Hepatic Pyruvate Kinase

QUANTITATIVE MEASUREMENTS OF PHOSPHORYLATION IN VITRO AND IN THE ISOLATED RAT HEPATOCYTE*

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The phosphorylation of rat liver pyruvate kinase was studied in isolated rat hepatocytes and with the crystalline enzyme. Hepatocytes were incubated with [32P]orthophosphate and isotopically labeled pyruvate kinase was quantitatively isolated using a resin-coupled antibody. The molar extent of phosphorylation of the enzyme was calculated from the amount of radioactivity in the enzyme and the specific radioactivity of [γ-32P]ATP determined simultaneously. A low steady state level of phosphorylation of pyruvate kinase was observed in hepatocytes incubated for 50 min in the absence of hormonal stimuli (0.4 P/tetramer). The activity ratio (enzyme activity at 1.8 mM P-enolpyruvate - fructose-1,6-P2/ +fructose-1,6-P2) for the low phosphate-containing form of the enzyme was 0.58. Exposure of the hepatocyte to 1 μM glucagon for 5-10 min increased the phosphorylation state to 1.7 P/tetramer and decreased the activity ratio by 60%. An inverse, linear relationship between the activity ratio and the phosphorylation state of the enzyme up to 2 P/tetramer was observed both in studies with the hepatocyte and with the crystalline enzyme phosphorylated using cAMP-dependent protein kinase from beef heart. In no situation was activity greater than 2 P/tetramer observed in the present investigation. With the crystallized enzyme, a single protein band migrating with an apparent M, = 62,000 was seen after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Isoelectric focusing in the presence of 8 M urea, however, revealed heterogeneity in the subunit composition. Five bands, three of which were not influenced by phosphorylation, could be resolved by isoelectric focusing. Despite the heterogeneity in the subunits with respect to phosphorylation, the purified enzyme was capable of binding 3.7 molecules of fructose-1,6-bisphosphate/tetramer. The results of this investigation differ from previous reports and suggest that some of the subunits of hepatic pyruvate kinase are modified such that they lose the capacity to be phosphorylated. Furthermore, nonphosphorylatable forms of the enzyme may occur in the intact cell and are not an artifact of purification.

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Administration of glucagon to the liver is accompanied by a rapid, reversible inhibition of Type L pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40). Several investigators have demonstrated that hormonal regulation of this key glycolytic enzyme involves a cAMP-mediated phosphorylation mechanism (1-3). Recently, investigation in our laboratory demonstrated that administration of adrenal glucocorticoids to rat hepatocytes maintained in primary culture is followed by a loss of regulation of pyruvate kinase by glucagon. Furthermore, the steroid hormone treatment decreases the ability to phosphorylate the enzyme with cAMP-dependent protein kinases (4). Although we currently do not understand the mechanisms by which the adrenal steroid hormones influence the regulation of liver pyruvate kinase, our observations raise questions concerning the existence of nonphosphorylatable forms of the enzyme.

Investigations with purified rat liver pyruvate kinase indicate the enzyme is a homotetramer (5, 6) and that phosphorylation by cAMP-dependent protein kinases occurs at a specific serine residue on each of the subunits, altering the enzyme activity (7-13). One recent report has suggested that the enzyme may also be phosphorylated at other sites through CAMP-independent mechanisms (14). It is possible that purification schemes employed for isolation of liver pyruvate kinase do not retain nonphosphorylatable forms of the enzyme and it is important to evaluate the phosphorylation state of the enzyme in the intact cell. Estimates of the maximum phosphorylation of Type L pyruvate kinase after administration of high concentrations of glucagon to isolated rat liver (15-17) or the intact rat (18) vary considerably, ranging from 0.3 to 3 mol of phosphate/mol of enzyme tetramer. Isotopic studies with intact liver cells have generally required several assumptions which remain to be verified. Also, it is not clear that the isolation procedures used in those investigations quantitatively retained the phosphorylation state of the enzyme after disruption of the cell.

In order to more clearly understand the hormonal regulation of rat liver pyruvate kinase, we undertook development of a quantitative procedure for estimating the phosphorylation state of the enzyme in rat hepatocytes under conditions previously established to be accompanied by hormonal regulation of the enzyme activity (19-21). This report summarizes our findings concerning phosphorylation of the enzyme in rat hepatocytes administered glucagon and epinephrine. The results obtained with the intact liver cell are correlated with those obtained with the purified enzyme phosphorylated in vitro using the cAMP-dependent protein kinase from beef heart.

MATERIALS AND METHODS

Preparation of Hepatocytes—Male Wistar strain rats weighing 250 to 350 g were obtained from Hilltop Laboratories, Scottdale, PA.
Hepatocytes were prepared by collagenase digestion of the liver as previously described (19). The cells were suspended to a final density of approximately 8 x 10^6 cells/ml and were incubated at 37°C in Krebs-Henseleit bicarbonate buffer containing 1.25 mM CaCl_2, 2.5% (w/v) dialyzed bovine serum albumin (19). For isotopic studies, [3P] orthophosphate was added to a final concentration of 1.2 mm (80 μCi/μmol). Incubation of the hepatocytes was terminated by adding 2.6 mM ammonium sulfate which preserves the phosphorylation state of pyruvate kinase (19).

**Isolation of Pyruvate Kinase Activity—**Pyruvate kinase activity was determined at pH 7.4 and 25°C (19, 21). Complete kinetic analysis of the enzyme was done by varying P-enolpyruvate and MgADP concentrations as previously reported (21). Hormonal regulation was assessed kinetically by measuring the activity ratio (Fru-1,6-P_2/Fru-1,6-P_2) at 1.8 mM P-enolpyruvate and 2.5 mM MgADP (19).

One unit of enzyme activity is defined as that catalyzing the formation of 1 μmol of pyruvate/min at 25°C.

**Immunologic Procedures—**Radiolabeled pyruvate kinase formed in hepatocytes was rapidly isolated using goat anti-pyruvate kinase serum prepared in the same manner except that the radioactive enzyme was isolated and purified by Dowex-1 Cl chromatography as described by Glynn and Chappell (28). [γ-3P]ATP was obtained commercially (New England Nuclear) or synthesized by the procedure of Penevsky (27) and purified by Dowex-1 Cl chromatography as described by Glynn and Chappell (28).

**Specific Radioactivity of ATP—**The specific radioactivity of phosphoenolpyruvate was determined by the procedure of Lowry et al. (29). The dilute acid solvent was chosen for this purpose to minimize dephosphorylation of the enzyme. Endogenous phosphate of ATP to form glucose-6-P. The reaction products were applied to a DEAE-cellulose column (5 ml bed volume) of DEAE-cellulose. The wash was extended by adding 0.3 mm NaCl to remove monophosphorylated intermediates. ATP was eluted with 0.2 n HCl and the ATP-containing fraction was rapidly neutralized with 1 ml Tris. ATP content of the effluent was determined enzymatically (30) and a portion of the reaction mixture was extracted with chloroform and hexokinase to quantitatively transfer the phosphorus of ATP to form glucose-6-P and washed with magnesium(NOD) as described by Stull and Bues (21). Amino acid analysis of the dialyzed and dried portions of the enzyme was conducted by AA Laboratories, Mercer Island, WA. Results are the mean of three determinations. Routine estimation of protein during the purification of pyruvate kinase was done by the procedure of Lowry et al. (32), with 90% of applied [3P]pyruvate kinase was recovered from the gel after electrophoresis. Peptide fragment analysis of radiolabeled pyruvate kinase was accomplished by digesting the protein with chymotrypsin (1 ng/ml in 2% SDS) and electrophoresis in 15% polyacrylamide gels as described by Cleveland et al. (24). Radioactivity in various bands of polyacrylamide gels was measured by cutting the gels into 2-mm slices, dissolving the slices in 0.2 ml of 2% periodic acid, and counting in 4 ml of aqueous counting fluid (ACS, Amersham). Isoelectric focusing was carried out in the presence of 8 M urea using the procedure of O'Farrell (25). The pH 3–10 range Ampholines were not supplemented with pH 6–8 Ampholines as recommended by O'Farrell (25). Elimination of the supplement resulted in a nonlinear pH gradient but enhanced the resolution of the various bands found in preparations of purified pyruvate kinase.

**In Vitro Phosphorylation of Purified Pyruvate Kinase—**Phosphorylation of purified pyruvate kinase was carried out at pH 7.6 in 3×M Tris/HCl, 0.97 mM [γ-3P]ATP (2.2 x 10^5 cpm/mmol), 0.08 mM cAMP, 10 mM MgCl_2 using cAMP-dependent protein kinase from bovine heart (Sigma Chemical Co.). The final incubation volume was 0.1 ml. After incubation, 0.025 ml aliquots were spotted on paper filter discs and placed in 10% trichloroacetic acid. Protein-bound radioactivity was then determined after washing the discs as described by Corbin and Reimann (26). Standard [γ-3P]pyruvate kinase was prepared in the same manner except that the radioactive enzyme was precipitated with 2.6 mM ammonium sulfate to remove excess ATP. [γ-3P]ATP was obtained commercially (New England Nuclear) or synthesized by the procedure of Penevsky (27) and purified by Dowex-1 Cl chromatography as described by Glynn and Chappell (28).

**Specific Radioactivity of ATP—**The specific radioactivity of phosphoenolpyruvate was determined by the procedure of Lowry et al. (29). The dilute acid solvent was chosen for this purpose to minimize dephosphorylation of the enzyme. Endogenous phosphate of ATP to form glucose-6-P. The reaction products were applied to a DEAE-cellulose column (5 ml bed volume) of DEAE-cellulose. The wash was extended by adding 0.3 mm NaCl to remove monophosphorylated intermediates. ATP was eluted with 0.2 n HCl and the ATP-containing fraction was rapidly neutralized with 1 ml Tris. ATP content of the effluent was determined enzymatically (30) and a portion of the reaction mixture was extracted with chloroform and hexokinase to quantitatively transfer the phosphorus of ATP to form glucose-6-P and washed with magnesium(NOD) as described by Stull and Bues (21). Amino acid analysis of the dialyzed and dried portions of the enzyme was conducted by AA Laboratories, Mercer Island, WA. Results are the mean of three determinations. Routine estimation of protein during the purification of pyruvate kinase was done by the procedure of Lowry et al. (32).
Hepatocytes from a fed rat were preincubated for 40 min with \[^{32}P\] orthophosphate. At 40 min, control solvent or 1 \(\mu\)M glucagon was added and the incubation was continued for 5 min. Extracts of the hepatocytes were incubated with 0.1 ml of anti-pyruvate kinase resin (Ab) or control resin (C) prepared with pre-immune serum. The activity of pyruvate kinase was measured before and after binding for 1 h. The amount of enzyme bound to the resin was calculated from these measurements. The washed resins were eluted with 2% SDS and the eluent was counted for radioactivity. Recovery of pyruvate kinase through the procedure was assessed by mixing purified \[^{32}P\] pyruvate kinase (originally 451 cpm/unit) with unlabeled tissue extract and carrying out the entire process. Recovery of the labeled purified enzyme is given in parentheses. Similar results were obtained in several other preparations of hepatocytes.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Glucagon</th>
<th>Resin</th>
<th>Pyruvate kinase added</th>
<th>Pyruvate kinase bound</th>
<th>cpm bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified [^{32}P]pyruvate kinase</td>
<td>Ab</td>
<td>6.6</td>
<td>0.5</td>
<td>6.1 (2751 cpm)</td>
<td>2592 (94%)</td>
</tr>
<tr>
<td>Purified [^{32}P]pyruvate kinase</td>
<td>Ab</td>
<td>6.6</td>
<td>0.6</td>
<td>6.0 (2706 cpm)</td>
<td>2625 (97%)</td>
</tr>
<tr>
<td>Cells</td>
<td>−</td>
<td>C</td>
<td>3.4</td>
<td>3.4</td>
<td>0</td>
</tr>
<tr>
<td>Cells</td>
<td>+</td>
<td>C</td>
<td>3.9</td>
<td>3.9</td>
<td>0</td>
</tr>
<tr>
<td>Cells</td>
<td>−</td>
<td>Ab</td>
<td>3.5</td>
<td>0.1</td>
<td>3.4</td>
</tr>
<tr>
<td>Cells</td>
<td>+</td>
<td>Ab</td>
<td>3.0</td>
<td>0.2</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Results

Rapid Isolation of \(^{32}P\)-labeled Pyruvate Kinase from Hepatocytes—To study the phosphorylation of pyruvate in the intact cell, hepatocytes were incubated with \[^{32}P\] orthophosphate. Radiolabeled pyruvate kinase was rapidly and quantitatively isolated from cell extracts using a one-step resin-coupled antibody procedure. As a control for non-specific binding, paired portions of cell extracts were reacted with control resins prepared with pre-immune serum which does not neutralize the pyruvate kinase activity (Table II). Specific binding of radiolabeled pyruvate kinase to the antiserum resin was calculated by subtracting non-specific binding (control resins) from total binding (antibody resins). This specific binding of radioactivity was then used to estimate the molar extent of phosphorylation. Non-specific binding of radioactivity to control resins was low compared to total radioactivity seen with the antibody resins (Table II). Recovery of pyruvate kinase through the procedure assessed by mixing purified \[^{32}P\]pyruvate kinase to tissue extracts was routinely found to be quantitative (Table II).

The radiolabeled proteins obtained from the antibody resins were subjected to SDS-polyacrylamide gel electrophoresis. Essentially, only one radiolabeled protein peak which migrated at the position of purified pyruvate kinase was observed (Fig. 1). To further characterize the radiolabeled protein obtained using the antibody resins, the extracts were digested with chymotrypsin in the presence of 2% SDS to form radio-labeled peptide fragments as described by Cleveland et al. (24). The peptide fragments separated by electrophoresis exhibited one major peptide fragment which was phosphorylated in response to glucagon or epinephrine. This major peptide fragment migrated at the same position as the labeled peptide obtained from the enzyme phosphorylated in vitro using a cAMP-dependent protein kinase (Fig. 1). The amount of radioactivity obtained in the pyruvate kinase band from hepatocytes incubated with no hormone was very low and peptide analysis of this condition was not routinely done.

Specific Radioactivity of ATP in Hepatocytes Incubated with \[^{32}P\]Orthophosphate—Upon addition of \[^{32}P\]orthophosphate to hepatocytes, a time-dependent increase in the specific radioactivity of the phosphate in the \(\gamma\) position of

\footnote{K.-H. Kim, personal communication.}
ATP is observed for approximately 30 min (Fig. 2). After that time, the specific radioactivity reached a steady state which was maintained for at least another 30 min. In 14 preparations of hepatocytes, the specific radioactivity of $[\gamma^{32}P]$ATP determined at steady state represented 54 ± 4% of the initial specific radioactivity of the phosphate added in the incubation medium. Addition of glucagon or epinephrine after preincubation of the hepatocytes with $[^{32}P]$orthophosphate for 40 min had no influence on the steady state specific radioactivity of ATP (Fig. 2).

**Basal Phosphorylation and Turnover of $[^{32}P]$Pyruvate Kinase**—Incubation of hepatocytes with $[^{32}P]$orthophosphate for 50-60 min in the absence of hormonal stimuli was consistently accompanied by a low level incorporation of radioisotope into pyruvate kinase although the high activity ratio of the enzyme was maintained. The basal incorporation of isotope into the enzyme slowed as the specific radioactivity of the ATP pool reached steady state (Fig. 2), suggesting that a small amount of phosphate is present in the enzyme and that it is in equilibrium with the ATP pool of the cell. Turnover of phosphate in the enzyme is readily apparent in hepatocytes to which glucagon is added at the same time as $[^{32}P]$orthophosphate (Fig. 2). In this situation, the kinetic response of the enzyme to glucagon was immediate; however, incorporation of isotope into the enzyme increased with time as the specific radioactivity of ATP increased. This observation further suggests that phosphorylation of the enzyme is not static. Rather, continuous phosphorylation-dephosphorylation of the enzyme must occur. Thus, changes in radiolabeled phosphate in the enzyme should reflect both a change in the steady state level of phosphorylation and the specific radioactivity of the ATP pool.

To estimate the molar extent of phosphorylation of pyruvate kinase, hepatocytes were routinely incubated with $[^{32}P]$orthophosphate for 40 min to allow the specific radioactivity of ATP to reach steady state. Incorporation of radiolabel into the enzyme and the specific radioactivity of $[\gamma^{32}P]$ATP were then determined. The extent of phosphorylation of the enzyme on a molar basis was estimated assuming measurements of the enzyme activity reflect the enzyme concentration (200 units/mg; see below). The results of several experiments examining the influence of 1 µM glucagon and 10 µM epinephrine on the kinetic activity ratio and phosphorylation state of pyruvate kinase in hepatocytes from fed rats using these assumptions are summarized in Table III. As previously noted, we consistently observed a low level of phosphorylation of the enzyme in hepatocytes incubated in the absence of hormones (approximately 0.4 mol of phosphate/mol of enzyme). Administration of 1 µM glucagon was accompanied by a 60% decrease in the activity ratio and a 4-fold increase in phosphorylation (to 1.7 mol of phosphate/mol of enzyme). On the other hand, 10 µM epinephrine (a weaker inhibitor of the enzyme) gave less than a 3-fold increase in the phosphorylation state (to 1.1 mol of phosphate/mol of enzyme).

Phosphorylation of pyruvate kinase in hepatocytes from fed rats in response to glucagon was quite rapid (Fig. 2) with half-maximal phosphorylation occurring with approximately 0.1 µM glucagon (Fig. 3). In two preparations of hepatocytes from rats fasted for 24 h, we found the quantitative extent of phosphorylation of pyruvate kinase to be essentially the same as that observed with hepatocytes from fed rats (Fig. 3).

**Kinetic, Immunologic, and Physical Properties of Purified Pyruvate Kinase**—The kinetic properties of purified pyruvate kinase were determined at pH 7.4 and 25 °C under conditions identical with those used for studying the enzyme in extracts from hepatocytes (19). In the presence of 100 µM Fru-1,6-P$_2$, the specific activity observed in five preparations of rat liver pyruvate kinase ranged from 190 to 230 units/mg. In the absence of Fru-1,6-P$_2$, the enzyme activity exhibited a sigmoidal dependence on P-enolpyruvate concentration with a half-maximal activity at 1.5 mM P-enolpyruvate and a Hill coefficient of 1.9 (see Table IV). Other kinetic properties examined are also summarized in Table IV. The kinetic prop-

**TABLE III**

Summary of pyruvate kinase phosphorylation in response to glucagon and epinephrine

<table>
<thead>
<tr>
<th>Addition</th>
<th>Activity ratio</th>
<th>Phosphate content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pMol/Unit</td>
<td>mol/250,000 g</td>
</tr>
<tr>
<td>Control (6)</td>
<td>0.58 ± 0.03</td>
<td>8.4 ± 0.6</td>
</tr>
<tr>
<td>Glucagon (6)</td>
<td>0.20 ± 0.02</td>
<td>34.0 ± 2.1</td>
</tr>
<tr>
<td>Epinephrine (5)</td>
<td>0.42 ± 0.04</td>
<td>22.7 ± 2.3</td>
</tr>
</tbody>
</table>
FIG. 3. Phosphorylation of pyruvate kinase incubated with various concentrations of glucagon. Hepatocytes from a fed rat (●, ○) and a rat starved 24 h (●, △) were preincubated 40 min with [32P]orthophosphate. Various concentrations of glucagon were added to the medium and the incubation was continued for 5 min. Radiolabeled pyruvate kinase, [γ-32P]ATP, and the activity ratio for pyruvate kinase activity at 1.8 mM P-enolpyruvate (open symbols) were determined. The phosphorylation state of the enzyme was calculated from these measurements (as picomoles of phosphate/unit of enzyme activity) and is presented (solid symbols).

TABLE IV
Kinetic properties of dephospho- and phosphopyruvate kinase

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dephosphopyruvate</th>
<th>Phosphopyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ (units/mg)</td>
<td>193</td>
<td>193</td>
</tr>
<tr>
<td>Phosphate content (mol/250,000 g)</td>
<td>0.34 ± 0.08 (4)</td>
<td>2.02 ± 0.09 (4)</td>
</tr>
<tr>
<td>$K_{M}$ for P-enolpyruvate (mM)</td>
<td>1.1</td>
<td>1.9</td>
</tr>
<tr>
<td>$K_{M}$ for Fru-1,6-P$_2$ (mM)</td>
<td>0.08</td>
<td>0.1</td>
</tr>
<tr>
<td>$K_{M}$ for ADP (mM)</td>
<td>0.56</td>
<td>0.46</td>
</tr>
<tr>
<td>$K_{M}$ for [Ala]$_3$ (μM)</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>$K_{M}$ for [ATP]$_{1/2}$ (mM)</td>
<td>12.0</td>
<td>2.8</td>
</tr>
</tbody>
</table>

* At 50 μM Fru-1,6-P$_2$.
* At 0.27 mM P-enolpyruvate.
* At 3.6 mM P-enolpyruvate.

Properties found for the enzyme purified by the modification of the procedure of Harada et al. (22) compare very well with those determined previously for the enzyme in fresh liver extracts (19, 21). Immunologically, the purified enzyme is also similar to that in fresh liver extracts. For three preparations of the purified enzyme, neutralization of the activity with goat antiserum required 15.5 ± 0.6 μl of serum/unit of pyruvate kinase activity. Immunotitration of the enzyme activity in liver extracts required 15.9 ± 0.3 μl of antiserum/unit (n = 42). The similarity in the immunologic properties of the enzyme from the two sources supports the suggestion that major proteolytic or other modification does not occur during purification (Table I).

Purified rat liver pyruvate kinase exhibited a single Coomassie blue-staining band after polyacrylamide gel electrophoresis in the presence of SDS (Fig. 4). The subunit molecular weight estimated from SDS-polyacrylamide gel electrophoresis was 62,250 (mean of seven determinations, data not shown). The minimum molecular weight estimated from amino acid analysis was 64,100 based on tryptophan content. Assuming 4 mol of tryptophan/subunit, the subunit molecular weight would be 66,600, a value which agrees well with estimates from SDS-polyacrylamide gel electrophoresis. The absorbivity of the enzyme in the ultraviolet spectrum is low due to the relatively low content of aromatic amino acids. The molar extinction coefficient

Hepatic Pyruvate Kinase

Electrophoresis

Isoelectric Focusing

pH 10 →

pH 6 →

pH 3 →

A B

C D E

Fig. 4. SDS-polyacrylamide gel electrophoresis and isoelectric focusing of purified rat liver pyruvate kinase. Purified rat liver pyruvate kinase was subjected to SDS-polyacrylamide gel electrophoresis using 7.5% acrylamide gels as described under "Materials and Methods." A, 10 μg of protein; B, 25 μg of protein. The enzyme was phosphorylated using cAMP-dependent protein kinase from beef heart to obtain maximum phosphorylation. The phosphorylated form (C) and phosphorylated form (E) were subjected to isoelectric focusing as described under "Materials and Methods." A mixture of the phosphorylated and dephosphorylated forms of the enzyme was also separated by isoelectric focusing (D).
at 280 nm was calculated to be $1.25 \times 10^8 \text{ M}^{-1}$, assuming the enzyme to be a tetramer composed of subunits with $M_r = 82,500$. The molar concentration of pyruvate kinase for the physical studies described below was determined using this extinction coefficient. Determination of the protein concentration of the purified enzyme in solution by the procedure of Lowry et al. (32), however, gave nearly identical results. Isoelectric focusing of the purified enzyme in the presence of 8 M urea revealed five Coomassie blue-staining protein bands, indicating a microheterogeneity not revealed by electrophoresis. The major band on electrofocusing was found at approximately pH 6.4. After phosphorylation with a CAMP-dependent protein kinase, this major band almost completely moved to pH 6.2. (Precise identification of the isoelectric pH of the various bands has been difficult due to the shallow pH gradient formed under the conditions applied.) Three other protein bands revealed by isoelectric focusing do not appear to be influenced by phosphorylation.

Although isoelectric focusing revealed structural heterogeneity in purified pyruvate kinase, each of the enzyme subunits appears capable of binding the allosteric activator, Fru-1,6-P$_2$. Fru-1,6-P$_2$ binding exhibited a sigmoidal dependence on Fru-1,6-P$_2$ concentration with half-maximal binding observed at approximately 1 mM Fru-1,6-P$_2$ (Fig. 5). The Scatchard plot of the binding data (see inset, Fig. 5) exhibited a bell-shaped curve, indicating positive cooperativity. Maximum binding of Fru-1,6-P$_2$ concentration with half-maximal binding observed at approximately 1 mM Fru-1,6-P$_2$ (Fig. 5). The Scatchard plot of the binding data (see inset, Fig. 5) exhibited a bell-shaped curve, indicating positive cooperativity. Maximum binding was found to be $3.68 \pm 0.40 \text{ mol of Fru-1,6-P}_2/250,000 \text{ g of pyruvate kinase (n = 5), indicating 1 binding site/subunit.}

**Phosphorylation of Purified Pyruvate Kinase—Chemical analysis of purified pyruvate kinase indicated a low endogenous organic phosphate content of 0.34 ± 0.08 mol/250,000 g of pyruvate kinase (n = 4). Maximum phosphorylation of the enzyme was accomplished by incubating the purified enzyme with ATP and CAMP in the presence of high concentrations of CAMP-dependent protein kinase from beef heart (Fig. 6). Complete phosphorylation was rapidly achieved at 37 °C with a protein kinase/pyruvate kinase ratio of 0.16 (Fig. 6). Increasing the amount of protein kinase above this ratio gave no further phosphorylation. With four separate preparations of pyruvate kinase, maximum phosphorylation resulted in a final phosphate content of 2.02 ± 0.09 mol of phosphate/250,000 g (Table IV). Isotopic determination of this extent of phosphorylation was confirmed by chemical phosphate analysis (not shown). Maximum phosphorylation of the enzyme caused an increase in the concentration of P-enolpyruvate required for half-maximal activity and an increase in the Hill coefficient ($n_H$, Table IV). Other kinetic properties of the enzyme were also influenced by phosphorylation (Table IV). Alkali-denatured pyruvate kinase is also phosphorylated by the CAMP-dependent protein kinase. Maximum phosphorylation, however, was not increased upon denaturation, indicating potential phosphorylation sites are not buried in the native enzyme.

**Correlation of Phosphorylation and Activity Ratio for Pyruvate Kinase—**To examine the relationship between phosphorylation of purified pyruvate kinase and the activity of the enzyme, partial phosphorylation was accomplished by incubating the purified enzyme with low concentrations of protein kinase for various time periods. Portions of the reaction mixture were taken for determination of phosphate content and another portion was used to measure the activity ratio at 1.8 mM P-enolpyruvate. As demonstrated in Fig. 7, an inverse linear relationship between the activity ratio and phosphate content ranging from 0.3 to 2.0 mol of phosphate/250,000 g was observed. Phosphorylation greater than 2 mol/250,000 g was not observed in the present investigation. The relationship between phosphorylation of pyruvate kinase and the activity ratio for the enzyme found with the purified enzyme is nearly identical with that found in studies with the intact hepatocyte (see Fig. 7 and Table III).

![Fig. 5. Fructose-1,6-bisphosphate binding to purified rat liver pyruvate kinase.](image-url)

**Fig. 5.** Fructose-1,6-bisphosphate binding to purified rat liver pyruvate kinase. 0.91 μM pyruvate kinase (tetramer) was incubated with various concentrations of [14C]Fru-1,6-P$_2$. The bound (B) and free (F) Fru-1,6-P$_2$ concentrations were determined by a nitrocellulose disc technique described under "Materials and Methods." The binding to Fru-1,6-P$_2$ to pyruvate kinase at various concentrations of free Fru-1,6-P$_2$ is presented. Scatchard analysis (inset) of the binding results was used to determine maximum binding.
phosphorylated events. Our findings are not in agreement with those of El-Maghrabi and phosphorylation state of the enzyme determined by CAMP-dependent protein kinase. In their investigation, phosphate analysis for rat liver pyruvate kinase showing up to 2 mol of phosphate/mol of pyruvate kinase after incubation with ATP and a CAMP-dependent protein kinase (Fig. 6, Table IV). Our results, however, are in agreement with their findings in that the complete kinetic response occurred with insertion of only 2 mol of phosphate in response to a CAMP-dependent protein kinase. El-Maghrabi et al. stated (see Ref. 14, p. 672) that “higher concentrations of protein kinase” were used to obtain phosphorylation at the fourth and fifth sites and that this extensive phosphorylation was not accompanied by further kinetic changes in the enzyme. It is possible that the extensive level of phosphorylation observed by those workers was due to phosphorylation at multiple sites on a subunit. Unfortunately, peptide fragment analysis was not carried out in their investigation to test that possibility. We found that very high concentrations of the CAMP-dependent protein kinase from beef heart gave no more than 2 mol of phosphate/mol of enzyme (Fig. 6). All attempts to obtain more extensive phosphorylation were unsuccessful. The inability to attain phosphorylation beyond 2 P/tetramer provides evidence for heterogeneity in the subunits which is substantiated by isoelectric focusing (Fig. 4). Although we do not currently understand the biochemical basis for this heterogeneity, the assumption that the enzyme is a tetramer composed of identical subunits must be re-evaluated.

We were concerned that the heterogeneity reflected proteolytic modification of the enzyme during the purification. The results from studies with the intact hepatocyte, however, would suggest that nonphosphorylatable forms of the enzyme also exist within the cell. This conclusion is based on the finding of somewhat less than 2 mol of phosphate/mol of pyruvate kinase after maximum regulation by glucagon (Table III). Ishibashi and Cottam (16) reported studies with rat hepatocytes suggesting that maximum regulation of the enzyme by glucagon was accompanied by less than 0.3 mol of phosphate/mol of pyruvate kinase, even less than that found in the present study. Those workers, however, did not directly measure the specific radioactivity of ATP but assumed that it was the same as the specific radioactivity of phosphate originally added to the cell suspension. The present investigation demonstrated that the steady state specific radioactivity of phosphate in the y position of ATP may be considerably less than that of [32P]orthophosphate added to the cells. Thus, the estimates of Ishibashi and Cottam (16) probably represent an underestimate of phosphorylation of the enzyme. Ljungstrom and Ekman (17) reported up to 3 mol of phosphate/mol of pyruvate kinase after administration of glucagon to rat liver slices. As previously discussed, early estimates of pyruvate kinase concentrations from that laboratory assumed $A_{260}$ = 1 and this value was used to calculate phosphorylation of the enzyme. The high extent of phosphorylation in liver slices calculated by those workers thus may represent an overestimate of the response to glucagon. Riou et al. (18) reported that administration of glucagon to the rat is accompanied by incorporation of approximately 1.4 mol of phosphate/mol of pyruvate kinase, a value very close to that found in the present study.

Although several assumptions are required to estimate the quantitative extent of phosphorylation of pyruvate kinase in the intact cell, the excellent agreement between results obtained with the intact cell and those from studies with the purified enzyme (Fig. 7) would suggest that the isotopic estimates provide a reasonable estimate of the phosphorylation state of the enzyme in the intact cell. Estimates with the intact cells require only two measurements taken from studies

![Diagram](http://www.jbc.org/)
with the purified enzyme. First, the molecular weight of the subunits was taken from SDS-polyacrylamide gel electrophoresis. Secondly, the relationship between enzyme concentration and its activity (200 units/mg) was taken from the value determined using the purified enzyme. The estimates of the enzyme molecular weight by electrophoresis appear to be straightforward and are supported by amino acid analysis. The extrapolation of the specific activity of the purified enzyme to the intact cell, however, must be scrutinized. Although several laboratories have reported a specific activity for rat liver pyruvate kinase in the range of 200 units/mg (5, 34), a wide range of values is available in the literature (7, 14, 22). It is difficult to compare the specific activity measured in various laboratories directly because of different conditions used to analyze the enzyme activity and to measure its concentration. The close correlation of the kinetic properties of the purified enzyme (Table IV) to those described for the enzyme in fresh extracts of hepatocytes in this laboratory under identical conditions (19, 21) supports the extrapolation of the specific activity determined for the purified enzyme to studies with tissue extracts. This conclusion is further supported by immunotitration of the enzyme from both sources (Table I).

The results of the present investigation strongly suggest that a significant fraction of Type L pyruvate kinase present within the hepatocyte is not phosphorylated in response to a fully effective dose of glucagon. This evidence for heterogeneity in the enzyme subunit composition supports previous observations concerning heterogeneity in the native enzyme (15, 37). We can only speculate at this time as to the basis for the presence of nonphosphorylatable forms of the enzyme. The present investigation does not distinguish if the heterogeneity reflects a mixed population of subunits within the enzyme tetramer or if two (or more) populations of the enzyme exist within the hepatocyte. Bergström et al. (35) and Ekman and Eriksson (36) reported that rat liver pyruvate kinase is susceptible to limited proteolysis resulting in removal of the phosphorylation site but with retention of catalytic activity. Those investigators further found that phosphorylation of the enzyme enhances susceptibility to proteolysis. Hall et al. (37) reported the existence of low molecular weight subunits of pyruvate kinase purified from fasted rats and that these subunits cannot be phosphorylated by a cAMP-dependent protein kinase. On the basis of these observations, the suggestion has been made that phosphorylation serves as a signal for proteolytic degradation of the enzyme (35, 37, 38). Red cell pyruvate kinase which is immunologically similar to the liver enzyme has been reported to undergo a series of proteolytic modifications during maturation of the reticulocyte. These proteolytic modifications alter the capacity of the enzyme for phosphorylation by a cAMP-dependent protein kinase (39, 40). Whether control of red cell pyruvate kinase can be related to that for the liver enzyme, however, is not clear at this time. The importance of proteolytic modification of liver pyruvate kinase to the physiological regulation of liver carbohydrate metabolism remains to be established.

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