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Kinetic analysis of inhibition of cAMP-dependent protein kinase catalytic subunit by the peptide–nucleoside conjugate AdcAhxArg$_6$

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Abstract

Kinetic analysis of the inhibition of the phosphorylation of Kemptide, (LRRASLG), catalyzed by the catalytic subunit of cAMP-dependent protein kinase, by a peptide–nucleoside conjugate inhibitor AdcAhxArg$_6$ was carried out over a wide range of ATP and peptide concentrations. A simple procedure was proposed for characterization of the interaction of this inhibitor with the free enzyme, and with the enzyme–ATP and enzyme–peptide complexes. The second-order rate constants, calculated from the steady-state reaction kinetics, were used for this analysis to avoid the complications related to the complex catalytic mechanism of the protein kinase catalyzed reaction.

Keywords: cAMP-dependent protein kinase; Bisubstrate analog inhibitor; Peptide phosphorylation

1. Introduction

Protein kinases (E.C.2.7.1.37) catalyze the transfer of the γ-phosphate group from an adenosine5′-triphosphate(ATP)[1]–magnesium complex to serine, threonine or tyrosine residues of various proteins to regulate their activity [1]. Although

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[1] *Abbreviations used:* Adc, adenosine-5′-carboxylic acid; Ahx, 6-aminohexanoic acid; Arg, L-arginine; ATP, adenosine5′-triphosphate; BSA, bovine serum albumin.

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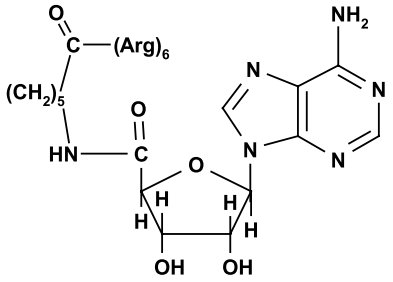
there is a large number of different protein kinases and these regulatory pathways can be found in a variety of biological systems [2], members of this enzyme family are closely related and have a conserved catalytic core with two lobes, linked by a short polypeptide. This conclusion is based on structure analysis of several individual protein kinases [3], including the catalytic subunit of the cAMP-dependent protein kinase, which is the best understood member of the protein kinase superfamily [4]. The larger lobe is responsible for molecular recognition of the peptide sequence, flanking the amino acid undergoing phosphorylation, and is recognized as one of the specificity determinants of protein kinases [5]. The role of this "primary" substrate specificity has been extensively studied with short synthetic peptide substrates and a considerable amount of data has been collected for different enzymes [6]. The smaller lobe interacts with ATP, which binds in the cleft between the lobes, where the fold of the nucleotide binding site is located [4].

For catalysis both substrates must be located in the enzyme active center, to allow direct transfer of the phosphate group from ATP to the residue of the peptide undergoing phosphorylation [7]. This requirement has been used in the design of bisubstrate analog inhibitors, which involve structural elements interacting with both binding sites and combine the specificity of the ATP site with that of the peptide binding site [8]. These elements are generally conjugated via a linker group to adjust their placement within the inhibitor molecule [9]. For inhibitor optimization it is important to characterize the interactions with the ATP and peptide binding sites separately. As the kinetic mechanism of the bisubstrate phosphorylation reaction could be rather complex, and seems to be different for distinct protein kinases [10], methods used for the kinetic analysis of protein kinases are not amenable for the screening of the bisubstrate inhibitors. Therefore, we developed a simplified procedure, involving the analysis of the second-order rate constants of the peptide phosphorylation reaction in the presence of the inhibitor. In this paper the kinetic approach was evaluated for the interaction of the cAMP-dependent protein kinase catalytic subunit with a nucleoside–peptide conjugate AdcAhxArg$_6$, shown in the Scheme 1.



Scheme 1.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP was obtained from Amersham (UK). Peptide LRRASLG (Kemptide) was synthesized in a stepwise manner on a 0.1 mmol scale using the Applied Biosystem peptide synthesizer (USA) as described elsewhere [11]. Inhibitor AdcAhxArg$_6$ was obtained by coupling of adenosine-5′-carboxylic acid (Adc) with hexa-arginine via linker NH(CH$_2$)$_5$C(O), and was purified and analyzed as described [9]. Phosphocellulose paper P81 was acquired from Whatman (UK). ATP, Tris–HCl, BSA, and H_3PO_4 were obtained from Sigma–Aldrich (USA). $MgCl_2$ was purchased from Acros (Germany). The catalytic subunit of cAMP-dependent protein kinase was a generous gift from Dr. P. Ek and Dr. M. Loog (Department of Medicinal Biochemistry and Microbiology, Uppsala University). The protein was expressed using plasmid Cat-pRSET B based on the T7 promoter expression system (Invitrogen). The purified catalytic subunit was obtained after a P-11 ion-exchanger step as described [12] except stepwise elution with 250 mM potassium phosphate (pH 6.5) was used. The enzyme stock solution was made up in buffer containing 50 mM Tris–HCl (pH 7.50) and 1 mg/ml BSA.

2.2. Assay of peptide phosphorylation

Peptide phosphorylation by the protein kinase catalytic subunit was carried out at 30 °C as described [13]. Briefly, the reaction mixture (final volume 100 μl, 50 mM TRIS–HCl, pH 7.5) contained peptide (Kemptide, LRRASLG) at various concentrations from 5 to 200 μM, [γ-^{32}P]ATP at various concentrations from 5 to 1000 μM, 10 mM of $MgCl_2$, and 0.17 μg/ml of the enzyme. In the inhibition assays the reaction mixture contained also 0.25, 0.5 or 1.0 μM of the inhibitor. The stock solution of the catalytic subunit of cAMP-dependent protein kinase was diluted 1000-fold in 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 points 10 μl aliquots were removed from the reaction mixture and spotted onto the pieces of phosphocellulose paper, which were subsequently immersed into the ice-cold 75 mM phosphoric acid to stop the reaction. The phosphocellulose papers were then washed four times with cold 75 mM H_3PO_4 (10 min each time) to remove excess [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. The values of the initial rate of the phosphorylation reaction (v_o) were calculated from the slopes of the product concentration vs time plots.

2.3. Data processing

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

3. Results

Kinetic measurements were carried out in distinct series of experiments where the concentrations of both substrates and the inhibitor were systematically changed. The range of concentration was 5–1000 μM for ATP and 5–200 μM for peptide. Inhibitor concentrations of 0.25, 0.5, and 1.0 μM were used. The array of kinetic data obtained is summarized in Fig. 1 in the form of the three-dimensional plot.

In the presence of the inhibitor the reaction rate decreased within the full concentration range of both substrates. Kinetic data in the absence of the inhibitor were previously determined and gave constants $K_a = 18 \pm 5\,\mu M$ and $K_b = 20 \pm 4\,\mu M$ [14]. The effect of substrate inhibition was characterized by $K_{aa} = 0.9 \pm 0.3\,mM$, and it was also shown that the formation of the complex EAAB was not relevant in this reaction.

Substrate inhibition of the peptide phosphorylation reaction by excess ATP was also observed in the presence of the bisubstrate analog inhibitor, and this effect was taken into consideration in the following kinetic analysis by introduction of the non-active ternary complex EAA into the kinetic model (Scheme 2). Because the inhibition of peptide phosphorylation by the excess of ATP is described by second-order rate constants, only the interaction of the substrate with the complex EA was characterized and no further analysis of the type of substrate inhibition was carried out.

The kinetic model and the data processing algorithm used in this study for inhibition of the cAMP-dependent protein kinase catalytic subunit by a bisubstrate analog inhibitor are discussed elsewhere [15]. Briefly, the reaction scheme was derived using the assumption that two substrates, ATP (substrate A in Scheme 2) and

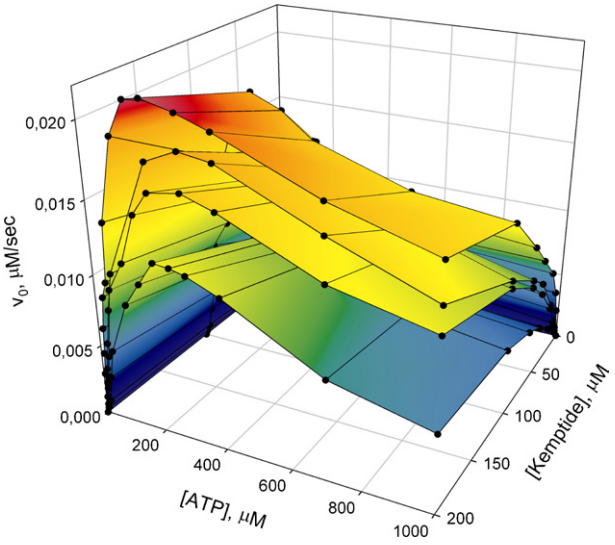
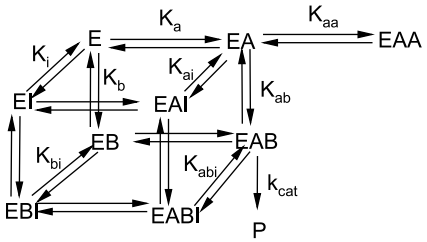


Fig. 1. Dependence of the initial rate of Kemptide (LRRASLG) phosphorylation by catalytic subunit of cAMP-dependent protein kinase on ATP and peptide concentration in the absence (top) and in the presence of inhibitor AdcAhxArg$_6$. Inhibitor concentrations (from top): [I] = 0 (control), [I] = 0.25 μM, [I] = 0.5 μM, and [I] = 1.0 μM.



Scheme 2.

peptide (substrate B in Scheme 2), bind simultaneously in the active site of the enzyme. Second, a random-order mechanism has been proposed for the reaction of the cAMP-dependent protein kinase catalytic subunit [14]. The present analysis uses the second-order rate constants (see also in [16]), so that the details of the catalytic mechanism are not important for processing and interpreting the data, and the reaction steps are summarized by a single catalytic rate constant k_{cat} in this kinetic model (Scheme 2).

The inhibitor can bind in complexes EI, EAI, EBI, and EABI. The latter complex may form if the inhibitor does not prevent binding of both substrates A and B.

The rate equation for the reaction Scheme 2 is presented as follows:

$$v_0 = \frac{k_{cat}[E_0][A][B]}{K_{ab}K_a\left(1+\frac{[I]}{K_i}\right)+K_{ab}\left(1+\frac{[I]}{K_{ai}}\right)[A]+\frac{K_{ab}K_a}{K_b}\left(1+\frac{[I]}{K_{bi}}\right)[B]+\left(1+\frac{[I]}{K_{abi}}\right)[A][B]+\frac{K_{ab}}{K_{aa}}[A]^2}, \quad (1)$$

where the parameters K_a, K_b, K_{ab}, K_i, K_{ai}, K_{bi}, K_{abi}, and K_{aa} are defined in Scheme 2.

For data processing the initial velocities were divided into subsets, where concentrations of ATP and inhibitor, or peptide and inhibitor, were fixed. Within these two subsets the plots of the initial velocity vs peptide concentration and initial velocity vs ATP concentration were used to calculate the $K_m^{app^A}$ and $K_m^{app^B}$ values as well as the maximal velocity (V^{app^A} and V^{app^B}) values, from which, in turn, the second-order rate constants $k_{II}^{app^A}$ and $k_{II}^{app^B}$, were calculated as proposed elsewhere [15]. Finally, the plots of $k_{II}^{app^B}$ vs ATP (substrate A) concentration and $k_{II}^{app^A}$ vs peptide (substrate B) concentration were analyzed by the following equations:

$$k_{II}^{app^B} = \frac{\frac{k_{cat}[E_0]\frac{K_{ai}}{K_{ab}}}{K_{ai}+[I]}[A]}{\frac{K_aK_{ai}}{K_{ai}+[I]}\left(1+\frac{[I]}{K_i}\right)+[A]+\frac{\frac{K_{ai}}{K_{aa}}}{K_{ai}+[I]}[A]^2} = \frac{X_i[A]}{Y_i+[A]+Z_i[A]^2}, \quad (2)$$

$$k_{II}^{app^A} = \frac{\frac{k_{cat}[E_0]}{\frac{K_{ab}}{K_a}K_{bi}K_b}[B]}{\frac{K_bK_{bi}\left(1+\frac{[I]}{K_i}\right)}{K_{bi}+[I]}+[B]} = \frac{Q_i[B]}{U_i+[B]} \quad (3)$$

and the complex parameters X_i, Y_i, Q_i, and U_i were obtained as functions of inhibitor concentration.

$$X_i = \frac{k_{cat}[E_0]\frac{K_{ai}}{K_{ab}}}{K_{ai} + [I]}, \tag{4}$$

$$Y_i = \frac{K_a K_{ai}}{K_{ai} + [I]}\left(1 + \frac{[I]}{K_i}\right), \tag{5}$$

$$Q_i = \frac{\frac{k_{cat}[E_0]}{K_{ab}K_a}K_{bi}K_b}{K_{bi} + [I]}, \tag{6}$$

$$U_i = \frac{K_b K_{bi}\left(1 + \frac{[I]}{K_i}\right)}{K_{bi} + [I]}. \tag{7}$$

The constants K_{ai}, K_{bi}, and K_i were calculated from the parameters presented above. The K_{ai} and K_{bi} values were obtained from the X_i vs [I] and Q_i vs [I] plots (Fig. 2) and are listed in Table 1.

Interaction of the free enzyme with the inhibitor was analyzed by using the plot of X_i/Y_i vs inhibitor concentration (Fig. 3). Similar results were obtained if the ratio Q_i/U_i was used. The results of these calculations are listed in Table 1.

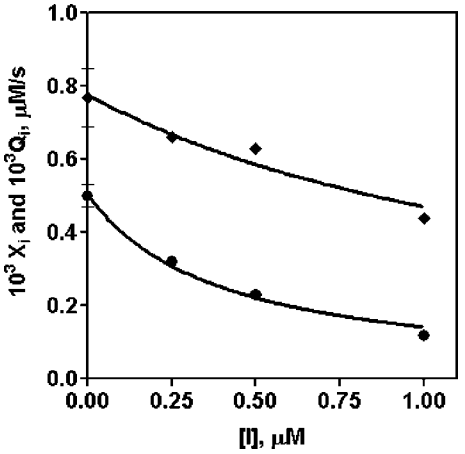


Fig. 2. Plot of the parameters X_i (◆) and Q_i (●) vs inhibitor concentration. These parameters were obtained as shown in Eqs. (4) and (6) in the text.

Table 1
Interaction of the bifunctional inhibitor AdcAhxArg$_6$ with the catalytic subunit of cAMP-dependent protein kinase in the absence (K_i) and in the presence of ATP (K_{ai}) and Kemptide (K_{bi})

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}	Not determined (estimated >10,000)

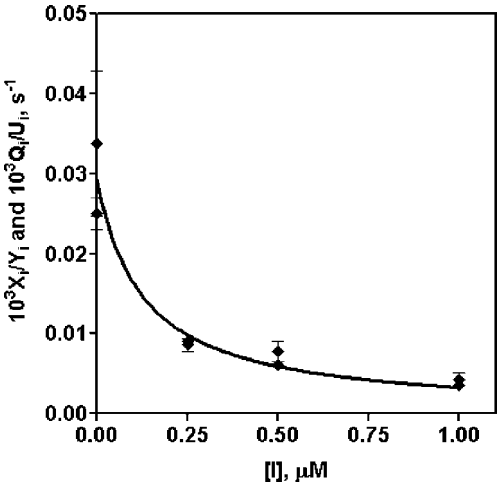


Fig. 3. Plot of parameters X_i/Y_i (◆) and Q_i/U_i (●) vs inhibitor concentration. The parameters X_i, Y_i, Q_i, and U_i were obtained as shown in Eqs. (4)–(7) in the text.

The constant K_{abi} could be estimated from the V^{app^A} values analogously to the procedure described above.

$$V^{app^A} = \frac{\frac{k_{cat}[E_0]K_{abi}}{K_{abi}+[I]}[B]}{\frac{K_{ab}\left(1+\frac{[I]}{K_{ai}}\right)}{\left(1+\frac{[I]}{K_{abi}}\right)}+[B]} = \frac{W_i[B]}{T_i+[B]}. \tag{8}$$

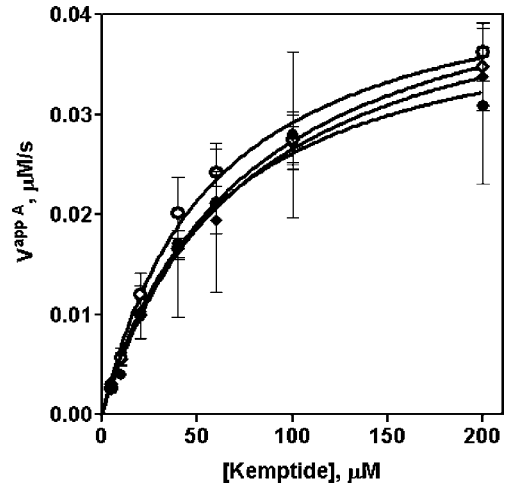


Fig. 4. Dependence of the V^{app^A} values on Kemptide (LRRASLG) concentration for the catalytic subunit of cAMP-dependent protein kinase-catalyzed peptide phosphorylation reaction, studied in the absence (○) and in the presence of inhibitor $AdcAhxArg_6$ at concentrations 0.25 μM (◆), 0.5 μM (◊), and 1.0 μM (●).

However, as shown in Fig. 4, the experimental plots obtained for V^{app^A} are rather similar and no systematic and statistically significant dependence of the W_i values upon [I] could be observed within the present concentration interval of the inhibitor. Taking into consideration the values of other constants of this kinetic model it is also estimated that the value of the constant K_{abi} should exceed 10 mM, and probably remains even higher.

4. Discussion

The constants K_i, K_{ai}, and K_{bi} characterize the interaction of the bisubstrate analog inhibitor with the free enzyme, the enzyme–ATP complex and the enzyme–peptide complex, respectively. The present results show that all three complexes are formed and could be characterized by appropriate kinetic parameters. Thus, the inhibitor AdcAhxArg$_6$ is not fully competitive against the substrates. On the other hand, binding of the inhibitor was affected by the presence of these substrates, as $K_i < K_{ai}$ and $K_i < K_{bi}$. This means that AdcAhxArg$_6$ is, indeed, a bifunctional inhibitor of cAMP-dependent protein kinase catalytic subunit, in agreement with the initial hypothesis used for the design of the molecule. At the same time the formation of the quaternary complex EABI could be excluded from the reaction scheme, as there is no statistically relevant effect of this complex on the kinetics of Kemptide phosphorylation by the protein kinase. In other words, occupation of both substrate-binding sites excludes the inhibitor from binding at the active site.

It was also observed that the interaction between the inhibitor AdcAhxArg$_6$ and the two substrates was not symmetrical. In the presence of ATP the effectiveness of the inhibitor decreased 11 times, as might be expected from simple competitive inhibition. In the presence of Kemptide the effectiveness of the inhibitor decreased only 3 times. This observation suggests that binding of the peptide fragment of the inhibitor molecule at the active site did not exclude the binding of the peptide substrate such that the appropriate binding sites are not completely overlapping. In other words, it seems likely that both molecules can fit simultaneously in the active site of the cAMP-dependent protein kinase catalytic subunit. For a better understanding of this observation, it will be necessary to obtain experimental data for other inhibitors having different peptide fragments or linker groups. The proposed kinetic approach can be used in this analysis, which is necessary for improving the theoretical and structural bases for the design of bisubstrate analog inhibitors of protein kinases.

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