Homogeneous preparations of rat liver L-type pyruvate kinase were found to contain about 3 mol of phosphate/mol of enzyme and were inactive as judged by a $K_{d5}$ for P-enolpyruvate of 1.2 mM. In the course of studies on the phosphate content of these purified preparations, it was shown that cyclic AMP-dependent protein kinase catalyzed the dephosphorylation of phospho- pyruvate kinase in the presence of MgADP. The equilibrium constant for this reverse reaction was 0.045. From this value, the free energy of hydrolysis ($AG^{0}_{	ext{MgADP}}$) of the phosphoprotein bond was calculated to be $-6.6$ kcal mol$^{-1}$. Using the reverse reaction, all but 1 mol of phosphate/mol of enzyme could be removed and the enzyme was activated with a $K_{d5}$ for P-enolpyruvate of 0.7 mM. The dephosphorylated enzyme could now be phosphorylated by protein kinase to the extent of 4 mol of $^{32}$P/mol of enzyme. This phosphorylated enzyme was inactive with a $K_{d5}$ for P-enolpyruvate of 1.4 mM. Thus, a large portion of the phosphate found in purified pyruvate kinase preparations was present in regulatory sites that are phosphorylated by the cyclic AMP-dependent protein kinase. One mole of P$_i$ per mol of enzyme was present in a site that is apparently not phosphorylated by cyclic AMP-dependent protein kinase. Changes from 90% to 40% of maximal enzyme activity occurred when the amount of enzyme-phosphate increased from 1.5 mol to 3 mol/mol of enzyme.

Physiological concentrations of the allosteric effectors, fructose bisphosphate and P-enolpyruvate, inhibited the rate of phosphorylation catalyzed by protein kinase of the dephosphorylated pyruvate kinase (1 mol of P$_i$/mol of enzyme). The negative allosteric effector, alanine, had only a small stimulatory effect by itself, but it relieved the inhibition by fructose bisphosphate and P-enolpyruvate. Much smaller effects of the various allosteric effectors were seen when pyruvate kinase containing 3 mol of P$_i$/mol of enzyme was used. These results suggest that allosteric effectors, in addition to regulating pyruvate kinase activity directly, may also act indirectly by modulating the phosphorylation state of the enzyme.

The activity of type L pyruvate kinase from rat liver can be inhibited by a cyclic AMP-dependent protein kinase-catalyzed phosphorylation reaction (1-6). Phosphorylation decreases the affinity of the enzyme for one of its substrates, P-enolpyruvate (1, 2, 6). Based on isotopic experiments, it has been estimated that protein kinase catalyzes the incorporation of 1 mol of phosphate/mol of pyruvate kinase subunit or 4 mol/mol of the tetrameric enzyme (1). However, the precise relationship of this phosphorylation to changes in enzyme activity is not known, principally because the amount of chemical phosphate present in pyruvate kinase preparations has not been determined. It is also not known whether the enzyme contains phosphate in sites that are not phosphorylated by cyclic AMP-dependent protein kinase.

In the present study, the amount of covalently bound phosphate has been determined in pyruvate kinase preparations purified to homogeneity from rat liver. In addition, we have found that the protein kinase-catalyzed phosphorylation of pyruvate kinase to be reversible. We have made use of this reverse reaction to study the relationship between the phosphorylation state of pyruvate kinase and its activity. The influence of various effectors of pyruvate kinase on enzyme phosphorylation was also investigated.

**Experimental Procedures**

**Methods**

**Purification of Hepatic Pyruvate Kinase**—The L-type enzyme was purified from rats that were fasted overnight and then fed a high carbohydrate diet for 72 h as previously described (7). The final elution of pyruvate kinase from the Blue Sepharose column was carried out using buffer that contained 16 mM P-enolpyruvate, 10 mM ADP, and 2 mM fructose 1,6-bisphosphate. The purified enzyme had a specific activity of about 300 units/mg of protein and showed a single band with a subunit molecular weight of 57,000 by sodium dodecyl sulfate disc gel electrophoresis. The purified enzyme preparations were dialyzed against 20 mM Tris-Cl, pH 7.0, 20% (v/v) glycerol, 2 mM 2-mercaptoethanol, 1 mM MgCl$_2$, and 0.1 mM phenylmethylsulfonyl fluoride (Buffer 1) in order to remove P-enolpyruvate, ADP, and fructose bisphosphate. They were then stored in the same buffer at 0-4°C for up to 1 month.

**Purification of the Catalytic Subunit of Rat Hepatic Cyclic AMP-Dependent Protein Kinase**—The procedure used was a modification of that described by Sugden et al. (8) for bovine liver. All buffers contained 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride unless otherwise mentioned. Livers from 20 to 30 rats were homogenized in a Waring Blender (3 x 30 s) in 3 to 4 volumes of 10 mM potassium phosphate, pH 7.5. The homogenate was centrifuged for 30 min at 30,000 $g$ and the resulting supernatant fraction filtered through glass wool and centrifuged for 60 min at 100,000 $g$. This fraction was then added to 400 ml of settled DEAE-Sephacel II buffer which was equilibrated with homogenizing buffer. The resulting slurry was stirred continually for 90 min and then washed in a Buchner funnel with 5 liters of homogenizing buffer and poured into a column (4.5 x 40 cm). Washing was continued until a constant $A_{280}$ was obtained. The catalytic subunit of cyclic AMP-dependent protein kinase was then eluted from the column with buffer containing 100 mM KCl. The pool of enzyme eluting at 0.2 M KCl was dialyzed for 6 h against Buffer 1 and then concentrated to 1 ml by using a Centricon-30 filter. The protein kinase-catalyzed phosphorylation of pyruvate kinase was inhibited by 100 mM KCl. The dialyzed enzyme was used in these studies for its ability to catalyze the reverse reaction and to increase the rate of release of phosphate from the pyruvate kinase-phosphate bond.

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† Investigator of the Howard Hughes Medical Institute.

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From the Department of Physiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232
protein kinase was eluted with 40 mM potassium phosphate, pH 6.3, that contained 0.1 mM cyclic AMP. The fractions that contained enzyme activity were pooled and applied to a hydroxylapatite (Bio-Gel HTP, Bio-Rad) column (2.5 x 7.5 cm) equilibrated with 40 mM potassium phosphate, pH 6.8. The column was washed with 2 bed volumes of buffer, then with 100 ml of 100 mM potassium phosphate, pH 6.8, and 600 ml of 350 mM potassium phosphate, pH 6.8. The fractions that contained enzyme activity, eluting at about 200 mM potassium phosphate, were pooled and concentrated by ultrafiltration (Diaflo PM10 membranes, Amicon) to 1 to 2 ml. It was then applied to a Sephadex G-100 superfine (Pharmacia) column (1.5 x 90 cm) equilibrated with 350 mM potassium phosphate, pH 6.8, that contained 1 mM diethiothreitol. After the enzyme fractions were concentrated by ultrafiltration, the gel filtration step was repeated. This procedure yielded a preparation of catalytic subunit with a specific activity of approximately 2 x 10^5 units/mg of protein. One unit of activity is defined as the amount of enzyme catalyzing the transfer of 1 pmol of ATP from [γ-32P]ATP to histone/min at 30°C. Only one protein band was detected after sodium dodecyl sulfate disc gel electrophoresis (data not shown). The purified catalytic subunit preparations were routinely concentrated by ultrafiltration to approximately 1 x 10^6 units/ml and stored at 0-4°C. The enzymatic properties of this catalytic subunit from both type I and type II isoforms of the rat liver cyclic AMP-dependent protein kinase will be referred to and will be discussed in the text of the enzyme throughout the text.

Pyruvate Kinase Assay—Enzyme activities were measured by a modification (10) of the method of Llerente et al. (9). The assay mixture contained 50 mM triethanolamine-HCl, pH 7.4, 10 mM KCl, 5 mM MgCl_2, 1 mM ADP, 0.15 mM NADH, 0.5 unit of lactate dehydrogenase, and various concentrations of P-enolpyruvate in a final volume of 1 ml. The assay mixture was incubated at 30°C for 5 min and 10 min, after the reaction started by the addition of enzyme. The results are expressed as the fraction of the maximum rate obtained with 4 mM P-enolpyruvate (v/v). The change in rate at submaximal concentrations of substrate was the best measurement of the interconversion of the active and inactive forms of the enzyme since they have the same V_max.

Pyruvate Kinase Activity Assay—The method of Corbin and Reinmann (10) was used for the measurement of cyclic AMP-dependent protein kinase activity except that the reaction mixture in a final volume of 50 μl contained 17 mM potassium phosphate, pH 6.8, 0.5 mg of histone (Sigma type IIA), 0.4 mM [γ-32P]ATP (20 to 500 pmol/ml), and 8 mM magnesium acetate. When the activity of liver homogenates or supernatant fractions were assayed, 1 μl cyclic AMP was also present. Incubations were done at 30°C and reactions started by the addition of enzyme. Twenty-microliter samples were removed at 10 and 20 min and applied to filter papers which were subsequently treated as previously described (10). The papers were counted for radioactivity by liquid scintillation in ACS (Amersham). Aliquots of the assay mixture were also counted to determine the specific radioactivity of the [γ-32P]ATP.

Phosphorylation of Rat L-type Pyruvate Kinase—Measurements of the rate of phosphorylation of pyruvate kinase catalyzed by protein kinase were carried out by incubating known concentrations of pyruvate kinase with 0.4 mM [γ-32P]ATP (200 to 500 pmol/ml), 5 mM MgCl_2, and 50 mM Tris HCl, pH 7, at 30°C, unless otherwise mentioned. Five- to ten-microliter aliquots of the assay mixture were removed at several time intervals and the incorporation of radioactive phosphate into pyruvate kinase determined as described for the assay of protein kinase activity.

Determination of Protein-bound Phosphate—The phosphate content of pyruvate kinase preparations was determined using the ashing procedure of Ames (11) and the phosphate assay of Itaya and Ui (12) except that 0.25 to 1 ml of enzyme protein) were added to 200 μg of bovine serum albumin and carried through the procedure only once instead of twice (13). The final concentration of trichloroacetic acid used was 20% (w/v) and the reaction was started by the addition of enzyme. Twenty-microliter samples were removed at several time intervals and the incorporation of radioactive phosphate into pyruvate kinase determined as described for the assay of protein kinase activity.

Activity of P-enolpyruvate kinase with 0.4 mM [γ-32P]ATP (200 to 500 pmol/ml), 5 mM MgCl_2, and 100 nmol glucose, and 10 units of hexokinase in Buffer I at pH 7 for 45 min or until the increase in the ratio of pyruvate kinase activities at 0 to 4 mM phosphoenolpyruvate had reached a constant value, i.e. maximal activation. The dephosphorylated enzyme, containing 0.7 to 1.0 mol of phosphate/mol of protein, was precipitated with 60% (NH_4)_2SO_4 in order to remove the ADP and glucose. After washing the precipitate once with 60% (NH_4)_2SO_4, the enzyme was resuspended in Buffer I. The enzyme was then phosphorylated to the extent of 4 mol of phosphate/mol of pyruvate kinase by incubation at 30°C for 60 min with 0.33 mM [γ-32P]ATP (400 to 1000 cpm/mmol), 5 mM MgCl_2, and 100 to 500 units of protein kinase. [γ-32P]Pyruvate kinase was separated from the protein kinase and [γ-32P]ATP by gel filtration on a Sephadex G-100 column (1.5 x 90 cm) (Pharmacia) equilibrated with Buffer I. The fractions were concentrated by ultrafiltration (Diaflo PM10 membranes, Amicon) and stored at 0-4°C. The [γ-32P]phosphorylated enzyme was stable for weeks and contained no endogenous phosphoprotein phosphatase activity and no detectable protein kinase activity.

Thin Layer Chromatography—The products of the dephosphorylation of [γ-32P]pyruvate kinase catalyzed by protein kinase in the reverse reaction were identified by thin layer chromatography on polyethyleneimine cellulose plates (Brinkmann MN PEI-cellulose 300) according to the method of Gonzalez and Geel (15). The chromagrams were cut into slices (20 x 1 cm) and the radioactivity counted by liquid scintillation in ACS cocktail (Amersham).

Calculation of Equilibrium Constant—For determination of the equilibrium constant (K_a) of the protein kinase-catalyzed reaction with pyruvate kinase as substrate, the following simplified equation was used:

\[
K_a = \frac{C_{ATP}}{C_{ADP}} \cdot \frac{C_{P_{\text{kinase}}}}{C_{\text{P_{kinase}}}}
\]

where C_{ATP} and C_{ADP} represent the concentrations of ATP and ADP, respectively, and C_{P_{\text{kinase}}} and C_{\text{P_{kinase}}} represent the concentrations of nonphosphorylated and phosphorylated forms of the enzyme substrate, respectively, at equilibrium. This expression is written for the reverse reaction and makes the assumption that all of the phosphorylation sites are equivalent.

Materials

Blue Sepharose CL-6B and DEAE-Sepharose were obtained from Pharmacia. Carrier-free [γ-32P]phosphoric acid was purchased from I.C.N. Pharmaceuticals and [γ-32P]ATP was prepared by the method of Walbo and Johnson (16). Yeast hexokinase and rabbit muscle lactate dehydrogenase were obtained from Boehringer Mannheim.

RESULTS

Phosphate Content and Kinetics of Purified Pyruvate Kinase—The chemical determination of covalently bound phosphate in eight different preparations of pyruvate kinase showed that the amount of phosphate ranged from 2 to 3.5 mol/mol of enzyme tetramer with a mean of 3.1 mol/mol of enzyme. It was found that all but 0.4 to 0.7 mol of phosphate/molecule of enzyme was alkaline labile. This enzyme will be referred to as native enzyme.

It has been reported that the activity of pyruvate kinase is inhibited by cyclic AMP-dependent phosphorylation (1, 4, 6). We investigated whether the phosphate found in the native enzyme is located in sites phosphorylated by cyclic AMP-dependent protein kinase by determining whether the enzyme was in a kinetically inactive form. The substrate dependency of a preparation of pyruvate kinase that contained 3.1 mol of P/mol of enzyme is shown in Fig. 1. The K_m for the enzyme with respect to P-enolpyruvate was 1.2 mM. When the native enzyme was incubated with protein kinase and ATP in vitro, an additional 1 mol of phosphate was incorporated/mol of enzyme, but the K_m for P-enolpyruvate only increased to 1.4 mM (Fig. 1). These K_m values are similar to those reported for pyruvate kinase that had been inactivated by a glucagon-induced phosphorylation mechanism in isolated hepatocytes (6). They suggest that endogenous phosphate was already present in the native enzyme in sites phosphorylated by protein kinase, i.e. regulatory sites.
In order to verify our hypothesis, it was necessary to demonstrate that removal of phosphate from the native enzyme resulted in its reactivation. Phosphate was removed from the enzyme by taking advantage of the reversibility of the protein kinase reaction (17). This reverse reaction with pyruvate kinase as substrate would be:

\[
P\text{-pyruvate kinase} + \text{ADP} \xrightleftharpoons{\text{protein kinase}} \text{pyruvate kinase} + \text{ATP}
\]

When the native enzyme that contained 3.1 mol of P\textsubscript{i}/mol of enzyme was incubated with Mg\textsubscript{ADP} and protein kinase, its phosphate content was reduced to 0.7 mol/mol of enzyme. The P\textsubscript{5}enolpyruvate concentration curve of the dephosphorylated enzyme was shifted to the left (Fig. 1) and the \(K_m\) for its substrate was reduced to 0.7 mM. This value is similar to that of the enzyme from rat hepatocytes incubated in the absence of glucagon (6). When the dephosphorylated enzyme was incubated with \([\gamma\text{-}^{32}\text{P}]\text{ATP}\) and protein kinase, 3.4 mol of \(^{32}\text{P}\) were incorporated/mmol of enzyme (Fig. 2) and the \(K_m\) for P\textsubscript{5}enolpyruvate was increased to 1.4 mM (data not shown). The initial rate of phosphorylation of the dephosphorylated enzyme was about 50-fold greater than that of the native enzyme. Thus, pyruvate kinase, as isolated by this method, contains up to 2 mol of P\textsubscript{i}/mol of enzyme in the regulatory site(s) that is phosphorylated by the cyclic AMP-dependent protein kinase.

**Characterization of the Reverse Reaction**—We have characterized the reversal of the protein kinase reaction with \([\gamma\text{-}^{32}\text{P}]\text{pyruvate kinase}\) as substrate. The requirement of ADP in the reaction is shown in Fig. 3. There was no loss of \(^{32}\text{P}\) from pyruvate kinase in the absence of ADP, while over 80\% of the radioactivity in the enzyme was lost in 15 min in the presence of 5 mM ADP. The \(K_m\) for ADP was about 200 \(\mu\text{M}\). The dephosphorylation of pyruvate kinase was completely blocked by the heat-stable inhibitor of cyclic AMP-dependent protein kinase (Fig. 3).

The nucleotide product of the reverse reaction with \([\gamma\text{-}^{32}\text{P}]\text{pyruvate kinase}\) as substrate was identified by thin layer chromatography (Table I). In the absence of ADP, the radioactivity remained bound to the enzyme. In the presence of ADP, there was a quantitative recovery of the radioactivity lost from \([\gamma\text{-}^{32}\text{P}]\text{pyruvate kinase}\) in ATP. In order to confirm that the labeled phosphate was present at the \(\gamma\) position of ATP, hexokinase and glucose were included as an ATP trap. In this case, the radioactive phosphate appeared as glucose 6-phosphate. These results indicate that the dephosphorylation of pyruvate kinase was via reversal of the protein kinase reaction and not due to the presence of a phosphoprotein phosphatase activity.

The pH profile of the initial rate of dephosphorylation as well as phosphorylation of pyruvate kinase by protein kinase was also determined (Fig. 4). The rate of dephosphorylation was almost constant from pH 5 to 6.5, while the rate declined at lower or higher pH values. In contrast, the rate of phosphorylation increased slowly from pH 5 to 7.5, but then rose steadily from pH 7.5 up to the highest pH value tested, pH 9. A similar pH profile for the phosphorylation of pyruvate kinase was reported by Berglund et al. (18). At no pH value did the incorporation exceed 4 mol of \(^{32}\text{P}\)/mol of enzyme (data not shown), which indicated that there was no phosphorylation of nonspecific sites at high pH values. In contrast, the pH optimum for histone phosphorylation was between 7.0 and 7.5 (data not shown). The reason for the difference in pH profiles for the forward and reverse reaction is unknown, but a similar difference was observed for phosphorylase kinase-catalyzed phosphorylation-dephosphorylation of phosphorylase (19).

**Determination of the Equilibrium Constant of the Protein Kinase Reaction with Pyruvate Kinase as Substrate**—The equilibrium constant for the protein kinase reaction was determined using \([\gamma\text{-}^{32}\text{P}]\text{pyruvate kinase}\) (4 mol of \(^{32}\text{P}\)/mol of...
was prepared as described under "Methods." Twenty micrograms of ["P"]pyruvate kinase (500 cpm/pmol) was incubated with 10 mM MgCl₂, 100 mM glucose, and 0.2 unit of hexokinase at 2000 units of rabbit muscle protein kinase inhibitor of 200 units of protein kinase. The reaction started by the addition (W)

None 10,505
ADP 3,250
ADP + hexokinase + glucose 2,650
Hexokinase + glucose 10,450

Table I
Identification of radioactive product of the reverse reaction
["P"]pyruvate kinase was incubated with protein kinase as described in Fig. 3. Aliquots of the reaction mixture were subjected to thin layer chromatography and radioactivity in the various compounds was analyzed as described under "Methods." For the phosphorylation reaction (●), 20 µg of ["P"]pyruvate kinase prepared as described under "Methods" was incubated for 15 min at 30°C with 5 mM ADP, 10 mM MgCl₂, 100 mM glucose, 0.2 unit of hexokinase, and 100 mM concentration of the appropriate buffer. The reaction was started by the addition of 200 units of protein kinase. For the phosphorylation reaction (O, ○), 30 µg of the dephosphorylated pyruvate kinase (see "Methods") was incubated with 0.4 mM [gamma-32P]ATP (340 cpm/pmol), 5 mM MgCl₂, and 100 mM concentration of the appropriate buffer at 30°C for 5 min. The reaction was started by the addition of 3 units of protein kinase. The buffers used were sodium acetate, pH 4.5, pH 5, and pH 5.5; 3-(N-Morpholino)ethane sulfonic acid, pH 6, pH 6.5, and pH 7; N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.5, pH 8, pH 8.5, and pH 9.

The Activity of Pyruvate Kinase during Dephosphorylation and Rephosphorylation Catalyzed by Protein Kinase—

enzyme) and dephosphorylated pyruvate kinase (0.7 mol of P₈/mol of enzyme) for the reverse and the forward reactions, respectively (Table II). The time necessary to reach equilibrium in the reverse reaction was 2 to 3 h, while the forward reaction required only 15 to 30 min under the conditions employed (data not shown). After equilibrium had been reached, the addition of more protein kinase did not alter the concentration of products or reactants. In both the forward and reverse reactions, the equilibrium position was maintained for 6 h. Using the equilibrium concentrations of the substrates and products after 4 h of incubation and Equation 1, an average value of 0.045 was obtained for the equilibrium constant (Table II).

The Activity of Pyruvate Kinase during Dephosphorylation and Rephosphorylation Catalyzed by Protein Kinase—

![Fig. 4. The effect of pH on the phosphorylation and dephosphorylation of pyruvate kinase. For the dephosphorylation reaction (●, ○), 20 µg of ["P"]pyruvate kinase prepared as described under "Methods" was incubated for 15 min at 30°C with 5 mM ADP, 10 mM MgCl₂, 100 mM glucose, 0.2 unit of hexokinase, and 100 mM concentration of the appropriate buffer. The reaction was started by the addition of 200 units of protein kinase. For the phosphorylation reaction (O, ○), 30 µg of dephosphorylated pyruvate kinase (see "Methods") was incubated with 0.4 mM [gamma-32P]ATP (340 cpm/pmol), 5 mM MgCl₂, and 100 mM concentration of the appropriate buffer at 30°C for 5 min. The reaction was started by the addition of 3 units of protein kinase. The buffers used were sodium acetate, pH 4.5, pH 5, and pH 5.5; 3-(N-Morpholino)ethane sulfonic acid, pH 6, pH 6.5, and pH 7; N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.5, pH 8, pH 8.5, and pH 9.](image)

![Table II
Determination of equilibrium position in the protein kinase reaction
Samples were incubated at 30°C for 4 h in 25 mM N-(Tris(hydroxymethyl)methyl-2-amino)ethanesulfonic acid, pH 6.7, 10 mM MgCl₂, 5000 units/ml of protein kinase, and various concentrations of ADP, ATP, ["P"]pyruvate kinase (659 cpm/pmol), and nonphosphorylated pyruvate kinase. Total volume of the incubation was 200 µl. In Experiment 5, [gamma-32P]ATP (400 cpm/pmol) was added in order to measure the amount of ["P"]pyruvate kinase produced. Five-microliter aliquots were taken at 0, 30, 60, 90, 120, 180, 240, 300, and 360 min and counted for ["P"]radioactivity as described under "Experimental Procedures." Ten-microliter aliquots were also taken at these times and added to 50 µl of 10 mM Tris, pH 7.0, and 1 mM EDTA and heated in a boiling water bath for 5 min. ATP and ADP were assayed in these samples by the luciferase method (20) and spectrophotometrically (21), respectively. The concentration of phosphorylated and nonphosphorylated pyruvate kinase was calculated from the specific activity of the ["P"]pyruvate kinase or [gamma-32P]ATP based on four sites/mol of pyruvate kinase.

<table>
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<th>Experiment</th>
<th>Time (h)</th>
<th>ATP (mM)</th>
<th>ADP (mM)</th>
<th>Pyruvate kinase (mM)</th>
<th>P-Pyruvate kinase (mM)</th>
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</table>

Average = 0.045

We made use of both the forward and reverse reactions to study the relationship between the amount of phosphate in pyruvate kinase and the activity of the enzyme. The activity of pyruvate kinase was measured at 0.4 mM and 4 mM P-enolpyruvate and the activity ratio (0.4/4) was expressed as a
percentage of the activity ratio of the maximally activated enzyme. In order to favor the dephosphorylation reaction, in these experiments the reverse reaction was run in the presence of hexokinase and glucose which act as an ATP trap. The time course of the dephosphorylation and reactivation of pyruvate kinase by protein kinase catalytic subunit and ADP is shown in Fig. 5A. Pyruvate kinase initially contained 3.1 mol of P_i/mol of enzyme and had 40% of the maximal activity. By 60 min, the enzyme appeared to be maximally active and contained approximately 1 mol of P_i/mol of enzyme. The major changes in activity occurred between 3 and 1 mol of P_i/mol of pyruvate kinase. Longer incubations or higher concentrations of protein kinase did not remove the remaining mole of phosphate or result in any further changes in enzyme activity (data not shown), suggesting that pyruvate kinase contained about 1 mol of P_i/mol in a nonregulatory site.

The time course of rephosphorylation and inactivation of the activated enzyme from Fig. 5A by protein kinase and ATP is shown in Fig. 5B. A similar inversely proportional relationship was seen during the rephosphorylation of pyruvate kinase as was seen during its dephosphorylation. The greatest decrease in enzyme activity (100% to 45%) occurred during the incorporation of 1 to 3 mol of P_i/mol of enzyme, while only small changes in activity occurred during the incorporation of greater than 3 mol of P_i/mol of enzyme. For example, the incorporation of 3.4 mol of P_i/mol of enzyme after 60 min resulted in an enzyme that had 37% of its maximum activity. When higher concentrations of protein kinase were used in the rephosphorylation reaction, the incorporation of phosphate reached a total of 5 mol/mol of enzyme or a net increase of 4 above the initial basal value (data not shown). However, the activity of the enzyme was only reduced to 30% of the maximum activity.

A composite of the results of 11 dephosphorylation-rephosphorylation experiments with four different preparations of pyruvate kinase is shown in Fig. 6. The major changes in activity (90% to 35% of the maximal activity) occurred between 1.5 and 3 mol of P_i/mol of enzyme, and the incorporation of greater than 3 mol of P_i/mol of enzyme did not further inhibit pyruvate kinase to any appreciable extent. Fully activated pyruvate kinase appeared to contain about 1 mol of P_i/mol of enzyme in a nonregulatory site or in a site not phosphorylated by the protein kinase. Treatment of this enzyme with NaOH showed that 0.3 to 0.6 mol of the phosphate was alkaline labile (data not shown).

**Influence of Allosteric Effectors on the Phosphorylation of Pyruvate Kinase**—We previously presented some evidence that fructose bisphosphate may have an indirect effect on the regulation of pyruvate kinase activity by influencing its phosphorylation (6). The influence of several allosteric effectors on the phosphorylation of the enzyme by protein kinase has been further characterized. The time course of the effect of physiological concentrations of P-enolpyruvate, alanine, and fructose bisphosphate on the phosphorylation of the dephosphorylated form of pyruvate kinase by protein kinase is shown in Fig. 7. The allosteric inhibitor, alanine, increased the initial rate of phosphorylation by only 20%, but

![Fig. 5. The time course of dephosphorylation-activation and rephosphorylation-inactivation of pyruvate kinase. A (dephosphorylation), 2 mg of native pyruvate kinase was incubated with 5 mM ADP, 10 mM MgCl₂, 100 mM glucose, and 20 units of hexokinase in Buffer I, pH 7, at 30°C. The reaction was started by the addition of 20,000 units of protein kinase. At the given times, aliquots were removed from the incubation mixture for the determination of pyruvate kinase activity (---) as described under “Methods.” B (rephosphorylation), 1 mg of pyruvate kinase that had been dephosphorylated at the end of the incubation in A was dialyzed overnight against 2 x 2,000 volumes of Buffer 1, pH 7.0, and then incubated with 2 mM ATP and 5 mM MgCl₂ in the same buffer at 30°C. The reaction was started by the addition of 100 units of protein kinase. Measurements of activity (---) and protein-bound phosphate (---) are described in A.](image)

![Fig. 6. The relationship of phosphate content of pyruvate kinase to enzymatic activity. The results of 11 dephosphorylation-rephosphorylation experiments such as that shown in Fig. 5 on four different preparations of pyruvate kinase are plotted as moles of P_i/mol of pyruvate kinase versus per cent of maximum activity. The curve was drawn to the best fit using the polynomial regression equation y = ax + bx² + cx³ + dx⁴ + ex⁵. Each symbol represents a different experiment.](image)

![Fig. 7. The effect of fructose bisphosphate, P-enolpyruvate, and alanine on the time course of phosphorylation of pyruvate kinase. Dephosphorylated pyruvate kinase (30 μg) was incubated with 0.33 mM [γ-³²P]ATP (200 to 400 cpn/mmol), 5 mM MgCl₂, and 50 mM Tris-HCl, pH 7.4, at 30°C with no additions (o—o), with 1 μM fructose bisphosphate (C—C), 1 μM P-enolpyruvate (Δ—Δ), or with 1 mM alanine (△—△). The reaction was started by the addition of 5 units of protein kinase.](image)
the allosteric activator, fructose bisphosphate, inhibited the initial rate by more than 80%. Phosphoenolpyruvate also inhibited the initial rate of phosphorylation, but was not as effective as fructose bisphosphate. In all cases, the rate of phosphorylation was linear only during the incorporation of the first mol of phosphate.

The effect of various concentrations of fructose bisphosphate and P-enolpyruvate on the initial rate of pyruvate kinase phosphorylation in the presence and absence of alanine is shown in Fig. 8. In the absence of alanine, the initial rate of phosphorylation of the dephosphorylated enzyme was inhibited 17% by 0.1 μM fructose bisphosphate (Panel A). Maximal inhibition (71%) was observed with 1 to 3 μM fructose bisphosphate, whereas 10 and 100 μM fructose bisphosphate resulted in 53% and 43% inhibition, respectively. Alanine (1 mM) appeared to relieve the inhibition by 0.1 to 10 μM fructose bisphosphate but had no effect at the highest concentration (100 μM). A similar relationship was seen when the effects of P-enolpyruvate were studied (Panel B). The initial rate of phosphorylation of pyruvate kinase was inhibited 24%, 42%, and 62% by 0.05, 0.1, and 1 mM P-enolpyruvate, respectively. The addition of alanine relieved the inhibition by 0.05 and 0.1 mM P-enolpyruvate and reduced by about 50% that seen with 1 mM P-enolpyruvate.

The influence of allosteric effectors on the phosphorylation of the native enzyme (3.1 mol of P/mol), on the other hand, was less pronounced (Fig. 8, Panels C and D). Ten- and one-hundred-fold concentrations of P-enolpyruvate and fructose bisphosphate, respectively, were necessary to elicit inhibitions similar to those seen with the dephosphorylated enzyme.

The inhibitory effect of fructose bisphosphate on the phosphorylation of dephosphorylated pyruvate kinase was greatest at pH values above 7 but was negligible at values below 7 (Table III). In contrast, the stimulatory effect of alanine was best seen at low pH values. Neither effector had any influence on the activity of the protein kinase with histone as substrate (data not shown).

![Phosphorylation-Dephosphorylation of Pyruvate Kinase](image)

**Fig. 8.** The effect of various concentrations of fructose bisphosphate and P-enolpyruvate on the rate of phosphorylation of pyruvate kinase in the presence and absence of alanine. Dephosphorylated (A and B) and native (C and D) pyruvate kinase were incubated as described in Fig. 7 in the presence of the indicated concentrations of fructose bisphosphate (A and C) or P-enolpyruvate (B and D) and either with (O) or without (●) 1 mM alanine.

**Table III**

Influence of pH on the effects of fructose bisphosphate and alanine on the rate of phosphorylation of pyruvate kinase

<table>
<thead>
<tr>
<th>pH</th>
<th>Rate of phosphorylation (pmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>6</td>
<td>3.99</td>
</tr>
<tr>
<td>7</td>
<td>5.09</td>
</tr>
<tr>
<td>8</td>
<td>10.12</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The cyclic AMP-dependent protein kinase-catalyzed reaction has been shown to be reversible with several phosphoprotein substrates (17, 19, 22). This reaction is also reversible with phosphopyruvate kinase as substrate (Figs. 4 and 5). We have characterized the reaction and have found the equilibrium constant (Equation 1) to be 0.045 at pH 6.7 and 30°C. From this value, it is possible to calculate the standard free energy of hydrolysis of the pyruvate kinase-phosphate bond using the following equation:

\[
\Delta G_{\text{rxn}} = -RT \ln K_{eq} = +1.83 \text{ kcal mol}^{-1}
\]

Assuming a value of -8.4 kcal mol\(^{-1}\) for the free energy of hydrolysis of the β, γ bond of ATP (23), the standard free energy of hydrolysis of phosphopyruvate kinase was calculated as follows:

\[
\Delta G_{\text{rxn}} = -8.4 \text{ kcal mol}^{-1} - 6.6 \text{ kcal mol}^{-1} = -15 \text{ kcal mol}^{-1}
\]

The high energy character of the enzyme-phosphate bond (−6.6 kcal mol\(^{-1}\)) is similar to that reported by Shizuta et al. (17) for the casein-phosphate bond. The significance of the high energy character of this bond is unknown, but its pres-
ence may be linked to conformational changes in pyruvate kinase that result in a lower affinity for its substrate.

Using the reverse reaction, we found that 2 of the 3 mol of phosphate found in the native enzyme were located in sites phosphorylated by protein kinase. The nature of the other mole of phosphate is uncertain. Also, it is uncertain whether the phosphate found in the homogeneous enzyme actually reflects the phosphorylation state of pyruvate kinase in vivo. Phosphorylation did not occur during the purification procedure since no changes in the activity ratio of the enzyme occurred and no labeled phosphate was found in the enzyme when [γ-32P]ATP was added to the homogenate. Furthermore, the administration of an α-adrenergic blocker (phenox-}

ybenzamine) or a β-adrenergic blocker (dl-propranolol) or both, prior to sacrifice of the rats (24), did not result in any change in the activity ratio of pyruvate kinase (data not shown). Thus, the 2 to 3.5 mol of P2/mol of enzyme were not incorporated as a result of sympathetic discharge at the time of sacrifice. Therefore, it is possible that pyruvate kinase contains appreciable amounts of phosphate and is partially inactivated in vivo.

There have been several reports that attempt to correlate the amount of phosphate incorporated into pyruvate kinase with changes in activity of the enzyme. Tajiri et al. (2) found that the incorporation by the cyclic AMP-dependent protein kinase of 1.5 to 2 mol of 32P/mol of the rat liver enzyme was sufficient to inhibit activity of the enzyme over 90%. On the other hand, when rat liver slices were incubated with 32P and glucagon, the incorporation of 3 mol of 32P/mol of enzyme caused only a 50% reduction in enzyme activity (3). When glucagon was administered to a rat in vivo, the incorporation of 1.4 mol of 32P/mol of enzyme resulted in a 39% reduction in the activity of the enzyme (7). Ljunghström et al. (25) found that pyruvate kinase from pig liver was only inhibited 25% when 1.5 mol of 32P were incorporated/mol of enzyme. These different results may be due to the fact that only the amount of radioactive phosphate incorporation was determined while the amount of endogenous phosphate in these various preparations was unknown. In this study, changes in pyruvate kinase activity were correlated with the amount of chemically determined phosphate in the enzyme. The presence of 2.5 mol of phosphate/mol of enzyme reduced activity of the enzyme by 50% when measured at submaximal substrate concentrations (Fig. 6). Changes from 90% to 40% of maximal activity occurred when the amount of chemically determined phosphate increased from 1.5 mol to 3 mol/mol of enzyme (Fig. 6). As noted above, there is about 1 mol of phosphate/mol of tetrameric enzyme in a site whose phosphorylation apparently is not catalyzed by cyclic AMP-dependent protein kinase. Thus, the incorporation of 2 mol of phosphate/mol of enzyme in the sites whose phosphorylation is catalyzed by cyclic AMP-dependent protein kinase resulted in essentially maximal inhibition of pyruvate kinase activity (Fig. 6). The incorporation of an additional 2 mol of phosphate by the protein kinase had little effect on enzyme activity.

The activity of phosphorylated pyruvate kinase is less sensitive to inhibition by fructose bisphosphatase and more sensitive to inhibition by alanine than is the dephosphorylated form of the enzyme (5). It has been reported that these allosteric effectors also can influence the rate of phosphorylation of pyruvate kinase. Berglund et al. (18) reported that the presence of alanine increased the rate of phosphorylation of the pig liver enzyme catalyzed by the cyclic AMP-dependent protein kinase but neither fructose bisphosphatase nor P-enolpyruvate had any effect. Eigenbrodt and Schoner (26) found that fructose bisphosphatase decreased and alanine increased the rate of phosphorylation of the K-type pyruvate kinase from chicken liver catalyzed by a cyclic AMP-independent protein kinase. In the present study, physiological concentrations of both fructose bisphosphatase and P-enolpyruvate decreased the initial rate of phosphorylation of the dephosphorylated form of rat liver enzyme (Figs. 7 and 8). Alanine by itself (Fig. 7) had little effect, but it relieved the inhibition by either fructose bisphosphatase or P-enolpyruvate (Fig. 8). These results are in good agreement with those obtained using Sephadex-treated hepatocyte extracts (27, 28). They suggest that pyruvate kinase is a better substrate for the cyclic AMP-dependent protein kinase when pyruvate kinase is allosterically inhibited than when it is allosterically activated. Consistent with this notion are the effect of pH and fructose bisphosphatase on the phosphorylation of the enzyme (Table III). At low pH values, the enzyme is in an active form (29, 30) and the rate of phosphorylation is low and unaffected by fructose bisphosphatase. When the pH shifts the enzyme into an inactive form (29, 30) and the rate of phosphorylation increases dramatically and is more sensitive to inhibition by fructose bisphosphatase. The differences between our results and those of Berglund et al. (18) may be due to species differences or to the presence of endogenous phosphate in their enzyme preparation.

We have recently demonstrated that the level of fructose bisphosphatase can affect the degree of phosphorylation of pyruvate kinase in the intact hepatocyte (6). These results, coupled with the present findings using a purified system, lend strong support to the hypothesis that regulation of pyruvate kinase activity is a complex function of direct heterotropic allosteric regulation, covalent modification, and of indirect effects of allosteric effectors on enzyme phosphorylation.

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