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Purification and Kinetic Properties of Human Liver Pyruvate Kinase[1]

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[1] This study has been summarized from doctorate thesis
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Summary

The kinetic properties of pyruvate kinase purified from human liver were investigated. Human liver pyruvate kinase was purified by three-step process involving ammonium sulphate precipitation, dialysis and Sephadex G-200 chromatography. L type of pyruvate kinase enzyme was able to be purified about 26.3-fold with 13.2% yield in human liver. Specific activity of the enzyme was found to be 2.6 U/mg protein. The optimal pH for human liver pyruvate kinase was found to be 7.4. K^+, Mg^{2+}, Mn^{2+} and Na^+ ions activated enzyme whereas Ca^{2+} ions inhibited it. The enzyme was regulated by FDP, ADP and pyruvate kinase activity was inhibited by alanine. The Km values for PEP and ADP were 1.2 and 0.3 mM respectively. 2,3-DPG and ATP were found to initially activate and subsequently inhibit the enzyme, as the concentration of 2,3-DPG and ATP increased.

Keywords: Human liver, pyruvate kinase, purification, kinetic properties.

İnsan Karaciğer Pirüvat Kinazının Saflaştırılması ve Kinetik Özellikleri[1]

Özet

İnsan karaciğer pirüvat kinazı saflaştırılarak kinetik özellikleri araştırılmıştır. İnsan karaciğer pirüvat kinazı amonyum sülfat ile çöktürme, dializ ve Sefadex G-200 kromatografiyi içeren 3 safha ile saflaştırılmıştır. L tip pirüvat kinaz enzimi insan karaciğer dokusunda %13.2 ürünle yaklaşık 26.3 kat saflaştırılabilmiştir. Enzimin spesifik aktivitesi 2.6 U/mg protein bulunmuştur. İnsan karaciğer pirüvat kinazı için optimal pH 7.4 olarak saptanmıştır. Ca^{+2} iyonu enzimi inhibe, K^+, Mg^{+2}, Mn^{+2} ve Na^+ iyonları ise aktive etmiştir. Pirüvat kinaz FDP, ADP ile düzenlenmiş, alanin ile inhibe edilmiştir. PEP ve ADP için Km değerleri sırası ile 1.2 ve 0.3 mM bulunmuştur. 2,3-DPG ve ATP konsantrasyon artışına bağlı olarak başlangıçta enzimi aktive daha sonra inhibe etmiştir.

Anahtar sözcükler: İnsan karaciğeri, pirüvat kinaz, saflaştırma, kinetik özellikler

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INTRODUCTION

Pyruvate kinase (E.C. 2. 7. 1. 40) is an enzyme of the glycolytic patway, which catalyses the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) in the presence of phosphoenolpyruvate (PEP)[1-4].

In mammalian tissues four distinct isoenzymes of pyruvate kinase is found: M_1-type, M_2-type (or K), L-type and R-type. These isoenzymes differ in their chemical, physical, kinetic, electrophoresis and immunological properties, as well as their tissue distributions[4,5].

The pyruvate kinase type M_1 is a dominating forms in skeletal muscle, heart muscle and brain tissue. The kinetic and regulatory properties of the M_1 type appear to be very different from the other three forms. Only M^1 isoenzyme is allosterically unregulated. This isoenzyme shows hyperbolic kinetics, is not activated by fructose-1, 6-diphosphate (FDP) and is inhibited by ATP, phosphocreatine and phenylalanine (Phe)[1,2,4].

The pyruvate kinase type M_2 exists in lungs, adipose tissue, leucocytes and renal medulla. M_2 (or K) isoenzyme displaying sigmoid kinetics with respect to PEP is activated by FDP, and is inhibited by L-alanine and Phe. The M_2 isoenzyme is electrophoretically and immunologically similar to the M_1 isoenzyme but kinetically similar to the L isoenzyme[6-8].

The pyruvate kinase type R exists in erythrocytes. It is immunologically and kinetically similar to the L isoenzyme. This isoenzyme is covalently regulated[5,9].

The pyruvate kinase type L is expressed in liver and renal cortex[2]. Kinetically, L isoenzyme shows co-operative binding of PEP and is allosterically activated by FDP and by 6-phosphogluconate. L-type pyruvate kinase is inhibited by alanine or glucagon stimulated phosphorylation[5,10,11]. The activity of the L-type pyruvate kinase in liver is regulated by phosphorylation and dephosphorylation in vivo in response to certain hormones such as glucagon and epinephrine[3,12]. The liver isoenzyme has been purified from the livers of rat, ox, chicken and human. Within the liver, L type pyruvate kinase is found in parenchyma cells and pyruvate kinase-M_2 in kupffer cells[5].

The aim of the present study was to purify L isoenzyme of pyruvate kinase from human liver and to obtain some kinetic properties and regulatory properties of the purified enzyme.

MATERIALS and METHODS

Purification: All purification steps were carried out at 0-4°C. Samples without pathological changes were used for enzyme determinations. Human liver obtained from autopsy specimens within 12 h of death was stripped of any vessels and the outer membrane. After washing with 0.9% NaCl to remove blood, specimens were stored at –20°C until assayed.

Tissues were thawed and homogenized in cold (2-4°C) 50 mM phosphate buffer (pH 7.5), containing 100 mM KCl, 6 mM mercaptoethanol with a volume of diluents to produce a 5% homogenate (w/v). The homogenates were centrifuged (Sorvall RC-5C) for 30 min at 5.500 g at +4°C.

Ammonium Sulphate $(NH_4)_2SO_4$ Saturation: Supernatant was brought to 40% of $(NH_4)_2SO_4$, stirred for 1 h and centrifuged at 5.500 g for 20 min at +4°C. The supernatant was saturated to 70% with $(NH_4)_2SO_4$, followed by centrifugation as above and precipitate was preserved. No pyruvate kinase activity was found in the supernatant.

Dialysis: The precipitate containing pyruvate kinase was suspended in 50 mM phosphate buffer (pH 7.5) in a total volume of 20 ml and dialysed for 24 h against the same buffer.

Sephadex G-200 Chromatography: The dialysate was centrifuged at 5.000 g for 40 min at +4°C and the enzyme was applied to the column of Sephadex G-200 equilibrated with 50 mM phosphate buffer (pH 7.5), containing 100 mM KCl, 6 mM mercaptoethanol. Fractions were collected and protein and enzyme activity was determined. The most active fractions were pooled.

Determination of Enzyme Activity: Pyruvate kinase activity was measured at 340 nm at 37°C according to the method of Beutler et al.[13]. The reaction rate was measured by the rate of decrease in absorbance at 340 nm. Enzyme assay was performed using spectrophotometer (Shimadzu UV 120-02) equipped with a recorder. One unit of activity was

defined, as the amount of enzyme required to oxidize 1 micro mole of NADH per min.

Protein Determination: The protein concentration was determined according to Lowry et al.[14].

RESULTS

The pyruvate kinase from human liver tissue was purified by precipitation with $(NH_4)_2SO_4$, dialysis and gel filtration. Table I summarize the results of a typical purification by this procedure. Human liver pyruvate kinase was purified 26.3-fold with 13.2% yield and showed specific activity of 2.6 U/mg protein (Table I).

To determine optimal pH for pyruvate kinase activity, various buffers were tested. These buffers were 1 M KH_2PO_4-K_2HPO_4 (pH 5-8), Tris-HCl (pH 7.1-8.9 pH), glicine-NaOH (pH 8.6-10.6). Pyruvate kinase activity was highest at Tris-HCl buffer (pH 7.1-8.9). The pH optimum for human liver pyruvate kinase was determined to be 7.4 (Figure 1). The effects of K^+, Mg^{2+}, Mn^{2+} and Na^+ on human liver pyruvate kinase activity are shown in Figures 2-5. Pyruvate kinase reaction velocity with respect to all ion concentration except Ca^{2+} was slightly hyperbolic profile. K^+, Mg^{2+}, Mn^{2+} and Na^+ had a stimulatory effect on the human liver pyruvate kinase whereas Ca^{2+} had inhibitory effect (Figure 6).

The V vs S plot for PEP and ADP of the purified enzyme fraction is shown in Figure 7 and 8. The pyruvate kinase type L of human liver exhibited normal sigmoid kinetics for PEP while it exhibited hyperbolic kinetics for ADP. At pH 7.4, Km values for PEP and ADP were 1.2 mM and 0.3 mM, respectively.

Table I. Summary of purification of pyruvate kinase from human liver.
Tablo I. İnsan karaciğer pirüvat kinazının saflaştırılması.

Step of purification	Protein (mg/ml)	Total Activity (Units)	Specific Activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	9.9	985	0.099	-	100
20 % $(NH_4)_2SO_4$ precipitate	7.9	849	0.11	1.08	86.2
40 % $(NH_4)_2SO_4$ precipitate	4.2	515	0.12	1.21	52.3
70 % $(NH_4)_2SO_4$ precipitate	1.8	280	0.16	1.58	28.4
Dialysis	0.45	155	0.34	3.48	15.7
Sephadex G-200	0.05	130	2.6	26.3	13.2

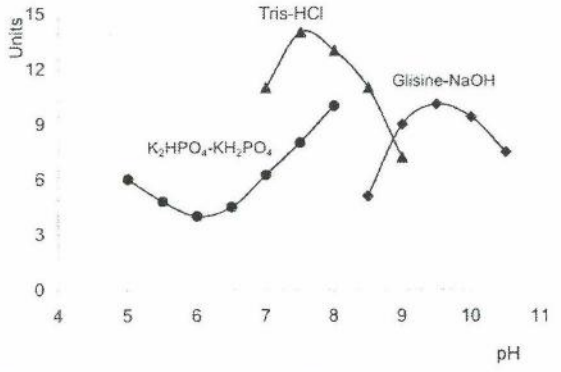

Figure 1. Optimum pH of human liver pyruvate kinase. Rection mixtures contained 1 M KCl, 0.1 M MgCl2, 0.05 M PEP, 0.03 M ADP, 0.01 M FDP, 0.002 M NADH, 60 U LDH. Final reaction voluma was 2 ml (•) K2HPO4-K2PO4 (▲) Tris-HCl (▪) Glisine NaOH

Şekil 1. İnsan karaciğer pyruvat kinazının optimum pH'sı. Reaksiyon karışımı 1 M KCl, 0.1 M MgCl2, 0.05 M PEP, 0.03 M ADP, 0.01 M FDP, 0.002 M NADH, 60 U LDH. Son reaksiyon hacmi 2 ml. (•) K2HPO4-K2PO4 (▲) Tris-HCl (▪) Glisine NaOH

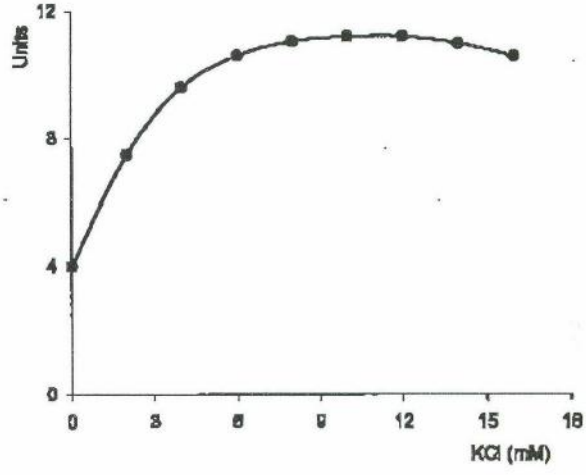

Figure 2. Effect of varying concentrations of K^+ ion on pyruvate kinase activity.

Şekil 2. Pirüvat kinaz aktivitesine farklı K^+ konsantrasyonlarının etkileri.

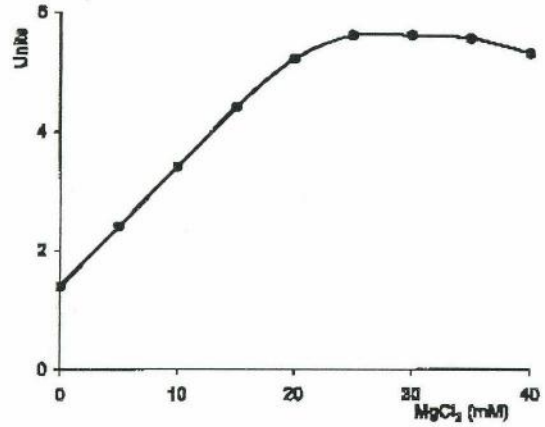

Figure 3. Effect of Mg^{2+} ion concentration on the activity of human liver pyruvate kinase.
Şekil 3. İnsan karaciğer pirüvat kinaz aktivitesine Mg^{+2} iyon konsantrasyonunun etkisi.

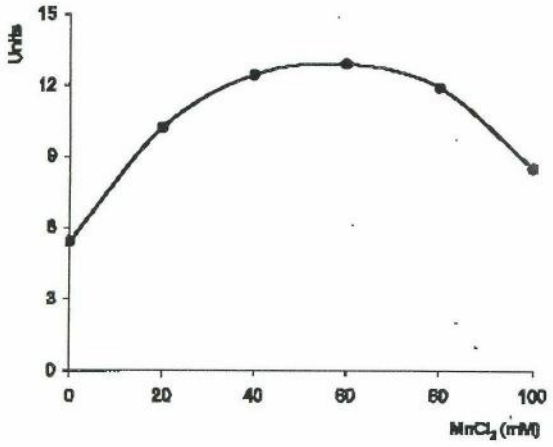

Figure 4. Effect of Mn^{+2} ion concentration on the activity of human liver pyruvate kinase.
Şekil 4. İnsan karaciğer pirüvat kinaz aktivitesine Mn^{+2} iyonkonsantrasyonunun etkisi.

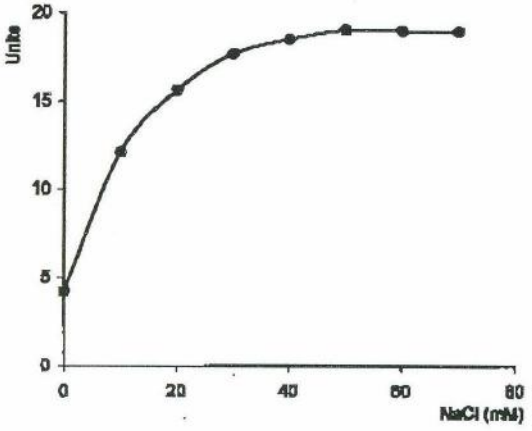

Figure 5. Effect of Na^{+} ion concentration on the activity of human liver pyruvate kinase.
Şekil 5. İnsan karaciğer pirüvat kinaz aktivitesine Na^{+} iyon konsantrasyonunun etkisi.

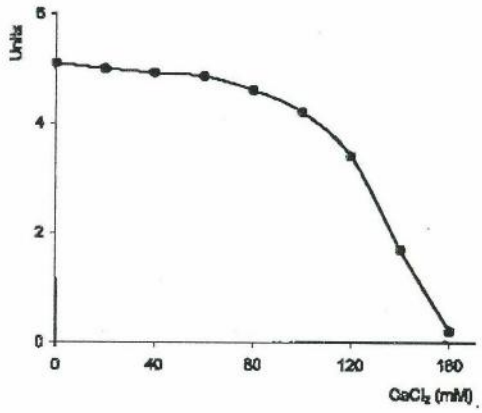

Figure 6. Effect of Ca^{2+} ion concentration on the activity of human liver pyruvate kinase.
Şekil 6. İnsan karaciğer pirüvat kinaz aktivitesine Ca^{+2} iyon konsantrasyonunun etkisi.

The enzyme was activated about 3.8-fold by 1.4 mM FDP when PEP concentration was kept at 5 mM (Figure 9). The Vmax values for PEP and ADP were 11.9 units and 13.9 units, respectively.

The influence of 2,3-diphosphoglycerate (2,3-DPG) and ATP on the enzyme activity depended on its concentration: at low concentrations they stimulated the activity, however at high concentrations they inhibited the enzyme activity (Figures 10, 11). Alanine was also found to inhibit pyruvate kinase (Figure 12).

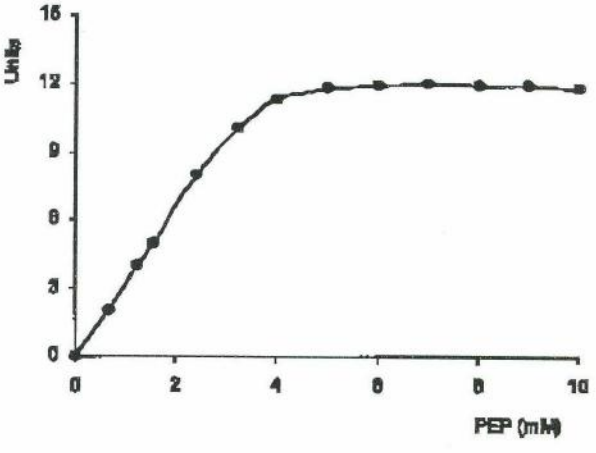

Figure 7. Michaelis-Menten plots for human liver in relation to PEP. Vmax: 11.9 U, Km: 1.2 mM.
Şekil 7. İnsan karaciğerinde PEP için Michaelis-Menten eğrisi Vmax: 11.9 U, Km: 1.2 mM.

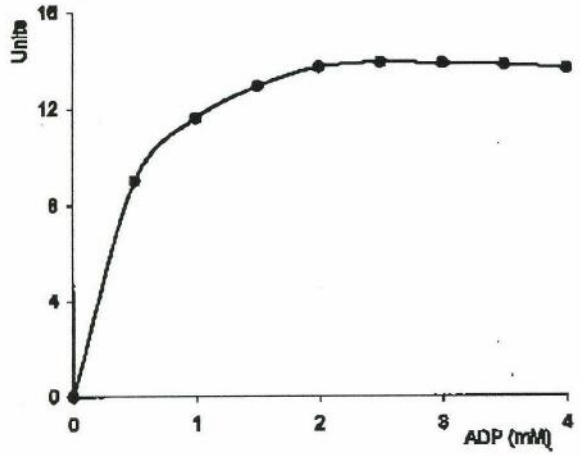

Figure 8. Effect of ADP concentration on reaction velocity for pyruvate kinase. Vmax: 13.9 U, Km: 0.3 mM.
Şekil 8. Pirüvat kinaz reaksiyon hızında ADP konsantrasyonunun etkisi. Vmax: 13.9 U, Km: 0.3 mM.

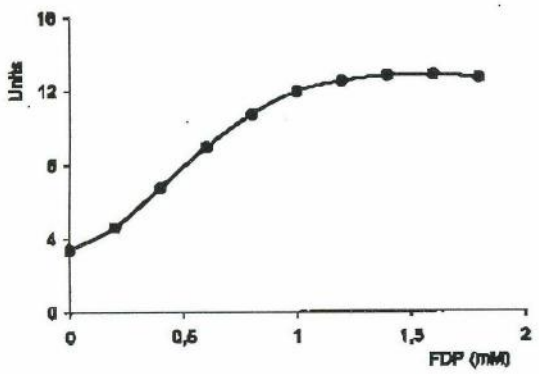

Figure 9. Effect of FDP on the activity of pyruvate kinase.
Şekil 9. Pirüvat kinaz aktivitesinde FDP'nin etkisi

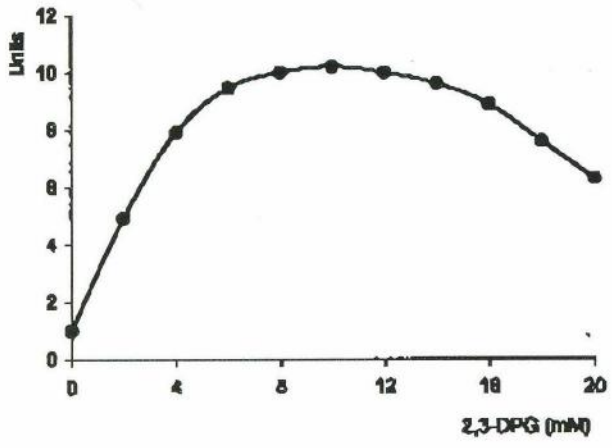

Figure 10. Effect of 2.3-DPG on the activity of pyruvate kinase.
Şekil 10. Pirüvat kinaz aktivitesinde 2.3-DPG'ın etkisi.

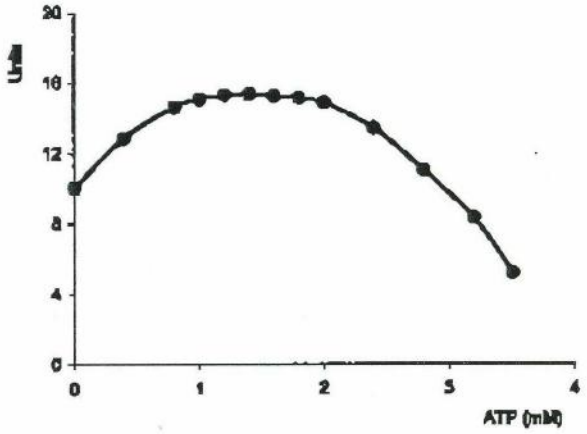

Figure 11. The effect of ATP on the activity of human liver pyruvate kinase.
Şekil 11. İnsan karaciğer pirüvat kinaz aktivitesinde ATP'nin etkisi.

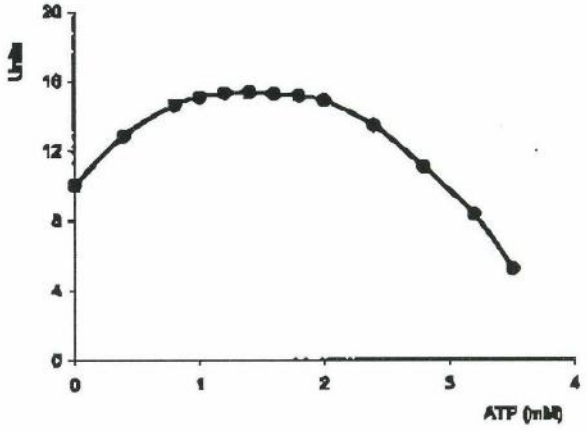

Figure 12. Sensivity of pyruvate kinase to various concentrations of alanine.
Şekil 12. Çeşitli alanin konsantrasyonlarına pirüvat kinazın duyarlılığı.

DISCUSSION

In order to isolate type L of pyruvate kinase from the liver, $(NH_4)_2SO_4$ saturation between 40-70 %, dialysis and gel filtration were used. This isoenzyme was purified 26.3-fold with 13.2 % yield. Specific activity of the enzymes was 2.6 U/mg protein. In order to isolate type L of pyruvate kinase from the mouse liver, Kedryna et al.[15] used $(NH_4)_2 SO_4$ saturation of 21-30 %.

The pH for human liver pyruvate kinase was determined to be 7.4. This value is within the reported pH values for pyruvate kinase from human tissues as 7.5 for human muscle and 8 for human erythrocyte. It is somewhat higher than the optimum of 6.5 reported

for pig liver[5,16,17].

We found that human liver pyruvate kinase required K^+, Mg^{2+}, Mn^{2+} and Na^+ ions for its activity. According to Beutler et al.[18], the stimulatory effect of K^+ is achieved by increasing the enzyme's affinity to ADP. The positive effect of K^+ and Mg^{2+} ions on human liver pyruvate kinase at suboptimal concentrations comes in agreement with that observed in human, amphibian and pigeon erythrocyte pyruvate kinase[16,19]. Pogson[20] demonstrated that FDP did not change the cooperatives of K^+ or Mg^{2+} kinetic in an FDP activated form of rat adipose tissue pyruvate kinase. Suelter et al.[21] found the binding of both monovalent and divalent cations studied exclusively of each other, to be cooperative. L. major pyruvate kinase, like the pyruvate kinase of T. cruzi, has a strict requirement for K^+, while its divalent cation requirement can to be satisfied by either Mg^{2+} or Mn^{2+}[22].

In crustacean muscle, enzyme is inhibited by Na^+ ions[23]. Ca^{2+} ions have inhibited human erythrocyte pyruvate kinase and the inhibitory effect of Ca^{2+} has partially overcome by K^+ [24]. K^+ and Ca^{2+} have a stimulatory effect on the amphibian erythrocyte pyruvate kinase whereas Na^+ has an inhibitory one[23]. Human liver pyruvate kinase was inhibited by Ca^{2+} ions and alanine.

In the enzyme from muscle or erythrocytes, Mn_2^+ ions can replace the more commonly used Mg^{2+} ions. However, the optimum concentration of Mn^{2+} is much lower (1 mM) than that of Mg^{2+} (7 mM), and inhibition by excess ions is much more marked with Mn^{2+}, although the maximum reaction velocities are similar for both ions[23]. Also human liver pyruvate kinase Mn^{2+} ion can replace the Mg^{2+} ion. But the optimal concentration of Mg^{2+} is much lower (25 mM) than that of Mn^{2+} (60 mM).

Significant sigmoid kinetics in relation to PEP was revealed only in the liver pyruvate kinase. The Km values found by various authors for L type of pyruvate kinase in respect to PEP are between 0.4-2.5 mM[15,25]. We found that Km value for human liver pyruvate kinase was 1.2 mM. Feliu et al.[26] observed that the Km (PEP) values for pyruvate kinase might change depending on the buffer composition or concentration, the presence of various electrolytes or effectors, temperature, storage period or even animal diet. Thus, the Km values for pyruvate kinase may be considered as comparable only when they are studied in the same conditions.

FDP, at low PEP levels, substantially activated L-type of pyruvate kinase from human, pig, ox[27]. This activation also occurred with the M_2 isoenzyme from chicken liver, pig kidney, pig lung and tumour, rat hepatoma and rat liver[28]. The activation of human liver pyruvate kinase by 1.2 mM FDP comes in agreement with the results obtained for human and amphibian erythrocyte pyruvate kinase[23]. El-Maghrabi et al.[28] suggested that FDP, apart from regulating pyruvate kinase activity directly, might also act indirectly by modulating the phosphorylation state of the enzyme.

The effect of 2,3-DPG on human liver pyruvate kinase may be similar to that in human erythrocytes[28]. It could be suggested that its physiological significance is related to the existence of the 2,3-DPG bypass in the red blood cell of the pigeon embryos[19]. According to Beutler et al.[18], the effect of 2,3-DPG on pyruvate kinase is merely due to the competition of 2,3-DPG with ADP for Mg^{2+} since they are both powerful magnesium-chelating compounds.

Pyruvate kinase inhibition by ATP has been reported for L type pyruvate kinase by several authors[3,4] The effect of ATP on the pyruvate kinase type L from human liver agrees with those described for the pyruvate kinase purified from rabbit skeletal muscle, bovine heart muscle, bovine brain and for partially purified pyruvate kinase from guinea-pig brain[1]. The influence of ATP on the enzyme activity depended on its concentration: at low concentrations (<1.6 mM) ATP stimulated the activity, however at concentrations >2 mM the inhibition of the enzyme activity was observed. Poole and Bloxham[29] showed alanine inhibited liver pyruvate kinase. Terlecki[1] reported alanine showed no inhibitory effect on the brain pyruvate kinase enzyme. In some tissues of humans and higher animals, allosteric regulation of pyruvate kinase controls glycolytic and gluconeogenic flux. Both the rate and the direction of the glycolytic enzyme sequence may be influenced by the feed-forward activation of pyruvate kinase by FDP, and the feedback inhibition of the same enzyme by ATP.

The biochemical properties reported in this paper support the concept that pyruvate kinase is kinetically suited to participate in the integrated glucose metabolism of the human liver.

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