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PHOSPHORYLATION OF PHOSPHOFRUCTOKINASE FROM SKELETAL MUSCLE : CORRELATIONS BETWEEN PHOSPHORYLATION AND MUSCLE FUNCTION

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Summary

The specific radioactivity of [^{32}P]-phosphate incorporated into muscle phosphofructokinase was in equilibrium with the specific radioactivity of the γ-phosphate group of ATP. The incorporation was independent of the presence of cycloheximide. The total content of covalently bound phosphate in phosphofructokinase was correlated with the functional state of the muscle from which the enzyme was purified. Muscle dissected *post mortem* led to phosphofructokinase containing less than 2 phosphate groups per tetramer. Muscle dissected *in vivo* gave phosphofructokinase with 4 phosphates per tetramer when kept at rest and 8 phosphates per tetramer when stimulated to contract.

INTRODUCTION

Phosphofructokinase has been purified from skeletal muscle as a phosphoprotein, containing between 1 and 3 phosphoserine residues per enzyme tetramer (1 - 3). Since changes in the catalytic or regulatory properties of the enzyme as result of the phosphorylation *in vitro* have not been observed until now, the physiological function of its phosphorylation has remained unclear. This study correlates the phosphorylation of phosphofructokinase with metabolic events, such as protein synthesis, and with the functional states of the muscle, rest and contraction. It is shown that the number of phosphorylated sites is increased during contraction and that the phosphorylation grade of the enzyme is distinctly higher if appropriate conditions for tissue preparation are used.

MATERIALS AND METHODS

Phosphofructokinase from rabbit skeletal muscle (4) and phosphofructokinase from mice muscle after *in vivo* labeling with [^{32}P]-orthophosphate was purified as described previously (1).
Protein concentrations were determined by the absorbance at 280 nm using an extinction coefficient of 1.08 for 1 mg phosphofructokinase per ml. For the determination of protein bound phosphate and radioactivity, the enzyme was precipitated with 10 % trichloroacetic acid, and after thorough washing with H_2O the precipitates were dissolved in formic acid. Phosphate was determined according to Ames (5).

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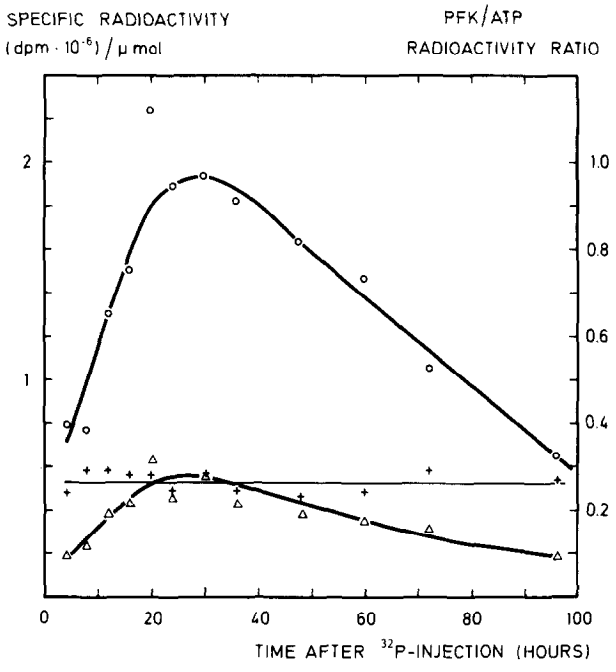

Fig. 1

Time course of the specific radioactivities (dpm/μmol) of the γ-phosphate of ATP (o —— o) and of phosphofructokinase (Δ —— Δ) from mice muscle after the injection of 4 mCi [^{32}P]-orthophosphate at zero time. The right hand scale refers to the ratio of specific radioactivities of ATP and phosphofructokinase (+ —— +).

The specific radioactivity of the γ-phosphate group of ATP was performed in the following steps: (1) Separation of ATP from other nucleotides and metabolites by chromatography on Dowex 1 X 8, (2) quantitative transfer of the phosphate group to glucose in the presence of hexokinase, and (3) separation of glucose 6-phosphate on Dowex 1 X 8. The chromatographic procedure and the principle of the specific radioactivity determination has been described previously (6).

RESULTS AND DISCUSSION

1. The synthesis of phosphofructokinase protein and phosphofructokinase phosphorylation.

The incorporation of [^{32}P]-orthophosphate which was intraperitoneally injected to mice, into the γ-phosphate group of ATP and into phosphofructokinase is shown as a function of time in Fig. 1. Despite the fact that the [^{32}P]-radioactivity in the serum reaches a maximum very rapidly, the incorporation of the phosphate into muscle is slow and leads to an optimum specific radioactivity of ATP and phosphofructokinase 20 to 25 hours after injection.

The specific radioactivities of phosphofructokinase and ATP were in almost constant relation between 4 and 96 hours after the injection of [^{32}P]-

phosphate. Compared to the slow incorporation of phosphate into muscle, the equilibration of the specific radioactivities of ATP and phosphofructokinase is rapid. This might suggest that the phosphorylation of phosphofructokinase is involved in a short-time process such as metabolic interconversion rather than in an irreversible modification of the enzyme. Taking into account, however, that the half-life of phosphofructokinase is in the range of 8 - 17 hours (7), a connection between phosphofructokinase synthesis and the phosphorylation of the enzyme protein cannot be ruled out completely. Therefore the influence of inhibitors of protein synthesis on the phosphorylation of phosphofructokinase was studied.

The incorporation of [1-^{14}C]-leucine into phosphofructokinase can be inhibited more than 90 % by a single dose of puromycin (0.25 mg/g body weight) or of cycloheximide (0.15 mg/g body weight). Though these doses are highly toxic to the animals, the levels of phosphofructokinase activity and the ATP concentrations in mice muscle were unchanged.

Table 1 shows the specific radioactivities of phosphofructokinase obtained from mice muscle under control conditions and after treatment with 6 doses of 0.4 mg cycloheximide per animal in intervals of three hours. This condition guaranteed a constant level of the protein synthesis inhibitor during the whole period of the experiment and rules out influences of different absorption kinetics of amino acid and phosphate.

The incorporation of L-leucine into the enzyme is reduced to 19 % of the controls in the presence of cycloheximide, whereas the phosphate incorporation is lowered only by 11 %. The ratios of the specific radioactivities of phosphofructokinase and ATP remained almost constant. The experiments demonstrate that the phosphorylation of phosphofructokinase occurs independent of the synthesis of the enzyme protein and must therefore be a reversible process.

2. The number of phosphorylated sites in phosphofructokinase at rest and after muscle contraction.

A constant relation between the specific radioactivity of phosphofructokinase and ATP, as shown in Fig. 1, would allow for the calculation of the number of phosphorylated sites of the enzyme. Since differences in the specific radioactivities of ATP in various compartments of the cell and rapid changes under different functional states of the muscle cannot be ruled out completely, the determination of protein-bound phosphate by a colorimetric method was preferred in this study.

Table 1

Incorporation of [1-^{14}C]-leucine and [^{32}P]-orthophosphate into phosphofructokinase from mice skeletal muscle in the presence of cycloheximide (6 doses of cycloheximide in intervals of 3 hours).

A. Incorporation of [1-^{14}C]-L-leucine

Condition	[^{14}C]-Radioactivity in PFK (dpm/μmol)	
Control	220 080 ± 33 230	(n = 3)
Cycloheximide	41 520 ± 14 740	(n = 3)
Cycloheximide/Control	0.19	

B. Incorporation of [^{32}P]-orthophosphate

Condition	[^{32}P]-Radioactivity in PFK (dpm/μmol)	$\frac{SR_{PFK}}{SR_{ATP}}$ ::
Control	138 940 ± 28 390 (n = 6)	0.20 ± 0.04
Cycloheximide	123 060 ± 48 280 (n = 6)	0.19 ± 0.04
Cycloheximide/Control	0.89	

:: SR: Specific radioactivity

In order to correlate the phosphate content of phosphofructokinase to muscle function, the enzyme was purified from muscles which were obtained under the following conditions: (1) resting muscle which was taken from the hind-limb of a rabbit in deep anesthesia and quickly frozen in liquid nitrogen, (2) muscle which had rhythmically contracted for 10 min induced by stimulation of the nerve and which was obtained as before, and (3) muscle which has been taken after sacrifice and stored on ice.

After extraction of soluble protein from the tissue by phosphate buffer containing KF and EDTA and purification of the enzyme, the contents of covalently bound phosphate were as listed in Table 2. The muscle which has been dissected and stored without precautions contained relatively low amounts of phosphate with 1 or 3 groups per tetramer. This value is close to that calculated for mice muscle phosphofructokinase on the basis of the specific radioactivity of ATP.

Dissecting the muscle from an anesthesized animal and immediate freezing of the muscle resulted in a higher phosphate content of about 4 groups per phosphofructokinase tetramer. Stimulation of the muscle led to a

Table 2

Number of phosphorylated sites per phosphofructokinase tetramer (m.w. 360 000) from rabbit skeletal muscle.

Condition	nmol Phosphate/nmol PFK	n*
Resting muscle *(post mortem)*	1.93 ± 1.1	13
Resting muscle *(in vivo)*	4.78 ± 0.9	8
Contracting muscle *(in vivo)*	7.92 ± 0.4	4

* number of independent preparations from different muscles

further increase to about 8 groups per tetramer. Assuming equal distribution of the phosphate groups over the subunits, this result suggests that at least two different phosphorylation sites are present in the enzyme.

The rapid and reversible phosphorylation of muscle phosphofructokinase which is correlated to the changes in the functional states of muscle suggests that phosphorylation plays a part in the regulation of the enzyme. Recent work (2, 3) has shown that the aggregated forms of phosphofructokinase have a higher phosphate content. As the aggregated phosphofructokinase is more active (8), this might provide a mechanism by which the control of phosphofructokinase by phosphorylation and dephosphorylation can occur.

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