-P, and cysteic acid were employed. Amino acids were detected with Cd-ninhydrin spray.

nant was present is indicated from the SDS-gel electrophoresis results in Fig. 2. The contaminant, which was slightly larger than phosphofructokinase, was also phosphorylated. The relative amount of the larger component increased in later fractions as did its [^{32}P] content (compare Fraction 9 with Fraction 8 in Fig. 2). For all subsequent studies only those fractions, usually the first two, with very high specific enzyme activity were used. The results from SDS-gels could be misleading when one considers that radiolabeled nucleotides and polynucleotides could co-migrate with the enzyme. To eliminate this possibility, the enzyme was subjected to a low pH electrophoresis in acetic acid, under which conditions nucleotides and proteins will migrate in opposite directions. These results are given in Fig. 3. More than 70% of the applied radioactivity was recovered in the single protein band.

We have observed that the high molecular weight contaminant observed on SDS-gel electrophoresis was removed when the enzyme was crystallized. To de-

termine whether the radioactivity co-crystallized with phosphofructokinase, unlabeled, twice-crystallized phosphofructokinase, 900 units, was mixed with 300 units of phosphorylated enzyme. After two days under conditions to promote crystallization (see Experimental Procedure), crystals were collected, dissolved, and assayed for radioactivity and enzyme activity. Of the initial enzyme, 51% of the enzyme activity and 50% of the radioactivity was recovered in the crystalline fraction.

To determine the extent of enzyme phosphorylation, it was necessary to measure the specific activity of the γ-position of ATP isolated from the muscle from which enzyme was prepared. The results from two different experiments are given in Table 1. On the basis of a protomer molecular weight of 80,000, only about one-eight of the protomers were phosphorylated. This value is considerably lower than that reported by Hofer and Furst (3) and is much closer to that of Hussey *et al.* (4) who analyzed phosphate content of rabbit phosphofructokinase.

Demonstration of Serine Phosphate. Prior to analysis for bound phosphate, care was taken to completely remove non-covalently bound ligands by carboxymethylation (16) followed by passage through Sephadex G 25 equilibrated with 6 M guanidine-HCl. The protein was subsequently dialyzed overnight against 1N acetic acid and then lyophilized. After acid hydrolysis, products were separated by high voltage paper electrophoresis according to the procedure of Bitte (17), using as internal standards serine phosphate, threonine phosphate, and cysteic acid. The results, given in Fig. 4, show serine phosphate was the only organic phosphate compound present.

Search for Phosphate in Pyruvate Kinase and Aldolase. The occurrence of a low level of phosphate in phosphofructokinase suggested that perhaps other muscle enzymes may contain low levels of serine phosphate. Recently, Lee *et al.* (18) have used ATP-Sepharose at pH 6.0 for purifying a variety of glycolytic enzymes, a procedure we adopted for the rapid isolation of aldolase and pyruvate kinase from a [^{32}P]-injected mouse. The initial effluent from an ATP-Sepharose column, free of phosphofructokinase, was

TABLE 1: CALCULATION OF PHOSPHATE CONTENT OF PHOSPHOFRUCTOKINASE (PFK)[a]

	Radioactivity in PFK cpm/unit	Specific activity of γ-P of ATP cpm/nmole	nmole P/Enzyme unit	Mole P/80,000 g PFK
A	25	2600	0.0096	0.12
B	70	6000	0.0117	0.15

[a] In A, 4.7 mCi, and in B, 7.2 mCi were injected 18 hrs prior to sacrifice. Radioactivity in PFK and in γ-position of ATP were determined as described in the Text. Calculation is based on a specific enzyme activity of 160.

adjusted first to 0.65 saturation of ammonium sulfate to precipitate aldolase and then to 0.95 saturation to precipitate pyruvate kinase. Each precipitate was dissolved in 10 mM sodium phosphate buffer at pH 6.0 and reapplied to separate ATP-Sepharose columns. The enzymes were eluted with linear gradients of ATP from 0 to 3 mM. Each enzyme was analyzed by SDS-gel electrophoresis and in the case of pyruvate kinase, a non-denaturing gel was used with an activity stain. In neither case was detectable radioactivity seen. Based on the sensitivity of the procedures, the phosphate content for either enzyme could not be higher than 0.006 moles per mole of protomer.

Concluding Remarks. The foregoing data support the idea that phosphofructokinase is only partially phosphorylated in mouse skeletal muscle. In the rabbit, the report by Hussey *et al.* (4) indicated that enzyme with varying degrees of phosphorylation could be partially resolved on chromatography and that no activity differences between fractions could be seen. We have also partially resolved mouse enzyme with differing degrees of phosphorylation upon DEAE-Sephadex chromatography and have yet found no differences in activity. The significance of phosphorylation of muscle phosphofructokinase remains to be established.

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