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IDENTIFICATION OF THE PHOSPHORYLATED SITES OF PHOSPHOFRUCTOKINASE FROM SKELETAL MUSCLE AFTER IN VIVO AND IN VITRO PHOSPHORYLATION

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Summary

Phosphofructokinase from mice muscle was radioactively labelled either *in vivo* by the injection of [^{32}P]-phosphate or *in vitro* by the incubation with cAMP-dependent protein kinase and [γ-^{32}P]-ATP. Two labelled peptides were obtained after tryptic digestion in either case showing that at least two sites were phosphorylated. Independent of the labelling method, the labelled peptides showed an analogous pattern on the peptide maps, indicating that both methods led to the phosphorylation of the same sites.

INTRODUCTION

Phosphofructokinase is phosphorylated *in vivo* (1) and by fractions of tissue extracts *in vitro* (2). Phosphorylation of the enzyme from kidney and muscle by cAMP-dependent protein kinase was also demonstrated (3, 4). On the other hand, no increase of the phosphate content of mice muscle phosphofructokinase has been found when the cAMP level in living mice was elevated by treatment with isoproterenol (5), though the interconversion of phosphorylase b to a was substantiated. This finding and the well-documented unspecificity of cAMP-dependent protein kinase for its protein substrate opened the questions whether this enzyme acts on the same sites of phosphofructokinase as those phosphorylated in the living tissue and whether this protein kinase is also responsible for the phosphorylation of phosphofructokinase *in vivo*.

This study describes the identification of the phosphorylated sites of phosphofructokinase which was purified from mice muscle after the incorporation of [^{32}P] *in vivo* and of phosphofructokinase which has been phosphorylated by cAMP-dependent protein kinase *in vitro*.

MATERIALS AND METHODS

Phosphofructokinase from mice muscle, labelled with [^{32}P] *in vivo*, was purified as described previously (1, 5). Cyclic-AMP-dependent proteinkinase from rabbit muscle and its catalytic subunit were isolated by the method of Beavo et al. (6). The protein kinase was assayed according to Corbin and Reimann (7).

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In vitro phosphorylation of phosphofructokinase was performed at pH 7.0 in the presence of [γ-^{32}P]-ATP (0.5 mM), $MgCl_2$ (10 mM), protein kinase (10 000 units/ml) and phosphofructokinase (4 mg/ml). From the incubation mixture phosphofructokinase was isolated by ammonium sulphate precipitations, heat treatment and DEAE-Sephadex chromatography as described before (5).

CNBr-Cleavage. 2 mg phosphofructokinase were dissolved in 220 µl sodium dodecylsulphate (4 % in 0.1 M NH_4HCO_3). The solution was heated to 95 °C for 5 min and, after cooling, 350 µl formic acid (95 %) and 0.5 mg CNBr were added. The mixture was stirred in an N_2 atmosphere at 20 °C in the dark for 48 hours with a further addition of 0.5 mg CNBr after 24 hours. Then the reaction mixture was dried, redissolved in 85 % formic acid, and chromatographed on Biogel P 30 (0.85 x 53 cm column; 85 % formic acid as eluant).

Tryptic Digestion. The phosphate containing fractions of the Biogel P 30 chromatography were freeze-dried, redissolved in 1.5 ml NH_4HCO_3 (0.1 M, pH 8.2) and incubated for 24 hours at 37 °C in the presence of 1 µg trypsin. An additional 1 µg trypsin was added to the incubation medium after the first 12 hours.

Peptide Mapping. Mapping of peptides was performed on cellulose thin layer plates (Merck, Darmstadt). In the first dimension, the plates were chromatographed in Butanol-1 - acetic acid - pyridin - H_2O (75 - 15 - 50 - 60 by volumes) followed by an electrophoresis in the second dimension (1 hour, 600 V) in a buffer containing 10 % pyridin and 1 % acetic acid (pH 6.1) (8).

RESULTS AND DISCUSSION

1. Phosphorylation of muscle phosphofructokinase by cAMP-dependent protein kinase.

Phosphofructokinase isolated from mice or rabbit skeletal muscle contains 1 to 3 phosphate groups per tetramer bound to serine residues, if the muscle has been obtained from the animals after sacrifice (1, 5, 9). These enzyme preparations incorporate [^{32}P]-phosphate from [γ-^{32}P]-ATP in the presence of peak I or peak II protein kinase prepared from rabbit skeletal muscle by the method of Beavo et al. (6). The reaction is cAMP-dependent and is not activated by Ca^{2+}. It becomes independent on cAMP if the catalytic subunits of the protein kinases are used for incubation instead of the holoenzyme.

The preparations of the catalytic subunits of protein kinases from peak I differed significantly from peak II in their relative ability to phosphorylate phosphofructokinase compared to histone phosphorylation. The catalytic subunit of peak I protein kinase phosphorylated phosphofructokinase with 2 % of its histone-phosphorylating activity, whereas it was only 0.9 % in the case of peak II catalytic subunit. The maximum amount of radioactive phosphate incorporated in that way corresponded to about one phosphate group per tetramer. It may be noted, however, that the reaction proceeded linearly with time only for about 1/5 of that incorporation and then slowed down.

We found no difference in the rate of incorporation of radioactive phosphate into phosphofructokinase from rabbit or mice muscle by the protein kinase from rabbit muscle. This is in correspondence to immunological studies which have shown great similarities of antigenic properties, thus suggesting wide structural homologies in these phosphofructokinases (10).

Considering the broad substrate specificity of cAMP-dependent protein kinase, the phosphorylation of purified phosphofructokinase by that enzyme *in vitro* does not necessarily imply that this proteinkinase is responsible for the phosphorylation of phosphofructokinase *in vivo* nor that identical amino acid residues are phosphorylated *in vivo* or *in vitro*.

2. Isolation of phosphorylated peptides of phosphofructokinase after *in vivo* and *in vitro* labeling.

In order to investigate the nature of the phosphorylated sites of phosphofructokinase, the radioactive peptides were isolated after the labeling of the enzyme with [^{32}P]-phosphate *in vivo* and after incubation of the purified enzyme with protein kinase in the presence of [γ-^{32}P]-ATP *in vitro*. The results are shown for mice muscle phosphofructokinase, yet, it should be noted that identical results were obtained with phosphofructokinase from rabbit muscle which has been labelled *in vitro* with protein kinase.

In the first step of analysis, the phosphofructokinase preparations labelled by different methods were split into peptides by CNBr treatment. The radioactive phosphate was in both cases eluted with 90 % recovery within single peaks from a Biogel P 30 column in the same region of large peptides. The fractions containing the [^{32}P]labelled peptides were then digested with trypsin and rechromatographed on Sephadex G-25 (0.6 x 50 cm column). The elution profiles of that chromatography are shown in Fig. 1. The phosphate-containing peptides are eluted as single symmetrical peaks. No difference could be detected between the molecular weights of the[^{32}P]-peptides with the two labeling modes. The chromatography fractions containing the radioactive peptides were collected, freeze-dried and subject to peptide mapping on thin-layer plates. The positions of the radioactive peptides were visualized by autoradiography as shown in Fig. 2 a and 2 b. Two radioactive peptide spots could be detected on each of the plates. They were distinctly separated in the chromatography and migrated in opposite directions in the subsequent electrophoreses which were performed at pH 6.1. The chromatographic and electrophoretic mobility of the two peptides was essentially the same for the peptides which were labelled *in vivo* or by protein kinase *in vitro*.

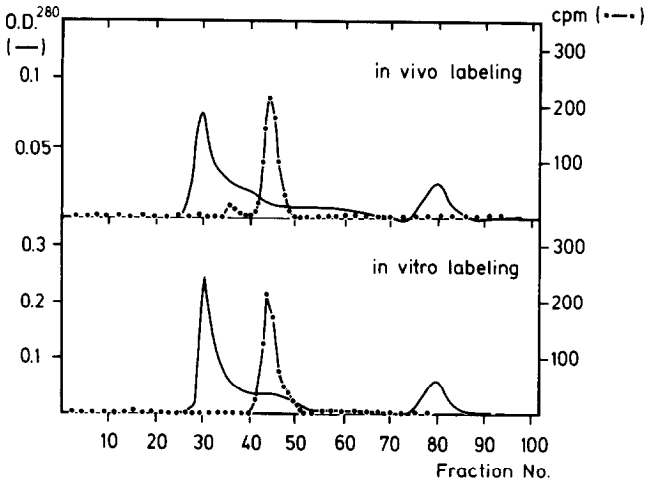

Fig. 1

Chromatography of tryptic peptides of the [^{32}P] containing CNBr fractions of phosphofructokinase labelled *in vivo* (upper) or in the presence of catalytic subunits of cAMP-dependent proteinkinase *in vitro* (lower) on Sephadex G-25 (column 0.6 x 50 cm; 0.1 M NH_4HCO_3).

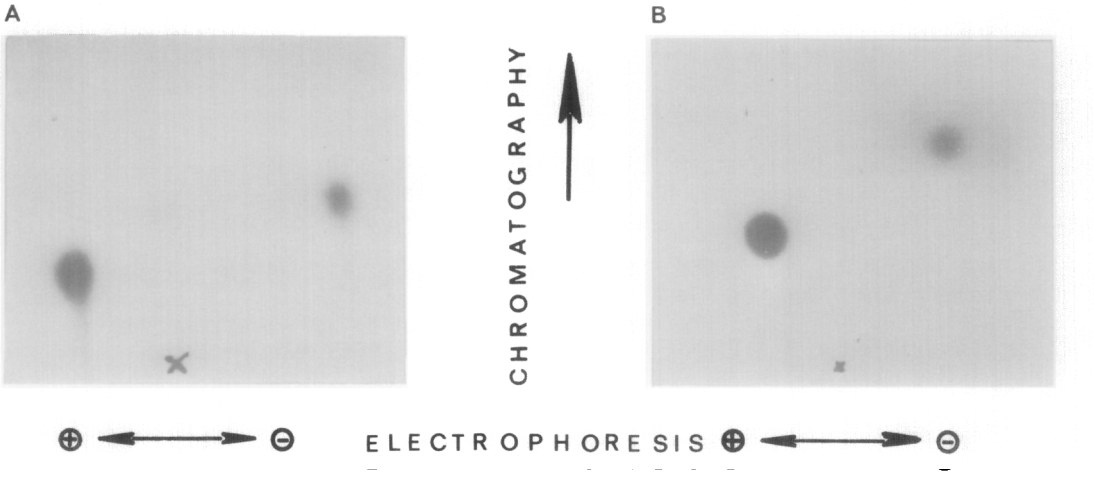

Fig. 2

Autoradiographies of the peptide maps of the [^{32}P] containing chromatography fractions from the Sephadex G-25 column. Fig. 2 a represents the peptides from the phosphofructokinase labelled *in vivo* and Fig. 2 b the peptides of phosphofructokinase which was treated with the catalytic subunit of cAMP-dependent protein kinase.

These experiments demonstrate that cAMP-dependent protein kinase phosphorylates the same sites of phosphofructokinase as those phosphorylated *in vivo*. The relatively high phosphorylating activity of the protein kinase with phosphofructokinase as substrate, as determined *in vitro*, also suggests that

the cAMP-dependent protein kinase is responsible for the phosphorylation of phosphofructokinase *in vivo*.

Moreover, two different phosphorylated peptides from phosphofructokinase have been revealed by peptide mapping. Since both peptides were eluted within a single symmetrical peak from Sephadex G-25 they must have very similar molecular weights, and the probability of incomplete cleavage by CNBr or trypsin as the cause of the diversity is greatly reduced. On a chemical basis, the heterogeneity of the phosphate sites can be shown only by the amino acid sequence of the peptides. On the other hand, it has been demonstrated that phosphofructokinase isolated from contracting muscle contains 8 phosphogroups per tetramer of enzyme (9). This finding obtained from a functional analysis of phosphofructokinase phosphorylation supports the view that the phosphorylated peptides, as visualized by two-dimensional autoradiography represent the two phosphorylation sites of skeletal muscle phosphofructokinase.

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