ISOLATION OF A PHOSPHORYLATED FORM OF PHOSPHOFRUCTOKINASE FROM SKELETAL MUSCLE

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1. Introduction

Phosphofructokinase plays an important role in the regulation of glycolysis. The outstanding characteristics of the control of phosphofructokinase are its cooperative saturation with substrate and the allosteric influences on phosphofructokinase activity of a variety of metabolites. In addition, an energydependent interconversion mechanism was indicated by the activation of yeast phosphofructokinase during the incubation of the enzyme in the presence of ATP and yeast extracts [1,2]. This hypothesis was supported by a recent publication of Brand and Söling [3] who demonstrated the precipitation of ^{32}P-containing phosphofructokinase by antibodies after incubation of fractionated rat liver extracts with [γ-^{32}P]ATP.

This study, which was performed independently, demonstrates that phosphofructokinase can in fact be isolated in a phosphorylated form from the skeletal muscle of mice.

2. Materials and methods

[^{32}P]Orthophosphate was obtained from Amersham Buchler (specific radioactivity 50–140 Ci/mg P). O-Phospho-L-serine and ribonuclease A and B were supplied by Sigma Chemical Co.. The other chemicals and biochemicals were from Boehringer Mannheim or Merck Darmstadt. Crystalline phosphofructokinase from rabbit muscle and γ-globulin fractions of antisera were prepared as described previously [4,5].

2.1. *SDS* electrophoresis on polyacrylamide gels*

Protein samples were precipitated by 10% TCA and thoroughly washed. After solubilization of the precipitates in 10% SDS (containing 5% mercaptoethanol) the samples were heated in a boiling water bath for 2 min. The electrophoreses were performed with 7.5% acrylamide gels using a buffer of 0.1 M Na-phosphate and 0.1% SDS (pH 7.3) (5 mA/tube). Bromophenol blue was used as reference. The electrophoreses were stopped when the reference had left the gels.

For autoradiographies, the gels were sliced in a longitudinal direction, dried on nylon gauze and exposed to Kodirex X-ray film for 4 weeks.

3. Results

3.1. *Precipitation of ^{32}P-containing protein by antibodies against muscle phosphofructokinase*

Antibodies against crystalline phosphofructokinase from rabbit muscle [5] show strong cross-reaction against phosphofructokinase from rat and mice muscle. About 90% of the rat and mice enzyme can be precipitated from crude extracts.

A considerable amount of radioactivity (300–500 cpm per unit of phosphofructokinase activity) could be precipitated from crude muscle extracts by antibodies when the animals were previously injected with 5 mCi of ^{32}P-orthophosphate. About one half of the radioactivity was insoluble in TCA or 1 M perchloric

Abreviations: SDS: sodium dodecyl sulphate. TCA: trichloroacetic acid.

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Table 1
Isolation of ^{32}P labelled phosphofructokinase from mice muscle

	Volume (ml)	Activity (units)	Protein (mg)	Spec. activity (units/mg)	Radioactivity (cpm)
Crude extract	110	696	773	0.9	$2.07 \cdot 10^9$
1st $(NH_4)_2SO_4$ prec.	15	487	47	10.4	$4.4 \cdot 10^7$
Heating 64°C	14	476	7.8	61	$4.0 \cdot 10^7$
2nd $(NH_4)_2SO_4$ prec.	7	424	4.7	90	$1.3 \cdot 10^6$
5th $(NH_4)_2SO_4$ prec.	7	380	4.3	88	$2.4 \cdot 10^5$
Chromatography + $(NH_4)_2SO_4$ prec.	5	171	1.1	155	$5.0 \cdot 10^4$

acid and migrated in the phosphofructokinase band on SDS electrophoresis. This band had the same R_f value as phosphofructokinase from rabbit muscle or purified phosphofructokinase from mice muscle. Control globulins were ineffective in precipitating radioactive material.

3.2. *Purification of ^{32}P-labelled phosphofructokinase*

In principle, the purification of phosphofructokinase from mice muscle followed the procedure described for the enzyme from rabbit muscle [4]. The purification procedure is summarized in table 1. The specific radioactivity of the intracellular inorganic phosphate was determined in an extract from heart muscle that had been prepared in the same way as the extract from skeletal muscle but the extraction buffer was replaced by water. The specific radioactivity of 1.6 Ci/mole P_i may be assumed as an upper limit of the specific radioactivity of phosphate bound to organic molecules.

The radioactivity found in the extracts from muscles corresponded to about 4% of the total radioactivity injected into the animals. As phosphofructokinase contained only a minor fraction of the total radioactivity, the purification of the enzyme was not paralleled by an increase in specific radioactivity. In the anion exchange chromatography, however, the appearance of phosphofructokinase in the eluate was indicated by a significant radioactive peak (fig.1). The main portion of ^{32}P was found to bind very strongly to DEAE Sephadex and was eluted in the nucleic acid fraction.

3.3. *SDS electrophoresis of purified ^{32}P-labelled phosphofructokinase*

Electrophoresis of the purified ^{32}P-labelled phosphofructokinase on polyacrylamide gels in the presence of 0.1 M phosphate and 0.1% SDS yielded a single band (fig.2A). The R_f value of this band was exactly the same as that of crystalline rabbit muscle

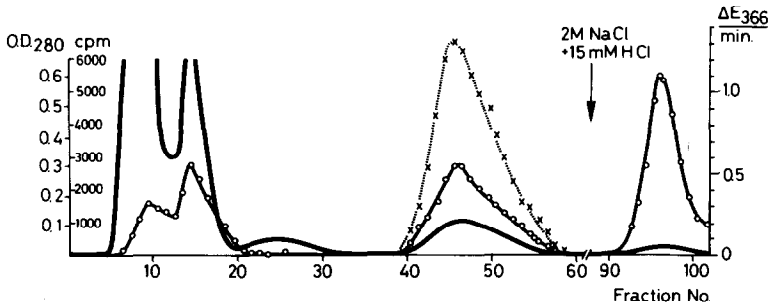

Fig.1. Preparative chromatography of phosphofructokinase from mice muscle on DEAE Sephadex A 50. (▬▬) Absorbance at 280 nm; (———) ^{32}P-radioactivity; (– – –) phosphofructokinase activity.

phosphofructokinase. Staining of the gels for nucleic acids with pyronine Y was negative.

Gels electrophoresed under the same conditions were also analyzed by autoradiography and counting in a liquid scintillation counter. The results are shown in fig.2B and 2C. The autoradiogram shows a single spot of radioactivity exactly at the position where phosphofructokinase was stained on the gel. This position was also identical with the peak of radioactivity found in the sliced gels by liquid scintillation counting.

As a control, phosphofructokinase from rabbit muscle, aldolase, and pyruvate kinase were incubated with a 1000-fold excess of ^{32}P radioactivity over the radioactivity applied to the gels in the experiments with mouse muscle phosphofructokinase. The control samples were treated in the same way as described above. No radioactivity could be detected after SDS electrophoresis by autoradiography or liquid scintillation counting.

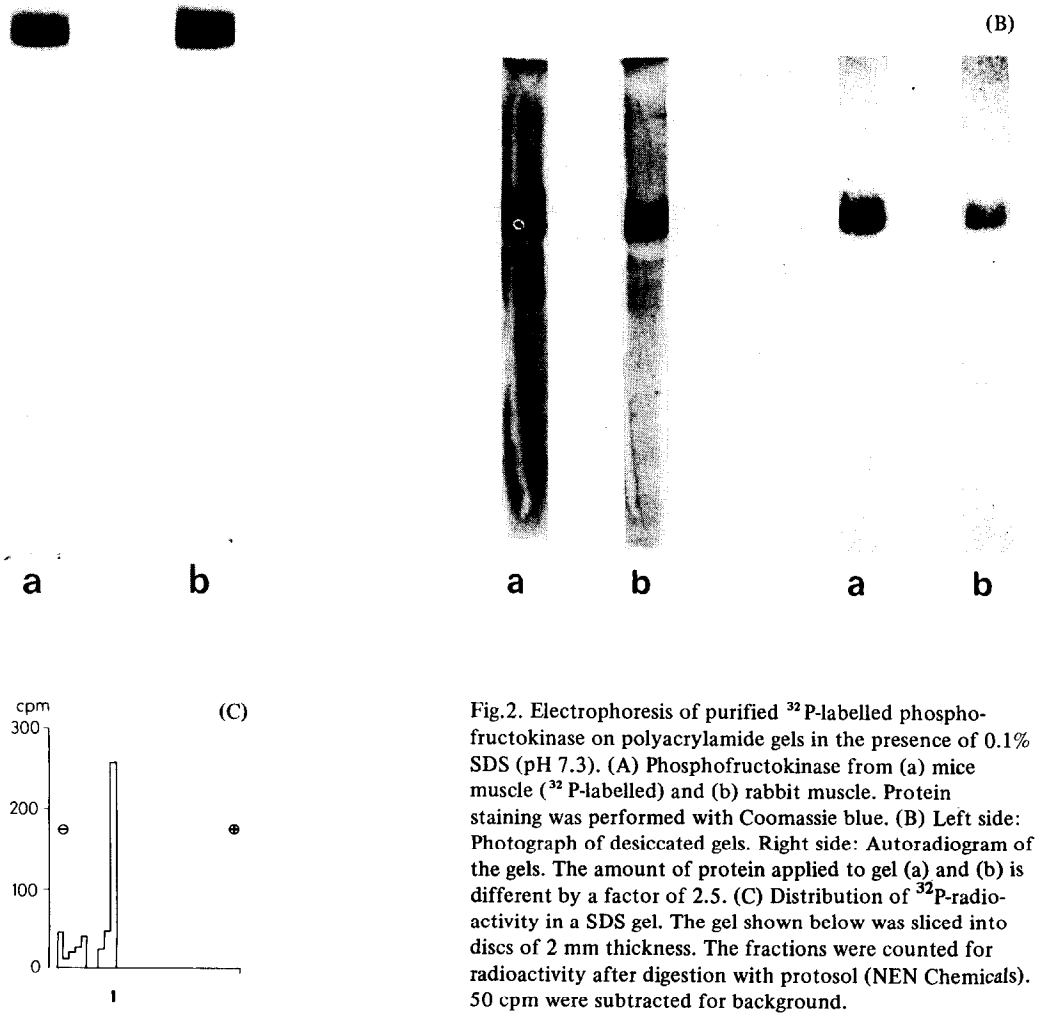

Fig.2. Electrophoresis of purified ^{32}P-labelled phosphofructokinase on polyacrylamide gels in the presence of 0.1% SDS (pH 7.3). (A) Phosphofructokinase from (a) mice muscle (^{32}P-labelled) and (b) rabbit muscle. Protein staining was performed with Coomassie blue. (B) Left side: Photograph of desiccated gels. Right side: Autoradiogram of the gels. The amount of protein applied to gel (a) and (b) is different by a factor of 2.5. (C) Distribution of ^{32}P-radioactivity in a SDS gel. The gel shown below was sliced into discs of 2 mm thickness. The fractions were counted for radioactivity after digestion with protosol (NEN Chemicals). 50 cpm were subtracted for background.

Table 2
Incubation of ^{32}P labelled phosphofructokinase with substrates or hydrolytic enzymes

Incubation	A	B	C	D
cpm supernatant	50	851	75	102
cpm pellet (protein bound)	860	59	890	790
% cpm in the supernatant	5.5	93.5	7.8	11.4

A: Incubation in the presence of 0.1 M K-phosphate (pH 7.6), 3 mM fructose 6-phosphate, 1 mM ATP and 10 mM $MgCl_2$ at 25°C for 15 min.
B: Incubation with 1 mg trypsin (ammonium carbonate buffer, pH 8.5)
C: Incubation with acid phosphatase (triethanolamine-HCl, pH 6.5)
D: Incubation with 1.0 mg ribonuclease A and 1.0 mg ribonuclease B (triethanolamine-HCl, pH 7.6)
Incubations B–D were performed at 37°C for 6 h. All incubations were stopped by 10% TCA.

3.4. *Effect of hydrolytic enzymes on ^{32}P-labelled phosphofructokinase*

More than 90% of the radioactivity of purified phosphofructokinase preparations could be precipitated by antibodies. Incubation of the enzyme in the presence of fructose 6-phosphate and ATP did not change the amount of radioactivity which was precipitable by antibodies or TCA. Obviously, an exchange of the ^{32}P on the protein with unlabelled phosphate did not take place during the catalytic reaction.

Incubation of labelled phosphofructokinase in the presence of ribonuclease A and B or in the presence of acid phosphatase had no effect on the amount of radioactivity which was precipitated by TCA. Treatment with trypsin, however, led to acid soluble products (c.f. table 2).

3.5. *Identification of the site of phosphate on phosphofructokinase*

Phosphofructokinase labelled with ^{32}P was hydrolyzed in the presence of 2 N HCl and the products were analyzed by high voltage electrophoresis at pH 2.3. The radioactive products and authentic O-phospho-L-serine migrated to the anode under these conditions. The radioactive material was separated

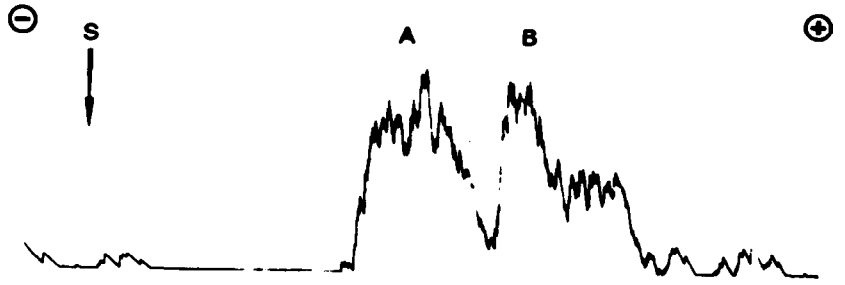

Fig.3. Electrophoresis of an acid hydrolysate of ^{32}P-labelled phosphofructokinase. The enzyme was precipitated with 10% TCA and hydrolyzed in 2 N HCl for 12 h at 120°C. The electrophoresis was performed on Whatman MM paper using 2 N acetic acid as buffer (2600 V, 20 mA, 90 min). Reference samples of commercial phosphoserine were run in parallel and stained with ninhydrin. The curve demonstrates the distribution of radioactivity after electrophoresis as evaluated with a chromatography scanner (Dr Berthold Laboratories, Wildbad, Germany). The arrow indicates the starting point. Peak A corresponds to the ninhydrin-positive spot of phosphoserine. Peak B corresponds to the position of inorganic phosphate.

into two fractions. The slower migrating moiety had the same electrophoretic velocity as phosphoserine (Peak A of fig.3). The faster migrating peak corresponded to the position of inorganic phosphate.

4. Discussion

As a maximum of one phosphate group per protomer of phosphofructokinase may be expected in a phosphorylated form of the enzyme and as the phosphate injected into the animals is diluted by the phosphate pool of the body, high initial radioactivities are necessary for detectability of phosphate bound to the enzyme. The specific radioactivity of phosphate in the cell had to be at least 0.5 Ci/mole phosphate. In our experience, this condition was met when about 0.2 mCi per g body weight was injected into an animal.

Assuming the upper limit of specific radioactivity of phosphate in phosphofructokinase to be the same as in inorganic phosphate, we found about 0.9 mole phosphate per 100 000 g of enzyme; this corresponds to about 80–90% phosphorylation of the purified enzyme. High initial levels of phosphorylation of the enzyme may explain difficulties of finding further phosphorylation of the enzyme by [γ-^{32}P]ATP in tissue extracts.

A single band of radioactivity has been detected in SDS electrophoreses of phosphofructokinase isolated using two different methods (1) precipitation of the enzyme from crude extracts by antibodies, (2) purification to electrophoretic homogeneity. In both cases, the radioactive bands were located at the position of phosphofructokinase protein. Any phosphorylated impurity other than phosphofructokinase would have had to show cross-reaction with antibodies against crystalline rabbit muscle phosphofructokinase as well as an identical behaviour to phosphofructokinase on ion exchange chromatography and SDS electrophoresis. An alternative possibility was the formation of a complex of an impurity with phosphofructokinase. But it is unlikely that such a complex would resist treatment with 10% SDS at 100°C. We could show that the binding of $^{32}P_i$ was destroyed by such treatment with SDS. Nucleic acids were not detectable on gels and moreover, complexes between proteins and nucleic acids are split by SDS.

Comparison of the electrophoretic mobility of the products of acid hydrolysis with authentic substances suggested the presence of an O-phospho-L-serine residue in the protein. This serine residue is not an intermediate in the catalytic reaction of the enzyme, as was shown by the lack of phosphate exchange in the presence of both substrates. The role of the phosphate residue on phosphofructokinase may therefore be in an interconversion mechanism. In fact, Brand and Söling [3] have provided evidence of such a mechanism in liver. The present study suggests that a similar regulation mechanism exists also for the muscle enzyme.

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