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KINETIC ANALYSIS OF PEPTIDE, ATP AND BIFUNCTIONAL INHIBITOR INTERACTION WITH PROTEIN KINASE A

Thesis for the degree of Master of Sciences in bioorganic chemistry

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Abbreviations

Chemical compounds

AdoC	adenosine-5'-carboxylic acid
Ahx	$NH(CH_2)_5C(O)$, aminohexanoic acid
Aoc	amino-octanoic acid
AMP	adenosine-5'-monophosphate
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
C	catalytic subunits of protein kinase A
cAMP	cyclic adenosine-3',5'-monophosphate
CDPK-1	calcium-dependent protein kinase 1
DTT	dithiothreitol
LRRASLG	Kemptide
MOPS	4-morpholinepropanesulfonic acid
NADH	nicotinamide adenine dinucleotide, reduced
PEP	phosphoenolpyruvate
PKA	protein kinase A, cAMP-dependent protein kinase
PKC	protein kinase C
PKI	heat-stable protein kinase inhibitor
P-site	protein or peptide binding site
R	regulatory subunit of protein kinase A
TRIS	tris(hydroxymethyl)-aminomethane

Abbreviations in Kinetic Schemes

A	ATP (substrate)
B	peptide (substrate)
I	inhibitor
E	enzyme

Introduction

Protein kinases (E.C.2.7.1.37) are enzymes involved in regulation of many cellular processes (see review in: (Graves & Krebs, 1999)). This regulation is based on highly specific chemical modification of cell proteins by phosphorylation of certain serine, threonine or tyrosine residues in structure of various enzymes or receptors (Boyer & Krebs, 1986). The phosphate group is transferred to the corresponding hydroxyl function of the substrate protein from ATP-magnesium complex and the phosphorylatable site is recognized by the protein kinase active center. In this recognition process the peptide sequence, flanking the phosphorylable amino acid, is evidently one of the specificity determining factors and governs selectivity the regulatory phosphorylation phenomena (Ho et al., 1988).

The role of these "local sequence elements" in specificity of protein kinases has been extensively studied with short peptide substrates, which resemble the amino acid sequence of the phosphorylatable sites in substrate proteins. It allows more explicit kinetic study of the phosphorylation reaction. The considerable amount of data has been obtained about variation of the local sequence specificity of different protein kinases. As the second substrate of the regulatory phosphorylation reaction is always ATP (more precisely its complex with magnesium ions), its recognition in the active site of different protein kinases seems to be rather universal (Hubbard, 1997; Adams, 2001).

Design of the active-site directed inhibitors of protein kinases has proceeded from structures of both of these substrates of the phosphorylation reaction. As general, ATP analogs have yielded more potent inhibition if compared with analogs of the substrate peptides, where the phosphorylatable amino acid is replaced by alanine or some other non-phosphorylatable amino acid (Kemp et al., 1994). On the other hand, the possibilities for tuning of the inhibitor selectivity seem to be rather limited if proceeding from compounds directed into the ATP binding site. Therefore a perspective trend of bisubstrate inhibitors has been proposed, where the effectiveness of the inhibitor binding into the ATP site is combined with wide spectrum of the specificity patterns of the peptide recognition site. Proceeding from this fact, the concept of bisubstrate inhibitors of protein kinases has been proposed and compounds consisting of two structural elements, resembling the peptide fragment and ATP, connected by a linker of appropriate length, have been designed (Ricouart et al., 1991). For more detailed understanding of the mechanism of interaction of these bifunctional inhibitors with the enzyme, as well as for their rational design, it is necessary to evaluate the effectiveness of interaction of these compounds with different regions of the enzyme active site, firsthand the binding sites for ATP and peptide. This kinetic analysis is, however, a rather complicated task, as the bisubstrate

mechanism of the phosphorylation reaction is a complex phenomenon and most probably involves besides the non-covalent binding and catalytic steps also rate-limiting conformational transitions (Lew et al., 1997a). Moreover, as the overall reaction mechanism seems to be different for different protein kinases, the sophisticated methods used for kinetic analysis of the phosphorylation reaction are not effective and handy for screening of the bisubstrate inhibitors.

In this work we suggest that the task of differentiation of the bisubstrate inhibitor interaction with the peptide and ATP sites of the enzyme active center can be characterized by a simplified procedure, using the second-order rate constants k_{II}, calculated as the ratio of the steady-state apparent catalytic parameters k_{cat} and K_m for the phosphorylation reaction. This approach was practically tested for protein kinase A inhibition by bifunctional inhibitor AdoC(Ahx)Arg_6 prepared by Professor Asko Uri and described previously (Uri et al., 1994). The main results of this study were published in three papers:

1. **Kuznetsov, A**., Oskolkov, N., Hansen, M., Järv, J. (2003) Steady-state kinetic analysis of protein kinase A interaction with peptide and ATP. *Proceedings of the Estonian Academy of Sciences. Chemistry*, **52**, No. 4, 165-177.
2. **Kuznetsov, A**., Väärtnõu-Järv, H., 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**, No. 4, 178-187.
3. **Kuznetsov, A**., Uri, A., Järv, J. Kinetic Analysis of Protein Kinase A Inhibition by a Peptide-Nucleoside Conjugate, Designed as Bisubstrate Analog Inhibitor. *Bioorganic Chemistry*. Manuscript.

Review of Literature

Functions of Protein Kinases

All cells contain networks of molecules that control information flow from their environment, as well as monitor their own status. Therefore it is not surprising that up to 5% of their genomes encode proteins responsible for these functions. Among these proteins one class of enzymes – the protein kinases – perform great part of this regulation (Woodgett, 2001). The function of protein kinases is catalysis of transfer of the γ-phosphate from ATP to one of the three amino acids in proteins: serine, threonine or tyrosine. This phosphorylation reaction may cause a variety of effects at the target proteins and alter virtually any aspect of their activity, specificity, stability, association or localization within the cell (Fig. 1) (Graves & Krebs, 1999). There is a great diversity of protein kinases in organisms. It has been estimated that 30-50% of the cellular proteins of an eukaryotic cell undergo phosphorylation *in vivo* (Pinna & Ruzzene, 1996). Protein kinases are predicted to constitute approximately 2.8% of the human genom (Venter et al., 2001).

The regulation of protein activities by phosphorylation is a reversible process, with the reverse direction catalyzed by protein phosphatases. The signals are transmissed from the plasma membrane to specific intracellular targets mostly through several phosphorylation events. Each of these steps, in order to maintain the exactness of the signaling flux, should possess high target recognition specificity and localization specificity.

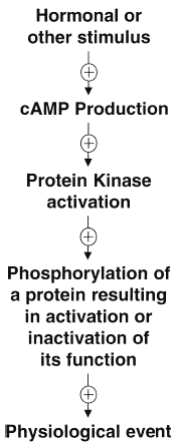


Figure 1. General pattern for regulation by cAMP and PKA (Graves & Krebs, 1999).

Protein kinases have been identified and characterized from various mammals,vertebrates, plants, fungi and microorganisms. The comparative studies have demonstrated that the basic aspects of protein phosphorylation have been maintained throughout the course of eukaryotic evolution (Hanks & Hunter, 1995).

The Protein Kinase Superfamily

The classification of protein kinases and the definition of the eukaryotic protein kinase superfamily were given in (Hanks et al., 1988). This classification is based on the phylogenetic trees derived from comparison of amino acid sequences in the kinase domain. It was found that the sequence similarity of the kinase domains was a good indicator of other common features of kinases, revealing five main families with related substrate specificity and mode of regulation. The five groups are listed below (Hanks et al., 1988):

1) The AGC group: cyclic nucleotide-regulated kinase family (PKA, PKG, PKC and others);
2) The CaMK group: calcium/calmodulin kinase family;
3) The CMGC group: cyclin-dependent kinases (Cdk), MAP, GSK3 and Clk families;
4) The PTK group: the protein-tyrosine kinase family;
5) The OPK group: the other protein kinase families.

Structural Elements of PKA

PKA is a heterotetrameric protein dissociating into a regulatory dimer (R_2) and two catalytic subunits (2C) in response to increased levels of the second messenger cAMP. This process frees active C-subunit that catalyses protein phosphorylation (Adams & Taylor, 1993). The first 3-dimentional structure of this enzyme was obtained for the catalytic subunit in complex with its peptide inhibitor PKI and MgATP (Fig. 2) (Knighton et al., 1991). Therefore PKA has become the most thoroughly studied representative of the protein kinase superfamily**.**

The C-subunit of PKA is comprised of two lobes (Fig. 2). The small lobe is associated with MgATP binding and composed primarily of β strands. The larger substrate-binding lobe is composed mostly of α helices. These two domains, connected by a small linker region, generate a binding pocket for ATP and the substrate. While the bound ATP is located between the domains, the inhibitor peptide remains on the periphery of the pocket. This fold seems to be present in many protein kinases (Knighton et al., 1991). In the binary complex with inhibitor or in the ternary complexes, the lobes adopt closed conformation. Such transitions

seems to be important: the open form is necessary to allow access of ATP to the catalytic site and release the product ADP; the closed form is necessary to bring residues into the correct conformation to promote catalysis (Olah et al., 1993).

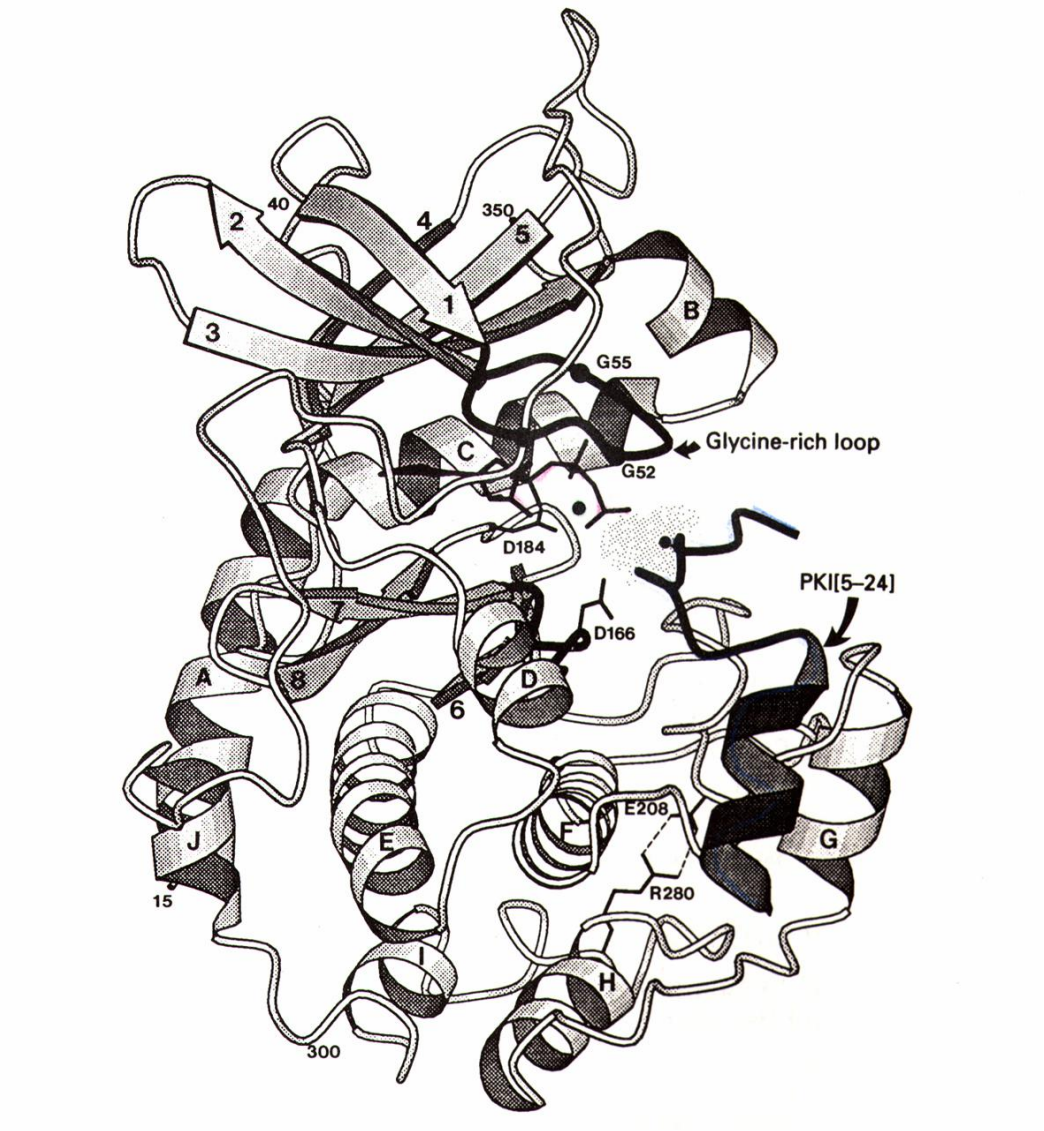


Figure 2. Ribbon diagram of C-PKA co-crystallized with ATP and a peptide inhibitor PKI [5-24] (Knighton et al., 1991).

Kinetic Studies on PKA

The reactions catalyzed by protein kinases require both ATP and a substrate protein/peptide and thus are bisubstrate reactions. These reactions could follow random or ordered mechanism (Cleland, 1977) with respect to peptide and ATP. PKA has been shown to follow random kinetic mechanism, if Kemptide (LRRASLG) is the peptide substrate. Firstly, non-competitive inhibition was observed in the presence of serine peptide analogue, guanethidine, or ADP (Cook et al., 1982). These results indicate that ATP and Kemptide can bind to PKA independently as expected in a random kinetic mechanism. Secondly, it was shown that the substrate peptide can bind prior to ATP, and γ-[^{32}P]ATP can bind prior Kemptide (Kong & Cook, 1988). These results are not possible unless ATP and Kemptide have unrestricted access to the active site, and the binding of one of these substrates does not exclude the binding of the other. On the other hand, it was also mentioned that PKA might have some preference for binding ATP prior to substrate (Cook et al., 1982; Grant & Adams, 1996). Longer substrate peptides have been designed that bind significantly better than ATP, but no studies have been performed to determine whether the reaction order is affected (Mitchell et al., 1995).

There are two control mechanisms over PKA activity. The first is the activity control by additional regulatory subunits. Another possibility is phosphorylation (autophosphorylation) of the enzyme catalytic subunit at the activation loop (Johnson et al., 1996). The crystal structure of PKA (Knighton et al.,1991) revealed the structural importance of Thr-197 in this phosphorylation and demonstrated possible roles of phosphorylation in activation.

Inhibitors of Protein Kinases

The role of protein kinases in cancer, inflammation and cardiovascular diseases provides a driving force for developing inhibitors as potential drugs. There are five main classes of reversible protein kinase inhibitors:

a) ATP-site inhibitors are compounds competing with ATP for its binding pocket in the enzyme active site cleft

b) Peptide-site inhibitors are compounds competing with the peptide or protein substrate for their binding site on the enzyme molecule

c) Bisubstrate inhibitors are inhibitors directed simultaneously into the ATP and peptide binding sites

d) Inhibitors targeting regulatory domains by acting on the sites of allosteric effectors

e) Inhibitors blocking the docking sites for anchoring proteins, inhibiting the specific localisation of a kinase (localisation inhibitors) (reviewed in: (Loog, 2001)).

Protein/Peptide Site Directed Inhibitors

These inhibitors compete with peptide or protein substrates for their binding site, and are peptides containing the elements of substrate binding motifs. Therefore these compounds can be named as pseudosubstrate inhibitors (Kemp et al., 1994). Firstly, in late 1970-s it was recognized that PKA could be inhibited by either its regulatory subunit, the protein kinase inhibitor PKI (also known as Walsh inhibitor) or synthetic peptide substrates that contain the substrate-like sequence RRNAI, where the phospho-acceptor serine is replaced by alanine. These inhibitors possess nanomolar K_i values (Walsh & Glass, 1991). According to the crystal structure of the PKA-ATP-PKI[5-24] complex (Knighton et al., 1991) and studies on structurally altered variants of PKI, it was found that arginine in position P–6 and phenylalanine in position P–11 play important role in tight binding of these inhibitors (Scott et al., 1986; Glass et al., 1989). Walsh and co-workers (Glass et al., 1989; Mitchell et al., 1995) have shown that the K_i for PKI[14-22]amide, a peptide containing the P-6 arginine, is 4-fold lower than the K_m for the corresponding serine containing substrate. On the other hand, however, the analogue of substrate LRRASLG (Kemptide, K_m = 17.2 μM (Lew et al., 1997a)), where serine was substituted by alanine, LRRAALG, has very low inhibitory effect with the K_i value in interval 2.39-0.25 mM (Mendelow et al., 1993; Whitehouse et al., 1983).

ATP Site Directed Inhibitors

The first discovered potent ATP-site directed inhibitor was staurosporine, a microbial alkaloid, isolated from *Streptomyces staurosporeus*. This inhibitor has the K_i values in the low nanomolar concentration range for several protein kinases, including PKA (Meggio et al., 1995), and it has remained in wide use in research as a potent universal inhibitor of protein kinase activity. Although the ATP binding pocket is conserved in protein kinases, the crystallographic studies coupled to computer modeling, has allowed the design of rather selective compounds interacting in this site (Furet et al., 1995). Various related compounds, developed on the basis of staurosporine, have relatively good selectivity for several kinases. Another historically important class is the inhibitors of H-series, including different derivatives of isoquinolinesulphon-amide, firstly reported by Hidaka et al**.** (1984).

Today, the crystal structures of the PKA-inhibitor complexes are available for staurosporine (Prade et al., 1997) and for three inhibitors belonging to the H-series (Engh et al., 1996). These crystallographic studies together with several other data confirm the existence of three specificity regions in the adenosine binding pocket: the “hydrophobic pocket”, the “ribose pocket”, and the “linker region”. The isoquinoline and dianalinophtalamide inhibitors bind in the ”ribose pocket” (Engh et al., 1996), while the “linker region” is utilized by the purine-based class of olomoucine inhibitors (Schulze-Gahmen et al., 1995). In general, there are two key components of the protein kinase structure that contribute to the specificity of ATP-site directed inhibitors: amino acid sequence diversity within the ATP binding cleft, and conformational diversity, which allows inhibitors to “adopt” and “lock” the protein kinase into a specific conformation.

Bisubstrate Analog Inhibitors

Design of bisubstrate analog inhibitors is based on combination of structural elements of both substrates into one inhibitor molecule, expecting that the enzymes active center recognize both structure fragments (Fig. 3) and the overall selectivity rises due to combination of these both components.

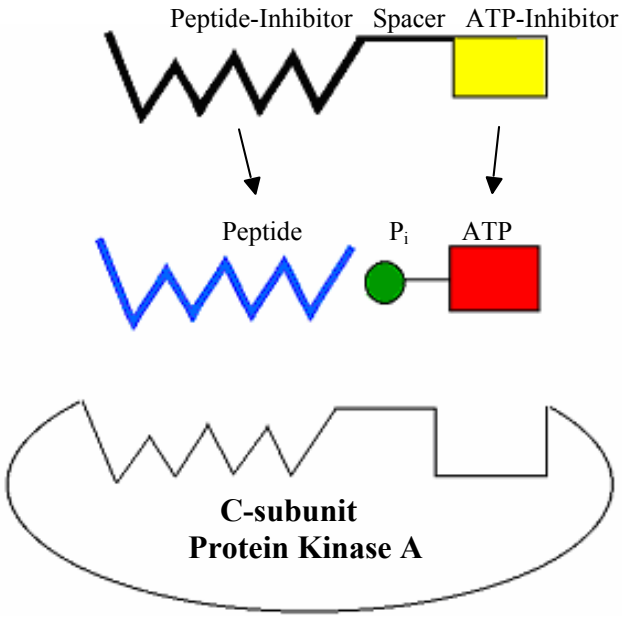


Figure 3. Principal action of bisubstrate analog inhibitor.

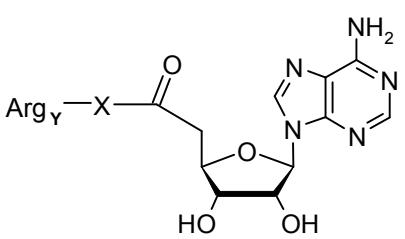
In general, a bisubstrate analog inhibitor of protein kinases should have the following characteristics for its optimal effect:

1. it should inhibit binding of both natural substrates in the active center, taking into consideration their mutual orientation and topology of the appropriate binding sites;
2. it should be specific against one particular protein kinase (this is especially important, due to the presence of overlapping natural substrates for a very large number of protein kinases);
3. it should show good bioavailability and low toxicity.

These strategies fall into the following categories:

1. sulfonamides and sulfonylbenzoyl derivatives,
2. carboxylic acid derivatives,
3. dipeptidyl derivatives,
4. phosphodiester derivatives,
5. peptide-nucleotide conjugates.

None of the strategies have still produced compounds which fully meet the characteristics for an optimal bisubstrate analog inhibitor for protein kinases, but substantial progress has been made in this direction (reviewed in: (Parang & Cole, 2002)). Especially promising seems to be the approach proposed by Uri and co-workers (Uri et al., 1994; Pehk & Uri, 1997), preparing series of nucleotide-peptide conjugates. Among these compounds several effective inhibitors of PKA were obtained (Loog et al., 1999). These inhibitors were synthesized by coupling of adenosine-5'-carboxylic acid (AdoC) with a peptide moiety via various linkers, such as β-alanine, β-aspartate, 2,3-diaminopropionic acids, and $NH(CH_2)_nC(O)$ (Fig. 4).



X = $NH(CH_2)_nC(O)$ (n = 1, 2, 3, 5, 7, 10)
β-alanine
β-aspartic acid
2,3-diaminopropionic acid
Y = 2, 4, 6

Figure 4. Proposed bisubstrate analog inhibitors (Uri et al., 1994; Pehk & Uri, 1997; Loog et al., 1999).

One of the most potent compounds, AdoC(Ahx)Arg_6 , inhibited PKA, PKC, and CDPK-1 with the IC_{50} values of 0.12 μM, 0.27 μM and 1.2 μM, respectively (Loog et al., 1999). All these kinases reveal clear preferences for positively charged amino acids (Muszynska et al., 1993; Kennelly & Krebs, 1991). Besides inhibition of the enzyme activity, these bisubstrate analog inhibitors were also tested for affinity chromatography (Loog et al., 2000).

The Aim of the Present Study

In this study we characterized interaction of the bifunctional inhibitor AdoC(Ahx)Arg_6 separately with the PKA binding sites for ATP and peptide, and also investigated into the possibility of simultaneous binding of these two substrates and the inhibitor molecule with the enzyme active center. The catalytic subunit of PKA was selected for these experiments as one of the most thoroughly investigated enzyme among protein kinases. The second-order rate constants k_{II}, calculated as ratio of the steady-state apparent catalytic parameters k_{cat} and K_m for the phosphorylation reaction, were used in the analysis, to avoid problems connected with the complicated catalytic mechanism of the reaction.

Materials and Methods

Chemicals. γ-[^{32}P] ATP was obtained from Amercham (UK). Phosphocellulose paper P81 was from Whatman (UK). ATP, TRIS/HCl, BSA and H_3PO_4 were from Sigma-Aldrich (USA). $MgCl_2$ was from Acros. Buffers were made using Mili-Q deionized water. Peptide LRRASLG (Kemptide) with purity of 78% was synthesized by Mats Hansen (Department of Neurochemistry and Neurotoxicology, Stockholm University). The catalytic subunit of protein kinase A was expressed and purified by Nikita Oskolkov (Department of Organical and Bioorganical Chemistry, Tartu University) and this procedure is described in (Kuznetsov et al., 2003). Inhibitor AdoC(Ahx)Arg_6 was synthesized by Research Professor Asko Uri (Department of Organical and Bioorganical Chemistry, Tartu University) and generously donated for this study.

Assay of peptide phosphorylation. Peptide phosphorylation by protein kinase A was carried out at 30°C in 100 µl reaction mixture composed as follows: 50 mM TRIS/HCl, pH 7.5, 10 mM $MgCl_2$, peptide substrate LRRASLG at concentration 5-200 µM (the stock solutions were prepared in 50 mM TRIS/HCl, pH 7.5); γ-[^{32}P]ATP at concentration 5-1000 µM (the stock solutions had specific radioactivity 60-200 cpm/pmol); 15 µL PKA solution in enzyme buffer containing 50 mM TRIS/HCl, pH 7.5 and 1 mg/mL BSA. This enzyme solution was made immediately before the experiments by 1000-fold dilution of protein kinase A stock solution.

The phosphorylation reaction was started by addition of the enzyme into the reaction mixture and peptide phosphorylation was followed by taking 10 µL aliquots of this mixture onto pieces of phosphocellulose paper. Then the reaction was immediately stopped through immersing of these pieces of paper into cold 75 mM H_3PO_4. The pieces of paper were washed four times with cold 75 mM H_3PO_4 (10 min each time) to remove excess of γ-[^{32}P]ATP and dried at 120°C for 25 min. The radioactivity bound to the paper was measured as Cherenkov radiation using a Beckman LS 7500 scintillation counter. Preceding from these data the product concentration vs. time plots were constructed (Fig. 5) and from these linear plots the values of the initial velocity of peptide phosphorylation reaction were calculated. This procedure is illustrated in Fig. 5.

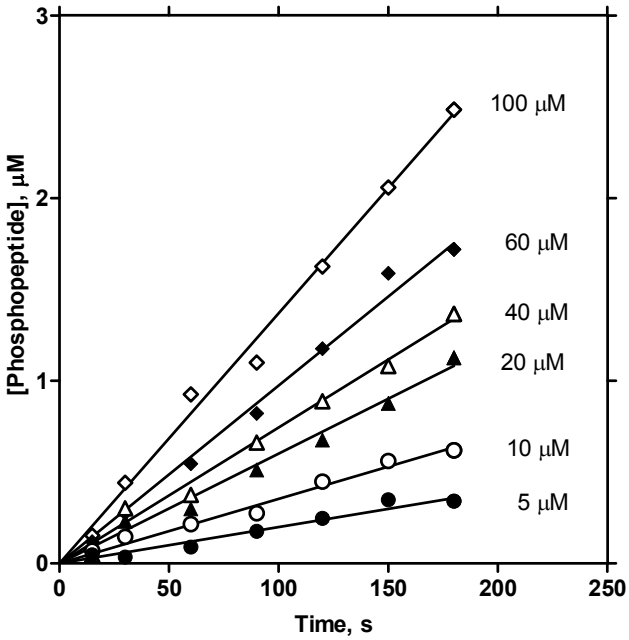


Figure 5. Determination of the initial rate of peptide phosphorylation by protein kinase A. Peptide (LRRASLG, Kemptide) concentration is shown in Figure, ATP concentration 700 μM, pH 7.5, temp. 30^{o}C, 50 mM TRIS/HCl buffer, 10 mM $MgCl_2$.

Data processing. Data processing was performed using the GraphPad Prism version 3.0 (GraphPad Software Inc., USA) and SigmaPlot version 8.0 software (SPSS Inc., USA). The values reported are given with standard errors.

Results and Discussion

Kinetic Analysis of Substrate Reaction

Kinetic measurements were carried out in distinct series, where concentration of one substrate was constant and concentration of the other substrate was systematically changed. The results of these experiments are listed and illustrated as the conventional rate-concentration plots in Appendix (Part 1, Table and figures). As summary of these data, the three-dimensional plot was designed to illustrate the kinetic behavior of the bisubstrate enzyme reaction (Fig. 6).

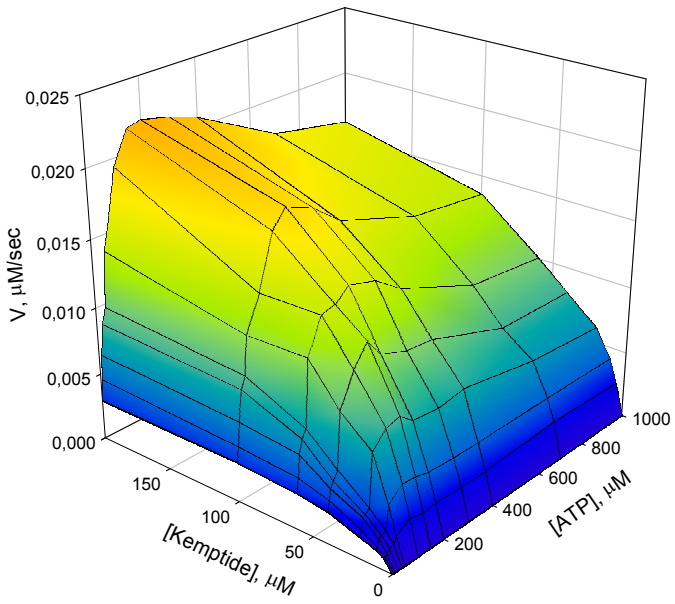


Figure 6. 3D-Plot of initial rate vs. ATP and peptide concentration for the Kemptide (LRRASLG) phosphorylation reaction by protein kinase A.

The shapes of the rate-concentration plots show in Appendix, as well as the tree-dimensional plot in Fig. 6 point to certain asymmetry in behavior of peptide substrate and ATP in this reaction. It is quite clear that some inhibitory effect reveals in the presence of large excess of ATP, while for the peptide counterpart of the reaction no substrate inhibition can be observed within the concentration interval used. This phenomenon of substrate inhibition at high ATP concentrations has not been mentioned before in literature.

Substrate Inhibition

Formally speaking, the phenomenon of substrate inhibition points to formation of complexes, which include two ATP molecules per enzyme and are less inactive regarding to the phosphopeptide formation if compared with the complexes with 1:1 stoichiometry. Therefore the phenomenon of substrate inhibition was further analyzed at constant peptide concentration in the form of semi-logarithmic plots v = f (log [A]), as recommended by Berezin et al. (1976). It can be seen in Fig. 7 that these plots are rather symmetrical at high and low ATP concentrations, pointing to the possibility of full inhibition of the reaction in the presence of the excess of this substrate. This is possible if the complexes with two ATP molecules do not lead to phosphorylation of peptide.

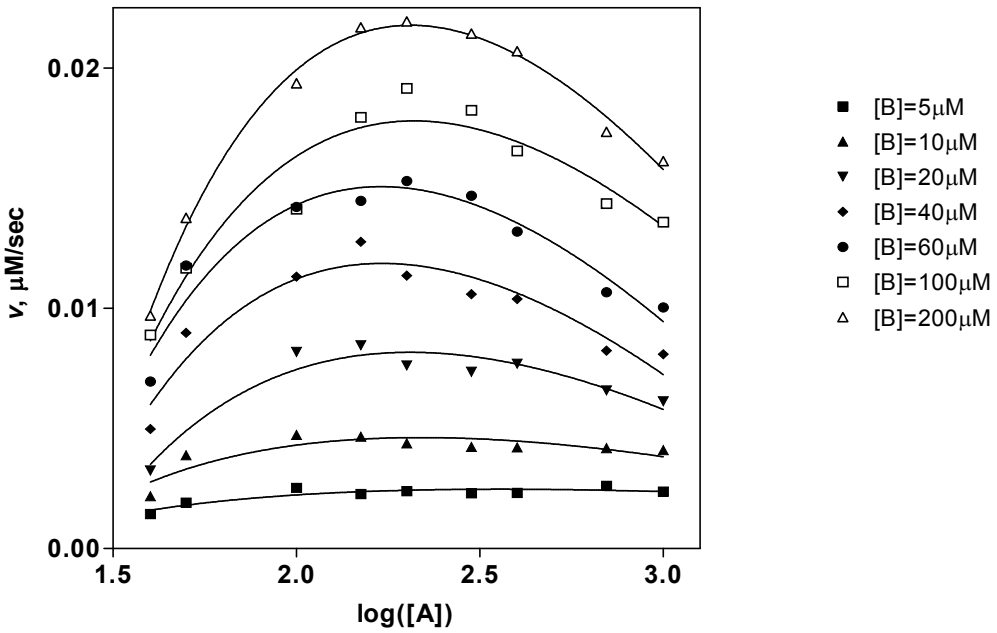
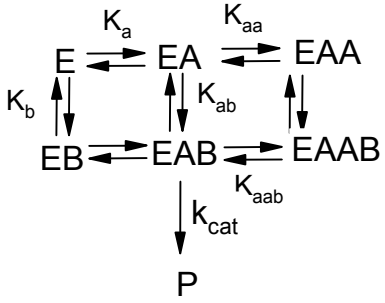


Figure 7. Semi-logarithmic plot for Kemptide phosphorylation by protein kinase A. The peptide concentrations are listed from bottom to top.

Kinetic Model for Substrate Reaction

For catalytic act the two substrates, peptide and ATP, should simultaneously bind in the enzyme active center. This ternary complex is formed reversibly and there are indications that the formation of both enzyme-peptide and enzyme-ATP complexes follows the random mechanism in the case of protein kinase A (Adams, 2001). Formally these equilibria can be presented by the following reaction scheme:



Scheme 1.

In this Scheme E stands for enzyme, A for ATP, B for peptide and P for product. As this study is focused on the analysis of the second-order rate constants, the details of the rate limiting catalytic steps and the accompanying conformational fluctuations as well as the diffusion-limited dissociation of the reaction products (see in (Lew et al., 1997b)) can be excluded from the analysis. Therefore the product formation is designed by the simplest possible way in Scheme 1.

The rate equation for the Scheme 1 can be obtained proceeding from the following relationships and equilibria,

$$v=k_{cat}[EAB] \tag{1}$$

$$[E_o]=[E]+[EA]+[EB]+[EAB]+[EAA]+[EAAB] \tag{2}$$

$$K_a=\frac{[E][A]}{[EA]} \tag{3}$$

$$[EA]=\frac{[E][A]}{K_a} \tag{4}$$

$$K_b=\frac{[E][B]}{[EB]} \tag{5}$$

$$[EB]=\frac{[E][B]}{K_b} \tag{6}$$

$$K_{ab}=\frac{[E][A][B]}{[EAB]K_a} \tag{7}$$

$$[EAB]=\frac{[E][A][B]}{K_aK_{ab}} \tag{8}$$

$$K_{aa}=\frac{[E][A][A]}{[EAA]K_a} \tag{9}$$

$$[EAA]=\frac{[E][A][A]}{K_aK_{aa}} \tag{10}$$

$$K_{aab}=\frac{[E][A][A][B]}{[EAAB]K_aK_{ab}} \tag{11}$$

$$[EA]=\frac{[E][A][A][B]}{K_aK_{ab}K_{aab}} \tag{12}$$

The overall rate equation for the kinetic Scheme 1 has the following form:

$$v=\frac{k_{cat}[E_0][A][B]}{K_{ab}K_a+[A]K_{ab}+[B]\frac{K_{ab}K_a}{K_b}+[A][B]+[A]^2[B]\frac{1}{K_{aab}}+[A]^2\frac{K_{ab}}{K_{aa}}} \quad (13)$$

This equation can be processed in two ways. Firstly, if ATP concentration (denoted as A) is a constant value within series of experiments, this equation can be simplified:

$$v=\frac{V_m^{app\,B}[B]}{K_m^{app\,B}+[B]} \quad (14)$$

and both complex parameters $V_m^{app\,B}$ and $K_m^{app\,B}$ can be presented as follows:

$$V_m^{app\,B}=\frac{k_{cat}[E_0][A]}{\frac{K_{ab}K_a}{K_b}+[A]\left(1+[A]\frac{1}{K_{aab}}\right)} \quad (15)$$

$$K_m^{app\,B}=\frac{K_{ab}\left(K_a+[A]\left[1+[A]\frac{1}{K_{aa}}\right]\right)}{\frac{K_{ab}K_a}{K_b}+[A]\left(1+[A]\frac{1}{K_{aab}}\right)} \quad (16)$$

Secondly, if peptide (denoted as B) concentration is constant, and the plot of the reaction rate v vs. substrate A (ATP) concentration is studied, the rate equation 13 can be presented as follows:

$$v=\frac{V_m^{app\,A}[A]}{K_m^{app\,A}+[A]\left(1+[A]\left[\frac{\frac{K_{ab}}{K_{aa}}+[B]\frac{1}{K_{aab}}}{K_{ab}+[B]}\right]\right)} \quad (17)$$

and the complex parameters $V_m^{app\,A}$ and $K_m^{app\,A}$ have the following meaning:

$$V_m^{app\,A} = \frac{V_m[B]}{K_{ab}+[B]} \tag{18}$$

$$K_m^{app\,A} = \frac{K_{ab}K_a}{K_b} \cdot \frac{K_b+[B]}{K_{ab}+[B]} \tag{19}$$

In both cases the second-order rate constants $k_{II}^{app\,B}$ and $k_{II}^{app\,A}$ can be obtained from the ratio of the appropriate V_m and K_m values. If we define $K' = \frac{V/[E_o]}{K_{ab}K_a}$, where $[E_o]$ stands for enzyme concentration, the equations for the second-order rate constants can be rewritten as follows:

$$k_{II}^{app\,B} = \frac{K'K_a[A]}{K_a+[A]\left(1+[A]\frac{1}{K_{aa}}\right)} \tag{20}$$

$$k_{II}^{app\,A} = \frac{K'K_b[B]}{K_b+[B]} \tag{21}$$

From these equations the K_a, K_{aa} and K_b values can be easily calculated, if the second-order rate constants are available for different concentrations of both substrates. These constants describe interaction of the first and the second ATP molecule and the peptide molecule with the free enzyme.

Kinetic Parameters for Substrate Reaction

Proceeding from the rate equation 13 and its modifications above, the kinetic constants $V_m^{app\,B}$ and $K_m^{app\,B}$ for different ATP concentrations and kinetic constants $V_m^{app\,A}$ and $K_m^{app\,A}$ for different Kemptide concentrations were measured. These results are listed in Appendix (Part 1). Further the plots of the second-order rate constants versus ATP (A) and

Kemptide (B) concentrations were analyzed, as show in Figs. 8 and 9, and the parameters K_a and K_b as well as K_{aa} were calculated and given in Table 1. The constants K_a and K_b are in rather good agreement with the results of other works, where both steady-state and pre-steady-state kinetic methods were used for kinetic analysis. The latter values are given in Table 2, where the meaning of the parameters corresponds to the reaction Scheme 1 in page 18.

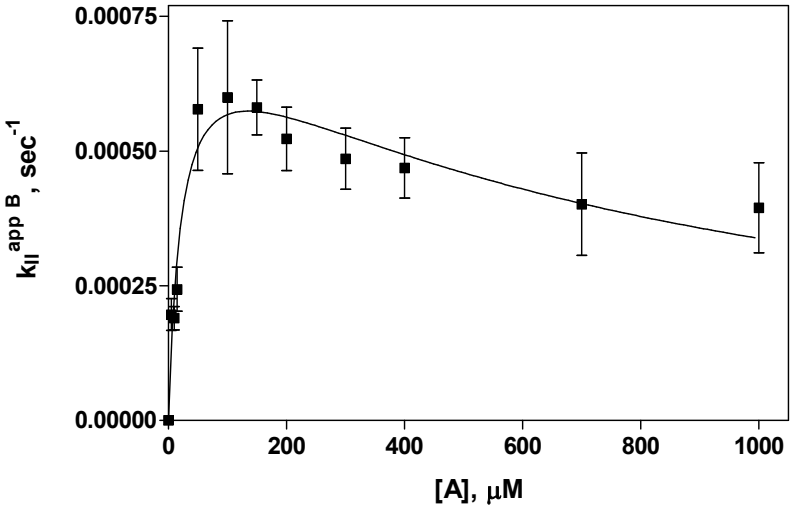


Figure 8. Plot of $k_{II}^{app\ B}$ vs. ATP concentration for Kemptide phosphorylation by protein kinase A.

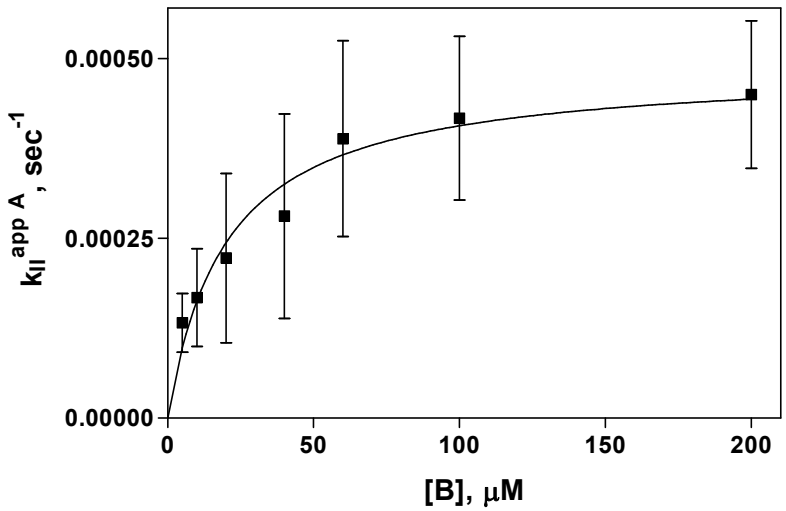


Figure 9. Plot of $k_{II}^{app\ A}$ vs. peptide concentration for Kemptide phosphorylation by protein kinase A.

Table 1. Effectiveness of ATP and Kemptide interaction with protein kinase A, characterized by means of the steady-state kinetic analysis of the phosphorylation reaction. Reaction conditions were: 50 mM TRIS, pH=7.5, 10 mM $MgCl_2$, temp. 30°C.

Substrate	Constants
ATP	K_a = 18,2 ±5,3 μM
	K_{aa} = 862±293 μM
Kemptide	K_b = 20,0±4,4 μM

Table 2**.** Kinetic constants for Kemptide phosphorylation by protein kinase A, compiled from literature.

Kinetic parameters	Constants	Reaction conditions	Reference
K_{ATP} (μM)	12,7 ± 0,5	50 mM MOPS, pH=7.0, 10 mM $MgCl_2$, 0.1 M KCl, 1 mM DTT, temp. 30°C	(Aimes et al., 2000)[a]
	12,9 ± 0,8	100 mM MOPS, pH=7.1, 10 mM $MgCl_2$	(Cook et al., 1982)[a]
	17,2 ± 1,8	1 mM PEP, 100 μM NADH, 12 U/mL lactate dehydrogenase, 4 U/mL pyruvate kinase, 20 mM MOPS, 50 mM NaCl, 9 mM $MgCl_2$, pH=7.0, temp. 23°C	(Lew et al., 1997b)[b]
	18,0 ± 3,1	1 mM PEP, 300 μM NADH, 12 U/mL lactate dehydrogenase, 4 U/mL pyruvate kinase, 100 mM TRIS, pH=8.0, 10-11 mM $MgCl_2$, temp. 24°C	(Adams & Taylor, 1992)[b]
$K_{Kemptide}$ (μM)	21,7 ± 0,7	50 mM MOPS, pH=7.0, 10 mM $MgCl_2$, 0.1 M KCl, 1 mM DTT, temp. 30°C	(Aimes et al., 2000)[a]
	24,7 ± 2,7	1 mM PEP, 100 μM NADH, 12 U/mL lactate dehydrogenase, 4 U/mL pyruvate	(Lew et al., 1997b)[b]
	65 ±11	1 mM PEP, 300 μM NADH, 12 U/mL lactate dehydrogenase, 4 U/mL pyruvate kinase, 100 mM TRIS, pH=8.0, 10-11 mM $MgCl_2$, temp. 24°C	(Adams & Taylor, 1992)[b]

[a] Obtained by the radioassay

[b] Measured spectrophotometrically

Substrate Reaction in the Presence of Bifunctional Inhibitor

In this part of the study the kinetic experiments were made in the presence of the bifunctional inhibitor at concentrations 0.25, 0.5 and 1.0 μM. The principal structure of the study was as in previous section, namely, the experiments were made in distinct series where concentration of one substrate was constant and concentration of the other substrate was systematically changed. The initial velocities measured under these conditions are listed and illustrated as conventional rate-concentration plots in Appendix (Part 2, Tables and Figures). As summary, the three-dimensional plot was designed to illustrate the kinetic behavior of the bisubstrate reaction in the presence of the inhibitor AdoC(Ahx)Arg_6 (Fig. 10). It can be seen that in the presence of the inhibitor systematic decrease of the reaction rate can be observed.

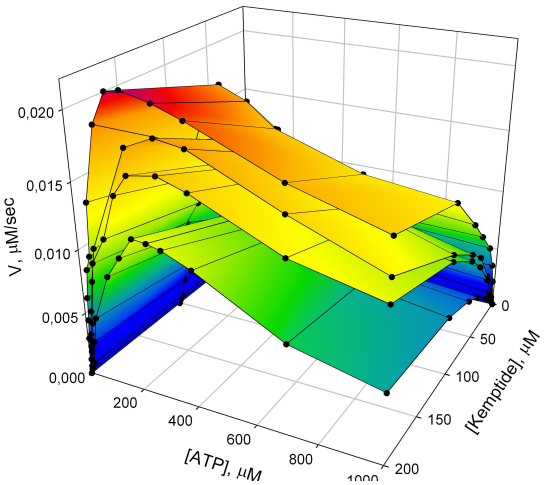
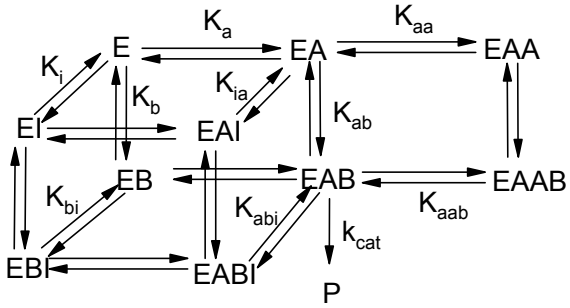


Figure 10. 3D-Plot for initial rate of Kemptide phosphorylation reaction by protein kinase A in the presence of inhibitor AdoC(Ahx)Arg_6. From top: control (no inhibitor), [I] = 0.25 μM, [I] = 0.5 μM, [I] = 1.0 μM.

Kinetic Model for Protein Kinase A Inhibition

The influence of the inhibitor was described by introduction of additional enzyme-ligand complexes, including the bifunctional inhibitor I (Scheme 2). This model includes besides the complex EI also the appropriate ternary complexes EAI and EBI. These ternary complexes result from the presence of the two distinct binding sites for substrates that should allow various combinations of the enzyme bound ligands. However, for more general analysis also

the complex including the enzyme, inhibitor and both substrates (EABI) was included. The formation of the latter complex might be possible if the inhibitor molecule does not fit explicitly into the active center and allows simultaneous binding of the substrates A and B. Although this situation seems to be rather unlikely, it may still occur, for example, due to too short or too long linker group in the bifunctional inhibitor molecule. The practical data analysis should reveal the feasibility of this complex formation and is one of the objectives of the kinetic analysis.



Scheme 2.

The rate equation for the Scheme 2 can be obtained proceeding from the following relationships and equilibriums, where K_a, K_{aa}, K_{aab}, K_b and K_{ab} were defined above, in the case of the kinetic analysis of the substrate reaction (see Scheme 1).

$$[E_O]=[E]+[EA]+[EB]+[EAB]+[EAA]+[EAAB]+[EI]+[EAI]+[EBI]+[EABI] \quad (22)$$

$$K_i = \frac{[E][I]}{[EI]} \quad (23) \qquad [EI] = \frac{[I]}{K_i}[E] = \frac{K_a K_{ab}[I]}{K_i [A][B]}[EAB] \quad (24)$$

$$K_{ai} = \frac{[EA][I]}{[EAI]} \quad (25) \qquad [EAI] = \frac{[I]}{K_{ai}}[EA] = \frac{K_{ab}[I]}{K_{ai}[B]}[EAB] \quad (26)$$

$$K_{bi} = \frac{[EB][I]}{[EBI]} \quad (27) \qquad [EBI] = \frac{[I]}{K_{bi}}[EB] = \frac{K_a K_{ab}[I]}{K_{bi} K_b [A]}[EAB] \quad (28)$$

$$K_{abi} = \frac{[EAB][I]}{[EABI]} \quad (29) \qquad [EABI] = \frac{[I]}{K_{abi}}[EAB] \quad (30)$$

The following rate equation can be obtained for the Scheme 2:

$$v=\frac{k_{cat}[E_0][A][B]}{K_{ab}K_a(1+\frac{[I]}{K_i})+K_{ab}(1+\frac{[I]}{K_{ai}})[A]+\frac{K_{ab}K_a}{K_b}(1+\frac{[I]}{K_{bi}})[B]+(1+\frac{I}{K_{abi}})[A][B]+\frac{1}{K_{aab}}[A]^2[B]+\frac{K_{ab}}{K_{aa}}[A]^2} \quad (31)$$

Firstly, if concentrations of substrate A (ATP) and inhibitor I remain constant, the plot of the initial velocity vs. substrate B concentration can be analyzed by the common hyperbolic function, derived from Eq. 31:

$$v=\frac{V_m^{app\,B}B}{K_m^{app\,B}+B} \quad (32)$$

In this equation the steady-state kinetic parameters $V_m^{app\,B}$ and $K_m^{app\,B}$ depend on A and I concentration and have the following meaning:

$$V_m^{app\,B}=\frac{k_{cat}[E_0][A]}{\frac{K_{ab}K_a}{K_b}(1+\frac{[I]}{K_{bi}})+(1+\frac{I}{K_{abi}})[A]+\frac{1}{K_{aab}}[A]^2} \quad (33)$$

$$K_m^{app\,B}=\frac{K_aK_{ab}(1+\frac{[I]}{K_i})+K_{ab}(1+\frac{[I]}{K_{ai}})[A]+\frac{K_{ab}}{K_{aa}}[A]^2}{\frac{K_{ab}K_a}{K_b}(1+\frac{[I]}{K_{bi}})+(1+\frac{[I]}{K_{abi}})[A]+\frac{1}{K_{aab}}[A]^2} \quad (34)$$

Similarly, if concentration of substrate B (peptide) and inhibitor I are kept constant within series of measurements, the rate equation for variable A can be presented in the form:

$$v=\frac{V_m^{appA}[A]}{K_m^{appA}+[A]+K_{aa}^{appA}[A]^2} \tag{35}$$

The apparent kinetic parameters of this equation, also reflecting the phenomenon of product inhibition by ATP excess (Kuznetsov et al., 2003), have the following meaning:

$$V_m^{appA}=\frac{k_{cat}[E_0][B]}{K_{ab}(1+\frac{[I]}{K_{ai}})+(1+\frac{[I]}{K_{abi}})[B]}=\frac{W_i[B]}{T_i+[B]} \tag{36}$$

$$K_m^{appA}=\frac{K_{ab}K_a}{K_b}\cdot\frac{K_b\left(1+\frac{[I]}{K_i}\right)+\left(1+\frac{[I]}{K_{bi}}\right)[B]}{K_{ab}\left(1+\frac{[I]}{K_{ai}}\right)+\left(1+\frac{[I]}{K_{abi}}\right)[B]} \tag{37}$$

$$K_{aa}^{appA}=\frac{\frac{K_{ab}}{K_{aa}}+\frac{[B]}{K_{aab}}}{K_{ab}(1+\frac{[I]}{K_{ai}})+(1+\frac{[I]}{K_{abi}})[B]} \tag{38}$$

From these apparent parameters the following k_{cat}/K_m ratios, which are equivalent to the appropriate second-order rate constants and are further denoted as k_{II}^{app}, can be obtained:

$$k_{II}^{appB}=\frac{\frac{k_{cat}[E_0]}{K_{ab}(1+\frac{[I]}{K_{ai}})}[A]}{K_a\frac{1+\frac{[I]}{K_i}}{1+\frac{[I]}{K_{ai}}}+[A]+\frac{1}{K_{aa}(1+\frac{[I]}{K_{ai}})}[A]^2}=\frac{X_i[A]}{Y_i+[A]+Z_i[A]^2} \tag{39}$$

$$k_{II}^{app\,A} = \frac{\dfrac{k_{cat}\left[E_0\right]}{K_{ab}K_a\left(1+\dfrac{[I]}{K_{bi}}\right)}[B]}{1+\dfrac{[I]}{K_i}} = \frac{Q_i[B]}{U_i+[B]} \qquad (40)$$
$$K_b\,\frac{}{1+\dfrac{[I]}{K_{bi}}}+[B]$$

If the dependence of $k_{II}^{app\,B}$ vs. [A] and $k_{II}^{app\,A}$ vs. [B] is analysed at various inhibitor concentrations, the parameters X_i, Y_i, Z_i, Q_i, U_i and W_i can be calculated and further used for determination of the equilibrium constants for binary and tertiary complexes formulated in Scheme 2. All these plots can be obtained from equations 39 and 40 for the second rate constants above.

$$X_i = \frac{k_{cat}[E_o]\dfrac{K_{ai}}{K_{ab}}}{K_{ai}+[I]} \qquad (41)$$

$$Y_i = \frac{K_aK_{ai}}{K_{ai}+[I]}\left(1+\frac{[I]}{K_i}\right) \qquad (42)$$

$$Q_i = \frac{k_{cat}[E_o]\dfrac{K_bK_{bi}}{K_aK_{ab}}}{K_{bi}+[I]} \qquad (43)$$

$$U_i = \frac{K_bK_{bi}}{K_{bi}+[I]}\left(1+\frac{[I]}{K_i}\right) \qquad (44)$$

$$W_i = \frac{k_{cat}[E_0]K_{abi}}{K_{abi}+[I]} \qquad (45)$$

It can be see that the dependences of X_i, Q_i and W_i upon the inhibitor concentration [I] are hyperbolic. But the plots of Y_i and U_i vs. [I] are more complicated functions (Eqs. 42 and 44). For simplification, the ratios X_i/Y_i and Q_i/U_i were used in the analysis, yielding hyperbolic plots for determination of K_i.

$$\frac{X_i}{Y_i} = \frac{k_{cat}[E_o]\dfrac{K_i}{K_a K_{ab}}}{K_i + [I]} \tag{46}$$

$$\frac{Q_i}{U_i} = \frac{k_{cat}[E_o]\dfrac{K_i}{K_a K_{ab}}}{K_i + [I]} \tag{47}$$

It can be seen that the plots X_i/Y_i and Q_i/U_i vs. [I] should be similar. This result is expected, as bifunctional inhibitor is assumed to interact simultaneously with both peptide and ATP binding sites. On the other hand, comparison of these experimental plots, calculated proceeding from different series of experiments, can be used as a mean of validation of the kinetic model under discussion.

Kinetic Parameters for Bifunctional Inhibitor

Proceeding from the kinetic model above the kinetic constants $V_m^{app\,A}$, $K_m^{app\,A}$ and $k_{II}^{app\,A}$, measured at different ATP concentrations, and the kinetic constants $V_m^{app\,B}$, $K_m^{app\,B}$ and $k_{II}^{app\,B}$, measured at different kemptide concentrations in the presence of various inhibitor concentrations, were determined and listed in Appendix (Part 2). The parameters X_i, Y_i, Q_i, U_i for each inhibitor concentration were determined and listed in Tab. 3. From these data the plots of X_i and Q_i vs. inhibitor concentration were constructed as shown in Figs. 11 and 12, and the plots for X_i/Y_i and Q_i/U_i vs. inhibitor concentration are shown in Fig. 13. From these plots the parameters K_{ai}, K_{bi}, K_i and K_{abi} were calculated and listed in Tab. 4.

Table 3. Kinetic parameters characterizing ATP and Kemptide interaction with protein kinase A in the presence of the inhibitor AdoC(Ahx)Arg_6.

[I], μM	$10^3 \cdot X_i$, μM/sec	Y_i, μM	$10^3 \cdot Q_i$, μM/sec	U_i, μM	$10^6 \cdot X_i/Y_i$, sec^{-1}	$10^6 \cdot Q_i/U_i$, sec^{-1}
0,00	0,77 ± 0,08	22,8 ± 6,5	0,50 ± 0,03	20,0 ± 4,0	23,2 ± 9,0	25,0 ± 2,0
0,25	0,66 ± 0,01	72,9 ± 2,2	0,32 ± 0,01	37,2 ± 2,3	6,2 ± 0,3	8,6 ± 0,8
0,50	0,63 ± 0,01	103,0 ± 0,4	0,23 ± 0,01	29,5 ± 5,0	7,8 ± 0,1	6,1 ± 1,3
1,00	0,44 ± 0,01	121,6 ±0,3	0,12 ± 0,01	27,7 ± 5,7	3,6 ± 0,1	2,8 ± 0,8

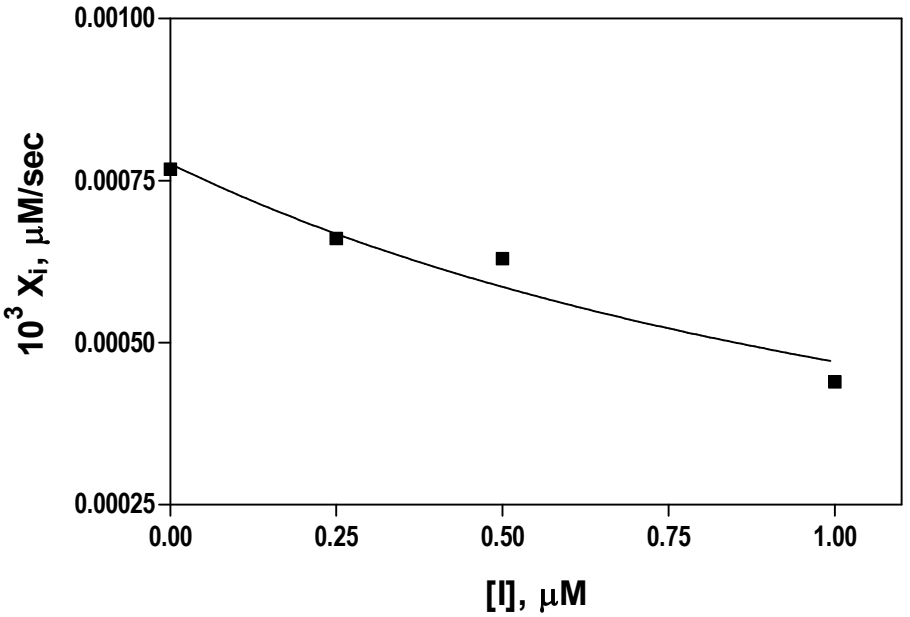


Figure 11. Plot of the parameter X_i vs. inhibitor concentration.

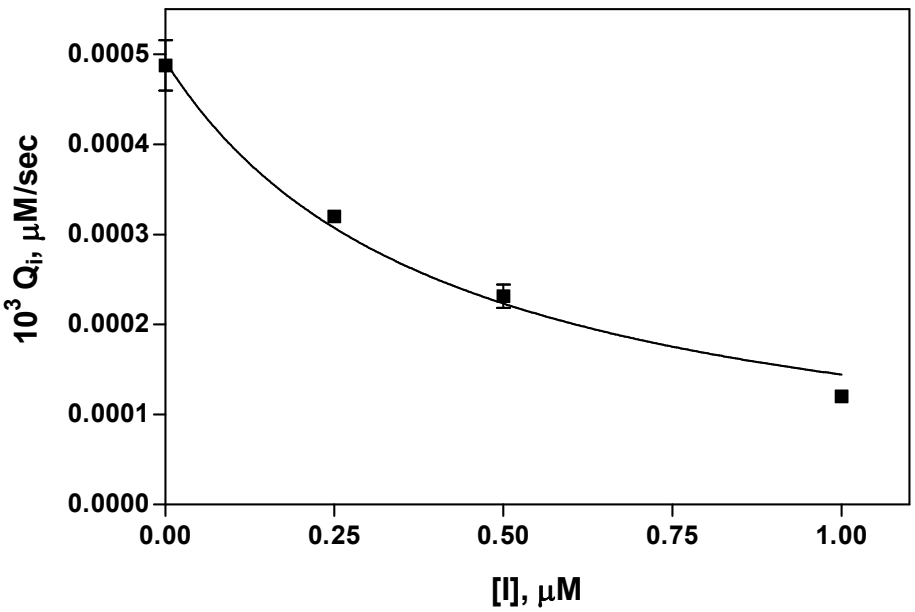


Figure 12. Plot of the parameter Q_i vs. inhibitor concentration.

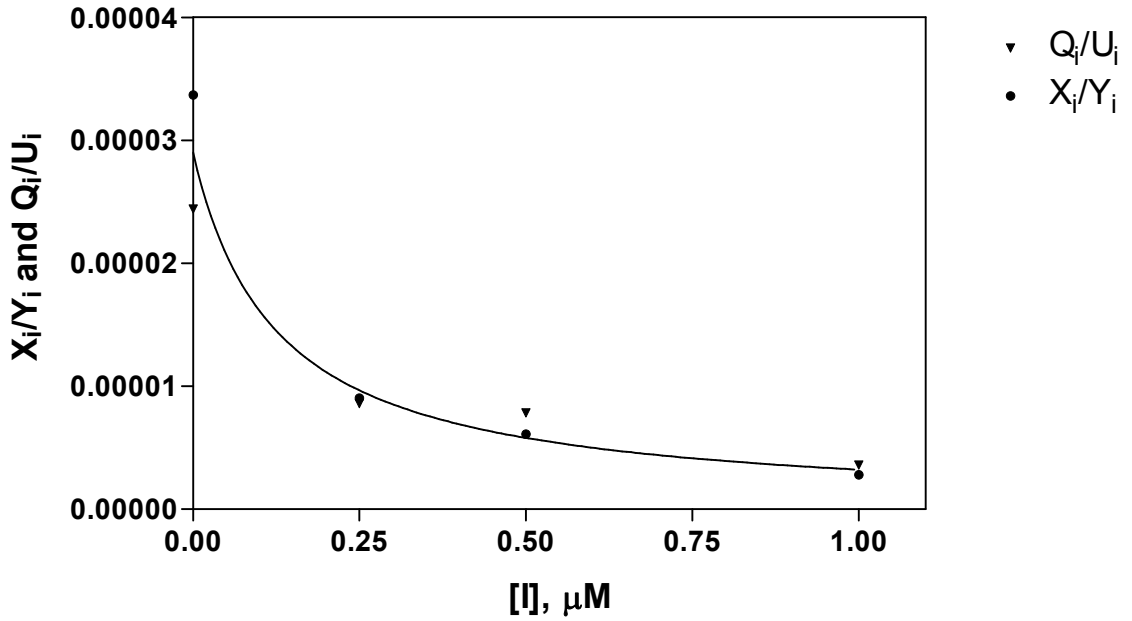


Figure 13. Plot of parameters X_i/Y_i and Q_i/U_i vs. inhibitor concentration.

Table 4. Kinetic parameters, characterizing interaction of the bifunctional inhibitor AdoC(Ahx)Arg_6 with protein kinase A in the presence of two substrates, ATP and Kemptide, calculated from the steady-state kinetic data of the peptide phosphorylation reaction.

Parameter	**Value of the parameter, μM**
K_i	0,13 ± 0,03
K_{ai}	1,54 ± 0,46
K_{bi}	0,41 ± 0,06
K_{abi}	>1000

Conclusions

The two main conclusions can be drawn from the results of the kinetic study of inhibition of the substrate reaction, catalyzed by protein kinase A, by inhibitor AdoC(Ahx)Arg_6.

1. The inhibitor competes with both substrates for their binding sites. This conclusion follows from the fact that $K_i < K_{ai}$ and $K_i < K_{bi}$, and in the presence of both substrates the effectiveness of interaction of the inhibitor with the binding site of the enzyme decreases. This means that the compound is, indeed, a bifunctional inhibitor (or bisubstrate analog inhibitor) of protein kinase A, in agreement with the initial hypothesis used to design the molecule.
2. The complex of the enzyme with three ligands, EABI, can be excluded from the analysis, as its formation is statistically irrelevant under the conditions used for the kinetic study. In other words, occupation of the both substrate binding sites is sufficient for exclusion of the inhibitor molecule from the enzyme active center.

Besides these two conclusions it can be also observed that competition between the inhibitor and the two substrates is not a symmetrical phenomenon. The effectiveness of interaction of this inhibitor with the enzyme is decreased in the presence of ATP more that 11 times that could be rather close to the competitive mechanism of inhibition. On the other hand, in the presence of Kemptide the effectiveness of the inhibitor binding is decreased only 3 times. This means that the binding of the peptide fragment of the inhibitor molecule with the enzyme active center is not highly exclusive for peptide binding. In other words, there is quite high probability that the both molecules can be fitted simultaneously in the active center. This non-exclusive binding should arise from the fact that the appropriate binding sites are not completely overlapping. For better understanding of this situation, experimental data for other inhibitors of different structure of the peptide fragment or the linker group are needed.

We suggest that the proposed kinetic approach can be used for extension of this analysis, necessary for improvement of the theoretical and structural background, used for design of the bisubstrate analog inhibitors of protein kinases.

Summary

1. Kinetics of Kemptide (LRRASLG) phosphorylation by protein kinase A (E.C.2.7.1.37) was studied in wide ATP and peptide concentration interval and kinetic approach was proposed to characterize the interaction of these both substrates with the enzyme active center. The kinetic analysis was made under steady-state conditions and was based on assumption of the random-order mechanism of binding of the substrates. This analysis was based on application of the second-order rate constants, which do not reflect the kinetic details of the catalytic steps as well as the accompanying conformational and diffusion-controlled phenomena. Inhibition of the reaction by excess of ATP was observed and taken into consideration in data processing.
2. Kinetic model for inhibition of the protein kinase A catalyzed phosphorylation of Kemptide by a bisubstrate analog inhibitor was proposed, taking into consideration that the inhibitor interacts with separate areas of the enzyme active center. This analysis was based on application of the second-order rate constants that simplified the data processing and interpretation of the results. Data processing algorithm was proposed for this kinetic model.
3. Kinetic analysis of protein kinase A inhibition by bifunctional inhibitor AdoC(Ahx)Arg_6 was made following the reaction scheme proposed. Effectiveness of the inhibitor interaction with the free enzyme and with both enzyme-substrate complexes was characterized by the appropriate dissociation constants. Also it was found that the enzyme active center cannot accommodate simultaneously the inhibitor molecule and both substrates.
4. The results obtained confirm that the inhibitor AdoC(Ahx)Arg_6 interacts with both ATP and peptide binding sites on the enzyme. This interaction, however, has weaker effect on binding of peptide substrate, if compared with ATP binding, pointing probably to some possibility of simultaneous location of the peptide fragment of the inhibitor molecule and peptide substrate in the enzyme active center.
5. The possibility of relatively simple characterization of details of protein kinase inhibition by the bifunctional inhibitors may be important for understanding the inhibition mechanism, and will supports the targeted design of these inhibitors.

Kokkuvõte

Proteiinkinaas A (E.C.2.7.1.37) katalüüsitud sünteetilise peptiidsubstraadi LRRASLG (Kemptiid) fosforüleerimise kineetikat uuriti statsionaarse reaktsiooni tingimustes ja substraatide kontsentratsiooni laias vahemikus.Töötati välja lihtne protseduur, et iseloomustada mõlema substraadi interaktsiooni ensüümi aktiivtsentriga olukorras, kus puudub detailne arusaam selle ensüümreaktsiooni katalüütilise mehhanismi kohta. ATP liia puhul ilmnes substraatpidurdus, mis võeti andmetöötlusel arvesse. Selle bisubstraatse ensüümreaktsiooni kineetika uurimine võeti ette eesmärgiga leida lihtne metoodika proteiinkinaaside substraatide ja pöörduvate inhibiitorite toime kvantitatiivseks iseloomustamiseks, lähtudes reaktsiooni statsionaarse kineetika andmetest ja kasutades analüüsiks bimolekulaarseid kiiruskonstante, mis võimaldab vältida reaktsiooni kiirust limiteeriva reaktsioonistaadiumi määramisega seotud probleeme.

Koostati kineetiline mudel proteiinkinaasi interaktsiooni kirjeldamiseks inhibiitoritega, mille molekul jäljendab bisubstraatsel reaktsioonil tekkivat kompleksi, kus ensüümiga on samaaegselt seotud nii ATP kui ka fosforüleeritav peptiid või valk. Selle mudeli põhjusel oli uuritud bifunktsionaalse inhibiitori AdoC(Ahx)Arg_6 toimet proteiinkinaas A katalüütilise alaühikuga. Selles mudelis on eeldatud võimalust, et inhibiitor on korraga seotud nii ATP kui ka peptiidi sidumistsentrites või siis ainult ühes neist tsentritest. Ensüümi moodustatud binaarsete ja tertsiaalsete komplekside iseloomustamine töös esitatud andmetöötluse algoritmi abil pakub huvi inhibeerimismehhanismi sügavama mõistmise seisukohast ja see võib olla vajalik inhibiitorite eesmärgipärasel konstrueerimisel.

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Appendix

KINETIC ANALYSIS OF PEPTIDE, ATP AND BIFUNCTIONAL INHIBITOR INTERACTION WITH PROTEIN KINASE A

1. Kemptide Phosphorylation by Protein Kinase A

Table 1. Initial velocity of Kemptide phosphorylation by protein kinase A, measured in the presence of various ATP and Kemptide concentrations.

Kemptide, μM / ATP, μM	Initial velocity, $10^3 \cdot v$, μM/sec						
	5	10	20	40	60	100	200
5	0,88 ± 0,05	1,23 ± 0,07	1,62 ± 0,05	2,38 ± 0,04	2,68 ± 0,06	2,93 ± 0,07	2,96 ± 0,25
10	0,93 ± 0,02	1,31 ± 0,04	2,04 ± 0,08	3,12 ± 0,12	3,74 ± 0,42	4,05 ± 0,11	4,50 ± 0,25
15	1,10 ± 0,06	1,84 ± 0,09	2,87 ± 0,08	3,90 ± 0,10	5,37 ± 0,16	5,81 ± 0,19	6,33 ± 0,16
25	1,29 ± 0,08	1,99 ± 0,25	2,96 ± 0,13	4,33 ± 0,18	6,37 ± 0,11	8,06 ± 0,41	8,59 ± 0,18
40	1,44 ± 0,12	2,18 ± 0,10	3,25 ± 0,06	4,97 ± 0,13	6,95 ± 0,19	8,89 ± 0,16	9,69 ± 1,10
50	1,91 ± 0,13	3,88 ± 0,22	7,11 ± 0,12	8,97 ± 0,54	11,80 ± 0,51	11,70 ± 0,56	13,80 ± 0,64
100	2,52 ± 0,16	4,73 ± 0,21	8,18 ± 0,51	11,30 ± 0,61	14,20 ± 0,73	14,10 ± 0,98	19,40 ± 0,57
150	2,28 ± 0,13	4,65 ± 0,27	8,46 ± 0,21	12,80 ± 0,77	14,50 ± 0,42	17,90 ± 0,80	21,70 ± 0,77
200	2,39 ± 0,23	4,38 ± 0,30	7,62 ± 0,51	11,40 ± 0,58	15,30 ± 0,55	19,20 ± 1,69	21,90 ± 1,83
300	2,31 ± 0,39	4,23 ± 0,68	7,36 ± 0,43	10,60 ± 0,77	14,70 ± 0,89	18,20 ± 0,81	21,40 ± 1,24
400	2,31 ± 0,14	4,21 ± 0,37	7,69 ± 0,37	10,40 ± 0,30	13,20 ± 0,31	16,50 ± 0,46	20,70 ± 0,57
700	2,61 ± 0,20	4,18 ± 0,36	6,59 ± 0,33	8,23 ± 0,43	10,70 ± 0,41	14,40 ± 0,59	17,30 ± 0,60
1000	2,37 ± 0,49	4,09 ± 0,47	6,13 ± 1,39	8,08 ± 0,38	10,00 ± 0,53	13,60 ± 0,79	16,10 ± 0,53

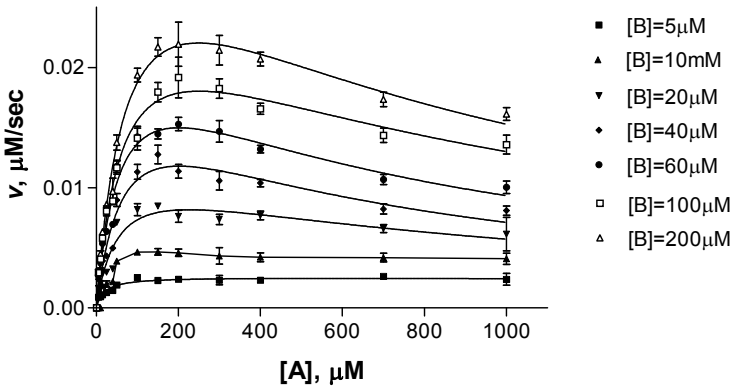
Table 2. Kinetic constants of Kemptide phosphorylation by protein kinase A in the presence of various ATP concentrations. Kemptide concentration was changed within each series of measurements.

ATP, μM	$10^3 \cdot V_m^{app\ B}$, μM/sec	$K_m^{app\ B}$, μM	$10^3 \cdot k_{II}^{app\ B}$, sec^{-1}
5	3,3 ± 0,1	16,9 ± 2,0	0,20 ± 0,03
10	5,2 ± 0,1	27,3 ± 2,4	0,19 ± 0,02
15	7,5 ±0,3	30,7 ± 3,9	0,24 ± 0,04
25	11,2 ± 0,3	50,7 ± 9,2	0,22 ± 0,06
40	12,6 ± 0,7	52,2 ± 7,8	0,24 ± 0,05
50	15,6 ± 0,7	27,1 ± 4,0	0,58 ± 0,01
100	21,7 ± 1,3	36,2 ± 6,3	0,60 ± 0,01
150	26,3 ± 0,6	45,3 ± 2,9	0,58 ± 0,05
200	28,5 ± 1,0	54,5 ± 4,4	0,52 ± 0,06
300	28,0 ± 0,9	57,6 ± 4,8	0,49 ± 0,06
400	26,0 ± 0,9	55,5 ± 4,7	0,47 ± 0,06
700	21,6 ± 1,4	53,9 ± 9,1	0,40 ± 0,10
1000	19,9 ± 1,2	50,4 ± 7,6	0,40 ± 0,08

Table 3. Kinetic constants for Kemptide phosphorylation by protein kinase A in the presence of various Kemptide concentrations. ATP concentration was changed within each series of measurements.

Kemptide, μM	$10^3 \cdot V_m^{app\ A}$, μM/sec	$K_m^{app\ A}$, μM	$10^3 \cdot k_{II}^{app\ A}$, sec^{-1}
5	2,6 ± 0,2	19,7 ± 4,7	0,13 ± 0,04
10	5,7 ± 0,6	33,7 ± 10,0	0,17 ± 0,07
20	1,2 ± 0,2	54,2 ± 19,5	0,22 ± 0,01
40	2,0 ± 0,4	71,9 ± 23,6	0,28 ± 0,01
60	2,4 ± 0,3	62,4 ± 14,4	0,39 ± 0,01
100	2,8 ± 0,2	66,0 ± 12,1	0,42 ± 0,01
200	3,6 ± 0,3	80,6 ± 12,0	0,45 ± 0,01

(a)



(b)

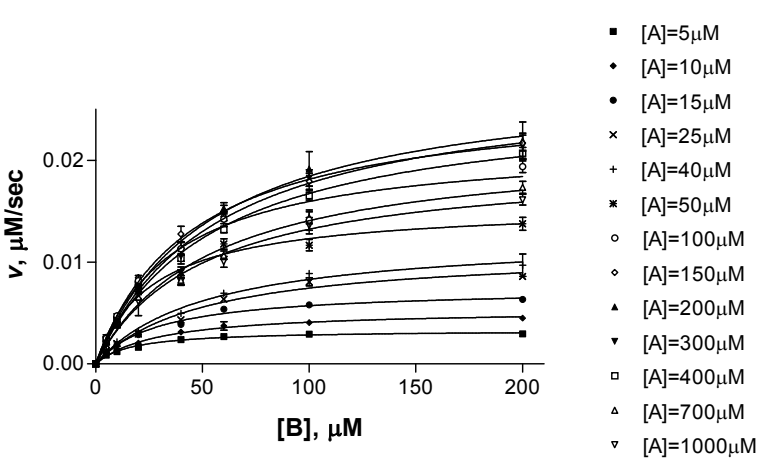
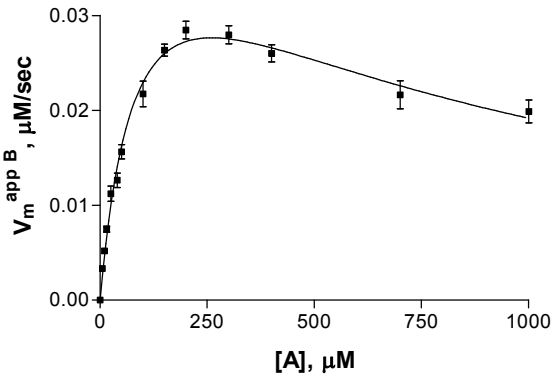


Figure 1. Protein kinase A catalyzed reaction of Kemptide (LRRASLG) phosphorylation.

(a)



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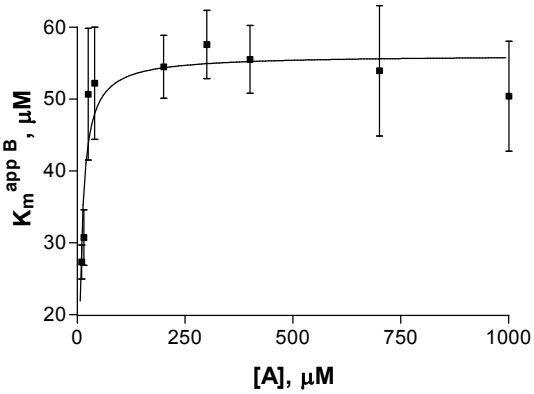
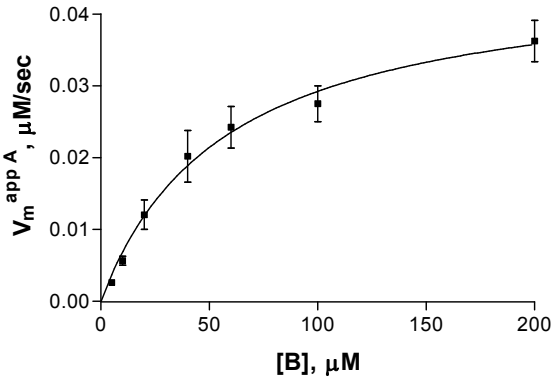


Figure 2. Plots of $V_m^{app\,B}$ and $K_m^{app\,B}$ vs. ATP concentration for Kemptide phosphorylation by protein kinase A.

(a)



(b)

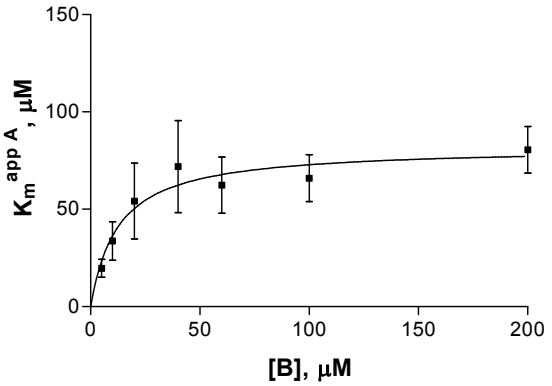


Figure 3. Plots of V_m^{appA} and K_m^{appA} vs. peptide concentration for Kemptide phosphorylation by protein kinase A.

2. Inhibition of the Protein Kinase A Catalyzed Phosphorylation of Kemptide by AdoC(Ahx)Arg_6

Inhibitor concentration 0.25 μM

Table 1. Initial velosity of Kemptide phosphorylation by protein kinase A in the presence of various ATP concentrations and 0.25 μM inhibitor AdoC(Ahx)Arg_6. Kemptide concentration was changed in each series of measurements.

Kemptide, μM / ATP, μM	Initial velocity, 10^3·v, μM/sec						
	5	10	20	40	60	100	200
5	0,19 ± 0,03	0,33 ± 0,02	0,54 ± 0,04	0,79 ± 0,07	0,93 ± 0,08	1,09 ± 0,18	1,25 ± 0,19
10	0,35 ± 0,06	0,62 ± 0,05	1,02 ± 0,21	1,50 ± 0,22	1,77 ± 0,13	2,09 ± 0,25	2,40 ± 0,11
15	0,49 ± 0,05	0,88 ± 0,08	1,45 ± 0,13	2,15 ± 0,23	2,55 ± 0,24	3,01 ± 0,19	3,48 ± 0,23
25	0,73 ± 0,13	1,31 ± 0,02	2,19 ± 0,16	3,27 ± 0,15	3,92 ± 0,27	4,66 ± 0,21	5,43 ± 0,38
40	0,99 ± 0,17	1,80 ± 0,31	3,03 ± 0,81	4,60 ± 0,55	5,56 ± 0,71	6,68 ± 0,83	7,86 ± 0,91
60	1,22 ± 0,14	2,23 ± 0,90	3,79 ± 0,72	5,85 ± 0,91	7,13 ± 1,31	8,66 ± 0,44	10,31 ± 1,42
200	1,45 ± 0,53	2,74 ± 0,64	4,90 ± 0,44	8,11 ± 0,63	9,84 ± 0,85	13,35 ± 0,80	17,01 ± 0,76
300	1,31 ± 0,54	2,49 ± 0,52	4,53 ± 0,64	7,68 ± 0,54	10,01 ± 1,01	13,20 ± 0,98	17,36 ± 2,71
400	1,16 ± 0,35	2,22 ± 0,21	4,08 ± 0,43	7,03 ± 0,65	9,26 ± 0,65	12,42 ± 1,55	16,69 ± 1,77
700	0,84 ± 0,32	1,62 ± 0,43	3,03 ± 0,18	5,38 ± 0,53	7,24 ± 0,34	10,02 ± 1,09	14,07 ± 1,38
1000	0,85 ± 0,10	1,26 ± 0,48	2,39 ± 0,92	4,29 ± 0,19	5,85 ± 0,72	8,24 ± 0,96	11,90 ± 0,82

Table 2. Kinetic constants for Kemptide phosphorylation by protein kinase A in the presence of 0.25 μM inhibitor AdoC(Ahx)Arg_6, calculated at various fixed ATP concentrations from v vs. Kemptide concentration plots.

ATP, μM	$10^3 \cdot V_m^{app\ B}$, μM/sec	$K_m^{app\ B}$, μM	$10^3 \cdot k_{II}^{app\ B}$, sec^{-1}
5	1,5 ± 0,01	34,6 ± 2,0	0,04 ± 0,003
10	2,8 ± 0,02	35,7 ± 0,4	0,08 ± 0,001
15	4,1 ± 0,04	37,0 ± 12,2	0,11 ± 0,04
25	6,5 ± 0,09	39,5 ± 26,7	0,16 ± 0,11
40	9,6 ± 0,20	43,0 ± 11,4	0,22 ± 0,06
60	12,7 ± 0,94	47,2 ± 8,2	0,27 ± 0,07
200	23,5 ± 0,80	75,8 ± 5,1	0,31 ± 0,03
300	25,3 ± 0,80	91,9 ± 5,7	0,28 ± 0,03
400	25,4 ± 0,80	104,7 ± 3,9	0,24 ± 0,02
700	23,6 ± 1,05	135,8 ± 11,3	0,17 ± 0,02
1000	21,4 ± 1,41	159,1 ± 8,2	0,13 ± 0,02

Table 3. Kinetic constants in precence of 0.25 μM AdoC(Ahx)Arg_6 inhibitor for Kemptide phosphorylation by protein kinase A at the presence of various Kemptide concentrations, calculated at various fixed Kemptide concentrations from v vs. ATP concentration plots.

Kemptide, μM	$10^3 \cdot V_m^{app\ A}$, μM/sec	$K_m^{app\ A}$, μM	$10^3 \cdot k_{II}^{app\ A}$, sec^{-1}
5	2,5 ± 0,3	59,2 ± 10,1	0,04 ± 0,01
10	5,8 ± 0,01	82,8 ± 1,9	0,07 ± 0,002
20	10,3 ± 0,03	90,4 ± 3,6	0,11 ± 0,01
40	16,8 ± 0,1	101,4 ± 6,4	0,17 ± 0,01
60	19,4 ± 0,7	97,1 ± 5,6	0,20 ± 0,02
100	27,0 ± 0,2	118,6 ± 12,3	0,23 ± 0,03
200	33,8 ± 0,3	129,8 ± 18,0	0,26 ± 0,04

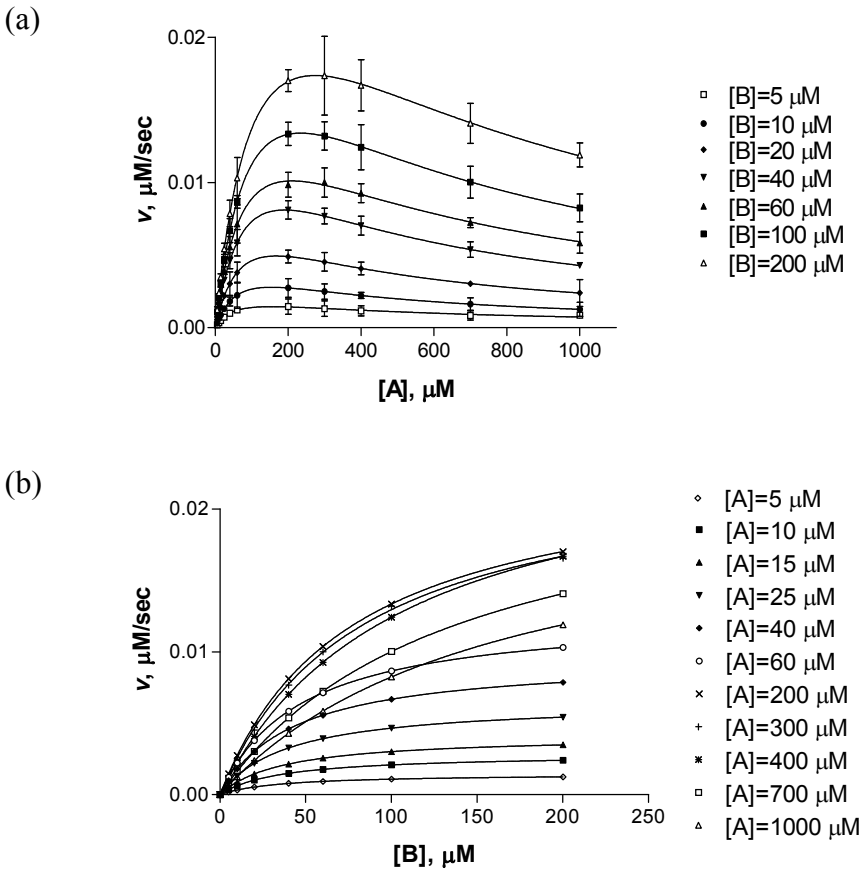
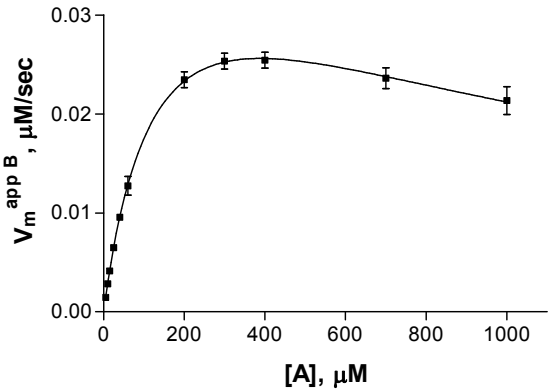
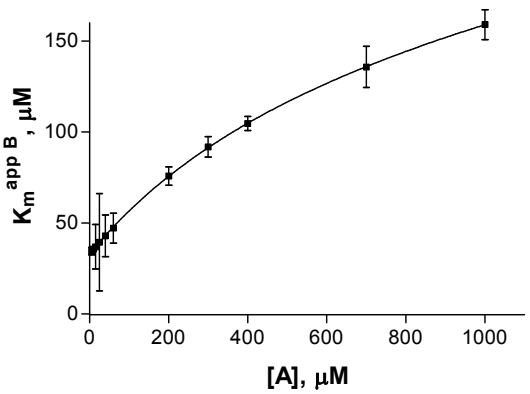


Figure 1. Protein kinase A catalyzed reaction of Kemptide (LRRASLG) phosphorylation in the presence of 0.25 μM inhibitor AdoC(Ahx)Arg_6.

(a)



(b)



(c)

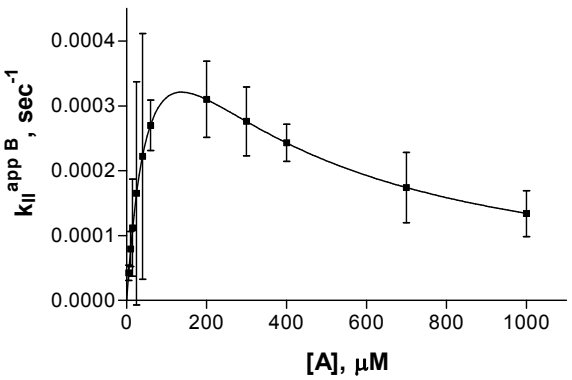


Figure 2. Dependences of kinetic parameters $V_m^{app\,B}$, $K_m^{app\,B}$ and $k_{II}^{app\,B}$ for Kemptide phosphorylation by protein kinase A in the precence of 0.25 μM inhibitor AdoC(Ahx)Arg_6 upon ATP concentration

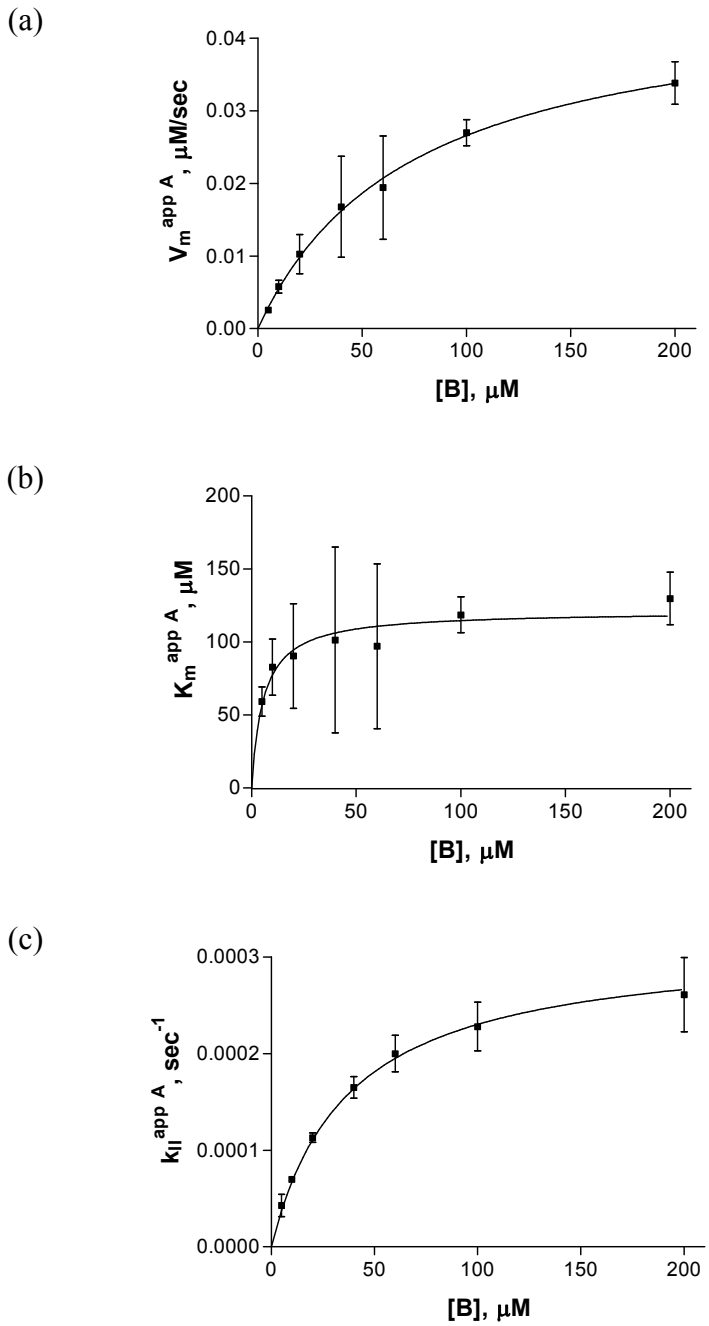


Figure 3. Dependences of kinetic parameters $V_m^{app A}$, $K_m^{app A}$ and $k_{II}^{app\ A}$ for Kemptide phosphorylation by protein kinase A in the precence of 0.25 μM inhibitor AdoC(Ahx)Arg$_6$ upon Kemptide concentration.

Inhibitor concentration 0.5 μM

Table 4. Initial velosity of Kemptide phosphorylation by protein kinase A in the presence of various ATP concentrations and 0.5 μM inhibitor AdoC(Ahx)Arg_6. Kemptide concentration was changed in each series of measurements.

Kemptide, μM / ATP, μM	Initial velocity, 10^3·v, μM/sec						
	5	10	20	40	60	100	200
5	0,12 ± 0,02	0,21 ± 0,06	0,32 ± 0,09	0,45 ± 0,06	0,52 ± 0,05	0,58 ± 0,03	0,65 ± 0,06
10	0,25 ± 0,03	0,45 ± 0,04	0,75 ± 0,03	1,12 ± 0,06	1,35 ± 0,08	1,61 ± 0,17	1,88 ± 0,13
15	0,36 ± 0,05	0,65 ± 0,12	1,09 ± 0,15	1,66 ± 0,25	2,00 ± 0,41	2,40 ± 0,12	2,83 ± 0,51
25	0,55 ± 0,01	0,99 ± 0,07	1,67 ± 0,17	2,53 ± 0,12	3,06 ± 0,23	3,68 ± 0,51	4,34 ± 0,21
50	0,92 ± 0,11	1,71 ± 0,22	3,00 ± 0,24	4,80 ± 0,37	6,00 ± 0,38	7,50 ± 0,32	9,23 ± 0,42
100	1,29 ± 0,27	2,36 ± 0,33	4,05 ± 0,31	6,29 ± 0,21	7,72 ± 0,49	9,42 ± 0,52	11,3 ± 0,56
150	1,48 ± 0,22	2,74 ± 0,30	4,79 ± 0,30	7,66 ± 0,51	9,57 ± 0,65	12,0 ± 0,40	14,7 ± 0,67
200	1,55 ± 0,19	2,89 ± 0,21	5,09 ± 0,38	8,24 ± 0,40	10,4 ± 0,64	13,1 ± 0,57	16,3 ± 0,98
300	1,54 ± 0,13	2,88 ± 0,20	5,10 ± 0,52	8,31 ± 0,31	10,5 ± 0,44	13,3 ± 0,94	16,7 ± 0,52
400	1,46 ± 0,90	2,74 ± 0,31	4,86 ± 0,99	7,93 ± 0,49	10,0 ± 0,29	12,8 ± 0,12	16,0 ± 1,33
700	1,19 ± 0,19	2,24 ± 0,76	3,97 ± 0,53	6,48 ± 0,72	8,21 ± 0,85	10,4 ± 1,00	13,1 ± 0,64
1000	0,96 ± 0,24	1,87 ± 0,35	3,35 ± 0,58	5,55 ± 0,24	7,11 ± 0,11	9,16 ± 0,84	11,7 ± 0,87

Table 5. Kinetic constants for Kemptide phosphorylation by protein kinase A in the presence of 0.5 μM inhibitor AdoC(Ahx)Arg_6, calculated at various ATP concentrations from v vs. Kemptide concentration plots.

ATP, μM	$10^3 \cdot V_m^{app\ B}$, μM/sec	$K_m^{app\ B}$, μM	$10^3 \cdot k_{II}^{app\ B}$, sec^{-1}
5	0,7 ± 0,1	25,0 ± 0,5	0,03 ± 0,01
10	2,3 ± 0,2	40,7 ± 0,9	0,06 ± 0,01
15	3,4 ± 0,4	43,0 ± 1,4	0,08 ± 0,01
25	5,3 ± 0,5	43,4 ± 1,1	0,12 ± 0,01
50	12,0 ± 0,4	60,0 ± 0,5	0,20 ± 0,01
100	14,1 ± 0,5	49,7 ± 0,8	0,28 ± 0,01
150	19,1 ± 1,3	59,7 ± 2,8	0,32 ± 0,04
200	21,6 ± 1,8	64,6 ± 1,3	0,33 ± 0,03
300	22,3 ± 0,9	67,6 ± 2,0	0,33 ± 0,05
400	21,5 ± 1,6	68,5 ± 4,4	0,31 ± 0,09
700	17,6 ± 1,2	68,6 ± 2,9	0,26 ± 0,04
1000	16,2 ± 1,1	76,7 ± 3,4	0,21 ± 0,08

Table 6. Kinetic constants for Kemptide phosphorylation by protein kinase A in the precence of 0.5 μM inhibitor AdoC(Ahx)Arg_6, calculated at various Kemptide concentrations fron the v vs. ATP concentration plots.

Kemptide, μM	$10^3 \cdot V_m^{app\ A}$, μM/sec	$K_m^{app\ A}$, μM	$10^3 \cdot k_{II}^{app\ A}$, sec^{-1}
5	3,0 ± 0,1	112,8 ± 3,1	0,03 ± 0,01
10	5,6 ± 0,1	113,5 ± 4,7	0,05 ± 0,01
20	10,0 ± 0,4	120,6 ± 8,4	0,08 ± 0,01
40	16,6 ± 1,1	132,3 ± 14,7	0,13 ± 0,02
60	21,2 ± 1,7	139,5 ± 19,2	0,15 ± 0,03
100	27,5 ± 2,9	150,8 ± 25,7	0,18 ± 0,05
200	34,8 ± 4,4	161,9 ± 33,4	0,21 ± 0,07

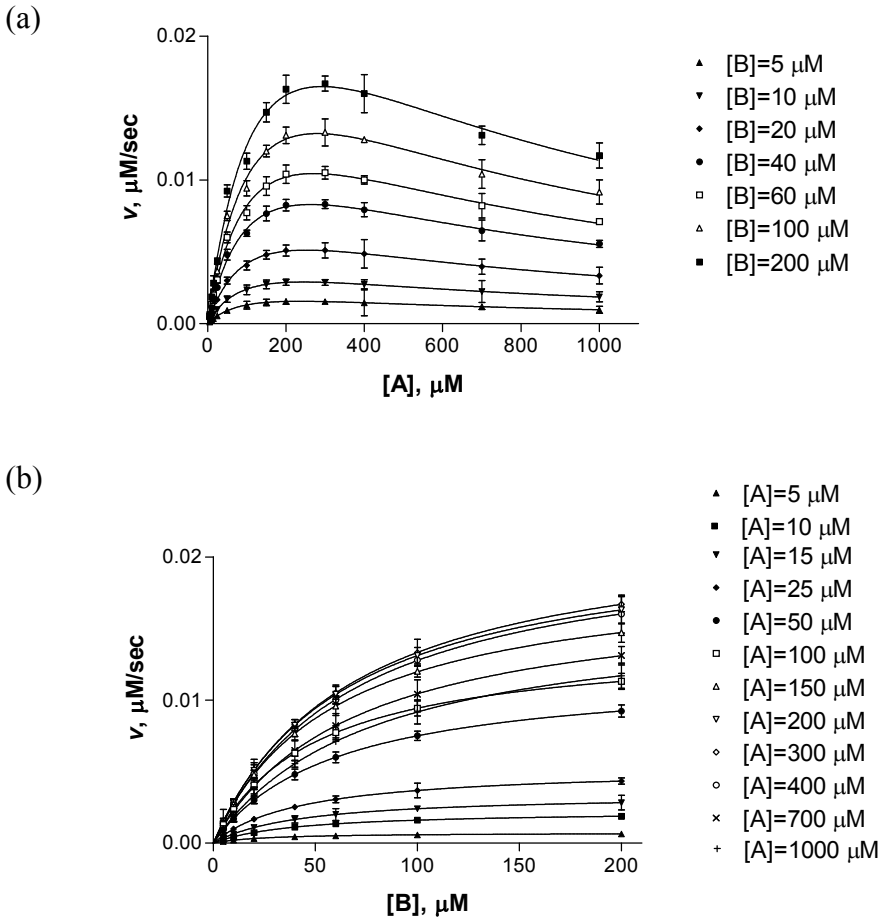


Figure 4. Protein kinase A catalyzed reaction of Kemptide (LRRASLG) phosphorylation in the precence of 0.5 μM inhibitor AdoC(Ahx)Arg$_6$.

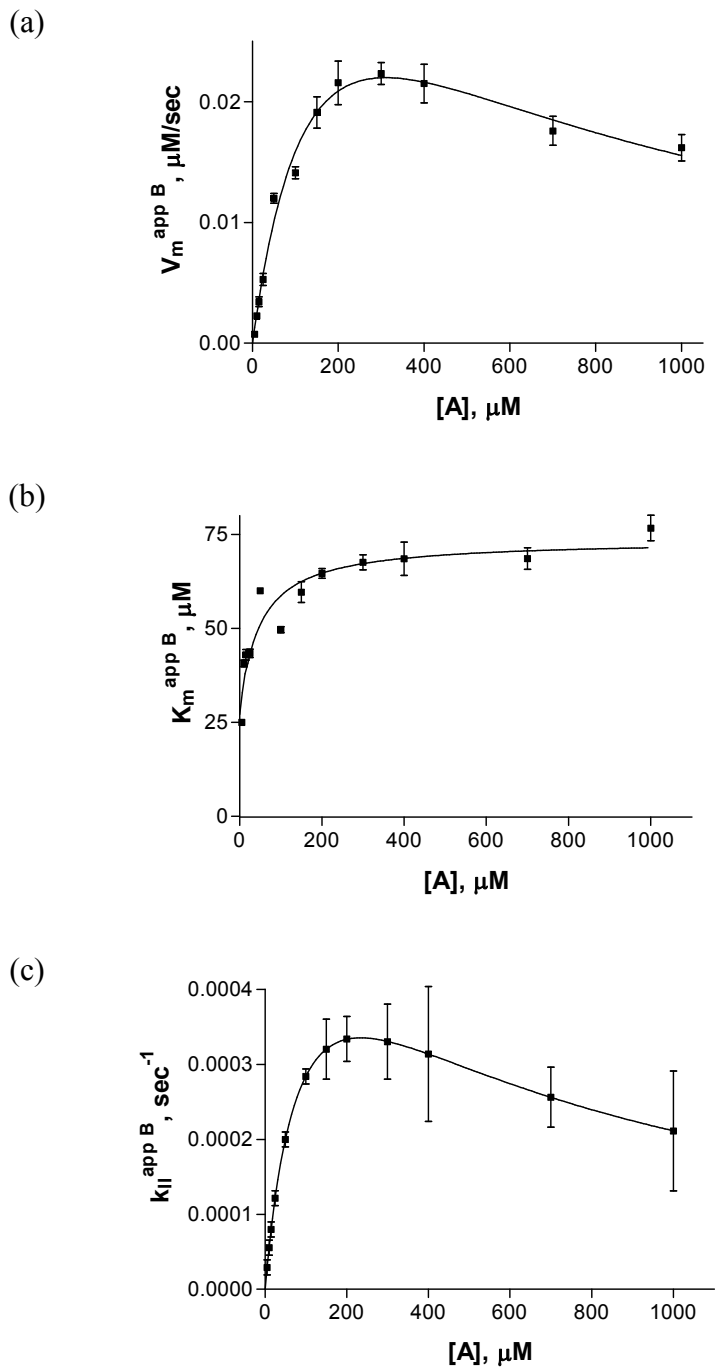


Figure 5. Dependences of kinetic parameters $V_m^{app B}$, $K_m^{app B}$ and $k_{II}^{app B}$ for Kemptide phosphorylation by protein kinase A in the precence of 0.5 μM inhibitor AdoC(Ahx)Arg$_6$ upon ATP concentration.

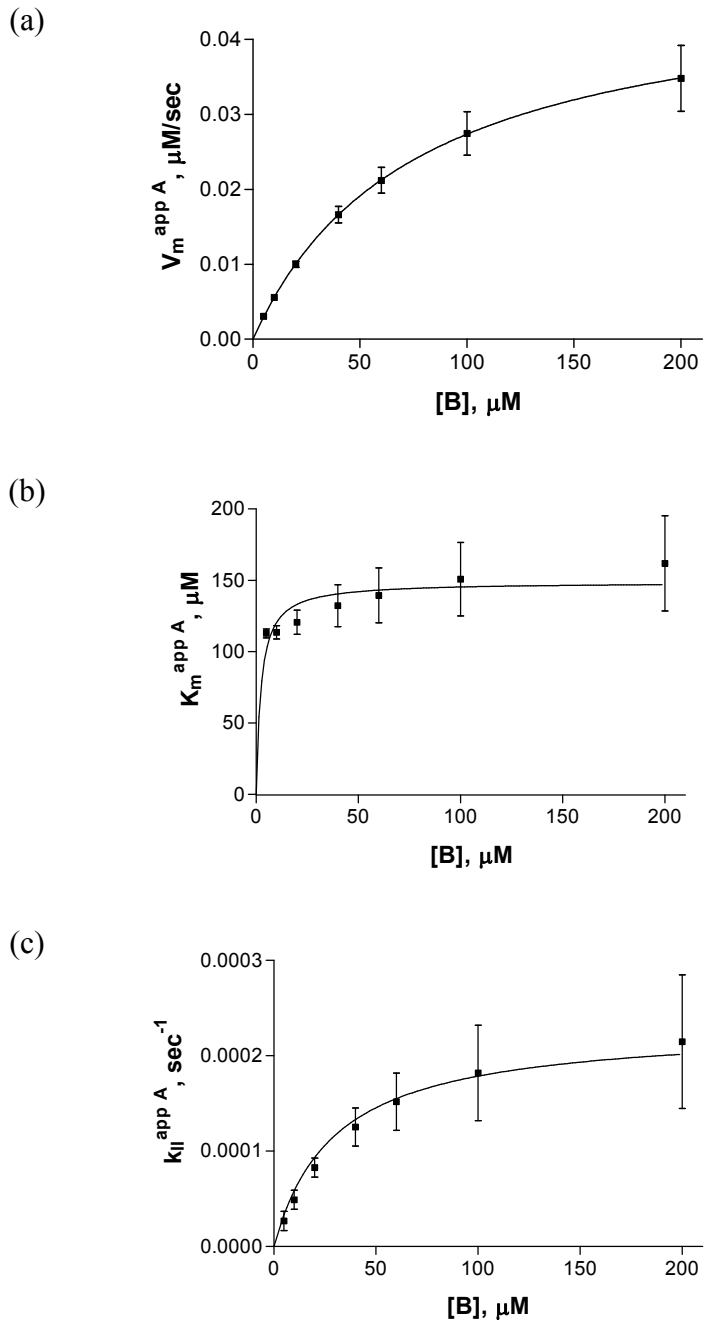


Figure 6. Dependences of kinetic parameters $V_m^{app\,A}$, $K_m^{app\,A}$ and $k_{II}^{app\,A}$ for Kemptide phosphorylation by protein kinase A in the precence of 0.5 μM inhibitor AdoC(Ahx)Arg_6 upon Kemptide concentration.

Inhibitor concentration 1.0 μM

Table 7. Initial velosity of Kemptide phosphorylation by protein kinase A in the presence of various ATP concentrations and 1.0 μM inhibitor AdoC(Ahx)Arg_6. Kemptide concentration was changed in each series of measurements.

Kemptide, μM / ATP, μM	Initial velocity, 10^3·v, μM/sec						
	5	10	20	40	60	100	200
5	0,06 ± 0,02	0,10 ± 0,03	0,16 ± 0,07	0,24 ± 0,06	0,28 ± 0,07	0,46 ± 0,11	0,47 ± 0,08
10	0,12 ± 0,04	0,21 ± 0,03	0,35 ± 0,09	0,54 ± 0,05	0,65 ± 0,03	0,91 ± 0,03	0,92 ± 0,07
15	0,16 ± 0,02	0,28 ± 0,02	0,45 ± 0,02	0,64 ± 0,03	0,75 ± 0,03	1,34 ± 0,04	1,35 ± 0,07
25	0,27 ± 0,02	0,50 ± 0,13	0,88 ± 0,10	1,42 ± 0,54	1,79 ± 0,41	2,15 ± 0,55	2,18 ± 0,59
40	0,39 ± 0,04	0,74 ± 0,05	1,33 ± 0,08	2,23 ± 0,10	2,87 ± 0,12	3,23 ± 0,16	3,30 ± 0,16
100	0,67 ± 0,18	1,27 ± 0,48	2,30 ± 0,35	3,86 ± 0,73	4,99 ± 0,71	6,10 ± 0,47	6,44 ± 0,66
150	0,77 ± 0,36	1,47 ± 0,48	2,67 ± 0,26	4,53 ± 0,43	5,88 ± 0,69	7,14 ± 0,98	7,77 ± 1,33
200	0,82 ± 0,12	1,58 ± 0,60	2,90 ± 0,24	4,99 ± 1,74	6,58 ± 0,82	7,48 ± 0,96	8,36 ± 1,32
250	0,84 ± 0,37	1,60 ± 0,76	2,93 ± 0,94	5,02 ± 1,58	6,59 ± 1,16	7,44 ± 1,38	8,49 ± 1,82
300	0,83 ± 0,50	1,57 ± 0,73	2,86 ± 1,42	4,85 ± 1,25	6,31 ± 2,13	7,21 ± 1,73	8,38 ± 1,19
400	0,80 ± 0,29	1,51 ± 0,43	2,76 ± 0,65	4,68 ± 1,70	6,10 ± 2,36	6,56 ± 0,99	7,82 ± 1,00
700	0,65 ± 0,38	1,32 ± 0,72	2,17 ± 0,80	3,56 ± 0,95	4,52 ± 1,13	5,38 ± 1,38	5,94 ± 1,29
1000	0,54 ± 0,15	1,15 ± 0,45	1,70 ± 0,79	2,94 ± 0,64	3,73 ± 0,95	4,30 ± 1,29	4,64 ± 1,07

Table 8. Kinetic constants for Kemptide phosphorylation by protein kinase A in the presence of 1.0 μM inhibitor AdoC(Ahx)Arg_6, calculated at various ATP concentrations from v vs. Kemptide concentration plots.

ATP, μM	$10^3 \cdot V_m^{app\ B}$, μM/sec	$K_m^{app\ B}$, μM	$10^3 \cdot k_{II}^{app\ B}$, sec^{-1}
5	0,6 ± 0,1	59,2 ± 3,9	0,01 ± 0,004
10	1,2 ± 0,7	45,8 ± 4,7	0,03 ± 0,02
15	1,9 ± 0,1	69,0 ± 4,3	0,03 ± 0,01
25	2,8 ± 0,9	38,2 ± 3,5	0,07 ± 0,02
40	4,2 ± 1,2	36,7 ± 2,4	0,11 ± 0,02
100	8,4 ± 1,4	46,4 ± 1,2	0,18 ± 0,02
150	10,1 ± 2,0	49,2 ± 1,8	0,21 ± 0,02
200	10,7 ± 2,0	46,7 ± 6,8	0,23 ± 0,03
250	10,8 ± 1,6	47,3 ± 7,1	0,23 ± 0,03
300	10,7 ± 0,8	48,9 ± 5,2	0,22 ± 0,02
400	9,8 ± 2,1	45,2 ± 6,9	0,22 ± 0,04
700	7,2 ± 1,1	42,2 ± 1,9	0,17 ± 0,02
1000	5,5 ± 0,9	37,4 ± 4,6	0,15 ± 0,02

Table 9. Kinetic constants for Kemptide phosphorylation by protein kinase A in precence of 1.0 μM inhibitor AdoC(Ahx)Arg_6, calculated at various fixed Kemptide concentrations from v vs. ATP concentration plots.

Kemptide, μM	$10^3 \cdot V_m^{app\ A}$, μM/sec	$K_m^{app\ A}$, μM	$10^3 \cdot k_{II}^{app\ A}$, sec^{-1}
5	1,7 ± 0,3	135,6 ± 13,9	0,01 ± 0,003
10	3,0 ± 0,1	121,7 ± 8,7	0,02 ± 0,002
20	7,4 ± 0,4	189,3 ± 16,8	0,04 ± 0,01
40	14,1 ± 1,4	226,8 ± 32,4	0,06 ± 0,02
60	21,3 ± 3,1	277,6 ± 54,2	0,08 ± 0,03
100	20,0 ± 8,3	196,5 ± 111,3	0,09 ± 0,05
200	30,9 ± 7,8	322,1 ± 76,3	0,10 ± 0,04

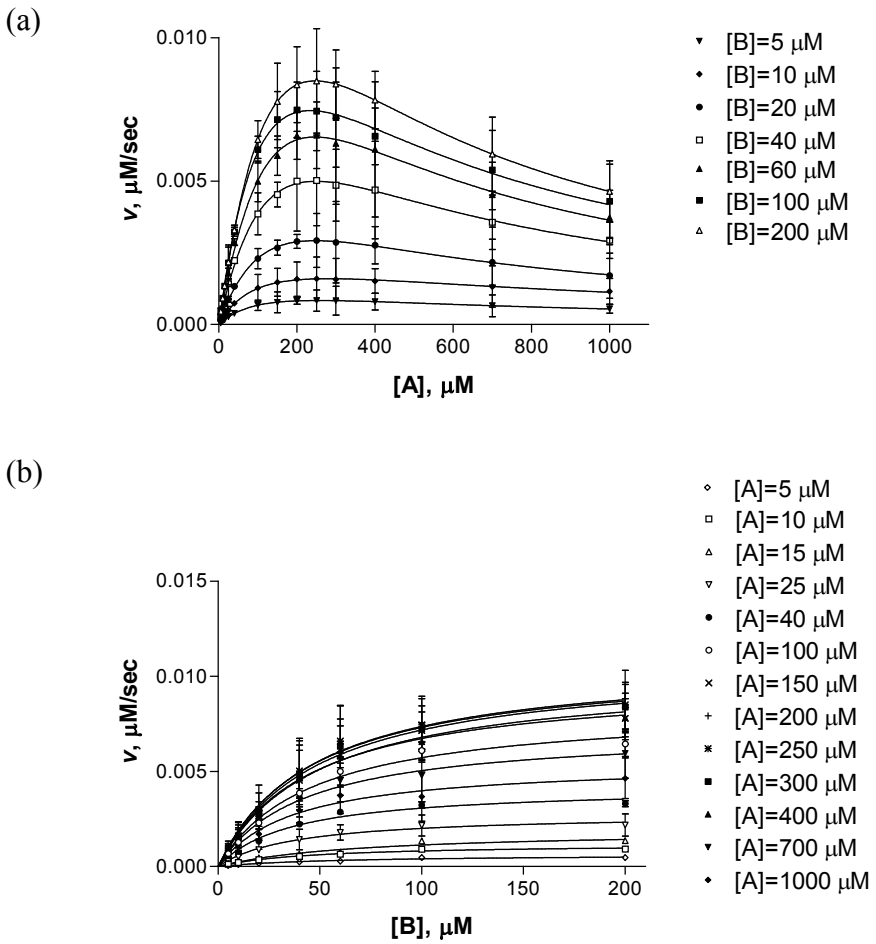
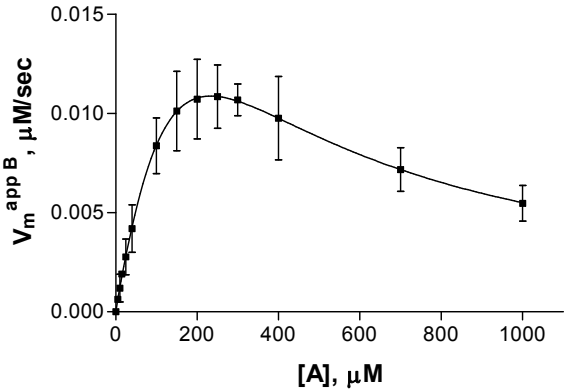
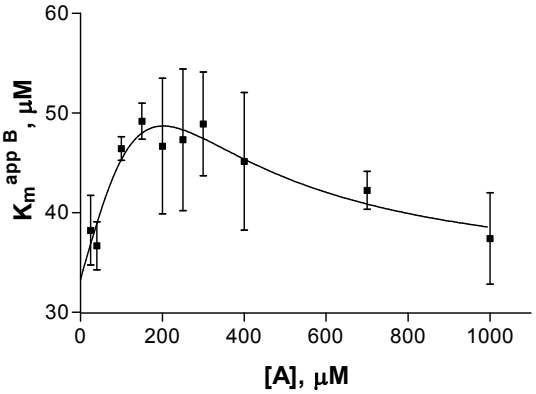


Figure 7. Protein kinase A catalyzed reaction of Kemptide (LRRASLG) phosphorylation in the precence of 1,0 μM inhibitor AdoC(Ahx)Arg$_6$.

(a)



(b)



(c)

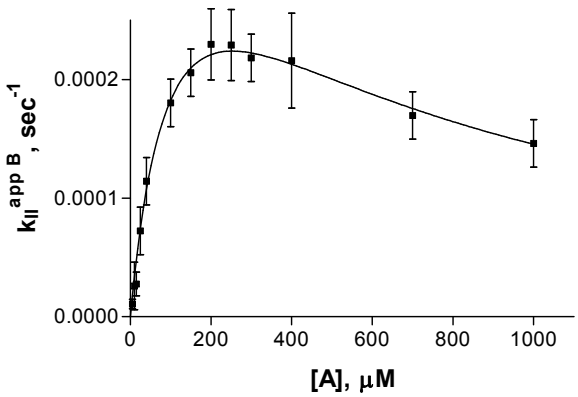


Figure 8. Dependences of kinetic parameters $V_m^{app\,B}$, $K_m^{app\,B}$ and $k_{II}^{app\,B}$ for Kemptide phosphorylation by protein kinase A in the precence of 1.0 μM inhibitor AdoC(Ahx)Arg$_6$ upon ATP concentration.

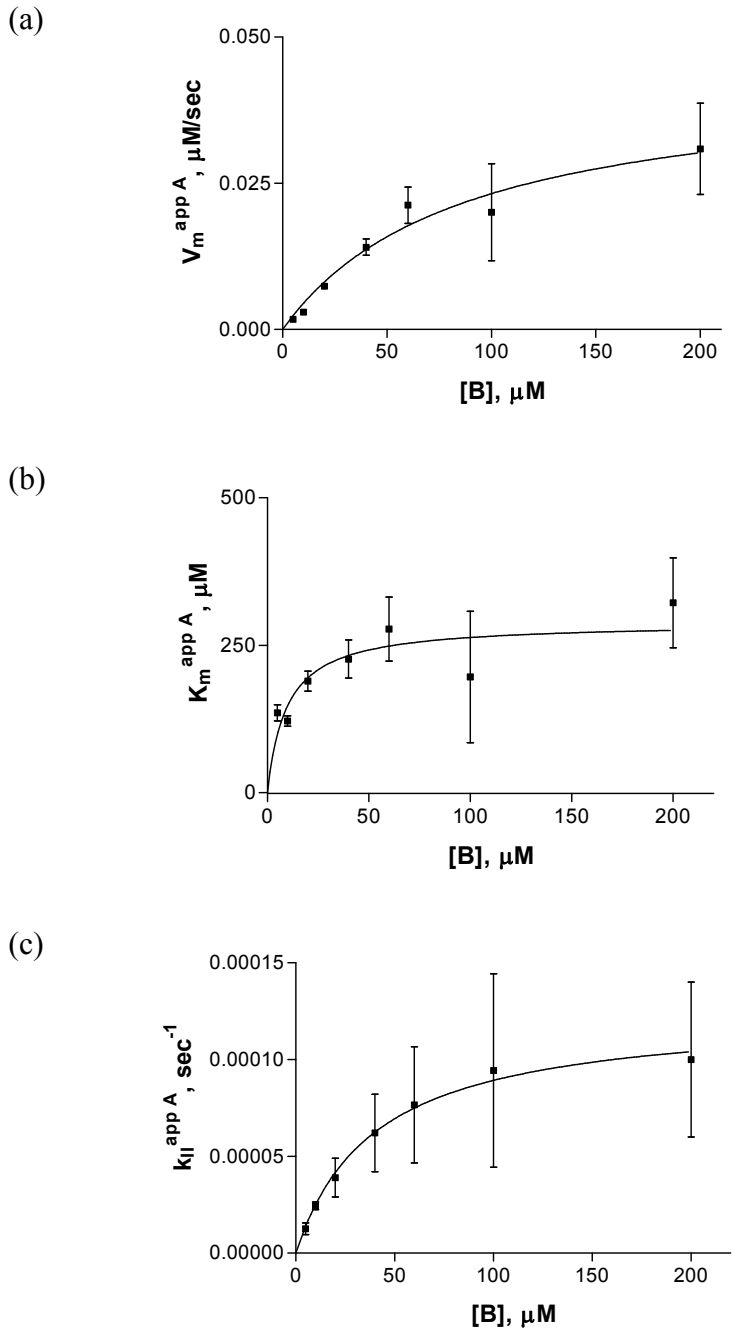


Figure 9. Dependences of kinetic parameters $V_m^{app\,A}$, $K_m^{app\,A}$ and $k_{II}^{app\,A}$ for Kemptide phosphorylation by protein kinase A in the precence of 1.0 μM inhibitor AdoC(Ahx)Arg$_6$ upon Kemptide concentration.