Metabolite-controlled phosphorylation of hepatic phosphofructokinase proceeds by cAMP-dependent protein kinase

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In hepatocytes ^{32}P-incorporation into rat liver phosphofructokinase is stimulated by glucose as well as by glucagon, the effects of both stimuli being prevented by L-alanine [Eur. J. Biochem. (1982) 122, 175]. The phosphopeptides of the enzyme derived from limited proteolysis by subtilisin and from exhaustive tryptic digestion were analyzed either by one-dimensional mapping on sodium dodecyl sulphate–polyacrylamide slab gels and by fingerprint mapping, respectively. It is shown that in vivo stimulation of ^{32}P-incorporation by glucose or by glucose plus glucagon results in identical phosphopeptide maps, and that these maps were identical with those obtained from phosphofructokinase phosphorylated in vitro with catalytic subunit of cAMP-dependent protein kinase. It is concluded that in the intact liver cell phosphofructokinase is phosphorylated by cAMP-dependent protein kinase but that the state of phosphorylation is modified by metabolite control.

Phosphofructokinase *Phosphorylation* *Peptide mapping* *cAMP-dependent protein kinase*

1. INTRODUCTION

Rat liver phosphofructokinase (PFK) is phosphorylated in vivo and it has been shown that this phosphorylation is not only increased by glucagon [1] but also by glucose [2,3]. Moreover, phosphorylation of PFK induced by glucagon seems to be dependent on the presence of glucose or glycogen and can be suppressed by the gluconeogenic substrate L-alanine [2]. Under the same conditions the phosphorylation of pyruvate kinase was exclusively dependent on the hormone and not affected by exogenous metabolic substrates.

On the other hand, liver and muscle PFK can be phosphorylated in vitro by incubation with cAMP-dependent protein kinase and ATP, and PFK phosphorylated in this way has been used to compare the kinetic properties of the phosphorylated and the dephosphorylated form of the enzyme [4]. Therefore the question arose: Is the in vivo phosphorylation of PFK catalyzed by a cAMP-dependent protein kinase under control of metabolites or is it catalyzed by a cAMP-independent protein kinase which can be stimulated by glucose or one of its metabolites? One way to approach this problem is to analyze the phosphorylated site(s) of the in vitro phosphorylated PFK and to compare it with the pattern obtained from PFK which had been phosphorylated in the living cell in the presence of either glucose or glucagon or both.

Here, we show that the phosphorylation patterns obtained in vivo under different conditions are identical with that obtained in vitro after phosphorylation with catalytic subunit of the cAMP-dependent protein kinase.

Abbreviations: SDS, sodium dodecyl sulphate; TPCK, L-1-tosylamide-2-phenylethyl chloromethylketone; PFK, phosphofructokinase

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2. MATERIALS AND METHODS

The catalytic subunit of cAMP-dependent protein kinase was prepared from beef liver as in [5] but stored in 20 mM potassium phosphate (pH 6.8) containing 1 mM EDTA and 5 mM mercaptoethanol/glycerol (1/1, v/v) at −20°C. Trypsin (type III, 2 × crystallized) and L-1-tosylamide-2--phenylethyl chloromethylketone (TPCK) were obtained from Sigma, agarose 0.5 m from Bio-Rad, subtilisin, auxiliary enzymes and biochemicals from Boehringer (Mannheim). Thin-layer cellulose plates (MN Polygram Cel 300, 0.1 mm) were from Machery und Nagel (Aachen).

2.1. *^{32}P-Labelling of phosphofructokinase in vitro*

PFK was prepared from fresh rat liver according to [6] with an additional gel filtration on agarose 0.5 m, equilibrated with 20 mM potassium phosphate (pH 7.6), 0.5 mM $MgCl_2$, 5 mM mercaptoethanol. This procedure resulted in specific activities of ~2–4 U/mg. PFK was assayed under optimized conditions as in [7]. The partially purified PFK (0.4 unit) was incubated with 500 U of purified catalytic subunit of cAMP-dependent protein kinase in a medium of 20 mM triethanolamine buffer (pH 7.2) containing (final conc.): 0.15 mM [^{32}P]ATP (200 nCi/nmol), 3.5 mM $MgCl_2$, 5 mM mercaptoethanol. After 60 min of incubation at 25°C, 'cold' ATP (10 mM final conc.) was added and PFK was precipitated by the addition of 1/4 vol. of 25% trichloroacetic acid. The pelleted protein was subjected to SDS–polyacrylamide (7.5%) gel electrophoresis as in [8].

2.2. *^{32}P-Labelling of phosphofructokinase in vivo*

PFK was radioactively labeled in hepatocytes from fed rats and isolated as in [2]: In principle, hepatocytes (170 mg/ml) were incubated for 40 min with [^{32}P]orthophosphate (1 mCi/g wet wt) in the presence of 20 mM glucose. When glucagon was used, it was added after the first 30 min of incubation. Soluble proteins were extracted by the addition of a digitonin containing 'stopping medium' and [^{32}P]PFK was isolated from the supernatant by antibody precipitation followed by SDS–polyacrylamide slab gel electrophoresis.

2.3. *Limited proteolysis of phosphorylated PFK in SDS–polyacrylamide gels*

Gel pieces containing the [^{32}P]PFK band were subjected to limited proteolysis in SDS–polyacrylamide slab gels as in [9] with 1.5 μg subtilisin/slot. After electrophoresis, the gel was transferred for 15 min into 12.5% trichloroacetic acid, followed by washing in 10% acetic acid, 10% 2-propanol for 30 min with three changes of this solution. Thereafter the gels were dried immediately and used for autoradiography.

2.4. *Tryptic fingerprinting*

Gel pieces containing [^{32}P]PFK were subjected to tryptic digestion as in [10] with the following modifications: The lyophilised gel pieces were reswollen and incubated in 1 ml solution containing 2 μg trypsin and 2.5 μg TPCK in 50 mM ammonium bicarbonate, 5 mM mercaptoethanol. After 12 h the supernatants were removed and exchanged against 0.5 ml 50 mM ammonium bicarbonate, 5 mM mercaptoethanol and incubated for another 4 h. This procedure was repeated until $\geq$ 80% of the radioactivity was eluted. The combined supernatants were again incubated for 3.5 h after addition of trypsin (2 μg) and TPCK (2.5 μg). The samples were then lyophilised and the remaining ammonium bicarbonate was completely removed in a vacuum oven at 60°C for 24 h. The resulting peptides were separated on cellulose thin-layer chromatography sheets as in [11] with electrophoresis in 10% (v/v) acetic acid, 1% (v/v) pyridine (pH 3.5) for 70 min at 500 V in the first dimension and chromatography in *n*-butanol/pyridine/acetic acid/water, 37.5:25:7.5:30 (by vol.) in the second dimension. The dried plates were washed twice with acetone and autoradiographed with Kodak-X-omat R film.

3. RESULTS AND DISCUSSION

3.1. *Limited proteolysis of liver PFK phosphorylated in vitro and in vivo*

The patterns of the phosphopeptides obtained after limited proteolysis of rat liver phosphofructokinase after in vitro and in vivo labelling are shown in fig.1. Under all conditions some of the radioactivity corresponding to small peptides migrated with the dye front, whereas the main radioactivity was found in a digestion product with

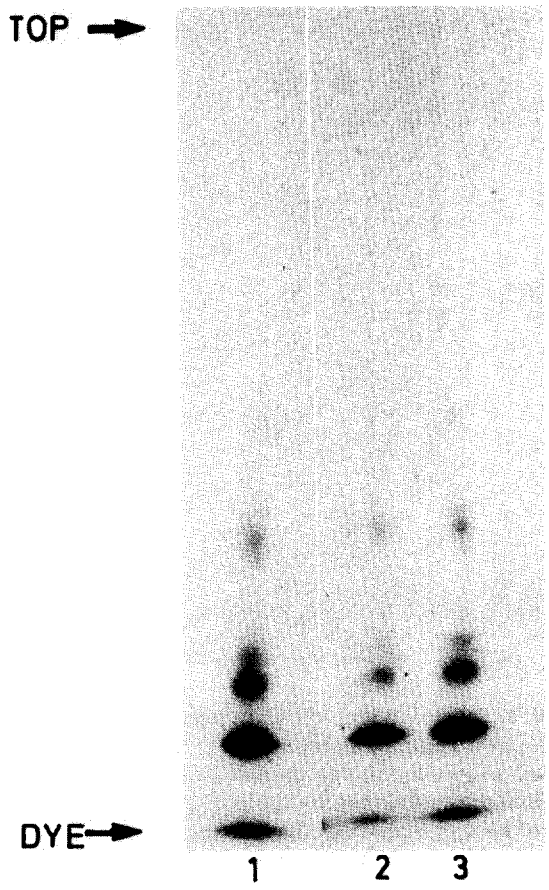

Fig.1. Autoradiograph showing the phosphopeptide pattern obtained upon digestion with subtilisin of [^{32}P]PFK. PFK was radioactively labeled in vitro with catalytic subunit of cAMP-dependent protein kinase and in hepatocytes incubated either in the presence of glucose alone or with glucose and glucagon. After separation on SDS–polyacrylamide gels, [^{32}P]PFK were cut out of the gel and subjected to limited proteolysis by subtilisin during electrophoresis on 15% SDS–polyacrylamide gels: (1) phosphopeptides of PFK phosphorylated in vitro; (2) phosphopeptides of PFK phosphorylated in hepatocytes in the presence of glucose alone; (3) phosphopeptides of PFK phosphorylated in hepatocytes in the presence of glucose and glucagon; in (2,3) 1 unit of in vivo labeled PFK was subjected to digestion.

app. M_r 7000–10000. The rest of the radioactivity was detected in 3–4 larger polypeptides. Trace 1 depicts the pattern of in vitro phosphorylated PFK, whereas traces 2 and 3 represent the phosphopeptide patterns of the in vivo labeled PFK. The PFK used in trace 2 had been phosphorylated in hepatocytes in the presence of glucose alone, while the PFK subjected to trace 3 had been phosphorylated in the same batch of hepatocytes but in the presence of glucose plus glucagon. Although the same amount of PFK (1 unit each) was subjected to limited proteolysis in trace 2 and trace 3, the radioactivity applied to trace 3 (1000 cpm) was somewhat higher than that subjected to trace 2 (730 cpm).

The additional radioactivity incorporated due to the action of glucagon was evenly distributed between all phosphopeptides. This indicates a higher degree of phosphorylation of the same sites phosphorylated in the presence of glucose alone rather than the phosphorylation of an additional site.

The patterns of the phosphopeptide resulting from in vivo and in vitro phosphorylation seem to be identical (compare trace 1 with traces 2 and 3).

3.2. *Tryptic fingerprintings*

In order to get a more well defined digestion, the gel pieces containing the [^{32}P]PFK were incubated with trypsin as in section 2. The extracted peptides were subjected to peptide mapping on thin-layer plates (fig.2). In addition to mapping separately [^{32}P]PFK obtained under the different conditions as shown in fig.2(A–C) equal amounts (in terms of ^{32}P-radioactivity) of in vitro and in vivo labeled PFK were mixed and also analysed (map A + B and map A + C). The in vitro phosphorylation of PFK by the catalytic subunit of the cAMP-dependent protein kinase (A) and the in vivo phosphorylation of PFK induced by glucose (B) or by glucose plus glucagon (C) resulted in identical phosphopeptide maps. The identity of the phosphopeptide spots is strongly supported by the combination experiments (A + B and A + C). From these results it must be concluded that the in vitro and the in vivo phosphorylations led to the same phosphorylated sites. (This confirms [12] where analogous peptide maps were found when PFK from mouse muscle was radioactively labeled in vitro by cAMP-dependent protein kinase or in vivo by injection of [^{32}P]phosphate.)

The identity of the phosphopeptide maps of in vitro and in vivo labeled PFK makes it likely that in vivo a cAMP-dependent protein kinase is responsible for PFK phosphorylation, be it in the

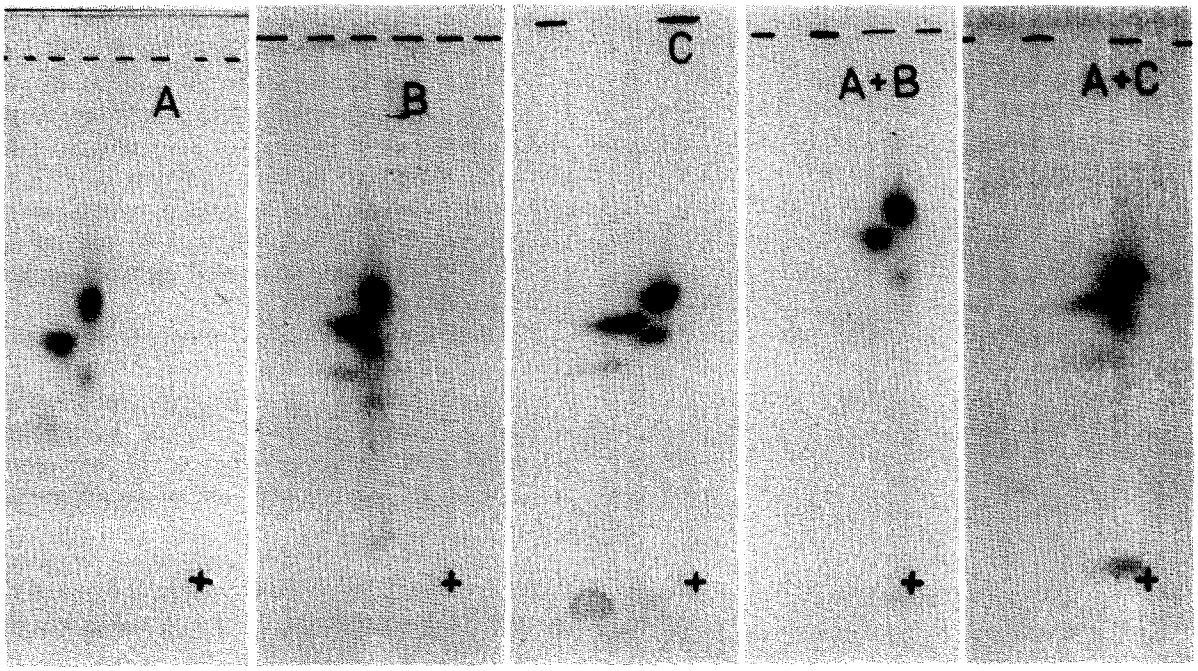

Fig.2. Autoradiographs showing tryptic fingerprints of [^{32}P]PFK labeled under different conditions. PFK was radioactively labeled in vitro and in vivo, and separated on SDS–polyacrylamide slab gels. [^{32}P]PFK were cut out of the gels and subjected to digestion by trypsin. Peptides were spotted on cellulose thin-layer plates [(+) origin] and were separated in two dimensions, first by electrophoresis in the horizontal dimension (negative pole left; positive pole right) and by chromatography in the vertical dimension: (A) fingerprint of PFK phosphorylated in vitro with catalytic subunit of cAMP-dependent protein kinase; (B) PFK was phosphorylated in hepatocytes incubated in the presence of glucose; (C) PFK was phosphorylated in hepatocytes incubated in the presence of glucose and glucagon; (A + B) and (A + C) were mixtures of the same amount of radioactivity in the phosphopeptides derived from PFK labeled under the conditions described.

presence of glucose or of glucagon. However, the phosphorylation of PFK is dependent on the metabolic state [2]:

(1) In liver cells from fed rats, both glucagon and glucose lead to maximal phosphorylation;
(2) In liver cells from starved rats glucagon or glucose alone increase ^{32}P-incorporation only to a limited extent, while the addition of both glucose and glucagon increases the phosphorylation to maximal values [2,3];
(3) The addition of L-alanine inhibits almost completely the effects of glucose or glucagon on PFK phosphorylation.

As under the same conditions the glucagon-induced phosphorylation of pyruvate kinase is independent from the nutritional state or from exogenous metabolites, the effects of metabolites on PFK phosphorylation cannot result from an effect of metabolites on cAMP-dependent protein kinase itself. Instead, we have to assume that a glucose-derived metabolite either renders PFK a better substrate for cAMP-dependent protein kinase or regulates the activity and/or the affinity of a protein phosphatase. Although the nature of the effective metabolite is not known yet, glucose and L-alanine should have an opposite effect on the concentration of this metabolite or affect PFK phosphorylation via different metabolites. Glucagon and glucose should not have an opposite effect (thus excluding fructose 2,6-bisphosphate as the effective intermediate) as glucagon leads to a decrease, whereas glucose leads to an increase in the concentration of this metabolite [14]. Moreover, lactate which is less effective than L-alanine in inhibition of PFK phosphorylation (unpublished), decreases the fructose 2,6-bisphos-

phate levels in hepatocytes more drastically than L-alanine [14].

Although the stimulation of PFK phosphorylation by glucagon is in line with a stimulation of cAMP-dependent protein kinase, the dependence of the glucagon effect on the nutritional state demonstrates that this effect is under further control, presumably by the binding of one or several metabolic ligands to PFK.

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