DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
81

DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
81

ALEKSEI KUZNETSOV

Allosteric effects in reactions catalyzed by the cAMP-dependent protein kinase catalytic subunit

TARTU UNIVERSITY
PRESS

Institute of Chemistry, Faculty of Science and Technology, University of Tartu, Estonia

Dissertation is accepted for the commencement of the Degree of Doctor of Philosophy in Bioorganic Chemistry on March 6th, 2009 by the Doctoral Committee of the Institute of Chemistry, University of Tartu.

Supervisor: Professor Jaak Järv (DSc)

Opponents: Associate Professor Per Jemth (PhD), Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden

Senior Research Scientist Vello Tõugu (PhD), Tallinn University of Technology, Department of Gene Technology, Tallinn, Estonia

Commencement: April 28th 2009 at 15:00
18 Ülikooli Str., room 204

Publication of this dissertation is granted by University of Tartu

ISSN 1406–0299
ISBN 978–9949–19–088–1 (trükis)
ISBN 978–9949–19–089–8 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimuse nr 83

CONTENTS

2

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers:

I **Kuznetsov, A.** and Järv, J (2009) Ligand structure controlled allostery in protein kinase A. *Central European Journal of Biology*, in press. doi: 10.2478/s11535-009-0012-6

II **Kuznetsov, A.** and Järv, J. (2008) Single-subunit allostery in kinetics of peptide phosphorylation by protein kinase A. *Proceedings of the Estonian Academy of Sciences*, ***57***, 247–254. doi: 10.3176/proc.2008.4.07

III **Kuznetsov, A.** and Järv, J. (2008) Allosteric Cooperativity in Inhibition of Protein Kinase a Catalytic Subunit. *The Open Enzyme Inhibition Journal*, ***1***, 42–47. doi: 10.2174/1874940200801010042

IV **Kuznetsov, A.**, Uri, A., Raidaru, G. and Järv, J. (2004) Kinetic analysis of inhibition of cAMP-dependent protein kinase catalytic subunit by the peptide-nucleoside conjugate AdcAhxArg(6). *Bioorganic Chemistry*, ***32***, 527–535. doi: 10.1016/j.bioorg.2004.05.004

V **Kuznetsov, A.**, Väärtnõu-Järv, H. and Järv, J. (2003) Kinetic model for protein kinase simultaneous interaction with peptide, ATP and bifunctional inhibitor. *Proceedings of the Estonian Academy of Sciences. Chemistry*, ***52***, 178–187.

Author's contribution

Paper I: The author has contributed in data processing and analysis and participated in preparation of the manuscript.

Paper II: The author planned and performed the experimental work, made data processing and participated in preparation of the manuscript.

Paper III: The author planned and performed the experimental work, made data processing and participated in preparation of the manuscript.

Paper IV: The author planned and performed kinetic experiments, made data analysis and participated in preparation of the manuscript.

Paper V: The author participated in development of the kinetic model, performed theoretical calculations and participated in preparation of the manuscript.

ABBREVIATIONS

α, β, γ, χ	interacting factors
A	ATP substrate
ADC	adenosine-5'-carboxylic acid, 1-(6-amino-9*H*-purin-9-yl)-1-deoxy-β-D-ribo-furan uronic acid (CAS 3415–0906)
ADP	adenosine-5'-diphosphate
Ahx	6-aminohexanoic acid
AMPPNP	5'-adenylylimido-diphosphate, non-hydrolyzable nucleotide analogue (CAS 72957-42-7)
ATP	adenosine-5'-triphosphate
cAMP	cyclic adenosine-3',5'-monophosphate
B	peptide substrate
BSA	bovine serum albumin
C	catalytic subunit of cAMP-dependent protein kinase
C-lobe	C-terminal lobe
CDPK-1	calcium-dependent protein kinase 1
E. coli	Escherichia coli
H7	1-(5-isoquinolinesulfonyl)-2-methylpiperazine (CAS 84477-87-2)
H8	*N*-{2-(methylamino)ethyl}-5-isoquinolinesulfonamide (CAS 84478-11-5)
H89	*N*-{2-[(*p*-bromocinnamyl)amino]ethyl}-5-isoquinoline-sulfonamide (CAS 127243-58-0)
HPLC	high performance liquid chromatography
I	inhibitor ligand
K_a, K_b, K_i, K_{L1}, K_{L2}	dissociation constants
k_{cat}	catalytic rate constant
k_{II}	second-order rate constant of the enzymatic reaction
K_m	Michaelis constant
KT5720	(8R, 9S, 11S)-(-)-9-hydroxy-9-hexoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo[a,g]cyclo-octa[cde]trinden-1-one (CAS 108068-98-0)
L_1	peptide ligand (substrate or inhibitor)
L_2	ATP and ATP analogs
LFE	linear free-energy relationship

MS	mass spectroscopy
Mw	molecular weight
N-lobe	N-terminal lobe
NMR	nuclear magnetic resonance
P-loop	conserved glycine-rich sequence motif
PDK1	3-phosphoinositide-dependent protein kinase
PKA	protein kinase A, cAMP-dependent protein kinase catalytic subunit
PKI	heat-stable protein kinase inhibitor
PrKX	human X chromosome encoded protein kinase
PrKY	Y homologue of the protein kinase PrKX
TRIS	tris(hydroxymethyl)-aminomethane
V	maximum velocity of the enzymatic reaction
v	initial rate of the enzymatic reaction

INTRODUCTION

Cooperation between structurally and/or functionally distinct parts of living systems is a fundamental phenomenon, which can be observed on the level of single biomolecules and whole ecosystems. In biochemistry the term "cooperativity" was initially formulated to describe behavior of multimeric proteins, where ligand binding at one subunit affects binding of the same ligand with other subunits (Koshland *et al.*, 1966, Monod *et al.*, 1965). Later, the term "allosteric cooperativity" was introduced to describe situation, where substrate binding is controlled by binding of some structurally distinct "effector" molecule. The idea about different shapes of these ligands was emphasized by the prefix "*allo*" that means "different" in Latin (Cui and Karplus, 2008, Koshland and Hamadani, 2002). Today, however, these terms are gradually replaced by a new word "allostery", stressing shift of the focus from ligand properties to properties of proteins, more precisely to feedback between binding properties of distinct binding sites. This feedback can be specified as indirect interaction between remotely bound ligands, mediated through the protein structure via network of interacting amino acids (Cui and Karplus, 2008, Gunasekaran *et al.*, 2004, Liang *et al.*, 2007, Tsai *et al.*, 2008). Following this definition, monomeric bisubstrate enzymes seem to be the simplest proteins, which could reveal allostery.

Proceeding from this widened definition of allostery, kinetic behavior and ligand binding properties of cAMP-dependent protein kinase catalytic subunit (EC 2.7.11.11) (ExPASy, *www.expasy.org/enzyme/2.7.11.11*), further denoted as PKA, were analyzed in this work. This protein is a highly dynamic monomeric enzyme, which transfers the γ-phosphate group from ATP to the phosphorylatable residue of protein or peptide substrates. For the in-line phosphoryl transfer reaction, ATP and peptide should simultaneously interact with the enzyme and form ternary complex that is an important prerequisite for manifestation of allostery. And finally, interaction of various substrates and inhibitors with this enzyme can be studied kinetically that allowed quantification of the allosteric effects in catalysis and opened good possibility for investigation into the dependence of this effect upon ligand structure. This analysis has provided unique information about the ligand structure induced inversion of allostery that might have implication for deeper understanding of several aspects of enzyme catalysis in general.

cAMP-DEPENDENT PROTEIN KINASE

Regulatory phosphorylation and protein kinases

Protein kinases catalyze the transfer of the γ-phosphoryl group of ATP to serine, threonine or tyrosine residues of proteins and this chemical modification regulates their various physiological functions, including catalytic properties of enzymes. Through this process, named as "regulatory phosphorylation", protein kinases play the key role in control of multiple signaling and metabolic pathways in living cell. It has been estimated that as much as half of all cellular proteins undergo regulatory phosphorylation *in vivo* (Pinna and Ruzzene, 1996, Williams and Cole, 2001). Therefore, this is probably the most important mechanism of regulation in eukaryotic cells. The phenomenon of protein phosphorylation was first discussed as a regulatory mechanism in 1955 by Krebs and Fischer (1955), who were awarded Nobel Prize for this discovery in 1992.

It has been shown that approximately 2% of genes may encode the protein kinase domains in eukaryotic genome and the occurrence of more than 500 different protein kinases could be predicted based on the human genome sequence (Hunter, 2000, Rubin *et al.*, 2000). It is generally accepted that there are many potential drug targets among these enzymes (Blume-Jensen and Hunter, 2001, Sridhar *et al.*, 2000), as trough regulation of their activity variety of cellular events can be controlled. This has caused extremely high interest of biochemists and pharma people in structure, functions and specificity of this class of enzymes, targeting in design of compounds that allow efficient and selective way of regulation of their activity.

Following the Hanks Classification (The Protein Kinase Resource, *www0.nih.go.jp/mirror/Kinases*), cAMP-dependent protein kinase (E.C.2.7.11.11) belongs to the AGC Group III of these enzymes (Hanks and Hunter, 1995, Manning *et al.*, 2002). For several reasons cAMP-dependent protein kinase has remained until today the best-characterized serine/threonine kinase (Akamine *et al.*, 2003), and it is often considered a "model" enzyme of the protein kinase superfamily (Seifert *et al.*, 2002, Taylor, 1989, Breitenlechner *et al.*, 2004). However, not only this fact is stressing importance of this enzyme for regulatory phosphorylation studies. Undoubtedly, this enzyme is ubiquitous for many physiological functions, including cell division and differentiation, building of cell morphology, apoptosis, glycolysis, and neuronal plasticity (Hanks and Hunter, 1995).

cAMP-dependent protein kinase is presented in cells as inactive tetramer consisting of two regulatory (R) and two catalytic (C) subunits and it is activated in response to increased level of the second messenger cAMP. Four cAMP molecules bind to the regulatory subunits of the holoenzyme and cause dissociation of the catalytic subunits, which are responsible for transfer of the γ-phosphoryl group of ATP to serine or threonine residues in substrate protein.

The possibility of separation of regulatory and catalytic subunits is a unique property of cAMP-dependent protein kinase, as in all other protein kinases the regulatory and catalytic parts are integrated into the same protein molecule.

$$R_2C_2 + 4\ cAMP \rightarrow R_2(cAMP)_4 + 2\ C \quad (1)$$

The catalytic subunit C is a monomeric water-soluble protein with Mw of 40,800 Da (Shoji *et al.*, 1983). This catalytically active protein, which is further denoted as PKA in this work, can be overexpressed and purified by the conventional methods of preparative biochemistry (Slice and Taylor, 1989, Yonemoto *et al.*, 1991) and therefore can be conveniently used for kinetic and binding studies *in vitro*. This approach allows also experiments with distinct isozymes of the catalytic subunit that is important for explicity of experimental data.

In mammalian tissues, several isozymes of PKA have been found. Initially Cα and Cβ forms of the catalytic subunit have been identified and their amino acid sequences differ approximately 7%. While the Cβ is a tissue specific isozyme, Cα is expressed in most cells and is the predominant isozyme (Chrivia *et al.*, 1988, Doskeland *et al.*, 1993, Shuntoh *et al.*, 1992). Following these discoveries the third form Cγ has been cloned (Beebe *et al.*, 1992, Gamm *et al.*, 1996), and thereafter also the PrKX and PrKY isozymes were described (Schiebel *et al.*, 1997). All these isozymes differ in their expression level, tissue distribution, substrate selectivity and functionality. But all these proteins share conserved core structure that approximately extends from residue 40 to residue 285 of their primary structure (Hanks *et al.*, 1988, Taylor *et al.*, 1992).

Structure of PKA

In the case of PKA the X-ray structures have been determined for the free enzyme (Knighton *et al.*, 1991a), for the enzyme complexes with peptide (Knighton *et al.*, 1991b) and with low-molecular ATP-analog inhibitor (Engh *et al.*, 1996). The first X-ray structure of ternary complex, containing PKA, ATP (more precisely ATP complex with magnesium ions) and the pseudosubstrate peptide inhibitor PKI[5–24] was defined by Knighton *et al.* (1991b). All these studies have revealed that PKA is comprised of a bilobal core, where smaller N-terminal lobe (N-lobe) and larger C-terminal lobe (C-lobe) can be clearly identified (Fig. 1). These structural elements can be observed also in the case of other protein kinases and are highly conserved in the case of PKA isozymes. The N-lobe is composed of five-stranded β-sheet and one α-helix and in apoenzyme this lobe is folded back to form the open conformation of the enzyme molecule. Most of the amino acid residues, which are considered essential for binding of ATP, are located within this domain and also in the

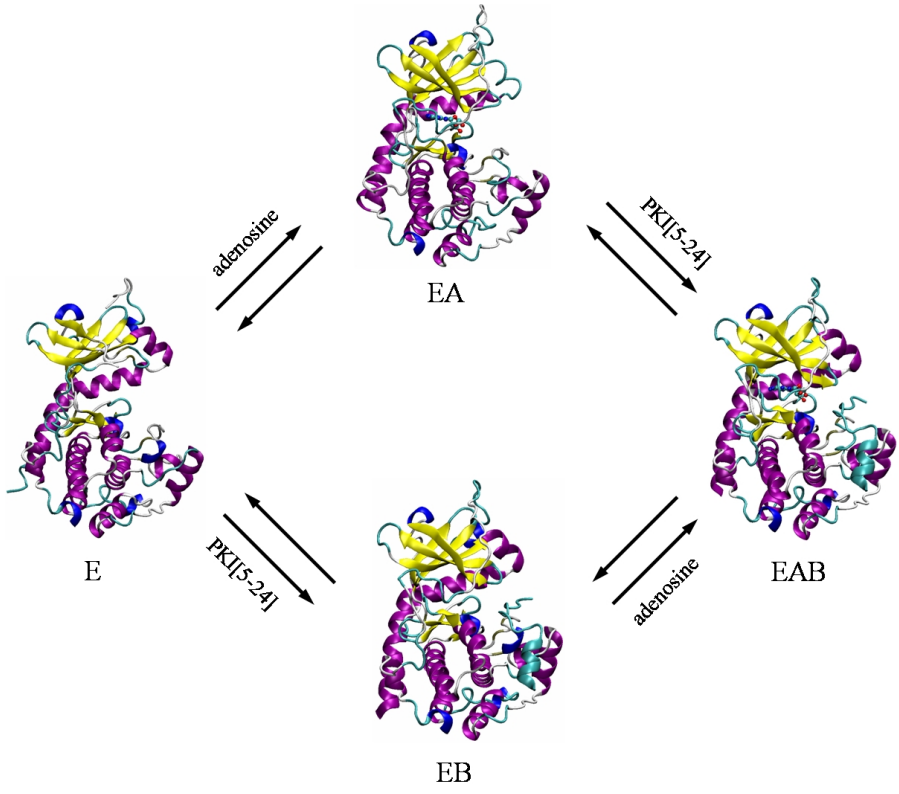


Figure 1. Illustration of passing between conformational states of apoenzyme and enzyme substrate complexes. The catalytic subunit is capable to form open and closed conformational states. The apoenzyme E (PDB ID 1J3H), the adenosine binary complex EA (1BKX), the binary complex with PKI[5–24] EB (1APM) and a complex with adenosine and PKI[5–24] EAB (1FMO) are shown.

linker strand, connecting the two domains. The complexes of PKA with ATP and AMPPNP (the non-hydrolysable analog of ATP) have revealed the detailed features of the ATP-binding site (Bossemeyer *et al.*, 1993, Zheng *et al.*, 1993). As all protein kinases are using ATP as substrate, they share this binding site structure. The adenine ring of ATP is staked directly against the linker part via two hydrogen bonds and there are no water molecules in this pocket when the adenine ring is present. Without nucleotide, however, two structured water molecules are present and they form the same hydrogen bonds that are made by the two nitrogen atoms of the adenine ring. This pocket is occupied in similar way in various kinase molecules (Davies *et al.*, 2007, Cherry and Williams, 2004), and these binding properties have been the major focus for design of small-molecule drugs in the case of protein kinases.

Most of the small lobe is highly dynamic structure without ligands, as shown by the temperature factors (Seifert *et al.*, 2002) and the difficulty of explicit

tracing the chain conformation in some regions, especially such as the tip of the glycine-rich loop (Akamine *et al.*, 2003). These transitions could be described as closing of the active site cleft (Taylor *et al.*, 1999), which opened structure is a characteristic feature of the free enzyme. This understanding was confirmed also by more recent direct NMR studies of PKA dynamics (Masterson *et al.*, 2008, Langer *et al.*, 2004).

The primary function of the large lobe is to provide the docking surface for peptide and thus to facilitate the phosphoryl transfer reaction. The C-lobe is predominantly helical, with the exception of a small highly conserved surface comprised of four strands that line the floor of the active site cleft. In contrast to the small lobe, the large lobe has quite stable structure. The stability of the large lobe is due to the solid hydrophobic core, which anchors the catalytic loop and the magnesium-positioning loop of the active site. Even if no peptide is present, as in the adenosine-enzyme binary complex, the conformation of this loop remained unchanged and the temperature factors were low (Narayana *et al.*, 1997a). On the other hand, the portion of the carboxyl terminal tail, which eventually clamps down onto the small lobe when ATP and peptide are bound, is quite disordered (Shaltiel *et al.*, 1998).

An important structural element of the large lobe is the catalytic loop. This loop is preceded by the conserved Arg165, the side chain of which forms a tight electrostatic contact with the phosphorylated Thr197. This interaction is essential for maintaining the active conformation of the kinase. The neighboring amino acid Asp166 is also conserved, and its putative role is to act as catalytic base in the phosphoryl transfer reaction (Madhusudan *et al.*, 1994). The β-strand of this lobe 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 neighboring phenylalanine, is one of the ligands in the magnesium ion coordination sphere, which in turn coordinates the β- and γ-phosphates of ATP. The DFG motif is followed by β-strand and the activation loop. The activation loop contains the previously mentioned Thr197, which auto-phosphorylation or phosphorylation by PDK1 kinase is essential for activity of PKA (Cheng *et al.*, 1998, Steinberg *et al.*, 1993). Most protein kinases possess threonine or tyrosine residues in the activation loop and this residue must be phosphorylated either auto-catalytically or by an activating kinase to obtain the active form of the enzyme (Johnson *et al.*, 1996). With PKA this phosphorylation occurs at Thr197 (Knighton *et al.*, 1991a), and this process is the second natural regulatory mechanism besides the enzyme activation by cAMP, used to control of activity of this enzyme in cell.

The three additional residues in the large lobe of the kinase core, which are conserved throughout the superfamily, act as general stabilizers of the structure. The Asp220 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). In summary, it can be concluded that at least some part of the active site is already formed in the apoenzyme

(Akamine *et al.*, 2003). On the other hand, the final formation of the active center should accompany substrate binding steps and perhaps even the phosphoryl transfer reaction, which takes place in the active site cleft, located between two domains of the protein. Changes in the mutual positioning of these domains can be observed even in the crystal structure of the enzyme, and should be significantly more intensive in solution. As shown by Gerstein *et al.* (1994), the minimal structural requirement for having efficient allosteric interaction between two binding sites is that these sites should be located in the interface between two domains, which may undergo conformational transitions like the hinge-bending motion. As summarized above, PKA matches perfectly with these requirements.

Substrate binding with PKA

In the absence of bound substrates the catalytic subunit exists in "open" state, where the conserved glycine-rich sequence motif (P-loop) is shifted and the molecule is opened for ATP intake (Taylor *et al.*, 1999). Binding of ATP is accompanied by closing of the cleft by the P-loop, which directly interacts with the nucleotide and thus functions as the nucleotide positioning motif. In addition to the hinge in the glycine-rich P-loop, overall sliding of the small lobe relative to the large lobe seems to accompany this ligand binding process (Taylor *et al.*, 2004).

Peptide substrate binds with the enzyme in an extended conformation across the front end of the ATP-binding pocket, close to the place where the γ-phosphate group of the enzyme-bound ATP should be located, and its correct positioning for the following catalytic step is assisted by the consensus motif around the phosphorylatable site, and by some remote parts, interacting with the secondary docking sites of the enzyme (Shabb, 2001). The response of the enzyme molecule to peptide binding can be observed as decrease of the α-helical content of its structure (49% to 31%) and concomitant increase in its β-structure (20% to 49%) (Reed and Kinzel, 1984a, Reed and Kinzel, 1984b, Reed *et al.*, 1985). This significant conformational reorganization is possible in the case of a highly dynamic enzyme molecule, characterized by the presence of large ensemble of different conformational states, which distribution can be changed by ligand binding (Gunasekaran *et al.*, 2004, Liang *et al.*, 2007, Ming and Wall, 2005). This property forms the structural basis for allosteric behavior of any enzyme (Masterson *et al.*, 2008). On the other hand, it is also clear that this dynamic behavior cannot be studied by X-ray analysis, but other methods, monitoring the protein properties in solution should be applied (Acharya and Lloyd, 2005, DePristo *et al.*, 2004). The conventional kinetic studies seem still to have solid position among these methods.

PKA reveals clear selectivity against ATP, as this nucleotide is the predominant, if not sole phosphate group donor in this peptide/protein phosphoryla-

tion reaction. More precisely, for effective ATP binding with PKA two Mg^{2+}-ions are needed: the first ion forms coordination bounds between β- and γ-phosphates and the second ion between α- and γ-phosphates of the nucleotide (Herberg *et al.*, 1999, Zimmermann *et al.*, 2008). The reasons for sharp specificity against this nucleotide can be explained by definite structure of adenine binding site in the active center of PKA.

Differently from nucleotide binding, molecular recognition of peptide fragments by the enzyme seems to allow significant variation in substrate structure. In general, three structural levels were used to determine this recognition process.

Firstly, PKA recognizes the peptide primary structure motifs around the phosphorylatable amino acid and this step is considered obligatory for the following reaction step. The basic ideas about this phosphorylation site specificity of PKA originate from papers published by Zetterqvist and Ragnarsson (Zetterqvist *et al.*, 1976, Zetterqvist and Ragnarsson, 1982) and were based on kinetic analysis of phosphorylation of peptides derived from structure of the phosphorylation site of L-type pyruvate kinase. The peptide RRASV was still efficiently phosphorylated by PKA. Therefore, this peptide was named as the "minimum substrate", stressing that this sequence should contain the whole set of specificity determinants, which are necessary for efficient phosphorylation of the peptide by PKA (Zetterqvist and Ragnarsson, 1982). The two arginines were exclusively important in this sequence since they could not be replaced even by lysine. Subsequent determination of structure of PKA complex with pseudosubstrate peptides was in good agreement with this consensus sequence motif (Taylor *et al.*, 1993, Tsigelny *et al.*, 1996).

Secondly, interaction between substrate and some docking-sites, apart of the primary binding site of the catalytic center, may occur (Kreegipuu *et al.*, 1998, Ubersax and Ferrell Jr, 2007). These interactions reveal in the case of long peptide substrates and may include, for example, additional binding of the N-terminal helix structure of these substrates with the docking site, or additional binding of the arginine residue in position -6 of the substrate primary structure, where 0 stands for position of the phosphorylatable amino acid (Kim *et al.*, 2007, Pinna and Ruzzene, 1996).

Thirdly, anchoring proteins may support fine targeting of the kinase to its substrate (Mochly-Rosen, 1995).

Attempts were made to rationalize peptide binding data with PKA by using structure-activity relationships (Järv and Ragnarsson, 1991). In some cases specificity of this enzyme could be quantitatively described by hydrophobicity, bulkiness and charge parameters. These studies also revealed that it was not possible to express the primary specificity of PKA for short peptides by one consensus sequence. The same conclusion was archived through statistical analysis of the known phosphorylation sites in natural substrates (Kreegipuu *et al.*, 1998).

Kinetic mechanism of PKA catalysis

The PKA catalyzed reaction involves two substrates – the phosphorylatable protein or peptide and ATP as the source of the phosphoryl group, which is transferred directly between the enzyme-bound substrates (Ho *et al.*, 1988, Lew *et al.*, 1997b). This means that both substrates must be bound with the enzyme before the catalytic step is possible. Binding of these two substrates with the enzyme may occur, in general, following the random or ordered mechanism. Random-order kinetic mechanism for substrate binding with PKA was defined by Kong and Cook (1988). Further, this aspect of substrate binding was studied by Cook *et al.* (1982) and Adams and Taylor (1992) and the results of these studies also supported the random substrate binding mechanism. Moreover, unrestricted access of ATP and kemptide to the active centre of PKA was also demonstrated by Kong and Cook (1988), in line with the random binding mechanism. At the same time it was mentioned that under some conditions the enzyme might have some preference for ATP binding first (Cook *et al.*, 1982, Grant and Adams, 1996), however even in this case the equilibrium ligand binding model seems to be applicable. The latter fact significantly simplifies interpretation of results of peptide phosphorylation kinetics.

The chemical mechanism of the peptide phosphorylation reaction has been analyzed by different physical methods (Ni *et al.*, 2005), suggesting the in-line phosphoryl transfer reaction for the catalytic step (Ho *et al.*, 1988, Madhusudan *et al.*, 1994). Further, the conventional methods of correlation analysis were used by Järv (1996) to analyze the transition state structure of this reaction. The results of these studies pointed to that the catalytic rate constant (or the maximal rate) of the process seem to characterize the chemical process rather than conformational transitions of the enzyme, or rate-limiting diffusion of reactants from the active centre. The latter possibility was discussed by Grant and Adams (1996), where the influence of viscosity of the reaction medium on kinetic parameters of peptide phosphorylation reaction was studied.

Keeping in mind these results, conclusion can be drawn that there is no final confidence about the meaning of the maximal velocity (or k_{cat}) values, measured for the PKA-catalyzed peptide phosphorylation reactions. Moreover, it was quite surprising to recognize that all kinetic studies of peptide phosphorylation, we were able to come across while writing this survey, were made under conditions, where concentration of one substrate (commonly ATP) was taken constant. In this experimental setup, kinetic data were processed by adopting rate equations derived for single-substrate reaction, without any attention on possible impact of such simplification on the physical meaning of kinetic parameters.

PKA inhibitors

It is generally believed that effective and selective inhibitors of protein kinases could be promising drug targets and this has generated wide interest in design of these compounds. The basic principles of protein kinase substrate specificity have inspired search for inhibitors, directed either into the ATP binding site or into the peptide binding site of these enzymes (Hanks and Hunter, 1995, Garcia-Echeverria *et al.*, 2000). In parallel, the concept of bisubstrate analog inhibitors was formulated (Ricouart *et al.*, 1991), assuming that specially designed ligands may interact simultaneously with binding sites of both substrates and through this reveal enhanced specificity and potency.

The list of ATP site directed inhibitors is rather long and includes several compounds, which effect can be observed in nanomolar concentration range. Among these inhibitors are microbial alkaloid staurosporine, extracted from *Streptomyces staurosporeus*, and its synthetic analogs (Meggio *et al.*, 1995); inhibitor KT5720 and its analogs, derived from alkoloid of the fungus *Nocardiopsis* (Kase *et al.*, 1987); balanol and its analogs, reviewed by Pande *et al.* (2008), and several purine derivatives (Gompel *et al.*, 2004). Isoquinolinesulphonamide based inhibitors were developed by Hidaka *et al.* (1984) and Reuveni *et al.* (2002). The most frequently used members of this series are compounds H89, H8 and H7 (Engh *et al.*, 1996, Hidaka *et al.*, 1984).

For several of these compounds, including H89, the structure of the inhibitor-PKA complex has been resolved by X-ray analysis and indeed, these inhibitors clearly dock in the adenine binding pocket (Engh *et al.*, 1996). This docking mechanism should be doubtlessly significant for effective binding of inhibitors, but may have negative drawback from their selectivity, as the adenine binding pocket seems to be quite conserved for distinct protein kinases. For example, this seems to be the case with inhibitors H89 and KT5720, which have been classified as potent competitive ATP antagonists (Kase *et al.*, 1987) and are marketed as specific PKA inhibitors. However, more recent data clearly do not agree with their high selectivity against PKA (Murray, 2008, Lochner and Moolman, 2006). On the other hand, in some other cases good selectivity of the ATP-site directed inhibitors has still been declared (Taylor *et al.*, 2004).

The list of peptide site directed inhibitors is less impressive. Regardless the substrate binding motifs were kept in mind in design of these inhibitors, binding effectiveness of short inhibitory peptides with PKA was rather bad and remained even in submillimolar concentration range (Bogoyevitch *et al.*, 2005). On the other hand, however, there are at least some natural peptides, which reveal extra high binding effectiveness and are very selective against PKA: the regulatory subunit of cAMP-dependent protein kinase and the thermostable inhibitor peptide PKI (Walsh and Glass, 1991). Proceeding from sequence of PKI different shorter peptides have been synthesized and denoted as PKI[6–22], PKI[5–24], PKI[14–22], where the numbers refer to the amino acid sequence of the parent compound. Many of these compounds were effective inhibitors of

PKA (Akritopoulou-Zanze, 2006, Mitchell *et al.*, 1995, Glass *et al.*, 1992, Cheng *et al.*, 1986, Wen and Taylor, 1994, Glass *et al.*, 1995). The extra high binding effectiveness of these peptides seems to be caused by interaction of their N-terminal part with some remote docking sites on PKA.

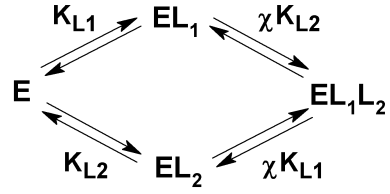
In bisubstrate analog inhibitors, the ATP site and peptide site directed structural elements are combined (Ricouart *et al.*, 1991). These parts are conjugated via a linker group to adjust their placement within the enzyme active center (Loog *et al.*, 1999, Shen *et al.*, 2005). Following this understanding many nucleotide-peptide conjugates have been synthesized (Uri *et al.*, 1994, Pehk and Uri, 1997). Indeed, these compounds revealed high potency and rather good selectivity against PKA. For example, one compound of this series of inhibitors, $AdcAhxArg_6$, inhibited PKA at submicromolar concentration, while approx 10 times less effective inhibition of CDPK-1 was observed (Loog *et al.*, 1999).

Another type of bisubstrate inhibitors was developed proceeding from chemical structure of a selective peptide substrate kemptide, where serine residue was is replaced by amino-alanine, which in turn was conjugated with nucleotide moieties (Hines and Cole, 2004, Medzihradszky *et al.*, 1994). These compounds inhibited PKA at micromolar concentrations, and behaved as competitive inhibitors versus ATP, but were clearly noncompetitive inhibitors versus peptide substrate (Parang and Cole, 2002). Therefore, it can be assumed that inhibitors may form diverse complexes with this enzyme, especially if the presence of two different substrate binding sites is considered and outstanding structural flexibility of PKA is kept in mind (Taylor *et al.*, 2004). Although this understanding seems to be appreciated in general, formation of these complexes has never been discussed more thoroughly. However, the asymmetric inhibition pattern mentioned above has clearly pointed to necessity of more detailed investigation into the mechanism of action of PKA inhibitors, taking into account the possibility of their interaction with both substrates, or perhaps more precisely, with their binding sites, recognizing the principle that it is virtually impossible to deduce the molecular mechanism of ligand binding solely from binding data (Henis and Levitzki, 1979).

Direct observation of PKA allostery

At the final stage of this project, publication by Masterson *et al.* (2008) appeared and reported their investigation into ligand binding properties of PKA, using NMR spectroscopy and the $^{2}H/^{15}N/^{13}C$-labeled PKA samples. This very straightforward analysis produced the first vivid picture of the complex changes of PKA conformation, occurring in the peptide backbone of the protein molecule during different steps of ligand binding. The enzyme was titrated with the ATP analog inhibitor AMPPNP and kemptide, and four distinct mutually transferring states of the protein were identified as illustrated by Eq. 2:

– the free enzyme (apo-protein), denoted as E,
– PKA complex with the nucleotide AMPPNP (intermediate-N), denoted as EL_1,
– PKA complex with kemptide (intermediate-S), denoted as EL_2, and
– PKA ternary complex with AMPPNP and kemptide yielding the "closed" structure and denoted as EL_1L_2.



(2)

The titration data allowed calculation of the full set of the dissociation constants listed in this scheme: K_{L1} = 39 μM, χK_{L1} = 12 μM, K_{L2} = 980 μM and χK_{L2} = 292 μM. These data allow quantification of the allosteric effect, denoted in this reaction scheme as χ (otherwise also named as interaction factor), as ratio of the appropriate dissociation constants. For this pair of ligands $\chi = 0.3$ that agrees with the presence of positive cooperativity in binding of these ligands.

These data were also compared with the conformational states identified by X-ray crystallography and the following conclusions were drawn. Without ligands this protein appeared to favor the conformation observed in the crystal structure of the apo state (the free enzyme), with small populations of the other states. If a ligand is added, the conformation is altered to favor the intermediate sate and becomes closer with the closed conformation of the protein. In this manner, the first ligand can drive the system close to the final state, facilitating binding of the second ligand.

Taking together, these results clearly suggest that PKA may interconvert between multiple dynamic conformational states, as has been speculated before. These structural changes are clearly connected with allosteric feedback between the ligand binding sites of this enzyme. It has become clear that effectiveness of binding of a ligand with this enzyme could be influenced by the presence of some other enzyme-bound ligand.

Secondly, these results also demonstrate that binding of ligands can be well analyzed by using equilibrium binding model. This significantly simplified interpretation of binding data and results of kinetic experiments, which could be used for analysis of allostery in PKA. It was not surprising that some data, which allowed estimation of allosteric effects, were occasionally found in earlier papers, and analytical survey of these data is given in the following part of this work.

OBJECTIVES OF DISSERTATION

The main objectives of this project were:

1. Adoption of existing methods of kinetic analysis of bisubstrate enzymatic reactions for investigation into PKA allostery.
2. Kinetic investigation into allostery in peptide phosphorylation reaction by PKA and using substrates of different structure and reactivity for this study, designed to reveal influence of substrate structure on allostery in the PKA catalysis.
3. Kinetic investigation into mechanism of PKA inhibition by compounds, designed to be ATP analog inhibitor, inhibitory peptide and bifunctional inhibitor, and concretization of interaction of these inhibitors with both substrates of the PKA catalyzed reaction.
4. Comparison of the results of these kinetic experiments with binding data, which could be found in literature and allow estimation of allostery in PKA binding properties. In this part of the project, the competitive list of literature data was compiled and analyzed.
5. Examination of possibilities for linking of the allostery effects with ligand structure and analysis of possibilities for application of LFE relationships for description of these phenomena in specificity of PKA.

MATERIALS AND METHODS

Chemicals

γ-[^{32}P]ATP was obtained from Amersham (UK) and the used samples had specific radioactivity 110 TBq/mmol. Peptide LRRAALG-NH_2 (Ala-kemptide amide) was synthesized in a stepwise manner on a 0.1 mmol scale using the Applied Biosystem peptide synthesizer (USA) as described elsewhere (Kuznetsov *et al.*, 2003). Peptide substrates RRYSV, RRASVA, LRRASLG (kemptide), RKRSRKE, LRKASLG, LARASLG and LRAASLG of purity above 95% were purchased from GL Biochem Ltd, (Shanghai, China) and where characterized by MS spectra and HPLC. ATP and inhibitor H89 (N-[2-((p-bromocinnamyl)amino)-ethyl]-5-isoquinolinesulfon-amide, 2 HCl) was obtained from Sigma-Aldrich (USA). Bisubstrate inhibitor $AdcAhxArg_6$ was a generous gift from Dr. Asko Uri (Institute of Chemistry, University of Tartu) and synthesis of this compound was described in (Loog *et al.*, 1999). Phosphocellulose paper P81 was acquired from Whatman (UK). All other chemicals (TRIS/HCl, BSA, H_3PO_4, $MgCl_2$) were obtained from Sigma-Aldrich (USA) or Acros (Germany) and were of highest grade available. Buffers were made using Mili-Q deionized water.

Enzyme

The catalytic subunit Cα of mouse cAMP-dependent protein kinase, recombinantly expressed in *E. coli*, 30 U/mg, 0.1 mg/ml, lot 040916, was obtained from Biaffin GmbH and Co KG (Germany). The enzyme preparation supplied by Biaffin GmbH was diluted 500 – 1000-fold in 50 mM TRIS/HCl buffer (pH 7.5) containing 1 mg/ml BSA, to obtain the stock solution for kinetic experiments. In some experiments, we used the catalytic subunit of the same isozyme donated by Dr. Mart Loog (Institute of Technology, University of Tartu). This enzyme was expressed using plasmid Cat-pRSET B (Narayana *et al.*, 1997b) in the T7 promoter expression system (Invitrogen/Inbio Ltd, USA/Estonia). The expression construct vector was a generous gift from Dr. S.S. Taylor (La Jolla, California). Purified catalytic subunit was obtained after a P-11 ion-exchanger step as except that instead of linear gradient stepwise elution with 250 mM potassium phosphate (pH 6.5) was used. The procedure was described in more detail in (Yonemoto *et al.*, 1991). The stock solution of this PKA preparation was prepared immediately before experiments in buffer containing 50 mM TRIS/HCl (pH 7.5) and 1 mg/ml BSA.

6

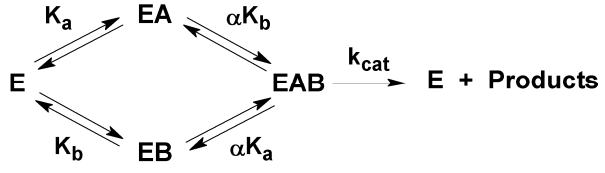
Assay of peptide phosphorylation

Initial rate of phosphorylation of peptide substrates by PKA was carried out at 30°C as described previously (Kuznetsov *et al.*, 2004, Roskoski, 1983). Briefly, the reaction mixture (final volume 100 µl, 50 mM TRIS/HCl, pH 7.5) contained γ-[^{32}P]ATP (concentration interval between 2.5 to 150 µM if not mentioned differently), peptide substrate (concentrations were depending on affinity of PKA for particular substrate), 10 mM of $MgCl_2$ and 0.015 – 0.10 µg/ml of the enzyme. The stock solution (15 µl) of the enzyme was added into the reaction mixture to initiate the phosphorylation reaction. At different time moments 10 µl aliquots were removed from the reaction mixture and spotted onto the pieces of phosphocellulose paper, which were subsequently immersed into ice-cold 75 mM phosphoric acid to stop the reaction. These pieces were then washed four times with cold 75 mM H_3PO_4 (10 min each time) to remove excess of γ-[^{32}P]ATP and were dried at 120°C for 25 min. The radioactivity bound onto the paper was measured as Cherenkov radiation using a Beckman LS 7500 scintillation counter. The values of the initial rate of the phosphorylation reaction, further denoted as v, were calculated from the slopes of the product concentration *vs* time plots.

In the PKA inhibition assays, the reaction mixture contained also inhibitors (25 and 50 nM of H89, 100 and 200 µM of LRRAALG-NH_2 and 1.0, 0.5 and 0.25 µM of AdcAhxArg$_6$). The stock solution of PKA was diluted into 50 mM TRIS/HCl buffer (pH 7.5) containing 1 mg/ml BSA, and 15 µl of this solution was added into the reaction mixture to initiate the phosphorylation reaction. At different time moments 10 µl aliquots were removed from the reaction mixture and the values of the initial rate of substrate phosphorylation were calculated as described above.

Kinetic formalism for PKA substrate reaction

The following analysis of substrate phosphorylation kinetics was based on understanding that the ternary complex EAB is formed between ATP, peptide and the enzyme, denoted as A, B and E, respectively. In this reaction scheme EA and EB stand for the intermediate enzyme-substrate complexes:



(3)

This reaction scheme presents affinity of the free enzyme for substrates A and B through the dissociation constants K_a and K_b, respectively, and characterizes mutual interaction of these binding sites through the interaction factor α. Definition of the latter parameter was introduced by Segel (1975). Following this definition, we have positive allosteric effect if $\alpha < 1$, and affinity of the enzyme for the second substrate increases if the first substrate has been bound beforehand. Differently, if $\alpha > 1$, the pre-bound substrate hinders binding of the next substrate. Only at $\alpha = 1$ ligands bind independently and there is no coupling between binding properties of their binding sites

For practical data processing we proceeded from the following rate equation derived for the reaction scheme (1),

$$v = \frac{V \dfrac{[A][B]}{\alpha K_a K_b}}{1 + \dfrac{[A]}{K_a} + \dfrac{[B]}{K_b} + \dfrac{[A][B]}{\alpha K_a K_b}} , \qquad (4)$$

where $V = k_{cat}[E_0]$. In general, the dependence of v upon [A] and [B] can be presented by a 3D plot, as demonstrated later in discussion of kinetic data. Following the rate equation (4) the apparent value of the Michaelis constant for ATP (A) should depend on peptide (B) concentration:

$$K_m^A = K_a \frac{1 + \dfrac{[B]}{K_b}}{1 + \dfrac{[B]}{\alpha K_b}} . \qquad (5)$$

By analogy, the Michaelis constants for peptides should depend on ATP (A) concentration:

$$K_m^B = K_b \frac{1 + \dfrac{[A]}{K_a}}{1 + \dfrac{[A]}{\alpha K_a}} . \qquad (6)$$

These plots of K_m^A *vs* [B] and K_m^B *vs* [A] were be used for calculation of the α values.

Although Eqs 5 and 6 can be used for one-time calculation of α and K_a or α and K_b, we have introduced a more reliable algorithm for characterization of

affinity of the free enzyme for substrates A and B. This algorithm uses the second-order rate constants of the enzymatic reaction, $k_{II}^{A}=\frac{V^{A}}{K_{m}^{A}}$ and $k_{II}^{B}=\frac{V^{B}}{K_{m}^{B}}$ respectively. These rate constants can be derived from Eq. 4 and have the following meaning:

$$k_{II}^{A}=\frac{\frac{V}{\alpha K_{a}}[B]}{K_{b}+[B]} \tag{7}$$

and

$$k_{II}^{B}=\frac{\frac{V}{\alpha K_{b}}[A]}{K_{a}+[A]}\ . \tag{8}$$

It is important to emphasize that the second-order rate constants can be determined from slope of the initial linear part of hyperbolic v *vs* substrate concentration plots. Therefore, these parameters can also be determined from kinetic experiments made at extra low substrate concentrations, where the initial velocity *vs* concentration plot can still be approximated by linear function. For example, at extra low concentration of substrate A, where also $[A] < K_{m}^{A}$ and $[A] << [B]$, the reaction occurs at pseudo-first order conditions and the rate equation 4 simplifies

$$v=\frac{\frac{V[B]}{\alpha K_{a}K_{b}}}{1+\frac{[B]}{K_{b}}}[A]=k_{II}^{A}[A] \tag{9}$$

and the rate constant k_{II}^{A} can be calculated by the conventional procedures of kinetic analysis. Analogously, at $[B] < K_{m}^{B}$ and $[B] << [A]$, the second-order rate constant k_{II}^{B} can be obtained from the simplified rate equation 10.

$$v = \frac{\frac{V[A]}{\alpha K_a K_b}}{1 + \frac{[A]}{K_a}}[B] = k_{II}^{B}[B] \qquad (10)$$

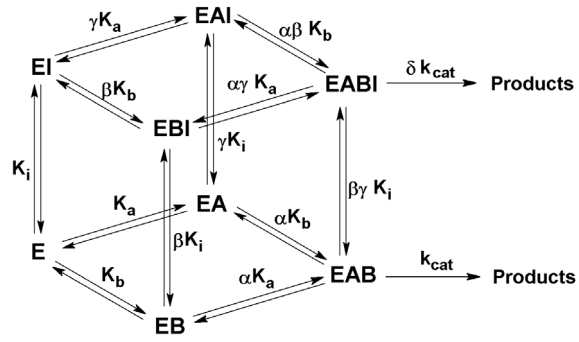
The latter method for calculation of the second-order rate constants is more reliable tool under conditions, where the reaction course can be affected by concentration-dependent effects like substrate inhibition.

The plots of k_{II}^{A} *vs* [B] and k_{II}^{B} *vs* [A] provide easy way for direct calculation of the values of K_b and K_a, which were thereafter used as constraints in calculation of the α values from K_m^A *vs* [B] and K_m^B *vs* [A] plots. As the results of these calculations did not depend on the actual value of the maximal rate, catalytic activity of the enzyme was estimated on milligram basis and these data were used for planning of experiments.

For the practical data analysis, the initial velocities of substrate phosphorylation reaction (v) were measured at various ATP (A) and peptide (B) concentrations. In most experiments, the arrays of these kinetic data were processed as two subsets. In one subset, initial v *vs* ATP concentration plots were used to calculate the parameters and K_m^A and k_{II}^A at various peptide concentration and these parameters were further plotted versus [B]. Analogously, K_m^B and k_{II}^B were obtained from v *vs* peptide concentration plots at different ATP concentration and further plotted versus [A].

Kinetic formalism for analysis of PKA inhibition

In this analysis we suggested that the inhibitor I may interact with the free enzyme (E) and with the enzyme-substrate complexes EA and EB, as shown in Eq. 11. Affinity of the free enzyme for substrates A and B and for ligand I is quantified by the dissociation constants K_a, K_b and K_i, respectively. For generality, two more interaction factors, denoted as β and γ, were defined. These interaction factors characterize allosteric feedback between the appropriate binding sites in formation of the ternary complexes EAI and EBI, respectively. Different combinations of these parameters characterize formation of the quaternary complex EABI. If the value of these interaction factors remains below unity, simultaneous binding of any of two ligands with the enzyme is enhanced in comparison with their binding with the free enzyme.



(11)

This influence is defined as positive allosteric effect. Differently, decrease in binding affinity takes place if the appropriate interaction factor is above unity, and this situation is defined as negative allosteric effect. These both cases should be classified as heterotrophic allosteric effects, as two different ligands are involved. Finally, the option of partial enzyme inhibition was considered in this reaction scheme by introducing additional reaction path proceeding from the complex EABI. The rate equation (12) was obtained for this general reaction scheme.

$$\frac{v}{[E_0]} = \frac{k_{cat}\left(\frac{[A][B]}{\alpha K_a K_b} + \frac{\delta[A][B][I]}{\alpha\beta\gamma K_a K_b K_i}\right)}{1 + \frac{[A]}{K_a} + \frac{[B]}{K_b} + \frac{[I]}{K_i} + \frac{[A][B]}{\alpha K_a K_b} + \frac{[A][I]}{\gamma K_a K_i} + \frac{[B][I]}{\beta K_b K_i} + \frac{[A][B][I]}{\alpha\beta\gamma K_a K_b K_i}} \qquad (12)$$

Simplification of this reaction scheme and the rate equation above could be made in the course the data processing. In this connection, the following options were kept in mind.

Firstly, for discrimination between the complete and partial inhibition mechanisms the reaction rate *vs* [I] plots were analyzed at some constant concentration of substrates A and B. In the case of the complete inhibition mechanism and the reaction rate should go down to zero at high inhibitor concentration.

$$\frac{\delta}{\beta\gamma K_i} = 0 \qquad (13)$$

This means that linear plot should be observed in coordinates 1/v and [I]. For the partial inhibition mechanism, however, the enzyme activity should reach some fixed value at high inhibitor concentration, where the reaction path via EABI becomes effective. This results in deviation of the 1/v and [I] plot from

linearity and can be used for diagnostic purposes of the reaction mechanism. It should be mentioned in advance that for all inhibitors studied in this work the complete inhibition mechanism was identified that significantly simplified the analysis.

Secondly, significance of formation of particular complexes can be analyzed using the appropriate dissociation constants, and these values can be used to diagnose reliability of formation of any of the enzyme-ligand complexes shown in this kinetic model. All states characterized by statistically uncertain K-values should be omitted from the analysis.

Thirdly, the particular feature of this kinetic analysis was application of the second-order rate constants, which physical meaning does not depend on the rate-limiting steps of the catalytic process, and upon other disturbances occurring in the catalytic steps. This advantage of the second-order rate constants of complex enzymatic reactions is well known (Eisenthal *et al.*, 2007, Koshland, 2002), but their practical application appeared to be not trivial for bisubstrate reaction. The general equations for the second-order rate constants, corresponding to the experimental conditions presented below were presented by Eqs 7 and 8.

Finally, for practical use we customized the rate equation (8) for the step-by-step data processing algorithm. Briefly, Eq. 12 was rearranged for two options, considering varied [A] at fixed [B] and [I], and varied [B] at fixed [A] and [I]. From these data-sets the second-order rate constants k_{II} were calculated from the apparent V and K_m values for different combinations of ligand concentrations, and were further used for calculation of the complex parameters X_i, Y_i, Q_i and U_i as defined below:

$$k_{II}^{app^B} = \frac{\dfrac{k_{cat}\left(1+\dfrac{\delta[I]}{\beta\gamma K_i}\right)}{\alpha K_b\left(1+\dfrac{[I]}{\gamma K_i}\right)}[A]}{\dfrac{K_a\left(1+\dfrac{[I]}{K_i}\right)}{\left(1+\dfrac{[I]}{\gamma K_i}\right)}+[A]} = \frac{X_i[A]}{Y_i+[A]} \qquad (14)$$

and

$$k_{II}^{app^A} = \frac{\dfrac{k_{cat}\left(1+\dfrac{\delta[I]}{\beta\gamma K_i}\right)}{\alpha K_a\left(1+\dfrac{[I]}{\beta K_i}\right)}[B]}{\dfrac{K_b\left(1+\dfrac{[I]}{K_i}\right)}{\left(1+\dfrac{[I]}{\beta K_i}\right)}+[B]} = \frac{Q_i[B]}{U_i+[B]} \quad . \qquad (15)$$

It is important to mention that the method for calculation of the second-order rate constants from experimental data, obtained under extra low substrate concentration (under the pseudo-first order conditions) can be effectively used also in inhibition studies.

The complex parameters X_i, Y_i, Q_i and U_i have the following meaning:

$$X_i = \frac{\frac{k_{cat}\,\gamma K_i}{\alpha K_b}\left(1+\frac{\delta[I]}{\beta\gamma K_i}\right)}{\gamma K_i + [I]} \quad , \tag{16}$$

$$Y_i = \frac{K_a\,\gamma K_i\left(1+\frac{[I]}{K_i}\right)}{\gamma K_i + [I]} \quad , \tag{17}$$

$$Q_i = \frac{\frac{k_{cat}\,\beta K_i}{\alpha K_a}\left(1+\frac{\delta[I]}{\beta\gamma K_i}\right)}{\beta K_i + [I]} \quad , \tag{18}$$

$$U_i = \frac{K_b\;\beta K_i\left(1+\frac{[I]}{K_i}\right)}{\beta K_i + [I]} \quad . \tag{19}$$

The hyperbolic plots of X_i and Q_i *vs* [I] were used for calculation of the inhibition constants βK_i and γK_i, while the K_i values were calculated from the Y_i/X_i *vs* [I] and U_i/Q_i *vs* [I] plots. For this analysis, however, these equations were simplified, as $\frac{\delta}{\beta\gamma K_i} = 0$, and the K_i values were calculated from the linear plots of Y_i/X_i *vs* [I] and U_i/Q_i *vs* [I] plots, as shown below:

$$\frac{Y_i}{X_i} = \frac{\alpha K_b\,K_a}{k_{cat}} + \frac{\alpha K_b\,K_a}{K_i\,k_{cat}}[I] \tag{20}$$

and

$$\frac{U_i}{Q_i} = \frac{\alpha K_a\,K_b}{k_{cat}} + \frac{\alpha K_a\,K_b}{K_i\,k_{cat}}[I] \quad . \tag{21}$$

Data processing

Calculations and statistical analysis of kinetic data were made using the GraphPad Prism (versions 4.0, GraphPad Software Inc., USA) and SigmaPlot (version 8.0, SPSS Inc., USA) software packages. The results of calculations were reported with standard errors.

RESULTS AND DISCUSSION

Allostery in PKA reaction with substrates

In our preliminary experiments, kinetics of the PKA catalyzed kemptide phosphorylation reaction was measured at ATP concentration interval from 5 to 1000 μM and peptide concentration interval from 5 to 200 μM (Fig. 2). The modest peptide concentrations were selected because of the preliminary information about substrate inhibition in this reaction (Whitehouse *et al.*, 1983). However, as seen in Fig. 2, significant substrate inhibition effect was revealed also with ATP. Theoretically this effect can be taken into account in the data processing algorithm, as discussed in our earlier paper (Kuznetsov *et al.*, 2003). However, in practice this analysis was rather complicated and caused significant loss of accuracy. As the estimated value for this substrate inhibition constant was approx 0.9 mM, it was reasonable to use lower ATP concentrations, not exceeding 150 μM, where the expected deviations from the hyperbolic kinetics remains low. On the other hand, if the phenomenon of substrate inhibition is not considered, significant distortion of the results of data processing can be observed. Not surprisingly, the second-order rate constants of the reaction are less sensitive against this distortion, especially if these parameters are determined under the pseudo-first order conditions, as described by Eqs 9 and 10.

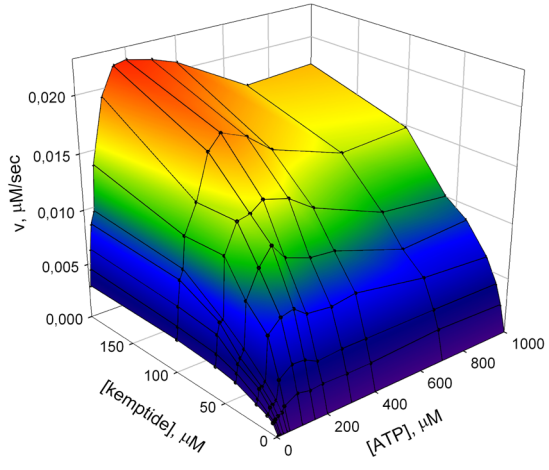


Figure 2. 3D-Plot of initial rate *vs* ATP and peptide concentration for the PKA catalyzed reaction of kemptide (LRRASLG) phosphorylation.

Following this annotation, kinetics of phosphorylation of seven peptides (see structures in Table 1) was studied in this work, using the algorithm presented above (Eqs 3–10). Accordingly, using this algorithm, interaction of peptides with the free enzyme was characterized by the dissociation constant K_b, and in parallel, affinity of PKA for ATP (K_a in Eq. 3) was determined from kinetic data for different peptides. These calculations were performed by using the second order rate constants as instructed by Eqs 7 and 8. The plots of k_{II}^{B} *vs* [A] and k_{II}^{A} *vs* [B] were indeed hyperbolic, as illustrated for LRRASLG (kemptide) and ATP in Fig. 3. As seen, the limited concentration interval was used for both substrates and the hyperbolic plots allowed reliable calculation of the K_a and K_b values.

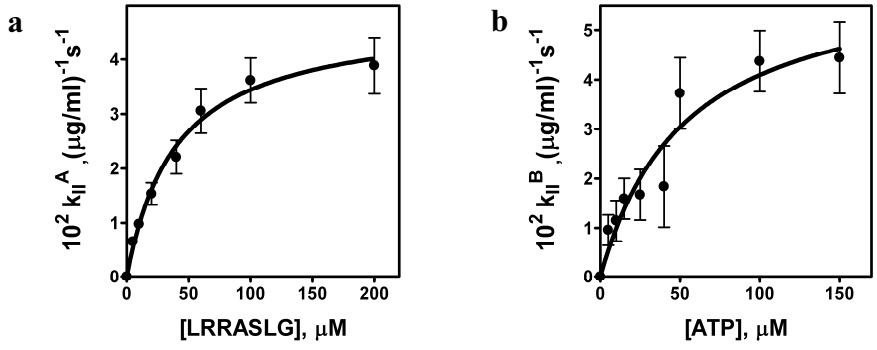


Figure 3. Calculation of the PKA affinity for kemptide (a) and ATP (b) by using the second-order rate constants of kemptide phosphorylation reaction.

The same procedure was used for all peptides studied and the results of these determinations were listed in Table 1. As the parameter K_a characterizes affinity of the free enzyme for ATP, it was not surprising that all these values, obtained from assays made with different peptides, coincided well with each other. Therefore, the mean value K_a = 49.7 μM was calculated from these results.

It is noteworthy that this K_a value is higher than the K_m values commonly reported for ATP in the PKA catalyzed reaction, where most of these parameters are ranging between 5 μM and 20 μM. However, this difference between K_a and K_m for ATP can be explained by Eq. 5, showing the dependence of the apparent K_m value for ATP upon peptide concentration. As bisubstrate kinetics has not been systematically studied with PKA, it seems now obvious that this additional influence should derange most if not all available kinetic data.

Differently from the results for ATP, affinity of PKA for peptides was rather diverse, and the K_b values were ranging from 2 μM to 6 mM for the selected different primary structure and reactivity were specially selected for this study

proceeding from their K_m values reported in literature (Kemp *et al.*, 1977, Wu *et al.*, 1994, Leader *et al.*, 1991, Prorok and Lawrence, 1989a, Glass and Krebs, 1979, Gibbs and Zoller, 1991). Diversity of the K_b values listed in Table 1 reveals that this selection was effective and the general principles of PKA substrate specificity, formulated from the Michaelis constants, hold also for constants K_b. However, likewise with the apparent K_m values for ATP, the parameters K_b cannot be directly compared with the appropriate Michaelis constants, as $K_b = K_m^B$ only if $\alpha = 1$. In all other cases, if the interaction factor α is different from unity, the apparent Michaelis constants for peptides should depend upon ATP concentration, as predicted by Eq. 6. Indeed, the plots of the K_m^B values upon ATP concentration were observed experimentally, as illustrated for kemptide phosphorylation reaction in Fig. 4. Therefore, the K_m^B *vs* [ATP] plots were also used for calculation of the α values, as instructed by Eq. 6.

As the next step of this study, the K_m^A values were determined for ATP at different peptide concentration. Similarly, the K_m^B values were determined for each peptide at different ATP concentration, as described by Eqs 5 and 6, respectively. This analysis revealed that the conventional Michaelis constants were indeed dependent upon the presence of the "second" substrate and these plots were further used for estimation of the α values. As the plots of K_m^B *vs* [A] and K_m^A *vs* [B] were separately analyzed for each pair of substrates, two α values were obtained from these independent sets of experimental data.

Table 1. Results of the kinetic analysis of phosphorylation of peptide substrates by PKA (the catalytic subunit of cAMP-dependent protein kinase). Meaning of the kinetic parameters is given in Eq. 3. Parameters are listed with standard errors.

	Peptide	**K_b µM**	**K_a µM**	**α_b**	**α_a**	**pα (average)**	**pK_b**
1	**RRYSV**	2.1±0.5	48±11	0.11±0.02	0.08±0.01	1.02±0.10	5.68±0.05
2	**RRASVA**	25±8	53±10	0.19±0.03	0.19±0.02	0.72±0.10	4.60±0.14
3	**LRRASLG**	40±5	51±14	0.36±0.04	0.37±0.03	0.44±0.07	4.39±0.05
4	**RKRSRKE**	117±14	49 ±10	0.52±0.06	0.46±0.05	0.31±0.05	3.92±0.06
5	**LRKASLG**	231±36	52±17	0.60±0.08	0.76±0.09	0.17±0.07	3.64±0.07
6	**LARASLG**	1880±541	45±13	1.2±0.2	1.6±0.3	–0.14±0.07	2.72±0.14
7	**LRAASLG**	6454±2328	49±23	3.5±0.6	2.5±0.4	–0.48±0.15	2.19±0.15

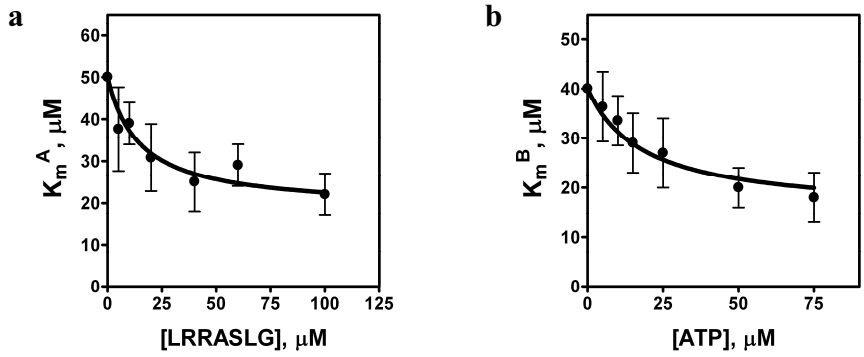


Figure 4. Calculation of the interaction factor α from K_m^A *vs* peptide concentration plot (a) and K_m^B *vs* ATP concentration plot (b) for the PKA catalyzed reaction of kemptide phosphorylation. At zero substrate concentration the K_a and K_b values were used.

Therefore, two values of the interaction factor for each ATP-peptide pair were listed in Table 1 as α_a and α_b, respectively. It can be seen that there was agreement between these results. The mean value of α was calculated from α_a and α_b for further analysis.

It can be seen in Fig. 5 that K_m^A *vs* peptide concentration plots could have rather different shapes, if different peptides are used as substrates. This divergence manifested also in the α values, which are changing from 0.09 for RRYSV to 3 for LRAASLG. Interestingly, the same peptides had the highest and the lowest binding effectiveness with the free enzyme, as seen from the appropriate K_b values in Table 1. Moreover, concurrent changes in the K_b and α values were also observed for other peptides (Table 1).

In summary, this method of allostery analysis is based on analysis of the plot of the apparent Michaelis constant on concentration of the second substrate. It is important to emphasize that this dependence can be observed experimentally, and these results do not depend upon the kinetic scheme or the mathematical algorithm applied for data processing. Surprisingly, we were unable to trace any systematic kinetic study of this type in literature, although the fact that rate of a bisubstrate enzyme reaction depends on concentration of both substrates is well known. On the other hand, however, the variation of the apparent K_m values can be seen also from data compiled from the literature for kemptide (Fig. 6).

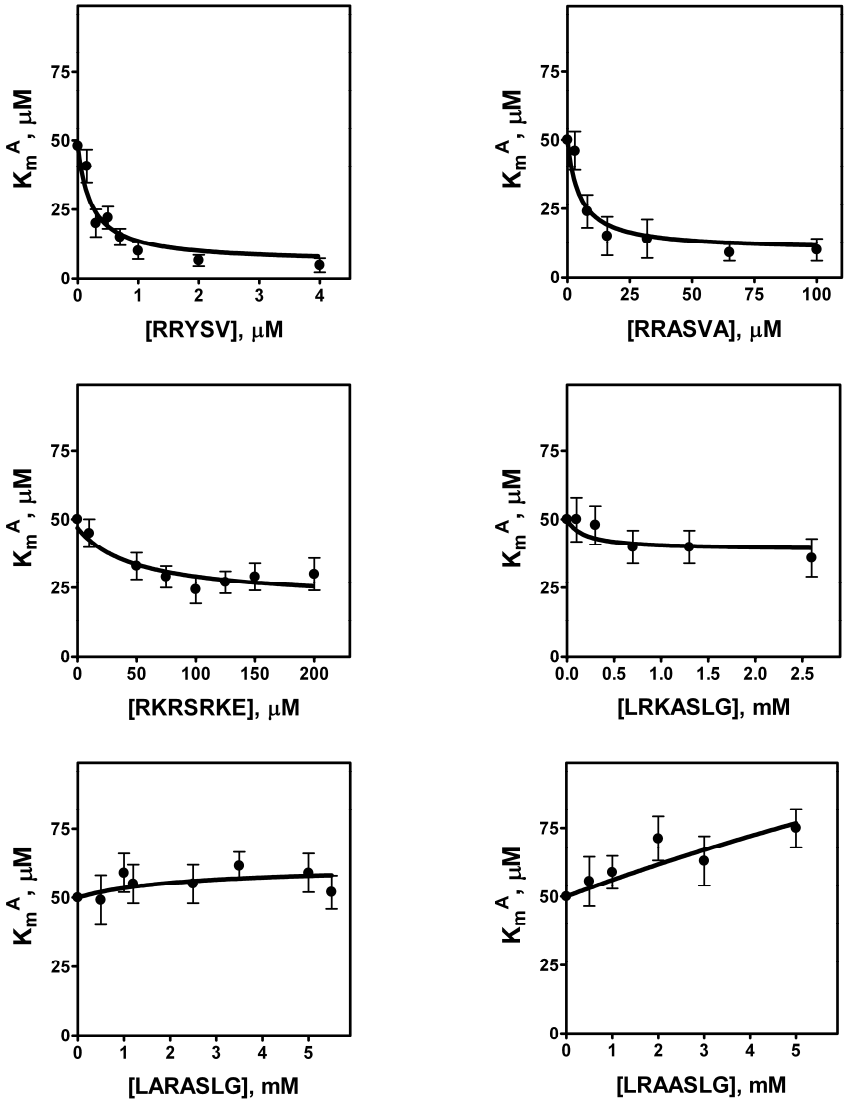


Figure 5. Influence of peptide concentration upon the value of the Michaelis constant for ATP, determined for the PKA catalyzed phosphorylation reaction of peptides. At zero substrate concentration the K_a values were used.

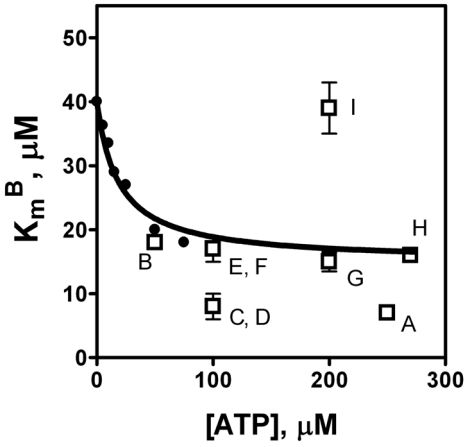


Figure 6. Comparison of the apparent Michaelis constants for kemptide phosphorylation from literature (squares) with our data (filled circles, as shown in Fig. 4). The literature data were taken from the following papers: A – (Feramisco *et al.*, 1980); B – (Prorok and Lawrence, 1989a); C – (Prorok and Lawrence, 1989b); D – (Roskoski and Ritchie, 1991); E – (Bramson *et al.*, 1985); F – (Cheley and Bayley, 1991); G – (Rascon *et al.*, 1994); H – (Kemp *et al.*, 1977); I – (Colbran *et al.*, 1992).

In summary, it is important to stress that similar procedure, where the Michaelis constants, obtained at zero and saturating substrate concentrations, were used for calculation of the interaction factor, has been initially suggested for allostery analysis of multimeric enzymes by Symcox and Reinhart (1992). However, these authors were unable to determine explicit values of the Michaelis constants under conditions, where substrate concentration is zero. Therefore, their analysis used these parameters determined simply at some low substrate concentration that caused uncertainty. We improved the procedure by applying the second-order rate constants, which allow explicit characterization of substrate interaction with the free enzyme. These parameters allow experimental determination of the enzyme affinity for substrate at its "zero concentration".

Allostery in inhibition of the PKA catalyzed reactions

In this work we investigated the influence of allosteric interactions between substrate binding sites and inhibitor binding site(s) on catalytic activity of PKA, and characterized formation of different complexes between the inhibitor molecule and the free enzyme, as well as between the inhibitor and the enzyme-substrate complexes. The particular feature of this kinetic analysis was application of the second-order rate constants, which advantages in analysis of these complex enzymatic reactions were discussed above. For practical

evaluation of the proposed method of kinetic analysis we studied interaction of PKA with three differently targeted reversible inhibitors:

- with H89 (N-[2-(p-bromo-cinnamylamino)ethyl]-5-isoquinolinesulfon-amide), the isoquinoline derivative, specially designed to block the ATP binding site of PKA,
- with non-phosphorylatable analog of kemptide amide, LRRAALG-NH_2, and
- with bisubstrate analog inhibitor AdcAhxArg$_6$, containing the nucleotide part and hexa-arginine, connected with each other via linker group.

Structures of these inhibitors were illustrated in Fig. 7.

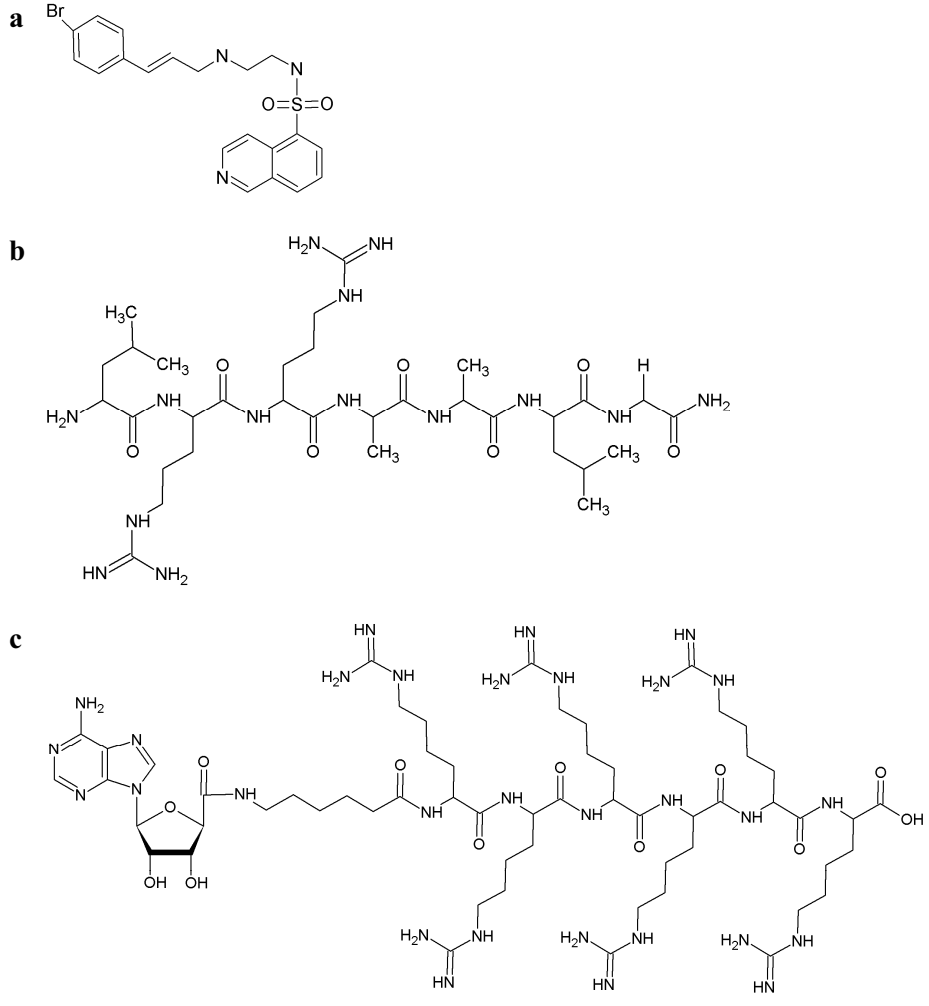


Figure 7. The structures of PKA inhibitors designed for different sites. ATP-site inhibitor H89 (a), peptide-site inhibitor LRRAALG-NH_2 (b) and bisubstrate analog inhibitor AdcAhxArg$_6$ (c) are shown.

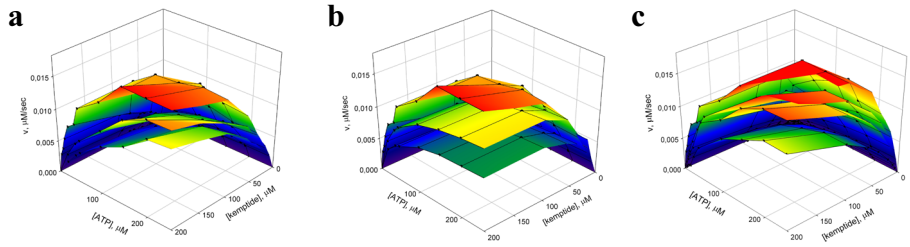


Figure 8. 3D plots for the PKA catalyzed reaction of kemptide phosphorylation in the presence of inhibitor H89 (a), peptide LRRAALG-NH_2 (b) and $AdcAhxArg_6$ (c). Concentration of inhibitor H89 was 0.05, 0.025 and 0 μM (starting from the bottom). Concentration of the peptide inhibitor was 200, 100 and 0 μM (starting from the bottom). Concentration of the bisubstrate inhibitor was 1.0, 0.5, 0.25 and 0 μM (starting from the bottom).

The arrays of the reaction rates (approx 400 data-points each) were obtained at various concentrations of both substrates and in the presence and absence of inhibitors and these data are illustrated as three-dimensional plots in Fig. 8, where colors change from blue to red to demonstrate the increase in reaction rate.

It can be seen that the phosphorylation rate decreased in the presence of both inhibitors, and the inhibition effect was dose-dependent. Moreover, in the case of $AdcAhxArg_6$, where data for high ATP concentrations were also involved, inhibition of the reaction by excess of substrate was clearly seen. To minimize the influence of substrate inhibition, the experimental data for high ATP concentration were omitted from our analysis (Fig. 8, c), although the mechanism of this phenomenon deserves special analysis. Further analysis of the inhibition data in coordinates 1/v *vs* [I] yielded linear plots for all inhibitors. This confirmed the complete inhibition mechanism for all these inhibitors and simplified the following data processing, where the kinetic formalism presented above was applied. From the arrays of the kinetic data shown in Fig. 8 the second order rate constants and further the complex parameters X_i, Y_i, Q_i and Y_i were calculated and used for determination of the K_i, βK_i and γK_i values as presented in the reaction scheme 11 above. The results of this analysis are listed in Table 2. Importantly, the Y_i/X_i *vs* [I] and U_i/Q_i *vs* [I] plots coincided well for both inhibitors. As the experimental data used for calculation of the Y_i/X_i and U_i/Q_i values were obtained from different kinetic experiments, this result could be taken as validation of the results obtained.

The constants K_i, βK_i and γK_i (Table 2) were used for calculation of coefficients β and γ, defined as interaction factors in the reaction scheme 11. In fact, these parameters compare effectiveness of the inhibitor binding with the free enzyme E and the EA complex (γ), or with the free enzyme E and the EB

Table 2. Interaction of inhibitors H89, LRRAALG-NH_2 and $AdcAhxArg_6$ with the free PKA (K_i), with the enzyme-ATP complex (γK_i), with the enzyme-kemptide complex (βK_i) and with the enzyme-ATP-kemptide ternary complex ($\beta\gamma K_i$). The meaning of the listed parameters is specified by the reaction scheme 11.

Parameter	Inhibitor		
	H89	**LRRAALG-NH_2**	**$AdcAhxArg_6$**
K_i, μM	0.009 ± 0.002	161 ± 32	0.13 ± 0.03
βK_i, μM	0.016 ± 0.003	77 ± 18	0.41 ± 0.06
γK_i, μM	0.08 ± 0.01	45 ± 10	1.54 ± 0.46
$\beta\gamma K_i$, μM	n.d.[a]	39 ± 20	n.d.[a]
β	1.8	0.5	3
γ	8.9	0.3	12

[a] n.d. not determined, because the dissociation constants were too large for reliable detection under the used experimental conditions.

complex (β), respectively. Consequently, these values quantitatively characterize allosteric interactions, which govern binding properties of the appropriate binding sites. We also introduced separate entry for the constant $\beta\gamma K_i$ in Table 2. This parameter quantifies the formation of the ternary complex EABI, and its value can be obtained from the experimental data, or calculated indirectly by combining the K_i, β and γ values. In this study, we were able to detect experimentally the formation of this quaternary complex in the case of LRRAALG-NH_2 (see Table 2), while similar value can be calculated from K_i, β and γ for this peptide. Consequently, the formation of the quaternary complex EABI was relevant for interaction of LRRAALG-NH_2 with PKA. In the case of two other inhibitors, however, the $\beta\gamma K_i$ value was not determined experimentally. Its estimation by combining the K_i, β and γ values yielded the approximate $\beta\gamma K_i$ values 0.14 μM and 4.6 μM, respectively. Indeed, at so high concentration of these effective inhibitors the rate of peptide phosphorylation cannot be monitored.

The constants K_i, βK_i and γK_i characterize interaction of the three inhibitors with the free enzyme and with the two enzyme-substrate complexes. The present results surprisingly show that formation of all these three complexes can be observed and characterized by the appropriate dissociation constants. Thus, none of these inhibitors can be described as purely competitive inhibitor, although in two cases the γ values were around 10, pointing to the inhibitors significantly interfered with ATP binding and could be considered being rather close to the "competitive" inhibition mechanism relatively ATP. However, H89 and $AdcAhxArg_6$ also blocked kemptide binding, yielding $\beta = 1.8$ and 3, respectively. In the case of $AdcAhxArg_6$ this is not surprising, as this inhibitor is designed as a bisubstrate analog inhibitor. However, the compound H89, which has been designed specifically as ATP site inhibitor, has the β value 1.8. Thus, this compound revealed at least some properties of bisubstrate inhibitors, affecting binding of both substrates. Consequently, the borderline between the

mono-substrate and bisubstrate inhibitors seems to be not very strict, or perhaps even not existing, at least as far as allosteric feedback between different ligand-binding sites of PKA is considered.

The situation observed with LRRAALG-NH_2 was rather different, as the constants βK_i and γK_i were smaller than the K_i value (Table 2). Thus, the binding effectiveness of this compound is increased in the presence of ATP ($\gamma = 0.3$) and kemptide ($\beta = 0.5$), pointing to positive cooperativity between the appropriate binding sites, which should simultaneously accommodate the peptide substrate, ATP and the peptide inhibitor. Indeed, in this case even the formation of the quaternary complex EABI was detected experimentally and characterized by the $\beta\gamma K_i$ value 39 μM. This seems to be a rather intriguing situation, as the substrate and the peptide inhibitor have very close sequence and it would be natural to assume that they are similarly recognized by the enzyme. On the other hand, however, the possibility of simultaneous interaction of protein kinases with several peptides or with several parts of the same protein may provide additional possibility for fine regulation of these enzymes and thus might have biological relevance.

This study has revealed that the influence of inhibitors on PKA interaction with substrates (ATP and kemptide) can be characterized figuratively in terms of the appropriate interaction factors. If the interaction factors have values above 1, simultaneous binding of two ligands is hindered and negative allostery is in operation. On the other hand, as the interaction factors can be calculated for each pair of ligands, these values present information about these interactions. For example, the results shown in Table 2 demonstrate that influence of inhibitors upon binding of ATP and kemptide was clearly asymmetric and this asymmetry depends on the nature of inhibitors. More complex inhibition pattern can be observed with the peptide inhibitor, where positive allostery was observed (Table 2). In summary, if compared with the classical inhibitor analysis, this approach provides more complete picture about the inhibition process, characterizing mutual interaction of the enzyme-bound ligands in terms of the interaction factors as defined by reaction schemes 2, 3 and 11. However, interaction factors can be calculated also from binding data, if affinity of the free enzyme and the enzyme substrate complex for this ligand has been determined.

Prior observations of allostery in ligand binding with PKA

In the following part of this work we have compiled experimental data from the literature, which also demonstrate the presence of allostery in PKA interaction with different ligands, and allow quantification of this effect, but were not analyzed following this concept beforehand. In this study, these binding data were analyzed according with the reaction mechanism presented in Eq. 2, where ATP or ATP-like ligands were denoted as L_1 and peptides were denoted as L_2. The allosteric effect was quantified as ratio of the dissociation constants for the ternary and binary complexes, respectively. Summary of these data are listed in Table 3 together with other results, obtained in this study.

Firstly, the influence of ATP on binding effectiveness of peptide inhibitors with PKA has been observed in many papers (Lew *et al.*, 1997a, Demaille *et al.*, 1977, Herberg *et al.*, 1994, Kuznetsov and Järv, 2008a, Whitehouse *et al.*, 1983, Whitehouse and Walsh, 1983), and initially this phenomenon has been described as "synergism" of ligand binding. Firsthand, such "synergistic" effect was observed in interaction of the catalytic and regulatory subunits of cAMP-dependent protein kinase. Without ATP the complex was characterized by the dissociation constant 125 nM (Herberg and Taylor, 1993). However, if interaction between the catalytic and regulatory subunits was assayed by surface plasmon resonance in the presence of excess of ATP (more precisely ATP–Mg complex), the value of the dissociation constant 0.04 nM was calculated from on-rate and off-rate kinetic data (Herberg *et al.*, 1994). Later, quite similar dissociation constant, equal to 0.1 nM, was published (Gibson and Taylor, 1997). From these data the χ values 0.0003 to 0.0008 can be calculated as ratio of the dissociation constants for the ligand-enzyme complex, denoted as K_{L2} in Eq. 2, and the dissociation constant for ligand-enzyme-ATP complex, denoted as χK_{L2} in the same scheme.

Similar situation has been observed with the heat-stable inhibitor protein PKI, which effectively binds with PKA. In the absence of ATP, the enzyme affinity for this inhibitor was characterized by the dissociation constant 2.3 μM (Herberg and Taylor, 1993). For the same compound, however, inhibition constants 2 nM (Demaille *et al.*, 1977) and 0.49 nM (Whitehouse and Walsh, 1983) were determined at saturating ATP and zero peptide substrate concentrations. Analogously, the interaction factors 0.0009 and 0.0002 were calculated from these binding data.

These results clearly demonstrate that stability of PKA complex with these potent inhibitors can be increased more than three powers of magnitude in the presence of ATP-Mg complex. However, Eq. 2 suggests that in the presence of these inhibitors similar increase should be observed also in ATP binding effectiveness, as both dissociation constants include the same interaction factor χ. Indeed, this enhancement of PKA affinity for ATP has been found experimentally, as the dissociation constant, ranging between 20 and 60 nM, was

determined for interaction of ATP with the enzyme-PKI complex (Whitehouse and Walsh, 1983). Using the dissociation constant 49 μM for ATP complex with the free catalytic subunit of this enzyme, as determined in this study, the interaction factor 0.0008 could be calculated from these data. This value agreed well with the results above, calculated from binding data for PKI.

Later, ATP binding with acrylodan-modified PKA catalytic subunit has been studied in the absence and in the presence of different peptide inhibitors, including the regulatory subunit and PKI (Lew *et al.*, 1997a). As labeling of this protein had no significant effect on catalytic properties of the enzyme, these data were also used for calculation of the values of interaction factors. Comparing binding effectiveness of ATP with the free enzyme and with the appropriate enzyme-inhibitor complexes, the χ values 0.0001 and 0.0005 were calculated for the regulatory subunit and PKI, respectively. This outcome was in good agreement with the results referred above, especially taking into consideration significant uncertainty limits of the available data. Further, using the similar approach, the χ values 0.0003 and 0.15 were calculated for PKI fragments PKI[5–24] and PKI[14–22]. And finally, Ala-kemptide binding with the enzyme has been assayed in the absence and presence of ATP, yielding the dissociation constants 230 μM and 320 μM, respectively (Whitehouse and Walsh, 1983). The χ value 1.4 was calculated from the latter data (Table 3).

Enhancement of ligand binding with PKA has been observed also in the presence of several ATP analogs, including AMPPNP, ADP and adenosine (Lew *et al.*, 1997a), and interaction factors were calculated from these data for different ligand-peptide pairs as listed in Table 3. In addition to these data, complex formation between protein kinase A, AMPPNP and kemptide was studied by using NMR spectroscopy and the dissociation constants for AMPPNP with the free enzyme and with the enzyme-kemptide complex were published (Masterson *et al.*, 2008). These data allow calculation of the χ value 0.3 for AMPPNP and kemptide pair. As all these ligands are recognized as ATP-site directed inhibitors, it seems to be natural that these results characterize allosteric properties related to this binding site.

Even provisional look at the results listed in Table 3 reveals that the allosteric effect, observed for the regulatory subunit, PKI and PKI[5–24] in combination with ATP, was much bigger if compared with similar effect observed in the presence ADP and AMPPNP, although binding effectiveness of all these nucleotides with the enzyme was rather similar. Thus, some additional interactions should govern the binding effectiveness of ATP and extra potent peptide inhibitors. These interactions were addressed more definitely by the following extra-thermodynamic analysis.

In summary, agreement between results of various experiments support the basic principle, formulated by Eq. 2 that the changes in enzyme affinity, induced by ligand binding, cannot be treated as “specific influence of ATP on peptide binding”, but have no “direction”, as the same interaction factor χ quantifies alteration of the enzyme affinity for both ligands L_1 and L_2. Certainly,

the term “synergistic binding” did not open this very important aspect of this phenomenon in the best way.

Allostery and ligand binding effectiveness

The results of our kinetic analysis of allostery in the PKA catalyzed substrate reactions (Table 1) and in inhibition of peptide phosphorylation reaction (Table 2), as well as the results of our analysis of literature data (Table 3) point to the fact that allostery depends on structure of ligands that in turn determines effectiveness of their binding with the enzyme. Therefore, it was natural to compare data about the binding effectiveness and allostery. This comparison reveals that more efficient ligand binding is accompanied by more significant allosteric effect, independently whether substrates or inhibitors are used. Considering this trend, the principle **“better binding: stronger allostery”** was formulated by us for the PKA catalyzed reactions. Intuitively, the existence of such interrelationship is not very surprising. Indeed, stronger ligand binding should cause major perturbation in the protein molecule.

It is interesting that this formulation is similar to the principle “better binding: better reaction”, advanced by J.R. Knowles for the α-chymoptrypsin catalyzed reactions in 1965 (Knowles, 1965). It is important that this similarity refers to similarity of the content of the phenomenon, as the statement “better binding: stronger allostery” compares also two distinct steps of enzyme catalysis. Firstly, we consider interaction of a ligand molecule with the free enzyme. Secondly, interaction of the same ligand with the pre-formed enzyme complex, containing another ligand, is considered. At the same time the principle “better binding: better reaction” was linking substrate binding effectiveness with the free energy of the transition state of the catalytic step. However, as the activation free energy of the catalytic step also includes interaction of the substrate transition state with the protein, clear analogy can be found between these formulations. Summing up, this analogy refers to enzyme ability to couple effectiveness of ligand binding with effectiveness of some following step of the catalytic process. Therefore, the principle “better binding: stronger allostery” could have rather general meaning for biocatalytic processes in general.

Different binding steps can be compared by using the LFE relationships, which set-up is based on the following principles. Interaction factors, denoted as α, β, γ or χ for different ligand combinations in reaction schemes 2, 3 and 11, are calculated as ratio of the appropriate dissociation constants. For example, the α value can be obtained from dissociation constants αK_b and K_b.

Table 3. Allosteric effects in PKA interaction with peptides, ATP and ATP-like ligands. K_i and K_b characterize inhibitor and substrate interaction with the free enzyme. Interaction factors were defined by Eqs 2 (χ), 3 (α), and 11 (γ), and have the same physical meaning independently of the symbol used.

Peptide		Protein kinase A affinity for peptide	Negative logarithm of interaction factor (pα, pγ or pχ)			
			ATP pK_i=4.6 [a]	**AMPPNP** pK_i=4.4 [b]	**ADP** pK_i=4.6 [a]	**Adenosine** pK_i=3.7 [a]
I	Protein kinase A regulatory subunit	pK_i=6.9 [c]	3.8 [a, d]	2.1 [a]	1.7 [a]	0.7 [a]
II	Heat-stable protein kinase inhibitor PKI	pK_i=5.6 [c]	3.3 [a, e, f]	1.5 [a]	1.3 [a]	0.5 [a]
III	PKI-fragment [5–24] TTYADFIASGR-TGRRNAIHD	pK_i=6.2 [g]	3.5 [a]	1.7 [a]	–	–
IV	PKI fragment [14–22] GRTGRRNAI	pK_i=5.2 [a]	0.8 [a]	0.7 [a]	1.2 [a]	–
V	Ala-kemptide-NH_2 LRRAALG-NH_2	pK_i=3.8 [h]	0.5 [h]	–	–	–
VI	Ala-kemptide LRRAALG	pK_i=3.6 [i]	–0.1 [i]	–	–	–
VII	RRYSV	pK_b=5.7 [j]	1.0 [j]	–	–	–
VIII	RRASVA	pK_b=4.6 [j]	0.7 [j]	–	–	–
IX	Kemptide LRRASLG	pK_b=4.4 [j]	0.4 [j]	0.5 [b]	–	–
X	RKRSRKE	pK_b=3.9 [j]	0.3 [j]	–	–	–
XI	LRKASLG	pK_b=3.6 [j]	0.2 [j]	–	–	–
XII	LARASLG	pK_b=2.7 [j]	–0.1 [j]	–	–	–
XIII	LRAASLG	pK_b=2.2 [j]	–0.5 [j]	–	–	–

[a] (Lew *et al.*, 1997a),
[b] (Masterson *et al.*, 2008),
[c] (Herberg and Taylor, 1993),
[d] (Herberg *et al.*, 1994),
[e] (Demaille *et al.*, 1977),
[f] (Whitehouse and Walsh, 1983),
[g] (Cheng *et al.*, 1985),
[h] (Kuznetsov and Järv, 2008a),
[i] (Whitehouse *et al.*, 1983),
[j] (Kuznetsov and Järv, 2008b).

As these dissociation constants are linked to the free energy of formation of enzyme-ligand complexes,

$$\Delta G = - RT \ln K = 2.3\ RT\ pK \quad , \tag{22}$$

the interaction factor α can be used for calculation of the free energy of allosteric interaction (ΔG_{allo}), which quantifies interactions between two enzyme-bound ligands. For substrates A and B:

$$\Delta G_{allo} = 2.3\ RT\ p\alpha \quad , \tag{23}$$

where pα is the negative logarithm of the α value. As logarithmic values of kinetic parameters are conventionally used in linear free-energy relationships instead of the appropriate ΔG values, the following correlations were also based on application of the negative logarithms of the interaction factors.

LFE relationships and allostery

The principle "better binding: stronger allostery" has been formalized in terms of LFE relationships. For example, interrelationships between pα and pK_b as well as between pγ and pK_i were presented by the following equations:

$$p\alpha = C_b + S_b\ pK_b \quad , \tag{24}$$

and

$$p\gamma = C_i + S_i\ pK_i \quad , \tag{25}$$

where C stands for intercept and S stands for slope of the plot.

Summary of these plots is shown in Fig. 9. It can be seen that data for peptide substrates, marked by filled circles, follow Eq. 24, and were characterized by the intercept value $C_b = -1.4 \pm 0.1$ and by the slope value $S_b = 0.43 \pm 0.03$. Data for inhibitory peptides, marked by empty circles, show a more complicated pattern and form very clearly two separate series. The first serie consists of three peptides, marked as compounds IV – VI in Table 3.

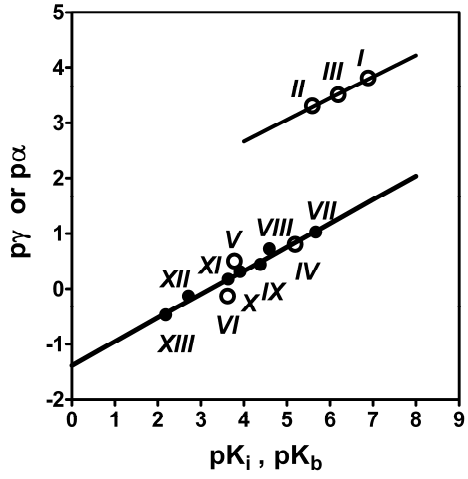


Figure 9. Linear free-energy relationship between pα or pγ, characterizing allosteric interaction of enzyme-bound ATP and peptides, and pK_a or pK_i values, characterizing affinity of the free protein kinase A for these ligands. The interaction factors α or γ were calculated from published data and numbers of compounds refer to Table 3. This table lists also references used for calculation of these interaction factors.

Data for these inhibitors fitted well into the correlation obtained for peptide substrates, and $C_i = C_b$ and $S_i = S_b$ in Eqs 24 and 25. This conclusion was confirmed by the results of processing of these consolidated data, yielding the slope value 0.43 ± 0.05 and intercept value -1.4 ± 0.2 ($r = 0.95$), which did not differ from the parameters calculated for peptide substrates alone. Consequently, the same mechanism should govern interrelationship between allostery and ligand binding effectiveness of these inhibitors and substrates.

It can be seen in Fig. 9 that data-points for the regulatory subunit, PKI and PKI[5–24], labeled as ligands I–III, decline from the common correlation obtained for substrate and inhibitory peptides. Of course, PKA has high affinity for these peptides, but still not sufficiently high to justify the pγ values above 3 logarithmic units. This means that the allosteric properties of these potent inhibitors should be enhanced through some additional interaction mechanism, which does not operate with other inhibitors and substrates. Proceeding from the common linear free-energy relationship for other ligands, these specific effects can be quantified by calculating deviations of the experimental pγ values from the correlation line. It appears that these deviations do not depend on inhibitor structure. Therefore, an extra term Z_i was included into the correlation equation 25 to consider this specific effect:

$$p\gamma = C_i + S_i\, pK_i + Z_i \quad . \qquad (26)$$

For most peptides $Z_i = 0$, and for the regulatory subunit, PKI and PKI[5–24] $Z_i = 2$. Although the physical background of this extra effect Z_i should emerge from some separate study, this way of definition and quantification of new interactions is undoubtedly an important merit of the extra-thermodynamic approach used.

Until now the influence of peptide structure on PKA allostery in the presence of ATP has been discussed. However, results compiled in Table 3 demonstrate that allosteric behavior can be observed also with ADP, AMPPNP and adenosine. To compare allosteric effects of these ligands, the correlation of $p\gamma$ *vs* pK_i for peptide inhibitors was analyzed. In this plot data for different ATP-site directed ligands were included (Fig. 10), where circles denote ATP, squares stand for AMPPNP and triangles for ADP. Two data-points for adenosine were marked with stars. Peptides were marked with arrows according with their pK_i values and the numbers of arrows refer to the list in Table 3.

Interestingly, a common relationship can be observed for allosteric effects, observed in the presence of different nucleotides, with exception of the free data-points for potent inhibitors and ATP. Following Eq. 25 this common linear correlation is characterized by $C_i = -2.0 \pm 0.4$ and $S_i = 0.57 \pm 0.07$ ($r = 0.94$). Interestingly, data for the three extra potent peptide inhibitors were also described by this relationship, if other nucleotides except ATP were involved in this analysis.

The common correlation shown for ATP, ADP and AMPPNP in Fig. 10 demonstrates that these nucleotides have similar capacity to induce allosteric effect. On the other hand, however, allosteric effects observed in the presence of adenosine were relatively small and the appropriate experimental data clearly

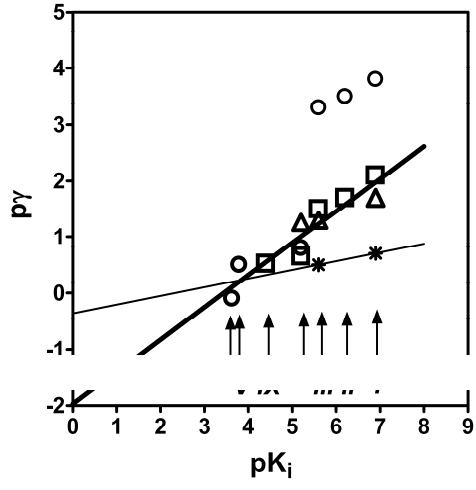


Figure 10. LFE relationship between $p\gamma$ and pK_i for peptide inhibitors, interacting with protein kinase A in the presence of ATP (circles), AMPPNP (squares), ADP (triangles) and adenosine (stars). Arrows mark affinity of protein kinase A for the used compounds and numbers refer to the list of peptides in Table 3.

deviated from the common correlation line obtained for other nucleotides. As affinity of the enzyme for adenosine is also lower, this result is in good agreement with the principle “better binding: stronger allostery”, stressing that not ligand structure, but its binding effectiveness with the enzyme is the key factor for triggering off the allosteric effect.

If the principle “better binding: stronger allostery” holds similarly for peptides and nucleotides, it can be expected that a common LFE relationship can be derived to describe allosteric effect for different pairs of these ligands in general, independently of their type of action. Therefore, we return to the reaction scheme 2, where allostery is defined by the interaction factor χ. Importantly, χ is equivalent of α, if data for ATP and peptide substrate are involved, or γ, if data for ATP and peptide inhibitors are involved. Using this general definition of interaction factors, the following expression for allosteric effects can be presented, where ligand L_1 means peptide (substrate or inhibitor) and L_2 denotes ATP or its congeners, nucleotides or other ATP-site directed ligands:

$$p\chi = C + S_{L1}\, pK_{L1} + S_{L2}\, pK_{L2} + Z_i \quad . \qquad (27)$$

This equation is, in fact, superposition of Eqs 24 and 26. The term Z_i has the same meaning as in Eq. 26 and stands for specific interaction, observed in the case of ATP in combination with the regulatory subunit, PKI and PKI[5-24]. For these ligands $Z_i = 2$, while for all other ligands $Z_i = 0$. If all data listed in Table 1 were processed by this correlation equation, the following results were obtained:

$C = -6 \pm 1$
$S_{L1} = 0.5 \pm 0.1$ (peptides)
$S_{L2} = 1.0 \pm 0.2$ (ATP and analogs)
$Z_i = 1.9 \pm 0.2$ (regulatory subunit, PKI and PKI[5–24] in combination with ATP).
$r = 0.981$, standard deviation 0.241

The scatter plot (calculated *vs* experimental $p\chi$ values) for this regression is presented in Fig. 11. This plot shows that the interaction factors can be effectively predicted for all ligand combinations listed in Table 3 and Eq. 27 provides statistically relevant presentation of the allosteric effect.

It can be seen that the sensitivity factors S_i are different for peptides and ATP site directed ligands, as different interactions govern ligand binding in these sites. Therefore, changes in nucleotide and peptide affinity should affect allostery differently.

Secondly, the results of this analysis indicate that allosteric phenomena in PKA do not depend on nature of the ligands involved, and can be equally

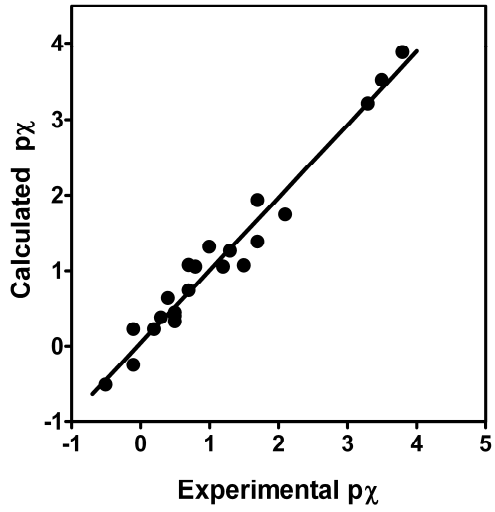


Figure 11. Scatter plot showing the interrelationship between the calculated and observed allosteric effects (pχ) for PKA interaction with different pairs of peptides and ATP-site directed ligands. All data listed in Table 3 were involved in this correlation and calculations were made by correlation equation 27. The results are: slope 0.96 ± 0.05, Y-intercept 0.04 ± 0.07, r = 0.980.

initiated by substrates and inhibitors, which specifically interact with the enzyme. This situation justifies once more the principle "better binding: stronger allostery".

Thirdly, this linear free-energy relationship indicates that allostery is exclusively governed by ligand binding effectiveness, except the three extra potent peptides. However, even in this case the slope of the pγ *vs* pK_i plot was close to the S_i value. Therefore, the same specificity determining factors, which govern ligand binding effectiveness, should also reveal in allostery. Formally this means that enzyme specificity is boosted by allostery, as the same structural fragment of ligand is recognized by the enzyme twice. Therefore, all kinetic parameters, which involve contribution of allostery, cannot be analyzed by the classical structure-activity relationships, based on presumption that effects of the specificity determining factors (or structural fragments) can be calculated additively. Contribution of a certain group of substrate molecule into its overall binding effectiveness depends on overall binding effectiveness of this molecule. And more generally, this contribution should also depend on binding properties of the second ligand, as the allosteric effect is governed by binding effectiveness of both compounds, as formalized by Eq. 27. Certainly, this situation complicates theoretical presentation of substrate specificity of PKA, and application of structure-activity relationships for prediction of kinetic parameters for allosterically regulated bisubstrate enzymes in general.

Interestingly, the role of flexibility of protein binding sites has been mentioned recently as possible source of outliers in structure-activity relationships, based on additive interaction models (Kim, 2007).

Finally, the extra-thermodynamic relationships discussed above allow presentation of the dependence of allostery upon ligand binding effectiveness by means of a continuous function. This means that the allosteric effect cannot be explained by shift between enumerate conformational states of the enzyme. More likely, the dynamic protein molecule may continually change its conformation and through these changes smoothly modulate the binding properties of its binding sites. Probably we observe in these experiments the shift of protein conformational populations, as suggested by Gunasekaran *et al.* (2004). Perhaps these changes can be compared by the non-specific solvation phenomena of organic molecules in different media. Certainly, this model of allostery presumes extra "soft" and highly dynamic protein structure, and complicates presentation of ligand recognition mechanism by counting the presence or absence of distinct interactions between ligand and protein, as is made in the conventional structural biology. Perhaps protein kinase A is an example of such highly dynamic protein.

Structure-induced inversion of allostery

It is not surprising that stronger deformation of PKA structure by more efficient ligand binding could result in bigger change in binding properties of another site, and in the case of positive allostery ($p\chi > 0$) binding of the first ligand enhances enzyme affinity for the second ligand. However, quantitative characterization of PKA allostery in terms of correlation equation 27 opens possibility for a more thorough analysis of this phenomenon.

It is evident from plots shown in Figs 9 and 10 that $p\alpha$ and $p\gamma$ may also have negative values. The same conclusion can be drawn from Eq. 27 for $p\chi$. This means that for certain combination of allosterically interacting ligands negative cooperativity can be revealed and these substrates or inhibitors hinder binding of each other. It is obvious that between the regions of positive cooperativity ($p\chi > 0$) and negative cooperativity ($p\chi < 0$) we must have $p\chi = 0$, and this specific point can be reached at some specific combination of the pK_{L1} and pK_{L2} values. Under these conditions the enzyme abolishes its allosteric properties and no mutual influence of ligands on their binding effectiveness should be observed. This situation can be denominated as ligand structure induced inversion of allostery. The conditions of this inversion can be specified by using Eq. 27. If we omit for simplicity the extra potent ligands and take $Z_i = 0$, the following interrelationship between pK_{L1} and pK_{L2} can be obtained to denote the inversion area, where $p\chi = 0$:

$$pK_{L1} = 12 - 2\,pK_{L2} \quad . \qquad (28)$$

For example, taking pK_{L2} = 4.6 (PKA affinity for ATP), pK_{L1} = 2.8 can be calculated from this equation. This value agrees with the X-axis intercept of the correlation line shown in Fig. 9 and denotes threshold for enzyme affinity for peptides, where inversion of allostery should take place in the presence of ATP. If enzyme affinity for a peptide remains under this threshold, binding of this substrate (or inhibitor) is hindered by allostery. This inversion of allostery can be observed experimentally, as some peptides listed in Table 1 are characterized by the α value above one.

The dependence of allostery upon ligand structure and especially the possibility of inversion of this effect from positive allostery to negative allostery may have principal importance for regulatory phosphorylation in general. Selectivity of this process is controlled not only by substrate binding, but also through the allosteric mechanism, which may additionally support or hinder enzyme interaction with substrates. This mechanism provides functionally flexible way for up- or down-regulation of enzyme activity and might have physiological importance, as phosphorylation of wrong substrate can be prevented even under conditions, where the enzyme is loaded with this substrate following the mass action law. Thus, the outcome of this allosteric regulation in monomeric enzymes is the same as defined by classical theories of allostery for multimeric enzymes: making the protein response more efficient if proper ligand binds. As protein phosphorylation affects significant part of the proteome, this mechanism of specificity boosting may have multiple implications in control of vital cellular processes.

CONCLUSIONS

1. Steady-state methods of kinetic analysis can be used as effective tools for description of the PKA catalyzed reactions with peptide substrates and inhibition of this enzyme, taking into consideration the bisubstrate catalytic mechanism of this reaction. These data allow determination of appropriate kinetic parameters and characterization of several complexes, formed between the enzyme and two substrates as well as between the enzyme, substrates and inhibitors. Formation of all these complexes can be discussed in terms of allostery. Application of the second-order rate constants simplified the process of this kinetic analysis.
2. Allostery governs the PKA catalyzed peptide phosphorylation reaction and these effects were quantified in terms of the "interaction factors". It was discovered that these allosteric effects depend on peptide structure and change in parallel with alteration of binding effectiveness of these substrates. This general trend has been summarized through formulation of the principle "better binding: stronger allostery".
3. Interaction of PKA with three different inhibitors includes formation of various complexes between these inhibitors and the enzyme, as well as between the inhibitors, substrates and the enzyme. Although the complex inhibition pattern revealed asymmetric interaction of these inhibitors with ATP and peptide substrate, there was no sharp distinction between the behavior of inhibitors, designed as "single-site directed" compounds or as bisubstrate analog inhibitors. In formation of various complexes with inhibitors also the allosteric properties of the enzyme were observed that gave rise to formulation of the principle "better binding: stronger allostery" also for inhibitors.
4. The main conclusions from the present kinetic analysis of PKA allostery were supported by results of survey of literature data, which also allowed quantitative characterization of PKA allostery in ligand binding processes. Taking together, all these data allowed clarification of the general pattern of allostery in this enzyme and advanced the existing understandings about its specificity.
5. Correlation between allosteric effect and binding effectiveness of ligands was formalized in terms of typical extra-thermodynamic analysis and the observed interrelationships between ligand binding and allostery were characterized by using the linear free-energy relationships. This analysis revealed the possibility that variation in ligand structure and binding effectiveness can result in inversion of the allosteric effect. This means that positive allostery, which is supporting ligand binding, can be changed into negative allostery for bad ligands. This phenomenon can have wide implication for theory of biocatalysis in general, and in the case of PKA seems to function as an additional specificity determining mechanism, which prevents phosphorylation of wrong substrates.

SUMMARY

In this dissertation survey of existing data about structure and functioning of PKA is given with special reference to dynamic properties of this enzyme. These data strongly emphasize the importance of experimental approaches, where these dynamic properties of the enzyme can be studied experimentally. Among these approaches methods of kinetic analysis of the enzyme-catalyzed reactions and their inhibition by reversibly binding ligands seem to have still solid status. As PKA catalyzed reactions are typical bisubstrate reactions, the appropriate kinetic models must be used in this case. However, it was revealed that in several cases this was not a trivial approach, especially in the case of reversible inhibition of this enzyme. Therefore kinetic formalism of this analysis was recapitulated with special reference to the importance of the second-order rate constants for this analysis. It was found that application of the second-order rate constants for analysis of bisubstrate enzymatic reaction may give good results and this approach was applied for studying of several aspects as substrate reaction as well as interaction of inhibitors with this enzyme.

The kinetic methods of analysis were used for study of allosteric cooperativity between peptide and ATP binding sites of PKA in reaction with seven substrates. The allosteric effect was quantified in terms of the interaction factor α that reflects the magnitude of the allosteric feedback between PKA binding sites for different peptides. The principle **"better binding: stronger allostery"** was formulated for this reaction. This interrelationship was further formalized in terms of a linear free-energy relationship. It was concluded that the enzyme affinity for good substrates can be additionally enhanced by allostery, and thus this effect is in use for discrimination between good and bad substrates.

More complicated kinetic analysis was made with inhibitors, including the ATP-site directed compound H89, peptide inhibitor LRRAALG-NH_2 and bisubstrate inhibitor AdoAhxArg$_6$. It was found that all these inhibitors revealed asymmetric interaction with the two substrates that was also described in terms of the interaction factors. In the case of PKA inhibition, the binding effectiveness of inhibitors was not governed only by structure of these molecules, but also depended on properties of substrates present. Binding effectiveness of the peptide LRRAALG-NH_2 was increased in the presence of ATP, pointing to the positive allostery. Accordingly, the formation of the quaternary complex between the enzyme, peptide inhibitor, ATP and peptide substrate was detected experimentally.

In the last part of this work, we compared our results with data described in other studies by using linear free-energy relationships. The common equation was derived to describe allosteric effect for different pairs of ligands directed to ATP and peptide binding sites. This approach was used independently whether the ligand binding with protein kinase A results in the phosphorylation reaction or blocks the active site. Results of this analysis revealed that the change in

ligand affinity caused different allostery. Formally this means that enzyme specificity for substrates and inhibitors can be boosted by allostery. This mechanism provides functionally flexible way for up- or down-regulation of enzyme activity and might have physiological importance. As protein phosphorylation affects significant part of the proteome, this mechanism of specificity boosting may have multiple implications in control of vital cellular processes.

14

SUMMARY IN ESTONIAN

Allosteerilised efektid cAMP-sõltuva proteiinkinaasi katalüütilise alaühiku reaktsioonides

cAMP poolt kontrollitava proteiinkinaasi katalüütilise alaühiku poolt katalüüsitavas reaktsioonis osaleb kaks substraati: ATP ja fosforüleeritav peptiid või valk. Katalüüsil toimub fosfaatrühma ülekanne ATP-lt peptiidile ning selleks peavad need erinevad substraadid seostuma samaaegselt ensüümiga. Kui neist ühe sidumine mõjutab teise sidumist, on tegemist allosteeria nähtusega. Samuti esineb allosteeria siis, kui omavahel toimivad eri sidumiskohtades asuvad substraat ja inhibiitor. Käesoleva töö eesmärgiks oli selliste allosteeriliste toimete uurimine cAMP-sõltuva proteiinkinaasi poolt katalüüsitavates reaktsioonides, võttes arvesse nende protsesside bisubstraatset iseloomu.

Allosteerilisi toimeid iseloomustati kvantitatiivselt nn interaktsioonifaktorite abil, mis võrdlevad ATP, substraadi või inhibiitori sidumise efektiivsust vabale ensüümile ja selle kompleksile ühega neist ligandidest. Sellise analüüsi teostamiseks oli vajalik arendada bisubstraatsete ensüümreaktsioonide kineetika analüüsi põhimõtteid. Kasutusele võeti ensüümkatalüüsi teist järku kiiruskonstandid, mis võimaldavad vältida reaktsiooni mehhanismist tuleneda võivaid komplikatsioone ja lubavad iseloomustada reaktsiooni toimumise tingimustes substraatide ja ligandide toimet vaba ensüümiga. Välja töötatud meetodeid rakendati mitme substraadi ja inhibiitori ning cAMP-sõltuva proteiinkinaasi katalüütilise alaühiku (täpsemalt hiire Cα isosüümi) katalüütiliste omaduste uurimiseks.

Ensüümi pöörduvatest inhibiitoritest valiti uuringuteks ATP sidumiskoha blokeerimiseks loodud inhibiitor H89, peptiidinhibiitor LRRAALG-NH_2 ja bisubstraat-kompleksi analoogina loodud inhibiitor AdcAhxArg$_6$, mis kujutas endast peptiidi ja nukleotiidi konjugaati. Kineetika uuringute abil kirjeldati nende ligandide sidumist vabale ensüümile, ensüüm-ATP kompleksile ja ensüüm-peptiidsubstraat kompleksile. Samuti uuriti kolme ligandi samaaegse seostumise võimalusi. Saadud andmetest lähtudes kirjeldati ensüümi inhibeerimisel ilmnevaid allosteerilisi efekte ning iseloomustati neid vastavate interaktsiooni-faktorite abil. Tulemused näitasid, et nende inhibiitorite toime ATP ja peptiidsubstraadi (kemptiid) suhtes on asümmeetriline, inhibiitori LRRAALG-NH_2 sidumine aga võimendub ATP juuresolekul. Inhibeerimisel ilmnevaid allosteeria efekte võrreldi ka sidumiskatsete andmetega. See võimaldas formuleerida põhimõttelise seaduspärasuse „parem sidumine: tugevam allosteria“.

Allosteerilised toimed ilmnesid ensüümkatalüüsil ka ATP ja peptiidsubstraadi vahel ning neid efekte iseloomustati interaktsioonifaktorite abil. Seejuures ilmnes, et substraatreaktsiooni korral muutub sidumistsentrite vaheline allosteeriline toime koos peptiidi sidumise efektiivsuse muutumisega. Seega kehtib

põhimõte „parem sidumine: tugevam allosteeria“ ka ensüümi poolt katalüüsitud substraatreaktsiooni korral. Avastatud seaduspärasus võib seega osutuda ensüümide toime üldiseks põhimõtteks. Allosteeria ja ligandi sidumise efektiivsuse vahelist seost õnnestus esitada nende protsesside vabaenergia sõltuvuse kujul. Nendes sõltuvsutes iseloomustati allosteerilist efekti interaktsioonifaktori negatiivse logaritmi kaudu ja ligandi sidumise efektiivsust kirjeldati tekkiva kompleksi dissotsiatsioonikonstandi negatiivse logaritmi kaudu.

Leitud vabaenergia sõltuvustest järeldati, et allosteerilised toimed sidumiskohtade vahel võivad olla kirjeldatud sama toimemehhanismi abil nii substraadi kui inhibiitori jaoks. Samas sõltub allosteeria ligandi sidumise tugevusest ning see efekt võib pöörduda vastassuunaliseks, kui ligandi sidumise tugevus langeb allapoole teatud piiri. Seega võib positiivne allosteeriline efekt asenduda negatiivse allosteerilise toimega. Selline allosteeria inversioon leiti erineva struktuuriga peptiidide fosforüleerimise kineetikat uurides ka eksperimentaalselt. Tehti järeldus, et avastatud nähtus suurendab ensüümi afiinsust heade substraatide suhtes ja vähendab ensüümi afiinsust halbade substraatide suhtes.

See järeldus näitab, et allosteeriat võib vaadelda kui ensüümkatalüüsi spetsiifilisust võimendavat mehhanismi ning ta täidab monomeersete bisubstraatsete ensüümide korral sama rolli, mida märgivad ära klassikalised multimeersete ensüümide jaoks loodud kooperatiivsuse mudelid: suurendab ensüümi toime efektiivsust kui seostub õige ligand. Kuivõrd proteiinkinaaside osavõtul toimuv regulatoorne fosforüleerimine haarab valdavat osa proteoomist, võib avastatud substraatspetsiifilisuse võimendamise mehhanism mängida olulist rolli mitmete eluprotsesside regulatsioonil

REFERENCES

Acharya, K. R. and Lloyd, M. D. (2005) The advantages and limitations of protein crystal structures. *Trends Pharmacol Sci*, ***26***, 10–14.

Adams, J. A. and Taylor, S. S. (1992) Energetic limits of phosphotransfer in the catalytic subunit of cAMP-dependent protein kinase as measured by viscosity experiments. *Biochemistry*, ***31***, 8516–8522.

Akamine, P., Madhusudan, Wu, J., Xuong, N. H., Ten Eyck, L. F. and Taylor, S. S. (2003) Dynamic features of cAMP-dependent protein kinase revealed by apoenzyme crystal structure. *J Mol Biol*, ***327***, 159–171.

Akritopoulou-Zanze, I. (2006) The identification of new protein kinase inhibitors as targets in modern drug discovery. *IDrugs*, ***9***, 481–487.

Beebe, S. J., Salomonsky, P., Jahnsen, T. and Li, Y. (1992) The C gamma subunit is a unique isozyme of the cAMP-dependent protein kinase. *J Biol Chem,* ***267***, 25505–25512.

Blume-Jensen, P. and Hunter, T. (2001) Oncogenic kinase signalling. *Nature,* ***411***, 355–365.

Bogoyevitch, M. A., Barr, R. K. and Ketterman, A. J. (2005) Peptide inhibitors of protein kinases-discovery, characterisation and use. *Biochim Biophys Acta,* ***1754***, 79–99.

Bossemeyer, D., Engh, R. A., Kinzel, V., Ponstingl, H. and Huber, R. (1993) Phosphotransferase and substrate binding mechanism of the cAMP-dependent protein kinase catalytic subunit from porcine heart as deduced from the 2.0 A structure of the complex with Mn2+ adenylyl imidodiphosphate and inhibitor peptide PKI[5–24]. *Embo J,* ***12***, 849–859.

Bramson, H. N., Thomas, N. E. and Kaiser, E. T. (1985) The use of N-methylated peptides and depsipeptides to probe the binding of heptapeptide substrates to cAMP-dependent protein kinase. *J Biol Chem,* ***260***, 15452–15457.

Breitenlechner, C., Gassel, M., Engh, R. and Bossemeyer, D. (2004) Structural insights into AGC kinase inhibition. *Oncol Res,* ***14***, 267–278.

Cheley, S. and Bayley, H. (1991) Kinetics and regulation of two catalytic subunits of cAMP-dependent protein kinase from Aplysia californica. *Biochemistry,* ***30***, 10246–10255.

Cheng, H. C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten, S. M. and Walsh, D. A. (1986) A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. *J Biol Chem,* ***261***, 989–992.

Cheng, H. C., van Patten, S. M., Smith, A. J. and Walsh, D. A. (1985) An active twenty-amino-acid-residue peptide derived from the inhibitor protein of the cyclic AMP-dependent protein kinase. *Biochem J,* ***231***, 655–661.

Cheng, X., Ma, Y., Moore, M., Hemmings, B. A. and Taylor, S. S. (1998) Phosphorylation and activation of cAMP-dependent protein kinase by phosphoinositide-dependent protein kinase. *Proc Natl Acad Sci USA,* ***95***, 9849–9854.

Cherry, M. and Williams, D. H. (2004) Recent kinase and kinase inhibitor X-ray structures: mechanisms of inhibition and selectivity insights. *Curr Med Chem,* ***11***, 663–673.

Chrivia, J. C., Uhler, M. D. and McKnight, G. S. (1988) Characterization of genomic clones coding for the C alpha and C beta subunits of mouse cAMP-dependent protein kinase. *J Biol Chem,* ***263***, 5739–5744.

Colbran, J. L., Francis, S. H., Leach, A. B., Thomas, M. K., Jiang, H., McAllister, L. M. and Corbin, J. D. (1992) A phenylalanine in peptide substrates provides for selectivity between cGMP- and cAMP-dependent protein kinases. *J Biol Chem,* ***267***, 9589–9594.

Cook, P. F., Neville, M. E., Jr., Vrana, K. E., Hartl, F. T. and Roskoski, R., Jr. (1982) Adenosine cyclic 3',5'-monophosphate dependent protein kinase: kinetic mechanism for the bovine skeletal muscle catalytic subunit. *Biochemistry,* ***21***, 5794–5799.

Cui, Q. and Karplus, M. (2008) Allostery and cooperativity revisited. *Protein Sci,* ***17***, 1295–1307.

Davies, T. G., Verdonk, M. L., Graham, B., Saalau-Bethell, S., Hamlett, C. C., McHardy, T., Collins, I., Garrett, M. D., Workman, P., Woodhead, S. J., Jhoti, H. and Barford, D. (2007) A structural comparison of inhibitor binding to PKB, PKA and PKA-PKB chimera. *J Mol Biol,* ***367***, 882–894.

Demaille, J. G., Peters, K. A. and Fischer, E. H. (1977) Isolation and properties of the rabbit skeletal muscle protein inhibitor of adenosine 3',5'-monophosphate dependent protein kinases. *Biochemistry,* ***16***, 3080–3086.

DePristo, M. A., de Bakker, P. I. W. and Blundell, T. L. (2004) Heterogeneity and inaccuracy in protein structures solved by X-ray crystallography. *Structure,* ***12***, 831–838.

Doskeland, S. O., Maronde, E. and Gjertsen, B. T. (1993) The genetic subtypes of cAMP-dependent protein kinase – functionally different or redundant? *Biochim Biophys Acta,* ***1178***, 249–258.

Eisenthal, R., Danson, M. J. and Hough, D. W. (2007) Catalytic efficiency and k_{cat}/K_m: a useful comparator? *Trends Biotechnol,* ***25***, 247–249.

Engh, R. A., Girod, A., Kinzel, V., Huber, R. and Bossemeyer, D. (1996) Crystal structures of catalytic subunit of cAMP-dependent protein kinase in complex with isoquinolinesulfonyl protein kinase inhibitors H7, H8, and H89. Structural implications for selectivity. *J Biol Chem,* ***271***, 26157–26164.

ExPASy (2009) *www.expasy.org/enzyme/2.7.11.11*.

Feramisco, J. R., Glass, D. B. and Krebs, E. G. (1980) Optimal spatial requirements for the location of basic residues in peptide substrates for the cyclic AMP-dependent protein kinase. *J Biol Chem,* ***255***, 4240–4245.

Gamm, D. M., Baude, E. J. and Uhler, M. D. (1996) The major catalytic subunit isoforms of cAMP-dependent protein kinase have distinct biochemical properties in vitro and in vivo. *J Biol Chem,* ***271***, 15736–15742.

Garcia-Echeverria, C., Traxler, P. and Evans, D. B. (2000) ATP site-directed competitive and irreversible inhibitors of protein kinases. *Med Res Rev,* ***20***, 28–57.

Gerstein, M., Lesk, A. M. and Chothia, C. (1994) Structural mechanisms for domain movements in proteins. *Biochemistry,* ***33***, 6739–6749.

Gibbs, C. S. and Zoller, M. J. (1991) Identification of electrostatic interactions that determine the phosphorylation site specificity of the cAMP-dependent protein kinase. *Biochemistry,* ***30***, 5329–5334.

Gibson, R. M. and Taylor, S. S. (1997) Dissecting the cooperative reassociation of the regulatory and catalytic subunits of cAMP-dependent protein kinase. Role of Trp-196 in the catalytic subunit. *J Biol Chem,* ***272***, 31998–32005.

Glass, D. B., Feller, M. J., Levin, L. R. and Walsh, D. A. (1992) Structural basis for the low affinities of yeast cAMP-dependent and mammalian cGMP-dependent protein kinases for protein kinase inhibitor peptides. *Biochemistry,* ***31***, 1728–1734.

Glass, D. B. and Krebs, E. G. (1979) Comparison of the substrate specificity of adenosine 3':5'-monophosphate- and guanosine 3':5'-monophosphate-dependent protein kinases. Kinetic studies using synthetic peptides corresponding to phosphorylation sites in histone H2B. *J Biol Chem,* ***254***, 9728–9738.

Glass, D. B., Trewhella, J., Mitchell, R. D. and Walsh, D. A. (1995) Conformationally constrained analogs of protein kinase inhibitor (6–22)amide: effect of turn structures in the center of the peptide on inhibition of cAMP-dependent protein kinase. *Protein Sci,* ***4***, 405–415.

Gompel, M., Leost, M., De Kier Joffe, E. B., Puricelli, L., Franco, L. H., Palermo, J. and Meijer, L. (2004) Meridianins, a new family of protein kinase inhibitors isolated from the ascidian Aplidium meridianum. *Bioorg Med Chem Lett,* ***14***, 1703–1707.

Grant, B. D. and Adams, J. A. (1996) Pre-steady-state kinetic analysis of cAMP-dependent protein kinase using rapid quench flow techniques. *Biochemistry,* ***35***, 2022–2029.

Gunasekaran, K., Ma, B. and Nussinov, R. (2004) Is allostery an intrinsic property of all dynamic proteins? *Proteins,* ***57***, 433–443.

Hanks, S. K. and Hunter, T. (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J,* ***9***, 576–596.

Hanks, S. K., Quinn, A. M. and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science,* ***241***, 42–52.

Henis, Y. I. and Levitzki, A. (1979) Ligand competition curves as a diagnostic tool for delineating the nature of site-site interactions: theory. *Eur J Biochem,* ***102***, 449–465.

Herberg, F. W., Dostmann, W. R., Zorn, M., Davis, S. J. and Taylor, S. S. (1994) Crosstalk between domains in the regulatory subunit of cAMP-dependent protein kinase: influence of amino terminus on cAMP binding and holoenzyme formation. *Biochemistry,* ***33***, 7485–7494.

Herberg, F. W., Doyle, M. L., Cox, S. and Taylor, S. S. (1999) Dissection of the nucleotide and metal-phosphate binding sites in cAMP-dependent protein kinase. *Biochemistry,* ***38***, 6352–6360.

Herberg, F. W. and Taylor, S. S. (1993) Physiological inhibitors of the catalytic subunit of cAMP-dependent protein kinase: effect of MgATP on protein-protein interactions. *Biochemistry,* ***32***, 14015–14022.

Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry,* ***23***, 5036–5041.

Hines, A. C. and Cole, P. A. (2004) Design, synthesis, and characterization of an ATP-peptide conjugate inhibitor of protein kinase A. *Bioorg Med Chem Lett,* ***14***, 2951–2954.

Ho, M., Bramson, H. N., Hansen, D. E., Knowles, J. R. and Kaiser, E. T. (1988) Stereochemical course of the phospho group transfer catalyzed by cAMP-dependent protein kinase. *J Am Chem Soc,* ***110***, 2680–2681.

Hunter, T. (2000) Signaling-2000 and beyond. *Cell,* ***100***, 113–127.

Järv, J. and Ragnarsson, U. (1991) Linear free energy relationships in cAMP-dependent protein kinase reactions with synthetic substrates. *Bioorg Chem,* ***19***, 77–87.

Järv, J., Sak, K., Eller, M., Ek, P., Engström, A. and Engström, L. (1996) Quantitative structure-activity relationships in the protein kinase C reaction with synthetic peptides derived from myelin basic protein. *Bioorg Chem,* ***24***, 159–168.

Johnson, L. N., Noble, M. E. and Owen, D. J. (1996) Active and inactive protein kinases: structural basis for regulation. *Cell,* ***85***, 149–158.

Kase, H., Iwahashi, K., Nakanishi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A. and Kaneko, M. (1987) K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem Biophys Res Commun,* ***142***, 436–440.

Kemp, B. E., Graves, D. J., Benjamini, E. and Krebs, E. G. (1977) Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. *J Biol Chem,* ***252***, 4888–4894.

Kim, C., Cheng, C. Y., Saldanha, S. A. and Taylor, S. S. (2007) PKA-I holoenzyme structure reveals a mechanism for cAMP-dependent activation. *Cell,* ***130***, 1032–1043.

Kim, K. H. (2007) Outliers in SAR and QSAR: is unusual binding mode a possible source of outliers? *J Comput Aided Mol Des,* ***21***, 63–86.

Knighton, D. R., Bell, S. M., Zheng, J., Ten Eyck, L. F., Xuong, N. H., Taylor, S. S. and Sowadski, J. M. (1993) 2.0 Å refined crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with a peptide inhibitor and detergent. *Acta Crystallogr D Biol Crystallogr,* ***49***, 357–361.

Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Ashford, V. A., Xuong, N. H., Taylor, S. S. and Sowadski, J. M. (1991a) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science,* ***253***, 407–414.

Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Xuong, N. H., Taylor, S. S. and Sowadski, J. M. (1991b) Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science,* ***253***, 414–420.

Knowles, J. R. (1965) Enzyme specificity: alpha-chymotrypsin. *J Theor Biol,* ***9***, 213–228.

Kong, C. T. and Cook, P. F. (1988) Isotope partitioning in the adenosine 3',5'-mono-phosphate dependent protein kinase reaction indicates a steady-state random kinetic mechanism. *Biochemistry,* ***27***, 4795–4799.

Koshland, D. E. (2002) The application and usefulness of the ratio k(cat)/K(M). *Bioorg Chem,* ***30***, 211–213.

Koshland, D. E., Jr. and Hamadani, K. (2002) Proteomics and models for enzyme cooperativity. *J Biol Chem,* ***277***, 46841–46844.

Koshland, D. E., Jr., Nemethy, G. and Filmer, D. (1966) Comparison of experimental binding data and theoretical models in proteins containing subunits. *Biochemistry,* ***5***, 365–385.

Krebs, E. G. and Fischer, E. H. (1955) Phosphorylase activity of skeletal muscle extracts. *J Biol Chem,* ***216***, 113–120.

Kreegipuu, A., Blom, N., Brunak, S. and Järv, J. (1998) Statistical analysis of protein kinase specificity determinants. *FEBS Lett,* ***430***, 45–50.

Kuznetsov, A. and Järv, J. (2008a) Allosteric cooperativity in inhibition of protein kinase A catalytic subunit. *Open Enzym Inhib J,* ***1***, 42–47.

Kuznetsov, A. and Järv, J. (2008b) Single-subunit allostery in kinetics of peptide phosphorylation by protein kinase A. *Proc Estonian Acad Sci Chemistry,* ***57***, 247–254.

Kuznetsov, A., Oskolkov, N., Hansen, M. and Järv, J. (2003) Steady-state kinetic analysis of protein kinase A interaction with peptide and ATP. *Proc Estonian Acad Sci Chemistry,* ***52***, 165–177.

Kuznetsov, A., Uri, A., Raidaru, G. and Järv, J. (2004) Kinetic analysis of inhibition of cAMP-dependent protein kinase catalytic subunit by the peptide-nucleoside conjugate AdcAhxArg(6). *Bioorg Chem,* ***32***, 527–535.

Langer, T., Vogtherr, M., Elshorst, B., Betz, M., Schieborr, U., Saxena, K. and Schwalbe, H. (2004) NMR backbone assignment of a protein kinase catalytic domain by a combination of several approaches: application to the catalytic subunit of cAMP-dependent protein kinase. *Chembiochem,* ***5***, 1508–1516.

Leader, D. P., Deana, A. D., Marchiori, F., Purves, F. C. and Pinna, L. A. (1991) Further definition of the substrate specificity of the alpha-herpesvirus protein kinase and comparison with protein kinases A and C. *Biochim Biophys Acta,* ***1091***, 426–431.

Lew, J., Coruh, N., Tsigelny, I., Garrod, S. and Taylor, S. S. (1997a) Synergistic binding of nucleotides and inhibitors to cAMP-dependent protein kinase examined by acrylodan fluorescence spectroscopy. *J Biol Chem,* ***272***, 1507–1513.

Lew, J., Taylor, S. S. and Adams, J. A. (1997b) Identification of a partially rate-determining step in the catalytic mechanism of cAMP-dependent protein kinase: a transient kinetic study using stopped-flow fluorescence spectroscopy. *Biochemistry,* ***36***, 6717–6724.

Liang, J., Kim, J. R., Boock, J. T., Mansell, T. J. and Ostermeier, M. (2007) Ligand binding and allostery can emerge simultaneously. *Protein Sci,* ***16***, 929–937.

Lochner, A. and Moolman, J. A. (2006) The many faces of H89: a review. *Cardiovasc Drug Rev,* ***24***, 261–274.

Loog, M., Uri, A., Raidaru, G., Järv, J. and Ek, P. (1999) Adenosine-5'-carboxylic acid peptidyl derivatives as inhibitors of protein kinases. *Bioorg Med Chem Lett,* ***9***, 1447–1452.

Madhusudan, Trafny, E. A., Xuong, N. H., Adams, J. A., Ten Eyck, L. F., Taylor, S. S. and Sowadski, J. M. (1994) cAMP-dependent protein kinase: crystallographic insights into substrate recognition and phosphotransfer. *Protein Sci,* ***3***, 176–187.

Manning, G., Whyte, D. B., Martinez, R., Hunter, T. and Sudarsanam, S. (2002) The protein kinase complement of the human genome. *Science,* ***298***, 1912–1934.

Masterson, L. R., Mascioni, A., Traaseth, N. J., Taylor, S. S. and Veglia, G. (2008) Allosteric cooperativity in protein kinase A. *Proc Natl Acad Sci USA,* ***105***, 506–511.

Medzihradszky, D., Chen, S. L., Kenyon, G. L. and Gibson, B. W. (1994) Solid-Phase synthesis of adenosine phosphopeptides as potential bisubstrate inhibitors of protein kinases. *J Am Chem Soc,* ***116***, 9413–9419.

Meggio, F., Donella Deana, A., Ruzzene, M., Brunati, A. M., Cesaro, L., Guerra, B., Meyer, T., Mett, H., Fabbro, D., Furet, P. and *et al.* (1995) Different susceptibility of protein kinases to staurosporine inhibition. Kinetic studies and molecular bases for the resistance of protein kinase CK2. *Eur J Biochem,* ***234***, 317–322.

Ming, D. and Wall, M. E. (2005) Quantifying allosteric effects in proteins. *Proteins,* ***59***, 697–707.

Mitchell, R. D., Glass, D. B., Wong, C. W., Angelos, K. L. and Walsh, D. A. (1995) Heat-stable inhibitor protein derived peptide substrate analogs: phosphorylation by cAMP-dependent and cGMP-dependent protein kinases. *Biochemistry,* ***34***, 528–534.

Mochly-Rosen, D. (1995) Localization of protein kinases by anchoring proteins: a theme in signal transduction. *Science,* ***268***, 247–251.

Monod, J., Wyman, J. and Changeux, J. P. (1965) On the nature of allosteric transitions: a plausible model. *J Mol Biol,* ***12***, 88–118.

Murray, A. J. (2008) Pharmacological PKA inhibition: all may not be what it seems. *Sci Signal,* ***1***, re4.

Narayana, N., Cox, S., Nguyen-huu, X., Ten Eyck, L. F. and Taylor, S. S. (1997a) A binary complex of the catalytic subunit of cAMP-dependent protein kinase and adenosine further defines conformational flexibility. *Structure,* ***5***, 921–935.

Narayana, N., Cox, S., Shaltiel, S., Taylor, S. S. and Xuong, N. (1997b) Crystal structure of a polyhistidine-tagged recombinant catalytic subunit of cAMP-dependent protein kinase complexed with the peptide inhibitor PKI[5-24] and adenosine. *Biochemistry,* ***36***, 4438–4448.

Ni, F., Li, W., Li, Y. M. and Zhao, Y. F. (2005) Analysis of the phosphoryl transfer mechanism of cAMP-dependent protein kinase (PKA) by penta-coodinate phosphoric transition state theory. *Curr Protein Pept Sci,* ***6***, 437–442.

Pande, V., Ramos, M. J. and Gago, F. (2008) The protein kinase inhibitor balanol: structure–activity relationships and structure-based computational studies. *Anticancer Agents Med Chem,* ***8***, 638–645.

Parang, K. and Cole, P. A. (2002) Designing bisubstrate analog inhibitors for protein kinases. *Pharmacol Ther,* ***93***, 145–157.

Pehk, T. and Uri, A. (1997) Synthesis and structural characterization of conjugates of adenosine and tetra-aspartate, novel analogs of ATP. *Bioorg Med Chem Lett,* ***7***, 2159–2164.

Pinna, L. A. and Ruzzene, M. (1996) How do protein kinases recognize their substrates? *Biochim Biophys Acta,* ***1314***, 191–225.

The Protein Kinase Resource (1998) *www0.nih.go.jp/mirror/Kinases*.

Prorok, M. and Lawrence, D. S. (1989a) Intrasubstrate steric interactions in the active site control the specificity of the cAMP-dependent protein kinase. *Biochem Biophys Res Commun,* ***158***, 136–140.

Prorok, M. and Lawrence, D. S. (1989b) Multiple arginine residues contribute to the increased efficacy of peptide substrates for the cAMP-dependent protein kinase. *Biochem Biophys Res Commun,* ***165***, 368–371.

Rascon, A., Degerman, E., Taira, M., Meacci, E., Smith, C. J., Manganiello, V., Belfrage, P. and Tornqvist, H. (1994) Identification of the phosphorylation site in vitro for cAMP-dependent protein kinase on the rat adipocyte cGMP-inhibited cAMP phosphodiesterase. *J Biol Chem,* ***269***, 11962–11966.

Reed, J. and Kinzel, V. (1984a) Ligand binding site interaction in adenosine cyclic 3',5'-monophosphate dependent protein kinase catalytic subunit: circular dichroic evidence for intramolecular transmission of conformational change. *Biochemistry,* ***23***, 968–973.

Reed, J. and Kinzel, V. (1984b) Near- and far-ultraviolet circular dichroism of the catalytic subunit of adenosine cyclic 5'-monophosphate dependent protein kinase. *Biochemistry,* ***23***, 1357–1362.

Reed, J., Kinzel, V., Kemp, B. E., Cheng, H. C. and Walsh, D. A. (1985) Circular dichroic evidence for an ordered sequence of ligand/binding site interactions in the catalytic reaction of the cAMP-dependent protein kinase. *Biochemistry,* ***24***, 2967–2973.

Reuveni, H., Livnah, N., Geiger, T., Klein, S., Ohne, O., Cohen, I., Benhar, M., Gellerman, G. and Levitzki, A. (2002) Toward a PKB inhibitor: modification of a selective PKA inhibitor by rational design. *Biochemistry,* ***41***, 10304–10314.

Ricouart, A., Gesquiere, J. C., Tartar, A. and Sergheraert, C. (1991) Design of potent protein kinase inhibitors using the bisubstrate approach. *J Med Chem,* ***34***, 73–78.

16

Roskoski, R., Jr. (1983) Assays of protein kinase. *Methods Enzymol,* ***99***, 3–6.

Roskoski, R., Jr. and Ritchie, P. (1991) Phosphorylation of rat tyrosine hydroxylase and its model peptides in vitro by cyclic AMP-dependent protein kinase. *J Neurochem,* ***56***, 1019–1023.

Rubin, G. M., Yandell, M. D., Wortman, J. R., Gabor Miklos, G. L., Nelson, C. R., Hariharan, I. K., Fortini, M. E., Li, P. W., Apweiler, R., Fleischmann, W., Cherry, J. M., Henikoff, S., Skupski, M. P., Misra, S., Ashburner, M., Birney, E., Boguski, M. S., Brody, T., Brokstein, P., Celniker, S. E., Chervitz, S. A., Coates, D., Cravchik, A., Gabrielian, A., Galle, R. F., Gelbart, W. M., George, R. A., Goldstein, L. S., Gong, F., Guan, P., Harris, N. L., Hay, B. A., Hoskins, R. A., Li, J., Li, Z., Hynes, R. O., Jones, S. J., Kuehl, P. M., Lemaitre, B., Littleton, J. T., Morrison, D. K., Mungall, C., O'Farrell, P. H., Pickeral, O. K., Shue, C., Vosshall, L. B., Zhang, J., Zhao, Q., Zheng, X. H. and Lewis, S. (2000) Comparative genomics of the eukaryotes. *Science,* ***287***, 2204–2215.

Schiebel, K., Mertz, A., Winkelmann, M., Glaser, B., Schempp, W. and Rappold, G. (1997) FISH localization of the human Y-homolog of protein kinase PRKX (PRKY) to Yp11.2 and two pseudogenes to 15q26 and Xq12-->q13. *Cytogenet Cell Genet,* ***76***, 49–52.

Segel, I. H. (1975) *Enzyme kinetics : behavior and analysis of rapid equilibrium and steady state enzyme systems,* New York: Wiley.

Seifert, M. H. J., Breitenlechner, C. B., Bossemeyer, D., Huber, R., Holak, T. A. and Engh, R. A. (2002) Phosphorylation and flexibility of cyclic-AMP-dependent protein kinase (PKA) using ^{31}P NMR spectroscopy. *Biochemistry,* ***41***, 5968–5977.

Shabb, J. B. (2001) Physiological substrates of cAMP-dependent protein kinase. *Chem Rev,* ***101***, 2381–2411.

Shaltiel, S., Cox, S. and Taylor, S. S. (1998) Conserved water molecules contribute to the extensive network of interactions at the active site of protein kinase A. *Proc Natl Acad Sci USA,* ***95***, 484–491.

Shen, K., Hines, A. C., Schwarzer, D., Pickin, K. A. and Cole, P. A. (2005) Protein kinase structure and function analysis with chemical tools. *Biochim Biophys Acta,* ***1754***, 65–78.

Shoji, S., Ericsson, L. H., Walsh, K. A., Fischer, E. H. and Titani, K. (1983) Amino acid sequence of the catalytic subunit of bovine type II adenosine cyclic 3',5'-phosphate dependent protein kinase. *Biochemistry,* ***22***, 3702–3709.

Shuntoh, H., Sakamoto, N., Matsuyama, S., Saitoh, M. and Tanaka, C. (1992) Molecular structure of the C[beta] catalytic subunit of rat cAMP-dependent protein kinase and differential expression of C[alpha] and C[beta] isoforms in rat tissues and cultured cells. *Biochim Biophys Acta,* ***1131***, 175–180.

Slice, L. W. and Taylor, S. S. (1989) Expression of the catalytic subunit of cAMP-dependent protein kinase in Escherichia coli. *J Biol Chem,* ***264***, 20940–20946.

Sridhar, R., Hanson-Painton, O. and Cooper, D. R. (2000) Protein kinases as therapeutic targets. *Pharm Res,* ***17***, 1345–1353.

Steinberg, R. A., Cauthron, R. D., Symcox, M. M. and Shuntoh, H. (1993) Auto-activation of catalytic (C alpha) subunit of cyclic AMP-dependent protein kinase by phosphorylation of threonine 197. *Mol Cell Biol,* ***13***, 2332–2341.

Symcox, M. M. and Reinhart, G. D. (1992) A steady-state kinetic method for the verification of the rapid-equilibrium assumption in allosteric enzymes. *Anal Biochem, 206*, 394–399.

Taylor, S. S. (1989) cAMP-dependent protein kinase. Model for an enzyme family. *J Biol Chem,* ***264***, 8443–8446.

Taylor, S. S., Knighton, D. R., Zheng, J., Ten Eyck, L. F. and Sowadski, J. M. (1992) Structural framework for the protein kinase family. *Annu Rev Cell Biol,* ***8***, 429–462.

Taylor, S. S., Radzio-Andzelm, E., Madhusudan, Cheng, X., Ten Eyck, L. and Narayana, N. (1999) Catalytic subunit of cyclic AMP-dependent protein kinase: structure and dynamics of the active site cleft. *Pharmacol Ther,* ***82***, 133–141.

Taylor, S. S., Yang, J., Wu, J., Haste, N. M., Radzio-Andzelm, E. and Anand, G. (2004) PKA: a portrait of protein kinase dynamics. *Biochim Biophys Acta,* ***1697***, 259–269.

Taylor, S. S., Zheng, J., Radzio-Andzelm, E., Knighton, D. R., Ten Eyck, L. F., Sowadski, J. M., Herberg, F. W. and Yonemoto, W. M. (1993) cAMP-dependent protein kinase defines a family of enzymes. *Philos Trans R Soc Lond B Biol Sci,* ***340***, 315–324.

Tsai, C. J., del Sol, A. and Nussinov, R. (2008) Allostery: absence of a change in shape does not imply that allostery is not at play. *J Mol Biol,* ***378***, 1–11.

Tsigelny, I., Grant, B. D., Taylor, S. S. and Ten Eyck, L. F. (1996) Catalytic subunit of cAMP-dependent protein kinase: electrostatic features and peptide recognition. *Biopolymers,* ***39***, 353–365.

Ubersax, J. A. and Ferrell Jr, J. E. (2007) Mechanisms of specificity in protein phosphorylation. *Nat Rev Mol Cell Biol,* ***8***, 530–541.

Uri, A., Järlebark, L., von Kugelgen, I., Schönberg, T., Undón, A. and Heilbronn, E. (1994) A new class of compounds, peptide derivatives of adenosine 5'-carboxylic acid, includes inhibitors of ATP receptor-mediatedresponses. *Bioorg Med Chem,* ***2***, 1099–1105.

Walsh, D. A. and Glass, D. B. (1991) Utilization of the inhibitor protein of adenosine cyclic monophosphate-dependent protein kinase, and peptides derived from it, as tools to study adenosine cyclic monophosphate-mediated cellular processes. *Methods Enzymol,* ***201***, 304–316.

Wen, W. and Taylor, S. S. (1994) High affinity binding of the heat-stable protein kinase inhibitor to the catalytic subunit of cAMP-dependent protein kinase is selectively abolished by mutation of Arg133. *J Biol Chem,* ***269***, 8423–8430.

Whitehouse, S., Feramisco, J. R., Casnellie, J. E., Krebs, E. G. and Walsh, D. A. (1983) Studies on the kinetic mechanism of the catalytic subunit of the cAMP-dependent protein kinase. *J Biol Chem,* ***258***, 3693–3701.

Whitehouse, S. and Walsh, D. A. (1983) Mg ATP^{2-}-dependent interaction of the inhibitor protein of the cAMP-dependent protein kinase with the catalytic subunit. *J Biol Chem,* ***258***, 3682–3692.

Williams, D. M. and Cole, P. A. (2001) Kinase chips hit the proteomics era. *Trends Biochem Sci,* ***26***, 271–273.

Wu, J., Ma, Q. N. and Lam, K. S. (1994) Identifying substrate motifs of protein kinases by a random library approach. *Biochemistry,* ***33***, 14825–14833.

Yonemoto, W. M., McGlone, M. L., Slice, L. W. and Taylor, S. S. (1991) Prokaryotic expression of catalytic subunit of adenosine cyclic monophosphate-dependent protein kinase. *Methods Enzymol,* ***200***, 581–596.

Zetterqvist, O. and Ragnarsson, U. (1982) The structural requirements of substrates of cyclic AMP-dependent protein kinase. *FEBS Lett,* ***139***, 287–290.

Zetterqvist, O., Ragnarsson, U., Humble, E., Berglund, L. and Engström, L. (1976) The minimum substrate of cyclic AMP-stimulated protein kinase, as studied by synthetic

peptides representing the phosphorylatable site of pyruvate kinase (type L) of rat liver. *Biochem Biophys Res Commun,* ***70***, 696–703.

Zheng, J., Knighton, D. R., ten Eyck, L. F., Karlsson, R., Xuong, N., Taylor, S. S. and Sowadski, J. M. (1993) Crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MgATP and peptide inhibitor. *Biochemistry,* ***32***, 2154–2161.

Zimmermann, B., Schweinsberg, S., Drewianka, S. and Herberg, F. W. (2008) Effect of metal ions on high-affinity binding of pseudosubstrate inhibitors to PKA. *Biochem J,* ***413***, 93–101.

ACKNOWLEDGMENTS

This study was carried out in the Institute of Chemistry at Tartu University, Estonia.

I wish to express my sincere gratitude to my supervisor Professor Jaak Järv for introducing me to the practical field of biochemistry and for his professional guidance, good advice and constant support. Being his student was a pleasant and valuable experience.

Special thanks to:
Dr. Asko Uri for providing inhibitor AdcAhxArg$_6$.
Dr. Mats Hansen from Stockholm University for providing peptide.
Dr. Mart Loog and MSc Nikita Oskolkov for expressing protein kinase A catalytic subunit.

The greatest thanks to colleagues for their help and critical remarks, and for warm and nice atmosphere in lab.

I thank all co-authors for their contributions to our joint papers.
I am very grateful to my parents, sister and all my friends for support and encouragement throughout the work.

I would like to express my sincere gratitude to all the people who have supported me throughout this study.

The present study was generously supported by grants the Estonian Science Foundation Grants 4632 and 5214, by the Estonian Ministry of Education and Science Grants 0182592s03 and 0180064s8 and by the Doctoral Program UTTP.

17

DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS

1. **Toomas Tamm.** Quantum-chemical simulation of solvent effects. Tartu, 1993, 110 p.
2. **Peeter Burk.** Theoretical study of gas-phase acid-base equilibria. Tartu, 1994, 96 p.
3. **Victor Lobanov.** Quantitative structure-property relationships in large descriptor spaces. Tartu, 1995, 135 p.
4. **Vahur Mäemets.** The ^{17}O and ^{1}H nuclear magnetic resonance study of H_2O in individual solvents and its charged clusters in aqueous solutions of electrolytes. Tartu, 1997, 140 p.
5. **Andrus Metsala.** Microcanonical rate constant in nonequilibrium distribution of vibrational energy and in restricted intramolecular vibrational energy redistribution on the basis of slater's theory of unimolecular reactions. Tartu, 1997, 150 p.
6. **Uko Maran.** Quantum-mechanical study of potential energy surfaces in different environments. Tartu, 1997, 137 p.
7. **Alar Jänes.** Adsorption of organic compounds on antimony, bismuth and cadmium electrodes. Tartu, 1998, 219 p.
8. **Kaido Tammeveski.** Oxygen electroreduction on thin platinum films and the electrochemical detection of superoxide anion. Tartu, 1998, 139 p.
9. **Ivo Leito.** Studies of Brønsted acid-base equilibria in water and non-aqueous media. Tartu, 1998, 101 p.
10. **Jaan Leis.** Conformational dynamics and equilibria in amides. Tartu, 1998, 131 p.
11. **Toonika Rinken.** The modelling of amperometric biosensors based on oxidoreductases. Tartu, 2000, 108 p.
12. **Dmitri Panov.** Partially solvated Grignard reagents. Tartu, 2000, 64 p.
13. **Kaja Orupõld.** Treatment and analysis of phenolic wastewater with microorganisms. Tartu, 2000, 123 p.
14. **Jüri Ivask.** Ion Chromatographic determination of major anions and cations in polar ice core. Tartu, 2000, 85 p.
15. **Lauri Vares.** Stereoselective Synthesis of Tetrahydrofuran and Tetrahydropyran Derivatives by Use of Asymmetric Horner-Wadsworth-Emmons and Ring Closure Reactions. Tartu, 2000, 184 p.
16. **Martin Lepiku.** Kinetic aspects of dopamine D_2 receptor interactions with specific ligands. Tartu, 2000, 81 p.
17. **Katrin Sak.** Some aspects of ligand specificity of P2Y receptors. Tartu, 2000, 106 p.
18. **Vello Pällin.** The role of solvation in the formation of iotsitch complexes. Tartu, 2001, 95 p.

19. **Katrin Kollist.** Interactions between polycyclic aromatic compounds and humic substances. Tartu, 2001, 93 p.
20. **Ivar Koppel.** Quantum chemical study of acidity of strong and superstrong Brønsted acids. Tartu, 2001, 104 p.
21. **Viljar Pihl.** The study of the substituent and solvent effects on the acidity of OH and CH acids. Tartu, 2001, 132 p.
22. **Natalia Palm.** Specification of the minimum, sufficient and significant set of descriptors for general description of solvent effects. Tartu, 2001, 134 p.
23. **Sulev Sild.** QSPR/QSAR approaches for complex molecular systems. Tartu, 2001, 134 p.
24. **Ruslan Petrukhin.** Industrial applications of the quantitative structure-property relationships. Tartu, 2001, 162 p.
25. **Boris V. Rogovoy.** Synthesis of (benzotriazolyl)carboximidamides and their application in relations with *N*- and *S*-nucleophyles. Tartu, 2002, 84 p.
26. **Koit Herodes.** Solvent effects on UV-vis absorption spectra of some solvatochromic substances in binary solvent mixtures: the preferential solvation model. Tartu, 2002, 102 p.
27. **Anti Perkson.** Synthesis and characterisation of nanostructured carbon. Tartu, 2002, 152 p.
28. **Ivari Kaljurand.** Self-consistent acidity scales of neutral and cationic Brønsted acids in acetonitrile and tetrahydrofuran. Tartu, 2003, 108 p.
29. **Karmen Lust.** Adsorption of anions on bismuth single crystal electrodes. Tartu, 2003, 128 p.
30. **Mare Piirsalu.** Substituent, temperature and solvent effects on the alkaline hydrolysis of substituted phenyl and alkyl esters of benzoic acid. Tartu, 2003, 156 p.
31. **Meeri Sassian.** Reactions of partially solvated Grignard reagents. Tartu, 2003, 78 p.
32. **Tarmo Tamm.** Quantum chemical modelling of polypyrrole. Tartu, 2003. 100 p.
33. **Erik Teinemaa.** The environmental fate of the particulate matter and organic pollutants from an oil shale power plant. Tartu, 2003. 102 p.
34. **Jaana Tammiku-Taul.** Quantum chemical study of the properties of Grignard reagents. Tartu, 2003. 120 p.
35. **Andre Lomaka.** Biomedical applications of predictive computational chemistry. Tartu, 2003. 132 p.
36. **Kostyantyn Kirichenko.** Benzotriazole — Mediated Carbon–Carbon Bond Formation. Tartu, 2003. 132 p.
37. **Gunnar Nurk.** Adsorption kinetics of some organic compounds on bismuth single crystal electrodes. Tartu, 2003, 170 p.
38. **Mati Arulepp.** Electrochemical characteristics of porous carbon materials and electrical double layer capacitors. Tartu, 2003, 196 p.

39. **Dan Cornel Fara.** QSPR modeling of complexation and distribution of organic compounds. Tartu, 2004, 126 p.
40. **Riina Mahlapuu.** Signalling of galanin and amyloid precursor protein through adenylate cyclase. Tartu, 2004, 124 p.
41. **Mihkel Kerikmäe.** Some luminescent materials for dosimetric applications and physical research. Tartu, 2004, 143 p.
42. **Jaanus Kruusma.** Determination of some important trace metal ions in human blood. Tartu, 2004, 115 p.
43. **Urmas Johanson.** Investigations of the electrochemical properties of polypyrrole modified electrodes. Tartu, 2004, 91 p.
44. **Kaido Sillar.** Computational study of the acid sites in zeolite ZSM-5. Tartu, 2004, 80 p.
45. **Aldo Oras.** Kinetic aspects of dATPαS interaction with $P2Y_1$ receptor. Tartu, 2004, 75 p.
46. **Erik Mölder.** Measurement of the oxygen mass transfer through the air-water interface. Tartu, 2005, 73 p.
47. **Thomas Thomberg.** The kinetics of electroreduction of peroxodisulfate anion on cadmium (0001) single crystal electrode. Tartu, 2005, 95 p.
48. **Olavi Loog.** Aspects of condensations of carbonyl compounds and their imine analogues. Tartu, 2005, 83 p.
49. **Siim Salmar.** Effect of ultrasound on ester hydrolysis in aqueous ethanol. Tartu, 2006, 73 p.
50. **Ain Uustare.** Modulation of signal transduction of heptahelical receptors by other receptors and G proteins. Tartu, 2006, 121 p.
51. **Sergei Yurchenko.** Determination of some carcinogenic contaminants in food. Tartu, 2006, 143 p.
52. **Kaido Tämm.** QSPR modeling of some properties of organic compounds. Tartu, 2006, 67 p.
53. **Olga Tšubrik.** New methods in the synthesis of multisubstituted hydrazines. Tartu. 2006, 183 p.
54. **Lilli Sooväli.** Spectrophotometric measurements and their uncertainty in chemical analysis and dissociation constant measurements. Tartu, 2006, 125 p.
55. **Eve Koort.** Uncertainty estimation of potentiometrically measured ph and pK_a values. Tartu, 2006, 139 p.
56. **Sergei Kopanchuk.** Regulation of ligand binding to melanocortin receptor subtypes. Tartu, 2006, 119 p.
57. **Silvar Kallip.** Surface structure of some bismuth and antimony single crystal electrodes. Tartu, 2006, 107 p.
58. **Kristjan Saal.** Surface silanization and its application in biomolecule coupling. Tartu, 2006, 77 p.
59. **Tanel Tätte.** High viscosity $Sn(OBu)_4$ oligomeric concentrates and their applications in technology. Tartu, 2006, 91 p.

60. **Dimitar Atanasov Dobchev**. Robust QSAR methods for the prediction of properties from molecular structure. Tartu, 2006, 118 p.
61. **Hannes Hagu**. Impact of ultrasound on hydrophobic interactions in solutions. Tartu, 2007, 81 p.
62. **Rutha Jäger.** Electroreduction of peroxodisulfate anion on bismuth electrodes. Tartu, 2007, 142 p.
63. **Kaido Viht.** Immobilizable bisubstrate-analogue inhibitors of basophilic protein kinases: development and application in biosensors. Tartu, 2007, 88 p.
64. **Eva-Ingrid Rõõm.** Acid-base equilibria in nonpolar media. Tartu, 2007, 156 p.
65. **Sven Tamp.** DFT study of the cesium cation containing complexes relevant to the cesium cation binding by the humic acids. Tartu, 2007, 102 p.
66. **Jaak Nerut.** Electroreduction of hexacyanoferrate(III) anion on Cadmium (0001) single crystal electrode. Tartu, 2007, 180 p.
67. **Lauri Jalukse.** Measurement uncertainty estimation in amperometric dissolved oxygen concentration measurement. Tartu, 2007, 112 p.
68. **Aime Lust.** Charge state of dopants and ordered clusters formation in CaF_2:Mn and CaF_2:Eu luminophors. Tartu, 2007, 100 p.
69. **Iiris Kahn**. Quantitative Structure-Activity Relationships of environmentally relevant properties. Tartu, 2007, 98 p.
70. **Mari Reinik.** Nitrates, nitrites, N-nitrosamines and polycyclic aromatic hydrocarbons in food: analytical methods, occurrence and dietary intake. Tartu, 2007, 172 p.
71. **Heili Kasuk.** Thermodynamic parameters and adsorption kinetics of organic compounds forming the compact adsorption layer at Bi single crystal electrodes. Tartu, 2007, 212 p.
72. **Erki Enkvist.** Synthesis of adenosine-peptide conjugates for biological applications. Tartu, 2007, 114 p.
73. **Svetoslav Hristov Slavov**. Biomedical applications of the QSAR approach. Tartu, 2007, 146 p.
74. **Eneli Härk.** Electroreduction of complex cations on electrochemically polished Bi(*hkl*) single crystal electrodes. Tartu, 2008, 158 p.
75. **Priit Möller.** Electrochemical characteristics of some cathodes for medium temperature solid oxide fuel cells, synthesized by solid state reaction technique. Tartu, 2008, 90 p.
76. **Signe Viggor.** Impact of biochemical parameters of genetically different pseudomonads at the degradation of phenolic compounds. Tartu, 2008, 122 p.
77. **Ave Sarapuu.** Electrochemical reduction of oxygen on quinone-modified carbon electrodes and on thin films of platinum and gold. Tartu, 2008, 134 p.
78. **Agnes Kütt.** Studies of acid-base equilibria in non-aqueous media. Tartu, 2008, 198 p.

79. **Rouvim Kadis.** Evaluation of measurement uncertainty in analytical chemistry: related concepts and some points of misinterpretation. Tartu, 2008, 118 p.
80. **Valter Reedo.** Elaboration of IVB group metal oxide structures and their possible applications. Tartu, 2008, 98 p.