Kinetic analysis of ligand-induced autocatalytic reactions

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Protein phosphorylation and limited proteolysis are two most common regulatory mechanisms involving the energy-dependent covalent modification of regulatory enzymes. In addition to modifying other proteins, many protein kinases and proteases catalyse automodification reactions (i.e. reactions in which the kinase or zymogen serves as its own substrate), and their activities are frequently regulated by other regulatory ligands. In the present study, a kinetic analysis of autocatalytic reaction modulated by regulatory ligands is presented. On the basis of the kinetic equation, a novel procedure is developed to evaluate the kinetic parameters of the reaction. As an example of an application of this method, the effects of calcium ions on the autoacatalytic activation of trypsinogen by trypsin is re-examined. The results indicate that the binding affinity for Ca^{2+}-bound trypsinogen to trypsin is at least two orders of magnitude higher than that for Ca^{2+}-free trypsinogen, and therefore that the effect of Ca^{2+} ions on $K_m{}^*$ values for trypsinogen is very much greater than that for the model peptides. Based on the experimental results, one possible molecular mechanism has been proposed.

Key words: autophosphorylation, limited proteolysis, protein kinase, trypsin, trypsinogen, zymogen activation.

INTRODUCTION

Enzyme-catalysed covalent modification is an important mechanism for the regulation of enzyme activity. In such a regulatory process, one enzyme acts to modify the activity of another by chemically modifying the target enzyme. Protein phosphorylation and limited proteolysis are two most common regulatory mechanisms involving the energy-dependent covalent modification of regulatory enzymes. The control of enzyme activity through a reversible covalent modification such as phosphorylation can have a variety of functions: (i) it can change the kinetic and allosteric properties of the enzyme; (ii) by necessity it introduces two interconverting enzymes into the system, which may themselves be subject to control mechanisms; and (iii) further increases in the regulatory potential of an enzyme can be obtained by introducing multiple phosphorylation or by linking two or more phosphorylation/dephosphorylation cycles in sequence [1].

Protein phosphorylation is the most prevalent form of post-translational covalent modification mechanism that cells employ to regulate enzyme activity. It is a means of superimposing the effect of a stimulus from outside the cell on the prevailing metabolic state within that cell. The enzymes responsible for catalysing this reaction are protein kinases. In addition to phosphorylating other proteins, many 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 other regulatory ligands [2]. The autophosphorylation reactions can be intramolecular or intermolecular. The 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 the protein kinase, and thereby provides an alternative means for regulating particular biological processes.

Proteolytic enzymes are normally synthesized and secreted as inactive precusors, which are activated at a physiologically appropriate time and place. These precusors are known as proenzymes, or zymogens. The zymogens must undergo an activation process, usually a limited proteolysis, to attain their catalytic activity. The active forms of zymogens usually have powerful physiological effects, and their synthesis in inactive form permits them to be safely stored until they are required. Zymogen activation is a phenomenon of great importance to our understanding of fundamental biochemical and physiological processes. They are involved in many physiological processes, such as digestion, metabolism, differentiation, immunity, blood coagulation, fibrinolysis, apoptosis and respose to injury [1,3–10]. When the activating enzyme and the activated enzyme coincide, the process is an autocatalytic zymogen activation. Physiological examples of these processes are the activation of trypsinogen, prekallikrein, pepsinogen and human blood coagulation factor XII by trypsin, kallikrein, pepsin and factor XIIa (a form of activated XII), respectively [1,11–13].

Recently, kinetic studies of the autoactivation of protein kinase and zymogen have been reported [14,15]. In the present paper, kinetic analysis of an autocatalytic reaction modulated by a regulatory ligand is presented. As an example of an application of this method, the effect of calcium ions on the autoactivation of trypsinogen by trypsin was re-analysed. Some years ago, Abita et al. [16] examined in detail the effect of Ca^{2+} in the trypsin-catalysed hydrolysis of the Lys–Ile bond in trypsinogen and several model peptides with sequences related to the N-terminal sequence of bovine trypsinogen. With trypsinogen, when the concentration of Ca^{2+} increases from 4 to 50 mM, K_m decreased by a factor 3 and k_{cat} was not changed. The trypsin-catalysed hydrolysis of the nonapeptide Val-Asp_4-Lys-Ile-Val-Gly was also Ca^{2+}-dependent, and K_m decreased by a factor of 4.3; the effect was very similar to that observed for trypsinogen. According to these observations, the authors suggested that the N-terminal hexapeptide is probably randomly arranged at the surface of trypsinogen and floats freely in the surrounding solvent. However, our results indicate that the K_m value for the trypsinogen activation was greatly overestimated in the previous study, and that the binding affinity for Ca^{2+}-bound trypsinogen to trypsin is at least two orders of magnitude higher than that for Ca^{2+}-free trypsinogen. Therefore, the effect of Ca^{2+} ions

Abbreviation used: TAME, *N*-α-*p*-tosyl-L-arginine methyl ester.

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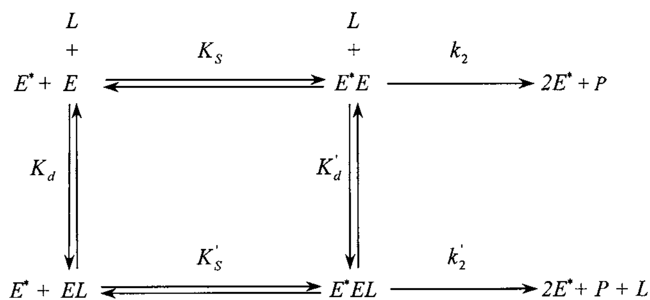

Scheme 1 Mechanism of the autocatalytic reaction

L represents regulatory ligand, E and E* represent unmodified and modified enzyme, respectively, and P is the product.

on $K_m{}^*$ values for trypsinogen is very much greater than that for the model peptide, Val-Asp$_4$-Lys-Ile-Val-Gly. Based on the experimental results, one possible molecular mechanism has been proposed.

THEORETICAL ANALYSIS

In the cases of the autocatalytic reactions, the unmodified enzyme serves as a substrate in reactions. The general mechanism of the intermolecular autocatalytic reaction of an enzyme can be written as shown in Scheme 1. In Scheme 1, L represents regulatory ligand, and E and E* represent unmodified and modified enzyme, respectively. It is assumed that the other substrates and products for modification, such as ATP, water, etc., are present at constant levels, and can therefore be included in the kinetic constants without loss of generality. 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 [17]. When there is equilibrium as far as EL, E*E and E*EL are concerned, i.e. when k_2 and k_2' are sufficiently small as not to disturb equilibrium, we then have

$$K_S = \frac{[E^*][E]}{E^*E}, \quad K_S{}' = \frac{[E^*][EL]}{[E^*EL]}$$
$$K_d = \frac{[E][L]}{[EL]}, \quad K_d{}' = \frac{[E^*E][L]}{[E^*EL]} \tag{1}$$

The total concentration of enzyme is

$$[T]_0 = [E]_0 + [E^*]_0 = [E] + [E^*] + [EL] + 2[E^*E] + 2[E^*EL] \tag{2}$$

where $[T]_0$ is the total concentration of trypsin plus trypsinogen, and $[E]_0$ and $[E^*]_0$ are the initial concentrations of the unmodified and modified enzyme respectively. Let

$$[E_T{}^*] = [E^*] + [E^*E] + [E^*EL] \tag{3}$$

From eqns (1)–(3), we have

$$\left(\frac{K_d{}' + [L]}{[L]}\right)[E^*EL]^2 - ([T]_0 + K_m{}^*)[E^*EL] + \frac{([T]_0 - E_T{}^*)[E_T{}^*][L]}{K_d' + [L]} = 0 \tag{4}$$

where

$$K_m{}^* = \frac{(K_d + [L])K_S{}'}{K_d{}' + [L]} \tag{5}$$

is the apparent Michealis–Menten constant for the intermolecular autocatalytic reaction. The solution of eqn (4) for [E*EL] is given by the quadratic formula as

$$[E^*EL] = \frac{[L]}{2(K_d{}' + [L])}\left\{[T]_0 + K_m{}^* - \sqrt{([T]_0 + K_m{}^*)^2 - 4([T]_0 - [E_T{}^*])[E_T{}^*]}\right\} \tag{6}$$

The rate of the modified enzyme formation is given by

$$\frac{d[E_T{}^*]}{dt} = k_2[E^*E] + k_2{}'[E^*EL] = \left(\frac{k_2 K_d{}'}{[L]} + k_2{}'\right)[E^*EL] = \frac{k_{cat}{}^*}{2}\left\{[T]_0 + K_m{}^* - \sqrt{([T]_0 + K_m{}^*)^2 - 4([T]_0 - [E_T{}^*])[E_T{}^*]}\right\} \tag{7}$$

where

$$k_{cat}{}^* = \frac{k_2 K_d{}' + k_2{}'[L]}{K_d{}' + [L]} \tag{8}$$

is the apparent turnover number (catalytic centre activity) for the intermolecular autocatalytic reaction. Eqn (7) can be rewritten as

$$\frac{2d[E_T{}^*]}{[T]_0 + K_m{}^* - \sqrt{([T]_0 + K_m{}^*)^2 - 4([T]_0 - [E_T{}^*])[E_T{}^*]}} = k_{cat}{}^*\,dt \tag{9}$$

To integrate this equation, put $x = 2[E_T{}^*] - [T]_0 + \sqrt{([T]_0 + K_m{}^*)^2 - 4([T]_0 - [E_T{}^*])[E_T{}^*]}$, so that

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

and

$$[T]_0 + K_m{}^* - \sqrt{([T]_0 + K_m{}^*)^2 - 4([T]_0 - [E_T{}^*])[E_T{}^*]} = 2[E_T{}^*] + K_m{}^* - x = \frac{(x + [T]_0)^2 - (K_m{}^* + [T]_0)^2}{2x} + K_m{}^* - x = \frac{(x - K_m{}^*)(x - K_m{}^* - 2[T]_0)}{2x} \tag{11}$$

Differentiation of eqn (10) with respect to x gives

$$d[E_T{}^*] = \frac{x^2 + 2K_m{}^*[T]_0 + K_m{}^{*2}}{4x^2}\,dx \tag{12}$$

Substitution of eqns (11) and (12) into eqn (9) yields

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

With the boundary condition $t = 0$, $[E_T{}^*] = [E^*]_0$, this integrates to

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

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where

$$x_0 = 2[E^*]_0 - [T]_0 + \sqrt{(K_m{}^* + [T]_0)^2 - 4[E^*]_0[E]_0} \quad (15)$$

MATERIALS AND METHODS

Bovine pancreatic trypsinogen, trypsin and TAME (N-α-p-tosyl-L-arginine methyl ester) were purchased from Sigma Chemical Co. The active-site normality of trypsin was 90%. Traces of chymotryptic activity would not be expected to interfere with the activation of trypsinogen, since the specificity does not fit the activation sites. All other chemicals were local products of analytical grade. The concentration of trypsinogen was determined by measuring the absorbance at 280 nm and using the absorption coefficient 33 600 $M^{-1} \cdot cm^{-1}$ [18].

Trypsin activity was routinely assayed by monitoring the increase in absorbance at 245 nm due to hydrolysis of TAME using a PerkinElmer spectrophotometer [19]. Initial rates of the reactions were determined from the linear slope of the progress curves obtained with a molar absorption coefficient ε_{245} of 595 $M^{-1} \cdot cm^{-1}$. All the assays were carried out at 30 °C and pH 8.1 in 40 mM Tris/HCl buffer. In kinetic studies of the autocatalytic conversion of trypsinogen into trypsin, aliquots of the incubation mixture of trypsinogen and trypsin were periodically removed and the activity of trypsin was determined at 30 °C. The assay system contained 40 mM Tris/HCl (pH 8.1), 1 mM TAME and 10 mM $CaCl_2$.

Since there are many sets of constants that give essentially the same curve, the individual fit for each set of experimental data to eqn (14) cannot give a reliable estimate for the kinetic parameters of the autocatalytic reaction. In practice, commercial trypsinogen always contains a trace amount of contaminating active enzyme. Therefore, 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. In this case, χ_0 in eqn (14) can be written as

$$x_0 = (2\alpha - 1)[T]_0 + \sqrt{(K_m{}^* + [T]_0)^2 - 4\alpha(1-\alpha)[T]_0{}^2} \quad (16)$$

and eqn (14) can then be treated as a function with two independent variables, $[E_T{}^*]$ and $[T]_0$, and three parameters, α, $K_m{}^*$ and $k_{cat}{}^*$. The problem of non-uniqueness of the estimated parameters can be solved by a global-analysis approach when a series of experiments is carried out at different initial concentrations of enzyme [15]. Unlike conventional fitting methods in which data corresponding to each fixed $[T]_0$ are fit individually, the kinetic parameters obtained by global analysis are constrained by the entire matrix of $[E_T{}^*]$ and $[T]_0$ simultaneously. By combining multiple experiments together in a single analysis, much more dramatic improvement in the fitting results can be obtained. In this study, we use a commercially available computer program for the non-linear regression data analysis, SigmaPlot 2000. SigmaPlot's non-linear curve fitter uses a least-squares procedure (Marquardt–Levenberg algorithm) to determine the parameters that minimize the sum of the squares of differences between the dependent variable in the equations and the observations.

RESULTS

As an example, the new method was used to analyse the calcium-induced autoactivation of trypsinogen by trypsin. Trypsinogen, the zymogen form of trypsin, is secreted into the duodenum by pancreatic cells. Trypsin catalyses the activation of trypsinogen in an intermolecular autocatalytic process. The conversion of trypsinogen into trypsin involves the removal of the N-terminal hexapeptide H_2N-Val-Asp-Asp-Asp-Asp-Lys [20]. This process is strongly stimulated by calcium ions. It has been shown that trypsinogen has two different binding sites for calcium ions while trypsin has only one. The high-affinity calcium-binding site ($K_d \approx 6.31 \times 10^{-4}$ M) is common to both trypsinogen and trypsin. The binding of Ca^{2+} on this site induces a stabilization of the conformation of these proteins which prevents the formation of inert proteins in the course of trypsinogen activation and protects trypsin against autolysis. The second site, with a much lower affinity for calcium ions ($K_d \approx 1.58 \times 10^{-2}$ M), is found only in the zymogen and has been assigned to the two aspartyl residues 13 and 14 neighbouring the important Lys-15–Ile-16 bond that is split during activation. Binding of calcium to this site accelerates very clearly the rate of hydrolysis by trypsin of the Lys–Ile bond [16,21,22].

Table 1 Effect of Ca^{2+} concentration on the steady-state kinetic parameters for trypsin-catalysed TAME hydrolysis

The kinetic experiments were carried out in 40 mM Tris/HCl buffer (pH 8.1) at 30 °C. $[E]_0 = 50$ nM and [TAME] = 50–1500 μM. k_{cat} and K_m were determined from the steady-state kinetic analysis.

$[Ca^{2+}]$ (mM)	k_{cat} (min^{-1})	K_m (μM)
0	3.33 ± 0.065	221 ± 17
10	3.86 ± 0.42	185 ± 79
30	4.06 ± 0.23	255 ± 50
50	3.32 ± 0.16	161 ± 32

To characterize the effect of calcium ions on the trypsin-catalysed reaction, the kinetic parameters of trypsin-catalysed TAME hydrolysis were determined first. The initial velocities for the hydrolysis of TAME by trypsin were measured under different Ca^{2+} concentrations at pH 8.1, 30 °C. By fitting the experimental data to the Michaelis–Menten equation, the values of k_{cat} and K_m were determined. The steady-state kinetic parameters for the trypsin-catalysed TAME hydrolysis are listed in Table 1. It can be seen from Table 1 that both k_{cat} and K_m are independent of the concentration of Ca^{2+}, indicating that binding of Ca^{2+} to trypsin is not required for activity of the enzyme. This result is in agreement with the experimental observation that calcium does not affect activation of a trypsinogen derivative in which the carboxylate groups are blocked [23].

In order to study the stimulation mechanism of calcium ions for the autoactivation of trypsin, the activation kinetics of trypsin was monitored at several fixed concentrations of Ca^{2+}. The trypsinogen was incubated with trypsin in 100 μl of reaction mixture containing 40 mM Tris/HCl (pH 8.0) and different concentrations of Ca^{2+} at 30 °C. At defined time intervals, an aliquot (5 μl) was taken from the reaction mixture and assayed for enzyme activity. Enzyme activity assays were carried out under kinetically valid conditions with TAME as a substrate. Figure 1 shows time courses for trypsin autoactivation in the presence of 10 and 50 mM calcium ions. The time required for activation increases at lower enzyme concentration, and the maximal trypsin activity was proportional to the total concentration of trypsin plus trypsinogen, indicating that the reaction went to completion in each case. As an example, fitting of eqn (14) to the experimental data in the presence of 10 mM Ca^{2+} is shown in Table 2. In Table 2 the reaction time t is the dependent variable, and $[E_T{}^*]$ and $[T]_0$ are the two independent variables. The best-fitting results were obtained with $\alpha = 0.047$, $K_m{}^* = 42.44$ μM and $k_{cat}{}^* = 0.041$ min^{-1} by using the non-linear regression analysis program SigmaPlot

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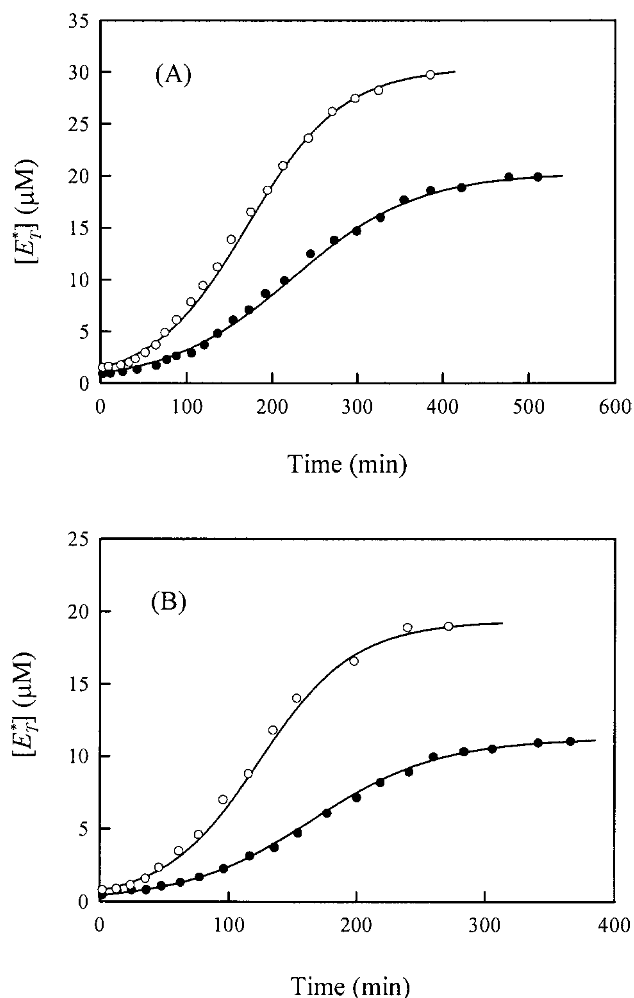

Figure 1 Autocatalytic activation of trypsinogen by trypsin

(**A**) Effect of trypsinogen concentration on the time course for autoactivation in the presence of 10 mM Ca^{2+} at 30 °C. The symbols represent the experimental data. The total concentrations of trypsinogen plus trypsin are (●) 20.29 μM and (○) 30.43 μM, respectively. The lines are the theoretical curves generated by using eqn (14) with $\alpha = 0.047$, $K_m{}^* = 42.44$ μM and $k_{cat}{}^* = 0.041$ min^{-1}. (**B**) Effect of trypsinogen concentration on the time course for autoactivation in the presence of 50 mM Ca^{2+} at 30 °C. The symbols represent the experimental data. The total concentrations of trypsinogen plus trypsin are (●) 11.26 μM and (○) 19.35 μM, respectively. The lines are the theoretical curves generated by using eqn (14) with $\alpha = 0.038$, $K_m{}^* = 14.47$ μM and $k_{cat}{}^* = 0.043$ min^{-1}.

2000. Similarly, when the time courses for trypsin activation at two different enzyme concentrations were analysed by a global-fitting procedure simultaneously according to eqn (14), the values of $K_m{}^*$, $k_{cat}{}^*$ and α could then be determined for each fixed concentration of Ca^{2+}. At the same concentrations of Ca^{2+}, the kinetic parameters so determined are quite close to those obtained by García-Moreno et al. [24], but are very different from those reported by Abita et al. [16]. In the presence of 50 mM Ca^{2+}, the value of $K_m{}^*$ was determined to be 14.5 μM, which is about 30 times lower than that obtained by Abita et al. [16] at 1 °C (400 μM).

Figure 2 shows the effect of increasing Ca^{2+} concentration on the kinetic parameters of trypsin autoactivation. The dominant effect of the Ca^{2+} concentration appears to be on $K_m{}^*$, but it has no significant effect on $k_{cat}{}^*$. On increasing the concentration of Ca^{2+}, $K_m{}^*$ decreases and approaches a limiting value. The kinetic parameters in the absence of Ca^{2+} cannot be measured experimentally since trypsinogen is very unstable under this condition. In the present study, the lowest concentration of Ca^{2+} used was 2.5 mM when the tight Ca^{2+}-binding site is almost

Table 2 Determination of kinetic parameters of trypsinogen autoactivation

Reaction time t is the dependent variable, and $[E_T{}^*]$ and $[T]_0$ are the two independent variables.

t (min)	$[E_T{}^*]$ (μM)	$[T]_0$ (μM)
2.1	0.90	20.29
76.8	2.27	20.29
172.6	7.09	20.29
214.4	9.88	20.29
299.3	14.68	20.29
354.8	17.68	20.29
422.2	18.84	20.29
510.5	19.88	20.29
2.1	1.50	30.43
88.3	6.12	30.43
119.0	9.43	30.43
212.6	20.98	30.43
270.4	26.20	30.43
324.8	28.22	30.43
385.4	29.74	30.43

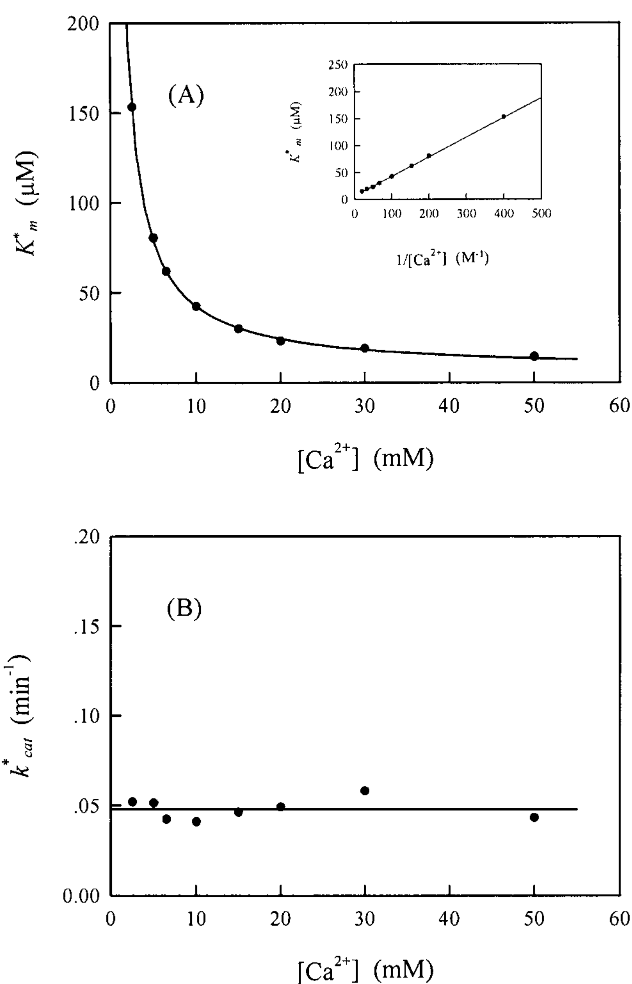

Figure 2 Effect of Ca^{2+} concentration on $K_m{}^*$ and $k_{cat}{}^*$

(**A**) Effect of Ca^{2+} concentration on $K_m{}^*$ for autoactivation of trypsinogen by trypsin. Inset: plot of $K_m{}^*$ versus $1/[Ca^{2+}]$. (**B**) Effect of Ca^{2+} concentration on $k_{cat}{}^*$ for autoactivation of trypsinogen by trypsin.

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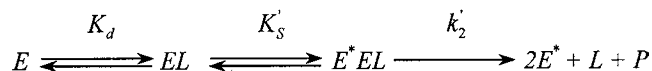

Scheme 2 Reaction mechanism of Ca^{2+}-induced trypsinogen auto-activation

saturated. By fitting eqn (5) to the experimental data, the parameters were determined to be $K_S' = 6.19 \pm 0.83\ \mu M$, $K_d = 58.79 \pm 9.61$ mM and $K_d' = -0.0026 \pm 0.0074$ mM. Because K_d' derived from the curve-fitting of the data to eqn (5) had a small negative value with a very large standard deviation, such that its confidence interval would include the value of zero, we suspected that K_d' might be in fact zero. In this case, the expressions of $K_m{}^*$ and $k_{cat}{}^*$ can be simplified to

$$k_{cat}{}^* = k_2' \tag{17}$$

$$K_m{}^* = \frac{(K_d + [L])K_S'}{[L]} \tag{18}$$

The apparent turnover number $k_{cat}{}^*$ is independent of [L] and the plot of $K_m{}^*$ against [L] is a descending curve. At very low [L] values the plot approaches the $K_m{}^*$ axis, and at very high [L] values it approaches a limiting value, K_S'. A straight line will be obtained if $K_m{}^*$ is plotted against 1/[L] as shown in the inset of Figure 2. When the experimental data of Figure 2 were fitted to eqn (18), a remarkable correspondence was observed. The continuous line in Figure 2 represents the best fit of the experimental data to eqn (18), yielding $K_S' = 5.96 \pm 0.5\ \mu M$ and $K_d = 61.67 \pm 8.96$ mM. Since K_S, K_S', K_d and K_d' are the true dissociation constants, our data suggest that the binding affinity for Ca^{2+}-bound trypsinogen to trypsin is at least two orders of magnitude higher than that for Ca^{2+}-free trypsinogen. It is to be noted that $K_d' K_S = K_d K_S'$ when K_d' is very small and K_S very large, and the enzyme E^* can only bind to EL to form the ternary complex E^*EL, i.e. E^* cannot form the complex E^*E, and the mechanism can be written as shown in Scheme 2.

DISCUSSION

As mentioned above, calcium ions have two diametrically opposite effects on trypsinogen. They markedly increase hydrolysis of the Lys–Ile bond near the N-terminus of the chain and almost totally suppress hydrolysis of certain other linkages, which in the absence of calcium are responsible for conversion of the precursor into inert proteins. The Lys–Ile bond in the inert proteins is not split by trypsin even in the presence of calcium. This double effect is due to the binding of the calcium at two different sites. The high-affinity Ca^{2+}-binding site in trypsin was first identified and described in detail by Bode and Schwager [25] based on analysis of crystal structure and the Ca^{2+} content of their crystal. Trypsinogen and trypsin have an apparently identical specific site for Ca^{2+} [21]. The binding of Ca^{2+} on this site induces a structural change and stabilizes protein towards thermal denaturation or autolysis, but it is not essential for enzyme activity of trypsin. A second Ca^{2+}-binding site exists only on the zymogen. This binding site is on the two N-terminal aspartyl residues of trypsinogen, Asp-13 and Asp-14. Binding of calcium at this site does not seem to involve any structural reorganization and has no effect on the rate of formation of inert proteins, but it is required for complete and efficient activation of trypsinogen [16]. There is no corroborative crystallographic evidence as yet, since Ca^{2+} was excluded from the crystallizing solution [26].

With trypsinogen, when the concentration of Ca^{2+} increases from 2.5 to 50 mM, $K_m{}^*$ decreases by a factor of 11 and $k_{cat}{}^*$ does not change. Extrapolation of the experimental data to zero and high Ca^{2+} concentration suggests that the $K_m{}^*$ value in the absence of Ca^{2+} is very much greater than that at saturating Ca^{2+} concentration. Therefore, the occupancy of the second site enhances greatly the binding affinity of trypsinogen to trypsin without changing the rate of decomposition of the trypsinogen–trypsin complex. Tryptic hydrolysis of the nonapeptide Val-Asp_4-Lys-Ile-Val-Gly and of the heptapeptide Val-Asp_2-Lys-Ile-Val-Gly have been shown to be Ca^{2+}-dependent [16,22]. However, Ca^{2+} ion binding to Asp-13 and Asp-14 together seems to be insufficient to explain the zymogen Ca^{2+} ion effect since the change in $K_m{}^*$ caused by Ca^{2+} binding to the N-terminal of trypsinogen is much greater than that to the model peptides. One possible explanation for this result would be that the N-terminal aspartyl residues make an important contribution to the stabilization of the zymogen by forming hydrogen bonds or salt linkages with other side chains of the protein. The binding of Ca^{2+} to the N-terminal of zymogen disrupts this interaction and results in exposure of the side chains, which provide an additional 'docking site' for trypsin binding, and therefore increase the binding affinity to trypsin.

Most protein kinases share a common molecular organization composed of a regulatory or inhibitory domain and a catalytic kinase domain having 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 [27,28]. Although the events which trigger activation of protein kinases are quite variable, a common molecular mechanism has been proposed [29]. A key feature in 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, a protein substrate. With some protein kinases, binding of ligand to the regulatory domain can stimulate autophosphorylation of the catalytic domain, and the autophosphorylation is required to fully activate the enzyme. For example, PAK1 (p21-activated protein kinase 1) exists in a closed conformation in the absence of Cdc42 due to an interaction between the inhibitory domain and the kinase domain [30]. The binding of Cdc42 to the inhibitory domain leads to a conformational change. The conformational change withdraws the inhibitory domain from the cleft of the kinase domain and releases the activation loop. The activation loop contains one critical phosphorylation site, Thr-423. In the absence of phosphorylation, this loop is either disordered or in a conformation that is not optimal for catalysis. Cdc42-stimulated autophosphorylation of Thr-423 in the activation loop will activate the enzyme. Therefore, the kinetic analysis described here is also applicable to study the ligand-induced autophosphorylation of protein kinases.

This work was supported in part by grants from NIH (R03TW01501), Natural Science Foundation of China (30270327) and the Ministry of Science and Technology of China (G1999075606).

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Received 8 September 2003/26 November 2003; accepted 23 December 2003
Published as BJ Immediate Publication 5 January 2004, DOI 10.1042/BJ20031365

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