Autophosphorylation kinetics of protein kinases

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Protein kinases play a central role in cellular signal transduction, by transmitting biochemical information between activated membrane-bound receptors and physiological target proteins. In addition to phosphorylating other proteins, almost all protein kinases catalyse autophosphorylation reactions (i.e. reactions in which the kinase serves as its own substrate). The autophosphorylation reactions can be intramolecular or intermolecular. In the present study, a detailed kinetic analysis of the intermolecular autophosphorylation reaction is presented. On the basis of the kinetic equations, a new procedure is developed to evaluate the kinetic parameters of the autophosphorylation

reaction. This method was used to analyse the intermolecular autophosphorylation of an S6/H4 kinase from human placenta. At a fixed ATP concentration of 0.125 mM, the apparent catalytic-centre activity (turnover number; k_{cat}) and apparent Michaelis–Menten constant (K_m) for the autophosphorylation reaction were determined to be 0.91 min⁻¹ and 0.86 μ M respectively.

Key words: intermolecular autocatalytic reaction, p21-activated protein kinase, phosphorylation, two-substrate reaction.

INTRODUCTION

Protein phosphorylation is one of the most important processes for cellular regulation and signal transduction in eukaryotic cells. The enzymes responsible for catalysing this reaction are the protein kinases. The protein kinases constitute a very diverse set of enzymes, and it is predicted that the human genome encodes more than 1000 protein kinases [1]. In protein phosphorylation reactions, the terminal (γ) phosphate group of ATP is transferred to specific serine and threonine residues by one class of protein kinases, and to specific tyrosine residues by another. Transfer of the γ -phosphate of ATP to a serine or threonine in the protein involves the introduction of a relatively high-energy phosphate group into the protein structure as compared with serine phosphate.

In addition to the phosphotransferase reaction between ATP and specific residues in other polypeptides, almost all protein kinases catalyse autophosphorylation reactions (i.e. reactions in which the kinase serves as its own substrate), and their activities are frequently regulated by phosphorylation and by other regulatory ligands. The autophosphorylation reactions can be intramolecular or intermolecular [2]. The autophosphorylation and activation mechanisms of some protein kinases have been carefully studied from different points of view [3,4]. However, a detailed kinetic analysis of the autophosphorylation reactions has yet to be carried out. The simplest intramolecular autophosphorylation is a first-order reaction, and therefore can be described by the single-exponential kinetic equation. In contrast, intermolecular autophosphorylation involves a bimolecular autocatalytic event. In addition to modulation by other regulatory ligands, the rate of intermolecular autophosphorylation is dependent on the concentration of protein kinase, and thereby provides an alternative means of regulating particular biological processes.

Since all phosphorylation reactions involve the participation of an ATP molecule in addition to the polypeptides undergoing phosphorylation, a number of mechanisms are possible for these two-substrate reactions. The kinetic mechanisms of some protein kinases with exogenous substrates have been studied. A rapid-

equilibrium Bi Bi mechanism was found to be involved in the phosphorylation reaction of rabbit muscle myosin light chain kinase [5], whereas steady-state kinetic analysis of the epidermal growth factor receptor kinase using a synthetic peptide suggested that catalysis occurs by an ordered Bi Bi reaction. In this model, the peptide binds to the catalytic site first, followed by ATP [6]. There have been numerous inconsistencies concerning the kinetic mechanism for the cAMP-dependent protein kinase-catalysed reaction [7–13]. Whitehouse et al. [12] have carefully analysed the reaction mechanism using MgATP, peptide substrate, substrate analogues and protein kinase inhibitor. They concluded that the steady-state kinetics follow an ordered Bi Bi mechanism in which ATP binds first [12,13].

Compared with reactions involving exogenous substrates, autophosphorylation reactions could be more complex, since the unmodified kinase molecules are phosphorylated by their own active forms. In the present study, a detailed kinetic analysis of the intermolecular autophosphorylation reaction is presented. On the basis of the kinetic equation derived, a new procedure is developed to evaluate the kinetic parameters of the autophosphorylation reaction. For the three most common cases, the two-substrate reaction mechanisms can be distinguished from studies of the influence of the concentration of ATP on the kinetic parameters. As an example, this method was used to analyse the intermolecular autophosphorylation of an S6/H4 kinase from human placenta [14]. At a fixed ATP concentration of 0.125 mM, the apparent Michaelis–Menten constant and catalytic-centre activity (turnover number) for the autophosphorylation reaction were determined to be 0.86 μ M and 0.91 min⁻¹ respectively.

THEORETICAL ANALYSIS

In an autophosphorylation reaction, the unphosphorylated enzyme serves as a substrate. The general mechanism of the intermolecular autophosphorylation of a protein kinase is shown in Scheme 1, in which E and E* represent unphosphorylated and phosphorylated enzyme respectively, and A and P represent substrate (ATP) and product (ADP) respectively. In this general model of the autophosphorylation reaction, E and A can bind

Abbreviation used: PAK2, p21-activated protein kinase 2.

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Scheme 1 Intermolecular autophosphorylation of a protein kinase

E and E* represent unphosphorylated and phosphorylated enzyme respectively, and A and P represent substrate (ATP) and product (ADP) respectively.

randomly to E*. Since the concentrations of protein substrate and enzyme are of the same order of magnitude, the steady-state assumption is not satisfactory in this case [15]. When there is equilibrium as far as E*A, E*E and E*EA are concerned, i.e. when the rate constant k_c is sufficiently small as not to disturb equilibrium, we then have:

$$K_A' = [E^*][A]/[E^*A] \quad (1a)$$

$$K_E' = [E^*][E]/[E^*E] \quad (1b)$$

$$K_A = [E^*E][A]/[E^*EA] \quad (1c)$$

$$K_E = [E^*A][E]/[E^*EA] \quad (1d)$$

where $K_A' = k_{-1}/k_1$, $K_E' = k_{-1}'/k_1'$, $K_A = k_{-2}/k_2$ and $K_E = k_{-2}'/k_2'$. It is to be noted that:

$$K_A K_E' = K_A' K_E \quad (2)$$

The total concentration of enzyme, $[T]_0$, is given by:

$$[T]_0 = [E]_0 + [E^*]_0 = [E] + [E^*] + [E^*A] + 2[E^*E] + 2[E^*EA] \quad (3)$$

where $[E]_0$ and $[E^*]_0$ are the initial concentrations of the unphosphorylated and phosphorylated enzyme respectively. Let:

$$[E^*]_T = [E^*] + [E^*A] + [E^*E] + [E^*EA] \quad (4)$$

From eqns (1)–(4), we have:

$$\begin{aligned}
 & \left(\frac{K_A + [A]}{[A]} \right) [E^*EA]^2 - ([T]_0 + K_m)[E^*EA] \\
 & + \frac{([T]_0 - [E^*]_T)[E^*]_T[A]}{K_A + [A]} = 0 \quad (5)
 \end{aligned}$$

where:

$$K_m = \{(K_A' + [A])K_E'\}/(K_A + [A]) \quad (6)$$

is the apparent Michaelis–Menten constant for the intermolecular autophosphorylation reaction. The solution of eqn (5) for $[E^*EA]$ is given by the quadratic formula as:

$$\begin{aligned}
 [E^*EA] = \frac{[A]}{2(K_A + [A])} \left\{ [T]_0 + K_m \right. \\
 \left. - \sqrt{([T]_0 + K_m)^2 - 4([T]_0 - [E^*]_T)[E^*]_T} \right\} \quad (7)
 \end{aligned}$$

The rate of formation of the phosphorylated enzyme is given by:

$$\begin{aligned}
 \frac{d[E^*]_T}{dt} = k_c[E^*EA] = \frac{k_{cat}}{2} \left\{ [T]_0 + K_m \right. \\
 \left. - \sqrt{([T]_0 + K_m)^2 - 4([T]_0 - [E^*]_T)[E^*]_T} \right\} \quad (8)
 \end{aligned}$$

Figure 1 Schematic plots of $[E^*]_T$ against reaction time for an auto-phosphorylation reaction: effects of K_m

The progress curves were generated by using eqn (14), with $[E]_0 = 2 \mu\text{M}$ and $[E^*]_0 = 0.1 \mu\text{M}$, as well as the following values for kinetic parameters: $k_{cat} = 1.0 \text{ min}^{-1}$ and $K_m = 1.0, 1.5, 2.0$ and $3.0 \mu\text{M}$ for curves 1–4 respectively.

where:

$$k_{cat} = k_c[A]/(K_A + [A]) \quad (9)$$

is the apparent catalytic-centre activity for the intermolecular autophosphorylation reaction. If we let $x = 2[E^*]_T - [T]_0 + \sqrt{([T]_0 + K_m)^2 - 4([T]_0 - [E^*]_T)[E^*]_T}$, we have:

$$[E^*]_T = \frac{(x + [T]_0)^2 - (K_m + [T]_0)^2}{4x} \quad (10)$$

Differentiation of this equation with respect to x gives:

$$\frac{d[E^*]_T}{dx} = \frac{x^2 + 2K_m[T]_0 + K_m^2}{4x^2} \quad (11)$$

Substitution of eqn (10) into eqn (8) yields:

$$\begin{aligned}
 \frac{d[E^*]_T}{dt} = \frac{k_{cat}}{2} \left\{ [T]_0 + K_m \right. \\
 \left. - \sqrt{([T]_0 + K_m)^2 - 4([T]_0 - [E^*]_T)[E^*]_T} \right\} \\
 = \frac{-k_{cat}[x - K_m][x - K_m - 2[T]_0]}{4x} \quad (12)
 \end{aligned}$$

Combining eqns (11) and (12), we obtain:

$$\frac{x^2 + 2K_m[T]_0 + K_m^2}{x[x - K_m][x - K_m - 2[T]_0]} dx = -k_{cat} dt \quad (13)$$

Eqn (13) can be integrated to give:

$$-k_{cat}t = \ln \frac{x}{x_0} + \frac{K_m + [T]_0}{[T]_0} \ln \frac{(x - K_m - 2[T]_0)(x_0 - K_m)}{(x_0 - K_m - 2[T]_0)(x - K_m)} \quad (14)$$

where:

$$x = 2[E^*]_T - [T]_0 + \sqrt{([T]_0 + K_m)^2 - 4([T]_0 - [E^*]_T)[E^*]_T} \quad (15)$$

and

$$x_0 = 2[E^*]_0 - [T]_0 + \sqrt{([T]_0 + K_m)^2 - 4[E^*]_0[E]_0} \quad (16)$$

Eqn (14) gives the S-shaped logistic curves typical of autocatalytic reactions. Figures 1 and 2 show the effects of K_m and k_{cat}

Figure 2 Schematic plots of $[E^*]_T$ against reaction time for an auto-phosphorylation reaction: effects of k_{cat}

The progress curves were generated by using eqn (14) with $[E]_0 = 2 \mu M$, $[E^*]_0 = 0.1 \mu M$, as well as the following values for kinetic parameters: $K_m = 1.5 \mu M$ and $k_{cat} = 0.6, 0.8, 1.0$ and 1.2 min^{-1} for curves 1–4 respectively.

Figure 3 Schematic representation of plots of k_{cat} and K_m against $[ATP]$ for different autophosphorylation mechanisms of a protein kinase

Note that k_{cat}^* and K_m^* represent k_{cat} and K_m respectively. See the text for details.

on the shapes of progress curves for the autophosphorylation reaction. As shown in these Figures, decreasing the K_m value or increasing the k_{cat} value will result in a decrease in the lag time for activation reaction.

It can be seen from eqns (6) and (9) that both k_{cat} and K_m are functions of the ATP concentration. The expression of k_{cat} is the equation of a rectangular hyperbola (Figure 3a). A plot of $1/k_{cat}$ against $1/[A]$ will give a straight line of slope K_A/k_c and intercept $1/k_c$ on the ordinate (inset of Figure 3a). From the intercept and slope of this plot, k_c and K_A can then be calculated. The shape of a plot of K_m against $[A]$ will be dependent on the relative magnitudes of the two dissociation constants. Three different kinds of behaviour are special cases of

Scheme 2 Mechanism for the formation of ternary complex E^*EA from the E^*A complex only

Scheme 3 Mechanism for the formation of ternary complex E^*EA from the E^*E complex only

eqn (6), and are illustrated in Figure 3(b). Curve 1 shows the behaviour when the value of K'_E is small compared with that of K_E (i.e. the affinity of the substrate E for free enzyme E^* is higher than for the enzyme–ATP complex E^*A); as $[A]$ increases from zero to infinity, K_m increases from K'_E to a limit of K_E . The opposite results are obtained if $K'_E > K_E$. Curve 3 of Figure 3(b) corresponds to a lower value of K'_E . In the event that both E^* and E^*A combine with E with equal affinity, eqn (6) reduces to $K_m = K'_E = K_E$, and the apparent Michaelis constant is therefore independent of $[A]$ (curve 2).

When the rate of formation of the ternary complex E^*AE from one of the binary complexes is negligible compared with the rate of its formation from the other, such a pathway is known as an ordered ternary-complex mechanism. As in the case of the random-order mechanism, such reaction pathways may also proceed by mechanisms in which one or both substrates are bound in an equilibrium manner. If the ternary complex E^*EA can be formed from only the E^*A complex, i.e. E is not allowed to form the complex E^*E , and equilibrium conditions apply for the binding of both substrates, the mechanism can be written as shown in Scheme 2. The expressions of k_{cat} and K_m are given by:

$$k_{cat} = k_c \quad (17)$$

$$K_m = \{K_E(K'_A + [A])\}/[A] \quad (18)$$

In this case, the apparent catalytic-centre activity k_{cat} is independent of $[A]$ (Figure 3c). The plot of K_m against $[A]$ is a descending curve. At very low $[A]$ values it approaches the K_m axis, and at very high $[A]$ values the plot approaches a limiting value, K_E (Figure 3d). A straight line will be obtained if K_m is plotted against $1/[A]$, as shown in the inset of Figure 3(d). According to the plot of k_{cat} against $[A]$, Scheme 2 can be easily distinguished from Scheme 1.

If the ternary complex E^*EA can be formed only from the E^*E complex, then the equilibria corresponding to the mechanism are shown in Scheme 3. In this case, the expressions of k_{cat} and K_m are:

$$k_{cat} = k_c[A]/(K_A + [A]) \quad (19)$$

$$K_m = K'_E K_A/(K_A + [A]) \quad (20)$$

Figures 3(e) and 3(f) show the effects of varying $[A]$ on the k_{cat} and K_m values. The expression of apparent catalytic-centre activity k_{cat} is hyperbolic; k_c and K_A can therefore be determined by the method mentioned above. It can be seen from Figure 3(f) that, as $[A]$ increase from zero to infinity, K_m decreases from K'_E to zero. Scheme 3 can be distinguished from Scheme 1 by plotting $1/K_m$ against $[A]$, since such a plot will give a straight line with slope $1/K'_E K_A$ and intercept $1/K'_E$ (inset of Figure 3f).

DETERMINATION OF KINETIC PARAMETERS

It can be seen from eqn (14) that any particular progress curve contains two kinetic parameters, k_{cat} and K_m . In theory, the fitting of data from a single progress curve to eqn (14) by the non-linear least-squares method will yield values for k_{cat} and K_m . However, eqn (14) is quite complex, and, due to the fact that experimental data always contain experimental uncertainties, may be difficult to treat by the conventional single-curve-fitting procedure. With a set of unfortunate starting values, the fitting procedure will converge to the wrong values or perhaps enter a non-convergent cycle. Therefore it is necessary to develop another approach to overcome this difficulty. In practice, many protein kinase preparations contain trace amounts of contaminating phosphorylated active enzyme. In these cases, the initial concentrations of enzyme species can be written as $[E^*]_0 = \alpha[T]_0$ and $[E]_0 = (1-\alpha)[T]_0$, where α is a constant. Thus $[T]_0$ and $[E^*]_0$ will be varied together at a constant ratio, and eqn (14) can then be treated as a function of the two independent variables, $[E^*]_T$ and $[T]_0$. If a group of experiments is carried out at different initial concentrations of enzyme, one can obtain a family of progress curves for various values of $[T]_0$. These autophosphorylation reaction curves can then be analysed by a global fitting procedure simultaneously. Unlike conventional fitting methods, in which data corresponding to each fixed $[T]_0$ are fitted individually, the kinetic parameters obtained by global analysis are constrained by the entire matrix of $[E^*]_T$ and $[T]_0$ simultaneously. As a consequence, when the reaction progress curves are analysed by a global fitting procedure using the non-linear regression method, the fitting results can be improved greatly.

Figure 4 represents an example of this problem. Because we cannot determine the 'true' values of parameters exactly in any real biological system, our example is based on a computer-generated experiment, where we have predetermined the values of all the kinetic parameters. Simulated data representing an

Figure 4 Three synthetic data sets with pseudo-random experimental uncertainty added

These data sets were generated by using eqn (14) with $K_m = 1.5 \mu\text{M}$, $k_{\text{cat}} = 0.8 \text{ min}^{-1}$ and $\alpha = 0.015$. The total enzyme concentration, $[T]_0$, is $1.2 \mu\text{M}$ ($\blacktriangle$), $2.0 \mu\text{M}$ ($\circ$) and $3.0 \mu\text{M}$ ($\bullet$). Gaussian-distributed pseudo-random noise was added with a standard deviation of 0.05. The data were globally fitted to eqn (14) simultaneously. The lines are the best-fitting curves generated by using eqn (14) with $K_m = 1.49 \mu\text{M}$, $k_{\text{cat}} = 0.81 \text{ min}^{-1}$ and $\alpha = 0.0141$.

experimental autophosphorylation system are shown in Figure 4. These data sets were generated according to eqn (14) with the parameters $K_m = 1.5 \mu\text{M}$, $k_{\text{cat}} = 0.8 \text{ min}^{-1}$ and $\alpha = 0.015$. Gaussian-distributed pseudo-random experimental uncertainty was added with an S.D. of 0.05. The standard non-linear least-squares analysis for each set of data cannot give the correct values of the kinetic parameters. The values determined by the conventional single-curve-fitting procedure were $K_m = 6.59 \pm 81.39 \mu\text{M}$, $k_{\text{cat}} = 2.48 \pm 26.11 \text{ min}^{-1}$ and $\alpha = 0.012 \pm 0.002$ for data set 1; $K_m = 16.27 \pm 88.21 \mu\text{M}$, $k_{\text{cat}} = 4.43 \pm 21.55$ and $\alpha = 0.014 \pm 0.0015$ for data set 2; and $K_m = 10.63 \pm 33.83 \mu\text{M}$, $k_{\text{cat}} = 2.64 \pm 6.65$ and $\alpha = 0.0132 \pm 0.001$ for data set 3. Thus the parameters as inferred from these data were substantially different from the true values, and had very large S.D.s. This problem can be solved by the global fitting procedure. When the three data sets were analysed simultaneously by the global fitting procedure using the non-linear regression method, the kinetic parameters determined were $K_m = 1.49 \pm 0.05 \mu\text{M}$, $k_{\text{cat}} = 0.81 \pm 0.02 \text{ min}^{-1}$ and $\alpha = 0.014 \pm 0.0005$. Thus the global analysis provides much better estimates for all the parameters than do conventional single-curve-fitting procedures. The goodness-of-fit is more accurately reflected in the error associated with each parameter, which in this case was 4% or less.

As an example, this method was used to analyse the intermolecular autophosphorylation of an S6/H4 kinase from human placenta. The S6/H4 kinase purified from human placenta catalyses phosphorylation of the S6 ribosomal protein, histone H4 and myelin basic protein. This enzyme has been identified as p21-activated protein kinase 2 (PAK2). PAK2 is the ubiquitous isoform of a family of protein kinases activated by the small G-proteins [14]. The small G-protein binding site is in the N-terminal regulatory domain, whereas the C-terminus consists of a highly conserved catalytic domain. *In vitro* activation of the p60 S6/H4 kinase requires removal of an autoinhibitory domain by mild trypsin digestion and autophosphorylation of the catalytic domain (p40 S6/H4 kinase). The two autophosphorylation sites in the catalytic domain have been determined. Site 1 phosphorylation occurs by an intramolecular mechanism, whereas site 2 autophosphorylation occurs by an intermolecular mechanism. Site 1 phosphorylation is most rapid, but activation does not occur until site 2 is autophosphorylated. Site 2 autophosphorylation of p40 S6/H4 kinase is required for enzyme activation, since it is the only site that exhibits autophosphorylation kinetics consistent with the activation kinetics of S6/H4 kinase. When the activation time course was determined at several enzyme dilutions, the time required for activation increased at greater enzyme dilutions (see Figure 4 of [14]). The lag time for activation was well correlated with the lag time for phosphorylation of site 2, but not of site 1. If we assume that the maximum p40 S6/H4 kinase activity is proportional to the real enzyme concentration in the reaction solution, the time courses for p40 S6/H4 kinase activation at three different enzyme concentrations can be analysed simultaneously by using the computer program SigmaPlot V3.0. At a fixed ATP concentration of 0.125 mM , the values of K_m , k_{cat} and α are determined to be $0.858 \pm 0.159 \mu\text{M}$, $0.905 \pm 0.099 \text{ min}^{-1}$ and 0.091 ± 0.007 respectively.

DISCUSSION

Since the initial discovery of phosphorylase kinase by Fischer and Krebs, reversible phosphorylation has been shown to be the most common type of post-translational modification of

proteins [3]. It is proposed that approximately one in every three proteins in mammalian cells is phosphorylated [16,17]. Along with allosteric modulation, protein phosphorylation is now recognized as a universal mechanism for regulating the function of proteins involved in many biological processes. The reaction of protein phosphorylation involving a protein kinase can be shown as:

Most protein kinases share a common molecular organization, composed of a regulatory or inhibitory domain and a catalytic kinase domain that has many of the conserved features. The holoenzymes are usually in an inactive or basally activated state, and cellular activation of protein kinases occurs as a result of conformational changes induced by binding of regulatory ligands, such as cyclic nucleotides, Ras, Rho, Ca^{2+} /calmodulin or cyclin, or phosphorylation by upstream protein kinases [18,19]. *In vitro*, the inactive holoenzymes can also be activated by limited proteolysis by proteases [20–23]. Although the events that trigger activation of protein kinases are quite variable, a common molecular mechanism has been proposed [24]. A key feature in the control of kinase enzymic activity is a regulated interaction between the inhibitory domain and the catalytic kinase domain. The regulatory ligands bind to the inhibitory domain and disrupt this interaction, thereby relieving initial inhibition of the kinase. The conformational changes at the active site will result in increased accessibility to MgATP, protein substrate or both [25–30].

In addition to phosphorylating other proteins, many protein kinases catalyse autophosphorylation reactions. The phosphate may be incorporated into either the catalytic domain or the regulatory domain(s) of the enzyme. Autophosphorylation of the regulatory domain generally occurs via an intramolecular reaction, and often leads to dissociation of this domain from the catalytic centre. In contrast, autophosphorylation of the catalytic domain is usually an intermolecular event. In some protein kinases, binding of ligand to the regulatory domain can stimulate autophosphorylation of the catalytic domain [31]. One of the most dynamic regions of the protein kinase core, the activation loop, typically contains one or more critical phosphorylation sites. In the absence of phosphorylation, this loop is either disordered or in a conformation that is not optimal for catalysis [32,33]. The activity of many protein kinases is regulated by the phosphorylation state of this activation loop [34–37]. Although the significance of autophosphorylation may be apparent in many cases, autophosphorylation may (like phosphorylation of some protein substrates) have no functional effect (i.e. silent phosphorylation) and could reflect the availability of a substrate sequence similar to that in actual target proteins, or the lack of absolute specificity. In the present study, we have considered the simplest case, in which the inhibitory domain of a protein kinase has been removed by limited proteolysis and only one autophosphorylation site on the catalytic domain is critical for kinase activity.

In deriving the expressions of the kinetic parameters of the autophosphorylation reactions, it was assumed that rapid equilibrium conditions apply for the binding of both substrates. This is because one of the substrate concentrations, $[E]$, is comparable with the enzyme concentration, $[E^*]$; in addition, the total enzyme concentration, $[E^*]_t$, increases during the course of autocatalytic reactions. In this case, the steady-state assumption may not be valid for deriving the kinetic equations [15]. However, since the step $E^*E\text{-ATP} \rightarrow 2E^* + \text{ADP}$ requires the breaking of a covalent bond, whereas the substrate-binding steps are simple dissociation processes, the rapid equilibrium conditions are

satisfied in many situations. Therefore the assumptions made to simplify the derivation of the equations do not invalidate their usefulness in analysing the fundamental characteristics of the autophosphorylation reaction.

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