IDENTIFICATION OF L-TYPE PYRUVATE KINASE AS A MAJOR PHOSPHORYLATION SITE OF ENDOGENOUS CYCLIC AMP-DEPENDENT PROTEIN KINASE IN RAT LIVER SOLUBLE FRACTION

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Received 12 February 1979

1. Introduction

The mechanism by which hormones such as glucagon and catecholamines stimulate gluconeogenesis in liver has as yet not been elucidated. Injection of glucagon or epinephrine has been reported to lead to a lowered activity of the glycolytic enzymes phosphofructokinase and pyruvate kinase, whereas the gluconeogenic enzyme fructose-1,6-diphosphatase shows an increased activity; all enzymes being measured under one particular condition [1,2]. The mechanism of these changes in enzyme activity has not been investigated. The most well-known action of glucagon on liver cells is the stimulation of cyclic AMP-dependent protein kinases. It is obvious to explain the action of glucagon on gluconeogensis by phosphorylation of the above-mentioned enzymes which results in a change of enzyme activity. This seems indeed to be the case for pyruvate kinase, as judged from in vitro studies (reviewed in [3]) and from in vivo experiments [4]. Phosphorylation of purified phosphofructokinase [5] and fructose-1,6-diphosphatase [6] with concurrent change in enzyme activity has been reported. Experimental evidence for in vivo regulation of these enzymes by phosphorylation–dephosphorylation reaction is up till now absent.

Phosphorylation of rat liver cell sap [7–9] or rat hepatocyte soluble proteins [10] has been studied in the presence of added exogenous protein kinase. The effect of glucagon and catecholamines on the phosphorylation of supernatant proteins has been studied [10] in intact hepatocytes where endogenous protein kinase was used. Although it was not proven, pyruvate kinase was indicated as one of the proteins of which the phosphorylation is greatly stimulated by these hormones. No evidence for a stimulated phosphorylation of fructose-1,6-diphosphatase was found. We investigated the cyclic AMP-dependent phosphorylation of rat liver soluble proteins by endogenous protein kinase(s). Phosphorylated proteins were analysed by SDS–polyacrylamide gel electrophoresis and subsequent autoradiography. With this system we found that L-type pyruvate kinase is the major protein which incorporates ^{32}P from [γ-^{32}P]ATP in a cyclic AMP-stimulated reaction. The phosphorylation of pyruvate kinase is greatly diminished in the presence of the substrate phosphoenolpyruvate and not influenced by the allosteric activator fructose-1,6-diphosphate. Phosphorylation of fructose-1,6-diphosphatase was not detectable in this system.

2. Materials and methods

2.1. *Preparation of soluble fractions*

Minced rat liver was homogenized (20% w/v) with a Potter Elvehjem homogenizer in 250 mM sucrose, 25 mM Tris–HCl (pH 7.5), 2 mM mercaptoethanol and centrifuged at 105 000 × *g* for 60 min at 4°C. A human liver sample was obtained from a 7 month old baby. The sample was frozen in liquid N_2 within 4 h after death. The sample was treated as above.

2.2. *Phosphorylation experiments*

Soluble fraction (10 μl) was preincubated for 1 min with 10 μl from a mixture containing 25 mM Tris–HCl (pH 7.5), 40 mM phosphate, 200 mM KCl and 10 mM theophylline at 37°C. Phosphorylation was started by adding 10 μl of a mixture of [γ-^{32}P]ATP, ATP and $MgCl_2$. The final concentration of $MgCl_2$ was 5 mM. ATP concentrations ranged from 10–100 μM (see figure legends). The specific activity of [γ-^{32}P]ATP was 3000 Ci/mmol (The Radiochemical Centre, Amersham). About 5 μCi/incubation was used. The reaction was stopped with 15 μl of a solution containing 0.3 M Tris–H_3PO_4 (pH 6.8), 3% SDS, 50 mM 2-mercaptoethanol and 20% glycerol. In a pilot study it was established that the inactivation of pyruvate kinase activity, measured at suboptimal phosphoenolpyruvate concentrations, was complete ⩽ 30 s with 10 μM ATP. At 100 μM ATP this inactivation occurred ⩽ 10 s. Samples were heated to 95°C for 5 min before electrophoresis.

2.3. *Electrophoresis*

SDS–polyacrylamide slab gel electrophoresis was performed as in [11] in 10% gels. Gels were 0.75 mm thick. Marker proteins were (mol. wt): phosphorylase (92.5 × 10^3), bovine serum albumin (67 × 10^3), catalase (60 × 10^3), ovalbumin (45 × 10^3), chymotrypsinogen (25 × 10^3). Marker proteins were mixed with an equal amount of the soluble fraction prior to electrophoresis. The gels were stained in a solution of 0.2% Coomassie brilliant blue R 250 in 50% methanol 3.5% acetic acid and destained in a mixture of 5% methanol and 7.5% acetic acid and subsequently vacuum dried. Autoradiographs were made by exposing the dried gel to Kodak XR-1 film. Scanning of the autoradiographs was performed with a Vitatron TLD 100 densitometer at 510 nm. Peak area was determined by weighing the cut out densitogram. Different exposure times were used to check linearity of amounts of radioactivity with optical density.

2.4. *Specific removal of proteins from a soluble fraction*

Antiserum against human liver L-type pyruvate kinase was raised in rabbit. The antiserum is monospecific against human liver L-type pyruvate kinase, but shows crossreactivity with L- and M-types pyruvate kinase of rat liver. In a concentration range of this antiserum and rat liver soluble fraction we detected 1 weak precipitation line in an Ouchterlony test. Antiserum against rat liver fructose-1,6-diphosphatase was raised in cavia. This antiserum showed 4 precipitation lines in an Ouchterlony test. Antiserum

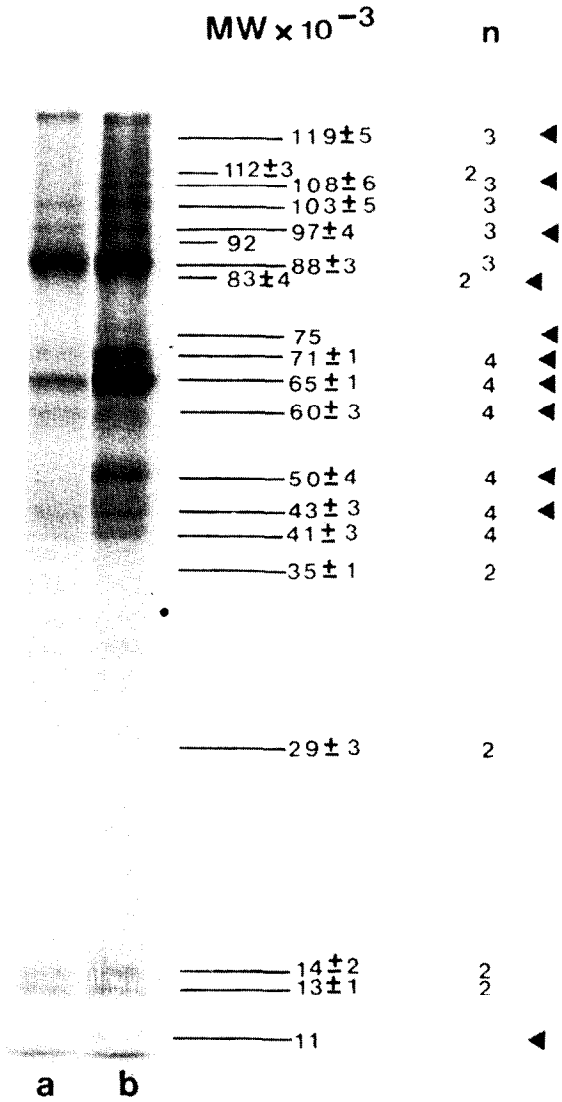

Fig.1. Cyclic AMP-dependent phosphorylation of rat liver soluble proteins as detected in an autoradiograph of a SDS–slab gel. Rat liver soluble fraction was phosphorylated as in section 2 with 10 μM ATP, for 30 s in the absence (a) or presence of 10 μM cyclic AMP (b). *n* denotes number of determinations; cyclic AMP-dependent increase in phosphorylation is indicated by (▲).

and control serum proteins were precipitated by 50% ammonium sulfate and after centrifugation dissolved in and subsequently dialysed against 0.1 M $NaHCO_3$, 0.5 M NaCl. The proteins were coupled to CNBr-activated Sepharose 4 B as described by the manufacturer (Pharmacia, Sweden). Soluble fractions were incubated with control serum- or antiserum-coupled Sepharose for 20 min at room temperature. Removal of enzyme was tested by measuring the remaining enzyme activity in the supernatant.

3. Results and discussion

3.1. *Phosphorylation pattern*

Rat liver soluble proteins separated by SDS–polyacrylamide gel electrophoresis contains 20 clear phosphorylation bands. The phosphorylation of at least 11 bands is stimulated by cyclic AMP as judged by scanning of the autoradiographs and visual interpretation, with the most pronounced effect on a protein with mol. wt ($65 \pm 1 \times 10^3$) (figs.1,2). A similar pattern was found with rat hepatocyte supernatant fraction upon incubation with protein kinase catalytic subunit [10].

3.2. *Identification of phosphorylation bands*

To detect pyruvate kinase in this pattern, we prepared a soluble fraction, from which pyruvate kinase was removed (see section 2). The major phosphorylation band disappeared specifically upon this treatment (fig.3). The possibility exists that M-type pyruvate kinase is responsible for this observation, because antiserum against human pyruvate kinase shows crossreactivity with L- and M-types pyruvate kinase of rat liver [12]. However, the same result was obtained with a soluble fraction from parenchymal cells which contains only L-type pyruvate kinase [16]. Furthermore, in human liver, this band with approximately the same molecular weight also disappeared after treatment with Sepharose-bound antiserum against L-type pyruvate kinase. In this latter case the antiserum is monospecific for L-type pyruvate kinase. From these experiments it must be concluded that the major phosphorylation band originates from the subunits of L-type pyruvate kinase.

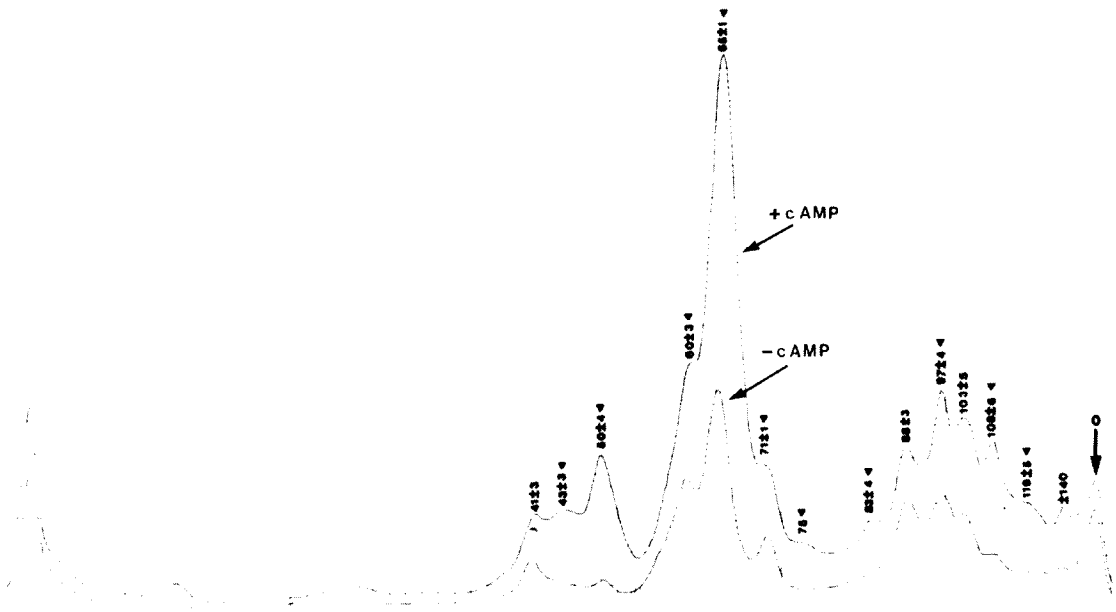

Fig.2. Cyclic AMP-dependent phosphorylation of rat liver soluble proteins. A typical example of a scanning of an autoradiograph is shown. For conditions see fig.1. The mol. wt $\times 10^{-3}$ is indicated. Cyclic AMP-stimulated bands are indicated by (▲).

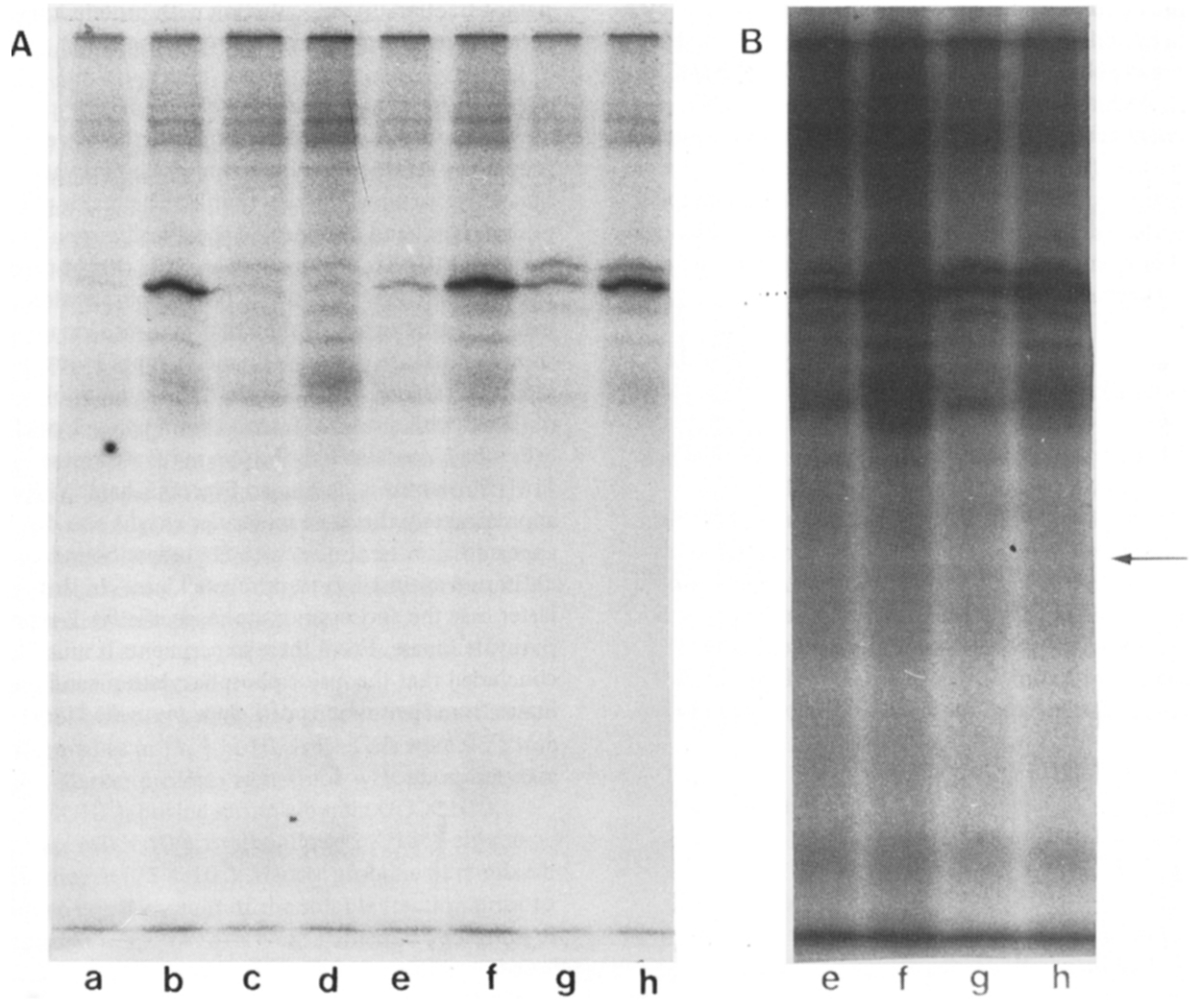

Fig.3.(A) Detection of pyruvate kinase and fructose-1,6-diphosphatase in SDS–slab gel electrophoresis of rat liver soluble proteins. Soluble fractions were incubated with sera bound to Sepharose and afterwards phosphorylated at 1 μM ATP for 10 s. Rabbit control serum-treated sample: (a) – cyclic AMP; (b) + 10 μM cyclic AMP. Rabbit antiserum against pyruvate kinase treated sample: (c) – cyclic AMP; (d) + 10 μM cyclic AMP. Cavia control serum treated sample: (e) – cyclic AMP; (f) + 10 μM cyclic AMP. Cavia antiserum against fructose-1,6-diphosphatase-treated sample: (g) – cyclic AMP; (h) + 10 μM cyclic AMP. (B) e–h were as in (A) but a longer exposure time was used. This experiment was also performed with 100 μM ATP for 2 min. The phosphorylation pattern was almost identical as shown here. Arrow indicates approximate mobility of fructose-1,6-diphosphatase subunits.

From this, direct evidence is obtained that the reported changes in kinetic parameters of pyruvate kinase in liver homogenates [13] or homogenates of parenchymal cells [14–16] upon incubation with MgATP and cyclic AMP are indeed parallelled by an increased phosphorylation state of the enzyme. The fact that pyruvate kinase is identified as a major phosphorylated protein is not necessarily surprising as the concentration of this enzyme in liver cells is high (± 2 μM, calculated from data in [17]). The concentration of fructose-1,6-disphosphatase is similar (calculated from data in [6]). However, no intensive phosphorylation bands are visible in the region of the subunit molecular weight of this enzyme (38×10^3

[6]) in SDS–gel electrophoresis (fig.1). Removal of low molecular weight components which could act as inhibitors of phosphate incorporation in this system, by gel filtration, did not influence the phosphorylation pattern. Phosphorylation of a fructose-1,6-diphosphatase-free soluble fraction did not lead to the disappearance of a phosphorylation band as compared to control serum treated soluble fraction (fig.3). It is not very likely that the non-monospecificity of the antiserum used impaired this result. The absence of a detectable phosphorylation band of fructose-1,6-diphosphatase makes it rather doubtful whether the reported in vitro phosphorylation of the purified enzyme [6] is involved in the hormonal regulation of gluconeogenesis.

3.3. *Effects of phosphoenolpyruvate and fructose-1,6-diphosphate on phosphorylation pattern*

It has been argued that the incubation conditions applied here in a crude cellular preparation is representative of native conditions [14]. We agree with this view because in these systems, probably because endogenous protein kinase is involved, the kinetic changes develop very quickly after addition of cyclic AMP [13,14] in contrast to experiments in a purified system [3] and the inactivation occurs at physiological cyclic AMP concentrations [13–15]. Great care has to be taken, however, when changes in kinetic parameters in these experiments are interpreted as changes in phosphorylation state. Especially the binding of very small amounts of the allosteric activator fructose-1,6-diphosphate to pyruvate kinase can influence the determination of changes in enzyme activity upon incubation, unless special precautions are taken [18]. The described system makes it possible to discriminate between effects upon the phosphorylation state of pyruvate kinase and changes in pyruvate kinase activity. As shown in table 1, phosphoenolpyruvate caused a large decrease in phosphate incorporation in pyruvate kinase. This agrees with experiments in which enzyme activity was used as a parameter for cyclic AMP-induced phosphorylation of pyruvate kinase [14,15]. Fructose-1,6-diphosphate at 1–50 μM, did not have any influence on the phosphorylation state of pyruvate kinase (table 1), in contrast to the effect upon enzyme activity, as reported [14,15]. The absence of an effect of fructose-1,6-diphosphate on the phosphorylation of pyruvate kinase agrees with experiments performed in a purified enzyme system [19]. It seems most likely that the reported inhibition by fructose-1,6-diphosphate of the cyclic AMP-dependent inactivation of pyruvate kinase activity is brought about by binding of the allosteric activator of pyruvate kinase, fructose-1,6-diphosphate. It is important to note the detection of a somewhat higher molecular weight of pyruvate kinase ($65 \pm 1 \times 10^3$) in SDS–gel electrophoresis of a rat liver soluble fraction compared with purified enzyme [3,17]. This difference may be explained by partial proteolysis of pyruvate kinase upon purification. The identification of pyruvate kinase as a major substrate for cyclic AMP-dependent protein kinase in vitro opens the possibility to study the relation between the phosphorylation state of pyruvate kinase and the reported inactivation of the enzyme upon starvation [18,20].

Table 1
Effect of phosphoenolpyruvate (PEP) and fructose-1,6-diphosphate (FDP) on phosphate incorporation in pyruvate kinase

Addition	– cyclic AMP	+ cyclic AMP (10 μM)
none	28	100
0.1 mM PEP	25	88
1.0 mM PEP	8	37
none	26	100
1 μM FDP	28	92
10 μM FDP	32	101
50 μM FDP	23	94

Results are expressed as % of phosphate incorporation without addition of metabolites in the presence of 10 μM cyclic AMP. Soluble fraction was pretreated by Sephadex G-25 filtration

Acknowledgements

Dr A. Kahn is thanked for a gift of antiserum against L-type pyruvate kinase, Dr A. van Tol for a gift of antiserum against fructose-1,6-diphosphatase and Dr H. R. de Jonge for stimulating discussion and advice in the phosphorylation experiments. Miss A. C. Hanson is thanked for typing the manuscript. The Netherlands Foundation for Fundamental Medical Research (FUNGO) is acknowledged for partial financial support (grant 13-39-18).

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