Kinetic Basis for Activation of CDK2/Cyclin A by Phosphorylation*

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The activation of most protein kinases requires phosphorylation at a conserved site within a structurally defined segment termed the activation loop. A classic example is the regulation of the cell cycle control enzyme, CDK2/cyclin A, in which catalytic activation depends on phosphorylation at Thr160 in CDK2. The structural consequences of phosphorylation have been revealed by x-ray crystallographic studies on CDK2/cyclin A and include changes in conformation, mainly of the activation loop. Here, we describe the kinetic basis for activation by phosphorylation in CDK2/cyclin A. Phosphorylation results in a 100,000-fold increase in catalytic efficiency and an approximate 1,000-fold increase in the overall turnover rate. The effects of phosphorylation on the individual steps in the catalytic reaction pathway were determined using solvent viscosimetric techniques. It was found that the increase in catalytic power arises mainly from a 3,000-fold increase in the rate of the phosphoryl group transfer step with a more moderate increase in substrate binding affinity. In contrast, the rate of phosphoryl group transfer in the ATPase pathway was unaffected by phosphorylation, demonstrating that phosphorylation at Thr160 does not serve to stabilize ATP in the ATPase reaction. Thus, we hypothesize that the role of phosphorylation in the kinase reaction may be to specifically stabilize the peptide phosphoacceptor group.

Cellular proliferation is controlled by a family of protein kinases in which the catalytic subunits are members of the cyclin-dependent kinase (CDK) family and the regulatory subunits are cyclins. To date, nine distinct CDKs in addition to eight different cyclins have been identified, in which different CDK/cyclin combinations serve to regulate distinct points in the mammalian cell division cycle. Although Cdc2 (CDK1)/cyclin B controls the transition of cells from the G2 to M-phase, the activities of CDK2/cyclin E and CDK2/cyclin A are critical for G1/S-phase transition and progression through S-phase, respectively (1). Since the critical role of the CDKs in cell cycle control has been well established, understanding the details of their regulation is now of fundamental importance.

The three-dimensional structures of several forms of CDK2 have been solved by x-ray crystallography. Like all protein kinases, CDK2 displays a globular fold consisting of two lobes, a smaller N-terminal lobe that is principally β-sheet and a larger C-terminal lobe that is principally α-helix. The bilobal interface constitutes the active site cleft into which the adenine ring of substrate ATP is deeply buried. The ATP γ-phosphate is directed toward the mouth of the active site where peptide and protein substrates bind and where phosphoryl group transfer occurs (for a review see Ref. 2). Located near the mouth of the active site is a conserved loop structure termed the activation loop (residues 146–166). This loop structure is present in all protein kinases (3), and phosphorylation at a conserved site within the activation loop is necessary for full activation of most protein kinases. In CDK2, this site is Thr160, phosphorylation of which is catalyzed by a heterologous kinase, CAK (cyclin-dependent kinase activating kinase) (4, 5).

Activation of CDK2 requires binding to its regulatory subunit, cyclin, in addition to phosphorylation at Thr160 (5). The structural consequences of cyclin binding to CDK2 and phosphorylation at Thr160 in CDK2 have been revealed by x-ray crystallography. Interaction with cyclin A results in the repositioning of an active site helix and the consequent alignment of key catalytic residues in CDK2, including the invariant residues Lys153 and Glu151, which function to stabilize the α- and β-phosphates of ATP, and Asp145, which chelates an essential Mg2+ ion also serving to stabilize ATP. Furthermore, the active site cavity of CDK2 is exposed upon cyclin binding by repositioning of the activation loop by over 15 Å (6,7). Subsequent phosphorylation of the CDK2/cyclin A complex at Thr160 (in CDK2) is associated with less dramatic changes in structure that are localized primarily to the activation loop (8). Nonetheless, the latter modification is associated with a dramatic increase in catalytic power.

Although abundant structural information regarding the activation of CDK2 is available, it is not known how the associated alterations in structure correlate with increased catalytic rate. In particular, it is not known which steps along the catalytic reaction pathway are altered in response to Thr160 phosphorylation to achieve kinase activation. Thus, it has not been possible to make a correlation between CDK structure and regulation. In this study, we describe the catalytic reaction pathway for both the unphosphorylated and Thr160-phosphorylated CDK2/cyclin A complexes and report the kinetic basis for activation by phosphorylation. Our results show that phos-
phorylation affects mainly the rate of chemistry, doing so by specific stabilization of the protein phosphoacceptor group.

**Materials and Methods**

Protein Expression and Purification

Unphosphorylated CDK2/cyclin A (non-p/CDK2/Cyclin A)—CDK2 was expressed as the full-length human CDK2 N-terminally fused to GST in pGEX-2T. Cyclin A was expressed as a truncated fragment of bovine cyclin A3, corresponding to human cyclin A residues Val119 to the end) fused to a C-terminal hexahistidine tag in pET21d (9). *Escherichia coli* BL21 (DE3) transformed with either GST-CDK2 or cyclin A-His6 were grown in SMD in LB to an A600 of 0.6–0.8. Expression of GST-CDK2 was then induced with IPTG (0.4 mM) at room temperature for 18 h, whereas expression of cyclin A-His6 was induced with IPTG (0.2 mM) at room temperature for 3 h. Cell pellets from 1 liter of each culture were separately resuspended in 10 ml of lysis buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM dithiothreitol, 1 mM MgCl2, and 0.5 mM phenylmethylsulfonyl fluoride, 100 mM CaCl2, 1 mM dithiothreitol, 0.1 mM EDTA, pH 7.4) containing enzyme and varying amounts of one substrate with the other held fixed. Assays on p-[Thr]32P/ATP specific radioactivity over time follows first-order kinetics with rate constant k = (rate of ADP formation)/[ATP]. It was assumed that the rate of ADP formation was effectively equal to the rate of ATP hydrolysis and that ADP generated from the kinase reaction was insignificant. The rate of ADP formation at a given concentration of ATP and enzyme was therefore calculated by solving for the Michaelis-Menten equation using K and kcat values of 90 µM and 10 min−1, respectively, for the ATPase reaction, which were determined in a spectrophotometric assay (see below; data not shown). The calculated rate constant corresponding to each ATP concentration was used to simulate a decay profile using the equation,

\[ y = y_0 - \frac{y_0 - k \cdot x^2}{K^2 + x^2} \]

where y is the ATP-specific activity at any time, y0 is the maximum ATP-specific activity, and k is the rate constant, as defined above. The average ATP-specific radioactivity at any time, t, was obtained by determining the ATP-specific activity time integral for the entire reaction time course, dividing by the total time. Calculations were performed using the program Scientist (Micromath Inc., Salt Lake City, UT). Reactions were initiated by the addition of [γ-32P]ATP (300–500 cpm/µmol) and allowed to proceed at 25 °C for 6 to 30 min, at which time reactions were terminated with 25% acetic acid. Reaction samples were assayed for kinase activity as the change in absorbance at 340 nm in a Shimadzu UV1601 spectrophotometer. The micromolar specific radioactivity of [γ-32P]ATP was determined by Cerenkov counting.

Steady-state kinetic parameters for the phosphorylation of Peptide 1 were determined from initial velocity data obtained from a matrix of several fixed ATP concentrations at varied peptide substrate concentrations. Equation 1 was globally fit to the data using the program Scientist (Micromath Inc.).

\[ v = V \cdot A - B \cdot \left( \frac{K_{mB} \cdot (A + K_{mB} \cdot (A + K_{mA} \cdot (A + B)) \cdot (A + B)}{K_{mB} \cdot (A + K_{mB} \cdot (A + B))} \right) \]  

(Eq. 1)

where v is the initial velocity, V is the maximal initial velocity, A and B are the concentrations of the fixed and varied substrates, respectively, Ki for Michaelis constant is the dissociation constant for A, and kcat was determined by dividing the maximal initial velocity by the enzyme concentration.

ATPase activity was monitored using a coupled spectrophotometric assay in which the regeneration of ATP from ADP and phosphoenolpyruvate catalyzed by pyruvate kinase is coupled to the reduction of nitro blue tetrazolium (NBT) by NADH, and the latter of which is catalyzed by lactate dehydrogenase (13). The Michaelis-Menten enzyme constants were determined by nonlinear least-squares analysis using the Michaelis-Menten equation, which was fit to the velocity data.

**Solvent Viscosity Studies**—Steady-state assays were carried out as described above in buffer containing varied sucrose or fructose. Relative solvent viscosity (ηrel) was determined from the following equation:

\[ ηrel = η - η_w = \frac{t_w}{t} \cdot ρ, \]

where η is the solvent viscosity, t is the solvent transit time measured by an Ostwald capillary viscometer, and ρ is the solvent specific gravity. The superscript “w” denotes the absence of viscosogen.

\[ dATP/dt = V \cdot ATP^{32P}/ATP^{32P} + K_{mATP} \cdot (1 + ATP/K_{mATP}) \]

and

\[ dATP/dt = V \cdot ATP^{32P}/ATP^{32P} + K_{mATP} \cdot (1 + ATP/K_{mATP}) \]

where k = V(1 + ATP/Km) and therefore:

\[ \text{ATPase} = k = (dATP/dt) \cdot ATP \]

Without viscosogen.
Mechanism of CDK2/Cyclin A Activation

RESULTS

Purification and Steady-state Kinetic Analysis—[p-Thr<sup>160</sup>]CDK2/cyclin A was produced by overexpression in either E. coli or Sf9 insect cells. Steady-state kinetic parameters for the phosphorylation of a peptide substrate displaying the canonical recognition motif XT/SPXKR (Peptide 1; PKTPKKAKKL) were determined by Michaelis-Menten analysis using a two-substrate model for sequential binding (see "Materials and Methods"). The data are shown in Fig. 1 in double reciprocal form for display. The concentration of enzyme was 2 nM. ATP concentrations were fixed at 20, 50, 100, 200, and 500 μM (from top to bottom). Kinetic parameters are reported in Table I.

![Fig. 1. Steady-state kinetic analysis of phosphorylation by [p-Thr<sup>160</sup>]CDK2/cyclin A. Initial velocities for the phosphorylation of Peptide 1 were determined as described under "Materials and Methods." A model describing two-substrate sequential binding was fit to the untransformed data using global nonlinear regression analysis. The experimental data and the fit were then transformed to the double reciprocal form for display. The concentration of enzyme was 2 nM. ATP concentrations were fixed at 20, 50, 100, 200, and 500 μM (from top to bottom). Kinetic parameters are reported in Table I.](image)

![Fig. 2. Anion exchange chromatography of (non-p)CDK2/cyclin A. (non-p)CDK2/cyclin A was produced by overexpression in E. coli and purified as described under "Materials and Methods." A, A<sub>ave</sub> of eluted protein and buffer conductivity from Uno Q anion exchange chromatography are shown. B, SDS-PAGE analysis of protein-containing fractions. (non-p)CDK2 and cyclin A migrate with the same relative mobility. Molecular mass markers are 97, 66, 55, 42/40, 31, 21.5, and 14.4 kDa from top to bottom. C, peptide kinase activity. Five μl of each fraction was assayed in the presence of 100 μM peptide and 1 μM [γ-<sup>32</sup>P]ATP (~300 dpm/pmol) for 10 min at 23 °C, as described under "Materials and Methods." Although two peaks of CDK2/cyclin A are observed in panel A, the steady-state kinetic parameters measured for both fraction 31 and the pool of fractions 31–34 were identical (not shown). The pool was thus used for all studies.](image)

(p-np)CDK2/cyclin A was expressed and purified from E. coli. SDS-PAGE analysis of fractions corresponding to the major protein peak eluting from the final chromatographic step is shown in Fig. 2. Each fraction was assayed for ATPase activity, which was found to correspond exactly to the profile of CDK2/cyclin A protein. However, analysis of the same fractions for peptide kinase activity revealed that, while the early fractions of the protein peak displayed low activity, the later fractions exhibited substantially higher kinase activity (Fig. 2, B and C). The basis for the high kinase activity in these later fractions is not known. For all studies on [un-P]CDK2/cyclin A, fractions free of the contaminating high kinase activity species were used (fractions 31–34, Fig. 2). In these fractions, peptide kinase activity corresponded exactly to the levels of CDK2/cyclin A protein and steady-state kinetic parameters, measured for both fraction 31 and fractions 31–34 pooled, were identical (not shown).

(p-np)CDK2/cyclin A in these fractions displayed low but measurable kinase activity. The kinase activity of (non-p)CDK2/cyclin A was linear for up to 2 h, demonstrating that autoactivation does not occur within this time frame. To test the possibility that the low level of activity may be attributable to partial denaturation, (non-p)CDK2/cyclin A was tested for activation by phosphorylation (see “Materials and Methods”). Incubation of (non-p)CDK2/cyclin A with MgATP and CAK resulted in a turnover rate (~9 s<sup>-1</sup>) corresponding to that of (p-Thr<sup>160</sup>)CDK2/cyclin A obtained after extensive purification (data not shown). These data indicate that all of the (non-p)CDK2/cyclin A was active and was therefore native. In addition, the maximal ATPase rates of (non-p)CDK2/cyclin A produced in E. coli (10 min<sup>-1</sup>) compared with insect cells (~14 min<sup>-1</sup>) were similar, as were their peptide kinase activities.

It was found that the maximal ATPase rate (10 min<sup>-1</sup>) was ~20-fold higher than the maximal rate of the peptide kinase reaction (0.5 min<sup>-1</sup>). This finding presented two problems for kinetic analysis. First, (non-p)CDK2/cyclin A binds ADP with a K<sub>d</sub> value of ~1 μM (14). Thus, under initial velocity conditions with respect to the kinase reaction, significant product inhibition would be expected to occur. Second, the fraction of ATP depleted at low initial ATP concentrations would be large even at small fractions of peptide phosphorylation, precluding kinetic measurement under initial velocity conditions. To circumvent these difficulties, an ATP-regenerating system was em-
ployed. In this system, ADP is efficiently removed from the reaction mix and the concentration of ATP remains constant. However, under these conditions, the \([\gamma^{32P}]ATP\)-specific radioactivity decays over the reaction time course as ATP is regenerated. This decay was accounted for in all analyses of peptide phosphorylation (see “Materials and Methods”). The corrected initial velocity data for the phosphorylation of Peptide 1 by (non-p)CDK2/cyclin A are shown in Fig. 3, and the optimized steady-state kinetic parameter values are reported in Table I. The turnover rate for (non-P)CDK2/cyclin A is 843-fold lower than that of \([p\text{-Thr}^{160}]\text{CDK2/cyclin A}\), whereas the \(K_m\) (peptide) is 137-fold higher. Thus, phosphorylation of CDK2/cyclin A results in an \(-100,000\)-fold increase in catalytic efficiency.

**Solvent Viscosity Studies**—The Michaelis-Menten parameters described above are composed of microscopic rate constants combined in a manner dependent upon the order of substrate addition. The steady-state data for both \([p\text{-Thr}^{160}]\text{CDK2/cyclin A}\) and (non-p)CDK2/cyclin A are consistent with both random and compulsorily ordered mechanisms.

\[
E^{ATP} + \text{Pept} \xrightleftharpoons[k_2]{k_1} E^{ATP}\text{Pept} \xrightleftharpoons[k_4]{k_3} E^{ADP}\text{Pept} \xrightleftharpoons[k_6]{k_5} E + \text{P-Pept} + \text{ADP}
\]

**Scheme 1.**

For both enzymes, however, if the kinetic mechanism is ordered, it is necessarily ordered with ATP binding first; this is true because the crystal structures of both \([p\text{-Thr}^{160}]\text{CDK2/cyclin A}\) and (non-p)CDK2/cyclin A were obtained with bound ATP alone. Furthermore, both enzymes display substantial ATPase activity in the absence of peptide or protein substrate.

Under saturating conditions of ATP, the catalytic mechanism of CDK2/cyclin A can therefore be described by Scheme 1. In this model, the catalytic efficiency is given by \(k_{cat}/K_m\) (peptide) = \(k_2 \times k_3/(k_{-2} + k_3)\), while the turnover rate is given by \(k_{cat} = k_2 \times k_3/(k_3 + k_4)\). To solve for the microscopic constants, \(k_2, k_{-2}, k_3, \) and \(k_4\), we employed steady-state solvent viscometric techniques, which allow separation of the diffusive \((k_2, k_{-2}, k_4)\) from non-diffusive steps \((k_3)\) (15–17). Initial velocity data for peptide phosphorylation by \([p\text{-Thr}^{160}]\text{CDK2/cyclin A}\) were obtained as a function of peptide substrate concentration at several concentrations of sucrose (Fig. 4A). At these sucrose concentrations, the maximum rate\(^3\) of ATP hydrolysis (12 min\(^{-1}\)) in the absence of peptide was constant, indicating that sucrose does not perturb the structure of the active site. Equation 2,

\[
v = E' \cdot (S/K_d' \cdot k_3 \cdot k_d/\eta) / (k_d/\eta + k_3 \cdot k_d/k_{-2} + S/K_d' \cdot (k_3 + k_d/\eta)) \quad \text{(Eq. 2)}
\]

which describes the effect of relative solvent viscosity on initial rate, was fit to the data, where \(E\) is the concentration of enzyme, \(S\) is the concentration of Peptide 1, \(\eta\) is the relative solvent viscosity, and \(K_d\) is the equilibrium dissociation constant for Peptide 1 binding to the E-ATP complex \((k_{-2} \cdot k_d)\). All other constants are defined in Scheme 1. The derived values for the kinetic constants are reported in Table I. Solvent viscosity studies performed on \([p\text{-Thr}^{160}]\text{CDK2/cyclin A}\) produced in insect cells revealed similar kinetic parameters \((K_d/\text{peptide}) = 20 \mu M, k_3 = 8 \text{ s}^{-1}, k_4 = 11 \text{ s}^{-1}\).

A similar solvent viscometric analysis was conducted on (non-p)CDK2/cyclin A. Although saturation with Peptide 1 could not be achieved because of the high \(K_m\) value for this substrate, subsaturating substrate concentrations equal to

\[
\begin{array}{c|c|c|c}
\text{Parameter} & \text{(non-p)CDK2/cyclin A} & \text{[p-Thr}\text{\textsuperscript{160}]\text{CDK2/cyclin A}} & \text{Fold change upon phosphorylation} \\
\hline
k_{cat} & 0.0083 \pm 0.0008 \text{ s}^{-1} & 7 \pm 0.7 \text{ s}^{-1} & \uparrow 843 \\
K_m(\text{peptide}) & 1100 \pm 400 \mu M & 8 \pm 0.7 \mu M & \downarrow 137 \\
K_m(\text{ATP}) & 55 \pm 5 \mu M & 6 \pm 6 \mu M & \\
K_m(\text{ATP})/K_m(\text{peptide}) & (7.5 \pm 3) \times 10^{-6} \mu M \text{ s}^{-1} & 0.9 \pm 0.12 \mu M \text{ s}^{-1} & \uparrow \sim 100,000 \\
k_{cat}/K_m(\text{peptide}) & \geq 0.083 \text{ s}^{-1} & 0.12 \pm 0.02 \mu M \text{ s}^{-1} & \geq 220 \text{ s}^{-1} \\
k_{-2}(\text{peptide}) & \geq 0.083 \text{ s}^{-1} & 22 \pm 4 \text{ s}^{-1} & \uparrow 2650 \\
k_3 & \geq 0.083 \text{ s}^{-1} & 11 \pm 1 \text{ s}^{-1} & \\
K_d(\text{peptide}) & 1100 \pm 400 \mu M & 25 \pm 3 \mu M & \downarrow 44 \\
\hline
\end{array}
\]

\(^3\) No change in rate was observed at 1 versus 2 mM ATP.
were obtained at 2 mM ATP. The concentration of enzyme was 1 nM.

The viscosity effects on catalytic efficiency nor turnover rate, respectively, was sensitive to solvent viscosity. The release rates for both substrate and product were obtained at 2 mM ATP. The concentration of enzyme was 2 nM. Identical results were obtained at 2 mM ATP. The concentration of enzyme was 1 mM. Identical results were obtained at 2 mM ATP. The concentration of enzyme was 1 μM. The "viscosity effect" on the reaction rate (kcat/Km) and turnover rate (kcat) are obtained by extrapolation to zero and infinite substrate concentration, respectively. kcat/Km and kcat relate to the individual rate constants in Scheme 1 as follows: kcat/Km = kcat/k - 1; kcat = kcat + kcat(25). A value for kcat/Km and kcat equal to zero implies that k - 1 >> kcat, and kcat << k, respectively.

0.36, 1.8, and 3.6 times the Km(peptide) revealed no viscosity effect on initial rates (Fig. 4B). Extrapolation to zero and infinite peptide substrate concentrations showed that neither the catalytic efficiency nor turnover rate, respectively, was sensitive to solvent viscosity. The release rates for both substrate peptide (k - 1) and product (kcat) can therefore be assumed to exceed the rate of chemistry (k - 1) by at least 10-fold (Table I).

Thus, phosphoryl group transfer in (non-p)CDK2/cyclin A is rate determining. A viscosometric analysis was also carried out on the ATPase reaction. No viscosity effect on the turnover rate was observed, suggesting that phosphoryl group transfer in the ATPase reaction is also rate determining (Table I). The lack of a viscosity effect on the steady-state kinetic parameters for both the kinase and ATPase reactions indicates that sucrose perturbs only the rates of diffusion.

The majority of protein kinases require phosphorylation at a conserved site within their activation loops for full catalytic activation (3). This site in CDK2/cyclin A is Thr160. Its phosphorylation by Cdk5 is of interest for at least two reasons: 1) it has been established that phosphorylation at this site is a critical regulatory mechanism for control of CDK2/cyclin A activity and progression through the cell cycle (5); 2) the structural basis for activation of CDK2 by both cyclin binding and phosphorylation is known (6–8), and the structure of [p-Thr160]CDK2/cyclin A bound to a synthetic peptide substrate has been solved (18). Given the abundant structural data, the consequences of phosphorylation at Thr160 on the specific steps for substrate binding and catalytic turnover have not been investigated. Thus, a correlation between atomic structure and the mechanism of regulation is not known. In this study, we have characterized the kinetic reaction pathway for the phosphorylation of a model peptide substrate by unphosphorylated CDK2/cyclin A as well as CDK2/cyclin A phosphorylated at Thr160 by Cdk5. The information reveals the kinetic basis for activation by phosphorylation in terms of the individual reaction steps.

We have employed a synthetic peptide (Peptide 1) as a model substrate in which the primary structure (PKTPKAKKAL) is patterned after a region of similar sequence found in histone H1 protein (19). The catalytic efficiency of Peptide 1 for [p-Thr160]CDK2/cyclin A is one of the highest reported for protein kinases (kcat/Km = 1 μM⁻¹ s⁻¹) and is similar to that of histone H1 protein (not shown). The lack of a viscosity effect on kcat/Km(peptide) for both [un-P]CDK2/cyclin A and [p-Thr160]CDK2/cyclin A demonstrates that during steady-state turnover Peptide 1 equilibrates rapidly with the active site of both enzymes. Thus, catalytic efficiency for the kinase activity in both cases is a function of only the affinity of peptide binding and the rate of phosphoryl group transfer (kcat/Km ≈ kcat/Km). Phosphorylation at Thr160 increases catalytic efficiency by ~100,000-fold, while the turnover rate is enhanced nearly 1000-fold.

The dramatic increase in catalytic efficiency is in part attributable to a moderate but significant increase in peptide binding affinity. The crystal structure of [p-Thr160]CDK2/cyclin A bound to AMPPNP and a peptide substrate (HHASPRK) (18) reveals the structural basis for interaction with peptide and protein substrates. Phosphorylation of CDK2/cyclin A at Thr160 results in displacement of the activation loop by 5.3–7.1 Å and rotation of the carbonyl oxygen of Val163 out of the so-called “P₁ binding pocket” (7, 8). This movement is necessary to accommodate binding of the essential proline residue found in the P₁ position (P₀ is the phosphorylation site) in all CDK2/cyclin substrates (18). In addition, the substrate lysine residue at position P₁ forms a hydrogen bond with the phosphate group in [p-Thr160]CDK2/cyclin A (18). Overall, phosphorylation of CDK2/cyclin A at Thr160 increases the binding affinity of Peptide 1 by ~10-fold.

Nonetheless, the large increase in both catalytic efficiency and turnover rate with respect to the kinase pathway is attributable mostly to an approximate 3000-fold increase in the rate of the phosphoryl group transfer step. In contrast, phosphorylation has no effect on the rate of ATP hydrolysis. Solvent viscosometric studies on the ATPase reaction of both [p-Thr160]CDK2/cyclin A and (non-p)CDK2/cyclin A show that phosphorylation affects neither the overall turnover rate nor the rate of the phosphoryl group transfer step (Table I).

The large rate enhancement of chemical reactions afforded by enzymes is classically accounted for by specific stabilization of the transition state with respect to the ground state Michael-
Phosphoryl group transfer may be separately controlled by cyclin binding at Thr160, respectively. Val163, which is relieved by phosphorylation at Thr 160. Each reaction of the steric interference from the carbonyl oxygen of orientation of the peptide substrate hydroxyl group as a consequence of the sequence transition-state structure for phosphotransfer in the kinase reaction. The dramatically slower rate of phosphotransfer to ADP that is slow. In addition, we cannot assess the effect that phosphorylation at Thr 160 has on the product dissociation rates, as the lack of a viscosity effect on $k_{cat}$ limited by product release ($k_4 = 11$ s$^{-1}$). However, our studies do not discern whether the slow step in this event is the release of ADP or phosphopeptide. If the dissociation rate of the phosphopeptide product is similar to, or greater than, that of the peptide substrate ($k_{-2} \geq 220$ s$^{-1}$), then it is the release of ADP that is slow. In addition, we cannot assess the effect that phosphorylation at Thr160 has on the product dissociation rates, as the lack of a viscosity effect on $k_{cat}$ in (non-p)CDK2/cyclin A permits only a lower limit value on the net rate of product release to be estimated ($k_4 \geq 0.083$ s$^{-1}$).

Studies addressing the role of autophosphorylation in the activation of cAMP-dependent protein kinase (PKA) have been carried out employing a nonphosphorylatable mutant as a model of the unphosphorylated enzyme (21). Although the site of phosphorylation in PKA (Thr197) is homologous to residue Thr160 in CDK2/cyclin A, clear differences exist between these enzymes in their kinetic mechanisms of activation. In particular, phosphorylated wild-type PKA, in comparison with PKA(T197A), displays no change in substrate binding affinity, a small increase in the overall rate of substrate turnover ($<10$-fold), and a moderate increase in the rate of the phosphoryl group transfer step ($<200$-fold) (21) (cf. Table I). The differences in activation of PKA versus CDK2/cyclin A may relate to their different physiological mechanisms of regulation. For example, reversible phosphorylation of the CDKs by CAK is critical to their function in cell cycle control (5), whereas the role of autophosphorylation in the regulation of PKA remains unclear (22, 23). Instead, the regulation of PKA is achieved mainly by association with either the RI or RII regulatory subunits. Comparison of CDK2/cyclin A with PKA (21) and the v-fps tyrosine kinase (24) does, however, reveal an apparent common theme, that phosphorylation of the activation loop in all cases serves mainly to enhance the rate of phosphotransfer with less influence on the affinity of substrate binding.

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