

TARTU UNIVERSITY  
Faculty of Physics and Chemistry  
Institute of Organic and Bioorganic Chemistry

**Ilona Faustova**

**Some Structural and Kinetic Aspects of L-Type Pyruvate Kinase  
Cooperativity**

*Thesis for Master Degree*

Supervisor: Professor Jaak Järv

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## ABBREVIATIONS

A = Ala – alanine

Acetyl-CoA – acetyl-coenzyme A

ADP – adenosine-5'-diphosphate

ATP – adenosine-5'-triphosphate

BSA – bovine serum albumine

cAMP – cyclic adenosine-3', 5'-monophosphate

CaM PK – Ca<sup>2+</sup>/calmoduline-dependent protein kinase

DTT – dithiothreitol

E = Glu – glutamine acid

FBP – fructose-1,6-bisphosphate

FPLC – fast protein liquid chromatography

K = Lys – lysine

L = Leu – leucine

LDH – lactate dehydrogenase

L-PK – L isoenzyme of pyruvate kinase found in rat liver

M1-PK – M1 isoenzyme of pyruvate kinase found in skeletal muscle

M2-PK – M2 isoenzyme of pyruvate kinase found in kidney, adipose tissues and lungs

NADH – nicotinamide adenine dinucleotide reduced form

NAD<sup>+</sup> – nicotinamide adenine dinucleotide oxidized form

PEP – phosphoenolpyruvate

PK – pyruvate kinase

PKA – protein kinase A

PK Lys(9) – rat liver pyruvate kinase mutant, arginine residue in position 9 is replaced by lysine

R-PK – R isoenzyme of pyruvate kinase found in erythrocytes

-RRASVA- = -Arg-Arg-Ala-Ser-Val- = -arginine-arginine-alanine-serine-valine- residue

TRIS – tris(hydroxymethyl)-aminomethane

Q = Gln – glutamine

## INTRODUCTION

Pyruvate kinase (PK, ATP-pyruvate -O-phosphotransferase, EC 2.7.1.40) is found in various organisms that metabolize sugars: bacteria, plants, and vertebrates. In mammalian tissues four different types of pyruvate kinase have been found. They are denoted as follows (Berg *et al.*, 2002; Munoz & Ponce, 2003):

- M1, which is found in skeletal muscle, heart and brain,
- M2, which is present in kidney, adipose tissue and lungs,
- type L (L-PK) exists in liver and
- R is present in erythrocytes

These enzymes control consumption of metabolic carbon for biosynthesis, and utilization of pyruvate for energy production (Mesecar & Nowak, 1997ab; Muirhead *et al.*, 1986). In liver tissues these both processes may occur simultaneously, and L-PK can be considered as a molecular switch between these metabolic pathways. Therefore the regulatory properties of this enzyme are of extreme importance for living organisms (Mesecar & Nowak, 1997ab; Jurica *et al.*, 1998; Valentini *et al.*, 2002).

Fine regulation of L-PK activity has been achieved by cooperative interaction of the enzyme with one of its substrates - phosphoenopyruvate (PEP). This cooperativity is known to be dependent on phosphorylation of the enzyme at the Ser12 residue of the N-terminal domain of L-PK subunit. As the result of phosphorylation, L-PK affinity against this substrate (PEP) decreases and the cooperative behavior of the catalytic reaction gets amplified (El-Maghrabi *et al.*, 1980, 1982; Pilkis *et al.*, 1980).

Historically, discovery of phosphorylation of L-PK at the serine residue in position 12 of the N-terminal domain has made significant contribution into understanding of the regulatory phosphorylation and substrate specificity of protein kinases in general (Hers & Van Schaftingen, 1984).

However, the structural events following phosphorylation of L-PK and the molecular basis of L-PK cooperativity against PEP are not well understood. Moreover, it is not clear whether phosphorylation amplifies cooperativity of L-PK catalysis, or this property is generated through the enzyme phosphorylation.

In this study we addressed some of these questions by comparative study of kinetic properties of phosphorylated and non-phosphorylated forms of L-PK, and also by investigating into catalytic properties of several mutant forms of L-PK, where the phosphorylation site around the Ser12 residue of the enzyme subunits was modified in positions 9, 10 and 13 of the N-terminal sequence. These mutants were investigated to mimic alteration of ionic status of this part of the protein molecule, by analogy with the phosphorylation of the regulatory domain.

Some results of this study have been published as follows:

Faustova I. and Järv J. Kinetic Analysis of Cooperativity of Phosphorylated L-Type Pyruvate Kinase. Proceedings of the Estonian Academy of Sciences (Chemistry), in press

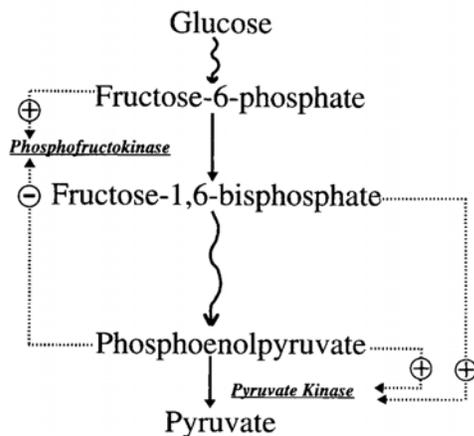
Faustova I., Kuznetsov A., Oskolkov N. and Järv J. (2005) Kinetic properties of some mutants of L-pyruvate kinase. In: *30<sup>th</sup> FEBS Congress and 9<sup>th</sup> IUBMB Convergence. Abstracts of Scientific Conference*, p.369. Budapest, Hungary

Faustova I. (2005) Effect of Net Charge of the Regulatory Domain Peptide on Catalytic Properties of L-pyruvate Kinase. In: *29<sup>th</sup> Estonian Chemistry Days. Abstracts of Scientific Conference*, p.11. Tallinn

# I. THEORETICAL PART

## 1. The role of pyruvate kinases

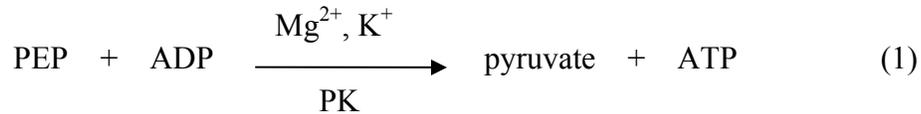
Pyruvate kinase (ATP-pyruvate -O-phosphotransferase, EC 2.7.1.40, PK) is the key enzyme in glycolysis and catalyzes the final step of this metabolic pathway, producing pyruvate and second ATP molecule from phosphoenolpyruvate and ADP (**Figure 1**) (Munoz & Ponce, 2003; Metzler, 2001).



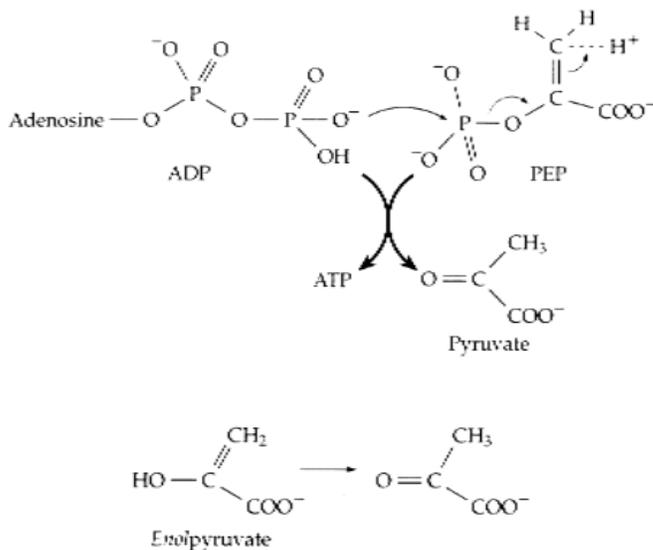
**Figure 1.** The role of pyruvate kinase in the regulation of glycolysis. PK is activated by phosphoenolpyruvate and fructose-1,6-bisphosphate. This metabolite is produced in the reaction catalyzed by phosphofructokinase, the other major regulatory glycolytic enzyme, whose activity is inhibited by phosphoenolpyruvate and activated by fructose-6-phosphate (Mattevi *et al.*,1996).

The similar glycolytic pathway is presented in both eukaryotes and prokaryotes. Product of this reaction – pyruvate – is further used in a number of other metabolic pathways. This fact stresses the importance of the product as well as the role of PK in primary metabolic intersection (Munoz & Ponce, 2003). This important position of PK in living organisms is the main reason for great interest of scientists in this enzyme, including its structure, catalytic mechanism and mechanisms of regulation of its activity.

In the pyruvate kinase catalyzed reaction the phosphoryl group from phosphoenolpyruvate (PEP) is transferred to ADP that leads to the formation of pyruvate and ATP:



The reaction of pyruvate kinase can be presented as two stages. The first is the transfer of the phosphogroup of phosphoenolpyruvate to MgADP and the second is the rapid conversion of the enolic form of pyruvate to keto form (**Figure 2**) (Muirhead *et al.*, 1986; Metzler, 2001).



**Figure 2.** Two stages of pyruvate kinase catalyzed reaction.

In most bacteria and lower eukaryotes only one form of PK can be found. But there are also some bacteria that have two isoforms of this enzyme. In plants pyruvate kinase is presented in two forms: cytoplasmic and plastid. In mammals there are four isoenzymes, as mentioned above: the M1 form (skeletal muscle, heart and brain), the M2 form (kidney, adipose tissue and lungs), the type L in liver and the type R in erythrocytes (Munoz & Ponce, 2003).

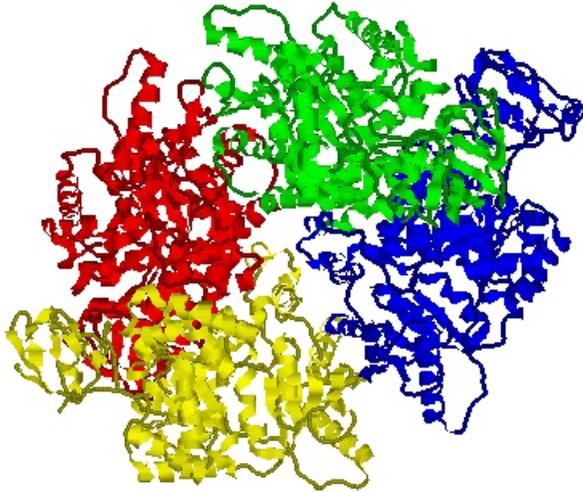
In mammals PK controls both the consumption of metabolic carbon for biosynthesis and the utilization of pyruvate for energy production. In muscle and brain tissues glucose is metabolized to CO<sub>2</sub> and water, or anaerobically to lactate for energy production. Differently, in liver tissues both the glycolysis and gluconeogenesis may occur simultaneously, although the main function of liver is gluconeogenesis: conversion of pyruvate into glucose or into fatty acids via acetyl-CoA. Therefore it is important to regulate pyruvate kinase activity, to prevent substrate cycling between phosphoenolpyruvate and pyruvate. In other words, PK can be considered as a switch between glycolysis and gluconeogenesis. And secondly, through regulation of pyruvate kinase the level of ATP in the cell can be under control (James & Blair, 1982; Mesecar & Nowak, 1997ab; Jurica *et al.*, 1998; Valentini *et al.*, 2000, 2002). Therefore PK plays an important role in functioning of mammalian organisms, including humans.

Deficiency of the human erythrocyte pyruvate kinase (R isoenzyme) is the most common cause of the hereditary non-spherocytic hemolytic anemia. PK has also potential use as a tumor marker, since the isoform Tu M2-PK is strongly over-expressed by tumor cells and released into body fluids, where they can be quantitatively determined. It was shown that the activity of pyruvate kinase in breast cancer cells is significantly increased compared to normal benign breast tissues. In neuroectodermal (brain) tumors shift from the M1 isozyme, the dominant form in the human brain, to the M2 isoenzyme occurs. There is also an interesting possibility to use thermophilic PKs as biosensors (Demina *et al.*, 1998; Mattevi *et al.*, 1996; Munoz & Ponce, 2003; Jurica *et al.*, 1998; Wang *et al.*, 2001).

## **2. Structure of pyruvate kinases**

Although PK is found in many organisms (cat, rabbit, yeast, bacteria, plants, mammals), all these enzymes (or isoenzymes) have rather similar structure. Moreover, although different pyruvate kinase forms, ranking from monomer to decamer, have been reported, it is generally accepted that the predominant and functionally active form is tetramer (Munoz & Ponce, 2003; Muirhead *et al.*, 1986; Jurica *et al.*, 1998; Wooll *et al.*, 2001).

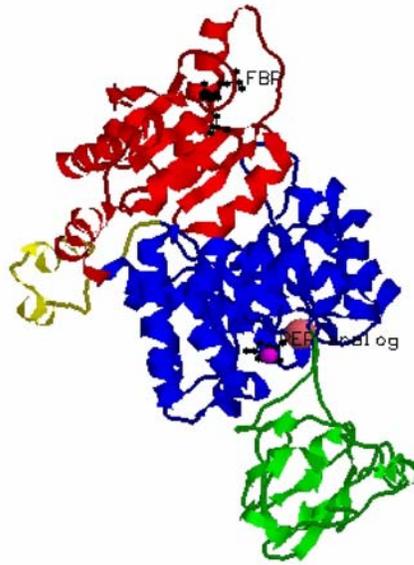
PKs from different organisms have an analogous structure; all of them are usually tetramers of four identical or very similar subunits (**Figure 3**) (Munoz & Ponce, 2003; Muirhead *et al.*, 1986; Jurica *et al.*, 1998; Wooll *et al.*, 2001).



**Figure 3.** The PK tetramer shown here has subunit 1 in blue, subunit 2 in yellow, subunit 3 in green, and subunit 4 in red (Protein Data Bank, PDB ID 1liu).

The molecular mass of the tetramer is about 200-250kDa (Valentini *et al.*, 2002). Each subunit consists of about 500 amino acid residues and has the molecular mass in range of 55-60kDa (Knowless *et al.*, 2001).

The subunits reveal relatively high degree of structural homology and can be divided into three principal domains (**Figure 4**). They are A, B, C and an additional small N-terminal domain. The last one doesn't exist in bacterial pyruvate kinases and in some types of mammalian PKs (Muirhead *et al.*, 1986; Valentini *et al.*, 2002, 2000). Also there are some PK isoenzymes having extended C-terminal domain, for example residues 476-587 of *Bacillus stearothermophilus* pyruvate kinase. All domains are connected to each other through covalent bonds. There is one covalent bond between N-terminal domain and domain A, two covalent bonds between domains A and B, and one covalent bond between domains A and C (Munoz & Ponce, 2003; Muirhead *et al.*, 1986).



**Figure 4.** The PK monomer shown here has A domain in blue, B domain in green, C domain in red, and N domain in yellow. The  $K^+$  and  $Mn^{2+}$  ions are shown in the active site with a substrate analog. The allosteric site in C domain shows FBP (Protein Data Bank, PDB ID 1liu).

Domain A has a classical  $(\alpha/\beta)_8$  topology, domain B is with an irregular  $\beta$  barrel and domain C can be characterized with  $\alpha/\beta$  organization. If there is an additional N-terminal domain, it is usually formed by helix-turn-helix motif (Mattevi *et al.*, 1996; Valentini *et al.*, 2000).

The active site of pyruvate kinase subunit is located in a pocket between A and B domains, closer to domain A. This active site binds PK substrate phosphoenolpyruvate and also positively charged ions: bivalent and monovalent cations (usually  $Mg^{2+}$  and  $K^+$ ), that are required for PK reaction (Muirhead *et al.*, 1986). This site contains three positively charged residues: lysine and two arginine, as well as four negatively charged side chains: two glutamic acid (Glu) and two aspartic acid (Asp) residues (in some species one Glu-residue can be absent). The second pyruvate kinase substrate ADP binds closer to the center of domain A near C domain (Munoz & Ponce, 2003).

Domains C and N are situated at sites of inter-subunit contact, so they can play essential roles in assembly and intermolecular communication (Wooll *et al.*, 2001). Domain C is responsible for allosteric properties of PK. One of the allosteric modulators for PK is fructose biphosphate. It binds to the effector binding pocket between domains A and C, more close to domain C. A cluster of positively charged residues characterizes this site. Also it is important to mention that not all pyruvate kinases are subject to allosteric control (Mattevi *et al.*, 1996; Munoz & Ponce, 2003).

The known regulatory property of domain N is that mammalian pyruvate kinase found in liver can be additionally regulated through phosphorylation (Mattevi *et al.*, 1996; Munoz & Ponce, 2003).

### **3. Mammalian isoenzymes**

In mammals glycolytic enzymes are present in all tissues and cells that utilize them in different ways. In muscles and brains this enzyme is used for energy production from glucose. Differently, the function of liver is mainly gluconeogenesis, where glucose is generated from compounds that consist of three carbon atoms, like pyruvate ( $\text{CH}_3\text{COCOO}^-$ ). When gluconeogenesis is stimulated by starvation, glycolysis must be suppressed (Munoz & Ponce, 2003). In order to fulfill these different demands in mammals, all the four isoenzymes of PK are needed. As mentioned, these isoenzymes are M1 found in muscles, heart and brain, M2 from adipose tissues, kidney and lungs, R presented in erythrocytes and L in liver (Wool *et al.*, 2001).

These PK isoenzymes have different kinetic properties that reflect the different metabolic requirements of the tissues. M1 type of pyruvate kinase shows hyperbolic Michaelis-Menten kinetics toward both substrates: PEP and ADP, and it cannot be allosterically regulated. M2, R and L isoenzymes show sigmoidal response toward PEP. Besides that these enzymes are allosterically regulated via binding of effectors like FBP and some other phosphorylated carbohydrates, which bind to the C domain of PK subunit. It means

that binding of the effector to the one site of enzyme subunit affects the substrate (PEP) binding in another PK subunit. Thus the binding of FBP to the C domain influences the binding of PEP to A domain of PK subunit. Also M2, R and L isoenzymes exhibit a sigmoidal kinetics toward the substrate PEP. This means that this substrate is at the same time homotropic cooperativity effector (Munoz & Ponce, 2003; Gunasekaran *et al.*, 2004; Koshland & Hamadani, 2002; Ainslie *et al.*, 1972).

Cooperativity of L type PK towards PEP depends additionally on phosphorylation on the N-terminal end of its subunit. Thus, the kinetic properties of types L and M1 are quite different: in the case of L-type affinity for substrate PEP is about 10 times less and affinity for inhibitor ATP is higher than these parameters of M1 type (Tanaka *et al.*, 1967). The second substrate of PK reaction is not involved in cooperative regulation of enzyme activity (Munoz & Ponce, 2003).

M1 and M2 isoenzymes are produced from the same gene by differential RNA transcription. This means that M1 and M2 are translated from different messenger RNAs. The difference in amino acid sequence between M1 and M2 type is preferably in the C domain of pyruvate kinase subunit, which constitutes the main region responsible for intersubunit contact and the allosteric regulators binding site lies in the same domain. These residues are common to M2 and L type, but not to the M1. Therefore the kinetic properties of M1 differ from all others isoenzymes, it is not allosterically regulated via binding of allosteric modulator to the C domain (Noguchi *et al.*, 1986; Friesen & Ching Lee, 1998).

R and L isoenzymes are encoded by the same gene. R and L type mRNAs are produced from a single gene by use of different promoters. The R type pyruvate kinase subunit is larger about 3500 Da than L type and it is proposed that it has an additional peptide located in N-terminal end of enzyme subunit. The L type is the only one isoenzyme of these four types, which can be regulated via phosphorylation on N-terminal end (Noguchi *et al.*, 1987; Friesen & Ching Lee, 1998).

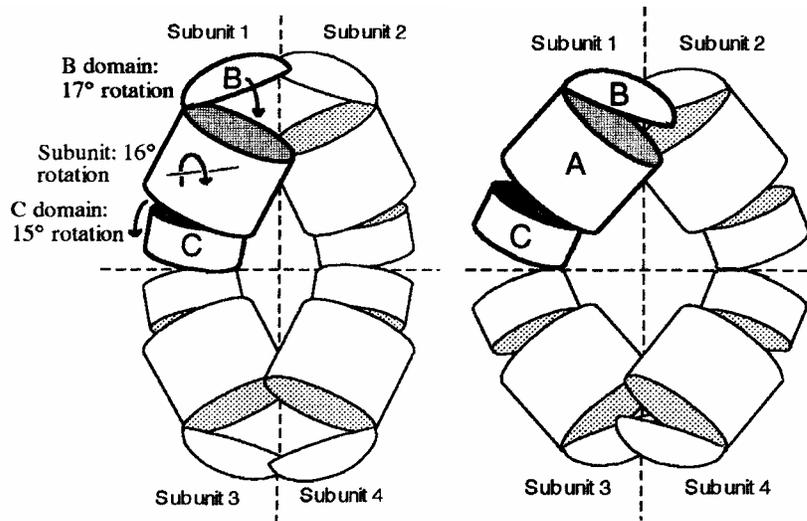
#### 4. Regulation of PK activity

In general, PKs require both monovalent and bivalent cations for their activity. Usually these ions are  $K^+$  and  $Mg^{2+}$ , but there are some bacterial pyruvate kinases that don't need monovalent cation for their activity (*C. glutamicum*, *Z. mobilis*, *E. coli* type II). Instead of  $Mg^{2+}$  and  $K^+$  another mono- and bivalents can be used, such as  $Mn^{2+}$ ,  $Co^{2+}$ ,  $NH_4^+$ .  $Na^+$  is effective only in the presence of FBP (Hunsley & Suelter, 1969). In general  $Mg^{2+}$  and  $K^+$  are considered as physiological activators. It is suggested that the role of  $Mg^{2+}$  is coordination of both pyruvate kinase substrates in the large active site pocket. It coordinates the phosphogroups of phosphoenolpyruvate and ADP before the transfer of PEP phosphogroup to ADP takes place.  $Mg^{2+}$  reduces the electrostatic repulsion between the phosphodonor (PEP) and the nucleophile ( $\beta$  phosphogroup of ADP). In its turn  $K^+$  coordinates the phosphoenolpyruvate carboxyl group, and in the presence of  $K^+$  the affinity of PK- $Mg^{2+}$  to PEP and to ADP- $Mg^{2+}$  increases.  $K^+$  binding is involved in acquisition of active conformation of the enzyme (Muirhead *et al.*, 1986; Mesecar & Nowak, 1997; Oria-Hernandez *et al.*, 2005).

The PK catalyzed reaction can be inhibited by ATP, which is the reaction product. However, this product inhibition can be counteracted by FBP, which is the positive allosteric effector of PK (Rozengurt *et al.*, 1969). The same reaction can be inhibited also by alanine and phenylalanine, which seems to be allosteric inhibitors. Allosteric regulation can be also observed if FBP in mammals or ribose 5-phosphate in most bacterial cells, activate PK (Rigden *et al.*, 1999). In the case of PK activation the allosteric transition mechanism has been discussed in more detail way.

Allosteric activation mechanism includes conformational change of PK from the inactive T-state to the active R-state. While the conversion of PK from inactive T- to active R-state occurs, rotation of the domains forming PK subunit and rotation of each subunit of tetrameric enzyme take place. Thus two kinds of movements can be observed: the rotation of domains B and C within every subunit and the rotation of every subunit within the tetramer (**Figure 5**). So the enzyme activation process involves the combination of

domain and subunit rotations. In this way all subunits and domain interfaces are modified. If the enzyme is in the absence of effector preferably in the active R-state, it doesn't need the allosteric activator for its activation and it cannot be allosterically regulated (Mattevi *et al.*, 1996; Valentini *et al.*, 2000).



**Figure 5.** The rotations of domains and subunits of PK tetramer, occurring on the conformational transition of pyruvate kinase inactive form T to the active form R. T-state is on the left (Mattevi *et al.*, 1996).

M1 PK, the only mammalian isoenzyme, which is not controlled by allosteric effectors. However, this isoenzyme can be converted into an allosteric enzyme by a single amino acid substitution: substitution of an amino acid Ala-398 with Arg results in the acquisition of allosteric properties (Ikeda *et al.*, 1997, 2000). This M1 mutant can be allosterically regulated by F1,6BP and exhibits sigmoidal kinetics toward PEP (the effect of this mutation on the ADP binding is minimal). So the Ala-398 residue of effector site is one of the most critical that allows PK to be preferably in active R-state (Ikeda *et al.*, 1997, 2000).

Allosterically activated pyruvate kinases generally show sigmoidal responses against cations and PEP. But if  $Mg^{2+}$  ions are substituted with  $Mn^{2+}$  ions, the sigmoidal kinetics toward PEP becomes the ordinary hyperbolic kinetics (Mesecar & Nowak; 1997). So it is proposed that  $Mn^{2+}$  can mimic the allosteric effect of PK heterotropic activator (FBP).

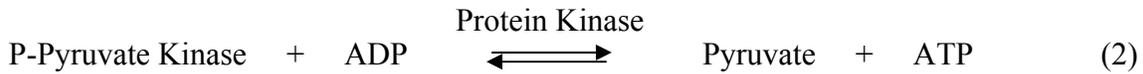
The  $Mg^{2+}$  activated enzyme exhibits hyperbolic dependence of velocities on PEP concentration in the presence of the allosteric regulator FBP. Also it was mentioned that  $Mn^{2+}$  activated enzyme follows an ordered sequential reaction mechanism where ADP is the first substrate which binds to pyruvate kinase, and the last product is pyruvate. Differently in the case of  $Mg^{2+}$  activated enzyme reaction follows the random kinetic mechanism in the presence of FBP (Mesecar & Nowak; 1997ab).

It was shown that in the absence of  $K^+$  ion the random kinetic mechanism was also changed to the ordered mechanism, but with the first substrate PEP (Oria-Hernandes *et al.*, 2005). The affinities for PEP and ADP as well as  $V_{max}$  were higher in the presence of  $K^+$  ion. Thus,  $K^+$  is involved in the acquisition of active conformation of PK, allowing PEP or ADP bind independently (random mechanism). If there is no  $K^+$  ion, ADP cannot bind to pyruvate kinase until PEP forms the competent active site (ordered mechanism) (Oria-Hernandes *et al.*, 2005).

Besides allosteric regulation by fructose-1,6-bisphosphate, L-PK can be additionally regulated via phosphorylation by protein kinase A on N-terminal domain of PK subunit. The phosphorylation takes place at the serine 12 residue, leading to the decreasing of affinity for PEP and also for the allosteric activator FBP. At the same time the affinity for ATP and alanine, which are both inhibitors of this enzyme, increases. Change of the degree of L-PK phosphorylation has no effect on the  $V_{max}$ . Thus in summary, phosphorylation favors the less active T-state of L-PK and also enhances the cooperativity of the catalytic reaction (El-Maghrabi *et al.*, 1980, 1982; Pilkis *et al.*, 1980).

It was shown that the native pyruvate kinase, if purified from rat liver contained 3 mole of phosphate per mole of enzyme. This PK was dephosphorylated using the reverse reaction of phosphorylation (eq.2) that occurs in the presence of cyclic AMP-dependent protein kinase (PKA) and MgADP. Using his reaction all but 1 mole of phosphate per mole of enzyme was removed. Due to dephosphorylation affinity of L-PK for PEP was increased about 1.7 times. Differently, if this enzyme was fully phosphorylated by protein

kinase A, 4 moles of phosphate per mole of enzyme was introduced. In this case affinity of L-PK for PEP decreased two times compared to dephosphorylated enzyme (El-Maghrabi *et al.*, 1980).



Pyruvate kinase can be phosphorylated not only by protein kinase A, but also by a  $\text{Ca}^{2+}$ /Calmoduline-dependent protein kinase. The difference between reaction catalyzed by PKA and by CaM PK is that if PKA phosphorylates pyruvate kinase only at Ser12 residue, the CaM PK catalyzes phosphorylation at two residues: the same serine and some threonine residue. When the affinity for PEP decreased 2 fold in the case of the phosphorylation by PKA, the phosphorylation of the same enzyme by CaM caused 3 fold decrease of affinity for PEP (Schworer *et al.*, 1985).

There is also effect of phosphorylation on cooperative behavior of PK. In one of works by El-Maghrabi *et al.* (1982) the kinetic properties of phosphorylated and dephosphorylated L-PK forms, containing 4.4 and 0.8 mole of phosphate per mole of enzyme, were compared. The cooperativity of phosphorylated enzyme was greater ( $n_H=3.1$ ) than cooperativity of dephosphorylated PK ( $n_H=2.1$ ). At the same time  $K_{0.5}$  for PEP changed from 0.38 to 0.09mM (El-Maghrabi *et al.*, 1982).

In summary, for all PK isoenzymes positive cooperativity has been observed. This means that binding of the first ligand makes the binding of the second ligand easier. Such cooperativity is important property of metabolic enzymes. It amplifies the sensitivity of the signal produced in the reaction. Thus, a very small change in ligand concentration can have a great effect on the output response in positively cooperative systems. For example, 9-fold increase the reaction rate of cooperative system can be easily caused by 3-fold increase in the ligand concentration, whereas the same change in reaction rate of non-cooperative system can be achieved through 81-fold increase in ligand concentration (Koshland & Hamadani, 2002). This property is important to achieve the sensitivity of a metabolic switch.

## II. EXPERIMENTAL PART

### 1. Chemicals

Phosphoenolpyruvate (PEP) tricyclohexylammonium salt, adenosin-5'-diphosphate disodium salt (ADP), L-Lactate dehydrogenase from rabbit muscle (LDH) and bovine serum albumin (BSA) fraction V were purchased from Boehringer Mannheim GmbH, Germany. Nicotinamide adenine dinucleotide reduced form disodium salt (NADH) was from Sigma Chemical Co. Tris(hydroxymethyl)-aminomethane (TRIS) and dithiothreitol was obtained from Sigma-Aldrich (USA). MgCl<sub>2</sub> and KCl were from Acros. The Milli-Q deionized water was used in all experiments.

### 2. Enzymes

L- type pyruvate kinase (L-PK) and its mutant forms, where the following N-terminal domain sequence -Arg-Arg-Ala-Ser(12)-Val-Ala- was modified, were expressed in *E. coli* and purified to homogeneity as described elsewhere (Loog *et al.*, 2005), and were kindly provided for the present study. All the enzymes were purified to homogeneity and this purity was checked by SDS-PAGE electrophoresis as described in (Loog *et al.*, 2005). In this study molecular mass of the enzymes was determined as described below.

The enzyme solutions were made by dilution of the stock solutions with 50mM TRIS-buffer (pH 7.4), containing 0.1% BSA. The enzyme concentration was measured spectrophotometrically by the absorbance of tryptophane (Trp), tyrosine (Tyr) and cysteine (Cys) at 280 nm as described by Aitken & Learmonth, 2002. From the primary structure of the protein (Noguchi *et al.*, 1987; Lone *et al.*, 1986) the number of these amino acid residues was evaluated (Trp - 3, Tyr - 10, Cys - 6) and on the basis of this composition the extinction coefficient 30590 M<sup>-1</sup>sm<sup>-1</sup> was derived.

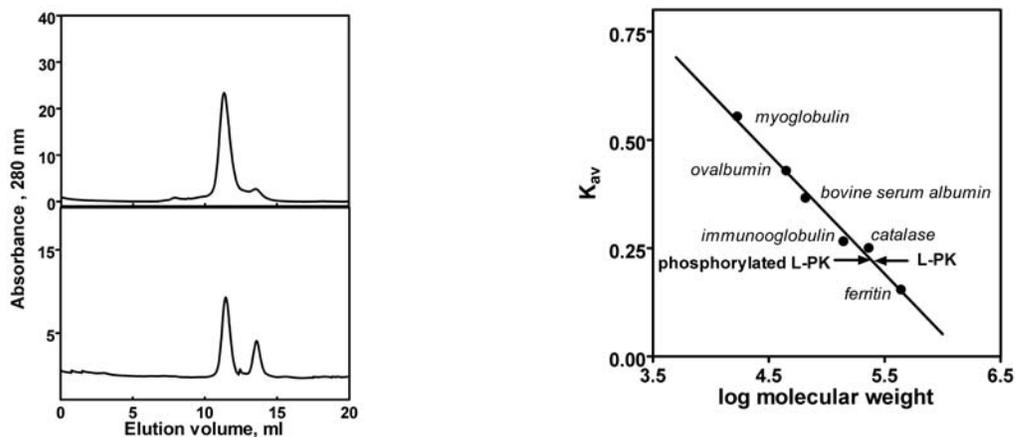
Phosphorylation of L-PK was made by Aleksei Kuznetsov and the samples of the phosphorylated enzyme were kindly provided for this kinetic study. Phosphorylation was made by using the catalytic subunits of the cAMP dependent protein kinase.

### **3. FPLC analysis of molecular mass of L-PK and the phosphorylated L-PK**

The fast protein liquid chromatography (FPLC analysis) was performed to control the molecular weight of enzymes. The protein sample (100-200  $\mu$ l) was applied to Superdex 200 HR 10/30 column and gel filtration was performed in 50 mM Tris/HCl buffer containing 150 mM NaCl at a flow rate 0.5 ml/min at room temperature, using ÄKTA FPLC system (Amersham Biosciences). For molecular weight determination were used following markers: myoglobin (MW = 17 000 Da), ovalbumin (MW = 45 000 Da), bovine serum albumin (MW = 66 000 Da), immunoglobulin (MW = 140 000 Da), catalase (MW = 232 000 Da) and ferritine (MW = 440 000 Da). Using the set of standard proteins the calibration curve as the relationship between  $K_{av}$  for each protein, that characterizes the elution volumes, and the logarithm of their respective molecular weights, was constructed (**Figure 6**).  $K_{av}$  was calculated using following equation (eq.3):

$$K_{av} = \frac{(V_e - V_0)}{(V_t - V_0)}, \quad (3)$$

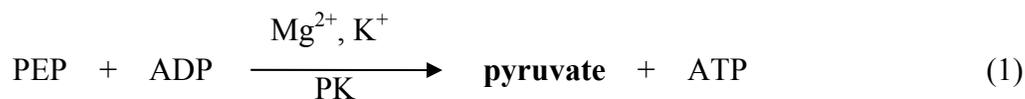
where  $V_e$  – elution volume for the protein,  $V_0$  – column void volume (elution volume for Blue Dextran),  $V_t$  – total bed volume. From the obtained calibration curve the molecular weights for enzymes were calculated.



**Figure 6.** FPLC analysis of recombinant L-PK before (at the top) and after (at the bottom) phosphorylation by PKA (the second peak of the phosphorylated L-PK chromatogram belongs to BSA). The insert shows the MW determination by using the following molecular weight markers (myoglobin, ovalbumin, bovine serum albumin, immunoglobulin, catalase and ferritin).

#### 4. Assay of L-PK activity

Activity of L-PK, its mutants and the phosphorylated form of L-PK was measured spectrophotometrically, using the method described earlier by Fujii & Miwa, 1987. This method is based on the coupling of two reactions: L-PK catalyzed reaction (eq.1) and an auxiliary LDH catalyzed reaction (eq.4).



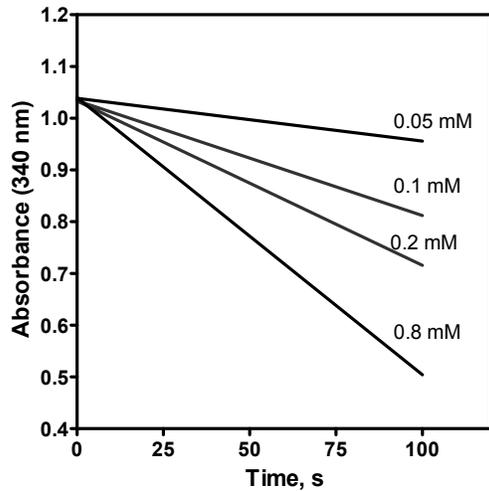
Firstly, L-PK catalyzes the formation of pyruvate and ATP (eq.1). Secondly, the pyruvate formed in this reaction is used by LDH to form L-lactate and converting simultaneously NADH into  $\text{NAD}^{+}$ . The latter change can be followed spectrophotometrically at  $\lambda =$

340nm by the consumption of NADH. Absorbance of the solution strongly decreases with the conversion of NADH into NAD<sup>+</sup> (extinction coefficient for NADH is  $\epsilon = 6220$  L/cm·mol at  $\lambda = 340\text{nm}$ ).

This method can be used for measurement of the L-PK catalyzed reaction only, if the rate of the second, LDH catalyzed reaction, is very fast comparing to the first reaction; only in these conditions the obtained data characterize L-PK properties. To prove, that we measure L-PK activity, the LDH concentration was varied, while all other concentrations were unchanged. In all these cases the apparent velocity of the L-PK catalyzed reaction was similar.

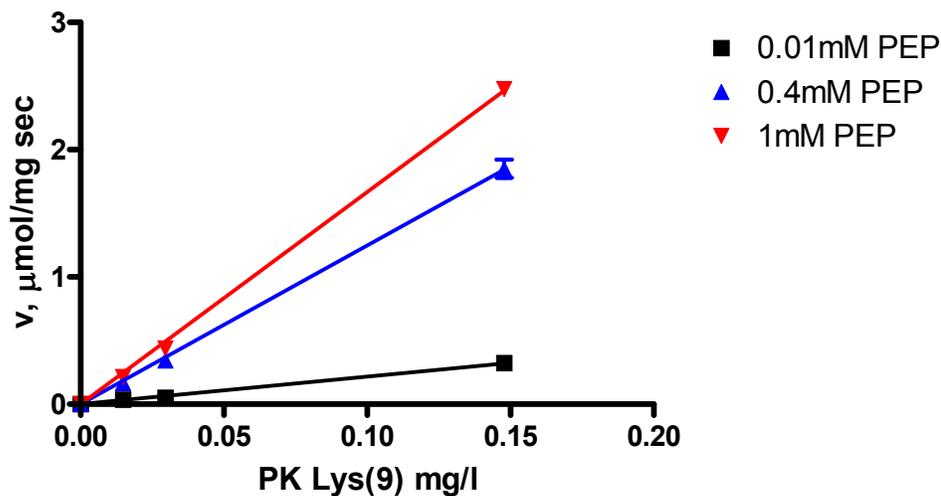
Kinetic measurements were made in 50 mM TRIS/HCl buffer (pH 7.4, 30°C). The reaction mixture contained 0,2mM NADH, 0,002mg/ml (1.5 units/ml) LDH, 100mM KCl, 10mM MgCl<sub>2</sub>, 0.1% BSA, 0.1mM DTT, 0.148 mg/l L-PK, L-PK-Pi or L-PK mutant. Substrate concentrations varied from 0.01 mM up to 10 mM for PEP, while the ADP concentration was hold constant = 1mM and from 0.01 mM to 6 mM for ADP, while the PEP concentration was constant, equal to 2mM.

The final volume of the reaction mixture was 1ml. The reaction was initiated by addition of L-PK, L-PK-Pi or L-PK mutant (960 $\mu$ l of reaction mixture + 40 $\mu$ l of PK) and the consumption of NADH was measured at 340nm using 1cm thermostated quartz cell. The consumption of NADH was followed by the decrease of the optical density of the reaction mixture. The initial velocities of the catalysis were monitored during 1-3 min, at  $\lambda=340\text{nm}$  by UV-VIS spectrophotometer: Unicam UV300, ThermoSpectronic, USA, integration time 0.25 sec, sampling interval 1 sec. Then the initial velocities for different substrate concentrations ( $v$ ) were calculated from the time-course of the absorbance ( $v =$  the slope of linear part of the kinetic curves) (**Figure 7**).



**Figure 7.** Spectrophotometric assay of L-PK activity: at 1 mM ADP and different PEP concentrations.

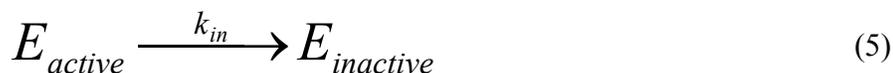
Also to control, that the change of the optical density of the assay mixture was caused by the enzymatic reaction, the measurement of the reaction rate at different enzyme concentrations was done (initial rate should be proportional to the enzyme concentration). The relationship between the initial velocity and L-PK concentration was linear (**Figure 8**), confirming that the change of the optical density of the assay mixture was caused by the pyruvate kinase reaction only.



**Figure 8.** The plot of the initial velocity against the enzyme (PK Lys(9)) concentration.

## 5. Enzyme stability

The stability of enzyme solutions in TRIS buffer, containing 0.1% BSA, was analyzed at 22°C. The decrease in activity of PK and its mutants was observed. The reaction mixture was the same as used for the activity assay. Enzyme activity was measured at different time intervals during 25-50 hours. For characterization of the inactivation process, the plot of the initial velocity versus time was constructed. Generally the initial part of the inactivation process is analyzed, assuming the first-order conversion of the active enzyme into inactive form (eq.5) (Сепп *et al.*, 1983):



The rate constant of the inactivation process ( $k_{in}$ ) can be calculated from the linear plot of  $\ln\left(\frac{v_0}{v_t}\right)$  vs time graph (eq.6) (Sepp *et al.*, 1983):

$$\ln\left(\frac{v_0}{v_t}\right) = k_{in} \cdot t \quad (6)$$

The initial parts of the inactivation process the  $\ln\left(\frac{v_0}{v_t}\right)$  vs time plots were linear, so the  $k_{in}$  values were calculated from the slopes.

## 6. Data processing

All data were analyzed by a non-linear least-squares regression analysis by using the GraphPad Prism version 4.0 (GraphPad Software Inc., USA), SigmaPlot 9.0 (Systat Software Inc., USA) and Microsoft Excel XP (Microsoft Corporation, USA). The values reported are given with standard errors.

### III. RESULTS AND DISCUSSION

#### 1. Properties of non-phosphorylated L-PK

In this study we have used L-PK sample, expressed in *E. coli*, as described by Loog *et al.* in 2005. Molecular properties of this enzyme were identical with the properties of the enzyme isolated from rat liver, as was shown by SDS electrophoresis by Loog *et al.*, 2005. In this study the same protein sample was additionally analyzed by gel-filtration on a Superdex 200 HR column. The results of this analysis are shown in **Fig 6**. The column was calibrated as described in Experimental part, and the molecular mass of the enzyme, equal to 245 551 Da, was calculated from these data. This value was in good agreement with the molecular mass calculated using the amino acid sequence of L-pyruvate kinase structure (Noguchi *et al.*, 1987; Lone *et al.*, 1986). As the elution profile obtained by measuring absorbance overlapped with the plot for enzyme activity, it was concluded that the catalytically active enzyme functioned under the assay conditions as tetramers.

This tetrameric enzyme was effectively phosphorylated by the catalytic subunit of cAMP-dependent protein kinase, as was shown by A. Kuznetsov in our lab. The absence of phosphorylation of the product, expressed in *E. coli*, is well understandable, as the expression system doesn't contain mammalian protein kinases. Application of  $\gamma[^{32}\text{P}]\text{ATP}$  as substrate for this phosphorylation reaction revealed that  $3.95 \pm 0.20$  phosphate groups were incorporated into the tetrameric L-PK structure (private communication). Thus, the protein purified from the expression system of *E. coli* could be defined as “non-phosphorylated L-PK”, and this enzyme is definitely distinct from the native L-PK samples purified from mammalian tissues. Further the kinetic properties of this unique L-PK sample were analyzed.

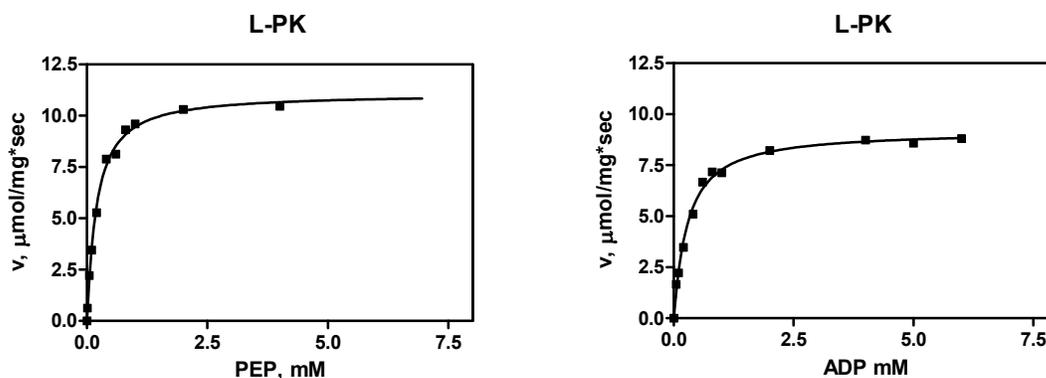
Results of this kinetic study are presented in **Figure 9** as plots of the reaction initial velocity over concentration of both substrates, PEP and ADP. In each of these plots concentration of the other substrate was kept constant (conditions see in Figure Caption). It can be seen from these results that interaction of both substrates with the enzyme can be described by the common Michaelis-Menten rate equation:

$$v = \frac{V [S]}{K_m + [S]} \quad (7)$$

where S is variable substrate, V – maximal velocity under these conditions,  $K_m$  is the Michaelis – Menten constant, characterizing the affinity of substrate to enzyme and equal to the concentration of substrate at which the half of maximal velocity will be achieved. This conclusion was additionally confirmed by data processing using the Hill rate equation (eq.8).

$$v = \frac{V [S]^n}{K_{0.5}^n + [S]^n} \quad (8)$$

where n is the cooperativity parameter (Hill coefficient), S is the variable substrate, V – the maximal velocity observed under the conditions used, and the constant  $K_{0.5}$  denotes the substrate concentration at which the reaction rate is half of the maximal rate. The results of data processing by this equation are listed in Table 1. It can be seen that the Hill parameter is not distinguishable from unity; this means that the cooperative regulation of the reaction rate by PEP and ADP cannot be observed, and indeed, the Hill rate equation (eq.8) can be simplified into the Michaelis-Menten rate equation (eq.7).



**Figure 9.** The kinetics of reaction of non-phosphorylated L-PK at variable PEP concentration and constant ADP concentration (1mM) (left panel) and at variable ADP concentration and constant PEP concentration (2mM) (right panel).

**Table 1.** Kinetic parameters for the Hill rate equation (8), calculated for non-phosphorylated L-PK (50 mM TRIS/HCl buffer, pH 7.4, 30°C).

Enzyme	PEP (at 1 mM ADP)			ADP (at 2 mM PEP)		
	V, μmol/mg s	K <sub>0.5</sub> , mM	n	V, μmol/mg s	K <sub>0.5</sub> , mM	n
L-PK	11.1±0.3	0.20±0.02	1.0±0.1	9.2±0.2	0.28±0.03	1.1±0.1

It can be seen in Table 1 that the V values are different as calculated from different plots. This is not surprising, as the reaction involves two substrates. This means the genuine reaction mechanism can be presented by the following reaction scheme (eq.9).



In this reaction scheme E is L-PK, A is ADP and B is PEP, and  $K_a$  and  $K_b$  are the appropriate substrate constants. If the random reaction mechanism is assumed as a more general case, the rate equation for this scheme can be presented as follows (eq.10):

$$v = \frac{V \frac{1}{\alpha} [PEP][ADP]}{K_a K_b + K_b [ADP] + K_a [PEP] + \frac{1}{\alpha} [PEP][ADP]} \quad (10)$$

The coefficient  $\alpha$  from this equation characterizes the randomness of the substrate binding sequence and is equal to unity, if both substrates interact independently with the enzyme active site. Processing of the kinetic data for L-PK reaction with PEP and ADP by the rate equation above (eq.10) yielded the following kinetic parameters:

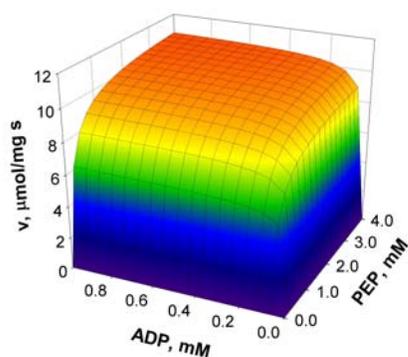
$$V = 15.8 \pm 0.5 \mu\text{mol/mg sec}$$

$$K_a = 0.26 \pm 0.03 \text{ mM}$$

$$K_b = 0.11 \pm 0.02 \text{ mM}$$

$$\alpha = 0.96 \pm 0.10$$

It can be seen that the results of data processing agree with the random mechanism of this two-substrate reaction, and the overall process can be characterized by three constants:  $V$ ,  $K_a$  and  $K_b$ . For illustration the 3D plot of the kinetic data for the L-PK catalyzed reaction is shown in **Figure 10**.



**Figure 10.** 3D plot of the kinetic data for non-phosphorylated L-PK.

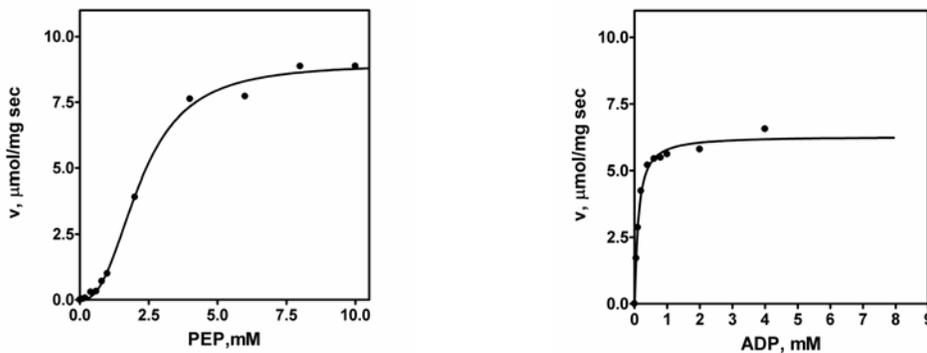
Thus, it can be concluded that the mammalian L-PK, expressed in *E. coli*, does not reveal cooperativity against PEP, and therefore differs from the samples of this enzyme extracted from mammalian tissues. We find this difference rather significant, as the tetrameric structure, which is associated with cooperative regulation of the kinases is present also in the case of this L-PK. On the other hand, the enzyme expressed in the bacterial system is not phosphorylated, that was found to be the reason for this difference, as shown below.

## 2. Properties of phosphorylated L-PK

The L-PK sample was phosphorylated and the phosphorylation reaction reached plateau after 20 min, pointing to the fact that the phosphorylatable sites of the enzyme were saturated by phosphate (A.Kuznetsov, private communication). This saturation corresponded to mol-per-mol phosphorylation of the subunits of the enzyme. In this study the molecular mass of the phosphorylated enzyme was determined by the gel filtration on Superdex 200 HR column. The results shown in **Figure 6** reveal that the protein, associated with the L-PK-Pi activity, eluted from the column similarly with the non-phosphorylated L-PK, providing the molecular mass values  $233\,293 \pm 15\,200$  Da. This is in agreement with the presence of the tetrameric structure of the enzyme under conditions, used for its activity assay. The second protein eluted after the tetrameric form

of the phosphorylated L-PK was bovine serum albumin. This protein was added to the stock solution of the cAMP-dependent protein kinase catalytic subunit for stabilization of the enzyme. Concentration of the catalytic subunit itself was too low to be detected in this experiment. Thus, in summary, the mole-per-mole phosphorylation of L-PK produces the tetrameric form of the phosphorylated enzyme. As the next step, kinetic properties of the phosphorylated L-PK were analyzed in more detail way, as phosphorylation of the enzyme affected significantly its kinetic and regulatory properties.

The data shown in **Figure 11** reveal that the main difference between the non-phosphorylated and phosphorylated L-PK can be associated with the sigmoidal dependence of the phosphorylated L-PK catalytic activity on PEP concentration, while no cooperativity can be observed in the case of ADP. This is in agreement with the generally accepted understanding that the rate of the L-PK catalyzed reaction is cooperatively regulated only by one of the substrates, PEP, while interaction of the other substrate ADP with the enzyme follows the common Michaelis-Menten type kinetics (Munoz & Ponce, 2003). This asymmetry in cooperativity of the bi-substrate reaction of the phosphorylated form of L-PK was confirmed by data processing, where the Hill rate equation (eq.8) was used in both cases. The results of the analysis are shown in Table 2.



**Figure 11.** Influence of PEP at constant ADP concentration (1 mM) and ADP at constant PEP concentration (2 mM) on the rate of reaction, catalyzed by the phosphorylated L-PK

**Table 2.** Kinetic parameters for the Hill rate equation (8), calculated for phosrylated L-PK (50 mM TRIS/HCl buffer, pH 7.4, 30°C).

Enzyme	PEP (at 1 mM ADP)			ADP (at 2 mM PEP)		
	V, μmol/mg s	K <sub>0.5</sub> , mM	n	V, μmol/mg s	K <sub>0.5</sub> , mM	n
Phosphorylated L-PK	9.0±0.2	2.2±0.1	2.5±0.2	6.3±0.2	0.11±0.01	1.1±0.2

It can be seen in this Table 2 that cooperativity of PEP was characterized by the n value 2.5. Also, it can be observed that phosphorylation of L-PK decreases its apparent affinity for PEP, by increasing the appropriate kinetic parameter  $K_m$  from 0.2 mM up to the  $K_{0.5}$  value 2.2 mM. Differently from PEP, phosphorylation of L-PK has only minor influence on interaction of the enzyme with its second substrate, ADP. Most importantly, in this case no cooperativity of the enzyme was posed, as the Hill coefficient for ADP was unity within its error limits (Table 2), and only slight increase in affinity for this substrate was observed.

It is obvious that the changes in catalytic properties of L-PK, generated by phosphorylation of the enzyme, can be understood if the bi-substrate reaction mechanism is taken into consideration. Due to obvious complications in explicit kinetic modeling of cooperatively regulated bi-substrate reaction, catalyzed by tetrameric enzyme, the kinetic data were analyzed proceeding from the rate equation for a non-cooperative bi-substrate random-order enzymatic reaction (eq.9) (Segel, 1975), where the reactive complex involves the enzyme, ADP and PEP. Taking into account the phenomenon of asymmetric cooperativity, the conventional bi-substrate kinetic model was adopted for the L-PK-Pi catalyzed reaction. As far as formation of the ternary complex is affected by PEP interaction with other subunits of the enzyme, the Hill parameter n was introduced into the rate equation for a bi-substrate reaction (eq.10), and the following equation was

obtained, where n stands for cooperativity parameter (Hill coefficient) for PEP:

$$v = \frac{\left( \frac{V[ADP]}{K_a + [ADP]} \right) [PEP]^n}{\left( \frac{K_a K_b + K_b [ADP]}{K_a + [ADP]} \right)^n + [PEP]^n} \quad (11)$$

In this equation V stands for the maximal velocity,  $K_a$  and  $K_b$  characterize interaction of ADP and PEP with the enzyme, and n is the Hill coefficient for PEP. It is important to emphasize that in this equation it is suggested that there is no cooperativity in the case of ADP and that substrates interact with the enzyme by the random mechanism. All data above agree with this suggestion.

Processing the kinetic data by the two-parameter equation (eq.11) provided the following results:

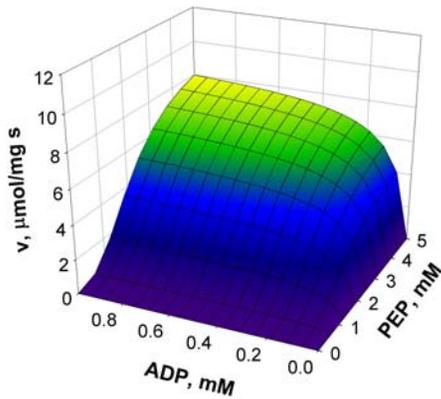
$$V = 9.6 \pm 0.7 \text{ } \mu\text{mol/mg s}$$

$$K_A = 0.10 \pm 0.05 \text{ mM (for ADP)}$$

$$K_B = 2.2 \pm 0.3 \text{ mM (for PEP)}$$

$$n = 2.5 \pm 0.3 \text{ (for PEP)}$$

For illustration, the 3D plot of the reaction rate on concentration of PEP and ADP is shown in **Figure 12**. It is obvious that in this case the constant V is independent on concentration of any of the substrates and thus represents the maximal rate of the overall reaction. Analogously, the constant  $K_a$  should explicitly characterize interaction of ADP with the enzyme, as this value can be obtained separately from the cooperative effect of PEP, characterized by the Hill coefficient n. The values of  $K_b$  and n are in good agreement with the n and  $K_{0.5}$ , obtained from data processing by the single-parameter equation (eq.8).



**Figure 12.** Illustration of the bi-substrate kinetic model for the phosphorylated L-PK catalyzed reaction with PEP and ADP.

Secondly, the sequential interaction model for cooperative effect of PEP on the phosphorylated L-PK catalyzed reaction was applied. This was possible because of asymmetric appearance of cooperativity in this bi-substrate reaction. This model assumes that interaction of substrate molecule with its binding site on one subunit affects binding properties of the remaining (“free”) subunits of the multimeric enzyme (Koshland & Hamadani, 2002; Koshland & Neet, 1968). Therefore, maximally four different levels of the enzyme affinity for PEP can be defined, which correspond to the four possible levels of enzyme saturation with this substrate. For simplification of the model and reduction of the number of variables, we assumed that the presence of another substrate, ADP, has no effect on PEP binding.

The four levels of the enzyme saturation with substrate S (S stands for PEP in this particular case) can be presented by the following schemes, where the substrate interaction with the first subunit is quantified by the dissociation constant  $K$ , affinity for the second substrate molecule is quantified by  $\alpha K$ , affinity for the third and fourth substrate molecules by  $\beta K$  and  $\gamma K$ , respectively. Formally this situation can be presented by the following reaction schemes (eq.12-15), assuming the sequential substrate interaction with the enzyme.



It is important to consider that the overall rate of the process depends on the total concentration of the enzyme-substrate complexes. However, the formation of these complexes is determined besides the equilibriums shown in schemes (eq.12-15) also by the probability factors. So, in the case of the tetrameric enzyme there are four ways to form ES complex from E, six ways to form the complex ES<sub>2</sub> from ES, four ways to form ES<sub>3</sub> from ES<sub>2</sub> and one way to form ES<sub>4</sub> from ES<sub>3</sub>. Taking into account these probability factors, and assuming that all complexes are in equilibrium, the following rate equation can be obtained for the cooperatively functioning tetrameric enzyme (eq.16):

$$v = V \frac{\frac{[S]}{K} + \frac{3[S]^2}{\alpha^2 K^2} + \frac{3[S]^3}{\beta^3 K^3} + \frac{[S]^4}{\gamma^4 K^4}}{1 + \frac{4[S]}{K} + \frac{6[S]^2}{\alpha^2 K^2} + \frac{4[S]^3}{\beta^3 K^3} + \frac{[S]^4}{\gamma^4 K^4}} \quad (16)$$

In this equation the maximal reaction rate V is determined by the total amount of the active sites participating in catalysis that is four times bigger than the analytical concentration of the enzyme (eq.17):

$$V_{MAX} = 4k[E]_{total} \quad (17)$$

Following the basic idea of stepwise formation of the enzyme-substrate complexes, the constraints that  $\alpha$ ,  $\beta$  and  $\gamma$  must be equal or greater than 1 were used in processing of the experimental data by eq.16. Under these conditions, the best fit of the experimental data for PEP was achieved at:

$$V = 10 \mu\text{mol/mg s}$$

$$K = 30 \text{ mM}$$

$$\alpha = 1.0$$

$$\beta = 0.1$$

$$\gamma = 0.1$$

It should be mentioned that variation of each of these parameters within 10% limit of their value clearly changed the goodness of the fit, characterized by sum of squares of deviations between the experimental and predicted values as well as by the multiple correlation coefficient ( $R_i = 0.998$  for the set of parameters above). Secondly, the goodness of the fit was significantly reduced if the complex  $ES_4$  alone, or both complexes  $ES_4$  and  $ES_3$ , were omitted from the analysis by taking  $\gamma = 0$  or  $\beta = \gamma = 0$ , respectively. Thus, the kinetic data agree with the tetrameric structure of the enzyme, and point to the fact that all the four different levels of the complex formation are necessary for modeling the positive cooperative regulation of the reaction velocity.

However, keeping in mind the bi-substrate nature of the reaction, it should be emphasized that for the catalytic step the presence of ADP in the enzyme-substrate complex is needed. Therefore, if the enzyme is not saturated by this second substrate, the constant  $K$  in eq.16 does not necessarily quantify affinity of L-PK-Pi for PEP. On the other hand,  $\alpha$ ,  $\beta$  and  $\gamma$  characterize the cooperative interactions between the enzyme subunits and therefore should be independent on the reaction conditions.

Similarly, the constant  $V$ , if calculated from one-parameter plots presented by eq.16, 7 or 8, is not a characteristic value for the bi-substrate reaction in general, but also depends on the affinity/concentration ratio of the second substrate. This cross-effect of substrate

concentration is clear from the results above, where the  $V$  values calculated from the rate versus concentration plots for ADP and PEP, are different. Therefore, for insight onto the mechanism of the L-PK catalyzed reaction, the bi-substrate nature of this reaction was observed above eq.10 and eq.11.

Following this sequential model, cooperativity of the enzyme is characterized by parameters  $\alpha$ ,  $\beta$  and  $\gamma$ . These parameters, also named as “interaction factors”, compare affinities of the first binding step with each of the following binding steps (Segel, 1975). Therefore, if  $\alpha = 1$ , there is no difference between the enzyme-substrate dissociation constants on the first and on the second steps. In other words, binding of the first substrate molecule has no effect on binding of the second molecule. This is the case observed for L-PK-Pi. Affinity of the enzyme for the third substrate molecule is increased, as  $\beta = 0.1$ .  $\gamma$  is also equal to 0.1. Thus, affinity of L-PK-Pi for the last two PEP molecules is similar. Most likely this means that binding of substrate molecules with two subunits of L-PK-Pi triggers off the conformational transition that changes the substrate binding sites on the two remaining subunits. These changes should be similar, to provide similar affinity for PEP. This change of binding properties is quantified by 10-fold decrease of the enzyme-substrate complex dissociation constants that is enough for generation of cooperativity of the enzyme. In terms of the substrate-protein interactions, this change is quite realistic and can be achieved by addition of one or two weak interactions between the substrate molecule and its binding site. As a result of these additional interactions small changes in concentration of PEP may have far significant effect on the rate of the process, if compared with the effect of substrate concentration on velocity according to the Michaelis-Menten type kinetics.

In summary, the kinetic analysis revealed that phosphorylation of L-PK generates cooperativity, and this property is not characteristic for the non-phosphorylated enzyme. In other words, phosphorylation makes possible that binding of two PEP molecules may increase approx 10-fold the binding affinity of the two remaining binding sites. This should follow some structural alteration of the enzyme, most probably on the level of the phosphorylated subunits. If this alteration can be explained through alteration of

electrostatic interactions, aroused by introduction of two negative charges of the phosphate group, it can be assumed, that all other possibilities of introduction of these charges do the same. Following this hypothesis, mutants of L-PK subunits with changed peptide structure around the phosphorylatable site at Ser 12 residue were prepared by Loog *et al.*, 2005 and kinetic properties of these enzymes were analyzed in this study.

### 3. Effect of mutations on L-PK kinetic properties

The mutants with the following sequence of N-terminal domain near phosphorylated site were analyzed in this study, proceeding from the idea that the ionic status of the N-terminal part of L-PK may mimic the effect of phosphorylation:

...Ala-Arg-Ala-Ser(12)-Val-Ala...

...Lys-Arg-Ala-Ser(12)-Val-Ala...

...Glu-Arg-Ala-Ser(12)-Val-Ala...

...Gln-Arg-Ala-Ser(12)-Val-Ala...

...Arg-Ala-Ala-Ser(12)-Val-Ala...

...Arg-Lys-Ala-Ser(12)-Val-Ala...

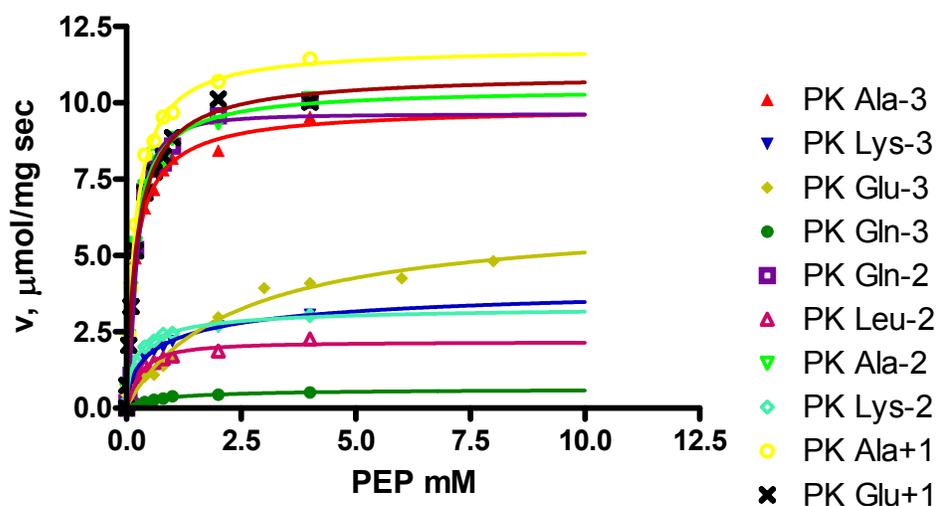
...Arg-Gln-Ala-Ser(12)-Val-Ala...

...Arg-Leu-Ala-Ser(12)-Val-Ala...

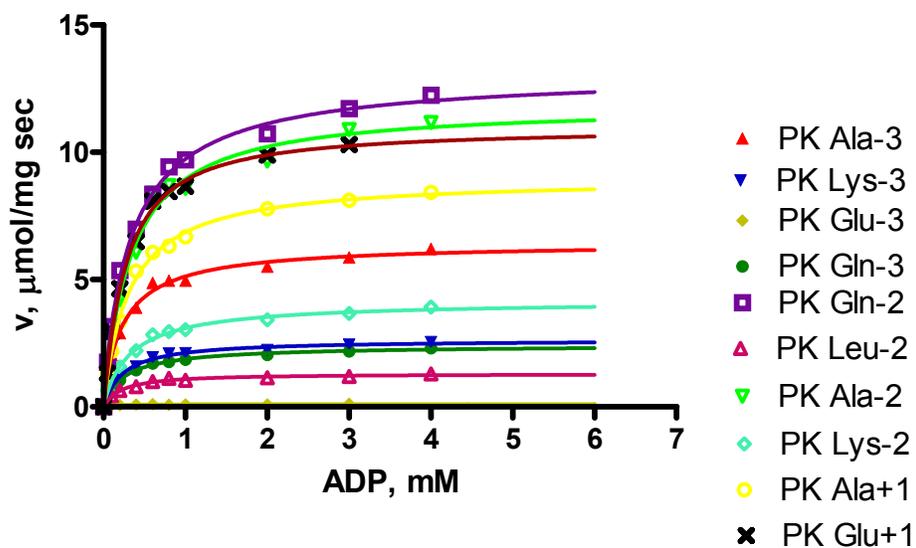
...Arg-Arg-Ala-Ser(12)-Ala-Ala...

...Arg-Arg-Ala-Ser(12)-Glu-Ala...

It can be seen that in these structures both, the cationic and anionic amino acids were used, together with non-ionic hydrophobic residues. For all these mutants the kinetic data were obtained at the same conditions as for wild type of L-PK. The activity of L-PK mutants was measured by variation of concentrations of both substrates, keeping the concentration of the second substrate constant (1mM for ADP and 2mM for PEP), and the following plots of the initial velocities vs substrate concentration were obtained (**Figures 13, 14**).



**Figure 13.** Influence of PEP at constant ADP concentration (1 mM) on the rate of reactions, catalyzed by mutants of L-PK as denoted by different colors (50 mM TRIS/HCl buffer, pH 7.4, 30°C).



**Figure 14.** Influence of ADP at constant PEP concentration (2 mM) on the rate of reactions, catalyzed by mutants of L-PK as denoted by different colors (50 mM TRIS/HCl buffer, pH 7.4, 30°C).

To characterize substrate interactions with enzymes these data were processed by the Hill rate equation (eq.8), and the cooperativity coefficients were evaluated (Table 3).

**Table 3.** Cooperativity coefficients  $n$  for wild type and mutant of L-PK-s

Sequence	Net charge of the phosphorylation site peptide	$n_H$	
		PEP	ADP
-RRAS <sup>12</sup> VA-	+2	1.0 ± 0.1	1.1 ± 0.1
-RRAS(P <sub>i</sub> )VA-	0	2.5 ± 0.2	1,1±0,2
-ARAS <sup>12</sup> VA-	+1	0.9 ± 0.1	0.9 ± 0.1
-KRAS <sup>12</sup> VA-	+2	0.6 ± 0.1	1.0 ± 0.1
-ERAS <sup>12</sup> VA-	0	0.9 ± 0.1	0.9 ± 0.3
-QRAS <sup>12</sup> VA-	+1	0.9 ± 0.1	0.9 ± 0.1
-RAS <sup>12</sup> VA-	+1	1.0 ± 0.1	1.0 ± 0.1
-RKAS <sup>12</sup> VA-	+2	0.7 ± 0.1	1.0 ± 0.1
-RQAS <sup>12</sup> VA-	+1	1.1 ± 0.1	0.9 ± 0.1
-RLAS <sup>12</sup> VA-	+1	1.2 ± 0.3	1.0 ± 0.1
-RRAS <sup>12</sup> AA-	+2	1.0 ± 0.1	1.0 ± 0.1
-RRAS <sup>12</sup> EA-	+1	1.0 ± 0.1	1.1 ± 0.1

The results show that Hill coefficient is not distinguishable from unity within error limits of all of the mutants. This means that the kinetics of substrate reaction, catalyzed by mutants of L-PK follows the ordinary Michaelis-Menten kinetics and is not controlled by positive cooperative influence of PEP as well as ADP. Only two mutants, where arginine was substituted with lysine in position 9 and 10, revealed the  $n$ -value somewhat less than unity. But the difference is rather small and we do not have explanation for such kinetic behavior of these mutant proteins.

Thus, as no one mutant exhibited positive cooperativity toward PEP, we can conclude that the cooperative effect of phosphorylation cannot be mimicked by the mutations in the phosphorylatable N-terminal site of pyruvate kinase.

#### 4. Stability of enzyme

The stability of phosphorylated L-PK, non-phosphorylated L-PK, and its mutants was analyzed under the same conditions as the kinetic measurements were made. The change in activity of enzymes was monitored at fixed substrate concentration during some time. Assuming that the initial part of the inactivation process follows the first-order conversion of the active enzyme into inactive form (eq.5) (Cern *et al.*, 1983), the  $k_{in}$  for each enzyme was found from the slope of the linear plots  $\ln\left(\frac{v_0}{v_t}\right)$  vs time. The first-order rate constants of the inactivation process, calculated for L-PK, L-PK-Pi and mutants are listed in **Table 4**.

**Table 4.** Inactivation constants for L-PK, L-PK-Pi and PK mutants. Charge shows the total charge of the mutated amino acid sequence (-**RRAS<sup>12</sup>VA**-) around phosphorylatable serine 12 residue.

Sequence	Net charge of the phosphorylation site peptide	$10^3 k_{in}, \text{min}^{-1}$
- <b>RRAS<sup>12</sup>VA</b> -	+2	$1.0 \pm 0.1$
- <b>RRAS(P<sub>i</sub>)VA</b> -	0	$0.9 \pm 0.3$
- <b>ARAS<sup>12</sup>VA</b> -	+1	$3.4 \pm 0.3$
- <b>KRAS<sup>12</sup>VA</b> -	+2	$0.93 \pm 0.02$
- <b>ERAS<sup>12</sup>VA</b> -	0	$6.0 \pm 0.4$
- <b>QRAS<sup>12</sup>VA</b> -	+1	$3.9 \pm 0.7$
- <b>RAAS<sup>12</sup>VA</b> -	+1	$2.4 \pm 0.2$
- <b>RKAS<sup>12</sup>VA</b> -	+2	$0.7 \pm 0.2$
- <b>RQAS<sup>12</sup>VA</b> -	+1	$2.4 \pm 0.1$
- <b>RLAS<sup>12</sup>VA</b> -	+1	$4.01 \pm 0.09$
- <b>RRAS<sup>12</sup>AA</b> -	+2	$0.8 \pm 0.1$
- <b>RRAS<sup>12</sup>EA</b> -	+1	$1.1 \pm 0.2$

It is seen from the obtained results that some mutations influenced on the stability of pyruvate kinase, while phosphorylation has no effect on the  $k_{in}$  of PK. The most sensitive to mutations was position 9, at the same time substitutions in position 13 had no effect on enzyme stability. Mostly the rate of inactivation increased in those L-PK mutants, where mutations involved the change of net charge of phosphorylatable site peptide.

It is interesting that in this case there seems to be some interrelationship between the protein stability and the net charge of its N-terminal part sequence, especially if the two arginines were replaced by other amino acids. So, in the presence of these 2 positive charges  $k_{in}$  is around  $10^{-3} \text{ min}^{-1}$ , while in the presence of 1 positive charge the  $k_{in}$  value remains between  $2.4 \cdot 10^{-3}$  and  $4 \cdot 10^{-3}$  and in the case of net charge 0  $k_{in}$  rises up to  $6 \cdot 10^{-3} \text{ min}^{-1}$ . On the other hand, not only this factor seems to determine stability of L-PK mutants, as phosphorylation of serine (additional negative charge) had no observable effect on enzyme stability. In summary, these data clearly show that mutations have some effect on enzyme stability and therefore also on enzyme structure. These structural changes are, however, not linked with the properties, which cause of cooperativity behavior of the enzyme.

## CONCLUSIONS

1. In this study kinetic properties of non-phosphorylated and phosphorylated L-PK were studied. These studies revealed that the non-phosphorylated form of L-PK does not reveal cooperativity towards PEP as well as ADP concentration, while the phosphorylated enzyme revealed cooperativity against PEP concentration. This means that cooperativity of the enzyme is generated by the phosphorylation reaction.

2. The kinetic mechanism of cooperative regulation of the phosphorylated form of L-PK was described in terms of the model of sequential substrate binding. It was found that binding of two substrates (PEP) occurs at similar affinity, while binding of the following two substrate molecules occurs more effectively. Enhancement of affinity of the binding sites through the cooperative mechanism was approx 10 times.

3. Kinetic properties of L-PK mutants with variable structure of the N-terminal part around the phosphorylatable Ser12 residue were studied. It was found that activity of all these enzymes was well described by the common Michaelis-Menten rate equation. This means that these mutants do not show cooperativity against PEP concentration, although the net charge of the N-terminal domain is changed by these mutations, like it happens by L-PK phosphorylation at the Ser12 residue. Thus, it was concluded that mutations on the N-terminal peptide do not mimic the phosphorylation effect. In other words, it means that the cooperativity of the substrate reaction cannot be induced by replacement of the amino acids, but selectively needs phosphorylation of the Ser12 residue.

4. Stability of non-phosphorylated and phosphorylated forms of L-PK and of all L-PK mutants studied was investigated under the same conditions as were used for kinetic assay. This analysis revealed that some mutations influenced the enzyme stability. The most sensitive part from this point of view was position 9, while the substitution of amino acid residue in position 13 had no effect on enzyme stability. In this case there seems to be interrelationship between stability of the mutant proteins and the net charge of the peptide sequence before the phosphorylatable Ser12 residue, pointing to the possibility that electrostatic interactions could be to some extent responsible for stabilizing of the enzyme structure.

# KOKKUVÕTE

## **L-tüüpi püruvaadi kinaasi kooperatiivsuse struktuurilised ja kineetilised aspektid.**

**Iona Faustova, magistritöö, 2006**

Püruvaat kinaas osaleb glükolüüsis ning seetõttu on kontroll selle ensüümi katalüütiliste omaduste üle väga tähtis selle metabolismi raja regulatsioonil. L – tüüpi püruvaadi kinaasi korral saavutatakse aktiivsuse peenem regulatsioon selle ensüümimolekuli seriini jäägi fosforüleerimise teel. Fosforüleerimine toimub valgumolekuli N-domeeni järjestuses ...Arg(9)-Arg(10)-Ala-Ser(12)-Val(13)... oleva Ser12 juures.

Selles töös uuriti mittefosforüleeritud L-tüüpi püruvaadi kinaasi kineetilisi omadusi ja võrreldi neid cAMP-sõltuva proteiinkinaasi poolt fosforüleeritud L-PK omadustega. Leiti, et mittefosforüleeritud ensüüm ei ilmuta kooperatiivsust oma substraadi (PEP) suhtes. Samas ilmneb selline kooperatiivsus fosforüleeritud ensüümi korral. Seega fosforüleerimine mitte ainult ei reguleeri L-PK kooperatiivsust, vaid põhjustab selle täiendava regulatsioonimehhanismi ilmumise. Samas ei ole fosforüleerimata ega fosforüleeritud ensüümi vormide katalüütiline aktiivsus kooperatiivselt reguleeritud ADP poolt.

Kineetika andmete analüüsil täpsustati ka fosforüleeritud L-PK poolt katalüüsitud bi-substraatse reaktsiooni kooperatiivsus kineetilist mudelit. Analüüsi tulemused näitasid, et fosforüleerimine alandab L-PK afiinsust PEP suhtes, kuna efektiivne substraatkonstant suurenes väärtuselt 0,11mM fosforüleerimata ensüümi korral väärtuseni 2,2mM fosforüleeritud ensüümi korral. Arvestades seda, et substraatide kooperatiivne toime on erinev ADP ja PEP korral, kasutati analüüsiks bi-substraatse reaktsiooni kineetilist mudelit, ning samuti substraadi järk-järgulise sidumise mudelit. Viimasel juhul selgus, et kahe PEP molekuli sidumine tekitab muutuse ensüümi afiinsuses ning ülejäänud kahe molekuli sidumine toimub umbes 10 korda efektiivsemalt, kui eelmiste substraatide sidumine.

Kuna fosforüleerimisel muutub valgumolekuli N-terminaalse osa summaarne laeng, siis kontrolliti selles töös ka hüpoteesi, et L-PK aktiivsuse regulatsioon on seotud summaarse laengu muutumisega N-domeenil. Selle hüpoteesi kontrollimiseks muudeti L-PK molekuli N-terminaalse domeeni laengut valgu punktmutatsioonide teel modifitseeritud produktis. Nendes produktides on valgu primaarses struktuuris asendatud positsioonidel 9, 10 ja 13 olevaid aminohapete jääke. Vastavad mutatsioonid on loodud Mart Loogi ja Nikita Oskolkovi poolt ning neid on kirjeldatud varem (Loog *et al.*, 2005). Käesolevas töös mõõdeti muteeritud ensüümide kineetikat, eesmärgiga kontrollida ülalloodud hüpoteesi. Selgus, et regulatoorse domeeni summaarse laengu muutumine mutatsioonide tõttu ei kutsunud esile ensüümi kooperatiivsust.

Lisaks reaktsioonikineetikale uuriti ka fosforüleeritud ja mittefosforüleeritud püruvaadi kinaasi ning viimase valgu mutantide stabiilsust, iseloomustades seda inaktivatsioonireaktsiooni kiiruskonstandiga  $k_{in}$ . Leiti, et mõned mutatsioonid mõjusid ensüümi stabiilsust. Ilmnes, et kõige tundlikum positsioon püruvaadi kinaasi inaktivatsiooni suhtes on 9. Samal ajal amiinohappe jääkide asendamine positsioonis 13 ei mõjunud ensüümi stabiilsust. Kuna ensüümide inaktivatsiooni kiirenemine oli seotud laengu muutumisega, saab järeldada, et elektrostaatilised interaktsioonid võivad vastutada ensüümi struktuuri stabilisatsiooni eest. Samas aga Ser 12 fosforüleerimine ei avaldanud mõju ensüümi inaktivatsioonile.

Seega kokkuvõtteks, vaatamata sellele, et mutatsioonid mõnedes positsioonides mõjutasid ensüümimolekuli stabiilsust ja seega ilmselt ka ensüümi struktuuri, need muutused ei põhjustanud substraatreaktsiooni kooperatiivsust, vaid selleks on vaja Ser12 jäägi fosforüleerimist.

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