The Mechanism of p21-activated Kinase 2 Autoactivation*

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The p21-activated kinases (PAKs) play an important role in diverse cellular processes. PAK2 is activated by autophosphorylation upon binding of small G proteins such as Cdc42 and Rac in the GTP-bound state. However, the mechanism of PAK2 autophosphorylation in vitro is unclear. In the present study, the kinetic theory of the substrate reaction during modification of enzyme activity has been applied to a study of the autoactivation of PAK2. On the basis of the kinetic equation of the substrate reaction during the autophosphorylation of PAK2, the activation rate constants for the free enzyme and enzyme-substrate complex have been determined. The results indicate that 1) in the presence of Cdc42, PAK2 autophosphorylation is a bipartite mechanism, with the regulatory domain autophosphorylated at multiple residues, whereas activation coincides with autophosphorylation of the catalytic domain at Thr-402; 2) the autophosphorylation reactions in regulatory domain are either a nonlimiting step or not required for activation of enzyme; 3) the autophosphorylation at site Thr-402 on the catalytic domain occurs by an intramolecular mechanism and is required for phosphorylation of exogenous substrates examined; 4) binding of the exogenous protein/peptide substrates at the active site of PAK2 has little or no effect on the autoactivation of PAK2, suggesting that multiple regions of PAK2 are involved in the enzyme-substrate recognition. The present method also provides a novel approach for studying autophosphorylation reactions. Since the experimental conditions used resemble more closely the in vivo situation where the substrate is constantly being turned over while the enzyme is being modified, this new method would be particularly useful when the regulatory mechanisms of the reversible phosphorylation reaction toward certain enzymes are being assessed.

Protein phosphorylation is the most important post-translational modification mechanism utilized by eukaryotic cells to regulate various signal transduction pathways. The enzymes responsible for catalyzing this reaction are protein kinases. It is now proposed that there exist over 1000 different protein kinases in mammals, and approximately one every three proteins in mammalian cells is phosphorylated (1–3). In protein phosphorylation reactions, the terminal (γ) phosphoryl group of ATP is transferred to specific serine and threonine residues by one class of protein kinases and transferred to specific tyrosine residues by another. Because of their central role in signal transduction, the regulation and function of protein kinases has been the subject of intense interest. Major insights for cellular signaling have been derived from studies of protein kinases, and it is common to view signaling pathways as cascades of protein kinase reactions.

In addition to the phosphotransferase reaction between ATP and specific residues in other polypeptides, almost all protein kinases catalyze autophosphorylation reactions (i.e. reactions in which the kinase serves as its own substrate), which are frequently modulated by regulatory ligands. The significance of autophosphorylation may be apparent in many cases, but, as with phosphorylation of some protein substrates, autophosphorylation may have no function effect (i.e. silent phosphorylation) and could reflect the availability of substrate sequence similar to that in the actual target proteins, or it may reflect the lack of absolute specificity (4). The autophosphorylation reactions can either be an intra- or intermolecular event, dependent upon the kinase. In some cases, both routes have been proposed for the same enzyme. The simplest intramolecular autophosphorylation is a first-order reaction and therefore can be described by the single exponential kinetic equation. In contrast, the intermolecular autophosphorylation is 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 for regulating particular biological processes (5).

The p21-activated kinases (PAKs) are members of a family of serine/threonine protein kinases defined by their interaction with the small GTPases, Cdc42 and Rac (6, 7). At present, six major members of the PAK family have been identified in mammalian cells (8). All PAKs consist of a C-terminal kinase domain and an N-terminal regulatory domain containing a GTPase binding domain and an inhibitory domain. PAKs play an important role in diverse cellular processes, including cytoskeletal dynamics, growth/apoptotic signal transduction through mitogen-activated protein kinases, and regulation of transcription factors (9–12). In addition, PAK2 is a substrate of caspase and may be cleaved and activated by it (13). The activated PAK2 is able to induce some of the morphological and biochemical changes characteristic of programmed cell death (14, 15). The requirement for PAK kinase activity in such diverse processes underscores the importance of understanding the molecular basis for regulation of PAK activity.

Based on the structural and architectural similarities, the PAK family can be categorized into two subgroups. The first group includes the closely related human PAK1, human PAK2,
mouse PAK3, and the corresponding rat homologues α-PAK, γ-PAK, and β-PAK, respectively (16–18). The most recently identified members of the PAK family are PAK4, PAK5, and PAK6, which fall into a second group of PAKs. PAK4, PAK5, and PAK6 are highly related to each other but show only ~50% identity to the GTPase binding domain and kinase domains of PAK1 to -3. Outside of this region, PAK4 to -6 are entirely different in sequence from other PAKs (19–21). Although, all PAKs can interact with GTPases, not all PAKs are activated by such interaction. The GTPase interaction of PAK1, PAK2, and PAK3 by itself leads to activation of PAK1 to -3, whereas binding of GTPases to PAK4, PAK5, and PAK6 does not lead to their activation, suggesting that GTPases regulate PAKs of the second group distinct from that of the first group (22).

PAK2 is activated by the binding of active Cdc42 and Rac to the GTPase binding domain, resulting in a conformational change and a subsequent autophosphorylation on several serine and/or threonine residues. However, there have been numerous inconsistencies concerning the mechanism of PAK2 autophosphorylation in vitro. Using the trypsin-cleaved PAK2, two autophosphorylation sites (Ser-197 and Thr-402) were identified. Based on the kinetic analysis of autophosphorylation reactions with enzyme dilution and the activation time course, Benner et al. (23) concluded that the phosphorylation of Thr-402 is most rapid, but activation does not occur until Ser-197 is autophosphorylated. The autophosphorylation of Thr-402 occurs by an intramolecular mechanism, whereas autophosphorylation of Ser-197 occurs by an intermolecular mechanism (23). In contrast, using the caspase 3-cleaved γ-PAK, Walter et al. (24) found that the phosphorylation of Thr-402 parallels the activity of the kinase and therefore suggested that autophosphorylation events of this threonine are closely coupled to the activation process. When MnATP is used instead of MgATP, both Thr-402 in the activation loop of γ-PAK and exogenous substrates cannot be phosphorylated by MnATP, substantiating the idea that the activation loop requires intermolecular phosphorylation (25). Similarly, Zenke et al. (26) also suggested that the intermolecular autophosphorylation of Thr-423 in PAK1 (equivalent to Thr-402 in PAK2) seems to be a key event for full activation of the enzyme.

In this study, we have applied the kinetic theory of substrate reaction during modification of enzyme activity (27, 28) to study the mechanism of PAK2 activation by autophosphorylation. On the basis of the kinetic equation of the substrate reaction during the autocatalytic activation of PAK2, all microscopic kinetic constants for the free enzyme and enzyme-substrate(s) complexes have been determined. Detailed analysis of the kinetic data enabled us to not only obtain the apparent rate constants for autoactivation of PAK2 activity from a single experiment but also to ascertain the effect of protein/peptide substrates on PAK2 autophosphorylation. The results indicate that 1) in the presence of Cdc42, PAK2 autophosphorylation is a bipartite mechanism, with the regulatory domain autophosphorylated at multiple residues, whereas activation coincides with autophosphorylation of the catalytic domain at Thr-402; 2) the autophosphorylation at site Thr-402 on the catalytic domain occurs by an intermolecular mechanism; 3) binding of the exogenous protein/peptide substrates at the active site of PAK2 has little or no effect on the rate of PAK2 autoactivation.

**EXPERIMENTAL PROCEDURES**

*Materials—*ATP, bovine brain acetone powder, phospho(enol)pyruvate, NADH, lactate dehydrogenase, pyruvate kinase, and urea (ultrapure) were purchased from Sigma. Fetal bovine serum, Grace’s insect cell culture medium, and yeastolate solution were purchased from Invitrogen; MOPS and Tris were purchased from Roche Applied Science. All peptides were synthesized using a standard protocol, purified by reverse-phase preparative high pressure liquid chromatography, and characterized by matrix-assisted laser desorption-ionization time-offlight mass spectrometry by ADI Inc. Other reagents were local products of analytical grade used without further purification. Double-deionized water was used throughout.

*Expression and Purification of Proteins—*GST fusion human PAK2 was cloned into baculovirus vector and expressed in SF9 cells. After 2–3 days of infection, culture SF9 cells containing GST-PAK2 were pelleted and resuspended in lysis buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.2% Triton X-100, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 2 mM dithiothreitol, 10% glycerol. The lysate was homogenized and centrifuged at 12,000×g for 15 min. The supernatant was applied onto a glutathione-Sepharose column, and the GST-PAK2 were eluted with buffer A (50 mM Tris, pH 8.0, 100 mM NaCl, 2 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol) containing 10 mM glutathione (reduced). The purity of PAK2 was examined by Coomassie Blue staining SDS-PAGE, and no other bands were observed. The enzyme was stored at ~70 °C and stable for at least 1 year. Protein concentrations were determined according to the method of Bradford with bovine serum albumin as a standard. A constitutively active Cdc42, GST-Cdc42L61, was expressed in *Escherichia coli* and purified according to standard procedures using the affinity matrix glutathione-Sepharose 4B (Amersham Biosciences).

The protein purity was judged to be over 95% by SDS-PAGE. The purified proteins were stored in 20% glycerol at ~70 °C. Myelin basic protein (MBP) was isolated from brain acetone powder based on a published protocol (29). Protein concentration determination was based on an extinction coefficient calculated from the amino acid composition of bovine MBP, ε280 = 10,800 cm⁻¹ M⁻¹ (30). Caspase 3 was kindly provided by Dr. Y. Shi (Princeton University, Princeton, NJ).

*Enzyme Assays—*Enzyme activity of PAK2 was determined with the MBP as a substrate using a coupled spectrophotometric assay except where indicated (31). The standard assay for PAK2 was carried out at 25 °C in 1.0 ml of reaction mixture containing 100 mM MOPS buffer, pH 7.4, 100 mM KCl, 10 mM MgCl₂, 200 μM NADH, 1 mM phospho(enol)-pyruvate, 20 units/ml lactate dehydrogenase, 15 units/ml pyruvate kinase, and different concentrations of substrates. Reactions were initiated by the addition of PAK2 to the reaction mixture. The progress of the reaction was monitored continuously by following the formation of NAD⁺ at 340 nm, on a Shimadzu UV2501PC14 spectrophotometer. The concentrations of ADP formed in the PAK2-catalyzed reaction were determined using an extinction coefficient for NADH of 6220 cm⁻¹ M⁻¹ at 340 nm. The concentrations of substrates and PAK2 are given in the text or in the figure legends. The concentrations of peptides were determined by turnover with the GST-PAK2 under conditions of limiting peptides.

*Kinetic Analysis—*In the presence of exogenous substrate, the general mechanism of the intermolecular autophosphorylation at low enzyme concentrations can be represented as follows,

\[
S \overset{k_+}{\rightarrow} E^* \overset{k_-}{\rightarrow} E^* + E^* \overset{k_2}{\rightarrow} E^* + P
\]

**SCHEME 1**
where $E$ and $E_0$ represent unphosphorylated and phosphorylated enzyme, and $S$ and $P$ represent exogenous substrate and its corresponding product, respectively. If we assume that 1) the enzymatic reaction is irreversible and not inhibited by the product formed, 2) the substrate concentration remains constant throughout the activity measurement, and 3) the steady state of the substrate reaction is rapidly established, application of the steady-state treatment to $E_0S$ gives rise to the equation,

$$K_m = \frac{[E]_0[S]}{[E_0S]} \quad \text{(Eq. 1)}$$

where $K_m = (k_{-1} + k_{2})/k_1$ is the Michaelis constant for the exogenous substrate. Given the following,

$$[E^+_0] = [E^+] + [E^0S] \quad \text{(Eq. 2)}$$

the total concentration of enzyme is as follows,

$$[T]_0 = [E^+] + [E^0S] + [E] = [E^+_0] + [E]^0 \quad \text{(Eq. 3)}$$

where $[E]^0$ and $[E]^0_0$ are the initial concentrations of the unphosphorylated and phosphorylated enzyme, respectively. From Equations 1–3, we have the following.

$$[E^+] = \frac{K_m([E^+_0] - [E]^0)}{K_m + [S]} \quad \text{(Eq. 4)}$$

$$[E^0S] = \frac{[E^+_0][S]}{K_m + [S]} \quad \text{(Eq. 5)}$$

The rate of the phosphorylated enzyme formation is given by the following,

$$\frac{d[E^+]}{dt} = k_{-1}[E^+][E] + k_{-1}E^0S[E]$$

$$= \frac{k_{-1}K_m + k_{+1}[S]}{K_m + [S]}[E^+_0][E]$$

$$= \frac{k_{obs}[E^+_0][E]}{(K_m + [S])[E] + ([T]_0 - [E^+_0])e^{-k_{obs}[T]_0}} \quad \text{(Eq. 6)}$$

where $k_{obs} = (k_{+1} + k_{-1}[S])/K_m + [S]$.

Equation 6 can be integrated to give the following.

$$[E^+] = \frac{[E]^0_0[T]_0}{[E^+_0] + ([T]_0 - [E]^0_0)e^{-k_{obs}[T]_0}} \quad \text{(Eq. 7)}$$

The rate of product formation while the enzyme is being modified is as follows.

$$\frac{d[P]}{dt} = k_{-1}[E^+][S] = \frac{k_{+1}[S][E^+_0]}{K_m + [S]}$$

$$= \frac{k_{+1}[S][E]^0_0}{(K_m + [S])[E]^0_0 + ([T]_0 - [E]^0_0)e^{-k_{obs}[T]_0}} \quad \text{(Eq. 8)}$$

The concentration of product formed at time $t$ is given by the equation,

$$[P] = \frac{\nu_s}{k_{obs}[T]_0} \ln [1 - \alpha (1 - e^{-\frac{\nu_s}{k_{obs}[T]_0}})] \quad \text{(Eq. 9)}$$

where

$$\alpha = \frac{[E]^0}{[T]_0} \quad \text{(Eq. 10)}$$

$$\nu_s = \frac{k_{+1}[S]}{K_m + [S]} \quad \text{(Eq. 11)}$$

$$k_{obs} = \frac{k_{+1}K_m + k_{-1}[S]}{K_m + [S]} \quad \text{(Eq. 12)}$$

Note that when the consumption of substrate is negligible during the course of the reaction, $k_{obs}$ can be regarded as apparent rate constant of the following reaction.
To study the mechanism of GST-PAK2 autoactivation by autophosphorylation, the activation kinetics of PAK2 was determined at several lower enzyme concentrations. The autoactivation of GST-PAK2 was carried out in 1.0 ml of reaction mixture containing 100 mM MOPS (pH 7.4), 10 mM MgCl$_2$, 2 mM dithiothreitol, 1.5 mM ATP, and 0.5 mM Cdc42L61 at 25 °C. At defined time intervals, an aliquot was taken from the reaction mixture and assayed for enzyme activity. Kinase activity assays were carried out under kinetically valid conditions with MBP as a substrate. Fig. 2 shows time courses for PAK2 activation by autophosphorylation. A slight lag time for activation was observed. When the activation time course was measured at several enzyme dilutions, the time required for activation increased at greater enzyme dilutions, suggesting that the activation process of PAK2 is related to the intermolecular autophosphorylation events. The maximum PAK2 kinase activity is proportional to the enzyme concentration in reaction solution. If only one autophosphorylation site on the catalytic domain of PAK2 is critical for kinase activity, the simplest mechanism to describe autocatalytic reaction at low enzyme concentrations is given by the following second-order mechanism,

$$E^* + E \rightarrow 2E^*$$

**REACTION 2**

where $k_{\text{cat}}$ is an apparent second-order rate constant for the full-length PAK2. The reaction rate is given by the equation,

$$\frac{d[E^*]}{dt} = k_{\text{cat}}[E^*][E]$$

(Eq. 14)

which can be solved to give the following,

$$\frac{1}{([E^*]_0 + [E])^ln([E^*]_0/[E])} = -k_{\text{cat}}t$$

(Eq. 15)

If the inactive PAK2 preparation contains trace amounts of phosphorylated active enzyme, the initial concentrations of active and inactive enzyme species can be written as $[E^*]_0 = a[T]_0$ and $[E]_0 = (1 - a)[T]_0$, where $a$ is a constant and $[T]_0$ is the total concentration of active plus inactive enzyme. Thus, Equation 15 can then be written as follows.

$$[E^*] = \frac{a[T]_0}{a + (1 - a)e^{-st/k_{\text{cat}}}}$$

(Eq. 16)

The time courses for PAK2 autoactivation at three different enzyme concentrations can be analyzed by global fitting procedure simultaneously. At a fixed ATP concentration of 1.5 mM, the values of $k_{\text{cat}}$ and $a$ were determined to be $1.5 \pm 0.1 \mu M^{-1} s^{-1}$ and $0.12 \pm 0.01$, respectively.

Collectively, the data show that in the presence of Cdc42L61, the autophosphorylation of PAK2 is a bipartite mechanism. First, the autophosphorylation of the enzyme at multiple sites occurs rapidly and precedes activation of the enzyme. The activation of the enzyme is correlated to a slow autophosphorylation at the critical site(s). Time course of autoactivation can be well described with a second-order reaction, suggesting that this reaction occurs by an intermolecular mechanism.

**Kinetics of Substrate Reaction during Autocatalytic Activation of PAK2**—Inside the cell, PAK2 is surrounded with various protein/peptide substrates. Therefore, it is of interest to obtain information about the effect of exogenous substrates on the autophosphorylation of PAK2. In the present study, we applied the kinetic theory of the substrate reaction during modification of enzyme activity (27, 28) to the study of the autophosphorylation process of PAK2. Because only a catalytic amount of enzyme is required, this method allows extraction of kinetic parameters for the autophosphorylation of PAK2 from its effect on the exogenous substrate reactions. To characterize the kinetic properties of PAK2 autophosphorylation, the preliminary experiment for an autophosphorylation system is to follow the full time course of the reaction as a function of enzyme concentration. MBP was used for PAK2 assay. The phosphorylation of MBP (169 amino acids) by PAK2 has been mapped to single site Ser-55 (25), and this protein was shown previously to be a good substrate for PAK2.

Fig. 3A shows the progress curves of product formation at different enzyme concentrations. The activation rates of PAK2 increase with increasing concentrations of PAK2. The progress curves start off the lag phases but go up with increasing time due to the activation of PAK2. When reaction time is sufficiently large, the curves approach straight lines. Preincubation of PAK2 with MgATP and Cdc42L16 led to a linear progress curve (see Fig. 3A, control). Such linearity indicates that 1) the coupling enzyme is in sufficient quantity to ensure actual measurement of the primary enzyme’s behavior and 2) the substrate consumption can be neglected, and the initial rate conditions are satisfied during activity measurement. The relaxation times were determined from the intersection of the asymptotes for the initial and final steady-state phases of progress curves. Indicated are those portions of the curve from which initial velocity and final velocity can be obtained. The abscissa value of the intercept of the two lines gives the value of the relaxation time. The inset of Fig. 3A shows a plot of the relaxation time, $\tau$, against the total concentration of enzyme. With an increase in the concentration of PAK2, the relaxation time decreases. The relaxation time decreased from 1000 to 250 s when the concentration of PAK2 was increased from 1.5 to 7.5 nM. If activation is determined by intramolecular autophosphorylation only, the activation progress curves can be described by a single exponential equation, and the relaxation time should be independent of the enzyme concentration. If activation is related to intermolecular autophosphorylation, the relaxation time will vary as a function of enzyme concentration. Under the conditions employed in the present study, the time-dependent behavior of enzyme-catalyzed reaction can be well described by Equation 9. The best fit curves in Fig. 3A
The full-length PAK2 contains multiple autophosphorylation sites, and therefore it is impossible to identify the critical phosphorylation site responsible for the enzyme activation. Walter et al. (24) have shown that in vitro, caspase 3 cleaves recombinant γ-PAK into two peptides; p27–121 contains the majority of the regulatory domain, and p34 212–524 contains 34 amino acids of the regulatory domain plus the entire catalytic domain (24). Following cleavage, both peptides become autoprophosphorylated with MgATP alone. Thr-402 was only detectable site of autophosphorylation in p34. To directly investigate the role of Thr-402 in PAK2 activation, kinetic experiments were carried out with caspase 3-cleaved p34 peptide. The full-length GST-PAK2 was incubated with caspase 3 for 30 min, and an aliquot was taken from the reaction mixture and added to the assay system. Fig. 3B shows the progress curves of MBP phosphorylation by caspase 3-cleaved PAK2. The shapes of progress curves are very similar to those obtained with the full-length PAK2 in the presence of Cdc42L61. Similarly, the relaxation time of activation decreases with increase in the concentration of enzyme (Fig. 3B, inset). When PAK2 was cleaved by caspase 3 and then incubated with MgATP alone for 20 min, the rate of phosphorylation of MBP by the cleaved and prephosphorylated PAK2 was linear with time (see Fig. 3B, control). No significant kinase activity (less than 1%) was detected in the absence of Cdc42L61 or caspase 3 (see Fig. 3B, curve 5). Because the enzyme concentration in the assay system is far less than the inhibition constant of the N-terminal domain ($K_i \sim 90 \, \text{nm}$), proteolysis would be expected to actually release the catalytic domain (35). Similarly, the kinetic parameters $v_{\text{obs}}/[T]_0$, $a$, and $k_{\text{obs}}$ were determined by fitting Equation 9 to the experimental data, and the average values are $v_{\text{obs}}/[T]_0 = 2.9 \pm 0.5 \, \text{s}^{-1}$, $a = 0.13 \pm 0.01$, and $k_{\text{obs}} = 1.2 \pm 0.1 \, \text{mM}^{-1} \, \text{s}^{-1}$, respectively. It can be seen that the kinetic parameters for the p34 peptide are comparable with those obtained with the full-length PAK2.

Recently, crystal structure of PAK1 in an autoinhibition conformation has been reported (36). X-ray crystallographic study suggested that PAKs exist as a dimer. The binding of Cdc42 to the regulatory domain or cleavage of N-terminal of PAK by a protease can disrupt this interaction and thereby dissociate the dimer. Thus, under the experimental conditions used here, all of the enzyme molecules will be in the monomeric form. Since the caspase 3-cleaved catalytic p34 peptide of human PAK2 contains only a single phosphorylation site, Thr-402, the mechanism of autophosphorylation of this site can be determined unambiguously. Taken together, our results indicate the following. 1) The caspase 3-cleaved enzyme has similar activity for exogenous substrate ($v_{\text{obs}}/[T]_0$) with the Cdc42-activated enzyme, and therefore the catalytic domain in the full-length PAK2 is almost completely released by binding of Cdc42 and/or phosphorylation of regulatory domain. 2) The fact that autophosphorylation at the multiple sites in regulatory domain does not affect the rate of autoactivation ($k_{\text{obs}}$) suggests that these reactions are either nonlimiting step or not required for activation of enzyme. 3) The enzyme preparation used here contains a small amount of endogenous phosphorylation at Thr-402 ($\alpha \sim 10\%$). This modification is necessary but not sufficient for activation, since no significant kinase activity was detected in the absence of Cdc42L61 (see Fig. 3B, curve 5). 4) Autophosphorylation of Thr-402 on the catalytic domain occurs by an intermolecular mechanism, and this reaction is required for enzyme activation.

**Influence of Exogenous Substrates on Autocatalytic Activation of PAK2**—Fig. 4A shows the time courses of enzyme-catalyzed reaction at the different MBP concentrations. From the progress curves of product formation, $v_{\text{obs}}$, $a$, and $k_{\text{obs}}$ can be

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**Fig. 3.** Time course of substrate reaction in the presence of different concentrations of PAK2 at 25 °C. A, the reaction mixture contained 100 mM MOPS buffer, pH 7.4, 100 mM KCl, 10 mM MgCl$_2$, 200 μM NADH, 1 mM phosphoenolpyruvate, 20 units/ml lactate dehydrogenase, 15 units/ml pyruvate kinase, 30 μM MBP, and 1.5 μM ATP. The full-length GST-PAK2 was added to the reaction mixture to start the reaction. Final concentrations of PAK2 were 7.5 nM (curve 1), 4.5 nM (curve 2), 3.5 nM (curve 3), and 2.5 nM (curve 4), respectively. In the control experiment, the full-length PAK2 was preincubated with Cdc42L61 and ATP. 7.5 nM fully activated PAK2 was used to initiate the reaction. The *inset* shows a plot of the relaxation time *versus* PAK2 concentration. B, the reaction mixture contained 100 mM MOPS buffer, pH 7.4, 100 mM KCl, 10 mM MgCl$_2$, 200 μM NADH, 1 mM phosphoenolpyruvate, 20 units/ml lactate dehydrogenase, 15 units/ml pyruvate kinase, 30 μM MBP, and 1.5 μM ATP. The full-length GST-PAK2 was incubated with caspase 3 (1 μM) for 30 min, and an aliquot was taken out and added to the reaction mixture to start the reaction. The enzyme concentrations were 7.5 nM (curve 1), 4.5 nM (curve 2), 3.5 nM (curve 3), and 2.5 nM (curve 4), respectively. In the control experiment, the full-length GST-PAK2 was preincubated with caspase 3 and ATP, 7.5 nM fully activated p34 peptide was used to initiate the reaction. The *inset* shows a plot of the relaxation time *versus* enzyme concentration. When the full-length GST-PAK2 was added to the reaction mixture directly, no significant kinase activity was observed (curve 5).

were obtained by nonlinear regression analysis from which the kinetic parameters, $v_{\text{obs}}/[T]_0$, $a$, and $k_{\text{obs}}$ can be determined. As expected, the kinetic parameters so determined are independent on the enzyme concentration at a fixed substrate concentration within experimental error (data not shown). The average values of the parameters are $v_{\text{obs}}/[T]_0 = 2.2 \pm 0.4 \, \text{s}^{-1}$, $a = 0.11 \pm 0.01$, and $k_{\text{obs}} = 1.2 \pm 0.2 \, \text{mM}^{-1} \, \text{s}^{-1}$.

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**Note:** The text includes scientific references and figures related to the study of PAK2 activation kinetics. The information is crucial for understanding the role of PAK2 in cellular processes and its potential involvement in various diseases. The study suggests that PAKs, particularly PAK2, can undergo autoprophosphorylation and that this process is affected by factors such as caspase cleavage and the binding of Cdc42. The kinetic analysis provides insights into the mechanisms of enzyme activation and autoinhibition, which are important for understanding the regulation of PAKs in cellular signaling pathways.
determined by fitting Equation 9 to the experimental data as described before. Fig. 4B shows the effect of the MBP concentration on the steady-state rate of the enzyme-catalyzed reaction. The steady-state velocity gives rise to the hyperbolic dependence on the MBP concentration. The MBP concentration at 25 °C and pH 7.4 in 100 mM MOPS buffer. Errors for individual parameter were obtained from the nonlinear regression fit of the data to the corresponding equations.

![Fig. 4](https://example.com/fig4.png)

**A** Time course of substrate reaction in the presence of different concentrations of MBP at 25 °C. A, the reaction was started by the addition of PAK2 to the reaction mixture. The final concentration of PAK2 was 2.5 nm. Concentrations of MBP were 20, 45, and 100 μM for curves 1–3, respectively. Other conditions were the same as in Fig. 3A. The data were fitted to Equation 9 to determine the kinetic parameters, \( k_{cat} \), \( k_{obs} \), and \( \alpha \). The solid lines correspond to the best fit of the data to Equation 9 by a nonlinear least-squares regression algorithm. B, plot of \( v_r \) versus MBP concentration for the autoactivation of PAK2. The \( v_r \) values were calculated from time courses of PAK2 autoactivation shown in Fig. 4A. The solid line represents the best fit to the data according to Equation 9. C, plot of \( k_{obs} \) versus MBP concentration for the autoactivation of PAK2. The \( k_{obs} \) values were calculated from time courses of PAK2 autoactivation shown in Fig. 4A. The solid line represents the best fit to the data according to Equation 9.

![Fig. 5](https://example.com/fig5.png)

**B** The values of \( k_{cat,0} \), \( k_{obs,0} \), and \( \alpha \) were determined as described previously. Fig. 5A shows the time courses of enzyme-catalyzed reaction at the different RAFtide concentrations. Similarly, from the progress curves of product formation, \( v_r, \alpha, \) and \( k_{obs} \) were determined as described previously. Fig. 5B shows the variation of steady-state rates of the PAK2-catalyzed reaction with RAFtide concentrations. The experimental data fit the Michaelis-Menten equation with a \( K_m \) of 37 ± 6 μM and \( h_2 \) of 3.8 ± 0.3 s⁻¹. Knowing the value of \( K_m \), the activation rate constants for the free enzyme and enzyme-substrate complex can be obtained from the relationship between \( k_{obs} \) and MBP concentration. Fig. 4C shows a dependence of \( k_{obs} \) on the substrate concentration. On increasing the concentration of MBP, \( k_{obs} \) decreases and approaches a non-zero constant. The Michaelis activation rate constants for the free enzyme, \( k_{cat,0} = 1.9 ± 0.1 \mu M^{-1} s^{-1} \), and for the enzyme-substrate complex, \( k_{cat,0} = 0.9 ± 0.1 \mu M^{-1} s^{-1} \), can then be obtained by fitting Equation 12 to the experimental data. All of the kinetic parameters for the autoactivation reaction of PAK2 are summarized in Table I. Surprisingly, the binding of MBP at the active site of PAK2 does not completely abolish the PAK2 autoactivation even at saturating concentrations of MBP. The microscopic activation rate constant for the free enzyme is about 2-fold greater than that for the enzyme-substrate complex. These data suggest that the binding of MBP only partially inhibits the rate of PAK2 autophosphorylation at site Thr-402.

Small synthetic peptides serve as efficient substrates for a number of protein kinases (37, 38). To study the effect of different substrates on the PAK2 autoactivation reaction, we used a synthetic peptide derived from Raf-1, RAFtide (residues 330–344, IRPRGQDRDSYSYWEI), as a Ser peptide model substrate. PAK2 is a positive regulator of Raf-1 activity through phosphorylation of Ser-338, an essential site for Raf-1 activation (39). Fig. 5A shows the time courses of enzyme-catalyzed reaction at the different RAFtide concentrations. Similarly, from the progress curves of product formation, \( v_r, \alpha, \) and \( k_{obs} \) were determined as described previously. Fig. 5B shows the variation of steady-state rates of the PAK2-catalyzed reaction with RAFtide concentrations. The experimental data fit the Michaelis-Menten equation with a \( K_m \) of 42 ± 11 μM, and \( h_2 \) of 8 ± 1 s⁻¹. Fig. 5C shows a dependence of on the substrate concentration (10–40 μM). It can be seen from this figure that the apparent activation rate constant is independent of the concentration of RAFtide, indicating that the presence of RAFtide had no effect on the rate of PAK2 autoactivation.

LIM kinase is one important downstream effector of PAKs, implicated in the regulation of actin cytoskeletal dynamics. Active PAKs can phosphorylate LIM kinase at Thr-508 within the activation loop and activate LIM kinase activity toward coflin/ADF in *vivo* (40). To study the effect of Thr peptide substrate on the PAK2 autoactivation reaction, LIMKtide, a synthetic peptide derived from LIM kinase LIMKtide (residues 500–514, KPDKKKRTVVGVNPY), which contains a PAK phosphorylation site, Thr-508, was used as a model of Thr peptide substrate. Fig. 6A shows the time courses of enzyme-catalyzed reaction at the different LIMKtide concentrations. The values of \( k_r, \alpha, \) and \( k_{obs} \) were determined by fitting Equa-
tion 9 to the experimental data. Fig. 6B shows the variation of steady-state rates of the PAK2-catalyzed reaction with LIMK-tide concentrations. The experimental data fit the Michaelis-Menten equation with a $K_m$ of 40 ± 8 µM and $k_2$ of 10 ± 1 s$^{-1}$. Fig. 6C shows a dependence of $k_{obs}$ on the substrate concentration (10–40 µM). The value of $k_{obs}$ increases with increase in the concentration of LIMKtide. The microscopic activation rate constants for the free enzyme and the enzyme-substrate complex were determined to be $k_{a0} = 1.9 \pm 0.1 \mu M^{-1} s^{-1}$ and

![Figure 5](image5.png)

![Figure 6](image6.png)
**Table II**

| Parameter | MBP | RAFlide | LINKide | PAKtide |
|-----------|-----|---------|---------|---------|
| \( k_{\text{on}} \) (s\(^{-1}\)) | 4 ± 0.4 | 8.7 ± 1.3 | 9.7 ± 2.6 | 15 ± 6 |
| \( K_{\text{m}} \) (µM) | 22 ± 5 | 30 ± 9 | 30 ± 10 | 520 ± 226 |
| \( k_{\text{cat}}/K_{\text{m}} \) (µM\(^{-1}\) s\(^{-1}\)) | 0.18 ± 0.04 | 0.29 ± 0.1 | 0.32 ± 0.14 | 0.03 ± 0.02 |

According to our definition, the parameter \( k_{\text{on}} \) determined from the autoactivation kinetics is equal to \( k_{\text{cat}}K_{\text{m}} \) for the phosphorylation of the isogenous substrate, the full-length PAK2. The second order rate constant \( k_{\text{cat}}K_{\text{m}} \) is a physiologically relevant parameter for the reaction of free enzyme with free substrate and reflects both binding affinity and catalytic efficiency. Therefore, it is interesting to compare the \( k_{\text{cat}}/K_{\text{m}} \) value for the full-length PAK2 substrate with that for its corresponding model peptide, PAKtide. The full-length PAK2 was a very good substrate with the \( k_{\text{cat}}/K_{\text{m}} \) value of 2 µM\(^{-1}\) s\(^{-1}\). In contrast, PAKtide had a lower overall rate as shown by the \( k_{\text{cat}}/K_{\text{m}} \) value of 0.03 µM\(^{-1}\) s\(^{-1}\). Thus, the catalytic efficiency of activated PAK2 for the full-length PAK2 is about 2 orders of magnitude higher than that of PAKtide. Since the \( k_{\text{cat}} \) value for PAKtide was within the range observed with other threonine/serine protein kinases for protein/peptide substrates, it is likely that in comparison with PAKtide, the higher \( k_{\text{cat}}/K_{\text{m}} \) value for the full-length PAK2 is due to a decrease in \( K_{\text{m}} \) rather than an increase in \( k_{\text{cat}} \). Therefore, our data suggest that PAKtide on its own binds weakly to the active site of PAK2, and the full-length PAK2 has a much higher binding affinity to the activated enzyme. The substrate recognition of the full-length PAK2 by the activated PAK2 may involve extensive protein-protein contacts in addition to the interactions that engage the phosphoamino acid residue.

**DISCUSSION**

The conventional method for determining rate constant of enzyme modification is to take aliquots from an enzyme/modifier incubation mixture at definite time intervals and assay for the enzyme activity. This method is laborious and not easily applied to fast reactions with a half-life of less than 1 min. Some years ago, a systematic study of modification kinetics was presented. In the past 2 decades, a large body of work has been carried out that deals with the kinetics of irreversible modification of enzyme activity in the presence of substrates. The kinetics of enzyme modification by small molecule irreversible modifiers in the presence of substrate have been treated in two different ways, depending upon whether a significant proportion of substrate is consumed in the enzyme-catalyzed reaction (47–50) or whether the substrate concentration can be considered to be essentially constant during the period of observation and, hence, set equal to its initial value in the derivation of integrated rate expression describing the time dependence of product formation (27, 28). The latter approach has been extended beyond the treatment of relatively simple cases of irreversible modification of an enzyme catalyzing one substrate reaction involving a single intermediate to include more complicated models (51–53). In comparison with the conventional method, the advantage of the progress curve method is not only its usefulness in the study of fast modification reaction but also its convenience in the study of substrate effects on enzyme modification.

Enzyme-catalyzed covalent modification of another enzyme 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 dephosphorylation are the most prevalent forms of posttranslational covalent modification mechanism that cells employ to regulate enzyme activity. Most cellular functions are regulated by protein phosphorylation, and almost every known cellular signaling pathway utilizes one or more protein kinases. Recently, the substrate reaction theory has been employed to study the influence of substrates on the dephosphorylation of glycogen phosphorylase a by protein phosphatase 1 and dephosphorylation of ERK2 by HePTP (54, 55). Since the experimental conditions used resemble more
closely the *in vivo* situation where the substrate is constantly being turned over while the enzyme is being modified by another enzyme, this new method would be particularly useful when the regulatory mechanism of the reversible phosphorylation reaction toward certain enzymes is being assessed.

In this study, we have applied the kinetic theory of the substrate reaction during modification of enzyme activity to investigate the autocatalytic activation of PAK2. The main motivation behind this study was our desire to establish an efficient system for studying the autophosphorylation of protein kinases in the presence of exogenous substrates. The obtained results are physiologically relevant, because inside the cell, protein kinases are surrounded with various protein/peptide substrates. Further understanding of the specific functional role of protein kinases in cellular signaling requires detailed investigation of protein kinases with physiological exogenous substrates. Analyses of the time courses of PAK2 autophosphorylation in the presence of various protein/peptide substrates suggest that substrate binding to PAK2 has different effects on the PAK2 autophosphorylation. The second-order rate constants for the activation of free PAK2 determined from these sets of experiments are similar to that determined directly by the conventional method. Operationally, the second-order rate constant ($k_{obs}$) is the same as the kinetic parameter ($k_2/K_m$), which measures the catalytic efficiency of the PAK2-catalyzed autophosphorylation. The high $k_2/K_m$ value indicates that full-length PAK2 is a highly efficient substrate for the active enzyme. Since only a catalytic amount of enzymes is required, this new method should facilitate further studies to determine the molecular basis for substrate recognition between kinases and target proteins.

There have been several previous investigations of differential PAK autophosphorylation under various activation conditions (25, 33, 56, 57). Gatti *et al.* (33) have reported that autophosphorylation of γ-PAK with MgATP alone takes place at Ser-19, Ser-20, Ser-55, Ser-192, and Ser-197. In the presence of Cdc42, additional autophosphorylation of γ-PAK occurs at Ser-141, Ser-165, and Thr-402. Thus, the later sites are selectively phosphorylated upon γ-PAK activation (33). The phosphorylation of Thr-402 seems to be critical for the catalytic function of the protein kinase (24, 58). King *et al.* (59) have shown that PAK1 phosphorylates other PAK1 molecules at the activation loop Thr-423 (equivalent to Thr-402 in γ-PAK) by an intermolecular autophosphorylation mechanism. In contrast, studies on human PAK2 as a trypsin-activated kinase suggested that the autophosphorylation of Thr-402 (site 1) occurs...
by a rapid intramolecular mechanism and precedes activation, and the catalytic domain needs to undergo a slower intermolecular autophosphorylation at Ser-197 (site 2) prior to conversion to its active form (23). Maximum activation of the trypsin-cleaved PAK2 appears to require a dual phosphorylation of Ser-197 and Thr-402, and therefore the two residues play distinct but complementary roles. It should be indicated that whereas a change in the kinetic behavior as a function of enzyme concentration is certainly diagnostic for an intermolecular process, the opposite situation could also occur under certain conditions. In the experiments of Benner et al. (23), the Thr-402 autophosphorylation occurred very fast and was completed in 10 min; therefore, the linearity of Thr-402 phosphorylation with enzyme dilution cannot exclude the possibility that this residue is actually phosphorylated by an intermolecular mechanism. This far there is no experimental evidence that can distinguish unambiguously between the intra- and intermolecular mechanism for the autophosphorylation of Thr-402.

To further address this issue, we presented a detailed kinetic analysis of PAK2 autoactivation. In the presence of Cdc42L61, the autophosphorylation of PAK2 is a bipartite mechanism, with the regulatory domain autophosphorylated at multiple residues, whereas activation coincides with autophosphorylation of the catalytic domain at Thr-402. The autophosphorylation event of the regulatory domain is either a fast nonlimiting step or not required for enzyme activation. Analysis of primary sequences around these phosphorylation sites suggests that the phosphoserine residues in the regulatory domain are not efficient exogenous substrates for intermolecular phosphorylation by PAK2 (25). Therefore, the rapid autophosphorylation rates suggest that these residues are phosphorylated probably by an intramolecular mechanism. Kinetic studies of PAK2 activation at different enzyme concentrations indicate that autophosphorylation of Thr-402 in the catalytic domain occurs by an intermolecular mechanism. These findings disagree with the autophosphorylation mechanisms of Ser-197 and Thr-402 proposed by Benner et al. (23) with trypsin-treated PAK2. One possible explanation for this difference is that limited trypsin digestion of PAK2 alters the autophosphorylation mechanism of Ser-197, since this residue is located at the N terminus of the trypsin-cleaved PAK2. In the full-length PAK2, the autophosphorylation of Ser-197 either occurs through a fast intramolecular mechanism and precedes phosphorylation of Thr-402, or it occurs by a slow intermolecular mechanism and has no effect on the PAK2 activation. In fact, the intermolecular autophosphorylation of Ser-197 by the trypsin-treated PAK2 (k_p/K_m = 1.2 × 10^4 M^{-1} s^{-1} at 0.125 mM ATP) is much slower than phosphorylation of Thr-402 and the exogenous substrates obtained in the present study, suggesting that this intermolecular phosphorylation of Ser-197 may not occur in the full-length PAK2 (5, 23).

From the results that we obtained, together with published structural and biochemical data, a working model for the molecular basis of PAK2 autoactivation can be proposed (Fig. 7). PAK2 exists in a closed conformation in the absence of Cdc42L61 due to an interaction between the inhibitory domain and the kinase domain. The amount of endogenous phosphorylation of Thr-402 in each enzyme preparation may vary. The active site in the kinase domain is not accessible for intermolecular phosphorylation of substrate when the PAK2 is in the closed conformation. In the presence of Cdc42L61, the interaction of Cdc42L61 with PAK2 leads to a conformation change in the inhibitory domain, resulting in the disinhibition of the catalytic domain and intramolecular autophosphorylation at several residues in the regulatory region. 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-402. In the absence of phosphorylation, this loop is either disordered or in a conformation that is not optimal for catalysis. Intermolecular autophosphorylation of Thr-402 in the activation loop will activate the enzyme. Even if the Cdc42L61 dissociates from its binding site, the activated PAK2 remains in an "open" state, allowing the intermolecular phosphorylation of substrates.

An unexpected observation in the present study is that the protein kinase activity of PAK2 toward the full-length PAK2 is not competitively inhibited by binding of the exogenous substrate at the active site. These data suggest that the binding of exogenous and isoeugenol substrate is not mutually exclusive. We have demonstrated that the PAKtide is a relatively poor substrate when compared with the full-length PAK2. Clearly, the phosphoacceptor sequence of PAKtide is not the sole determinant for high affinity PAK2 binding, and other factors must contribute to the full-length PAK2 recognition by the activated enzyme. For many protein kinases, additional contributions to protein kinase target selection are provided by "docking sites" on substrates that are separate from the phosphoacceptor residues (60). Moreover, some protein kinases use domains or subunits other than the catalytic domain to aid in substrate recognition (61–63). We know from structural studies that the inhibitory domain packs against the kinase domain with a hydrophobic interface reinforced by peripheral polar contacts. Binding of Cdc42L61 results in exposure of the hydrophobic interface, making it accessible for intermolecular phosphorylation. Therefore, it is likely that a second substrate binding site of the full-length PAK2 is located in this region of the activated enzyme.

In summary, we have shown that the kinetic theory of substrate reaction during modification of enzyme activity can be applied to study the autophosphorylation reaction of protein kinases. Using this approach, we have shown that PAK2 is a highly efficient enzyme toward its physiological substrate, full-length PAK2. Further, we have shown that although the autophosphorylation of Thr-402 in PAK2 is an intermolecular process, it is not blocked by binding of exogenous protein/peptide substrates. This method should be generally applicable to the study of protein kinases that are involved in regulating the activity of other enzymes whose activity is altered by phosphorylation. The main advantages of this approach include the following: 1) only a small amount of the protein kinases are required, and 2) the results are directly physiologically relevant. These initial studies should provide a foundation for future structural and mechanistic work of protein kinases.

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