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Substrate specificity of protein kinase A in reaction with protein substrates

Thesis for the degree of Master of Sciences in bioorganic chemistry

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Tartu, 2005

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ABBREVIATIONS

PKA	catalytic subunit of cAMP-dependent protein kinase (protein kinase A)
L-PK	L-type pyruvate kinase
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
C	catalytic subunits of protein kinase A
cAMP	cyclic adenosine-3',5'-monophosphate
CDPK-1	calcium-dependent protein kinase 1
DTT	dithiothreitol
LRRASLG	Kemptide
MOPS	4-morpholinepropanesulfonic acid
NADH	nicotinamide adenine dinucleotide, reduced
PEP	phosphoenolpyruvate
PKA	protein kinase A, cAMP-dependent protein kinase
PKC	protein kinase C
PKI	heat-stable protein kinase inhibitor
P-site	protein or peptide binding site
R	regulatory subunit of protein kinase A
TRIS	tris(hydroxymethyl)-aminomethane

INTRODUCTION

Protein kinases (E.C.2.7.1.37) are enzymes, which catalyze phosphorylation of substrate proteins by transfer of the γ -phosphate of ATP to the acceptor amino acid. Through this reaction activity of the target enzymes is modified and therefore the phosphorylation process is used to control biochemical events in living cell (Graves and Krebs, 1999; Manning et al, 2002). The phosphorylation modulates the activity of many proteins and protein kinases play a key role in multiple signaling and regulatory phenomena in cell. It has been estimated that as much as 20-50% of all the cellular proteins undergo phosphorylation *in vivo* (Pinna and Ruzzene, 1996). On the other hand, there is great diversity of protein kinase forms involved in eukaryote signaling and according to the human genome analysis 518 putative protein kinase genes were identified (Manning et al, 2002). This makes them the largest enzyme family in eukaryotic cells.

Despite the large amount of sequence data for protein kinases, only a small fraction of these enzymes has been characterized on protein level and substrate specificity has been investigated for even a fewer number. Therefore investigation into specificity of several “model” enzymes has been very important for development of our understandings of the principles, used by protein kinases for selection of right substrates that in turn, is the basis of correct signaling and cell regulation (Pinna and Ruzzene, 1996). Protein kinase A is undoubtedly one of these “model” enzymes, used for different fundamental studies, including systematic analysis of substrate specificity (Taylor et al, 1993; Kemp et al, 2003).

These studies have revealed that the phosphate group is transferred to the corresponding hydroxyl function of the substrate protein from ATP-magnesium complex and the phosphorylatable site is recognized by the protein kinase active center. In this recognition process the peptide sequence, flanking the phosphorylatable amino acid, is evidently one of the specificity determining factors and governs selectivity the regulatory phosphorylation phenomena (Johnson, 2001). The role of these “local sequence elements” in specificity of protein kinases has been extensively studied with short peptide substrates, which resemble the amino acid sequence of the phosphorylatable sites in substrate proteins (Pearson and Kemp, 1991) and the consensus sequences for a number of protein kinases were defined (Kennelly and Krebs, 1991). However, statistical analysis of the known phosphorylation sites in proteins revealed that many of these sites did not contain the complete consensus sequence (Kreegipuu et al, 1998). Therefore it is still not clear if the primary specificity of protein kinases against protein substrates can be directly derived from the data for model peptides. In

the present work we directly addressed this question by comparing specificity of cAMP-dependent protein kinase catalytic subunit (PKA) in reaction with a series of site-directed mutant protein substrates and synthetic peptides. This enzyme is a classical model for the whole protein kinase family [5] and its specificity against peptide substrates has been thoroughly studied [1,3,6,7]. The mutant proteins were derived from L-type pyruvate kinase (L-PK), which is one of the physiological substrates of PKA (Engström et al, 1982), and structure of the phosphorylation site located at Ser residue in position 12 of the primary structure of this protein has been used for definition of the “minimum substrate” for PKA [9].

The obtained kinetic data confirmed the basic substrate specificity requirements for PKA, i.e. the preference of arginine in P-2 and P-3 and hydrophobic residue in P+1 position. Comparison of the obtained kinetic data with available data on similar structural alterations in peptide substrates revealed that amino acid alterations in peptides cause stronger effects on the phosphorylation rate than the corresponding alterations in protein substrate L-PK. This finding indicates that the importance of the primary structure specificity profile around the phosphorylation site in protein substrates may be overestimated, if based on the specificity analysis for peptide substrates.

Results of the work have been published in the following paper:

Loog M, **Oskolkov N**, O'Farrell F, Ek P, Järv J., Comparison of cAMP-dependent protein kinase substrate specificity in reaction with proteins and synthetic peptides. *Biochim Biophys Acta*. 2005 Mar 14; 1747(2):261-6.

REVIEW OF LITERATURE

1. Protein kinases

Classification of protein kinases and definition of the eukaryotic protein kinase superfamily were provided by Hanks, Quinn and Hunter (Hanks et al, 1988). The common motif for all protein kinases is the homologous “kinase domain”, also known as the “catalytic domain”. This part of these enzymes consists of 250-300 amino acid residues. The kinase domain contains 12 conserved subdomains and it folds to closely similar three-dimensional core structures in different protein kinases (reviewed in Hanks and Hunter, 1995). The classification given by Hanks et al. (1988) was based on the phylogenetic trees derived from alignment of kinase domain amino acid sequences. It was found that the sequence similarity of the kinase domains revealed four main families with related substrate specificities and modes of regulation. These four groups are listed below together with few representatives:

1. The AGC group with mainly basic amino acid specificity determinants, including the cyclic nucleotide-regulated protein kinase family with the well known representative PKA, the diacylglycerol-activated/phospholipid-dependent protein kinase C family and the RAC family of kinases.
2. The CaMK group covers the family of kinases regulated by Ca^{2+} /calmodulin. The plant Ca^{2+} -dependent protein kinases, studied in one of the papers of this thesis also belong to this group.
3. CMGC group includes family of cyclin-dependent kinases (Cdks), the MAP kinase family and casein kinase II.
4. PTK group: the protein –tyrosine kinase group

However, several kinases still fall outside these major groups and are difficult to classify into defined subsections.

The first 3-dimensional structure of a protein kinase was determined for the catalytic subunit of protein kinase A (cAMP-dependent protein kinase, PKA) in complex with peptide inhibitor PKI and MgATP, in 1991 (Knighton et al, 1991). Later additional structures of PKA in complex with different ligands were published (Kim et al, 2005; Wu et al, 2004) and these data are discussed with reference to the dynamic aspects of this protein in catalysis (Taylor et al, 2004; Taylor et al, 1999). Therefore PKA has become the most thoroughly studied representative of the protein kinase superfamily, and is generally recognized as a model enzyme of this class.

2. Catalytic domain of protein kinase A

Catalytically active enzyme (C) is formed through dissociation of the regulatory subunit (R) from the catalytic subunit in the presence of cAMP. In fact, the inactive holoenzyme of PKA is a tetramer consisting of two regulatory and two catalytic subunits (Tao et al, 1970).



The schematic representation of the catalytic and regulatory subunits is given in **Fig. 1**.

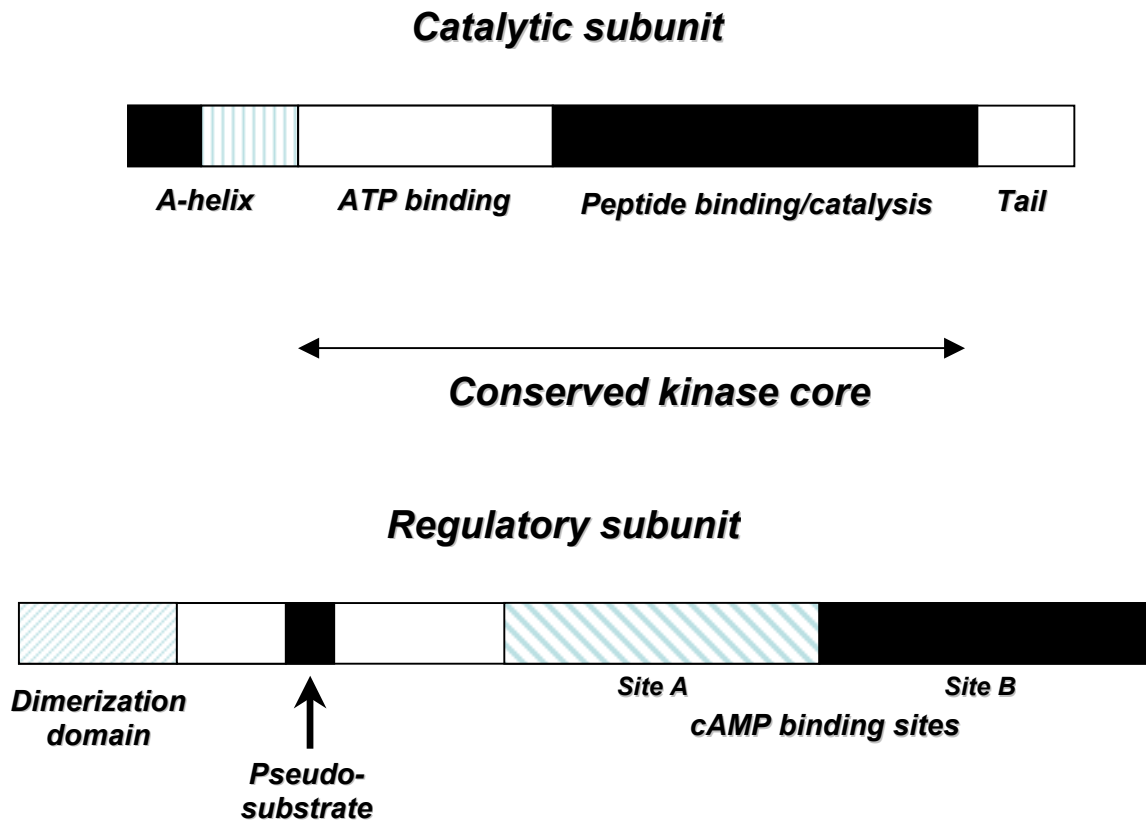


Figure 1. Schematic view of the catalytical and regulatory subunits.

The general three-dimensional architecture is shown in form of the ribbon diagram in **Fig 2**. Both the open and closed conformations are shown in this figure (Shaltiel et al, 1998).

The catalytic subunit consists of a smaller upper lobe and a larger lower lobe. The small lobe is built mostly by antiparallel β -sheets and is responsible for ATP binding. The loop between the beta sheets 1 and 2 of the small lobe, having the motif Gly50-X-Gly52-X-X-Gly55, is called the phosphate anchor since it is binding the phosphate groups of ATP *via* several hydrogen bonds (Bossemeyer et al, 1993). The third beta strand contains a lysine residue (Lys72), which is conserved throughout this superfamily. This lysine coordinates the

α - and β -phosphates of the ATP and also forms a salt bridge with Glu91 located in the middle of helix C. The adenine ring of ATP binds in a confined, mostly hydrophobic pocket, located in the cleft under the β strands 1 and 2. Most of the interactions of ATP, except the contacts with the γ -phosphate and the hydrophobic interactions with the adenine ring, are associated with the small lobe.

The large lobe is predominantly α -helical and is associated with catalysis and peptide binding. An important structural element of the large lobe is the catalytic loop connecting the β strands 6 and 7. The conserved Arg165, which side chain forms a tight electrostatic contact with phosphorylated Thr197, precedes the loop. This interaction is essential for maintaining the active conformation of the kinase. The neighbouring amino acid, the conserved Asp166,

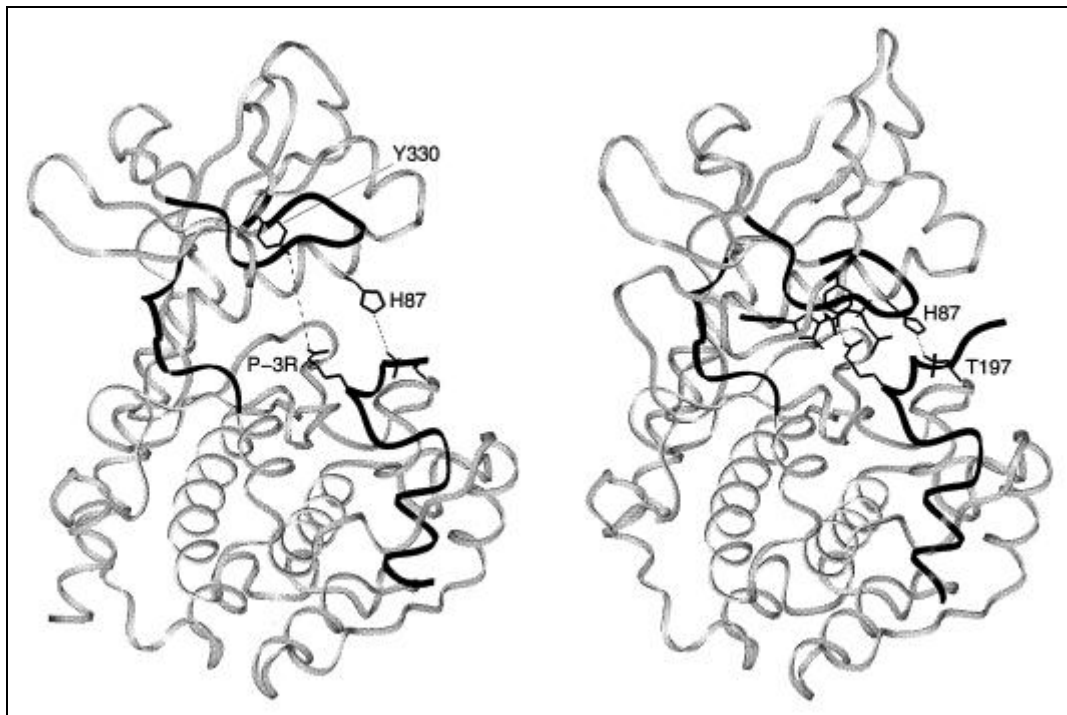


Figure 2. Open (*left*) and closed (*right*) conformations of the catalytic subunit of PK A. The open conformation is shown as complex with and PKI (5–24). The closed conformation is shown as ternary complex of the catalytic subunit with Mn₂ATP and PKI (5–24). The distances between the His-87 side chain and Thr-197 phosphate and between the Tyr-330 and the P-3 Arg of the inhibitor peptide serve as quantitative indicators for the degree of “openness.” The glycine-rich loop (residues 47–56), the linker strand (residues 121–127), and PKI (5–24) are highlighted in black (Shaltiel et al, 1998).

has been proposed to act as catalytic base in the reaction of phosphoryl transfer (Madhusudan et al, 1994).

β -strand 8 is followed by a conserved Asp184-Phe185-Gly186 (DFG) motif or the Mg^{2+} binding loop. The Asp184, correctly positioned by the anchoring hydrophobic interactions of the neighbouring phenylalanine, is one of the ligands of the magnesium ion coordination sphere, which in turn coordinates the β - and γ -phosphates of ATP. The DFG motif is followed by beta strand 9 and the activation loop. The activation loop contains the above mentioned Thr197, which phosphorylation by autophosphorylation or by PDK1 kinase (Cheng et al, 1998) is essential for the activity of PKA. The three additional residues in the large lobe of the kinase core, which are conserved throughout the superfamily have their role as general stabilizers of the structure. The Asp 220 forms hydrogen bonds to the backbone nitrogen atoms of Arg165 and Tyr164, and thereby stabilizes the catalytic loop, while Glu208 and Arg280 form a buried ion pair (Knighton et al, 1993).

The large lobe contains also the binding regions for binding of the peptide and protein substrates. Binding of peptide pseudosubstrate inhibitor TTYADFIAS**GRTGRRNA***IHD, where A* denotes the position for the phosphorylatable serine residue in the appropriate substrate peptide, is facilitated by contacts between consensus motif amino acid side chains, highlighted in bold in the sequence, and the binding site. The residues of the enzyme that are important for recognition of the basic specificity determinants in PKI (as well as other peptide substrates) are as follows:

- Glu170 and Glu230 for arginine in position -2 from the phosphorylatable serine (or A* as above),
- Asp328 and Glu127 for arginine in position -3 from the phosphorylatable serine (or A* as above),
- Glu203 and Tyr204 for position -6 arginine,
- Leu198, Pro202 and Leu205 form hydrophobic pocket accommodating the side chain of position +1 amino acid (Ile in the peptide above),
- Tyr235, Pro236 and Phe239 form another hydrophobic pocket for accommodation of the amino acid residue in position -11.

Activity of PKA is controlled besides the regulatory subunit association, based on interaction of the catalytic subunit with the with the pseudosubstrate type sequence RRNAI,

also by phosphorylation at the Thr 197 residue in the activation loop (Moore et al, 2002) and this absolutely essential for the enzyme activity.

3. Catalytic mechanism

The reactions catalyzed by protein kinases require both ATP and a substrate protein/peptide and thus are bisubstrate reactions. These reactions could follow random or ordered mechanism with respect to peptide and ATP. PKA has been assumed to follow predominantly random kinetic mechanism, if Kemptide (LRRASLG) is the peptide substrate.

Firstly, non-competitive inhibition was observed in the presence of serine peptide analogue, guanethidine, or ADP (Cook et al., 1982). These results indicate that ATP and Kemptide can bind to PKA independently as expected in a random kinetic mechanism.

Secondly, it was shown that the substrate peptide can bind prior to ATP, and γ -[³²P]ATP can bind prior Kemptide (Kong and Cook, 1988). These results are not possible unless ATP and Kemptide have unrestricted access to the active site, and the binding of one of these substrates does not exclude the binding of the other. On the other hand, it was also mentioned that PKA might have some preference for binding ATP prior to substrate. (Cook et al., 1982; Grant and Adams, 1996). It was also proposed that this sequential binding mechanism with ATP binding first might be followed also under physiological conditions (Adams et al, 1992). In the same study it was found that k_{cat} was very sensitive to viscosity, indicating that ADP release and not the chemical step is rate limiting.

Some years ago also the structure of the transition state for peptide phosphorylation reaction was discussed. Initially this discussion was based on QSAR analysis of the phosphorylation reaction of peptide substrates with modified structure around the hydroxyl group. This analysis has lead to the conclusion that the hydroxyl group seems to loose proton in the rate-limiting step of the catalytic process (Järv, 1996). Later the crystal structure of the transition state mimic was determined by using AlF_3 as model for the gamma-phosphorus atom of ATP (Madhusudan et al, 2002). This analysis provided evidence of the in-line mechanism of the phosphoryl transfer in the active center. Also, this mechanism is in agreement with the QSAR study, but in both cases the acceptor of hydroxyl group proton is not revealed.

4. Substrate specificity of PKA

Substrate specificity of protein kinases is determined on three structural levels. Firstly, protein kinases exhibit site-specificity, which means that they are able to recognize primary structure motifs around the phosphorylatable amino acid. This recognition is considered as obligatory for the following reaction step. Secondly, interactions between substrate and so-called “docking site”, apart of the primary binding site of the catalytic centre, may occur. Thirdly, anchoring protein(s) may support targeting of the kinase to its substrate. In the present study the primary specificity of PKA in reaction with protein substrates is analysed.

The basic ideas about the phosphorylation site specificity of PKA originate from seventies. For protein kinase A these studies started in seventies (Zetterqvist et al, 1976; Zetterqvist and Ragnarsson, 1982) and were based on peptides derived proceeding from the sequence of the phosphorylation site of L-type pyruvate kinase. It was found that the peptide sequence RRASV was still efficiently phosphorylated by PKA with K_m in the low micromolar range and the structure RRASV was denoted as “minimal substrate” for PKA, *i.e.* the shortest substrate structure containing the whole set of important specificity determinants for efficient phosphorylation by the protein kinase (Zetterqvist and Ragnarsson, 1982). The two arginines were postulated to be exclusive substrate specificity determinants for recognition by PKA since they could not be replaced even by lysine.

These studies resulted in definition of a minimal consensus sequence Arg-Arg-X-Ser/Thr-Y, for PKA, where X denoted any amino acid and Y is a big hydrophobic amino acid. Determination of the three-dimensional structures of PKA-pseudosubstrate complexes (Taylor et al, 1993; Tsigelny et al, 1996) is in agreement with the substrate recognition mechanism of PKA.

An attempt to rationalize specificity data for PKA was made by using QSAR (Järv and Ragnarsson, 1991). It was found that the specificity requirements of PKA could be quantitatively described by hydrophobicity, bulkiness and charge parameters. These studies also revealed that it is not possible to express the primary specificity of protein kinases by any defined and rigid consensus sequence, since it may have far more complex character. The same conclusion was achieved through statistical analysis of the known phosphorylation sites in proteins, which do not contain fixed consensus sequence (Kreegipuu et al, 1998). Therefore it is still not clear if recognition of protein substrates by PKA can be modeled by data obtained for short peptides.

5. Objectives of the present work

In the present work we directly addressed the question of PKA substrate specificity against protein substrates. For this reason kinetics of phosphorylation of protein substrates by cAMP-dependent protein kinase catalytic subunit (PKA) was studied under bimolecular conditions of the phosphorylation reaction. The protein substrates were obtained by site-directed mutagenesis around phosphorylation site of L-type pyruvate kinase (L-PK). This enzyme is one of the physiological substrates of PKA (Engström et al, 1982) and the peptide sequence around its phosphorylation site located at Ser-12 residue has been used for definition of the “minimum substrate” for PKA (Zetterqvist and Ragnarsson, 1982). Amino acids with basic, acidic, small and big hydrophobic and also with hydrophilic side chains were systematically introduced in the positions -3, -2 and +1 of the phosphorylation motif RRAS(12)V of L-PK and the purified mutated proteins were tested as substrates for PKA. Relative velocities of phosphorylation of these proteins were measured under the second-order rate conditions and the obtained data were compared with the appropriate kinetic data for peptide substrates.

MATERIALS AND METHODS

Chemicals - [γ -³²P]ATP was from Amersham Pharmacia Biotech. DE-52 ion-exchanger was from Whatman. Peptide substrate for protein kinase A, RRASVA was synthesized at the Department of Medical Biochemistry and Microbiology, Uppsala University. Hydroxyapatite, polyacrylamide/bis 29:1, low molecular weight reference proteins and Coomassie Brilliant Blue R-250 were obtained from BioRad. The restriction enzymes were from Amersham Pharmacia Biotech and Fermentas. The Ultima Taq polymerase was from Perkin Elmer. All other chemicals were of the highest purity commercially available.

Protein kinase A - The expression construct vector Cat-pRSETb for expression of catalytic subunit of protein kinase A was a generous gift from Dr. S.S. Taylor (La Jolla, California). The vector was transformed into *E.coli* strain BL21 (DE5) and the expression was induced at OD₆₀₀ between 0.55 and 0.80 by adding 0.5 mM final concentration of IPTG. The cells were grown additionally for 6 hours, harvested and the pellets were completely frozen in liquid nitrogen in small portions and stored then at -70°C. Partially purified catalytic subunit was obtained after a P-11 ion-exchanger step as described in (El-Maghrabi et al 1980) with the exception that instead of a linear gradient a stepwise elution with 250 mM potassium phosphate (pH 6.5) was used. The purity of eluted fractions and efficiency of chromatography were checked by SDS polyacrylamide electroforesis.

Generation of expression vector for L-PK - The cDNA of L-PK was a generous gift from Professor Noguchi, University of Tokyo, Japan. For production of L-PK in *E.coli* the cDNA of L-PK was cloned into the NdeI/EcoRI site of the polycloning region of a plasmid expression vector pRSETb (Invitrogen). The obtained vector construct was denoted as pRSETb-LPK (**Fig. 8**) and was about 4.5 kb large of which 2.9 kb was vector fragment and 1.6 kb the full length cDNA of rat L-PK. The NdeI site was inserted prior to the start codon and the EcoRI site was inserted adjacent to the stop codon by PCR using flanking primers containing the restriction site motifs.

Site-directed mutagenesis of L-PK - The mutations were introduced into the positions Arg-9, Arg-10 and Val-13 in the phosphorylation motif LRRAS¹²VA in L-PK. The corresponding mutants are later in the text denoted as mutants in position -3, -2 and +1, showing their

upstream or downstream positions from the phosphorylatable Ser-12. The mutagenesis was performed using PCR with complementary mutagenic oligonucleotide primers and the pRSETb-LPK construct as template. The sense and antisense primers had nine complementary nucleotides at both sides from the mutated codon and there were 1-3 mismatched nucleotides at the codon position depending on the desired mutation. The sequence of the sense and antisense primers for position -3 were GGATACCTTXXXCGTGCGAGT and ACTCGCACGXXXAAGGTATCC, respectively, for position -2, TACCTTCGAXXXGCGAGTCTG and CAGACTCGCXXXTCGAAGGTA, respectively, and for position +1, CGTGCGAGTXXGCTCAACTG and CAGTTGAGCXXACT CGCACG, respectively. The mutated codon is denoted as XXX. The other two primers with annealing positions at both sides of the polycloning site were denoted as forward primer I with a sequence GCTGCAGCTTCTGTGACACGTATTGCTGGCCTT TTGCTCACATGTTC and annealing site about 300 bp upstream from the start codon and reverse primer CTAGTTATTGCTCAGCGGTGGC, which corresponds to the reverse sequencing primer for pRSETb vector and has antisense annealing site at the other side of the polycloning region. The first PCR amplification reaction was performed using the forward I primer/antisense primer and sense primer/reverse primer in separate tubes. In the second PCR reaction the gel purified PCR fragments of the first PCR were mixed and used for generation of template for production of full length mutated cDNA of L-PK by the 10 min initial extending step at 72°C. At the end of this step the temperature was risen to 95°C and 2 µl of 25 µM forward primer II and the reverse primer were added and PCR cycles were proceeded for generation of mutated L-PK cDNA. The size of the obtained PCR product was around 2.1 kilobases and it was restricted with NdeI and EcoRI enzymes yielding a fragment of 1720 bases corresponding to the full length cDNA of L-PK. This fragment was ligated into the NdeI/EcoRI site of the pRSETb vector and the efficiency of mutagenesis was determined by sequencing.

Overexpression and purification of wt and mutated forms of L-PK - E.coli BL21(DE-3) cells were transformed with wt or mutated forms of vector construct pRSETb-LPK and 2ml overnight cultures were grown in LB medium containing 100 µg/ml ampicillin. From this culture an aliquot of 1/2000 (v/v) was added into 1 liter YT-medium (8 g of Tryptone, 5 g of yeast extract and 5 g of NaCl in 1 liter, pH 7.4, with 100 µg/ml ampicillin) in a 2 liter flask. The culture was grown at 37°C until the optical density at 600nm reached 0.55 to 0.8 and the

expression of L-PK was induced by addition of IPTG to a final concentration of 135 $\mu\text{g/l}$ (0.5 mM). Immediately after, the temperature of the incubator was set to 25°C accompanied with the opening of the incubator for faster temperature change. After 8-12 hours of shaking, the cells were harvested by centrifugation at 6000g for 11 min. The pellet was washed with PBS solution and centrifuged as above. In cases when the purification did not immediately follow the pellets were stored at -70°C . All purification steps were performed at 4°C. The overexpressed cells (about 5 grams) were thawed, if frozen, to room temperature, resuspended in 20 ml of homogenization buffer containing 250 mM sucrose, 20 mM potassium phosphate, 2mM EDTA, 1mM EGTA, 0.1 mM DTT, 0.1 mM PMSF, pH 7.2 and lysed with French Press at 2000-3000 p.s.i. twice. The cell suspension was centrifuged for 1 hour at 10000 g. The pH and conductivity of the supernatant were measured and adjusted to the same values as for buffer B (20 mM potassium phosphate/ 15% glycerol pH 7.2). The supernatant was applied to a system of three subsequently connected 5 ml DE-52 columns, equilibrated by buffer B and 10 column volumes of buffer B was used for wash. Each column was eluted with a 30-ml linear gradient combined of equal volumes of 20 mM and 175 mM potassium phosphate buffers, pH 7.2 containing 15% glycerol. Pyruvate kinase activity, conductivity and absorbance at 280 nm were measured for the fractions. Fractions containing pyruvate kinase activity were pooled and dialyzed overnight against 1 mM potassium phosphate, 30% glycerol, pH 7.0, and the material was applied onto a 5 ml OH-apatite column equilibrated with the same buffer. The column was washed with 10 columns volumes of equilibration buffer and the proteins were eluted with a 30-ml linear gradient combined of 1 mM and 80 mM potassium phosphate, also containing 50 mM KCl, 30% glycerol, and pH 7.0. The fractions containing pyruvate kinase activity were pooled and freezeed at -20°C . The purity of the pyruvate kinase was estimated using SDS-PAGE. The protein concentration was determined by the absorbance at 280 nm.

Pyruvate kinase activity assay – Kinase activity assay was measured spectrophotometrically and it based on the biochemical reaction which mediates in living cells. Pyruvate kinase activates the transfer from a phosphoryl group of phosphoenolpyruvate to ADP (**Fig. 3**), which results in ATP and pyruvate formation. Lactate dehydrogenase catalyses the reaction between pyruvate and NADH, which results in 1-lactate and NAD^+ formation. NADH has high molar extinction coefficient at 340 nm ($6.22 \cdot 10^6 \text{ cm}^2$) compared to NAD^+ and this difference is used for monitoring the enzyme reaction in time (**Fig. 4**).

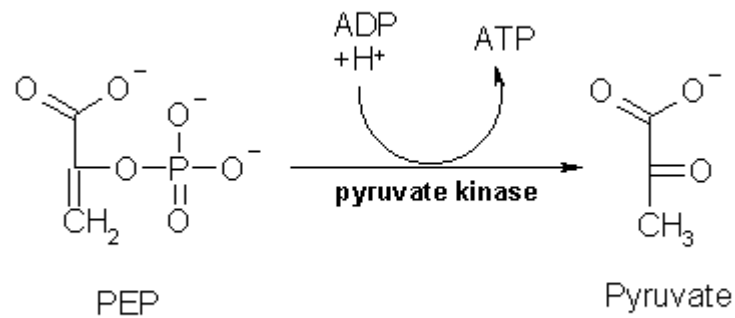


Figure 3. The last step of glycolysis converting phosphoenolpyruvate to ADP.

The assay medium, adjusted to pH 7.33, contained 5 mM potassium phosphate buffer, 0.1 mM dithiothreitol, 0.1% albumin, 100 mM KCl, 1 mM ADP, 5 mM MgCl₂, 0.15 mM NADH and 1.5 units/ml of lactate dehydrogenase. It was checked that the coupling enzyme was not rate limiting. The final concentration of phosphoenol pyruvate in the assays during the

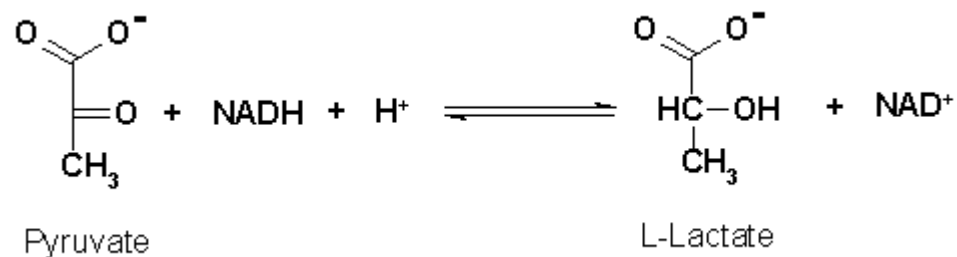


Figure 4. The reaction of pyruvate and NADH

purifications was 2 mM. In the kinetic determinations, the phosphoenolpyruvate concentration was varied from 0.05 – 5 mM. The initial velocity data were fit to the Hill equation:

$$\frac{v_0}{V_{\max}} = \frac{1}{1 + (K_{0.5}/[S])^n}$$

for determination of the apparent Michaelis constants ($K_{0.5}$), Hill coefficients (n) and maximal velocity (V_{\max}).

Phosphorylation assay of peptide substrate RRASVA, wild-type and mutated forms of pyruvate kinase by protein kinase A -Phosphorylation of peptide substrate RRASVA and pyruvate kinase forms was performed in an assay mixture consisting of 100 mM potassium

phosphate buffer (pH 7.5), 18% glycerol, different concentrations of pyruvate kinase, 100 μM [$\gamma\text{-}^{32}\text{P}$] ATP, 10 mM MgCl_2 and PKA. The reaction was performed at room temperature and it was initiated by addition of ATP. At different time points 20 μl aliquots were taken from the incubation mixture and spotted onto the pieces of phosphocellulose paper, which was subsequently immersed into the ice cold 75 mM phosphoric acid and then washed four times 10 minutes with the same acidic solution. The papers were then dried and the bound radioactivity was counted. The concentration of PKA was adjusted for each pyruvate kinase form so that the initial velocities of the reaction could be measured. The concentration of the phosphorylation sites in every pyruvate kinase form was estimated each time in parallel experiment for 100% phosphorylation by using excess of PKA and long incubation times. In these cases, after the incubation, three aliquots were taken at 5-10 minutes intervals to ensure that the phosphorylation process was complete. For peptide substrate RRASVA the K_m , V_{\max} and apparent second order rate constant V_{\max}/K_m (further denoted as k_{II}) were calculated from the ordinary hyperbolic Michaleis-Menten equation:

$$v_0 = \frac{V_{\max} [S]}{(K_m + [S])}$$

Since in the varied concentration range, each of the pyruvate kinase forms showed linear relationship of initial velocity vs pyruvate concentration, showing that the highest concentration used was still under the K_m value, the apparent second order rate constants k_{II} were calculated from the linear slopes of these relationships:

$$v_0 = \frac{V_{\max}}{K_m} [S] = k_{II} [S]$$

where v_0 is initial velocity and $[S]$ the concentration of available phosphorylation sites in pyruvate kinase.

RESULTS

1. Expression and purification of wild-type and mutated forms of L-type pyruvate kinase in *E.coli*

In this work 18 pyruvate kinase mutants were constructed using the PCR-based method of site-directed mutagenesis described in the experimental section. The overexpression levels of different mutants in *E.coli* varied relatively much and the ratio of the expressed protein in soluble vs insoluble fraction of the bacterial culture homogenate was individual for each mutant. Only in one case, for mutant Phe-2, there was no desired protein found in soluble fraction, while commonly it was found in the both soluble and membrane fractions. In few cases the majority appeared in the soluble fraction. Except for the mutant Phe-2, all the mutants were expressed at reasonably high levels in the soluble fraction, in the range of 2-5 mg per 1 liter culture media, being sufficient for phosphorylation studies after the purification.

The purification protocol was initially designed for purification of the wild-type pyruvate kinase but it worked equally well for most of the mutants. In the first step of the purification three subsequently connected DE-52 columns were used. This proved to be useful as the bulk of the low pI proteins were saturating the first DE-52 column while the pyruvate kinase was migrating to the second and mainly into the last column. The combined eluted material from the second and last column contained the wt pyruvate kinase at an 80-90% of purity and covered at least 70% of the whole pyruvate kinase amount loaded. Such effect was achieved by keeping the individual column size low, with capacity less than the protein pool to be bound, and the ionic strength of loading just under the eluting conditions of pyruvate kinase (around 40 mM potassium phosphate).

As for the wt pyruvate kinase, the mutants Ala-3, Gln-3, Leu-3, Glu-3, Lys-3, Ala-2, Leu-2, Glu-2, Gln-2, Phe-3, Ala+1, Arg+1, Glu+1 and Phe+1 were bound to the second and third columns of the triplicate column system. All of these mutants except Gln-2 were found to be more abundant in the elution of the third column compared to the second one. The larger fraction of Gln-2 was found in the second column. Three mutants Lys-2, Lys+1 and Leu+1 did not bind to the DE-52 column system and most of their activity was found in the wash. The wash of Lys-2 and Leu+1 was reloaded to a new 10 ml of DE-52 column equilibrated with lower ionic strength and higher pH buffer (5mM potassium phosphate/30% glycerol pH 7.5). In these conditions, the mutated proteins were bound to the DE-52 column and after the elution the purification was continued as usual. The mutant Lys+1 was found to be already in

a relatively purified state in the non-bound wash fraction from DE-52. The wash was concentrated and dialyzed against the equilibration buffer of the OH-apatite column and thereafter applied to the OH-apatite column.

The hydroxyapatite step was successful for all mutants except Lys-3 but the flow through and wash fractions were concentrated and found to be more than 95% pure. The purity of the other purified proteins was higher than 98% in most cases as estimated visually from SDS-PAGE, where the L-PK appears at position corresponding to 56 kD exemplified in Fig.5 together with the wt enzyme purified from rat liver according to (Humble et al, 1975).

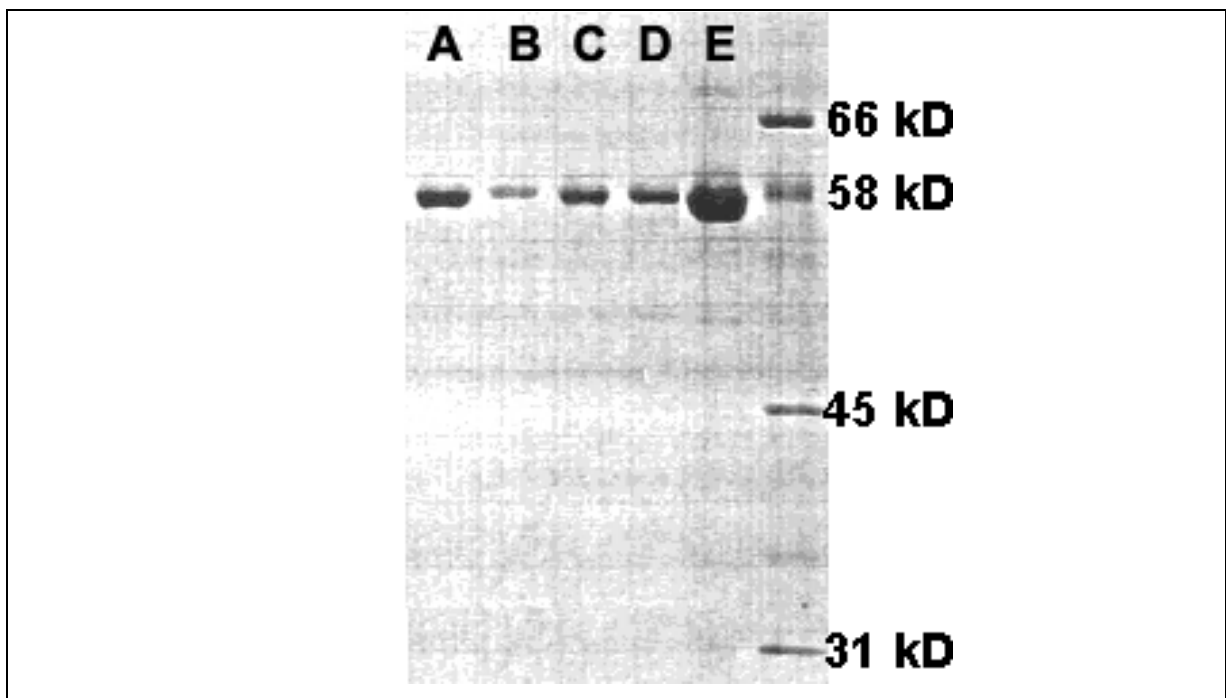


Figure 5.

SDS-PAGE gel showing purified recombinant L-type pyruvate kinase (L-PK, lane A), mutated forms of L-PK with Ala in positions 9, 10 and 13 (lanes B, C and D) and L-PK isolated from rat liver (lane E). The right lane shows molecular weight markers (BSA, bovine liver catalase, ovalbumin and carbonic anhydrase).

2. Comparison of kinetic properties of recombinant L-PK and L-PK purified from rat liver

For kinetic characterization of the recombinant wild-type L-PK the initial velocities were measured over the range of 0.5 to 5 mM phosphoenol pyruvate concentrations and the

obtained results were compared with the same data obtained in parallel experiments for L-PK purified from rat liver. Additionally, the phosphorylated forms of both pyruvate kinase forms were produced by incubation of the enzymes in the presence of protein kinase A and MgATP for a time period that was sufficient for phosphorylation to the maximal extent, and the kinetic properties of the obtained forms were studied. Both the non-phosphorylated and phosphorylated enzymes showed sigmoidal kinetic pattern in respect to phosphoenolpyruvate (Fig. 6).

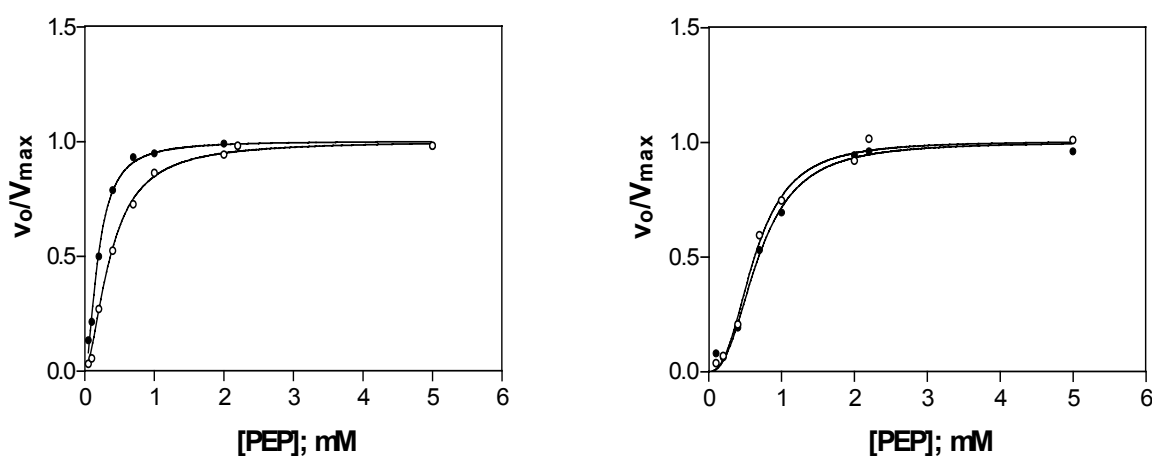


Figure 6

Kinetic comparison of recombinant L-PK and L-PK isolated from rat liver in non-phosphorylated (left) and phosphorylated (right) states. Open and filled circles represent L-PK from rat liver and recombinant L-PK respectively. The assay conditions were as described in Materials and Methods.

In the case of the non-phosphorylated forms, nearly a twofold difference in affinity towards phosphoenolpyruvate was observed, the corresponding in $K_{0.5}$ values being 0.20 and 0.37 mM for recombinant and rat enzyme respectively. For the phosphorylated kinase forms the $K_{0.5}$ values for phosphoenolpyruvate were found to be rather close for both enzymes, with values of 0.69 and 0.64 mM for recombinant and rat enzyme, respectively (Fig. 3 right). The specific activity of recombinant enzyme was estimated to be 545 units per mg and for the rat liver enzyme 409 units per mg. The values in relatively good agreement with previously reported value of 366 units/mg reported in (Ekman et al, 1976).

3. Phosphorylation of peptide substrate RRASVA, recombinant L-PK and rat liver L-PK by protein kinase A

The recombinant L-PK, rat L-PK and a peptide substrate RRASVA, based on the phosphorylation site of L-PK were tested as substrates of PKA. For RRASVA both K_m and V values could be measured. For pyruvate kinases the k_{II} was determined from the slope of the initial velocity vs substrate concentration relationship. The values of the kinetic constants are presented in Table 1. Both pyruvate kinase forms showed lower phosphorylation rate than the short peptide substrate. The recombinant L-PK had slightly higher value of k_{II} than rat L-PK. The mole per mole phosphorylation experiment revealed 64.5 % phosphate incorporated per mole of rat L-PK subunit and the corresponding value for recombinant L-PK was close to 100%.

Table 1. Kinetic parameters for protein kinase A catalyzed phosphorylation of peptide substrate RRASVA, recombinant L-PK and L-PK purified from rat liver.

Substrate	K_M μM	V_{MAX} $\mu mole/min/mg$	k_{II} $l/min/mg$
RRASVA	21.2±3.2	7.80±0.49	0.368
Recombinant L-PK	Nd	nd	0.158 ± 0.003
Purified L-PK	Nd	nd	0.126 ± 0.005

4. Phosphorylation of mutated forms of L-PK by protein kinase A

The k_{II} values were estimated for all purified mutant forms of pyruvate kinase and are presented in Table 2 in a scale normalized relatively to the wt sequence. In both positions –2 and –3 the k_{II} values of the arginines were nearly two orders of magnitude higher than other varied amino acids, except lysine. Lysine had been stronger specificity determinant in position –2 than in –3. In position +1 the hydrophobic amino acids were preferred. Phe showed the strongest effect in this position showing even higher k_{II} value than the wt Val. Lower end values for varied set in this position were obtained for charged amino acid Arg, and Glu, and Lys.

Table 2. Normalized second-order rate constants k_{II}^{norm} for phosphorylation of mutated forms of L-type pyruvate kinase by catalytic subunit of the cAMP-dependent protein kinase. In each mutant protein one amino acid was replaced in position 9, 10 or 13 of the L-type pyruvate kinase primary structure. Normalization of the constants was made relatively the phosphorylation rate of the wild type L-type pyruvate kinase with Arg residues in positions 9 and 10 and Val in position 13 of the protein sequence, $k_{II}^{norm} = k_{II}^{app(mutant)} / k_{II}^{app(wt)}$.

Amino acid	k_{II}^{norm}		
	Position 9	Position 10	Position 13
Ala	0.013±0.004	0.011±0.004	0.18±0.05
Arg	1	1	0.07±0.03
Gln	0.04±0.01	0.013±0.004	not determined
Glu	0.011±0.003	0.010±0.004	0.049±0.015
Leu	0.012±0.002	0.015±0.004	0.47±0.09
Lys	0.07±0.02	0.26±0.08	0.16±0.04
Phe	0.021±0.007	not determined	1.6±0.4
Val	not determined	not determined	1

DISCUSSION

L-type pyruvate kinase has served as one of the first model systems for an enzyme regulated by phosphorylation (Ekman et al, 1976; El-Magarabi et al, 1980). Phosphorylation of the serine-12 residue by protein kinase A lowers the affinity of pyruvate kinase towards its substrate, phosphoenolpyruvate and thereby down regulates the rate of the last step of glycolysis. In the present work, for the first time, the recombinant form of L-type pyruvate kinase was produced by overexpression in *E.coli*. The recombinant kinase was successfully purified and its kinetic behaviour was characterised by determination of initial velocities at varied phosphoenol pyruvate concentrations. The recombinant form of L-PK was compared with the form purified from rat liver, with respect to this kinetic property. Nearly twofold difference in $K_{0.5}$ values between the non-phosphorylated forms of the enzymes was observed, but the two enzymes were similar in their phosphorylated state. An explanation of this finding could be attributed to the fact that L-PK purified from liver contains a certain amount of covalently attached phosphate in its Ser-12 phosphorylation site. This fact has been reported previously in study of by El-Maghrabi et al (1980) where it was shown that L-PK isolated from rat liver might contain about 0.5 mole of phosphate per mole of pyruvate kinase. The partially phosphorylated state of rat L-PK was confirmed also indirectly by the fact that the maximal phosphorylation state obtained after phosphorylation by PKA was 0.65 mole of radioactive phosphate per mole of freshly prepared enzyme. The same value for recombinant L-PK was close to hundred percent.

Moreover, the depth of phosphorylation 0.6 moles of phosphorus per mole protein has been found also in this study if rat L-PK, purified from rat liver by the same protocol as described by Ljungstrom et al (1974), was used. Proceeding from these facts, the shift of the initial velocity vs phosphoenolpyruvate curve, in case of rat L-PK, can be easily understood.

The phosphorylation rate of the PKA catalysed reaction for the rat L-PK was also slightly lower than that of recombinant L-PK, which is in agreement with the findings of El-Maghrabi et al (1980) showing that the higher the phosphorylation state of L-PK is, the lower the phosphorylation rate will be. Such cooperative feedback regulation of phosphorylation rate possible avoids full phosphorylation of L-PK under physiological conditions, keeping the ratio of phosphorylated and non-phosphorylated states around unity, where the phosphorylation gives the highest effect on L-PK activity.

The other characteristics studied, the molecular weight and specific activity, were similar for rat L-PK and recombinant L-PK forms, confirming that the novel recombinant

pyruvate kinase and the analogously produced mutated forms are reliable model system for studying the structural requirements of protein kinase A and L-PK mutual recognition factors. Finally, the recombinant L-PK preparation, being a homogenous pool of non-phosphorylated enzyme, could be a good model for kinetic studies in general, since the available kinetic data on L-PK allosteric regulation are collected from rat L-PK, which is most likely a mixture of differently phosphorylated forms of the enzyme and thus is not a well defined system.

Study on the phosphorylation of mutated forms of pyruvate kinase confirmed the defined consensus sequence for PKA substrates, RRASB. The shape of the obtained specificity profile for positions -3, -2 and +1 was similar with the ones obtained from the peptide library studies reported previously (Songyang et al 1994). For the position +1, the structural alterations in L-PK caused sharper effects that obtained from the library study but the overall specificity profile, preferring the large hydrophobic amino acids in this position was found to be similar in the two studies. It can be concluded that the same specificity determinants obtained in the studies with synthetic peptide substrate models are valid also for recognition of protein substrate L-PK by PKA.

For comparative analysis, the logarithms of normalized k_{II} values for mutants and for corresponding amino acid alterations in peptide substrates, available in literature, were plotted in Fig 7. These pairs of substrates include:

- alanine and lysine alterations in both -2 and -3 positions in peptide substrates corresponding to the phosphorylation site of bovine L-PK,
- the version of “Kemptide” LRRASLG (Kemp et al, 1977),
- the alanine substitutions in positions -2 and -3 in peptide LRRNSI (Adams and Taylor, 1993),
- lysine substitutions in positions -2 and -3 in peptide GRTGRRNSI (Mitchell et al, 1995), derived from the structure of peptide inhibitor PKI,
- alterations in +1 position in L-PK analog RRASV [12] and on a myeline basic protein based peptide substrate KRPSXREKA (Zetterqvist et al, 1976).

Linear regression analysis of the relationship between the two data sets revealed correlation with R^2 value of 0.901 and a regression line slope of 0.59 (**Fig 4**). This linear relationship, with a slope different from 1, shows that amino acid alterations in peptides cause stronger effects on phosphorylation rate than the corresponding alterations in protein substrate. But the overall sequence of amino acid preferences remained the same in the case of both peptide and protein substrates.

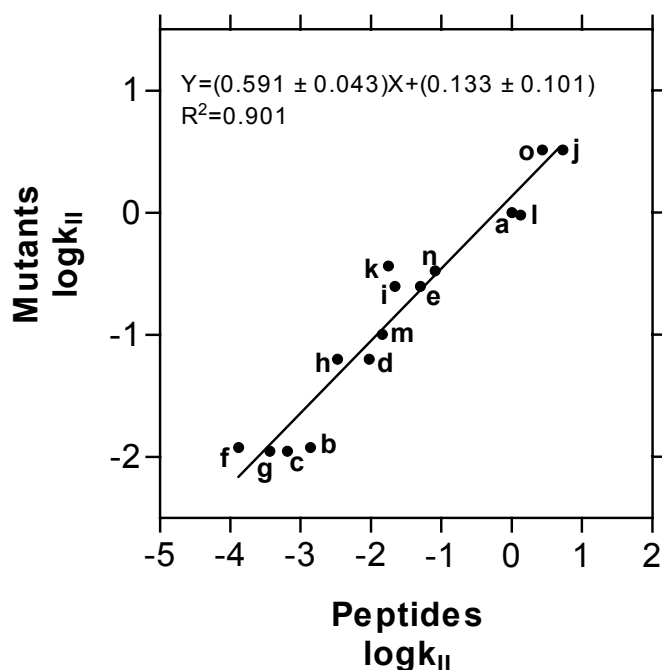


Figure 7.

Comparison of amino acid substitution effects on the phosphorylation rates of protein kinase A peptide substrates and mutated forms of L-PK. The k_{II} values for the peptide substrates were obtained from literature (see references in text) and normalized relatively to one peptide of every used peptide set – RRASVA, LRRASLG, GRTGRRNSI, RRASV, myelin basic protein based peptide substrate analog KRPSVRAKA - containing the amino acid corresponding to the amino acid in L-PK in the varied position. Together with the wild type L-PK, these peptides had $\log k_{II}$ values equal to zero and they are indicated on the figure by letter a. The other letters correspond to the following structurally varied peptides of different peptide sets: LARASLG - b, LRAASLG - c, LKRASLG - d, LRKASLG - e, LARNISI - f, LRANSI - g, RRASF - j, KRPSARAKA - k, KRPSLRAKA - l, KRPSERAKA - m, KRPSKRAKA - n, KRPSFRAKA - o and for the corresponding L-PK mutants with the same substitutions.

This finding indicates that the consensus sequence, at least for PKA, is not as strict requirement as would have been predicted from the peptide models. Additionally, taking into account the data from the statistical analysis (Kreegipuu et al, 1998) where it was shown that only about 69% of protein substrates of PKA have Arg residue in position -3 and only 59% of

PKA protein substrates contain Arg in position -2, although this is required by the consensus sequence RRXSX for this kinase. It can be suggested that other structural factors, possibly nonspecific interactions between the surfaces of two encountering proteins, play important role in protein phosphorylation events. Thus, the absence of critical consensus motif can possibly be compensated by these additional interactions.

In summary, although the similar specificity pattern (consensus motif) holds for “primary” specificity of PKA against protein and peptide substrates, the specificity factors play somewhat weaker role in the former case and the rules used by this enzyme for recognition of the phosphorylation sites in proteins seem to be “looser” if compared with short peptides.

SUMMARY

Specificity of protein kinase A was studied using site-directed mutagenesis in the phosphorylation site of a protein substrate, L-type pyruvate kinase, and the obtained data were compared with the data from the previous studies on structurally altered peptide substrates. Amino acids with basic, acidic, small and big hydrophobic and also with hydrophilic side chains were introduced by mutagenesis into each of the three important positions of consensus sequence motif for of protein kinase A, $R_3R_2XSV_{+1}$. These mutants of L-type pyruvate kinase were tested as substrates for PKA and the comparison of the obtained kinetic data with the available data on similar structural alterations in peptide libraries and separately tested peptide substrates revealed that amino acid alterations in peptides caused stronger effects on the phosphorylation rate than the corresponding alterations in protein substrate L-PK, while the overall sequence of amino acid preferences remained largely the same in the case of both peptide and protein substrates. This finding indicates that the consensus sequence, at least for PKA, is not as strict requirement as would have been predicted from the peptide models and other structural factors, and possibly nonspecific interactions between the surfaces of two encountering proteins, play important role in protein phosphorylation events. Thus, the absence of critical consensus motif in protein substrates can in some extent be compensated by the additional interactions and it may be questioned if the importance of a consensus motif defined on the basis of peptide studies is not exaggerated.

KOKKUVÕTE

Nikita Oskolkov. Proteiinkinaas A substraatspetsiifilisus reaktsioonil valksubstraatidega. Magistritöö. Tartu Ülikooli orgaanilise ja bioorgaanilise keemia instituut

Proteiinkinaas A spetsiifilisust uuriti proteiinsubstraate kasutades. Need substraadid saadi lähtudes L-tüüpi püruvaadi kinaasist koht-suunatud mutageneesi teel. Substraatide seeria saamiseks kasutati aluselisi, happelisi, hüdrofoobseid ja hüdrofiilseid aminohappeid, mis viidi püruvaadi kinaasi fosforüleerimise tsentrisse seriin 12 ümbruses. Vastavalt ettekujutustele proteiinkinaas A konsensusjärjestuse motiivist tehti asendused substraativalgu peptiidijärjestuses $R_{-3}R_{-2}XSV_{+1}$ positsioonides -3 , -2 ja $+1$. Kokku konstrueeriti 18 mutantvalku, mis puhastati ning mida testiti PKA substraatidena. Nende valkude jaoks mõõdetud fosforüleerimisreaktsiooni kineetilisi andmeid võrreldi varasematest uuringutest saadud andmetega peptiidsubstratide jaoks. See analüüs näitas, et aminohapete muutused peptiidides põhjustasid tugevamaid efekte fosforüleerimise reaktsiooni kiirusele kui sarnased muudatused proteiinsubstraadis. Samal ajal jäi aminohapete eelistuse järjestus peptiidide ja proteiinsubstraatide korral samaks. See tulemus näitab, et peptiidsubstraatide ja valksubstraatide suhtes on proteiinkinaas A spetsiifilisus põhimõtteliselt sarnane, kuid tundlikkus spetsiifilisust määravate struktuurifaktorite ning üldiselt ka konsensusjärjestuse suhtes on suurem esimesel juhul. Võimalik, et valksubstraatide molekulaarse äratundmise korral mängivad olulist rolli ka mittespetsiifilised interaktsioonid kahe kokkupuutuva valgumolekuli vahel. Seega võib kriitilise konsensusmotiivi puudumist proteiinsubstraatides mõningas ulatuses kompenseerida täiendavate interaktsioonidega.

ACKNOWLEDGMENTS

I wish to express my greatest gratitude to my supervisor Jaak Järv for his excellent and professional guidance. Being his student was a good and valuable experience.

Particularly I would like to extend my gratitude to:

Mart Loog for providing good ideas and,
Pia Ek for getting excellent practical skills.

Everybody in my group and around me: Åsa Haglund, Aleksei Kuznetsov and other people from the Institute of Organic and Bioorganic Chemistry, Tartu University, Estonia and Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden for providing the pleasant working atmosphere and their help.

Tartu University for giving a solid basis of knowledge and practical experience for further studies and work.

I deeply appreciate the support of my parents and my closest friends.

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APPENDIX

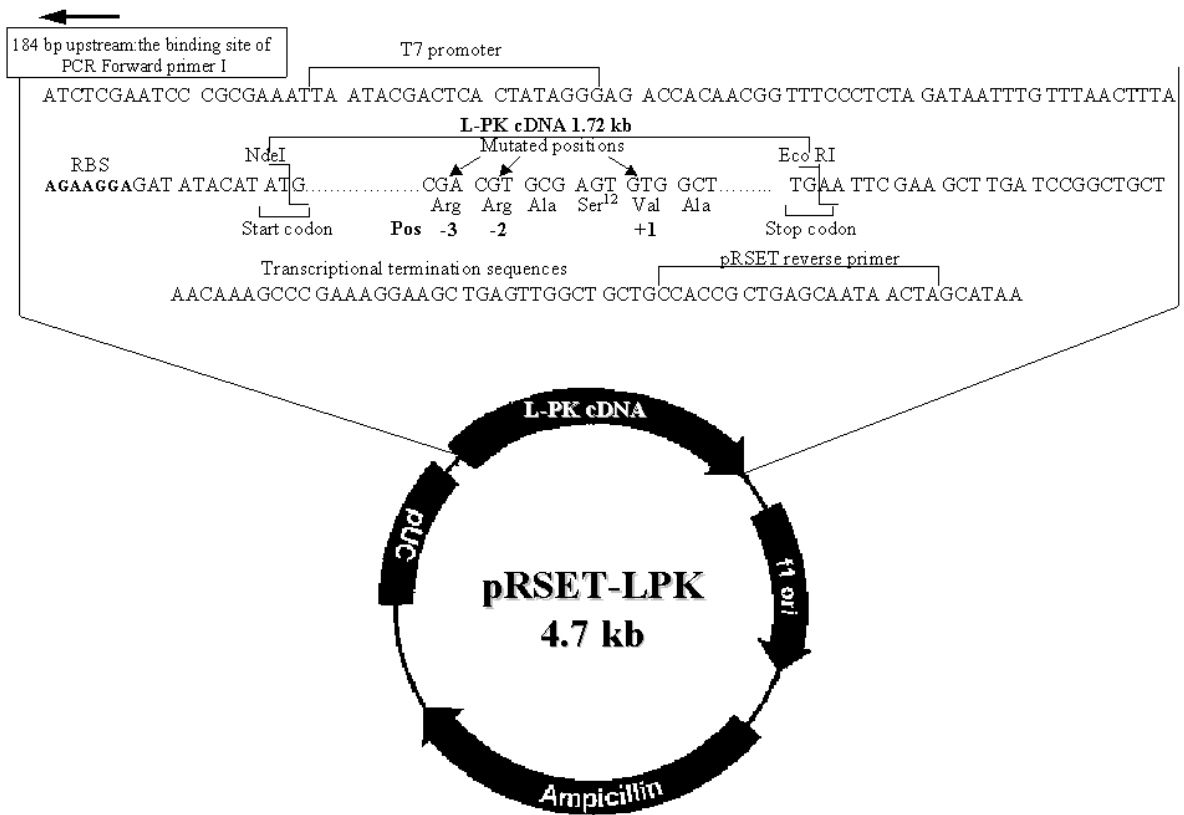


Figure 8:

The pRSET-LPK expression vector construct