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Regulatory role of L-type pyruvate kinase N-terminal domain

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Regulatory role of L-type pyruvate kinase N-terminal domain

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Paper I **Faustova I.** and Järv J. (2006) Kinetic analysis of cooperativity of phosphorylated L-type pyruvate kinase. Proc. Estonian Acad. Sci. Chem., 55, 4, 179–189.

Paper II **Faustova I.,** Kuznetsov A., Juronen E., Loog M. and Järv J. (2010) Phosphorylation is switch of L-type pyruvate kinase allostery. CEJB, 5, 135–142.
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Paper III **Faustova I.,** Loog M. and Järv J. (2012) Probing L-Pyruvate Kinase Regulatory Phosphorylation Site by Mutagenesis. Protein J., 31(7), 592–7.
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Paper IV **Faustova I.** and Järv J. (2013) Interaction of Non-Phosphorylated Liver Pyruvate Kinase with Fructose 1,6-Bisphosphate and Peptides that Mimic the Phosphorylatable N-Terminus of the Enzyme. Protein & Pept. Lett., 20, 1200–1203.
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Author contributions:

Paper I Responsible for experimental work and data analysis. Participated in preparation of the manuscript.

Paper II Responsible for kinetic experiments and data analysis. Participated in preparation of the manuscript.

Paper III Responsible for kinetic experiments and data analysis. Participated in preparation and submission of the manuscript.

Paper IV Responsible for all performed kinetic experiments and data analysis. Participated in preparation and submission of the manuscript.

LIST OF ABBREVATIONS

Amino acids	–	IUPAC single letter code
AC_{50}	–	the amount of activator needed to activate a process by half
Acetyl-CoA	–	acetyl-coenzyme A
ADP	–	adenosine-5'-diphosphate
ATP	–	adenosine-5'-triphosphate
BSA	–	bovine serume albumine
cAMP	–	cyclic adenosine-3', 5'-monophosphate
CaM PK	–	Ca^{2+}/calmoduline-dependent protein kinase
DTT	–	dithiothreitol
FBP	–	fructose-1,6-bisphosphate
FPLC	–	fast protein liquid chromatography
IC_{50}	–	the amount of inhibitor needed to inhibit a process by half
$K_{0.5}$	–	the substrate concentration at which the reaction rate is half of maximal reaction rate (V_{max})
K_{ADP}	–	$K_{0.5}$ for ADP, the concentration of substrate adenosine-5'-diphosphate (ADP) at which the reaction rate is half of V_{max}
k_{cat}	–	catalytic rate constant
K_m	–	Michaelis constant
K_{PEP}	–	$K_{0.5}$ for PEP, the concentration of substrate phosphoenolpyruvate (PEP) at which the reaction rate is half of V_{max}
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
n	–	Hill coefficient describes cooperativity
NADH	–	nicotinamide adenine dinucleotid reduced form
NAD^+	–	nicotinamide adenine dinucleotid oxidized form
PEP	–	phosphoenolpyruvate
PK	–	pyruvate kinase
PKA	–	protein kinase A catalytic subunit
R-PK	–	R isoenzyme of pyruvate kinase found in erythrocytes
TRIS	–	tris(hydroxymethyl)-aminomethane
UV-VIS	–	ultraviolet visible
v	–	initial rate of enzymatic reaction
V^{app}	–	the apparent maximal reaction rate
V_{max} or V	–	maximal reaction rate

1. INTRODUCTION

The activity of enzymes can be controlled on the level of individual proteins through non-covalent binding of effector molecules or cooperatively by substrate binding, and also through covalent protein modification, most often by regulatory phosphorylation at serine, threonine or tyrosine residues. In many cases these different regulation mechanisms may function together, especially in the case of enzymes that play key functions in the crossing points of metabolic pathways and therefore need more sophisticated control mechanisms.

A typical example of the complexity of enzyme regulation can be found in the case of pyruvate kinase from liver tissue, which participates in the last step of glycolysis, and like other pyruvate kinase isozymes, synthesises ATP from ADP and phosphoenolpyruvate (PEP). However, differently from other tissues, liver tissue is responsible not only for glycolysis, but also for gluconeogenesis, and this fact calls for coordination and even reciprocal regulation of these two metabolic pathways, making one inactive while the other is active. One of the checkpoints of this regulatory mechanism lies at the crossover of the phosphoenolpyruvate/pyruvate transformation catalysed by the liver-type pyruvate kinase. Thus the tissue-specific regulatory properties of this enzyme play an important role in glucose metabolism.

Like most known pyruvate kinase isozymes, the enzyme extracted from liver tissue also reveals cooperativity towards phosphoenolpyruvate binding, is activated by fructose 1,6-bisphosphate, and is inhibited by ATP and alanine (Munoz & Ponce, 2003; Flory *et al*., 1974). However, differently from all other pyruvate kinases the liver enzyme is also regulated by phosphorylation of its N-terminal domain (Munoz & Ponce, 2003; Tanaka *et al*., 1967). This modification decreases the activity of the enzyme by increasing the K_{PEP} value and the Hill coefficient n for phosphoenolpyruvate. Although on the phenomenological level this regulatory effect has been known since the 1970s (Ekman *et al*., 1976), its mechanism and structural background as well as the role of the phosphorylatable N-terminal regulatory peptide of the protein in general have not been established.

One reason for this uncertainty is the absence of the crystallographic structural data of the N-terminal end of the protein, as due to significant flexibility, the positioning of the amino acids of this peptide chain has not been determined. Therefore, it was challenging to investigate the regulatory mechanism by using the kinetic approach in combination with computer modelling of protein structure and docking sites. This analysis has provided unique information about the regulation of the enzyme activity and might have implications for deeper understanding of the regulatory phosphorylation phenomena in general.

2. PYRUVATE KINASES

2.1. Occurrence and functions

Pyruvate kinases (ATP-pyruvate-O-phosphotransferase, EC 2.7.1.40, PK) are enzymes involved in the glycolytic metabolic pathway, which is the crucial component of energy metabolism in cells and carbon metabolism in general. These enzymes catalyse the final step of glycolysis, transferring the phosphoryl group of phosphoenolpyruvate (PEP) to ADP, producing pyruvate and ATP (Bugg, 2001; Mesecar & Nowak, 1997ab; Sowadski & Epstein, 2001):

$$\text{PEP} + \text{ADP} \xrightarrow[\text{PK}]{\text{Mg}^{2+},\ \text{K}^{+}} \text{pyruvate} + \text{ATP} \qquad (1)$$

The actual role of pyruvate kinases depends on the needs of the tissue, and these enzymes may control consumption of metabolic carbon for biosynthesis and also utilisation of pyruvate for energy production. In muscle and brain tissues glucose is metabolised for energy production to CO_2 and water, or to lactate under anaerobic conditions. However, in liver tissue both glycolysis and gluconeogenesis may occur simultaneously, although the main function of the liver is gluconeogenesis, conversion of pyruvate into glucose or into fatty acids via acetyl-CoA may occur. In mammals about 90% of blood glucose is produced by gluconeogenesis in the liver. Therefore, it is important to regulate pyruvate kinase activity, to prevent substrate cycling between phospho-enolpyruvate and pyruvate. Subsequently PK can be considered as a switch between glycolysis and gluconeogenesis. And moreover, the level of ATP in the cell can be controlled through regulation of pyruvate kinase activity (Fenton & Hutchinson, 2009; James & Blair, 1982; Mesecar & Nowak, 1997ab; Jurica *et al*., 1998; Valentini *et al*., 2000, 2002). This option is illustrated in Figure 1.

Due to multiple functional requirements different isozymes of PK can be found in higher organisms, while bacteria and lower eukaryotes have only one form of PK. However, there are some bacteria that have two isoforms of this enzyme. Plants have 2 isoforms of PK, located in cytoplasm and plastids, respectively.

In mammalian tissues 4 PK isozymes are known, and these proteins are encoded by 2 genes (M and L loci) (Munoz & Ponze, 2003). These isozymes have been denoted by the following abbreviations:

M1 – found in skeletal muscle, heart and brain,
M2 – found in kidney, adipose tissue and lungs,
L – extracted from liver
R – found in erythrocytes

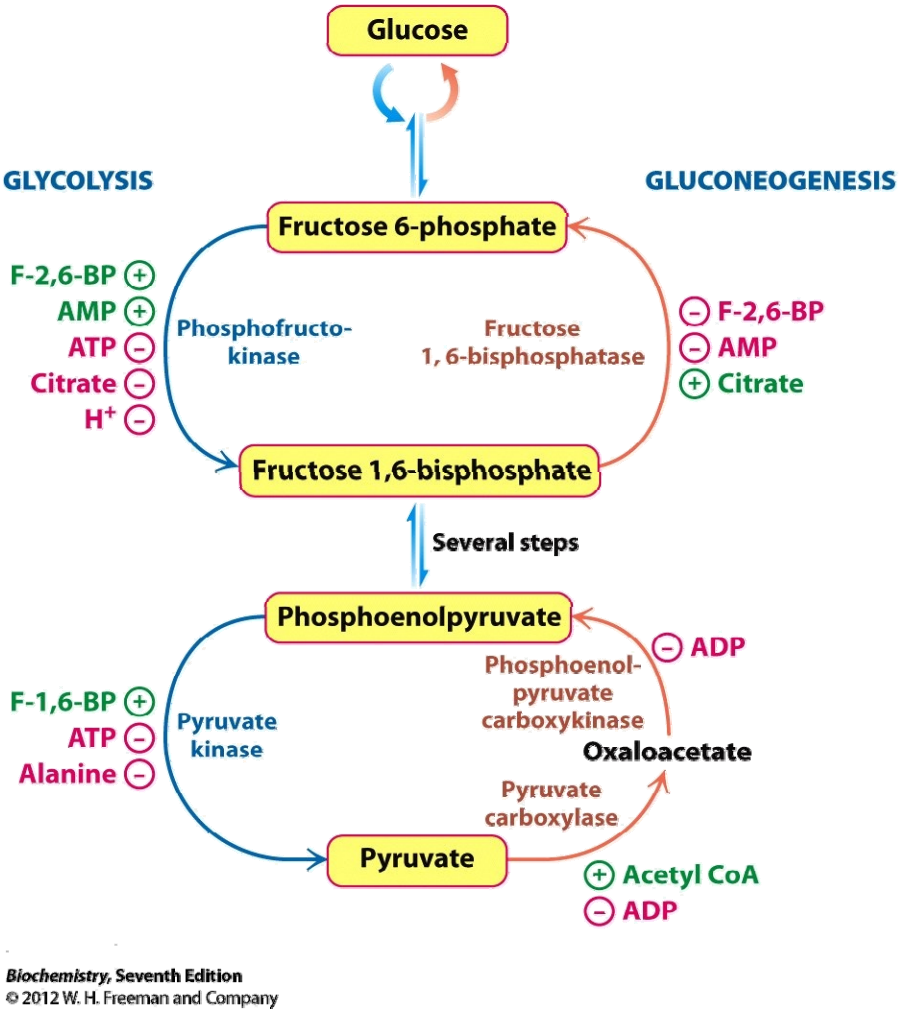


Figure 1. Reciprocal Regulation of Glycolysis and Gluconeogenesis (Biochemistry 2012, seventh edition, Freeman and Company, reproduced by permission)

The isozymes M1 and M2 are encoded by one gene with transcription by two different mRNAs. Similarly L and R isoenzymes are produced from the same gene by using different promoters (Noguchi *et al*., 1987). All these isozymes have similar structure and comparable functions, but their kinetic and regulatory properties may still be different, and these differences are governed by the specific metabolic requirements of the expressing tissue, as was stressed above.

It is generally accepted that only the M1 isozyme follows hyperbolic Michaelis-Menten kinetics, while the activity of the M2, R and L subtypes reveals more complex regulatory patterns. This pattern includes homotropic regulation by one of the substrates of this reaction, PEP, and heterotrophic regulation by different signal molecules, among which FBP has central impor-

tance (Munoz & Ponze, 2003; Mattevi *et al*., 1996; Valentini *et al*., 2000; Jurica *et al*., 1998). In the case of homotropic regulation, substrate is at the same time an effector of the multimeric enzyme. In other words, the binding of the substrate molecule to the multimeric enzyme influences binding of the next molecule of the same substrate. Heterotrophic regulation means that binding effectiveness of a substrate molecule is changed in the presence of an effectors molecule, which binds to a distinct effector binding site (Berg, 2012; Bindslev, 2008; Monod *et al*., 1965) So, in the presence of the allosteric regulator FBP, pyruvate kinases exhibit hyperbolic dependence of reaction velocity on PEP concentration (Ekman *et al*., 1976).

The activity of L-type PK can be additionally regulated through phosphorylation of the serine 12 residue of its N-terminal domain. As a result of phosphorylation the affinity of PEP to L-PK decreases and cooperativity coefficient n increases. All other types of pyruvate kinases lack this regulatory mechanism (El-Maghrabi *et al*., 1980, 1982; Pilkis *et al*., 1980, Muirhead *et al*., 1986). Historically, discovery of phosphorylation of L-PK has made a significant contribution to development of the concept of regulatory phosphorylation in general (Hers & Van Schaftingen, 1984). However, the molecular mechanism of L-PK activity regulation is still not well understood, and several aspects of this phenomenon will be discussed later in this dissertation.

Taken together, the diversity of regulation mechanisms of pyruvate kinase activity seem to be important for their functioning as the key enzymes regulating the glycolytic pathway and thus controlling both the energetic and metabolic statuses of living cells.

2.2. Structure of pyruvate kinases

The primary structure of pyruvate kinases is highly conserved among different organisms, and a significant similarity can also be observed in the spatial structure of these enzymes. The predominant and functionally active form of these enzymes is tetrameric (Figure 2), but they can also exist in monomeric up to decameric form (Munoz & Ponze, 2003). In mammalian tissues all functionally active isozymes are tetrameric and they consist of four similar subunits.

The molecular mass of the tetrameric PKs is around 220-250 kDa (Valentini *et al*., 2002), and each subunit consists of about 500 amino acid residues and has a molecular mass of 55–60 kDa (Knowless *et al*., 2001).

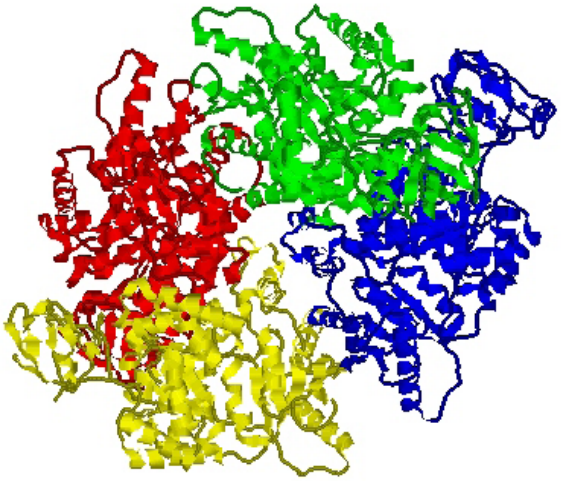


Figure 2. The PK tetramer shown here has subunit 1 in blue, subunit 2 in yellow, subunit 3 in green, and subunit 4 in red (Protein Data Bank, PDB ID 1liu).

Each subunit of PK can be divided into 3 domains – A, B and C (Figure 3). In addition, some types of PK have a relatively small N-terminal domain. In the tetrameric enzyme structure these domains are located in such a way that the N-terminal domain and the sequence that links domains A and C are at the centre of the molecule (Pendergrass *et al*., 2006). All domains are connected to each other through covalent bonds: there is one covalent bond between the 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).

The tetrameric structure of these enzymes can be divided into four parts, separated by inter-subunit planes, which mark the contacting surfaces of the interacting subunits (Munoz & Ponze, 2003, Jurica *et al*., 1998, Muirhead *et al*., 1986).

Domain A is characterised by $(\alpha/\beta)_8$ topology, domain B by an irregular β barrel and domain C can be characterised with α/β organisation. An additional the N-terminal domain is usually formed by a helix-turn-helix motif (Mattevi *et al*., 1996; Valentini *et al*., 2000), in some cases its structure has not been determined by X-ray analysis due to the high flexibility of this peptide. This is the case for the liver-type isozyme studied in this work.

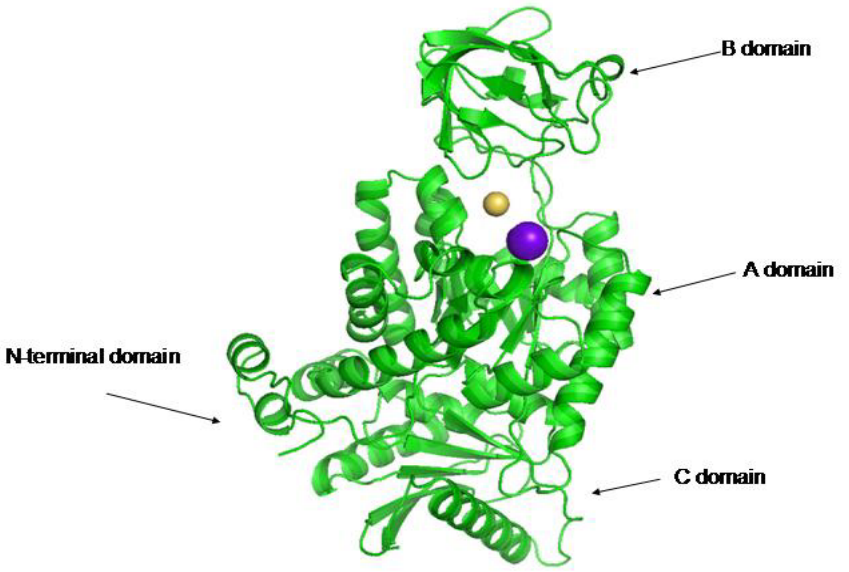


Figure 3. The PK subunit structure, calculated proceeding from data in the Protein Data Bank (PDB ID 2VGB). The K^+ and Mn^{2+} ions are denoted by violet and yellow spheres, respectively, and the position of amino acid 26, which is linked to the N-terminal domain, is denoted by the arrow, while amino acids 1–25 of this regulatory domain are not shown.

The active site of the pyruvate kinase subunit is located in a pocket between A and B domains. The PEP binding site is located in domain A, along with the binding sites for monovalent and bivalent cations (usually Mg^{2+} and K^+), which are required for the PK catalysed reaction (Muirhead *et al*., 1986). This site contains three positively charged residues: lysine and two arginine residues, as well as four negatively charged side chains: two glutamic acids (E) and two aspartic acids (D). In some species one E-residue can be absent. The second pyruvate kinase substrate ADP binds closer to the centre of domain A. The same location is used for binding of ATP, which is the product of the reaction and also acts as an inhibitor in some types of PK (Munoz & Ponce, 2003).

Domains C and N are situated in sites of inter-subunit contact, so they can play essential role in assembly and intermolecular communication (Wooll *et al*., 2001). The allosteric effector FBP binds to the pocket located between the A and C domains, closer to the C domain. This site is characterised by a cluster of positively charged residues (Mattevi *et al*., 1996; Munoz & Ponce, 2003). Thus domain C is responsible for regulation of PK activity by this allosteric modulator.

Another structural element participating in allosteric regulation of L-type PK is the N-terminal domain, as the activity of this isozyme can be regulated through phosphorylation of Ser 12 residue located in the following peptide sequence of this domain (Mattevi *et al*., 1996; Munoz & Ponce, 2003):

MEGPAGYLRR[10]A**S**VAQLTQEL[20]GTAFF … (2)

There are some differences in the amino acid sequence between the M1 and M2 types, mostly located in the C domain of the pyruvate kinase subunit. This domain is probably responsible for the allosteric behaviour of the enzyme induced by the allosteric activator FBP, as the binding site of this activator lies in the same domain. These structural differences also manifest in the kinetic properties of these enzymes, as M1 is not allosterically regulated via binding of the allosteric modulator to the C domain (Noguchi *et al*., 1986; Friesen & Ching Lee, 1998).

The primary structure of the R and L isozymes are very highly conserved, while the R type pyruvate kinase subunit is about 3500 Da larger. This difference is connected with the length of the N-terminal domains, as this peptide is 31 amino acid residues longer in the case of the R-type isoenzyme. The crystal structure of the R-type enzyme is available, but without revealing the positions of 49 amino acids belonging to this highly flexible N-terminus. Analogously, in the currently available crystal structure of human L-PK the first 26 amino acid residues of the N-terminus are not defined. Therefore, it is unclear how this peptide is located and how its phosphorylation may affect the catalytic properties of L-PK, as suggested in several papers (Noguchi *et al*., 1987; Friesen & Ching Lee, 1998, Pendergrass *et al*., 2006, Fenton & Tang, 2009; Prasannan *et al*., 2012).

Some differences between amino acid compositions can also be seen in the binding region of allosteric effectors in the C domain (Jurica *et al*., 1998). For example, differences in amino acid sequence were observed in the allosteric rabbit kidney PK and non-allosteric muscle PK and these differences were mostly located in the C domain and probably participate in inter subunit interactions (Lee, 2008). Comparing the structures of the M1 and M2 enzymes, encoded by M gene with alternative splicing, it was also found that the non-allosteric M1 isozyme and the allosterically regulated M2 isoenzyme differ by the presence of a glutamic acid residue in the region of the FBP binding site, and it was supposed that this negatively charged amino acid either prevents negatively charged FBP binding or mimics it (Jurica *et al*., 1998).

2.3. Catalysis and binding steps

As was mentioned above, pyruvate kinases catalyse the formation of ATP and pyruvate in the last step of glycolysis. This catalytic reaction can be presented by two steps. The first step is the transfer of phosphoenolpyruvate phospho-group to MgADP, and the second step is a rapid conversion of enolpyruvate into its keto form (Muirhead *et al*., 1986; Metzler, 2001). Schematically these steps are shown in Figure 4.

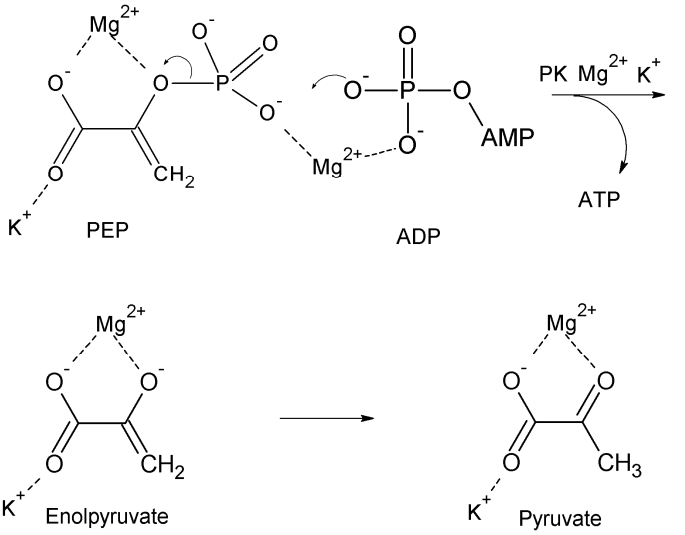


Figure 4. Two steps of pyruvate kinase catalysed reaction

However, the rate of these catalytic steps, quantified by k_{cat} (or V_{max}) value, seems not to be controlled by factors, which regulate the observed activity of pyruvate kinases (Munoz & Ponze, 2003). Therefore, in most cases this activity regulation should occur via control of the binding steps of substrates. This conclusion is valid for the influence of the allosteric effector FBP, which regulates the activity of mammalian M2, R and L type pyruvate kinases, and also the activity of some prokaryotic and eukaryotic PKs (Mesecar & Nowak, 1997b; Munoz & Ponze, 2003; Dombrauckas *et al*., 2005).

However, most interestingly, the survey of the existing data demonstrates that even this regulation is asymmetric, as only the binding effectiveness of PEP is subjected to alterations, while the binding effectiveness of ADP is in most cases practically not affected. This fact may have importance from the point of metabolic turnover and can be used by a cell for more sensitive control over the glucose level.

2.4. Transitions between T-state and R-state

Two states of PK are distinguished: the less active T-state and the more active R-state. The M1 isozyme is mostly in active R state and therefore its activity is not controllable by allosteric ligands. Other isozymes obviously undergo conversion between the T and R states. These transitions were described in the case of Leishmania pyruvate kinase, for which different ligand complexes were studied by X-ray analysis and the accompanying structural changes were characterised (Morgan *et al*., 2010). However, the main structural principles of these transitions have already been described in earlier papers.

For conversion from T to R state two types of rotations are observed. Firstly, rotation of domains B and C within every subunit can be detected. Secondly, rotation of the whole subunit within the tetramer occurs. During this transition domains undergo modification of their relative orientation up to 29°. As a result of these changes, the PEP binding site is distorted or vice versa, depending on the direction of the transition (Mattevi *et al*., 1996; Valentini *et al*., 2000). This situation is illustrated in Figure 5.

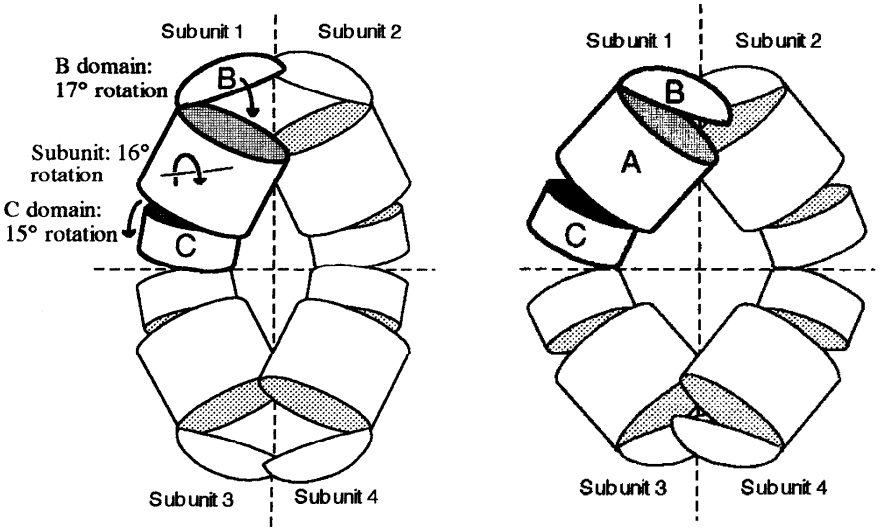


Figure 5. The rotations of domains and subunits of the PK tetramer, occurring on the conformational transition of pyruvate kinase inactive form T to the active form R. T-state is on the left (Mattevi *et al*., 1996).

Ching Lee investigated the R and T states of rabbit muscle pyruvate kinase. He found that PEP binding is accompanied by rotation of the B domain relatively to the A domain. This rotation leads to closure of the cleft between these domains, and locks the enzyme in the R-state. A similar effect was also observed in the presence of ADP (Lee, 2008). However, binding of phenylalanine causes opening of the same cleft. As a result of this, the less active state is stabilised and phenylalanine acts as an inhibitor of the M2 isozyme, characterised by the IC_{50} value 0,24 mM (Morgan *et al*., 2013). On the other hand, the well-known allosteric activator FBP shifts this equilibrium toward the active R-state, and this effect was described by AC_{50} = 7μM for the M2 isozyme. Most interestingly, the activity of the enzyme activated by FBP is very similar to the activity of the M1 isozyme, which is in the fully active form at all times (Morgan *et al*., 2013).

Transitions between T and R states can also be induced by differences in the primary structure of proteins. So, the differences between the primary structures of rabbit muscle PK and kidney PK include 22 amino acid residues. All these

residues are not located in the active site region, but can be found in the C-domain region, which participates in inter-subunit contact. This change in a relatively small number of amino acid significantly changes the allosteric properties and binding constants of ligands: the muscle PK stays preferably in the active R-state, while the kidney PK prefers the inactive T state (Lee, 2008).

Enzyme transition from the more active R state to the less active T state by allosteric ligand can be observed as enzyme inhibition. On the other hand, however, inhibition may also occur through shifting equilibrium between tetrameric and monomeric enzyme forms. The thyroid hormone (triiodo-l-thyronine, T3) stabilises the inactive monomeric form of M2-PK, and this inhibition effect was characterised by the IC_{50} value 78nM (Morgan *et al*., 2013).

2.5. Effect of ligands on pyruvate kinase activity

The inhibitory effect of amino acids is a common phenomenon for all 4 mammalian isozymes. However, particular inhibitory amino acids may be different for different enzyme subtypes. For example, the activity of the M1 subtype can be inhibited by various hydrophobic amino acids, and phenylalanine is clearly an allosteric inhibitor of this enzyme, causing a decrease of the enzyme affinity toward PEP. Another amino acid, alanine, elicits a negligible allosteric response in the case of the M1 subtype, but increasing hydrophobicity of the compound, for example by the addition of methyl groups, increased the role of the allosteric inhibition. Differently from M1 and M2 isoenzymes R- and L-types of PK can be allosterically inhibited by ATP (Feksa *et al*., 2003; Williams *et al*., 2006).

The affinity of PK for allosteric regulators depends on pH. For example, the increase of pH from 6.5 to 8.0 caused a lowering of the L-PK affinity towards PEP and FBP, but at the same time slightly increased affinity for inhibitors like alanine and ATP. The allosteric effect of ATP and FBP increased at higher pH values, while the effect of alanine was not affected by pH (Fenton & Hutchinson, 2009).

It is known that the pyruvate kinase catalysed reaction requires the presence of both bivalent and monovalent cations, most commonly Mg^{2+} and K^{+}, which are considered as physiological activators. But there are bacterial PKs that don't need the presence of K^{+} (Munoz & Ponce, 2003).

It is remarkable that not only Mg^{2+} and K^{+}, but also other monovalent and bivalent cations can be used as activators: for example Mn^{2+}, Co^{2+}, NH_4^{+}, and also Na^{+}. However, this replacement of cations is possible only in the presence of FBP (Hunsley & Suelter, 1969; Mesecar & Nowak, 1997ab; Muirhead *et al*., 1986; Munoz & Ponze, 2003).

Two bivalent cations per subunit are needed in the case of non-allosteric muscle pyruvate kinase. One of these ions binds directly to the enzyme, interacting with side chains of glutamic acid and aspartic acid (positions 271

and 295, respectively, rabbit muscle enzyme), which are situated in the A-domain, and are completely conserved in PKs from different sources (Munoz & Ponze, 2003, Larsen *et al*., 1994). Another ion doesn't interact with the enzyme and coordinates phosphoryl groups of nucleotides.

The role of bivalent cations was described in literature as follows: Mg^{2+} reduces the electrostatic repulsion between the phosphodonor (PEP) and the β phosphogroup of ADP, acting as a nucleophile. It was suggested that Mg^{2+} coordinates the phosphogroups of phosphoenolpyruvate and ADP in the large active site pocket and then the transfer of phosphogroup from PEP to ADP takes place (Muirhead *et al*., 1986; Mesecar & Nowak, 1997ab; Oria-Hernandez *et al*., 2005).

It was suggested that the monovalent cation K^+ binds to glutamine and glutamic acid residues (positions 328 and 363, respectively, cat muscle PK). These residues are conserved in different PKs, but glutamine can be substituted by asparagines in some cases (Muirhead *et al*., 1986; Munoz & Ponze, 2003). The spherical electron density of K^+ lies in the pocket, formed by the amino acids N-74, S-76, D-112 and T-113, rabbit PK. N-74 and D-112, which are completely conserved in all PKs (Larsen *et al*., 1994; Munoz & Ponze, 2003). It was also noticed that PKs, where T-113 and E-117 are replaced by L and K, don't need K^+ for activity. Therefore, it was suggested that the protonated ε-amino group of K can perform the role of an internal monovalent cation (Laughlin & Reed, 1997; Munoz & Ponce, 2003).

In spite of the fact that the role of K^+ is not totally understood, it was demonstrated that K^+ is involved in the formation of the active conformation of the enzyme (Oria-Hernandez *et al*., 2005). It was also proposed that K^+ participates in the coordination of the PEP carboxyl group and in phosphate group transfer (Muirhead *et al*., 1986). When the monovalent cation is absent, ADP cannot bind to an enzyme without primary binding of the second substrate PEP. Therefore, it was concluded that in the absence of K^+ the binding process follows an ordered mechanism with PEP binding as the first substrate. Otherwise the reaction follows a random kinetic mechanism (Oria-Hernandez *et al*., 2005; Muirhead *et al*., 1986; Mesecar & Nowak, 1997ab).

In summary, it can be concluded that the presence of Mg^{2+} and K^+ is needed for the sigmoid shape of the reaction initial rate versus PEP concentration. However, in the presence of FBP the Mg^{2+} activated enzyme follows hyperbolic kinetics toward PEP. If Mg^{2+} ions are substituted by Mn^{2+} PK shows hyperbolic kinetics toward PEP. Therefore, it can be suggested that Mn^{2+} ions can mimic the allosteric effect of the heterotropic activator FBP. Moreover, it was also shown that the Mn^{2+} activated L-PK was not allosterically regulated by FBP, and doesn't require monovalent cations for its activity. If monovalent cations Na^+ or Li^+ are used instead of K^+, the allosteric response to FBP binding decreases (Mesecar & Nowak, 1997ab; Fenton & Ponce, 2003; Alontaga & Fenton, 2009).

2.6. Point mutations and pyruvate kinase allosteric regulation

Among the four mammalian isoenzymes only M1 does not reveal cooperativity, and its affinity for PEP was characterised by the K_{PEP} value 0.049 mM (Ikeda *et al*., 1997). However, substitution of A-398 by R in domain C generated cooperative regulation of the enzyme activity by PEP, characterised by the Hill coefficient 2.7 and the K_{PEP} value 0.41 mM for this substrate (Ikeda *et al*., 1997). At the same time the affinity of the mutant enzyme for ADP was the same as in the case of the wild type enzyme. More interestingly, the mutant form was allosterically regulated by FBP, although this effect was not observed in the case of the wild type enzyme. It was concluded that this single point mutation shifted the equilibrium between the T and R states towards the T-state, while the wild type enzyme with A in position 398 is preferably in the R state (Ikeda *et al*., 1997).

Alternatively, an attempt was made to change the allosterically regulated enzyme into a non-allosteric enzyme by introducing point mutations into the same region of rat M2 PK, and this experiment was successful. Replacement of C 423 residue by L in the allosterically regulated rat M2 isozyme resulted in the loss of both homotropic and heterotropic allosteric effects observed in the case of PEP: the Hill coefficient decreased from 2.3 to 1.3 and the apparent K_{PEP} decreased from 0.18 mM to 0.047 mM. And again, the binding effectiveness of the second substrate ADP was not affected by this point mutation (Ikeda & Noguchi, 1998).

For a more systematic analysis of the interrelationship between allosteric regulation of PK activity by FBP and point mutations, the full list of mutated proteins and their catalytic properties was compiled (Fenton & Blair, 2002). This analysis revealed that in the case of yeast PK mutation E392A eliminated all effects of cooperativity and allosteric regulation. At the same time mutation R369A had only moderate effect on allostery, although both amino acids E392 and R369 are located in the same C-C interface between two subunits of the tetrameric enzyme. The same analysis revealed that seven mutations made at the A-C interface within the same subunit had no influence on allostery. Further, mutation T311M was done in the interface A-A between two subunits and this change decreased the affinity of PK for PEP and FBP. Another mutation in the same location, Q299N, made ADP binding controllable by FBP. Finally, point mutations T403K, T406R, A458K made in the vicinity of the FBP binding site revealed that the introduction of a positive charge into these positions had a minor effect on FBP binding, while FBP binding effectiveness was strongly reduced in T403E and R459Q mutants (Fenton & Blair, 2002).

Rabbit muscle PK and rabbit kidney PK have a region that contains 22 different amino acids, and several point mutations were made in this part of the protein molecule (Lee, 2008). In the mutant S402P of rabbit muscle enzyme, only partial appearance of enzyme cooperativity was observed (Lee, 2008).

Further, the T340M mutant that is characteristic of patients with pyruvate kinase deficiency was studied. It was found that the activity of mutated PK decreased twice, and the mutant became more sensitive to the inhibitory effect of phemylalanine, but was not regulated by FBP (Lee, 2008, Cheng *et al*., 1996).

Taken together, these results demonstrate that the allosteric regulatory effects should embrace different parts of the protein molecule, and single modifications may be sufficient for switching on and switching off cooperativity and allosteric regulation, especially if the ionic status of the binding site is affected.

2.7. Regulatory phosphorylation of L-type pyruvate kinase

In the case L-type pyruvate kinase an additional regulatory mechanism exists. This regulation is connected with phosphorylation on the N-terminal domain of this enzyme. The phosphorylation reaction takes place at the serine residue in position 12 of this sequence and down regulates the enzyme activity by reducing the binding effectiveness of PEP without significant effect on the V_{max} value. At the same time no effect was observed in the case of ADP binding. Phosphorylation of the regulatory N-terminal also decreases the binding effectiveness of the allosteric ligand FBP, while the affinity of the enzyme for inhibitors ATP and alanine increases. Therefore, it was concluded that phosphorylation shifts equilibrium between the active R state and inactive T state of the liver enzyme toward the less active T-state, and enhances cooperativity of the catalytic reaction (El-Maghrabi *et al*., 1980, 1982; Pilkis *et al*., 1980).

The dependence of L-PK activity upon the degree of phosphorylation of the enzyme and lowering of the enzyme affinity for PEP by this regulatory event was already described in 1974 (Ljungström *et al*., 1974). Further studies confirmed the interrelationship between the extent of protein phosphorylation and its affinity for PEP. So, the enzyme purified from rat liver contained 3 moles of phosphate per mole of tetrameric enzyme and was characterised by the K_{PEP} value 1.2 mM. Further, in the presence of 1 mole of phosphate per mole of tetrameric enzyme the K_{PEP} value was 0.7 mM, and in the case of the fully phosphorylated enzyme (4 mole of phosphate per mole of tetramecic enzyme) the K_{PEP} value 1.4 mM was obtained (El-Maghrabi *et al*., 1980). The increase in the K_{PEP} values was accompanied by an increase in the Hill coefficient, characterising the cooperativity of the system. Moreover, phosphorylation also decreased the binding effectiveness of the allosteric regulator FBP (El-Maghrabi *et al*., 1982).

2.8. Role of N-terminal domain in L-PK activity

The activity of L-PK can also be reduced by limited proteolysis of the N-terminal domain, and this effect was first described in 1978 (Bergström *et al*., 1978), and a more detailed study of proteolytic truncation of the N-terminal part of L-PK confirmed these results (Fenton & Tang, 2009). It was found that shortening of the N-terminal end (see Eq. 2) by 6 amino acids had practically no effect on PEP binding, while removal of the following 7 to 12 amino acids reduced PEP binding effectiveness. Further truncation had again no additional effect on PEP binding effectiveness (Fenton & Tang, 2009). Thus truncation and phosphorylation seem to have a rather similar influence on the catalytic properties of L-PK, and it was even claimed that truncation of the N-terminus peptide mimics the effect of phosphorylation, decreasing the similarly affinity of the enzyme toward PEP (Fenton & Tang, 2009).

Further, the phosphorylatable S12 residue was substituted by aspartic acid residue, and the kinetic properties of the S12D mutant were studied. It was found that this mutant indeed mimicked the kinetic properties of phosphorylated L-PK by decreasing PEP binding effectiveness, which became comparable to that of the phosphorylated enzyme. Moreover, the removal of the initial 10 residues of S12D had no effect on the apparent K_{PEP} value (Fenton & Tang, 2009).

On the basis of these results a hypothesis was proposed that the N-terminus of non-phosphorylated enzyme interacts with the main body of L-PK and activates the enzyme by increasing its affinity for PEP (Fenton & Tang, 2009). Phosphorylation, proteolytic truncation and S12D mutation obviously hampered this interaction and reduced PEP binding effectiveness. Most significantly, in the presence of peptides that were derived from the protein N-terminus, the activity of the S12D mutant enzyme was increased, pointing to the possibility that these peptides mimic interaction of the N-terminal domain with the protein main body, and increase its affinity for PEP (Prasanan *et al*., 2012). The oxidation of Cys 436 residue of the main body of PK also resulted in a decrease of the enzyme affinity for PEP, pointing to the fact that this oxidation may prevent the interaction of N-terminus with the protein main body (Holyoak *et al*., 2013).

3. OBJECTIVES OF DISSERTATION

The main objectives of this work were:

1. Investigation of the kinetic properties of non-phosphorylated L-type pyruvate kinase.

2. Investigation of the role of the N-terminal regulatory domain in activity of L-type pyruvate kinase by using point mutations around phosphorylatable serine residue.

3. Investigation of the interaction of non-phosphorylated pyruvate kinase with ligands that evoke allosteric effects in the case of the phosphorylated enzyme.

4. Kinetic study of the catalytic properties of the phosphorylated L-type pyruvate kinase.

4. MATERIALS AND METHODS

4.1. Chemicals

Adenosin-5'-diphosphate disodium salt (ADP), phosphoenolpyruvate (PEP) tricyclohexylammonium salt and bovine serum albumin (BSA) fraction V, were purchased from Boehringer Mannheim GmbH, Germany. Nicotinamide adenine dinucleotide reduced form disodium salt (NADH) and fructose-1,6-biphosphate trisodium salt (FBP) was from Sigma Chemical Co. Tris(hydroxymethyl)-aminomethane (TRIS), dithiothreitol (DTT) and protein molecular mass markers (bovine serum albumin, bovine liver catalase, ovalbumin and carbonic anhydrase) were obtained from Sigma-Aldrich (USA). $MgCl_2$ and KCl were from Acros. [γ-32P]ATP was obtained from Amersham (UK) (110 TBq/mmol). The DE-52 ion-exchanger was from Whatman and hydroxyapatite from BioRad. Peptides RRASVA, RRAAVA and RRAS(Pi)VA were purchased from GL Biochem (Shanghai) Ltd (China), sale confirmation number GLS-P060704. These samples were characterised by HPLC and ESI-MS data and had purity above 95%. All other chemicals of highest purity grade available were purchased from Sigma-Aldrich (USA). The Milli-Q deionized water was used in all experiments.

4.2. Enzymes

The recombinant expressed catalytic subunit of mouse cAMP-dependent protein kinase (PKA, 0.1 mg/ml, 30 U/mg, Lot 040916) was obtained from Biaffin GmbH and Co KG (Germany). Before experiments the protein solution was diluted 300 – 1000-fold in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mg/ml BSA, and the stock solution was kept in an ice bath until use.

Lactate dehydrogenase from rabbit muscle (LDH) was purchased from Boehringer Mannheim GmbH, Germany. Solution of this enzyme was diluted about 400 times to 0.002 mg/ml (1.5 units/ml).

The wild type and mutant forms of rat liver pyruvate kinase were over-expressed in *E.coli* and purified by Mart Loog and Nikita Oskolkov in Uppsala University. This procedure and obtained products were described in detail before (Loog *et al*., 2005), and the proteins were kindly provided for this study. The point mutations were introduced into the N-terminal domain of L-PK at positions 9, 10 and 13 near the phosphorylatable S12 residue:

The wild type protein:
MEGPAGYL**RR**[10]**AS**[12]**VA**QLTQEL[20]GTAFF…

Mutant proteins:
MEGPAGYL**A**[10]**R**[10]**AS**[12]**VA**QLTQEL[20]GTAFF…
MEGPAGYL**K**[10]**R**[10]**AS**[12]**VA**QLTQEL[20]GTAFF…
MEGPAGYL**E**[10]**R**[10]**AS**[12]**VA**QLTQEL[20]GTAFF…
MEGPAGYL**Q**[10]**R**[10]**AS**[12]**VA**QLTQEL[20]GTAFF…
MEGPAGYL**RA**[10]**AS**[12]**VA**QLTQEL[20]GTAFF…
MEGPAGYL**RK**[10]**AS**[12]**VA**QLTQEL[20]GTAFF…
MEGPAGYL**RQ**[10]**AS**[12]**VA**QLTQEL[20]GTAFF…
MEGPAGYL**RL**[10]**AS**[12]**VA**QLTQEL[20]GTAFF…
MEGPAGYL**RR**[10]**AS**[12]**A****A**QLTQEL[20]GTAFF…
MEGPAGYL**RR**[10]**AS**[12]**E****A**QLTQEL[20]GTAFF…

The structure of proteins was verified by DNA sequencing, all the enzymes were purified to homogeneity and this purity was checked by SDS-PAGE electrophoresis as was described in (Loog *et al.,* 2005). The enzyme solutions were made by dilution of the stock solutions with 50 mM TRIS-buffer (pH 7.4), containing 0.1% BSA.

4.3. Protein concentration

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. On the basis of the primary structure of the protein (Noguchi *et al*., 1987; Lone *et al*., 1986) protein the number of these amino acid residues was evaluated (Trp – 3, Tyr – 10, Cys – 6) and according to this composition the extinction coefficient 30590 $M^{-1}sm^{-1}$ was calculated for the L-PK subunit.

4.4. SDS-PAGE electrophoresis

The analysis was done according to the Laemmli protocol modified by O'Farrell in 12% SDS-polyacrylamide gel (O´Farrel, 1975). After electrophoresis visualisation of proteins was performed by colloidal staining in 1.6% ortho-phosphoric acid, 8% ammonium sulphate, 0.08% of Coomassie brilliant blue G-250 and 20% methanol. Destaining was carried out in water. Protein molecular mass markers were bovine serum albumin, bovine liver catalase, ovalbumin and carbonic anhydrase from Sigma-Aldrich.

4.5. L-PK phosphorylation

The phosphorylation of L-PK was carried out at 30°C in a 50 mM TRIS/HCl buffer (pH 7.5), containing 100 μM of γ-[^{32}P]ATP (specific radioactivity 380 cpm/pmol), 10 mM $MgCl_2$, 0.967 mg/ml of PKA and BSA (1 mg/ml). The phosphorylation reaction was started by the addition of PKA to the reaction mixture, and L-PK phosphorylation was followed by analysing samples taken from this reaction mixture at appropriate time moments. Firstly, 10 μl of these aliquots were applied to pieces of phospho-cellulose paper P81 (Whatman, UK) and immersed in ice-cold phosphoric acid (75 mM). Then the pieces of paper were washed 4 times for 10 minutes with additional amounts of phosphoric acid, dried at 120ºC for 25 minutes and counted for bound radioactivity on a scintillation counter RacBeta 1219 OK (USA). Secondly, at the end of the reaction, the last sample was taken into a Sephadex G50 mini-column (bed volume 0.5 ml) and the phosphorylated protein was separated from the excess of [γ-^{32}P]ATP by gel-filtration. The eluent was collected drop-wise on pieces of Whatman filter paper, dried at 120ºC and counted for radioactivity as described above.

4.6. FPLC analysis

Fast protein liquid chromatography (FPLC analysis) was performed to determine the molecular weight of the enzymes. The protein sample (100–200 μl) was applied to a Superdex 200 HR 10/30 column and gel filtration was performed in a 50 mM Tris/HCl buffer containing 150 mM NaCl at a flow rate 0.5 ml/min. at room temperature, using an ÄKTA FPLC system (Amersham Biosciences, Sweden). Markers used for molecular weight determination were: myoglobulin (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). The calibration curve was constructed using the set of standard proteins. It is the relationship between K_{av} for each protein that characterises the elution volumes and the logarithm of their respective molecular weights, (Figure 6). K_{av} was calculated using the following equation (Eq. 3):

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

where V_e – elution volume for the protein, V_0 – column void volume (elution volume for Blue Dextran), V_t – total bed volume. The molecular weights for all enzymes were calculated from the obtained calibration curve. Comparison of molecular masses of the phosphorylated and non-phosphorylated L-PK was shown in Figure 6. It can be seen that both these proteins were in tetrameric form. Similar conclusion was drawn for the other mutants investigated.

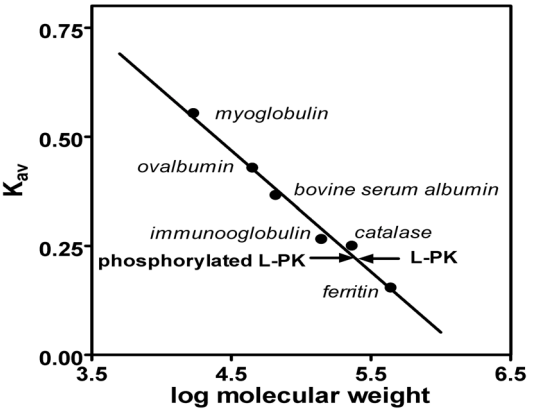


Figure 6. Molecular weight determination of phosphorylated and non-phosphorylated L-PK by using FPLC and the following molecular weight markers: myoglobulin, ovalbumin, bovine serum albumin, immunoglobulin, catalase and ferritine.

4.7. Assay of L-PK activity

Activity of L-PK was measured spectrophotometrically, adopting the procedure described before (Fuji & Miwa, 1987). Initial velocities *v* of the enzyme reaction were determined at various concentrations of substrates based on coupling of the L-PK catalysed reaction of ATP synthesis with the LDH-catalysed NADH oxidation. So firstly, L-PK catalyses the formation of pyruvate and ATP. Secondly, the pyruvate formed in this reaction is used by LDH to form L-lactate simultaneously converting NADH into NAD^+. This NADH consumption can be followed spectrophotometrically, as absorbance of the solution strongly decreases ($\Delta\varepsilon$ = 6220 L/cm·mol, λ=340 nm). To use this method the rate of the second process, if compared with the L-PK catalytic reaction, must be much faster. This issue was checked by using different LDH concentrations at similar conditions of the L-PK catalysis, and no change in the apparent velocity of the L-PK catalysed reaction was observed.

Kinetic measurements of the initial velocity of the catalysis were made at 30ºC in 1 cm thermostated quartz cells at λ =340 nm using a UV-VIS Unicam UV300 spectrophotometer (ThermoSpectronic, USA). The time-course of the reaction was monitored for 1–3 min. using a sampling interval of 1 s, integration time 0.25 s. The reaction was initiated by the addition of 40 µl of L-PK solution into 0.960 ml of the reaction mixture.

The assay medium in 50 mM TRIS/HCl buffer (pH 7.5, 30ºC) contained: 0.2 mM NADH, 0.002 mg/ml (1.5 units/ml) LDH, 100 mM KCl, 10mM $MgCl_2$, 0.1% BSA, 0.1 mM DTT, 0.148 mg/l L-PK. Substrate concentrations varied from 0.01 mM up to 10 mM in the case of PEP and from 0.01 mM to 6 mM of ADP.

Before kinetic assays, enzyme stability was analysed in the assay buffer without substrates and no denaturation was observed during 3 min., the same time interval used for the kinetic assay.

4.8. Processing of kinetic data

The initial velocities (v) of L-PK reaction were calculated from the time-course of the absorbance (Figure 7). The relationship between the initial velocity and L-PK concentration was linear, pointing to the fact that the change of the optical density of the assay mixture was caused by the enzymatic reaction (Paper I).

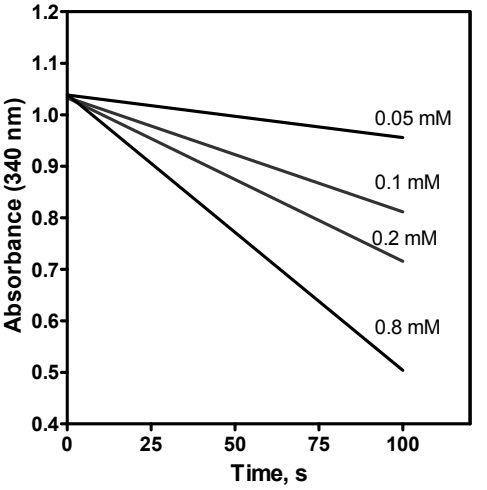


Figure 7. Spectrophotometric assay of L-PK activity: at 1 mM ADP and different PEP concentrations.

To characterise the catalytic properties of enzymes the dependences of the v values upon the concentration of both substrates were analysed and kinetic parameters calculated. In routine assays the concentration of one substrate was kept constant (v vs. PEP at 1 mM ADP and v vs. ADP at 2 mM PEP), and the initial velocities vs. concentration plots for the second substrate were analysed by the conventional Hill equation (Segel, 1975; Варфоломеев & Гуревич, 1999):

$$\frac{v}{V^{app}} = \frac{[S]^n}{K_{0.5}^n + [S]^n} \quad (4)$$

where S stands for concentration of the variable substrate, V^{app} is the apparent maximum rate constant, whose value depends on the concentration and kinetic properties of the second substrate, $K_{0.5}$ characterises the concentration of the

variable substrate at $v = 0.5\ V^{app}$ and n stands for the Hill coefficient, characterising the cooperativity of the process.

In the absence of cooperativity, $n = 1$, the reaction follows the common Michaelis-Menten rate equation (Segel, 1975; Варфоломеев & Гуревич, 1999):

$$v = \frac{V \cdot [S]}{K_m + [S]} \tag{5}$$

The same equation was used to process data measured in the presence of FBP or peptides in the reaction mixture, and the apparent K_{PEP}^{app} values were obtained in the case of the inhibition effect:

$$K_{PEP}^{app} = K_{PEP}\left(1 + \frac{[I]}{K_i}\right) \tag{6}$$

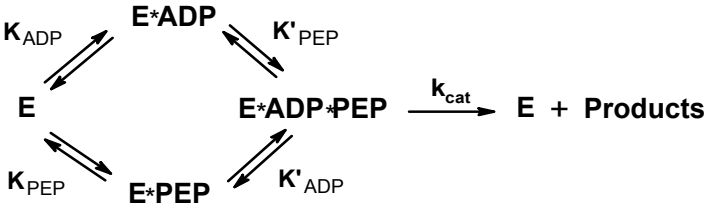
where $[I]$ stands for inhibitor concentration and K_i for inhibition constant. In separate experiments the initial reaction rates v were assayed at 0.8 mM PEP and 1 mM ADP concentrations in the presence of various concentrations of FBP or peptides and the v vs. I concentration plots were used for calculation of the K_i values:

$$\frac{v}{V} = \frac{\frac{K_i}{K_{PEP}[PEP]}}{\left(1 + \frac{[PEP]}{K_{PEP}}\right) \cdot K_i + [I]} \tag{7}$$

In the case $n = 1$ the kinetic data for this bi-substrate reaction were analysed together by the following rate equation (Segel, 1975):

$$v = \frac{k_{cat}[E_0][PEP][ADP]}{K'_{PEP}K_{ADP} + K'_{PEP}[ADP] + K'_{ADP}[PEP] + [ADP][PEP]} \tag{8}$$

In this rate equation E stands for the enzyme and the parameters k_{cat}, K_{PEP}, K'_{PEP}, K_{ADP} and K'_{ADP} correspond to the following reaction scheme:



(9)

where $K'_{PEP} = \alpha K_{PEP}$, $K'_{ADP} = \alpha K_{ADP}$ and α characterizes the randomness of the substrate binding sequence.

Data processing was performed with the non-linear least-squares regression analysis method by using GraphPad Prism version 4.0 (GraphPad Software Inc., USA) and Sigma Plot (Systat Software, Inc. SigmaPlot for Windows). The values of kinetic parameters were reported with standard errors.

5. RESULTS AND DISCUSSION

5.1. Molecular properties of L-PK expressed in *E.coli*

Molecular properties of the expressed enzyme were determined by SDS-PAGE electrophoresis (Figure 8) and by using fast protein liquid chromatography (FPLC) as shown in Figure 6, and the data obtained were compared with the results for the enzyme extracted from the liver tissue. It can be seen in Figure 8 that the molecular weight of the recombinant L-PK was the same as of the L-PK isolated from rat liver and corresponded to the value 56–59 kDa. Further, the FPLC analysis demonstrated that the MW value of 245 kDa for the recombinant enzyme was in good agreement with the previously reported MW values for the tetrameric form of the enzyme (Albe *et al*., 1990), and were also in agreement with the MW value 245,551 Da calculated from the amino acid sequence of the L-pyruvate kinase structure (Noguchi *et al*., 1987; Lone *et al*., 1986). Taken together, these analyses demonstrated that the enzyme existed in tetrameric form in the assay buffer.

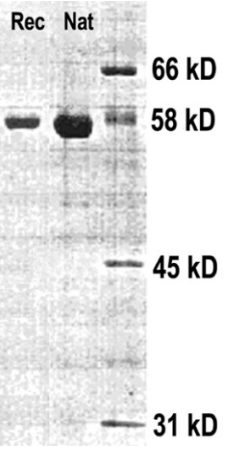


Figure 8. SDS-PAGE gel shows purified recombinant L-type pyruvate kinase (Rec) and L-PK isolated from rat liver (Nat). The right lane shows molecular weight markers: BSA, bovine liver catalase, ovalbumin and carbonic anhydrase.

Non-phosphorylated proteins were obtained from the used expression system, as bacterial cells do not contain protein kinases similar to the eukaryotic AGC family. This assumption was confirmed by the fact that the purified protein was efficiently phosphorylated by the cAMP-dependent protein kinase catalytic subunit and 4 phosphate groups were incorporated into one tetrameric protein molecule as seen in Figure 9, as a single serine residue at position 12 of the N-terminal domain of each subunit is phosphorylated by this protein kinase (El-Maghrabi *et al*., 1980, 1982; Schworer *et al*., 1985; Muirhead *et al*., 1986).

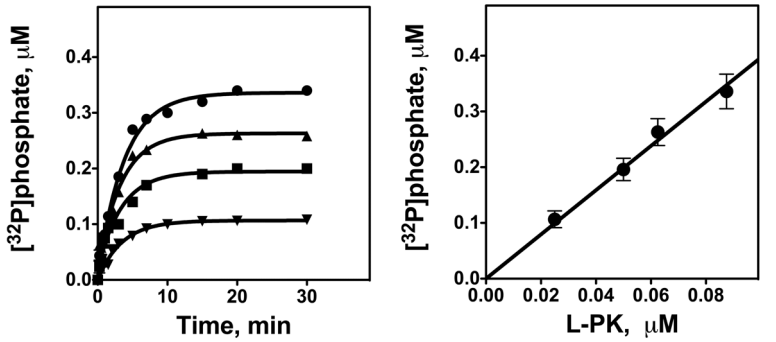


Figure 9. Determination of the stoichiometry of the phosphorylation of recombinant rat L-PK expressed in *E.coli*. Samples were phosphorylated until saturation in the presence of [γ-^{32}P]ATP (left panel) and concentration of radioactivity incorporated into this protein was compared with concentration of the enzyme (right panel). The slope of the linear plot was 3.97±0.09 in agreement with 1:1 stoichiometry of the phosphorylation of subunits of the tetrameric enzyme

Phosphorylation of the *E.coli*-expressed L-PK had no influence on the tetrameric structure of this enzyme, as the protein eluted similarly with the non-phosphorylated L-PK from the Superdex 200 HR 10/30 column (Figure 6).

Taken together, the enzyme expressed in *E.coli* was not phosphorylated, and it was stoichiometrically phosphorylated in the presence of protein kinase A. This phosphorylation reaction had no effect on the molecular properties of the protein, as both phosphorylated and non-phosphorylated enzymes existed in tetrameric form.

5.2. Catalytic properties of L-PK expressed in *E.coli*

The non-phosphorylated recombinant enzyme was catalytically active in the conventional L-PK assay system and the relationships of the initial rate vs. ADP and PEP concentration were determined (Figure 10). It can be seen that the obtained plots were hyperbolic, indicating that the non-phosphorylated enzyme was not cooperatively regulated by PEP as well as by ADP concentration. Therefore, the kinetic data for the non-phosphorylated L-PK were processed by rate equation 8, derived for a common bi-substrate enzyme reaction, taking into account that $K'_{PEP} = \alpha K_{PEP}$, $K'_{ADP} = \alpha K_{ADP}$. In this equation the coefficient α characterises the randomness of the substrate binding sequence (Segel, 1975), and the following results were obtained:

$V = 15.8 \pm 0.5$ μmol/mg·s
$K_{ADP} = 0.26 \pm 0.03$ mM
$K_{PEP} = 0.11 \pm 0.02$ mM
$\alpha = 0.96 \pm 0.10$

The α value was close to 1 that was in agreement with the random substrate binding model.

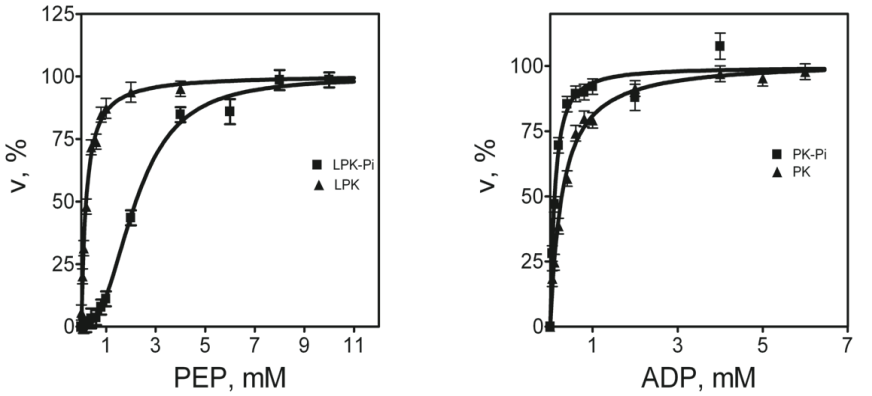


Figure 10. Comparison of kinetic properties of non-phosphorylated (▲) and stoichiometrically phosphorylated (■) L-PK at variable PEP concentrations (left panel, ADP concentration 1 mM) and at variable ADP concentrations (right panel, PEP concentration 2 mM) in 50 mM Tris-HCl buffer, pH 7.4, 30ºC.

After the recombinant L-PK was stoichiometrically phosphorylated its catalytic properties revealed significant changes. It can be seen that PEP binding followed sigmoid function, while ADP binding still followed hyperbolic dependence (Figure 10). Analysing the kinetic data for phosphorylated L-PK by equation 4, $K_{0.5}$ values for PEP and ADP and also the Hill coefficient for both substrates were obtained. These results were as follows:

For PEP: $K_{0.5} = 2.2 \pm 0.1$ mM $\quad n = 2.5 \pm 0.2$ for PEP

For ADP: $K_{0.5} = 0.11 \pm 0.01$ mM $\quad n = 1.1 \pm 0.2$, for ADP

Taken together, these kinetic data demonstrated that phosphorylation lowered the affinity of the enzyme for PEP, while it had no effect on the affinity of the enzyme for the second substrate ADP. This asymmetric influence was in good agreement with all previously published works (El-Maghrabi *et al*., 1980; Schworer *et al*., 1985). However, in parallel with this change, phosphorylation also changed the cooperativity of the system.

For further analysis of the cooperativity of phosphorylated L-PK toward PEP we used the sequential substrate binding model. Positive cooperativity assumes that binding of one PEP molecule to multimeric enzyme affects binding properties of the remained subunits (Koshland & Neet, 1968). For simplification of the model we considered that the presence of another substrate, ADP, has no effect on PEP binding. So the interaction of PEP with 4 subunits in the tetrameric enzyme can be presented by the following reaction scheme:

$$E + S \overset{K}{\rightleftharpoons} ES \xrightarrow{k} E + \text{products} \tag{10}$$

$$ES + S \overset{\alpha K}{\rightleftharpoons} ES_2 \xrightarrow{k} ES + \text{products} \tag{11}$$

$$ES_2 + S \overset{\beta K}{\rightleftharpoons} ES_3 \xrightarrow{k} ES_2 + \text{products} \tag{12}$$

$$ES_3 + S \overset{\gamma K}{\rightleftharpoons} ES_4 \xrightarrow{k} ES_3 + \text{products} \tag{13}$$

where E stands for enzyme and S is substrate (PEP). The substrate interaction with the first enzyme subunit is quantified by the dissociation constant K, affinity for the second substrate molecule is quantified by αK, affinity for the third and fourth substrate molecules by βK and γK, respectively. So α, β and γ characterise the interactions between the enzyme subunits.

The probability factors must be also considered, as in the case of the tetrameric enzyme there are four ways to form an ES complex from E, six ways to form the complex ES_2 from ES, four ways to form ES_3 from ES_2 and one way to form ES_4 from ES_3. Taking into account these probability factors, and assuming that all complexes are in equilibrium, the following rate equation 14 can be obtained for the cooperatively functioning tetrameric enzyme (Segel, 1975):

$$v = V \frac{\frac{[S]}{K} + \frac{3[S]^2}{\alpha^2 K^2} + \frac{3[S]^3}{\beta^3 K^3} + \frac{[S]^4}{\gamma^4 K^4}}{1 + \frac{4[S]}{K} + \frac{6[S]^2}{\alpha^2 K^2} + \frac{4[S]^3}{\beta^3 K^3} + \frac{[S]^4}{\gamma^4 K^4}} \tag{14}$$

In this equation V is determined by the total amount of the active sites, in the case of tetrameric enzyme 4 times bigger than the analytical enzyme concentration (Eq. 15):

$$V_{MAX} = 4k[E]_{total} \tag{15}$$

Using equation 14, the cooperativity of L-PK was characterised by α, β and γ parameters, comparing the affinity of the binding steps:

α = 1.0
β = 0.1
γ = 0.1

As $\alpha = 1$, binding of the first substrate molecule had no influence on the binding of the second substrate. But for binding of the third substrate molecule the affinity of the enzyme increased. The same increase was also observed in the case of the fourth PEP molecule. This means that binding of the first two molecules increases the affinity of the enzyme for binding the next two substrates, as assumed in the cooperativity model describing the tetrameric molecule as the "dimer of dimers" (Fenton & Hutchinson, 2009).

5.3. Kinetics of phosphorylation of expressed L-PK

The time course of phosphorylation of non-phosphorylated L-PK by PKA is shown in Figure 11. It was found that the phosphorylation reaction followed the exponential rate equation:

$$C_t = C_\infty \exp(-k_{obs}t) + C_0 \qquad (16)$$

where C_t stands for radioactivity incorporated into the protein at time moment t, C_∞ is the maximum amount of protein-bound radioactive phosphorus, C_0 stands for background of the bound radioactivity and k_{obs} is the observed rate constant of the reaction. Under the experimental conditions used in this work, the level of the non-specifically bound radioactivity C_0 was small if compared with the specifically bound radioactivity C_∞, and therefore the C_∞ value, calculated from the kinetic curve in Figure 11 agreed well with the experimental "plateau" values of the phosphorylation reaction shown in Figure 9.

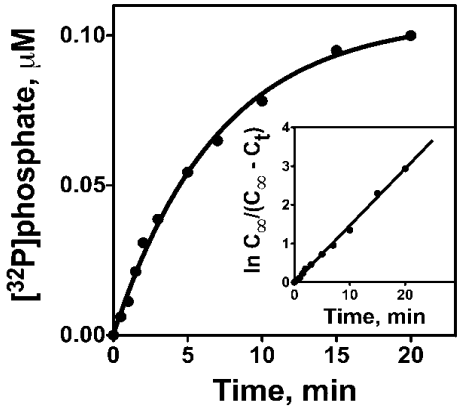


Figure 11. Kinetic analysis of the phosphorylation of L-PK expressed in *E.coli* by the cAMP dependent protein kinase catalytic subunit in the presence of 100 μM γ-[^{32}P]ATP, 30°C, 50 mM TRIS/HCl buffer (pH 7.5). The time-course of incorporation of radioactivity into protein (C_t) was approximated by exponential function and the linear transform of this plot was shown in intercept.

The exponential rate equation 16 refers to the pseudo-first-order conditions of the phosphorylation reaction, where the substrate (L-PK) concentration is small and remains below the $K_{0.5}$ value for this substrate. Therefore, the observed rate constants k_{obs} calculated from the kinetic curves in Figure 9 (left panel) were similar and yielded the mean k_{obs} value 0.29 ± 0.05 $min.^{-1}$. Moreover, the conclusion about the pseudo-first-order conditions was also supported by the statistically relevant linear plot between the initial rate values of the L-PK phosphorylation reaction the substrate (L-PK) concentration. And finally, the conclusion was in agreement with the $K_{0.5}$ value 17 μM estimated for the L-PK phosphorylation reactions by PKA as was shown by Pilkis *et al.,* 1980.

Taken together, the kinetic data indicated that all four phosphorylatable sites of the tetrameric protein structure were phosphorylated randomly, and the phosphorylation of one subunit did not affect the phosphorylation of the following subunits.

5.4. Interrelationship between phosphorylation and cooperativity

The regulatory phosphorylation reaction occurs at the S12 residue of the N-terminal part of L-PK (Mattevi *et al*., 1996; Muirhead *et al*., 1986), and protein extracted from liver tissue contained 2–3.5 moles of phosphate per tetrameric enzyme molecule (El-Maghrabi *et al*., 1980, 1982; Pilkis *et al*., 1980; Berglund *et al*., 1977). Therefore, additional phosphorylation of the enzyme was possible and this caused a decrease of the PEP binding affinity and led to a change in the sigmoid shape of the initial velocity vs. PEP plots. Formally this change resulted in higher values of the Hill coefficient *n* in Eq. 4 (El-Maghrabi *et al*., 1980, 1982; Schworer *et al*., 1985).

For example, the enzyme extracted from the liver contained 3 moles of phosphate per mole of tetrameric L-PK and was characterised by K_{PEP} = 1.15 mM and n = 2.0 (El-Maghrabi *et al*., 1980). Complete phosphorylation of this enzyme raised the K_{PEP} value up to 1.4 mM and increased the n value up to 2.2 (El-Maghrabi *et al*., 1980). In another study, K_{PEP} =1.1 mM and n = 2.5 were reported for the completely phosphorylated enzyme (Schworer *et al*., 1985). And finally, K_{PEP} = 2.2 mM and n = 2.5 were obtained for the stoichiometrically phosphorylated enzyme in this study.

Partial dephosphorylation of the enzyme, leaving approximately 1 mole phosphate per tetrameric L-PK molecule, decreased the K_{PEP} value to 0.6 mM (El-Maghrabi *et al*., 1980) and resulted in a shallow Hill plot characterised by n = 1.8. The latter value was calculated by Eq. 4 from data published by El-Maghrabi *et al.* (El-Maghrabi *et al*., 1980). Analogously, K_{PEP} = 0.55 mM and n = 1.5 were obtained from data published by Schworer *et al.* (Schworer *et al*., 1985).

All these results clearly point to the presence of an interrelationship between the L-PK affinity for PEP and the level of phosphorylation of this protein, and also between the Hill coefficient *n* and the phosphorylation level. Both of these dependences are shown in Figure 12.

The results in Figure 12 demonstrate that phosphorylation can be specified as a "switch" between cooperative and non-cooperative forms of L-PK. This conclusion supports the suggestion made by Muirhead in 1990 (Muirhead, 1990) that the N-domain of L-PK may have a functional role in the transmission of allosteric structural changes in pyruvate kinases. Later, a significant role of the N-terminus of L-PK in allosteric control of activity of this enzyme was also suggested by Fenton and Tang (Fenton & Tang, 2009).

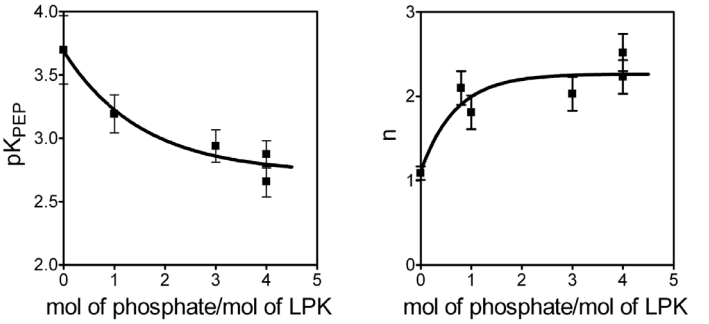


Figure 12. Dependence of the L-PK affinity for PEP (pK_{PEP}) (left panel) and plot of the Hill coefficient (*n*) for PEP binding with L-PK (right panel) upon the level of L-PK phosphorylation.

The plot obtained for Hill coefficients revealed that a clear change in cooperativity accompanied the phosphorylation of a single subunit in the tetrameric structure, as this modification increased the *n* value from 1 to 2, while the following phosphorylation of other sites increased the *n* value up to 2.2 or 2.5. However, phosphorylation of the second, third and fourth subunits still decreased PEP binding effectiveness, while a more than 10-fold difference can be observed between the K_{PEP} values of the non-phosphorylated and the stoichiometrically phosphorylated enzyme. In parallel with these major changes in PEP binding, ADP binding was practically not affected by phosphorylation.

It should be emphasised that the experimentally determined value of the phosphorylation level of L-PK reflects only the average overall stoichiometry, while the relative abundances of the enzyme molecules carrying one, two, three or four phosphate groups are determined by the kinetic mechanism of the reaction. This mechanism can be specified proceeding from the fact that the time course of the overall reaction was well described by a single exponential function. This result can be explained by two distinct kinetic models.

Firstly, it is possible that the whole population of phosphorylation sites is homogeneous and all four subunits of the L-PK molecule are phosphorylated

independently and at equal rates. This means that formation of the enzyme molecules carrying one, two, three or four phosphate groups is a consecutive process, and the mono-phosphorylated tetramers should prevail at the beginning of the process.

Secondly, however, the same exponential time course of phosphorylation can be explained by the rate-limiting phosphorylation reaction at the first subunit and fast phosphorylation of the other subunits of the tetrameric protein. Following this model, phosphorylation of ¼ of the sites means that ¼ of L-PK molecules are fully phosphorylated, while the rest are not phosphorylated. In the latter case the resulting initial velocity versus the PEP concentration plot should be a sum of hyperbolic and sigmoid plots, where the hyperbolic impact of the non-phosphorylated enzyme would influence the shape of the curve up to relatively high mole-per-mole phosphate levels. Such abnormality of the shape was not observed in previous studies.

In contrast, in the case of the random phosphorylation of equal sites the entirely non-phosphorylated form should disappear relatively fast, and as cooperativity is concurrent with the addition of the first phosphate per tetramer, the initial velocity versus the PEP curve becomes non-hyperbolic at early stages of phosphorylation.

Taken together, phosphorylation of the N-terminal peptide of L-PK initiates cooperativity of the enzyme towards PEP concentration, and this mechanism is most probably switched on by the addition of the first phosphate of the four, needed for the stoichiometric phosphorylation of the tetrameric protein.

5.5. Interrelationship between N-domain structure and activity of L-PK

On the phenomenological level, the dependence of L-PK activity upon phosphorylation was described in 1974 (Ljungström *et al*., 1974, Ekman *et al*., 1976). Further, it was observed that the enzyme activity could be decreased by limited proteolysis of the N-terminal peptide (Bergström *et al*., 1978). The recent study of proteolytic truncation of the N-terminal part confirmed these results, demonstrating that removal of amino acids in positions from 7 to 12 reduced PEP binding effectiveness (Fenton & Tang, 2009). Thus truncation and phosphorylation seem to have a rather similar influence on L-PK binding properties.

On the basis of truncation experiments it has been suggested that the N-terminal peptide of L-PK might interact with the main body of this protein, inducing in this way the active state of the enzyme. Correspondingly, interruption of this interaction by truncation of the N-terminal peptide should convert the enzyme into the low affinity state against PEP (Fenton & Tang, 2009). Interestingly, the possibility of such internal equilibria and their role in

regulation of enzyme activity was already discussed in general terms by G. Weber in 1972 (Weber, 1972).

As the amino acids, whose removal affects PEP binding effectiveness, are located before the phosphorylation site on S12, we decided to probe the functioning of the L-PK regulatory phosphorylation site by point mutations in positions 9, 10 and 13, and alanine, leucine, lysine, glutamine and glutamic acid were introduced into these positions (Table 1), changing apropos the ionic charge of this domain, and thus indirectly simulating a change of the ionic status of the N-domain peptide, as happens in the case of phosphorylation.

All these mutants were able to catalyse the synthesis of ATP and their catalytic properties were characterised by the initial rate vs. PEP concentration plots at 1 mM ADP concentration, and by similar plots for different ADP concentrations at 2 mM PEP. The data obtained for both substrates were analysed by rate equation 4, which allowed calculation of the apparent affinity constant $K_{0.5}$ and the Hill coefficient n. The results of these calculations are listed in Table 1. It can be seen from these data that the Hill coefficient was equal to 1 for all mutants, pointing to the fact that the interaction of PEP and ADP with these enzymes was not cooperatively regulated. In other words, the dependence of the initial velocity upon substrate concentration was well described by the common hyperbolic rate equation, and this fact is illustrated in Figure 13 where the consolidated kinetic data for L-PK mutants are shown.

Table 1.

L-PK N-terminal domain fragment	k_{cat} **µmol/mg*sec**	K^{app}_{PEP}, **mM**	K^{app}_{ADP}, **mM**	n **for PEP**	n **for ADP**
L-PK Mutants					
-<u>A</u>RAS^{12}VA-	12,0±0.2	0.19±0.03	0.24±0.05	0.9±0.1	0.9±0.1
-<u>K</u>RAS^{12}VA-	9.5±0.2	0.32±0.05	0.24±0.04	0.6±0.1	1.0±0.1
-<u>E</u>RAS^{12}VA-	14.2±0.7	2.3±0.4	0.20±0.06	0.9±0.1	0.9±0.3
-<u>Q</u>RAS^{12}VA-	13.6±0.2	0.39±0.08	0.27±0.04	0.9±0.1	0.9±0.1
-R<u>A</u>AS^{12}VA-	13,1±0.4	0.18±0.04	0.33±0.08	1.0±0.1	1.0±0.1
-R<u>K</u>AS^{12}VA-	9.1±0.3	0.17±0.04	0.33±0.06	0.7±0.1	1.0±0.1
-R<u>Q</u>AS^{12}VA-	15,5±1.0	0.31±0.06	0.33±0.03	1.1±0.1	0.9±0.1
-R<u>L</u>AS^{12}VA-	11,6±0.2	0.29±0.07	0.21±0.03	1.2±0.3	1.0±0.1
-RRAS^{12}<u>A</u>A-	15,6±0.2	0.19±0.03	0.31±0.05	1.0±0.1	1.0±0.1
-RRAS^{12}<u>E</u>A-	15,2±0.5	0.22±0.04	0.27±0.05	1.0±0.1	1.1±0.1
Wild type non-phosphorylated L-PK					
-RRAS^{12}VA-	15.8±0.5	0.11±0.02	0.28±0.02	1.1±0.1	1.1±0.1
Wild type phosphorylated L-PK					
-RRAS^{12}(P$_i$)VA-	9.6±0.7	2.2±0.1	0.11±0.01	2.5±0.2	1.1±0.2

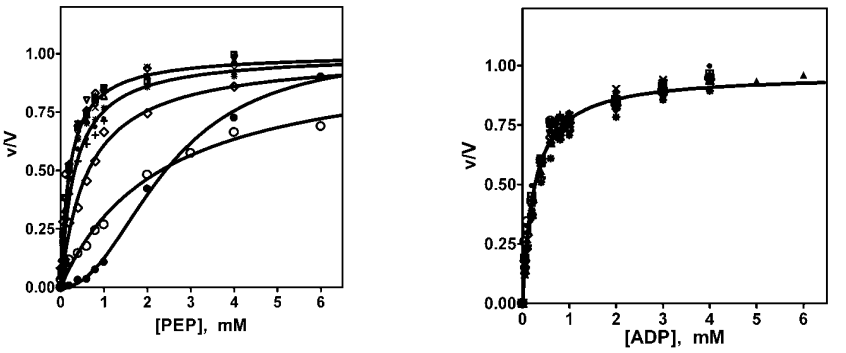


Figure 13. Effect of PEP and ADP concentration on the rate of ATP synthesis catalysed by L-PK and its mutants. The following enzymes were used: non-phosphorylated L-PK (×); phosphorylated L-PK (●); L-PK mutants R9E (○); R9K (◊); R9A (■); R9Q (□); R10Q (+); R10L (♦); R10A (❋); R10K (+); V13A (▽); V13E (△).

It can be seen in Table 1 that mutations made in the regulatory part of L-PK had a different influence on the interaction of ADP and PEP with the enzyme. As the K_{ADP}^{app} values were similar in the case of all mutants, and were also close to the same parameter for the wild type of non-phosphorylated and phosphorylated L-PK, it can be concluded that the binding site of this substrate was not affected by these mutations. On the other hand, however, some mutations affected the enzyme affinity for PEP, and this effect was clearly dependent upon the structure of the introduced amino acids. If smaller shifts in the K_{PEP}^{app} values were observed with mutants R9Q, R9K, R10Q and some others, this effect was large when arginine residues were replaced by glutamic acid. It can be seen from Table 1 that this mutation (R9E) increased the K_{PEP}^{app} value more than 20-fold, and again, without any effect on ADP binding.

In summary, some mutations in the N-terminal domain of L-PK specifically affected the PEP binding site, and the effect was dependent on the nature of the inserted amino acid. Differently from PEP, the binding of ADP was not influenced by the same mutations.

It is noteworthy that a similar asymmetric change of the catalytic properties of L-PK was also caused by phosphorylation, as this modification significantly decreased the enzyme affinity for PEP and had practically no effect on ADP binding (Table 1). From this point of view, mutations made in the N-terminal domain of L-PK and phosphorylation of this domain had a similar influence, and could be correlated with the ionic status of the N-terminal domain of L-PK, governed by two arginine residues in positions 9 and 10. Replacement of one arginine with glutamic acid changed the net charge of the regulatory domain by –2 charge units. A similar change was also caused by phosphorylation, pointing to the possibility that the L-PK affinity for PEP could be governed by electrostatic interactions.

The ionic status of the regulatory domain was also affected by other mutations. For example, the number of positive charges of the N-domain was changed by –1 charge unit in mutants, where non-ionic amino acids alanine, leucine and glutamine were used to substitute the arginine residue in position 9 or 10. The same change in the ionic status of the regulatory N-domain was also achieved by replacement of the non-ionic valine 13 by glutamic acid. It can be seen from Table 1 that all these replacements had a relatively low impact on the enzyme affinity for PEP. On the other hand, if arginine was replaced by lysine there was no change in the net charge of the regulatory domain, but still the effectiveness of PEP binding was changed. Consequently, the binding effectiveness of PEP cannot be unequivocally linked with the ionic charge of the N-terminal part of the protein.

The activity of the phosphorylated L-PK was cooperatively regulated by PEP and this effect was characterised by the Hill coefficient $n = 2.5$. At the same time the mutant R9E, as well as other mutants studied, revealed no cooperative feedback and the appropriate kinetic data were adequately described by the Hill coefficient 1 (Table 1). Therefore, the down-regulation of enzyme affinity for PEP and switching on cooperativity for this substrate should be governed by two different mechanisms. However, in both these mechanisms the regulatory pattern has remained asymmetric for PEP and ADP.

5.6. Asymmetric regulation of PEP and ADP binding

The fact that phosphorylation of the N-domain of L-PK and the point mutations (R9E) had an asymmetric influence on ADP and PEP binding indicated that the appropriate changes made in the regulatory domain should differently affect the binding sites of these substrates. Indeed, it has been described that binding of these substrates occurred in somewhat different areas of the enzyme molecule (Munoz & Ponze, 2003; Mattevi *et al*., 1996; Jurica *et al*., 1998; Muirhead *et al*., 1986). However, the binding sites of both substrates are still in the same domain of the protein and remain close to each other. Moreover, the close location of the enzyme-bound ADP and PEP is also required for the direct transfer of the phosphate group from PEP to ADP in the chemical step of the catalysis (Muirhead *et al*., 1986).

We carried out computer modelling of ADP and PEP docking with the L-PK subunit. The model of this protein (Figure 14) was built up proceeding from structural data for the R isozyme (Valentini *et al*., 2002) and omitting 25 amino acids of the flexible N-terminal domain, as this structure was not determined by X-ray analysis. The position of the amino acid number 26 is shown by the arrow in Figure 14. One bivalent (Mg^{2+}) and one monovalent (K^{+}) cation, depicted by the yellow and violet spheres in Figure 14, were included in the protein model. Positioning of these ions should define the location of the catalytic site of L-PK,

as their participation is important for the phosphoryl transfer reactions (Muirhead *et al*., 1986). Therefore, it was not surprising that the calculated docking sites for ADP and PEP labelled the same area of the protein molecule as illustrated in Figure 14.

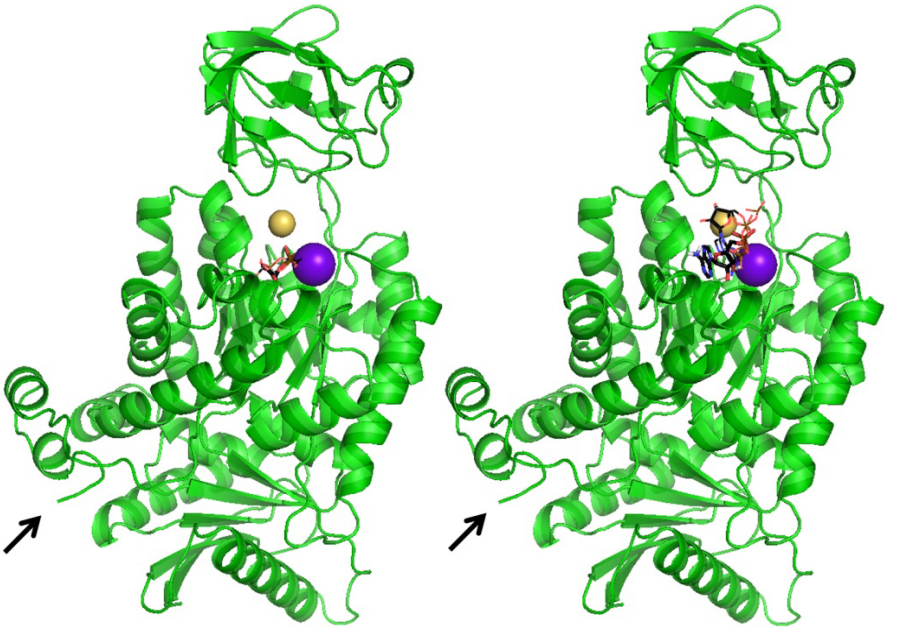


Figure 14. Computer modelling of PEP (left) and ADP (right) docking with the L-PK subunit. The protein model was built up starting from amino acid no. 26 (shown by the arrow), as the coordinates of preceding amino acids have not been determined due to significant flexibility of this part of the protein. The locations of Mg^{2+} (yellow sphere) and K^{+} (violet sphere) were shown in the catalytic site. Three energetically close docking poses were shown for each substrate and the bold structure referred to the best complex. The atoms of the ligands were marked as follows: carbon – black, oxygen – red, nitrogen – blue, and phosphorus – brown.

The computer modelling of ligand docking yielded several possible complexes that were ranked by the values of binding energy. It can be seen from Figure 14 that the binding poses obtained for both substrates marked quite definitely the catalytic site of the enzyme, while the most stable complexes from this list were shown in bold. Certainly, further calculations should be made to obtain the more precise structure of these complexes, also considering the dynamics of the protein molecule (Totrov & Abagyan, 2008), and inter-subunit interactions. However, even the present tentative docking model confirms the close positioning of PEP and ADP in the active centre of L-PK (Figure 14).

The N-terminal regulatory domain of L-PK is located at a considerable distance from the substrate binding sites, but still its phosphorylation at S12, as well as introduction of mutations in positions 9, 10 and 13, altered the binding

effectiveness of PEP with the enzyme. This regulation could not be explained by the conventional structural models of allostery, as these models assume transfer of conformational changes from the regulatory site to the substrate site by changing interactions between certain pairs (or sets) of amino acids, implying a trimmed and even somewhat strained protein structure. This regular structure is missing in the case of the N-domain of L-PK. Therefore**,** the transition of the regulatory effects to the active site of L-PK, and its targeted manifestation in the case of only one substrate, seems to define some alternative mechanism of allosteric control.

This mechanism could be based on the noticeable flexibility of the regulatory N-domain, as was initially proposed in (Bergström *et al*., 1978), and further discussed in (Fenton & Tang, 2009). Notoriously, this hypothesis agreed well with the results of probing the L-PK phosphorylation site by mutational analysis, as different degrees of the regulatory effect were achieved. Following this hypothesis it was suggested that the flexible N-terminal peptide of L-PK could, indeed, bind to the main body of the protein and this interaction was assumed to be responsible for high-affinity PEP binding. Truncation, phosphorylation and mutation of the N-terminal regulatory domain seem to interfere with this interaction and reduce the enzyme affinity for PEP.

5.7. Interaction of FBP and model peptides resembling the N-terminal domain of L-PK with the non-phosphorylated enzyme

Firstly, it was investigated whether the activity of the non-phosphorylated L-PK could be regulated by FBP, and the activity of the enzyme was monitored in the presence of this ligand. These results revealed no activation, but slight reversible inhibition, characterised by the K_i value 24 ± 3 μM was observed (Figure 15). Most interestingly, inhibition of the L-PK catalysed reaction by the excess of FBP has also been observed in earlier studies using L-PK extracted from rabbit liver (Irwing & Williams, 1973). However, as in this case the phosphorylated enzyme was also activated by FBP, and the observed dependence of the reaction rate versus FBP concentration was characterised by the sum of activation and inhibition effects. Therefore, the inhibition effect was seen after the activation effect had levelled off. Thus the inhibition phenomenon seems to be similar in the case of the phosphorylated and non-phosphorylated enzymes, and probably demonstrates that FBP may also compete for the PEP binding site, as both ligands have phosphate groups recognised by the active site of L-PK.

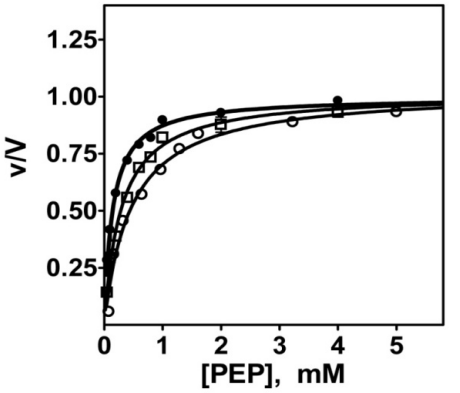


Figure 15. Influence of 60μM FBP (○) and 100 μM phosphopeptide RRAS(Pi)VA (□) on the catalytic properties of the non-phosphorylated L-PK at different PEP concentrations (● – control curve). Both these ligands did not induce cooperativity of the enzyme, but revealed slight inhibition of the reaction.

Secondly, the interaction of non-phosphorylated L-PK with short peptides that mimic the phosphorylatable N-terminal domain of the enzyme was studied. We used peptide RRASVA, which corresponds to fragments 9–14 of the L-PK N-domain, and RRAAVA, which corresponds to the point-mutated phosphorylatable peptide. It was found that neither of these peptides influenced the activity of non-phosphorylated L-PK (Figure 16), while similar peptide fragments enhanced PEP binding with the L-PK mutant resembling the phosphorylated enzyme (Prasannan *et al*., 2012).

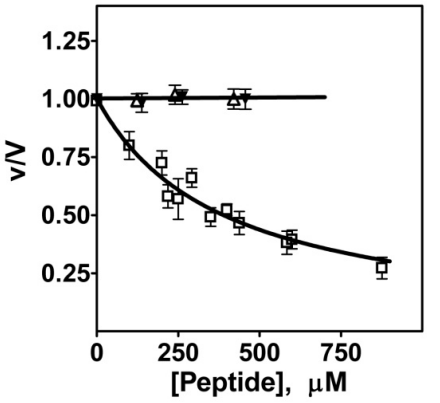


Figure 16. Influence of peptides RRASVA (Δ), RRAAVA (▼) and phosphorylated peptide RRAS(Pi)VA (□) on the activity of the non-phosphorylated L-PK. Concentrations of ADP and PEP in the assay mixture were 1.0 mM and 0.8 mM, respectively.

Finally, the influence of the phosphorylated peptide RRAS(Pi)VA on the catalytic properties of non-phosphorylated L-PK was studied. It was found that in the presence of 0.4 mM phosphopeptide a slight shift of PEP binding effectiveness was observed. The meaning of this phenomenon is more clearly demonstrated in Figure 16, where data about inhibition of the non-phosphorylated L-PK by RRAS(Pi)VA, characterised by the K_i value 47±7 μM, were shown. On the other hand, the ligand RRAS(Pi)VA did not induce cooperativity of the enzyme against PEP, although the initial purpose of this experimental set-up was to analyse this possibility.

Taken together, the present results demonstrate that the non-phopshorylated L-PK is not an allosterically regulated enzyme, and all allosteric regulation mechanisms known in the case of the phosphorylated L-PK should be switched on through phosphorylation of the regulatory N-terminal peptide of this protein. On the other hand, both phosphorylated ligands, FBP and RRAS(Pi)VA, revealed an inhibitory effect, which was probably connected with competition of these molecules with PEP for its binding site in the active site of the enzyme.

5.8. Putative docking site for the N-domain peptide on the main body of protein

The hypothesis that there may be a specific binding site for the N-terminal peptide on the main body of L-PK was probed by a computer simulation of docking of specific peptides: RRASVA and ERASVA, resembling the phosphorylation site of the wild-type enzyme and of its mutant R9E, respectively. Moreover, the phosphorylated peptide RRAS(Pi)VA was also used for the docking study. However, several energetically and geometrically different binding poses were obtained for each of these peptides. In all cases the most efficient poses were clearly grouped within the same region of the protein surface, thus labelling their most probable binding site.

It can be seen in Figure 17 that binding of RRASVA occurred close to the active centre of the enzyme. Interestingly, the docking site for the peptide ERASVA, which corresponds to the regulatory domain of the R9E mutant, was also located in the same region. Moreover, similar results were obtained for other mutant peptides. Therefore**,** these peptides could indeed govern PEP binding, while the effectiveness of this influence should be dependent upon their amino acid composition. The absence of a similar influence on ADP binding can be explained by stronger “anchoring” of this substrate in the active centre, perhaps via the protein-bound magnesium ion, as the stability of the ADP-Mg complex characterised by the pK_{diss} value 3.7 (Belaich & Sari, 1969) exceeds the stability of the PEP-Mg complex characterised by pK_{diss}=2.2 (Wold & Ballou, 1957).

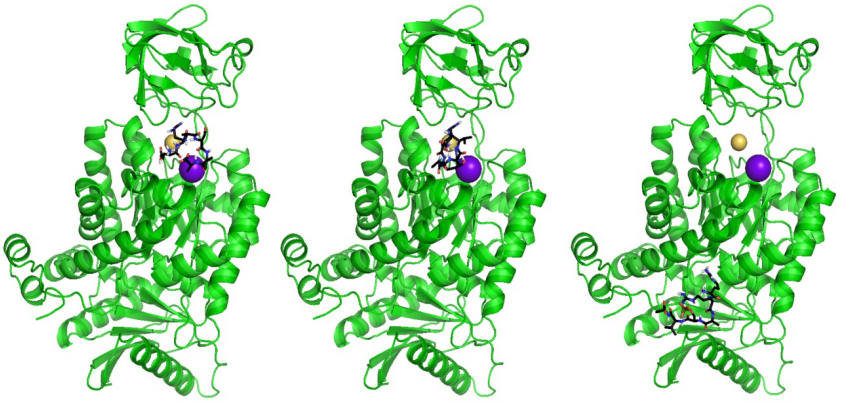


Figure 17. Computer modelling of docking of peptide fragments RRASVA (left), ERASVA (centre) and phosphopeptide RRAS(Pi)VA (right) with the L-PK subunit. The protein model described in Figure 14 was used. The docking poses of the lowest energy were shown for each peptide. Atoms of the ligands were marked as follows: carbon – black, oxygen – red, nitrogen – blue, and phosphorus – brown.

On the other hand, however, the phosphorylated peptide RRAS(Pi)VA clearly preferred a different binding site located outside the enzyme active centre (Figure 17). This means that phosphorylation should shift this peptide from one binding site into another, which is located close to the C domain. If a similar shift takes place in the case of the enzyme molecule, this structural transition could function as a switch for L-PK cooperativity.

This second docking site was partially overlapping with the binding site for the allosteric regulator FBP (Munoz & Ponze, 2003; Mattevi *et al*., 1996; Jurica *et al*., 1998). Following the results of this docking study, the allosteric effect of this ligand may be explained by competition between the phosphorylated N-terminal peptide and FBP for the second binding site on domain C, as displacement of the phosphorylated N-domain peptide from this site by FBP may restore the structural requirements for effective PEP binding. However, further discussion of the structural and mechanistic aspects of L-PK regulation by phosphorylation needs more thorough modelling, taking into account the protein dynamic properties and inter-subunit interactions within its tetrameric structure.

6. CONCLUDING REMARKS

Among the four pyruvate kinase isoenzymes, denoted as M1, M2, R and L, only M1 reveals no cooperativity against phosphoenolpyruvate (PEP) concentration. However, here we show that the non-phosphorylated form of L-type pyruvate kinase (L-PK) is also a non-cooperative enzyme against PEP, while the cooperativity and other allosteric catalytic properties of this enzyme are switched on through phosphorylation of this protein by cAMP-dependent protein kinase. The level of cooperativity of the enzyme, characterised by the Hill coefficient, depends on the phosphorylation stoichiometry, but the allosteric mechanism as such is engaged by phosphorylation of the first subunit of the tetrameric enzyme, and further phosphorylation only modulates this effect. The discovered switching between non-allosteric and allosteric forms of this enzyme and the possibility of modulating the allostery by phosphorylation seems to be important for understanding the interrelationship between allostery and the regulatory phosphorylation phenomenon in general, and may also have some implication for further analysis of the regulation of glycolysis in the liver.

Cooperativity of the non-phosphorylated L-PK cannot be induced in the presence of peptides that mimic the phosphorylated or non-phosphorylated N-domain sequence of the protein, and also by the introduction of additional negative charges to the protein structure around the phosphorylatable serine residue in the protein regulatory domain. At the same time these variations in protein structure may influence the enzyme affinity for PEP, pointing to the possibility that the regulatory domain may interact with the protein main body and thereby govern the enzyme activity, as was initially suggested by A. Fenton and Tang (Fenton & Tang, 2009). We have also identified that the docking site of the N-domain may well be in the region of the PEP binding site and thus this interaction may support PEP binding. Phosphorylation obviously hinders formation of this intramolecular complex and therefore reduces PEP binding effectiveness. The stability of this complex also seems to be affected by mutations in the N-domain peptide and shifting this equilibrium could explain the alteration of PEP binding effectiveness with the mutant enzymes. However, differently from phosphorylation, the cooperativity of the enzyme cannot be switched on by these mutations.

Some structural aspects of L-PK regulation were discussed proceeding from the results of mutation analysis and computer modelling of binding of the regulatory domain fragments with the main body of the enzyme. These results demonstrate that the cooperativity and allosteric properties of L-PK seem to be governed by some additional mechanism. Most likely it is based on the mutual competition of FBP and the phosphorylated N-domain for the same binding site on the C subunit of the enzyme.

7. SUMMARY

Pyruvate is an essential substrate in metabolic processes, and regulation of the activity of pyruvate kinases is essential for control over the energy and carbon fluxes in living cells. In liver tissue, where both of these glycolysis and glyconeogenesis pathways are in function, the tissue-specific pyruvate kinase (L-PK) is activated homotropically by phosphoenolpyruvate (PEP) and heterotropically by fructose-1,6-bisphosphate (FBP), and both of these effects depend on phosphorylation of the N-terminal domain of the enzyme. In this dissertation the regulatory role of the N-terminal domain has been studied by using conventional methods of protein chemistry and chemical kinetics along with computational ligand docking analysis.

The results of this study revealed that the activity of the non-phosphorylated L-PK was characterised by a common hyperbolic Michaelis-Menten plot in the case of PEP (K_{PEP} = 0.11 mM) and the activity of the enzyme was not regulated by FBP. The cooperativity of the enzyme for PEP was switched on by phosphorylation of the protein by cAMP-dependent protein kinase. The stoichiometrically phosphorylated enzyme, containing four moles of phosphate per tetrameric L-PK molecule, was characterised by K_{PEP} = 2.2 mM and the Hill coefficient n = 2.5. It was also observed that enzyme cooperativity was mostly engaged by phosphorylation of the first subunit in the tetrameric protein, while further phosphorylation only modulated this effect. Further, it was found that the activity of the non-phosphorylated L-PK was not elevated by FBP, and this ligand acted as a relatively weak reversible inhibitor. As the phosphorylated L-PK is a subject of significant allosteric activation by FBP, it was concluded that phosphorylation should also function as a molecular switch in the case of the allosteric properties of L-PK.

The regulatory phosphorylation occurs in the serine residue in position 12 of the N-terminal sequence MEGPAGYLRR^{10}A**S**^{12}VAQLTQEL^{20}GTAFF, and in this study the influence of point mutations around the phosphorylation site in positions 9, 10 and 13 on the catalytic properties of the mutants was studied. Amino acids A, L, Q and E were introduced into these positions to alter the net charge of the regulatory domain and to check whether redistribution and/or introduction of new ionic groups can mimic the effect of serine phosphorylation. It was found that some of these mutations affected the catalytic activity of the enzyme by reducing the effectiveness of phosphoenolpyruvate binding, simulating thus the effect of regulatory phosphorylation. This result was in agreement with the ideas suggested by A. Fenton & Tang (Fenton & Tang, 2009) that the flexible N-domain may interact with the main body of the enzyme affecting substrate binding, and this interaction is interfered with by phosphorylation. However, differently from phosphorylation, the cooperativity of the enzyme was not switched on by these mutations in the N-domain.

Finally, following the suggestion that the N-domain interacts with the main body of the L-PK molecule, computational analysis was made to identify

putative binding sites. The docking site of the N-domain peptide RRASVA was found in the vicinity of the enzyme active centre, while docking of the phosphorylated analogue RRAS(Pi)VA occurred on the C domain of the L-PK molecule, in a site overlapping with the allosteric site for FBP binding. This means that formation of these intramolecular complexes may indeed change equilibrium between the active R-state and the less active T-state of the enzyme, while induction of cooperativity by the N-domain phosphorylation could be connected with phosphopeptide intramolecular binding with the enzyme allosteric site.

The phenomenon of switching between non-allosteric and allosteric forms of L-PK and the possibility of modulating of the enzyme allostery by phosphorylation may have some implication for further understanding of the regulation of glycolysis in the liver.

8. SUMMARY IN ESTONIAN

L-tüüpi püruvaadi kinaasi N-terminaalse domeeni regulatoorne roll

Püruvaat on oluline metabolismi substraat ja seetõttu on püruvaadi kinaaside aktiivsuse regulatsioon oluline raku energia- ja süsinikuvoogude suunamise seisukohast. Maksas, kus samaaegselt toimivad need mõlemad metabolismi rajad, esineb koe-spetsiifiline püruvaadi kinaaas (L-PK), mis on aktiveeritud homotroopselt selle ensüümi substraadi fosfoenoolpüruvaadi (PEP) poolt ning heterotroopselt fruktoos-1,6-bisfosfaadi (FBP) poolt. Samas on need mõlemad efektid omakorda reguleeritud selle ensüümi N-terminaalse domeeni fosforüleerimise kaudu. Käesolevas töös uuriti selle regulatoorse fosforüleerimise nähtuse tagamaid, kasutades selleks klassikalisi valgukeemia meetodeid ning keemilise kineetika rakendusi koos ligandide seostumise modelleerimisega arvutil.

Töö tulemused näitasid, et mitte-fosforüülitud L-PK poolt katalüüsitud reaktsioon on PEP korral iseloomustatud tavalise hüperboolse Michelis-Menteni reaktsioonikiiruse võrrandiga (K_{PEP} = 0.11 mM). Samas lülitas cAMP-sõltuva proteiinkinaasi poolt katalüüsitud valgu fosforüülimine sisse L-PK kooperatiivsuse PEP suhtes ning ka selle ensüümi aktiivsuse allosteerilise regulatsiooni. Nii iseloomustas stöhhiomeetriliselt fosforüleeritud ensüümi, kus ühe valgu tetrameerse molekuli kohta esines neli fosfaatrühma, K_{PEP} väärtus 2.2 mM ja Hilli koefitsient n = 2.5. Samuti ilmnes, et kooperatiivsed omadused lülitusid sisse tetrameerse valgu esimese subühiku fosforüleerimisel, kusjuures edasine fosfaatrühmade lisamine ainult moduleeris seda efekti. Samuti leiti, et mitte-fosforüülitud L-PK aktiivsust ei mõjutanud ka allosteeriline aktivaator FBP. Kuna fosforüleeritud L-PK aktiivsus suureneb oluliselt allosteerilise aktivaatori FBP juuresolekul, siis järeldati, et fosforüleerimine toimib ka selle ensüümi allosteerilise regulatsiooni molekulaarse lülitina.

Regulatoorne fosforüülimine toimub seriini nr 12 juures, mis asub N-terminaalses valgujärjestuses MEGPAGYLRR10AS12VAQLTQEL20GTAFF. Selle valgujärjestuse rolli selgitamiseks uuriti fosforüülitava seriini ümbruses asuvate aminohapete punktmutatsioonide mõju valgu katalüütilistele omadustele. Muutused olid positsioonides 9, 10 ja 13 ning asenduseks kasutati aminohappeid A, L, Q ja E, mis võimaldas muuta selle domeeni summaarset laengut ja uurida, kas ioonsete rühmade ümberjaotumine ja uute ioonsete rühmade sisseviimine võib imiteerida seriini jäägi fosforüülimise efekti. Ilmnes, et mõned asendused mõjutasid valgu aktiivsust, vähendades selle afiinsust PEP suhtes ning sellega imiteerisid fosforüülimist. Saadud tulemus on kooskülas A. Fentoni ja kaastöötajate poolt pakutud oletusega (Fenton & Tang, 2009; Holyoak *et al*., 2013), et N-domeen võib seostuda valgumolekuli mingis muus piirkonnas ja selliselt mõjutada substraadi sidumist. Selle sisemolekulaarse kompleksi lõhub aga domeeni fosforüülimine. Samas aga ilmnes tõsiasi, et N-

domeeni struktuuri ja kogulaengu muutmine ei indutseeri ensüümi kooperatiivsust PEP suhtes.

Lähtudes oletusest, et N-domeen võib tänu oma paindlikkusele toimida valgumolekuli teiste osadega, teostati arvutil modelleerimise teel võimalike sidumiskohtade otsing. Selleks uuriti regulatoorse domeeni fragmendi RRASVA ja selle fosforüülitud analoogi RRAS(Pi)VA seostumist L-PK alaühikuga. Leiti, et RRASVA seostumine võib toimuda ensüümi aktiivtsentri piirkonnas ning see võib tõepoolest olla oluline PEP sidumise seisukohast. Samal ajal toimus fosfopeptiidi sidumine subühiku C-domeenil, kus seostub allosteeriline ligand FBP. Seega võib sisemolekulaarsete komplekside teke tõepoolest nihutada tasakaalu ensüümi aktiivse R-vormi ja vähemaktiivse T-vormi vahel. Samas võib kooperatiivsuse sisselülitamine toimuda fosforüleeritud N-domeeni seostumisel L-PK allosteerilises tsentris.

Molekulaarne lülitusmehhanism allosteerilise ja mitte-allosteerilise L-PK vormide vahel, mis põhineb regulatoorse domeeni fosforüülimisel, võib omada olulist tähtsust maksas toimuva glükoosi metabolism mõistmisel.

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15. 30th FEBS Congress. The Protein World. 02–07 July 2005 Budapest, Hungary. Poster presentation.
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Publikatsioonide loetelu:
1. Kuznetsov, A.; Faustova, I; Järv, J. Computational simulation of ligand docking to L-type pyruvate kinase subunit. Submitted for CBAC
2. Kõivomägi, M.; Örd, M.; Iofik, A.; Valk, E.; Venta, R.; Faustova, I.; Kivi, R.; Balog, E.R.M., Rubin, S. M. and Loog, M. (2013) Multisite phosphorylation networks as signal processors for Cdk1. Nature Structural & Molecular Biology, in press.

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