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Steady-state kinetic analysis of protein kinase A interaction with peptide and ATP

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Abstract. Kinetics of Kemptide (LRRASLG) phosphorylation by protein kinase A (E.C.2.7.1.37) was studied in a wide ATP and peptide concentration interval and a simple procedure was proposed to characterize the interaction of these two substrates with the enzyme active centre. The kinetic analysis was made under steady-state conditions and was based on the assumption of the random-order mechanism of binding of the two substrates. Inhibition of the reaction by excess of ATP was observed and considered in data processing. A procedure was designed for analysis of the interaction mechanism of protein kinase reversible inhibitors with the enzyme proceeding from the steady-state kinetic data.

Key words: protein kinase A, peptide phosphorylation, kinetic mechanism, bi-substrate reaction.

INTRODUCTION

Protein kinases (E.C.2.7.1.37) are enzymes involved in the regulation of many cellular processes [1]. This regulation is based on highly specific chemical modification of cell proteins by phosphorylation of certain serine, threonine, or tyrosine residues in the structure of various enzymes [2]. The phosphate group is transferred to the corresponding hydroxyl function of the substrate protein from the ATP–magnesium complex and the phosphorylatable site is recognized by the protein kinase. In this recognition process the peptide sequence, flanking the phosphorylatable amino acid, is evidently one of the specificity determining factors that governs selectivity of the regulatory phosphorylation phenomena [3].

The role of these “local sequence elements” in the 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, but allow

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more explicit kinetic study of the phosphorylation reaction, and considerable amount of data has been obtained about the variation of the local sequence specificity of different protein kinases [4]. As the second substrate of the regulatory phosphorylation reaction is always an ATP complex with magnesium ion(s), its recognition in the active site of different protein kinases seems to by rather universal [5, 6].

The design of the active-site directed inhibitors of protein kinases has proceeded from structures of both of these substrates. In general, ATP analogues have yielded more potent inhibition compared with peptide analogues, where the phosphorylatable amino acid is replaced by alanine or some other non-phosphorylatable amino acid [7]. On the other hand, the possibilities for tuning the inhibitor selectivity seem to be rather limited if proceeding from compounds directed into the ATP binding site. Therefore bi-substrate inhibitors have been proposed, where the effectiveness of the inhibitor binding into the ATP site is combined with a wide spectrum of the peptide specificity patterns. Until recently different types of such compounds have been designed and described as rather effective inhibitors of several protein kinases [8]. However, for understanding the inhibition mechanism as well as for rational design of the protein kinase bi-substrate inhibitors, it is necessary to evaluate the effectiveness of the interaction of these compounds with the enzyme not only on the IC_{50} level, but also on the level of the true dissociation constants. The latter task is, however, complicated, as the bi-substrate mechanism of the phosphorylation reaction is complex and involves besides binding and catalytic steps rate-limiting conformational transitions [9]. Moreover, the reaction mechanism seems to be different for different kinases and its elucidation needs application of rather sophisticated methods.

In this paper we suggest a solution of this problem using the second-order rate constants k_{II} of the peptide phosphorylation reaction. These constants can be determined under the steady-state conditions and calculated as the ratio of the apparent kinetic parameters k_{cat} and K_m. This approach was practically tested for protein kinase A reaction with its specific peptide substrate LRRASLG (Kemptide) [10].

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.

Enzyme

The catalytic subunit of protein kinase A was expressed using plasmid pRSET B based on the T7 promoter expression system (from Invitrogen). The target

gene responsible for coding the catalytic subunit of the enzyme was cloned into this vector. The desired bacteria were expressed in the presence of antibiotics as the vector has gene coding ampicillin resistance of the cells. So, other bacteria without target gene died during the expression. The expression was performed at 37 °C in YT medium, containing the antibiotic, at vigorous shaking till the absorbance at A_{600} reached 0.55–0.8 OD units. The following induction was done with IPTG (Isopropyl b-D-1-thiogalactopyranoside) and the culture was allowed to grow for an additional 6 h. The cells were harvested in centrifuge by spinning at 4068 × *g* for 4 min.

Enzyme purification

The harvested cells were sonicated in ice-cold Tris/HCl/EDTA buffer and then the homogenized solution was centrifuged to remove pellet. The catalytic subunit of protein kinase A was purified on the P-11 phosphocellulose column using isocratic elution. Purity of the protein was assayed by SDS-PAGE electrophoresis.

Peptide synthesis

Peptide LRRASLG (Kemptide) was synthesized in stepwise manner on 0.1 mmol scale using Applied Biosystem (USA) Model 431A peptide synthesizer. Solid phase synthesis used dicyclohexyl-carbodiimide/hydroxy-benzotriazole activation strategy. *tert*-Butyloxycarbonyl amino acids were coupled as hydroxy-benzotriazole esters to a *t*-Boc-Gly-PAM [4-(oxymethyl)-phenylacetamido-methyl] resin (0.6 mmol of amino groups/g, Neosystem, Strasbourg, France) to obtain C-terminal free acid containing peptide. The peptides were finally deprotected and cleaved from the resin with liquid HF at 0 °C for 60 min. Deprotection of the side-chains, cleavage of the peptides, and purification on HPLC have been described earlier in detail [11]. Peptide purity was >99% as demonstrated with HPLC on an analytical Nucleosil 120-3 C_{18} 0.4 cm × 10 cm reverse-phase HPLC-column. The molecular mass of the synthetic peptide was determined with a Plasma Desorption Mass Spectrometer (Applied Biosystems), and the calculated value was obtained.

Assay of peptide phosphorylation

Peptide phosphorylation by protein kinase A was carried out at 30 °C in 100 μL reaction mixture composed as follows: peptide substrate at concentrations from 5 to 200 μM (the stock solutions were prepared in 50 mM TRIS/HCl, pH 7.5); γ-[^{32}P]ATP at concentrations from 5 to 1000 μM (the stock solutions had specific radioactivity 60–200 cpm/pmol); 10 μL of 100 mM $MgCl_2$ solution; 15 μL of the enzyme solution in buffer containing 50 mM TRIS/HCl (pH 7.5); and 1 mg/mL BSA. The enzyme solution was prepared immediately before experiments by a 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 the 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. Proceeding from these data the product concentration vs. time plots were constructed and the initial velocities of peptide phosphorylation reaction were calculated.

Data processing

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

RESULTS

Kinetic data

Kinetic measurements were carried out in distinct series where the concentration of one substrate was constant and the concentration of the other substrate was systematically changed. The results of these experiments are shown in Fig. 1a and b as conventional rate–concentration plots. As summary of these data the three-dimensional plot was designed to illustrate the kinetic behavior of this bi-substrate reaction (Fig. 1c).

The shapes of the rate–concentration plots in Figs. 1a and 1b reveal certain asymmetry for peptide and ATP. Some inhibitory effect is revealed in the presence of excess of ATP, while for the peptide counterpart of the reaction no substrate inhibition was observed within the concentration interval used.

Substrate inhibition

Formally speaking, the phenomenon of substrate inhibition points to the formation of complexes that include two ATP molecules per enzyme and are less inactive regarding the phosphopeptide formation compared with the complexes with 1 : 1 stoichiometry. Therefore, the phenomenon of substrate inhibition was further analysed at a constant peptide concentration in the form of the semi-logarithmic plot $v = f(\log[A])$, as recommended by Berezin et al. [12]. It can be seen in Fig. 2 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. Thus enzyme–substrate complexes including two ATP molecules should not yield phosphopeptide.

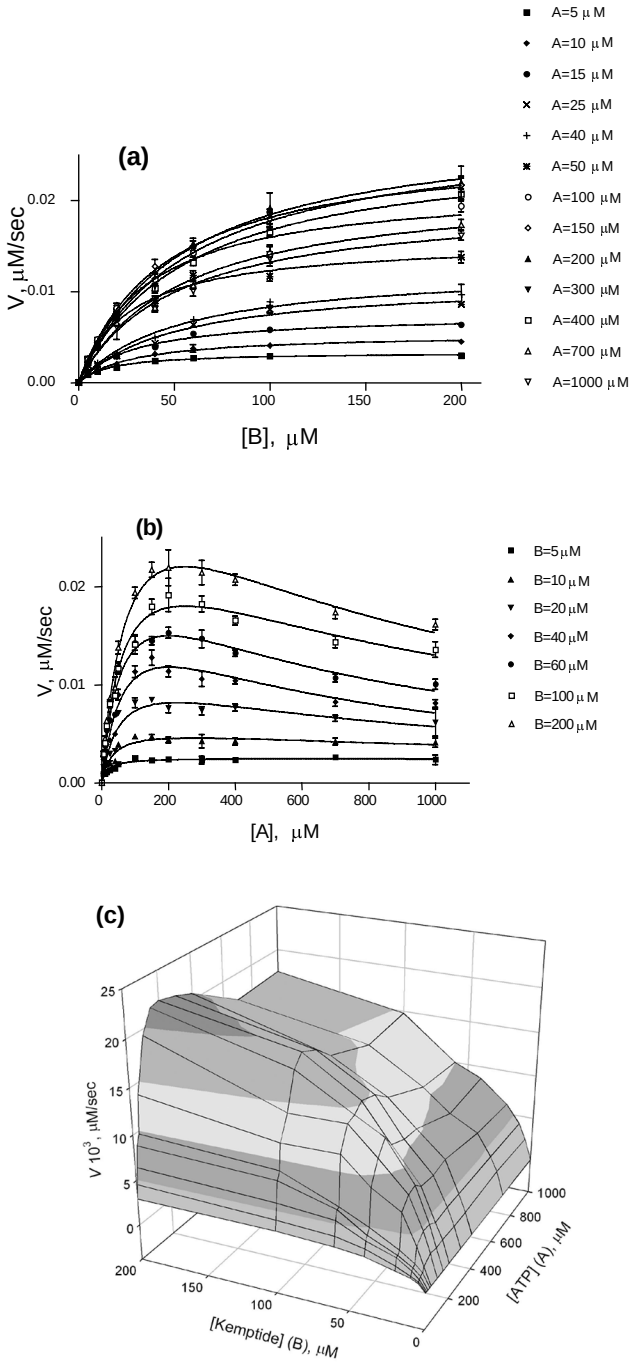


Fig. 1. Protein kinase A catalysed reaction of Kemptide (LRRASLG) phosphorylation. a – The Michaelis-Menten plots for peptide (Kemptide, B) in the presence of fixed ATP concentrations; b – the same plots for ATP (A) in the presence of fixed peptide concentrations; c – summary of plots a and b.

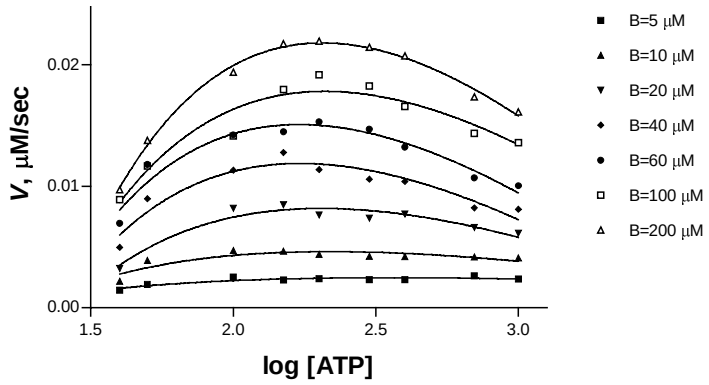
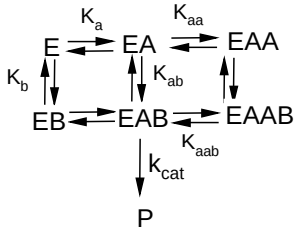


Fig. 2. Semi-logarithmic plot for Kemptide phosphorylation by protein kinase A. Different peptide concentrations were used to construct the dependences.

Reaction scheme

For catalytic act the two substrates, peptide and ATP, should simultaneously bind in the enzyme active centre. 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 [6]. Formally these equilibriums can be presented by the following reaction 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 accompanying conformational fluctuations as well as the diffusion-limited dissociation of the reaction products (see in [9]) can be excluded from the analysis. Therefore the product formation is designed by the simplest possible way in the reaction scheme under consideration.

Rate equations for substrate reaction

The rate equation for scheme 1 can be obtained proceeding from the following relationships and equilibriums:

$$v = k_{cat}[EAB], \tag{2}$$

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

$$[E] = \frac{K_a}{[A]}[EA] = \frac{K_a K_{ab}}{[A][B]}[EAB], \tag{4}$$

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

$$[EB] = \frac{[B]}{K_b}[E] = \frac{K_a K_{ab}}{K_b[A]}[EAB], \tag{6}$$

$$K_{ab} = \frac{[EA][B]}{[EAB]}, \tag{7}$$

$$[EA] = \frac{K_{ab}}{[B]}[EAB], \tag{8}$$

$$K_{aa} = \frac{[EA][A]}{[EAA]}, \tag{9}$$

$$[EAA] = \frac{[A]}{K_{aa}}[EA] = \frac{K_{ab}[A]}{K_{aa}[B]}[EAB], \tag{10}$$

$$K_{aab} = \frac{[EAB][A]}{[EAAB]}, \tag{11}$$

$$[EAAB] = \frac{[A]}{K_{aab}}[EAB], \tag{12}$$

and

$$[E_0] = [E] + [EA] + [EB] + [EAB] + [EAA] + [EAAB]. \tag{13}$$

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

$$v = \frac{k_{cat}[E_0][A][B]}{K_a K_{ab} + K_{ab}[A] + \frac{K_a K_{ab}}{K_b}[B] + [A][B] + \frac{1}{K_{aab}}[A]^2[B] + \frac{K_{ab}}{K_{aa}}[A]^2}. \tag{14}$$

It can be further processed in two ways. First, if ATP concentration (denoted as $[A]$) is constant within a series of experiments, the plot of the reaction velocity v vs. peptide concentration $[B]$ can be analysed:

$$v = \frac{\dfrac{k_{cat}[E_0][A]}{\dfrac{K_a K_{ab}}{K_b} + [A] + \dfrac{1}{K_{aab}}[A]^2}[B]}{\dfrac{K_a K_{ab} + K_{ab}[A] + \dfrac{K_{ab}}{K_{aa}}[A]^2}{\dfrac{K_a K_{ab}}{K_b} + [A] + \dfrac{1}{K_{aab}}[A]^2} + [B]} = \frac{V_m^{app^B}[B]}{K_m^{app^B} + [B]}. \quad (15)$$

The complex parameters $V_m^{app^B}$ and $K_m^{app^B}$ have the following meaning:

$$V_m^{app^B} = \frac{k_{cat}[E_0][A]}{\dfrac{K_a K_{ab}}{K_b} + [A] + \dfrac{1}{K_{aab}}[A]^2}, \quad (16)$$

$$K_m^{app^B} = \frac{K_a K_{ab} + K_{ab}[A] + \dfrac{K_{ab}}{K_{aa}}[A]^2}{\dfrac{K_a K_{ab}}{K_b} + [A] + \dfrac{1}{K_{aab}}[A]^2}. \quad (17)$$

Secondly, if peptide concentration ([B]) is constant, and the plot of the reaction rate v vs. ATP concentration ([A]) is studied, the rate equation (14) can be presented as follows:

$$v = \frac{\dfrac{k_{cat}[E_0][B]}{K_{ab} + [B]}[A]}{\dfrac{K_a K_{ab}\left(1 + \dfrac{[B]}{K_b}\right)}{K_{ab} + [B]} + [A] + \dfrac{\left(\dfrac{1}{K_{aab}}[B] + \dfrac{K_{ab}}{K_{aa}}\right)}{K_{ab} + [B]}[A]^2} = \frac{V_m^{app^A}[A]}{K_m^{app^A} + [A] + K_{aa}^{app}[A]^2}. \quad (18)$$

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

$$V_m^{app^A} = \frac{k_{cat}[E_0][B]}{K_{ab} + [B]}, \quad (19)$$

$$K_m^{app^A} = \frac{K_{ab} K_a}{K_b}\left(\frac{K_b + [B]}{K_{ab} + [B]}\right). \quad (20)$$

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 $K' = (V_m/[E_0])/K_{ab}K_a$, where $[E_0]$ stands for enzyme concentration, the equations for the second-order rate constants can be presented as follows:

$$k_{II}^{app^B} = \frac{K'K_a[A]}{K_a + [A] + \frac{1}{K_{aa}}[A]^2}, \tag{21}$$

$$k_{II}^{app^A} = \frac{K'K_b[B]}{K_b + [B]}. \tag{22}$$

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

Kinetic parameters

Proceeding from the rate equation (14) and its modifications above, the kinetic constants $V_m^{app^A}$, $K_m^{app^A}$, and $k_{II}^{app^A}$ for different ATP concentrations and kinetic constants $V_m^{app^B}$, $K_m^{app^B}$, and $k_{II}^{app^B}$ for different Kemptide concentrations were measured. These results are listed in Tables 1 and 2. Further the plots of the second-order rate constants vs. ATP (A) and Kemptide (B) concentrations were analysed, as shown in Figs. 3 and 4, and the parameters K_a and K_b as well as K_{aa} were calculated. These values are given in Table 3.

Table 1. Kinetic constants for Kemptide phosphorylation by protein kinase A in the presence of various ATP concentrations. Kemptide concentration was changed in each series of measurements

ATP, μM	$10^3\ V_m^{app^B}$, μM/sec	$K_m^{app^B}$, μM	$10^3\ k_{II}^{app^B}$, s^{-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 2. Kinetic constants for Kemptide phosphorylation by protein kinase A in the presence of various Kemptide concentrations. The ATP concentration was changed within each series of measurements

Kemptide, μM	10^3 $V_m^{app\ A}$, μM/sec	$K_m^{app\ A}$, μM	10^3 $k_{II}^{app\ A}$, s^{-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

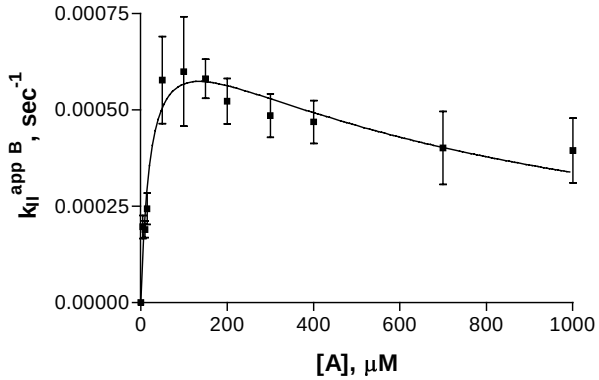


Fig. 3. Second-order rate constants of peptide (Kemptide) phosphorylation by protein kinase A as a function of the ATP (A) concentration.

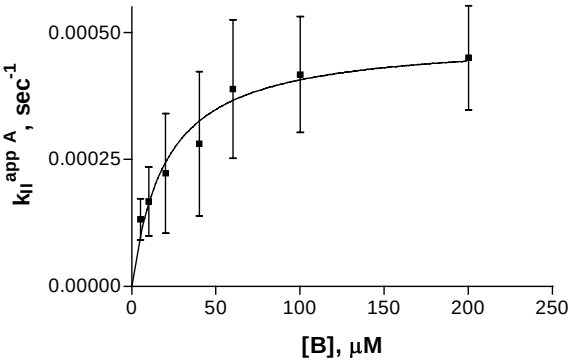


Fig. 4. Second-order rate constants of peptide (Kemptide) phosphorylation by protein kinase A as a function of the peptide (B) concentration.

Table 3. Effectiveness of ATP and Kemptide interaction with protein kinase A, characterized by means of the steady-state kinetic analysis of the phosphorylation reaction

Substrate	Constant
ATP	$K_a = 18 \pm 5\ \mu M$ $K_{aa} = 862 \pm 293\ \mu M$
Kemptide	$K_b = 20 \pm 4\ \mu M$

DISCUSSION

Steady-state kinetic analysis was used to characterize the interaction of peptide substrate and ATP with protein kinase A. This analysis is based on the application of 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. On the other hand, the parameters K_a and K_b obtained from this analysis characterize the interaction of both substrates with the free enzyme. Therefore, if the same approach is extended for the characterization of protein kinase inhibitors, only the "competitive" component of the inhibition process can be used for the characterization of these ligands.

The constants K_a and K_b are in rather good agreement with results of other works, where both steady-state and pre-steady-state kinetic methods were used for kinetic analysis. So, the K_m values $12.7 \pm 0.5\ \mu M$ [13], $12.9 \pm 0.8\ \mu M$ [14], $17.2 \pm 1.8\ \mu M$ [15], and $18 \pm 3.1\ \mu M$ [16] were published for ATP and $21.7 \pm 0.7\ \mu M$ [13], $24.7 \pm 2.7\ \mu M$ [15], and $65 \pm 11\ \mu M$ [16] were published for Kemptide.

The phenomenon of the inhibition of peptide phosphorylation at very high ATP concentrations has not been described before. Although the formal reaction scheme involves the complexes denoted as EAA and EAAB, probably different ways of the formation of these complexes can be proposed. For example, if dimerization of ATP–Mg complexes takes place at a high concentration of this substrate [17], the enzyme can interact with these complexes instead of the reactive ATP–Mg complex. However, this should have no influence on data analysis or on the meaning of the constants K_a and K_b.

Finally, it is important to mention that the standard multi-parameter non-linear regression analysis was tested for data processing immediately by Eq. 14. However, these attempts revealed that the results of data processing were very sensitive to the closeness of the initial estimates of the true values of the parameters. This hampered practical application of the analysis.

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Proteiinkinaas A katalüüsitud peptiidi ja ATP vahelise reaktsiooni statsionaarse kineetika analüüs

Aleksei Kuznetsov, Nikita Oskolkov, Mats Hansen ja Jaak Järv

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 bi-substraatse 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.