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Kinetic model for protein kinase simultaneous interaction with peptide, ATP, and bi-functional inhibitor

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Abstract. A kinetic model for protein kinase inhibition by bi-functional inhibitors, resembling the bi-substrate–enzyme complex and interacting with both ATP and peptide binding sites in the enzyme active centre, was analysed. The model provides a possibility for analysis of the enzyme–ligand interactions on the basis of the second-order rate constants, calculated from the steady-state kinetic data of the protein kinase catalysed phosphorylation reaction. The experimental data should be obtained at a wide range of peptide, ATP, and the bi-functional inhibitor concentration. The kinetic equations allow characterization of the enzyme–substrate–inhibitor ternary complexes as well as the enzyme–inhibitor binary complex by the appropriate kinetic parameters. A data processing algorithm is proposed for their calculation. The results can be useful for understanding the inhibition mechanism, including the possibilities of simultaneous interaction of substrates and inhibitor in the active centre. This information is important for designing new inhibitors.

Key words: protein kinase A, peptide phosphorylation, bi-functional inhibitor, kinetic mechanism, steady-state kinetics.

INTRODUCTION

Protein kinases (E.C.2.7.1.37) catalyse protein phosphorylation. In this reaction the phosphorylatable fragment and the ATP–magnesium complex should be simultaneously bound in the enzyme active centre. On the basis of this fact the concept of bi-substrate inhibitors has been developed in the case of these enzymes [1]. These inhibitors consist of two structural fragments, which resemble the peptide fragment and ATP and are supposed to bind in the appropriate binding sites. For the best fit with the active site these fragments are connected by a linker

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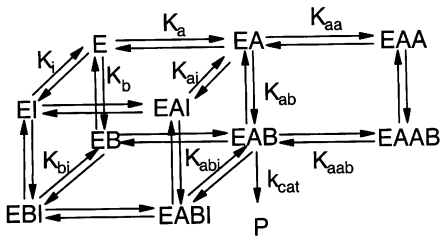
group of appropriate length [2, 3]. For detailed understanding of the mechanism of the interaction of these bi-functional inhibitors with the enzyme it is important to evaluate the effectiveness of the interaction of these compounds with the free enzyme, but also separately in the binding sites for ATP and peptide and reveal the possibilities of simultaneous binding of these ligands with the protein. This kinetic analysis is, however, a rather complicated task, as the bi-substrate 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 [4]. Moreover, as the overall reaction mechanism seems to be different for different protein kinases [5], the sophisticated methods used for kinetic analysis of the phosphorylation reaction are not handy for the screening of the bi-substrate inhibitors. Therefore we propose a simplified kinetic approach by using the second-order rate constants k_{II} of the peptide phosphorylation reaction, calculated as the ratio of the apparent kinetic parameters k_{cat} and K_m [6]. In this paper the kinetic model for protein kinase interaction with peptide, ATP, and bi-functional inhibitor is analysed.

KINETIC SCHEME

The kinetic scheme involves the enzyme complexes with substrates A (ATP) and B (peptide) and with the bi-functional inhibitor I. This model includes the appropriate ternary complexes and for generalization also the multicomplex including the enzyme, inhibitor, and both substrates. The ternary complexes result from the presence of two distinct binding sites for substrates, which should allow various combinations of the enzyme bound ligands. The multicomplex formation is possible if the inhibitor molecule does not fit explicitly the active centre, for example, due to a too short or a too long linker group. Practical data analysis should reveal the feasibility of this complex formation and is one of the objectives of kinetic analysis.

As the following experimental study will be based on the use of protein kinase A, the present reaction scheme takes into consideration also the phenomenon of substrate inhibition by the excess of ATP (substrate A), assuming the formation of both EAA and EAAB complexes. On the other hand, for simplicity the full inhibition scheme is assumed for this reaction. Thus the overall reaction scheme for product (P) formation can be presented as follows:



(1)

It is important to stress that the following analysis will be performed for the second-order rate constants, which significantly simplifies the experimental and analytical procedures.

RATE EQUATION

The rate of the reaction (1) can be presented as follows:

$$v = k_{cat}[EAB]. \quad (2)$$

Taking into consideration all the equilibriums shown in scheme 1

$$K_a = \frac{[E][A]}{[EA]}, \quad (3)$$

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

$$K_b = \frac{[E][B]}{[EB]}, \quad (5)$$

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

$$K_{ab} = \frac{[EA][B]}{[EAB]}, \quad (7)$$

$$[EA] = \frac{K_{ab}}{[B]}[EAB], \quad (8)$$

$$K_{aa} = \frac{[EA][A]}{[EAA]}, \quad (9)$$

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

$$K_{aab} = \frac{[EAB][A]}{[EAAB]}, \quad (11)$$

$$[EAAB] = \frac{[A]}{K_{aab}}[EAB], \quad (12)$$

$$K_i = \frac{[E][I]}{[EI]}, \tag{13}$$

$$[EI] = \frac{[I]}{K_i}[E] = \frac{K_a K_{ab}[I]}{K_i[A][B]}[EAB], \tag{14}$$

$$K_{ai} = \frac{[EA][I]}{[EAI]}, \tag{15}$$

$$[EAI] = \frac{[I]}{K_{ai}}[EA] = \frac{K_{ab}[I]}{K_{ai}[B]}[EAB], \tag{16}$$

$$K_{bi} = \frac{[EB][I]}{[EBI]}, \tag{17}$$

$$[EBI] = \frac{[I]}{K_{bi}}[EB] = \frac{K_a K_{ab}[I]}{K_{bi} K_b[A]}[EAB], \tag{18}$$

$$K_{abi} = \frac{[EAB][I]}{[EABI]}, \tag{19}$$

$$[EABI] = \frac{[I]}{K_{abi}}[EAB], \tag{20}$$

and the distribution of the enzyme between different complexes,

$$[E_0] = [E]+[EA]+[EB]+[EAB]+[EAA]+[EAAB]+[EI]+[EAI]+[EBI]+[EABI], \tag{21}$$

the following rate equation can be obtained for the initial rate of substrate phosphorylation:

$$v = \frac{v[A][B]}{K_{ab}K_a\left(1+\frac{[I]}{K_i}\right)+K_{ab}\left(1+\frac{[I]}{K_{ai}}\right)[A]+\frac{K_{ab}K_a}{K_b}\left(1+\frac{[I]}{K_{bi}}\right)[B]+\left(1+\frac{[I]}{K_{abi}}\right)[A][B]+\frac{1}{K_{aab}}[A]^2[B]+\frac{K_{ab}}{K_{aa}}[A]^2}, \tag{22}$$

where $V = k_{cat}[E_0]$. It can be seen that for data processing an extensive bank of experimental data is needed, including the dependences of the initial rate upon the concentrations of all three reagents – A, B, and I.

DATA PROCESSING ALGORITHM

The procedure proposed for the analysis of the kinetic scheme (1) is based on the measurement of the initial velocity of the phosphorylation reaction at various concentrations of A, B, and I. For simplicity, these experiments can be grouped so that the concentration of two ligands is fixed and the concentration of one of the ligands is changing. This allows systematic step-by-step data processing as described in the following algorithm.

First, if the concentrations of substrate A (ATP) and inhibitor I remain constant, the plot of the initial velocity vs. substrate B concentration can be analysed by the common hyperbolic function, derived from Eq. 22:

$$v = \frac{V^{app^B} B}{K_m^{app^B} + B}. \tag{23}$$

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

$$V^{app^B} = \frac{k_{cat}[E_0][A]}{\frac{K_{ab}K_a}{K_b}\left(1+\frac{[I]}{K_{bi}}\right)+\left(1+\frac{[I]}{K_{abi}}\right)[A]+\frac{1}{K_{aab}}[A]^2}, \tag{24}$$

$$K_m^{app^B} = \frac{K_a K_{ab}\left(1+\frac{[I]}{K_i}\right)+K_{ab}\left(1+\frac{[I]}{K_{ai}}\right)[A]+\frac{K_{ab}}{K_{aa}}[A]^2}{\frac{K_{ab}K_a}{K_b}\left(1+\frac{[I]}{K_{bi}}\right)+\left(1+\frac{[I]}{K_{abi}}\right)[A]+\frac{1}{K_{aab}}[A]^2}. \tag{25}$$

Similarly, if the concentration of substrate B (peptide) and inhibitor I are kept constant within a series of measurements, the rate will depend on the concentration of substrate A, and can be presented as follows:

$$v = \frac{V^{app^A}[A]}{K_m^{app^A} + [A] + K_{aa}^{app^A}[A]^2}. \tag{26}$$

The apparent kinetic parameters of this equation, reflecting also the phenomenon of product inhibition by ATP excess [6], have the following meaning:

$$V^{app^A} = \frac{k_{cat}[E_0][B]}{K_{ab}\left(1+\frac{[I]}{K_{ai}}\right)+\left(1+\frac{[I]}{K_{abi}}\right)[B]}, \tag{27}$$

$$K_m^{app^A} = \frac{K_{ab}K_a}{K_b}\frac{K_b\left(1+\frac{[I]}{K_i}\right)+\left(1+\frac{[I]}{K_{bi}}\right)[B]}{K_{ab}\left(1+\frac{[I]}{K_{ai}}\right)+\left(1+\frac{[I]}{K_{abi}}\right)[B]}, \tag{28}$$

$$K_{aa}^{app^A} = \frac{\frac{K_{ab}}{K_{aa}}+\frac{[B]}{K_{aab}}}{K_{ab}\left(1+\frac{[I]}{K_{ai}}\right)+\left(1+\frac{[I]}{K_{abi}}\right)[B]}. \tag{29}$$

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 as a function of the inhibitor concentration:

$$k_{II}^{app^B} = \frac{\frac{k_{cat}[E_0]}{K_{ab}\left(1+\frac{[I]}{K_{ai}}\right)}[A]}{K_a\frac{1+\frac{[I]}{K_i}}{1+\frac{[I]}{K_{ai}}}+[A]+\frac{1}{K_{aa}\left(1+\frac{[I]}{K_{ai}}\right)}[A]^2} = \frac{X_i[A]}{Y_i+[A]+Z_i[A]^2}, \tag{30}$$

$$k_{II}^{app^A} = \frac{\frac{k_{cat}[E_0]}{\frac{K_{ab}K_a}{K_b}\left(1+\frac{[I]}{K_{bi}}\right)}[B]}{K_b\frac{1+\frac{[I]}{K_i}}{1+\frac{[I]}{K_{bi}}}+[B]} = \frac{Q_i[B]}{U_i+[B]}. \tag{31}$$

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, and U_i can be calculated and further used for the determination of the equilibrium constants for binary and tertiary complexes formulated in scheme 1.

ENZYME TERNARY COMPLEXES WITH SUBSTRATES AND BI-FUNCTIONAL INHIBITOR

It can be seen that all the plots obtained from equations for the second order rate constants above are hyperbolic.

$$X_i = \frac{k_{cat}[E_0]}{K_{ab}\left(1+\frac{[I]}{K_{ai}}\right)} = \frac{\frac{k_{cat}}{K_{ab}}K_{ai}[E_0]}{K_{ai}+[I]}, \tag{32}$$

$$Y_i = K_a\frac{1+\frac{[I]}{K_i}}{1+\frac{[I]}{K_{ai}}} = \frac{K_aK_{ai}\left(1+\frac{[I]}{K_i}\right)}{K_{ai}+[I]}, \tag{33}$$

$$Z_i = \frac{1}{K_{aa}\left(1+\frac{[I]}{K_{ai}}\right)} = \frac{\frac{K_{ai}}{K_{aa}}}{K_{ai}+[I]}, \tag{34}$$

$$Q_i = \frac{k_{cat}[E_0]}{\frac{K_{ab}K_a}{K_b}\left(1+\frac{[I]}{K_{bi}}\right)} = \frac{\frac{k_{cat}[E_0]}{K_{ab}K_a}K_{bi}K_b}{K_{bi}+[I]}, \tag{35}$$

$$U_i = K_b\frac{1+\frac{[I]}{K_i}}{1+\frac{[I]}{K_{bi}}} = \frac{K_bK_{bi}\left(1+\frac{[I]}{K_i}\right)}{K_{bi}+[I]}. \tag{36}$$

This means that the plots X_i vs. [I], Y_i vs. [I], Z_i vs. [I], Q_i vs. [I], and U_i vs. [I] can be used and for calculation of the parameters K_{ai} and K_{bi}, which were the targets of the analysis, as they characterize separately the interaction between the both enzyme–substrate complexes (EA and EB) and the inhibitor molecule, i.e. formation of the ternary complexes EAB, EAI, and EBI.

For characterization of the interaction of the free enzyme with the inhibitor the ratios Q_i/U_i and X_i/Y_i obtained at different inhibitor concentrations can be used, as illustrated for the latter case:

$$\frac{X_i}{Y_i} = \frac{\dfrac{k_{cat}[E_0]}{K_a K_{ab}} K_i}{K_i + [I]}. \qquad (37)$$

It is understandable that after several transformations the errors of the obtained parameters K_i, K_{ai}, and K_{bi} may be rather significant. On the other hand, these error values can be kept under control if the amount of the initial data is large enough and the velocity measurements are precise and do not scatter.

FORMATION OF THE EABI COMPLEX

Although the probability of multicomplex EABI formation seems to be low, this should be demonstrated on the basis of experimental data. The necessary analysis can be made proceeding from Eqs. 24 or 27. In the latter case Eq. 27 can be transformed:

$$V^{app^A} = \frac{\dfrac{\dfrac{k_{cat}[E_0]}{\left(1 + \dfrac{[I]}{K_{abi}}\right)}[B]}{K_{ab}\left(1 + \dfrac{[I]}{K_{ai}}\right)}}{\dfrac{}{\left(1 + \dfrac{[I]}{K_{abi}}\right)} + [B]} = \frac{W_i[B]}{T_i + [B]}. \qquad (38)$$

The parameter W_i can be calculated at different inhibitor concentrations and the constant K_{abi} defined in the reaction scheme 1 can be obtained from the W_i vs. [I] plot:

$$W_i = \frac{k_{cat}[E_0]K_{abi}}{K_{abi} + [I]}. \qquad (39)$$

INFLUENCE OF PRODUCT INHIBITION

The phenomenon of product inhibition, observed in the protein kinase A catalysed phosphorylation reaction of Kemptide, and caused by excess of ATP [6], additionally complicates inhibition analysis. Therefore it was interesting to estimate the influence of the substrate inhibition effect on the data analysis, primarily on the level of the second-order rate constants $k_{II}^{app^B}$. For this purpose Eq. 30 and its simplified form, where the term $\{1/K_{aa}[1 + ([I]/K_{ai})]\}[A]^2$ was omitted, were simulated in Fig. 1. In this simulation the following conditions

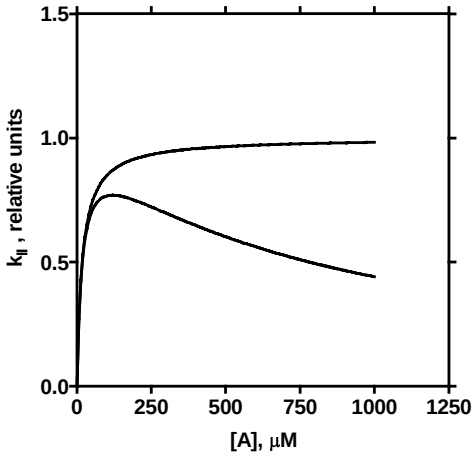


Fig. 1. Simulation of the effect of substrate inhibition for the second-order rate constants of peptide phosphorylation reaction as a function of the substrate A (ATP) concentration. In this simulation the following conditions were used: $K_a = 18$ μM and $K_{aa} = 862$ μM [6].

were used: $[I] = 0$, $K_a = 18$ μM, and $K_{aa} = 862$ μM [6]. Substrate A concentration was raised up to 1000 μM, as used in experiments [6]. It can be seen that these theoretical curves overlap at rather low substrate A (ATP) concentrations, and approximately 10% differences can be observed at concentrations above 200 μM. The same conclusion is valid for experiments made in the presence of the inhibitor, as all parameters of Eq. 30 have similar dependence upon I concentration. Thus the phenomenon of product inhibition should be taken into consideration in the analysis of the inhibition data if ATP concentration exceeds 200 μM.

SUMMARY

The data processing algorithm proposed in this report allows characterization of interactions taking place between separate areas of the protein kinase active centre and the bi-functional inhibitor. First, the constant K_{ai} provides the possibility of characterizing the interaction of the inhibitor molecule with the binding site for peptide, as the ATP binding site is occupied by an ATP–magnesium complex. Similarly, the constant K_{bi} should characterize the inhibitor interaction with the ATP binding site, measured in the presence of the bound peptide. Thirdly, the constant K_i characterizes the interaction of the bi-functional inhibitor with the free enzyme and thus should provide information about the fit of the both inhibitor fragments within the active centre. And finally, if the fit is not very good, all the three ligands can be simultaneously bound with the enzyme, forming the multiligand complex EABI. This complex is characterized by the parameter K_{abi}, as defined in the reaction scheme 1.

We expect that this analysis of interactions of the enzyme with different ligands is important for understanding the inhibition mechanism and will support the targeted design of bi-functional inhibitors.

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Kineetiline mudel proteiinkinaasi interaktsiooni iseloomustamiseks peptiidi, ATP ja bifunktsionaalse inhibiitoriga

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Koostati kineetiline mudel, mis kirjeldab proteiinkinaasi interaktsioone inhibiitoriga, mille molekul jäljendab bisubstraatsel reaktsioonil tekkivat kompleksi, kus ensüümiga on samaaegselt seotud nii ATP kui ka fosforüleeritav peptiid ja/või valk. Selles mudelis on eeldatud võimalust, et inhibiitor on korraga seotud nii ATP kui ka peptiidi sidumistsentrites või siis ainult ühes neist tsentritest. Mudel põhineb reaktsiooni algkiirustest leitud bimolekulaarsete kiiruskonstantide analüüsil ja võimaldab vältida reaktsiooni kiirust limiteeriva reaktsioonistaadiumi määramisega seotud probleeme. Ensüümi moodustatud binaarsete ja tertsiaarsete komplekside iseloomustamine töös esitatud andmetöötluse algoritmi abil pakub huvi inhibeerimismehhanismi sügavama mõistmise seisukohast ja see võib olla vajalik inhibiitorite eesmärgipärasel konstrueerimisel.