Glucagon-stimulated Phosphorylation of Pyruvate Kinase in Hepatocytes*

Hiromi Ishibashi‡ and G. Larry Cottam
From the Department of Biochemistry, The University of Texas Health Science Center at Dallas, Dallas, Texas 75235

A 32P-labeled pyruvate kinase (L-type isozyme) was elicited in response to glucagon and has been isolated from suspensions of hepatocytes incubated with inorganic [32P]phosphate. The pyruvate kinase in crude extracts of hepatocytes was stabilized by ammonium sulfate fractionation, 30% glycerol, and 50 mM KF and the enzyme was isolated by immunoprecipitation with anti-liver pyruvate kinase immunoglobulin. In the absence of glucagon, incorporation of 32P into pyruvate kinase was linear for up to 60 min. However, addition of 1 μg glucagon to hepatocytes incubated in the presence of inorganic [32P]phosphate resulted in a 3-fold increase in the incorporation of 32P into pyruvate kinase within 5 min. Immunoelectrophoresis of the hepatocyte extracts followed by autoradiography demonstrates that 32P is covalently bound to the dissociated subunits of pyruvate kinase. Incubation of the hepatocytes in 1 μM glucagon resulted in a 60% decrease of pyruvate kinase activity when assayed in subsaturating concentrations of phosphoenolpyruvate and in the absence of the activator fructose 1,6-bisphosphate. Furthermore, the pyruvate kinase in glucagon-treated hepatocytes has a decreased affinity for phosphoenolpyruvate and a decreased affinity for fructose 1,6-bisphosphate. Both the rapid decrease in pyruvate kinase activity and the rapid increase in phosphorylation of the enzyme in response to addition of glucagon are consistent with phosphorylation of pyruvate kinase being a hormone-sensitive biochemical modification to regulate its activity in vivo.

Glucagon, epinephrine, and cyclic AMP are known to stimulate the rate of hepatic glucose synthesis from pyruvate and lactate (1). Taunton and co-workers (2-4) investigated the effects of glucagon on several enzymes of the glycolysis and gluconeogenesis pathways in liver and reported that glucagon given intravenously resulted in rapid changes in the activity of several enzymes including a rapid decrease in hepatic pyruvate kinase (EC 2.7.1.40) activity. Subsequently, a decrease in liver pyruvate kinase (L-type) activity in response to glucagon has been reported using perfused livers (5), hepatocytes isolated from rat livers (6-11), and intact rats (11). Taunton and co-workers (2-4) also reported that administration of glucagon resulted in a dramatic increase in the cyclic AMP level prior to or concomitant with the decrease in the pyruvate kinase activity. In 1974, Ljungström et al (19) demonstrated the in vitro phosphorylation of purified liver pyruvate kinase by ATP catalyzed by a cyclic AMP-activated protein kinase, which resulted in a decrease of the catalytic activity of the enzyme at low concentrations of phosphoenolpyruvate (12, 13). Thus, those authors suggested the possible role of phosphorylation of pyruvate kinase as a mechanism to regulate the enzyme activity. Whether phosphorylation of pyruvate kinase is a hormone-sensitive mechanism to regulate its catalytic activity in vivo is the next important question and led to the series of experiments reported here.

In this investigation, inorganic [32P]phosphate is incubated with hepatocytes in the presence and absence of glucagon. The effect of glucagon on the incorporation of inorganic [32P]phosphate into pyruvate kinase and the alteration of the catalytic activity and kinetic parameters are reported.

EXPERIMENTAL PROCEDURES

The materials and methods used are described in detail in the miniprint supplement following this paper.

RESULTS

Tables and figures are in the miniprint supplement following this paper.

Rapid Preparation of Stable Pyruvate Kinase Extracts from Hepatocytes—To investigate whether phosphorylation of pyruvate kinase occurs in hepatocytes in response to a glucagon challenge, a rapid extraction procedure is required that stabilizes the catalytic activity and chemical integrity of the enzyme and that would minimize the activity of endogenous phosphatases and proteolytic enzymes. Table I outlines such a procedure for rapid extraction of a stable pyruvate kinase (L-type) from hepatocytes. Hepatocytes contain only the L-type isoform (29, 30). The procedure of sonication of the hepatocytes in 50 mM KF, centrifugation at 10,000 x g, a 50% ammonium sulfate precipitation of the enzyme, and a subsequent 100,000 x g centrifugation in 30% glycerol and 50 mM KF quickly isolates and stabilizes the pyruvate kinase from the hepatocytes. Furthermore, this procedure yields some purification of the enzyme with high recovery of enzyme activity. The pyruvate kinase isolated in this manner results in a stable enzyme activity that is essentially unaltered for at

* This work was supported, in part, by The Robert A. Welch Foundation Grant I-381 and National Institutes of Health Grant AM19031. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC. Section 1734 solely to indicate this fact.

‡ Permanent address, First Department of Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan.

1 Portions of this paper (including Figs. 1 to 8 and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78-627, cite author(s), and include a check or money order for $1.50 per set of photocopies.
Phosphorylation of Pyruvate Kinase

least 5 days and approximately 80% of the initial activity is present even after storing this rather crude extract for 20 days at 4°C (Fig. 1). In contrast, the enzyme activity is rapidly lost in either a 10,000 x g supernatant fraction or a 100,000 x g supernatant fraction of the sonicated hepatocyte solution. In addition to stabilizing the pyruvate kinase catalytic activity, the procedure outlined is effective in a preliminary isolation of 32P-labeled pyruvate kinase from the large amount of nonspecific radioactivity present. As seen in Table I, the Fraction II extract contains only a small proportion of the total radioactive activity in the original cell suspension, yet it contains a large percentage of the pyruvate kinase.

Effect of Addition of Glucagon to Hepatocytes on the Kinetic Parameters of Pyruvate Kinase—Administration of glucagon decreases the pyruvate kinase activity and results in altered kinetic parameters for liver pyruvate kinase. Incubation of hepatocytes with 1 μM glucagon for 10 min at 37°C results in a 50 to 60% decrease in the pyruvate kinase activity in Fraction II extracts when the enzyme is assayed in the absence of fructose 1,6-bisphosphate and at low phosphoenolpyruvate concentration (see Table II). This point will be discussed in more detail in Fig. 6. On the other hand, when the enzymes are assayed at saturating levels of phosphoenolpyruvate or in the presence of 1 mM fructose 1,6-bisphosphate, no difference in Vₘₐₓ for pyruvate kinase is observed. However, glucagon treatment of hepatocytes apparently results in a pyruvate kinase with a lowered affinity for both phosphoenolpyruvate and fructose 1,6-bisphosphate. As seen in Fig. 2A, incubation of hepatocytes with glucagon results in an increase in the apparent Kₐₜ values for phosphoenolpyruvate from 0.58 to 1.10 mM when assayed in the absence of fructose 1,6-bisphosphate. There is no difference in the apparent Kₐₜ values for phosphoenolpyruvate of 0.08 mM when assayed in 1 mM fructose 1,6-bisphosphate. When assayed at 0.1 mM phosphoenolpyruvate, the apparent Kₐₜ value of 0.16 μM for fructose 1,6-bisphosphate increases to 0.35 μM for pyruvate kinase extracted from glucagon-treated hepatocytes (Fig. 2B).

Effect of Glucagon on Incorporation of Phosphosphate into Pyruvate Kinase—the basal rate of incorporation of inorganic [32P]phosphate from the hepatocyte incubation medium in the absence of exogenous hormones into total cytosolic protein and into immunoprecipitable pyruvate kinase is illustrated in Fig. 3. The incorporation of [32P] into total protein (trichloroacetic acid-precipitable) in Fraction I extracts was linear for up to 60 min. The incorporation of [32P] into immunoprecipitable pyruvate kinase in Fraction II preparations was also linear during this time period. Quantitative removal of pyruvate kinase from Fraction II extracts was accomplished by the addition of 50 μl of goat anti-liver pyruvate kinase immunoglobulin per enzyme unit of pyruvate kinase activity when assayed in the presence of 1 mM fructose 1,6-bisphosphate (Fig. 4).

Addition of 1 μM glucagon to the hepatocyte incubation mixture rapidly increased the rate of 32P incorporation into immunoprecipitable pyruvate kinase. As seen in Fig. 5, the specific radioactivity of [32P]-labeled pyruvate kinase is increased approximately 3-fold over the basal incorporation within 4 to 5 min after the addition of glucagon. Addition of glucagon to hepatocytes preincubated with inorganic [32P]phosphate for various times up to 40 min always resulted in a 3-fold stimulation of [32P] incorporation into pyruvate kinase when compared to controls run in the absence of glucagon. The glucagon-stimulated incorporation of [32P] into pyruvate kinase occurs at a rate similar to that observed for the decrease in enzyme activity. As seen in Fig. 6 (Δ), the pyruvate kinase activity (assayed in the absence of fructose 1,6-bisphosphate at low phosphoenolpyruvate concentration) rapidly decreased by 60% within 2 to 4 min after the addition of 1 μM glucagon to the hepatocyte suspension. In contrast, the pyruvate kinase activity in hepatocytes incubated in the absence of glucagon (C) did not change during incubation up to 60 min.

To further investigate whether the 32P detected in the pyruvate kinase-immune complex is associated with pyruvate kinase, immunoelectrophoresis of Fraction I extracts of hepatocytes was carried out (Fig. 7). Upon staining, formation of a single precipitin line is observed which suggests only one major antigenic species exists in the Fraction I extracts that is reacting with the anti-liver pyruvate kinase immunoglobulin. Furthermore, the formation of a single coincident band via autoradiographic techniques demonstrates specific localization of 32P in the pyruvate kinase-antibody complex.

The question of whether 32P is covalently bound to pyruvate kinase was investigated by SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis of [32P]-labeled pyruvate kinase immune complex isolated from hepatocytes incubated with 1 μM glucagon yields two major protein bands of 23,500 and 50,000 daltons which correspond to IgG light chains and heavy chains, respectively (Fig. 8, Gel a). However, an autoradiogram of the only one radioactive band (Fig. 8, Gel c) which is easily distinguishable from the IgG protein bands and coincides with the pyruvate kinase subunit protein band of 59,000 daltons (14) (Fig. 8, Gel b). Furthermore, greater than 90% of the radioactivity applied to the SDS-polyacrylamide gel is recovered in this one band upon quantitation by slicing the gel and counting the individual slices in a scintillation counter (Fig. 8, bottom). These data suggest that glucagon induces a rapid covalent incorporation of 32P into pyruvate kinase.

**DISCUSSION**

In the present study, a phosphorylated pyruvate kinase (L-type isozyme) was isolated after incubation of hepatocytes with inorganic [32P]phosphate. The extent of phosphorylation was increased 3-fold upon the addition of 1 μM glucagon to the hepatocyte suspension. Furthermore, the maximum extent of phosphorylation of pyruvate kinase and the maximum inhibition of the enzyme activity occurred within a very short time (4 to 5 min) after exposure to the hormone. Ljungström et al. (12) originally demonstrated that purified liver pyruvate kinase is phosphorylated by ATP in the presence of a cyclic AMP-dependent protein kinase. This phosphorylation decreased the pyruvate kinase activity and altered the Kₐₜ values for phosphoenolpyruvate and fructose 1,6-bisphosphate (13). These kinetic changes were reversed by histone phosphatase (31). These in vitro studies strongly suggest a phosphorylation-dephosphorylation mechanism for regulation of liver pyruvate kinase. However, there has been no evidence that phosphorylation actually occurs in vivo until recently when [32P]-labeled pyruvate kinase was isolated after incubation of liver slices with inorganic [32P]phosphate (32) and from livers of rats injected with inorganic [32P]phosphate (33). The present study was performed with isolated hepatocytes—sequences of a protein species that is sensitive to hormones, the environment is easily controlled, and multiple samples can be run using the hepatocytes from the same liver. In addition, hepatocytes contain only L-type pyruvate kinase (29, 30), which is the isozyme regulated by hormones, whereas a whole liver contains both L-type and M-type pyruvate kinase isozymes (34, 35).

The change in pyruvate kinase activity in response to the glucagon challenge occurs very rapidly (within 4 to 5 min),

The abbreviations used are SDS, sodium dodecyl sulfate; RCTA, ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid.

---

8768

To further investigate whether the 32P detected in the pyruvate kinase-immune complex is associated with pyruvate kinase, immunoelectrophoresis of Fraction I extracts of hepatocytes was carried out (Fig. 7). Upon staining, formation of a single precipitin line is observed which suggests only one major antigenic species exists in the Fraction I extracts that is reacting with the anti-liver pyruvate kinase immunoglobulin. Furthermore, the formation of a single coincident band via autoradiographic techniques demonstrates specific localization of 32P in the pyruvate kinase-antibody complex.

The question of whether 32P is covalently bound to pyruvate kinase was investigated by SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis of 32P-labeled pyruvate kinase immune complex isolated from hepatocytes incubated with 1 μM glucagon yields two major protein bands of 23,500 and 50,000 daltons which correspond to IgG light chains and heavy chains, respectively (Fig. 8, Gel a). However, an autoradiogram of the only one radioactive band (Fig. 8, Gel c) which is easily distinguishable from the IgG protein bands and coincides with the pyruvate kinase subunit protein band of 59,000 daltons (14) (Fig. 8, Gel b). Furthermore, greater than 90% of the radioactivity applied to the SDS-polyacrylamide gel is recovered in this one band upon quantitation by slicing the gel and counting the individual slices in a scintillation counter (Fig. 8, bottom). These data suggest that glucagon induces a rapid covalent incorporation of 32P into pyruvate kinase.

**DISCUSSION**

In the present study, a phosphorylated pyruvate kinase (L-type isozyme) was isolated after incubation of hepatocytes with inorganic [32P]phosphate. The extent of phosphorylation was increased 3-fold upon the addition of 1 μM glucagon to the hepatocyte suspension. Furthermore, the maximum extent of phosphorylation of pyruvate kinase and the maximum inhibition of the enzyme activity occurred within a very short time (4 to 5 min) after exposure to the hormone. Ljungström et al. (12) originally demonstrated that purified liver pyruvate kinase is phosphorylated by ATP in the presence of a cyclic AMP-dependent protein kinase. This phosphorylation decreased the pyruvate kinase activity and altered the Kₐₜ values for phosphoenolpyruvate and fructose 1,6-bisphosphate (13). These kinetic changes were reversed by histone phosphatase (31). These in vitro studies strongly suggest a phosphorylation-dephosphorylation mechanism for regulation of liver pyruvate kinase. However, there has been no evidence that phosphorylation actually occurs in vivo until recently when [32P]-labeled pyruvate kinase was isolated after incubation of liver slices with inorganic [32P]phosphate (32) and from livers of rats injected with inorganic [32P]phosphate (33). The present study was performed with isolated hepatocytes which provides an experimental system that is sensitive to hormones, the environment is easily controlled, and multiple samples can be run using the hepatocytes from the same liver. In addition, hepatocytes contain only L-type pyruvate kinase (29, 30), which is the isozyme regulated by hormones, whereas a whole liver contains both L-type and M-type pyruvate kinase isozymes (34, 35).

The change in pyruvate kinase activity in response to the glucagon challenge occurs very rapidly (within 4 to 5 min),

The abbreviations used are SDS, sodium dodecyl sulfate; RCTA, ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid.
yet it is a transient effect. Furthermore, the kinetic changes in pyruvate kinase induced by glucagon treatment disappear very rapidly in crude liver extracts (5) and the phosphatase activity in liver homogenates rapidly dephosphorylates a phosphorylated pyruvate kinase (31). This necessitated development of the procedure for rapid extraction of pyruvate kinase from the large amount of nonspecific radioactive activity present. The pyruvate kinase is stable in these extracts and retains the effect of glucagon for at least 24 h. This allows phosphorylated pyruvate kinase to be quantitatively isolated by immunoprecipitation.

There are several problems which might arise in the use of immunoprecipitation techniques to identify and quantitate 32P-labeled pyruvate kinase. One problem is the possibility of nonspecific radioactivity associated with the antigen-antibody complex. This could result from either prolonged incubation of the antigen-antibody complex. There is also the possibility of nonspecific trapping of labeled proteins in the antigen-antibody complex. To minimize these problems, the 32P-labeled pyruvate kinase was isolated from stabilized hepatocyte extracts (Fraction II) which isolates the 32P-labeled pyruvate kinase from the majority of nonspecific radioactive activity and the immunoprecipitates were carefully washed through 1.0 M sucrose containing small amounts of detergents. Immmunoelectrophoresis of crude hepatocyte homogenates (Fraction I) reveals 32P associated with the pyruvate kinase precipitin band and suggests that the phosphorylation actually occurred in the hepatocyte during incubation and not during the subsequent procedures. SDS-gel electrophoresis of the solubilized immune complex suggests that the majority of the radioactivity derived from the immune complex is covalently bound and specifically associated with the pyruvate kinase subunits.

Since the basal incorporation of 32P into pyruvate kinase increased continually during the incubation times used in this investigation, an exact stoichiometry of 32P-labeled pyruvate kinase induced by glucagon has not been determined. However, a stoichiometry can be estimated by using the specific radioactivity of inorganic phosphate in the incubation mixtures (50 μCi/1.18 μmol of inorganic phosphate) and a specific activity of the purified enzyme of 200 enzyme units (EU) mg⁻¹ and a molecular weight of 220,000 (9, 10). Thus, after a 40-min incubation of hepatocytes with inorganic [32P]phosphate, one can estimate the incorporation to be 0.29 mol of phosphate/mol of pyruvate kinase in the sample incubated with glucagon and approximately 0.1 in the control (Fig. 5).

Acknowledgments—We would like to thank Dr. E. R. Hall and Mrs. V. McCully for preparation of the liver pyruvate kinase and assistance throughout this study. The secretarial assistance of Marie Rotondi, Marty Parkey, and Barbara Lewis is appreciated.

REFERENCES

The references are tabulated in the miniprint supplement found on p. 9770.
Phosphorylation of Pyruvate Kinase

Fig. 1: The stability of the pyruvate kinase activity in tissue storage at 4°C was assessed from isolated hepatocytes. The enzyme activity is plotted as a percentage of the initial specific activity observed with fresh tissue. The enzyme activity was expressed as units of activity per milligram of protein.

Fig. 2: Effect of the phosphomonoesterase concentration on the pyruvate kinase activity in tissue storage at 4°C was assessed from isolated hepatocytes. The enzyme activity is plotted as a percentage of the initial specific activity observed with fresh tissue. The enzyme activity was expressed as units of activity per milligram of protein.

Fig. 3: Fraction I-leucyl kinase activity in tissue storage at 4°C was assessed from isolated hepatocytes. The enzyme activity is plotted as a percentage of the initial specific activity observed with fresh tissue. The enzyme activity was expressed as units of activity per milligram of protein.

Fig. 4: Demonstration of liver pyruvate kinase immunoblotting. Samples of Fraction I and Fraction II were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with a specific antibody against liver pyruvate kinase and then reacted with a secondary antibody conjugated to horseradish peroxidase. The immunoblot was developed using an enhanced chemiluminescence kit.
Phosphorylation of Pyruvate Kinase

Fig. 3. Stimulation of the incorporation of \(^{32}P\) from pyruvate kinase upon incubation of glycogen with phosphoglycogen. N-p-nitrocellulose (2) (50 mg/100 ml) were incubated with glycogen (200 mg/ml) at 37°C for 30 min. A 1 ml aliquot of glycogen was added to the incubation mixture. In the test tubes, 0.5 ml of the glycogen solution was added to the incubation mixture and the reaction was started by addition of an equal volume of an ice-cold solution containing 0.5 M KCl, 0.1 M EGTA, and 1 M NaCl. The samples were immediately assayed and the reaction was started by addition of the reaction mixture. After 5 min, the reactions were stopped by addition of an equal volume of an ice-cold solution containing 0.5 M KCl, 0.1 M EGTA, and 1 M NaCl. The samples were assayed for total phosphorylation activity in the presence of 1 mM phosphoenolpyruvate and 1 mM phosphoenolpyruvate. A sample of glycogen was quantitatively precipitated at 4°C overnight after addition of 10% TCA-0.01% phosphoethanolamine (50 ml). The precipitated glycogen was used for the assay of glycogen phosphorylase. The glycogen phosphorylase activity was assayed by the method of Selman et al. (13). The reaction was expressed in units as the activity required to release 1 unit of glycogen per hour.

Fig. 4. Effect of glycogen on the activity of liver pyruvate kinase. A 40 mg/ml solution of glycogen in 0.01 M Tris-HCl buffer (pH 7.8) was incubated with 1 nkat of pyruvate kinase (50 µM/100 ml) for 5 min. The glycogen solution was added to the incubation mixture and the reaction was started by addition of an equal volume of an ice-cold solution containing 0.5 M KCl, 0.1 M EGTA, and 1 M NaCl. The samples were assayed for total phosphorylation activity in the presence of 1 mM phosphoenolpyruvate and 1 mM phosphoenolpyruvate. A sample of glycogen was quantitatively precipitated at 4°C overnight after addition of 10% TCA-0.01% phosphoethanolamine (50 ml). The precipitated glycogen was used for the assay of glycogen phosphorylase. The glycogen phosphorylase activity was assayed by the method of Selman et al. (13). The reaction was expressed in units as the activity required to release 1 unit of glycogen per hour.
Glucagon-stimulated phosphorylation of pyruvate kinase in hepatocytes.
H Ishibashi and G L Cottam


Access the most updated version of this article at
http://www.jbc.org/content/253/24/8767.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/253/24/8767.citation.full.html#ref-list-1