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KINETIC STUDIES WITH L-TYPE PYRUVATE KINASE FROM RATS EITHER FED A HIGH CARBOHYDRATE, LOW PROTEIN DIET OR STARVED

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Summary

Rat liver pyruvate kinase (ATP : pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) was purified to homogeneity from animals fed a high carbohydrate, low protein diet prior to killing and from animals starved 72 h prior to killing. The liver pyruvate kinase purified from the fed rats always has a specific activity around 220 International Units per mg of protein, whereas the maximum specific activity observed with enzyme purified from the starved rats was 80 units/mg. On polyacrylamide gel isoelectric focusing carried out in the absence of fructose-1,6-bisphosphate, the protein from fed rats resolved into one major band at pH 5.8—6.0 and into an apparent doublet at pH 5.2—5.3. The majority of the protein from starved rats focused into a broad band at pH 5.8—6.0. When isoelectric focusing was carried out in the presence of 5 μM fructose-1,6-bisphosphate, both proteins focused at pH 5.2—5.3. The enzymes isolated from both fed and starved rats differed in three kinetic parameters. Firstly, in the difference in specific activities. Secondly, in the absence of fructose-1,6-bisphosphate the $K_{0.5}$ value for phospho*enol*pyruvate was 0.96 mM for the enzyme from fed rats and was 1.6 mM for the enzyme from starved rats. In the presence of 1 mM fructose-1,6-bisphosphate the $K_{0.5}$ was 0.15 mM phospho*enol*pyruvate for both enzymes. Thirdly, at a phospho*enol*pyruvate concentration of 0.1 mM the $K_{0.5}$ values for fructose-1,6-bisphosphate were 0.05 μM for the enzyme from fed rats and 0.13 μM for the enzyme from starved rats. The enzymes from fed rats and from starved rats yielded the same kinetic values for ADP (K_m = 0.4 mM), for phospho*enol*pyruvate (K_m = 0.15 mM) in the presence of 1 mM fructose-1,6-bisphosphate, for fructose-1,6-bisphosphate ($K_{0.5}$ = 1 μM) in the presence of 10 mM L-alanine, and for L-alanine (K_I = 10 mM), when assayed with 1 μM fructose-1,6-bisphosphate and 0.1 mM phospho*enol*pyruvate.

Introduction

The very high levels of pyruvate kinase (ATP : pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) found in livers of rats maintained on a high carbohydrate, low protein diet fall to much lower levels upon starvation over a period of 2—4 days [14]. Yet liver glycogen is depleted 5—10 h after onset of fasting [5]. Since the blood glucose level is maintained during this fasting period [5], an increase of carbohydrate flux through gluconeogenesis must occur within this 5—10 h of fasting. Since the potential total liver pyruvate kinase activity far exceeds the maximum rate of gluconeogenesis, inhibition of the liver pyruvate kinase in vivo is a potential point for a metabolic control. Furthermore, any inhibition must occur within the same time scale as the maximum onset of gluconeogenesis; otherwise, the carbohydrate flux through gluconeogenesis would be impaired.

Alteration in the kinetic properties of hepatic pyruvate kinase has been reported for rat livers [6,7] and isolated hepatocytes [8,9] in response to glucagon. Glucagon administration results in a decreased level of liver pyruvate kinase activity. Since glucagon administration results in an increased level of cyclic AMP which in turn would activate protein kinase it was postulated that a phosphorylation-dephosphorylation mechanism could be involved in the regulation of pyruvate kinase (L type) in vivo [10]. Phosphorylation of the L-type pyruvate kinase in vitro with a cyclic AMP stimulated protein kinase has been reported [10]. In addition, the phosphorylation alters the kinetic parameters of liver pyruvate kinase [11].

If an in vivo regulation of liver pyruvate kinase does occur, one might be able to demonstrate the presence of at least two different species of the enzyme by comparison of the enzymes isolated from animals under extremes of glycolytic and gluconeogenic carbohydrate flux. In an attempt to detect electrophoretic or kinetic differences that might indicate the presence of two different species of liver pyruvate kinase we purified the enzyme from two groups of rats differing only in their pre-killing dietary states. The first group of rats was fed a high carbohydrate, low protein diet ad lib. and thus would have a minimal gluconeogenic carbohydrate flux. The second group of rats was starved for 72 h prior to death and would have a maximal gluconeogenic carbohydrate flux. The purified enzymes were then compared by electrophoretic and kinetic parameters.

Materials and Methods

Albino male Holtzman rats, average weight 400 g, were used for both enzyme preparations. The high carbohydrate, low protein diet fed to one group of rats comprise by weight 85% sucrose, 10% casein, 4% Rogers and Harper inorganic salt mix, and 1% B vitamin complex dry mix (all but sucrose purchased from Teklad Mills, Madison, Wisc.). Rats were fed this powdered diet ad lib. for 6 days prior to killing. The fasted rats were starved for 72 h prior to sacrifice. To synchronize the beginning of the fasting period the rats were administered intragastrically 2 ml of a 40 g percent sucrose solution.

Rat liver pyruvate kinase was purified following the procedure outlined by Ljungström, et al. [10] with the following modifications. Fructose-1,6-bisphosphate was not added to solutions during the purification. The hydroxylapatite column of the Ljungström procedure was replaced by a CM-Sephadex column procedure [4,12].

The pyruvate kinase activity was routinely assayed by the spectrophotometric procedure of Bücher and Pfleiderer [13] as modified by Tietz and Ochoa [14]. In the coupled assay the pyruvate generated is converted to lactate by NADH and excess lactic dehydrogenase with the decrease in NADH followed by 340 nm. The standard reactions were conducted at 30° C and comprised 100 μmol Tris · HCl, pH 7.4, 100 μmol KCl, 4 μmol $MgCl_2$, 1 μmol phospho*enol*pyruvate, 0.15 μmol NADH, 5 μg lactic dehydrogenase, 1 μmol fructose-1,6-bisphosphate, liver pyruvate kinase and 2 μmol adenosine diphosphate in a total volume of 1.0 ml. Alternative concentrations are noted in the Figure legends. The reaction was started by the addition of adenosine diphosphate after a 2 min preincubation at 30° C. Enzyme activity is expressed in enzyme units per mg of protein with one unit of enzyme catalyzing the formation of a one micromole of pyruvate per minute under the defined conditions. The protein concentration was measured via the procedure of Lowry et al. [15] using ovalbumin as a standard.

Disc gel electrophoresis was carried out with three different gel systems and separation pH values; 7% polyacrylamide, pH 9.5, 7.5% polyacrylamide, pH 8.0, and 7.5% polyacrylamide, pH 6.5 as described by Maurer's gel system 1a, 6 and 13 with their associated electrode buffer solutions [16]. The protein was routinely preincubated with 5 mM fructose-1,6-bisphosphate for 10 min at room temperature prior to electrophoresis and 0.1 mM fructose-1,6-bisphosphate was included in the upper buffer. The gels were run at 3 mA per tube of 4 mm diameter for 2 h at 4° C and were stained with Amido Black.

Analytical thin layer polyacrylamide gel isoelectric focusing was accomplished with the LKB 2117 Multiphore Electrophoresis Kit. A 5% polyacrylamide gel matrix (11.5 × 25 × 0.2 cm) was used with the pH gradient of 5.0—8.5. The pH gradient was determined by measuring the pH of homogenized segments of the gel (1 cm in the dimension parallel to pH gradient) with a Radiometer pH meter Model 26. The isoelectric focusing gels were run overnight at 4° C at 250 V. The gels were stained with a solution containing 0.75 g Coomassie Brilliant Blue, 225 ml methanol, 465 ml water, 22.5 g sulphosalicylic acid and 75 g trichloroacetic acid, and destained in water : methanol : acetic acid (8 : 3 : 1) solution. To detect catalytic activity in the gel, the gel was sectioned into 0.5-cm segments in the dimension parallel to the pH gradient. These segments were incubated for 2 min at 30° C in 1 ml of the standard assay mixture containing no ADP. The reaction was then started by addition of 2 μmol of ADP.

Results

Purification of pyruvate kinase Type L

Pyruvate kinase Type L was purified from livers of both rats that had starved 72 h and from rats that had been fed the high carbohydrate diet. The isolation

procedure of Ljungström et al. [10] was used with a slight modification. Their hydroxyapatite column was omitted and a CM-Sephadex column was used at that step [4,12]. This procedure yielded an apparently homogeneous preparation of liver pyruvate kinase as judged by disc gel electrophoresis and high speed analytical ultracentrifugation [4]. The liver homogenates from rats fed a high carbohydrate diet contained around 200 enzyme units per g of liver while those from starved (72 h) rats contained around 20 units/g of liver [12]. An overall yield of 5—10% was usual for the purification procedure. The purified enzyme was concentrated to at least 1 mg/ml and stored at 4° C in 6 mM potassium phosphate buffer, pH 7.0, containing 30% of glycerol and 0.1 mM dithiothreitol. When stored in this manner about 50% of the enzyme activity was lost over a three week period. The enzyme isolated from rats fed the high carbohydrate diet had a specific activity around 220 units/mg of protein while the enzyme isolated from rats that had been starved for 72 h a specific activity of 80 units/mg. Disc gel electrophoresis carried out in the presence of fructose-1,6-bisphosphate at pH values of 9.5, 8.0, and 6.5 yielded a single protein band for each of the purified enzymes with identical electrophoretic mobilities. Electrophoresis run in the absence of fructose-1,6-bisphosphate yielded several protein bands. Isoelectric focusing in the absence of fructose-1,6-bisphosphate resolved the enzyme from fed rats into two protein components (Fig. 1A). The major component focused at pH 5.8—6.0. However, a significant portion of the protein focused at pH 5.2—5.3 and was composed of two closely spaced bands. In the absence of fructose-1,6-bisphosphate almost all of the enzyme from the fasted animals focused at pH 5.7—6.0 with only a faint spot visible at pH 5.2—5.3. When isoelectric focusing was carried out in the presence of 5 μmol fructose-1,6-bisphosphate (Fig. 1B) the majority of both the "fed" and "starved" protein focused at pH 5.2—5.3 and only a trace of protein focused at pH 5.7—6.0. Pyruvate kinase activity could be assayed using slices of the gel and the catalytic activity was coincident with the major protein bands.

Fig. 1. Polyacrylamide gel isoelectric focusing of liver pyruvate kinase from fed vs. starved rats. Gel A was run in the absence of fructose-1,6-bisphosphate. Gel B was run in the presence of 5 μM fructose-1,6-bisphosphate: f, 10 μg of the enzyme from fed rats; s, 10 μg of the enzyme from starved rats. The pH gradient is given to the left of the gels. Pyruvate kinase activity was demonstrable in 0.5 cm gel sections comparable to all five areas of protein indicated by stain. Conditions of electrophoresis and methods of pH and activity determination given in Materials and Methods.

Comparison of kinetic parameters for purified liver pyruvate kinase from fed and starved rats

Phosphoenolpyruvate. Fig. 2 illustrates the Hill plots obtained using purified liver pyruvate kinase from both fed (A) and starved (B) rats as a function of the phospho*enol*pyruvate concentration. Both enzymes had sigmoidal kinetics in the absence of added fructose-1,6-bisphosphate (closed circles) and yield values, for phospho*enol*pyruvate, of $K_{0.5}$ = 0.96 mM with n (Hill coefficient) of 1.8 and $K_{0.5}$ = 1.6 mM and n = 1.5 for the "fed" and "starved" enzymes, respectively. In the presence of 1 mM fructose-1,6-bisphosphate (open circles) both enzymes exhibited hyperbolic kinetics resulting in an apparent K_m of 0.15 mM and n = 0.9 for phospho*enol*pyruvate.

Adenosine diphosphate. Fig. 3 shows the enzyme activity of liver pyruvate kinase for fed (A) and starved (B) rats at various ADP concentrations and a phospho*enol*pyruvate concentration of 1 mM. Although the maximum specific activity observed with both the "fed" and "starved" enzymes was lower when the assays were carried out in the absence of fructose-1,6-bisphosphate (closed circles), the curves obtained with both enzymes were hyperbolic and yielded the same K_m value of 0.4 mM. Addition of 1 mM fructose-1,6-bisphosphate (open circles) to the reaction mixture increased the observed activity for both enzymes 2—3-fold but did not alter the observed K_m = 0.4 mM value.

Fructose-1,6-bisphosphate. As seen in Fig. 3, 1 mM fructose-1,6-bisphosphate did not alter the Michaelis constant for ADP for either enzyme. Yet, in Fig. 2, 1 mM fructose-1,6-bisphosphate changed the velocity vs. phospho*enol*pyruvate concentration curves for both enzymes from sigmoidal to hyperbolic resulting in an apparent K_m value of 0.15 mM and a Hill coefficient of 0.9 for both enzymes. At 0.5 mM phospho*enol*pyruvate, upper level of its physiological

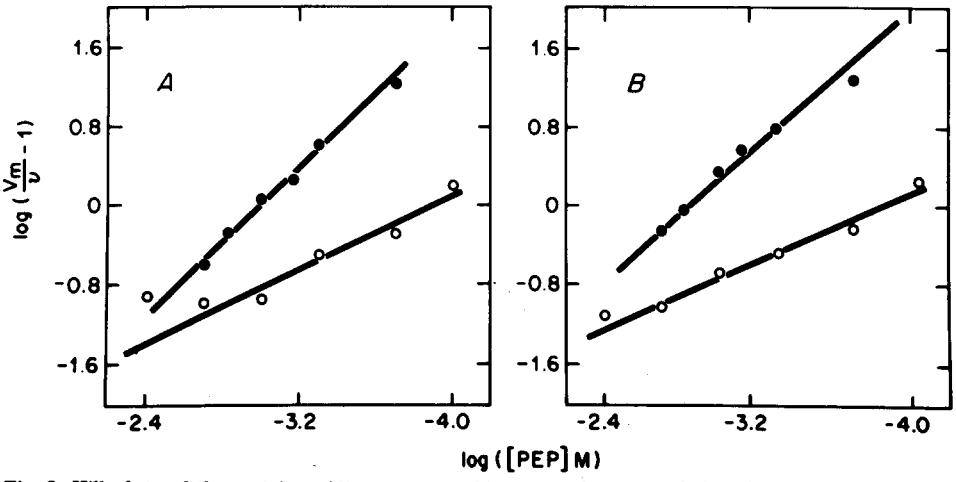

Fig. 2. Hill plots of the activity of liver pyruvate kinase as a function of phospho*enol*pyruvate (PEP) concentration for the enzyme purified from fed rats (A) and starved rats (B). Assays were run in the absence (•) or presence (○) of 1 mM fructose-1,6-bisphosphate. In the absence of fructose-1,6-bisphosphate, values of $K_{0.5}$ = 0.96 mM, n = 1.8 and $K_{0.5}$ = 1.6 mM, n = 1.5 were obtained from A and B, respectively. Using 1.0 mM fructose-1,6-bisphosphate both enzymes yield values of K_m = 0.15 mM, n = 0.9 for phospho*enol*pyruvate. Other assay conditions are in Materials and Methods.

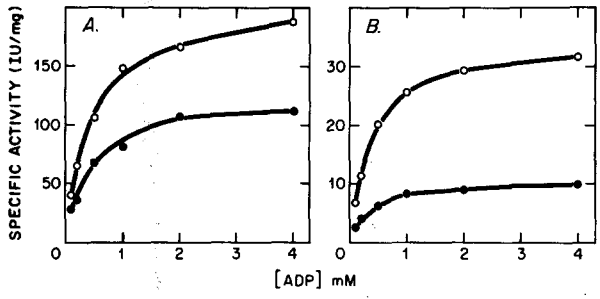

Fig. 3. Activity of liver pyruvate kinase as a function of ADP concentration for enzyme purified from fed rats (A) and from starved rats (B) in the absence (•) and presence (○) of 1 mM fructose-1,6-bisphosphate added to the assay solution. Assays contain 1 mM phospho*enol*pyruvate and other conditions as discussed in Materials and Methods.

range [11—14], 1 mM fructose-1,6-bisphosphate increases the observed activity for both enzymes approximately 7—9-fold (see Fig. 2). Fig. 4 shows the fructose-1,6-bisphosphate activation curves for the enzymes from both fed (closed circles) and starved rats (open circles) when assayed with 0.1 mM phospho*enol*pyruvate. A fructose-1,6-bisphosphate concentration of 0.05 μM was required for half-maximal activation of the enzyme from fed animals while a concentration of 0.13 μM was required for half-maximal activation of the enzyme from starved animals. Both enzymes were fully activated by 1 μM fructose-1,6-bisphosphate. Furthermore, Hill plots of the activation yielded Hill coefficients of 1.0 for both enzymes. Similar kinetic constants were obtained

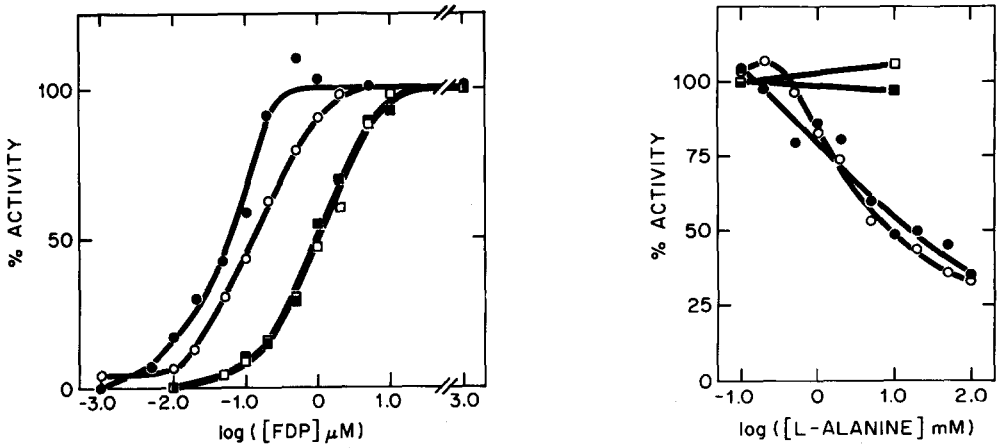

Fig. 4. Activity of liver pyruvate kinase as a function of fructose-1,6-bisphosphate concentration for the enzymes purified from fed rats (closed symbols) vs. starved rats (open symbols) without added L-alanine (circles) and with 10 mM L-alanine (squares). Reaction rates at the given fructose-1,6-bisphosphate concentration are expressed as the percentage of the rate obtained at 1.0 mM fructose-1,6-bisphosphate for each enzyme preparation. Concentration of phospho*enol*pyruvate used for all assays was 0.1 mM. Other assay conditions are given in Materials and Methods.

Fig. 5. Activity of liver pyruvate kinase as a function of L-alanine concentration for the enzyme purified from fed rats (•) vs. starved rats (○) at 2.0 μM fructose-1,6-bisphosphate and for the enzyme from fed rats (■) and from starved rats (□) at 1.0 mM fructose-1,6-bisphosphate. Concentration of phospho*enol*-pyruvate was 0.1 mM. Other assay conditions are given in Materials and Methods.

by using the enzyme which has been purified only through the ammonium sulfate fractionation. By using the enzyme purified only through the ammonium sulfate step, the concentration of fructose-1,6-bisphosphate required to give half-maximal activation was measured at various times of starvation. The $K_{0.5}$ value for fructose-1,6-bisphosphate of 0.05 μM was obtained up to 12 h after fasting. Between 12 and 24 h the $K_{0.5}$ value increased to 0.13 μM and remained at this level for up to at least 120 h of fasting.

L-Alanine. Fig. 5 shows the inhibition of liver pyruvate kinase at various L-alanine concentrations when assayed in the presence of 2 μM (circles) or 1 mM (squares) fructose-1,6-bisphosphate. In 2 μM fructose-1,6-bisphosphate 50% inhibition of both the "fed" enzyme (closed circles) and "starved" enzyme (open circles) was obtained with 10 mM L-alanine. In contrast, no inhibition of either enzyme was observed with 10 mM L-alanine when assayed in the presence of 1 mM fructose-1,6-bisphosphate.

Thus in the presence of low fructose-1,6-bisphosphate concentrations L-alanine inhibits both the enzyme purified from the livers of fed rats and the one purified from starved rats. Fig. 4 illustrates that when 10 mM L-alanine was added to the assay mixture containing 0.1 mM phospho*enol*pyruvate a shift of the fructose-1,6-bisphosphate activation curve to the right was observed with both enzymes. The $K_{0.5}$ for fructose-1,6-bisphosphate activation in the presence of 10 mM L-alanine was 1 μM for both the enzymes purified from "fed" (closed squares) and "starved" (open squares) rats. The apparent V was not altered and the Hill coefficients (n = 1.1) were not changed.

Fig. 6 illustrates the phospho*enol*pyruvate activation of liver pyruvate kinase purified from rats fed the high carbohydrate diet. When assays were carried out in the absence of fructose-1,6-bisphosphate (●) the kinetic constants of $K_{0.5}$ = 1.3 mM and n = 2.0 were obtained. In the presence of 1 mM fructose-1,6-bisphosphate (○) the values were $K_{0.5}$ = 0.07 mM and n = 0.9 for this set of

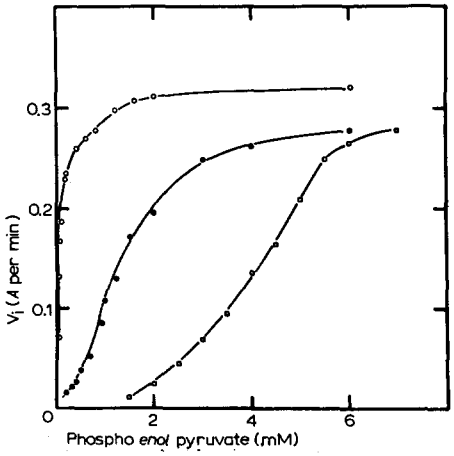

Fig. 6. Phospho*enol*pyruvate activation of liver pyruvate kinase purified from rats fed the high carbohydrate, low protein diet. Assay conditions are the standard conditions as given in Materials and Methods minus fructose-1,6-bisphosphate, except where modified as indicated without additions (●), with 1 mM fructose-1,6-bisphosphate (○), or with 10 mM L-alanine (□).

data. In 10 mM L-alanine (□) the activation constant for phospho*enol*pyruvate increased to $K_{0.5}$ = 4 mM and $n \approx 4.5$.

Discussion

The object of this study was to try to detect differences in the nature of liver pyruvate kinase from rats subjected to differing dietary stresses. The need to rapidly inhibit liver pyruvate kinase activity under the stress of starvation has been pointed out [17—20] yet an adequate description of a rapidly acting in vivo control has not been reported. One possible explanation of the decrease in pyruvate kinase activity during gluconeogenesis is a decrease in the total amount of liver pyruvate kinase. However, upon starvation, the rapid onset of gluconeogenesis [5] is not matched by a similar rapid decrease in the total liver pyruvate kinase activity or protein in liver homogenate [1—4]. A second explanation could be a rapid transient modification of the activity of liver pyruvate kinase to modify the carbohydrate flux from glycolysis to gluconeogenesis. This could result either from changes in metabolite concentration or from modification of the protein molecule such as by oxidation [21] or phosphorylation [10]. In this report liver pyruvate kinase was purified from rats either starved or fed a high carbohydrate, low protein diet. The electrophoretic and kinetic parameters for the two purified enzyme solutions were compared to see whether starvation resulted in detectable alteration of the enzyme molecule.

The liver pyruvate kinase isolated from either fed or starved rats appeared to be homogeneous as judged by disc gel electrophoresis under three different sets of conditions. Furthermore, the purified proteins were not distinguishable under these conditions. Upon isoelectric focusing in the absence of fructose-1,6-bisphosphate the protein from starved rats focused into one band with an isoelectric point between 5.8—6.0. The major fraction of protein from fed rats focused at pH 5.8—6.0, yet some protein focused at pH 5.2—5.3 and was composed of two closely spaced bands. In contrast, in the presence of fructose-1,6-bisphosphate, the protein from both the starved and the fed rats focused at pH 5.2—5.3. These results are consistent with the isoelectric focusing of pig liver pyruvate kinase [22]. Two protein bands were seen upon isoelectric focusing of purified pyruvate kinase from pig liver: a major band at pH 6.1 and another band appearing at pH 5.3. In the presence of fructose-1,6-bisphosphate, only the band at pH 5.3 remained. They concluded that the pH 6.1 band represented the enzyme containing no bound fructose-1,6-bisphosphate and that the pH 5.3 band represented the enzyme with 2 tightly bound moles of fructose-1,6-bisphosphate per mole of protein. Ibsen and Trippet [23] have reported that two major bands for pyruvate kinase activity were observed at pH 5.5 and 5.7 upon isoelectric focusing of crude extracts of rat liver. Human liver pyruvate kinase also had two interconvertible forms with isoelectric points at pH 5.85 in the presence of fructose-1,6-bisphosphate and pH 6.28 in its absence [24]. It is apparent that fructose-1,6-bisphosphate binding to liver pyruvate kinase causes a decrease in the p*I* value for the enzyme. In the absence of fructose-1,6-bisphosphate addition to the enzyme and gel prior to electrofocusing there appears to be more of the fructose-1,6-bisphosphate bound

enzyme from the livers of fed rats than from the livers of starved rats. Even in the presence of fructose-1,6-bisphosphate, there appears to be a slight difference in the pattern of localization for the enzyme from fed rats when compared to the enzyme from starved rats.

There were three kinetic differences between the enzymes purified from the fed vs. the starved rats: (1) their different specific activities; (2) their different $K_{0.5}$ values for phospho*enol*pyruvate; and (3) their different activation constants for fructose-1,6-bisphosphate. The specific activity of the purified liver pyruvate kinase from rats fed the high carbohydrate diet was always greater than 200 units of enzyme/mg protein. In contrast, a specific activity of 80 units of enzyme/mg protein was the maximum obtained from rats that had been starved 72 h. It has been shown that liver pyruvate kinase may be phosphorylated in vitro by ATP, and a cyclic AMP stimulated protein kinase [10]. Furthermore, changes in the kinetic properties of liver pyruvate kinase in perfused livers [6,7], isolated hepatocytes [8,9] and purified enzyme [11,12] are consistent with the concept of hormonal regulation of liver pyruvate kinase by such a postulated phosphorylation. However, since there is little, if any, difference in the phosphorylated and nonphosphorylated enzymes when using 1 mM fructose-1,6-bisphosphate and 1 mM *P-enol*pyruvate [11] the differences in specific activity observed in this paper cannot be explained by such a mechanism. However, the differences could be a result of other covalent modification for instance the oxidation observed by van Berkel et al. [21].

The increase in the $K_{0.5}$ value of phospho*enol*pyruvate from 0.96 mM—1.6 mM (in the absence of added fructose-1,6-bisphosphate) when comparing the enzyme isolated from rats fed the high carbohydrate diet with the enzyme from starved rats is similar to the increase seen when comparing nonphosphorylated and phosphorylated enzymes [11]. Upon addition of 1 mM fructose-1,6-bisphosphate the cooperativity observed with phospho*enol*-pyruvate was eliminated and the value of $K_{0.5}$ = 0.15 mM was obtained. The activation constants for fructose-1,6-bisphosphate are $K_{0.5}$ of 0.06 μM (nonphosphorylated) and $K_{0.5}$ of 0.13 μM (phosphorylated) as reported by Ekman, et al. [11]. The activation constants for fructose-1,6-bisphosphate found in this study are $K_{0.5}$ = 0.05 μM (high carbohydrate diet) and $K_{0.5}$ = 0.13 μM (starved) for the purified enzymes in the presence of 0.1 mM phospho*enol*pyruvate. These results would be consistent with a phosphorylation of the enzyme in vivo in response to starvation but should not be considered definite proof.

Other than the differences noted, every area of kinetic performance examined showed that the enzymes from fed and starved rats were identical. The two enzymes had the same Michaelis constant for ADP, the same Michaelis constant for phospho*enol*pyruvate in the presence of 1 mM fructose-1,6-bisphosphate, the same activation constant for fructose-1,6-bisphosphate in the presence of 10 mM L-alanine, and the same inhibition constants for L-alanine. The kinetic constants reported in this paper are in reasonable agreement with other published values [3,11,23,25—29]. It is likely that there is no single event that controls the pyruvate kinase activity in vivo. In all probability the sum of changes in metabolite and effector concentrations, protein conformational changes as well as covalent events such as phosphorylation and protein turnover all play a role in in vivo regulation.

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