Volume 218, number 1, 1–6 FEB 04789 June 1987

# Phosphorylation of type-L pyruvate kinase in intact hepatocytes

## Localisation of the phosphorylation site in response to both glucagon and the $Ca^{2+}$-linked agonist phenylephrine

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Received 14 April 1987

Pyruvate kinase is one of the enzymes which can be phosphorylated by stimulation of the cell with either glucagon or $Ca^{2+}$-linked hormones. Whether these two classes of hormones phosphorylate the same site on the enzyme is unclear. Our results demonstrate that isolation of [$^{32}$P]phosphorylated type-L pyruvate kinase from glucagon-treated hepatocytes followed by aspartyl-prolyl cleavage yields a [$^{32}$P]phosphorylated peptide of $M_r$ 17000. This fragment is also phosphorylated in response to the $Ca^{2+}$-mediated agonist phenylephrine.

Pyruvate kinase; Phosphorylation; Glucagon; Phenylephrine; Phosphorylation site; Aspartyl-prolyl deavage

## 1. INTRODUCTION

Pyruvate kinase (ATP:pyruvate $O^2$-phosphotransferase, EC 2.7.1.40) plays an important role in the control of hepatic metabolism, since regulation of its activity can influence the rates of gluconeogenesis and of glycolysis [1].

Liver pyruvate kinase can be phosphorylated, with a resultant inactivation, by a cAMP-dependent protein kinase [2]. In vivo experiments have shown partial inactivation of rat type-L pyruvate kinase (L-PK) within a few minutes of intravenous injection of glucagon [3]. There is now clear evidence that phosphorylation of the enzyme is stimulated by glucagon both in vivo and in vitro [1].

Recently, studies have demonstrated that hormones can regulate hepatic carbohydrate metabolism through two distinct pathways. Glucagon appears to act via cAMP and cAMP-dependent protein kinase, whilst $\alpha$-adrenergic agonists (such as vasopressin and angiotensin II) are thought to utilize a $Ca^{2+}$-linked pathway that is independent of cAMP [4,5].

Pyruvate kinase is one of the enzymes which can be phosphorylated by stimulation of the cell with either glucagon or $Ca^{2+}$-linked hormones [6]. At the present time, it is unclear if L-PK is phosphorylated at the same site by the two different hormonal effector systems. By comparison the rabbit muscle glycogen synthetase is known to possess multiple sites for phosphorylation [7]. Phosphorylation of L-PK by calcium/calmodulin-dependent protein kinase II has been shown to modify both serine and threonine residues in a 3–4 kDa CNBr fragment near the amino-terminus [8].

We have used immunological techniques to isolate L-PK from hepatocytes exposed to dif-

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*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/87/$3.50 

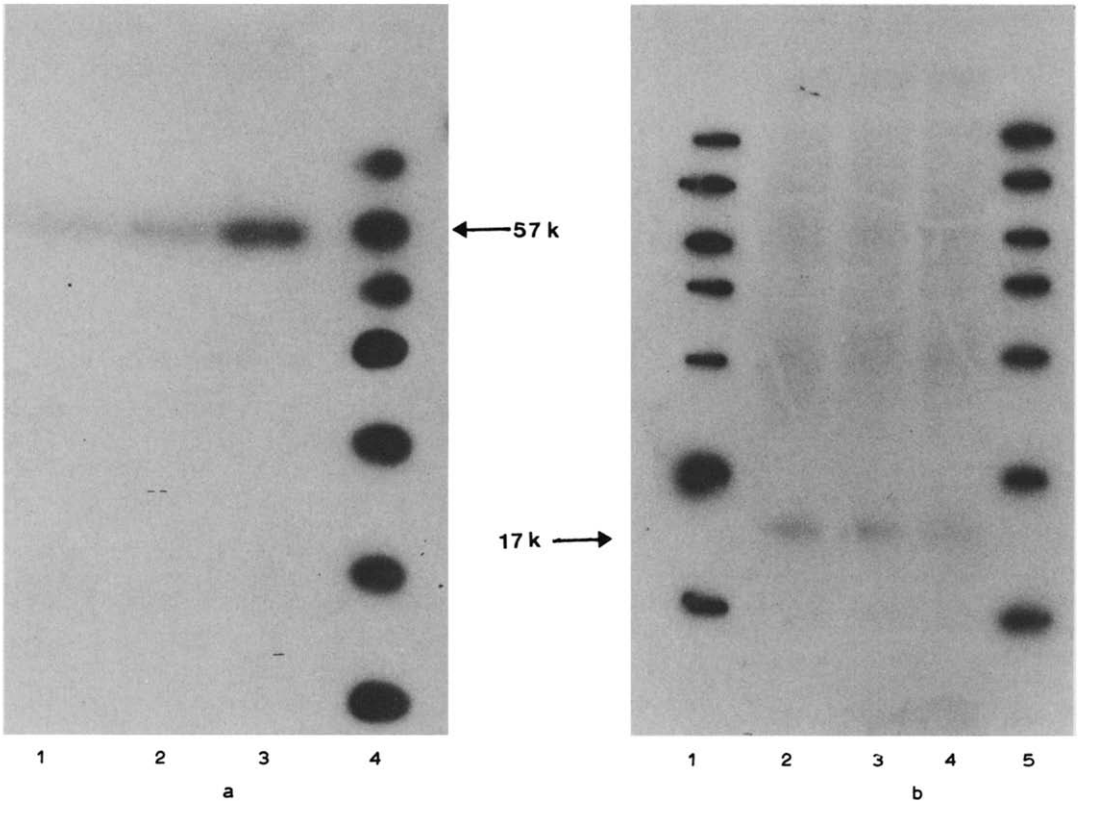


Fig.1. Autoradiographs of SDS-PAGE gels of phosphorylated L-PK before (a) and after (b) aspartyl-prolyl cleavage. (a) Varying amounts (0.5, 1, 2 $\mu$g) of protein obtained by Sepharose-Procion blue MX-R chromatography were applied to tracks 1–3. Track 4 contains reference markers (68, 57, 49, 35, 29, 18, 14 kDa). (b) Tracks 2–4 each contain 1.2 $\mu$g L-PK after aspartyl-prolyl cleavage. Tracks 1 and 5 contain the same reference markers as in (a).

ferent hormones in an attempt to study the phosphorylation state of the enzyme.

## 2. MATERIALS AND METHODS

### 2.1. *Enzyme isolation*

L-PK was purified from livers of high-carbohydrate diet fed rats [9] and used to raise anti L-PK antibody. The partially purified immunoglobulin was raised in sheep and prepared according to Hudson and Hay [10]. Rat liver cytosolic protein kinase was partially purified essentially by the method of Chen and Walsh [11]. [$^{32}$P]Phosphorylated L-PK was isolated either by affinity chromatography using a Sepharose-Blue MX-R column [9] or by immunoprecipitation [12]. Cleavage of aspartyl–prolyl bonds in L-PK was effected by hydrolysis in 75% formic acid [13].

### 2.2. *Isolation and incubation of hepatocytes*

Hepatocytes were isolated from high-carbohydrate diet fed rats following perfusion with collagenase [14]. Incubation of cells followed the protocol described by Postle and Bloxham [12]. The incubation was terminated by adding an equal volume of buffer A (50 mM potassium phosphate, 100 mM KCl, 50 mM NaF, 5 mM EDTA and 1 mM DTT, pH 7.4) saturated with ammonium sulphate. The cells were homogenized and the protein precipitate collected by centrifugation at 10000 × $g$ for 15 min at 4°C. The protein pellet was dissolved in buffer A alone. Insoluble protein and cell debris were removed by centrifugation (10000 × $g$ for 15 min) and the supernatant collected. This supernatant, containing >80% of the L-PK present in the cell extract, was dialysed against buffer A overnight.

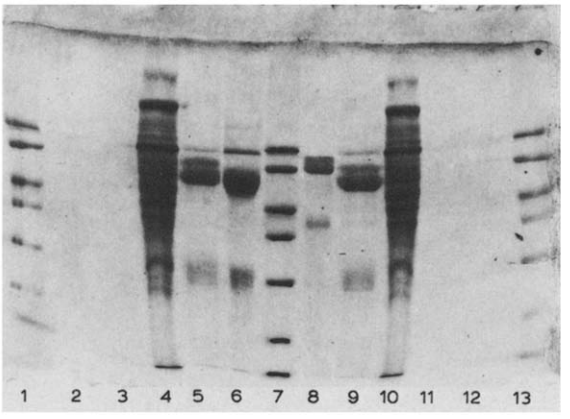


Fig.2. SDS-PAGE of proteins present at different stages of isolation by immunoprecipitation of L-PK. Tracks 4 and 5 refer to protein originating from glucagon-treated hepatocytes whilst tracks 9 and 10 originated from control hepatocytes. The proteins present after the 50% ammonium sulphate fractionation are shown in tracks 4 and 10, and those remaining after immunoprecipitation of this fraction are shown in tracks 5 and 9. Track 6 contains the anti L-PK immunoglobulin alone and track 8 contains muscle PK as a marker. Tracks 1, 7 and 13 contain molecular mass markers as used in fig.1. The gel was stained for protein using Coomassie brilliant blue R-250.

## 3. RESULTS

### 3.1. *Phosphorylation of L-PK in vitro by cAMP-dependent protein kinase*

L-PK was phosphorylated using partially purified protein kinase in vitro. Approx. 50 units of the L-PK was dialysed overnight against 5 mM potassium phosphate (pH 7.2), 5 mM magnesium acetate, 20% (v/v) ethylene glycol and 1 mM EDTA. The enzyme was then incubated in the presence of 17 mM potassium phosphate, pH 6.8; 6 $\mu$M cAMP, 0.3 mM [$\gamma$-$^{32}$P]ATP (10 Ci), 6 mM magnesium acetate in a final volume of 3 ml. 200 $\mu$l protein kinase (12 mg/ml) as its ammonium sulphate suspension was added and the mixture incubated at 37°C for 15 min. Subsequently, [$^{32}$P]phosphorylated L-PK was isolated by affinity chromatography on a Sepharose-Blue MX-R column (fig.1a). The fractions containing [$^{32}$P]phosphorylated L-PK were pooled and then concentrated using a small DEAE column. The labelled enzyme was subjected to aspartyl-prolyl cleavage in 75% formic acid. These results showed that the majority of the label was incorporated into a 17 kDa fragment (fig.1b). However, a trace of a phosphorylated peptide of 14 kDa was also present.

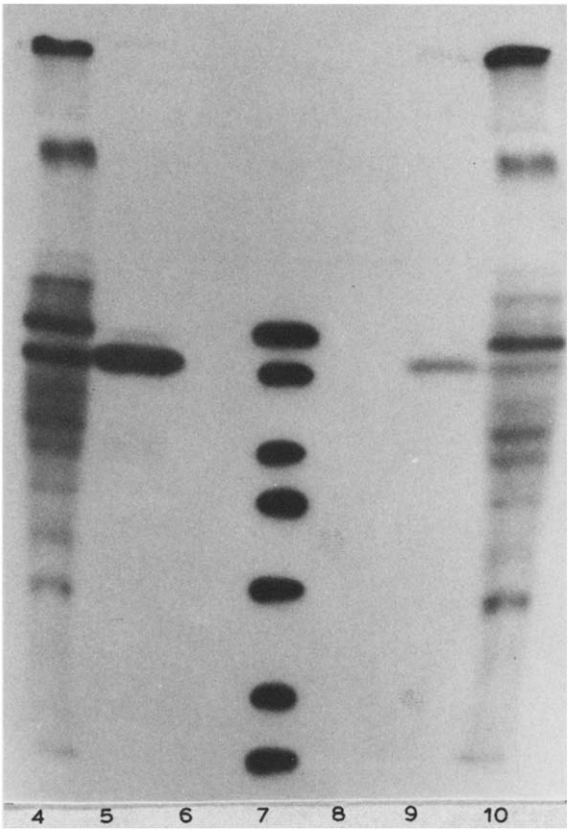


Fig.3. Localisation of protein-bound $^{32}$P by autoradiography. This figure shows the central section (tracks 4–10) of fig.2 after autoradiography.

### 3.2. *Effect of glucagon and phenylephrine on phosphorylation of L-PK in hepatocytes*

Glucagon acts via activation of adenylate cyclase to increase cAMP levels within the cell; the effect on protein phosphorylation being mediated via cAMP-dependent protein kinase. Phenylephrine acts, predominately, via the $\alpha$-adrenergic receptor to alter intracellular $Ca^{2+}$ levels, the effects on protein phosphorylation being mediated via a distinct $Ca^{2+}$-linked protein kinase. The effect of the latter enzyme on pyruvate kinase is unknown.

Hepatocytes were isolated and the cells in-

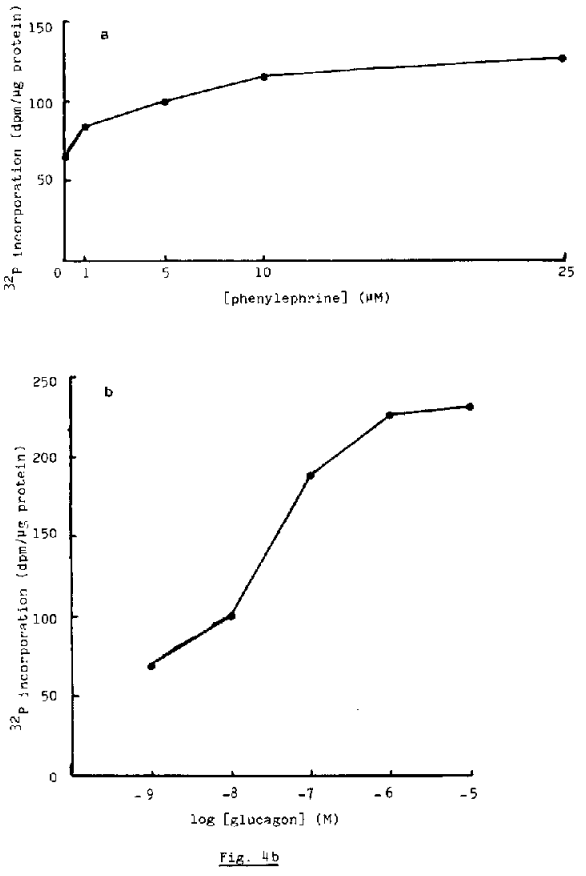


Fig.4. Incorporation of $^{32}P$ into L-type pyruvate kinase in response to varying concentrations of (a) phenylephrine or (b) glucagon. Phosphorylated pyruvate kinase was isolated from hepatocytes, incubated with varying concentrations of either phenylephrine or glucagon, by immunoprecipitation. Each immunoprecipitate was analysed by SDS-PAGE, and the bands corresponding to pyruvate kinase excised and counted, after NCS solubilisation, in a liquid scintillation counter. The incorporation of $^{32}P$ is reported as a function of agonist added. In the absence of any added compounds the background incorporation was 70 dpm/$\mu$g protein.

cubated in low phosphate (0.1 mM) Krebs-Henseleit buffered saline containing 100 $\mu$Ci $^{32}PO_4^{3-}$ per ml hepatocytes (20–25 mg protein/ml cell). After 45 min pre-incubation, the cells were exposed to either 1 $\mu$M glucagon or 10 $\mu$M phenylephrine for 10 min. Thereafter the L-PK in the cells was isolated as described in section 2.2.

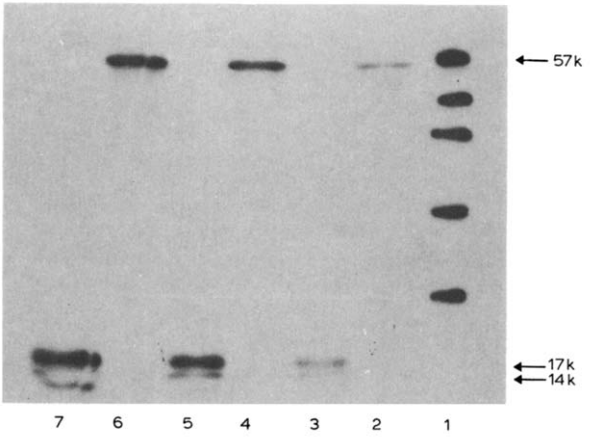


Fig.5. Phosphorylated L-PK isolated from either glucagon or phenylephrine stimulated hepatocytes, before and after aspartyl-prolyl (D/P) cleavage. Tracks: 1, molecular mass marker; 2, enzyme isolated from control hepatocytes; 3, after D/P cleavage of the protein in track 2; 4, enzyme isolated from hepatocytes incubated with 10 $\mu$M phenylephrine; 5, after D/P cleavage of the protein in track 4; 6, enzyme isolated from hepatocytes treated with 1 $\mu$M glucagon; 7, after D/P cleavage of the protein in track 6.

Antibody was used to isolate [$^{32}P$]phosphorylated L-PK from the solubilized ammonium sulphate pellet. Fig.2 shows that a specific protein of 57 kDa was obtained from both glucagon-treated and control hepatocytes as assessed by SDS-PAGE. After autoradiography (fig.3) it was demonstrated that the protein-bound $^{32}P$ was associated with the L-PK. The incorporation of $^{32}P$ into L-PK was stimulated in the presence of 1 $\mu$M glucagon by 3.5–4-fold over that of the control.

When phenylephrine was used an increase in the amount of $^{32}P$ associated with the L-PK was also seen. The incorporation showed a dose dependency; this is illustrated in fig.4a for phenylephrine and fig.4b for glucagon. From the dose-response curve we can see that the maximal incorporation of $^{32}P$ is different for the two substances, being 4- and 2-fold for glucagon and phenylephrine, respectively. The isolated [$^{32}P$]phosphorylated L-PK was subjected to aspartyl-prolyl cleavage. This demonstrated that both glucagon and phenylephrine cause phosphorylation of a 17 kDa polypeptide (fig.5). On average 90% of the label was

located in this fragment; the remaining 10% of the label was associated with a 14 kDa fragment.

## 4. DISCUSSION

Garrison and Wagner [6] have demonstrated that pyruvate kinase is one of the proteins which can be phosphorylated by either cAMP-dependent or $Ca^{2+}$-dependent protein kinases. Our results show by direct assay that 1 $\mu$M glucagon and 10 $\mu$M phenylephrine result in the maximal incorporation of $^{32}P$ into pyruvate kinase, respectively. However, the efficiency of the $Ca^{2+}$-linked agonist is observed to be less than that of glucagon, i.e. the incorporation of $^{32}P$ into pyruvate kinase stimulated by phenylephrine is approximately half of that seen with glucagon. The reason for this is still not clear. In fact, the mechanism by which the phosphorylation of pyruvate kinase is increased by $Ca^{2+}$-linked agonists is still not understood. It is possible that the calcium stimulates a $Ca^{2+}$-sensitive kinase or inhibits a protein phosphatase.

Garrison et al. [15] have indicated that neither phosphorylase kinase nor protein kinase C, two known $Ca^{2+}$-dependent protein kinases, appears to mediate this phosphorylation in hepatocytes. A recent report has demonstrated that pyruvate kinase is phosphorylated in vitro by a $Ca^{2+}$/calmodulin-dependent protein kinase [8], which may account for the observed phosphorylation in hepatocytes in response to agents which mobilize calcium, e.g. epinephrine, vasopressin, angiotensin II and A23187 [6].

Blackmore and Exton [16] reported that the effects of the $Ca^{2+}$-linked hormones on phosphorylase are mediated by phosphorylase kinase. Recent studies, however, indicate that their effects on pyruvate kinase are not mediated through phosphorylase kinase [17]. The exact mechanism by which hormones regulate protein phosphatases in liver is not known.

Pyruvate kinase has been shown to be phosphorylated by either cAMP or $Ca^{2+}$-mediated hormones in hepatocytes. Whether these two different sorts of hormone phosphorylate the same site of the enzyme is not clear. By comparison the rabbit muscle glycogen synthetase is known to possess multiple sites for phosphorylation [7]. Site 1 is phosphorylated by cAMP-dependent protein kinase, whilst site 2 is phosphorylated by either cAMP- or $Ca^{2+}$-sensitive protein kinases. Our results demonstrated that isolation of [$^{32}P$]phosphorylated L-PK from glucagon treated or as a result of in vitro phosphorylation using cAMP-dependent protein kinase followed by aspartyl-prolyl cleavage yields a [$^{32}P$]phosphorylation site located in a 17 kDa peptide fragment. This same fragment is also phosphorylated in response to the $Ca^{2+}$-mediated agonist phenylephrine. In addition to the 17 kDa peptide, we also observed a small amount of a 14 kDa fragment.

The complete nucleotide sequence for rat liver pyruvate kinase has been determined [18,19] and the amino acid sequence of the enzyme predicted. These results indicate that there are two aspartyl-prolyl sites in the enzyme, and the protein should be cleaved into three fragments of 16.9, 28.5 and 13.5 kDa. The N-terminal of the enzyme is located in the 16.9 kDa peptide and the C-terminal in the 13.5 kDa peptide. The major phosphorylation site would therefore appear to be near the N-terminal for both the cAMP-dependent [20] and $Ca^{2+}$-linked protein kinases. The identity of the minor fragment is, however, more problematical. It could either demonstrate a second, minor site near the C-terminal of the enzyme or it could arise through further cleavage of the 17 kDa phosphorylated polypeptide fragment in the acidic conditions used. The relationship between these polypeptides is still under investigation.

## ACKNOWLEDGEMENTS

Y.C.H. would like to thank T. Wilkinson for helpful discussions during the course of this work and Betty Draper for typing the manuscript.

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