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Kinetic Mechanistic Studies of Cdk5/p25-Catalyzed H1P Phosphorylation: Metal Effect and Solvent Kinetic Isotope Effect

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Abstract

Cdk5/p25 is a member of the cyclin-dependent, Ser/Thr kinase family and has been identified as one of the principle Alzheimer's disease-associated kinases which promote the formation of hyperphosphorylated tau, the major component of neurofibrillary tangles. We and others have been developing inhibitors of cdk5/p25 as possible therapeutic agents for Alzheimer's disease (AD). In support of these efforts, we examine the metal effect and solvent kinetic isotope effect on cdk5/p25-catalyzed H1P (a histone H-1-derived peptide) phosphorylation. Here, we report that a second Mg^{2+} in addition to the one forming the ATP-Mg complex is required to bind to cdk5/p25 for its catalytic activity. It activates cdk5/p25 by demonstrating an increase in k_{cat} and induces a conformational change that favors ATP binding but has no effect on the binding affinity for the H1P peptide substrate. The binding of the second Mg^{2+} does not change the binding order of substrates. The reaction follows the same rapid equilibrium random mechanism in the presence or absence of the second Mg^{2+} as evidenced by initial velocity analysis and substrate analogue and product inhibition studies. A linear proton inventory with a normal SKIE of 2.0 ± 0.1 in the presence of the second Mg^{2+} was revealed and suggested a single proton transfer in the rate-limiting phosphoryl transfer step. pH-profile revealed a residue with a pK_a of 6.5 that is most likely the general acid-base catalyst facilitating the proton transfer.

One of the pathological hallmarks of Alzheimer's disease (1,2) is neurofibrillary tangles (NFT). The major component of NFTs is hyperphosphorylated tau, the microtubule-associated protein (3). Cdk5/p25 has been identified as one of the principle AD-associated kinases due to the increased activity of cdk5/p25 in the AD brain and its ability to phosphorylate tau at multiple sites (4–6). Cdk5/p25 is a proline-directed Ser/Thr kinase and a member of the cyclin-dependent kinase family involved in cell cycle regulation. Unlike other members of the family that require association with a cyclin for activity, cdk5 associates with the activator protein p35. The resultant complex cdk5/p25 of the neuronal-specific processing of p35 to p25 by calpain likely plays a role in the pathogenesis of AD (5–7).

With our understanding of the role of cdk5/p25 in the AD brain, discovery and design of inhibitors of this enzyme have become a goal in the search for drugs to treat AD. Understanding the mechanism of cdk5/p25 is critical for the design of inhibitors. We previously reported a random kinetic mechanism for cdk5/p25 catalysis (8). In this article,

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we reveal additional features of cdk5/p25 catalysis discovered by probing the metal, pH, and solvent isotope effects.

Many kinases have been reported to bind to a second metal ion for catalytic activity in addition to the one required to form the nucleotide-metal complex. Mg^{2+} is considered the physiological metal of kinases due to its high concentration in the cell compared with other divalent metal ions, although catalytic activity is detected also with Mn^{2+} , Co^{2+} , and, Cd^{2+} (9–10). Herein we report that (I) cdk5/p25-catalyzed H1P phosphorylation requires the binding of a second Mg^{2+} for its catalytic activity; (II) the binding of the second Mg^{2+} induces a conformational change that does not limit the access of either substrate, although it favors ATP binding; and (III) in the presence of the second Mg^{2+} the phosphor transfer step is the rate-limiting step and associated with a single proton transfer, which is facilitated by a general acid-base catalyst.

MATERIALS AND METHODS

Materials

Adenosine 5'-triphosphate (ATP), phosphoenolpyruvate, magnesium chloride, NADH, 2-(N-morpholino)propanesulfonic acid (MOPS), trichloroacetic acid (TCA), bovine serum albumin, pyruvate kinase type II from rabbit muscle, and lactate dehydrogenase type II from bovine heart were purchased from Sigma (St. Louis, MO). Dithiothreitol (DTT) was from Fluka (St. Louis, MO). Histone H1-derived peptide PKTPKKAKKL (H1P) was purchased from Anaspec (San Jose, CA). H1P analogue H1P^{Ala} (PKAPKKAKKL) was purchased from American peptides (Sunnyvale, CA). $[\gamma\text{-}^{33}\text{P}]\text{-ATP}$ was from PerkinElmer (Boston, MA). Cdk5/p25 was generously provided by Dr. Ken Kosik of the University of California, Santa Barbara.

Kinetic Analysis of H1P Phosphorylation by Cdk5/p25

The kinase assay was conducted in buffer containing 20 mM MOPS (pH 7.5), 1 mM DTT, BSA 0.5 mg/ml, $MgCl_2$, H1P (PKTPKKAKKL), ATP, and $[\gamma\text{-}^{33}\text{P}]\text{-ATP}$. The reactions were conducted in triplicate, initiated by the addition of 7.8 nM cdk5/p25, and incubated at RT for 30 min. The reaction was stopped by the addition of 10 mM EDTA and the mixture was transferred to a multiscreen PH filtration plate (Millipore, Billerica, MA) and washed six times with 75 mM H_3PO_4 . The filters were removed and the samples were counted with a scintillation counter. Background reaction was conducted in the absence of H1P. In all cases, reaction progress curves for production of phospho-protein were linear over at least 45 minutes, and allowed calculation of initial velocities.

Buffer Salts for pH Dependencies

In all of these kinetic studies, reaction solutions contained 20 mM buffer salt, 50 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT, 0.5 mg/ml BSA. The following buffer salts were used: MES at pH 5.5–6.5, PIPES at pH 6.5–7.0, HEPES at pH 7.0–8.0, HEPBS at pH 8.5–9.0, and CHES at pH 9.5–10.0.

Data Analysis

Data were analyzed by nonlinear least-squares, using either Sigma-Plot or Grafit software packages. Standard kinetic mechanisms for two-substrate reactions and their rate equations are shown below: Ping-Pong:

$$v = \frac{k_{\text{cat}} [E][A][B]}{K_A [B] + K_B [A] + [A][B]} \quad (1)$$

K_A and K_B are Michaelis constants. Rapid Equilibrium Ordered:

$$v = \frac{k_{\text{cat}} [E][A][B]}{K_A K_B + K_B [A] + [A][B]} \quad (2)$$

K_A and K_B are substrate dissociation constants from EA and EB, respectively. Rapid Equilibrium Random/Steady-State Ordered:

$$v = \frac{k_{\text{cat}} [E][A][B]}{\alpha K_A K_B + \alpha K_A [B] + \alpha K_B [A] + [A][B]} \quad (3)$$

Replot equations for random mechanism:

$$(k_{\text{cat}})_A = k_{\text{cat}} \left(\frac{[B]}{\alpha K_B + [B]} \right) \quad (4)$$

$$(k_{\text{cat}}/K_m)_A = \frac{k_{\text{cat}}}{\alpha K_A} \left(\frac{[B]}{K_B + [B]} \right) \quad (5)$$

For rapid equilibrium systems, K_A , K_B , αK_A , and αK_B are substrate dissociation constants from EA, EB, and EAB. For steady-state systems, K_A is the substrate dissociation constant from EA and αK_A and αK_B are Michaelis constants. See Segel for definitions of mechanisms, substrate dissociation constants, and α (11).

RESULTS

Initial Velocity Studies

The effect of free Mg^{2+} on cdk5/p25-catalyzed H1P phosphorylation was studied by the use of initial velocity studies as discussed by Cook (12–13). Experiments were performed in which the initial velocities were measured as a function of H1P concentration, at several fixed concentrations of MgATP (331, 166, 83, 42, 21 μM) and at a single fixed concentration of free Mg^{2+} at 0.2 mM (Figure 1A). This experiment was repeated at four concentrations of free Mg^{2+} , 0.6, 1.3, 2.5, and 5 mM. Magnesium ions in excess of that needed to bind ATP were assumed to be free Mg^{2+} and the dissociation constant of MgATP (14.3 μM) (14) was used to calculate the concentrations of MgATP and free Mg^{2+} . Extra care and calculation were taken in order to keep the concentration of free Mg^{2+} as a constant for the entire range of MgATP concentration. Each data set was subjected to global analysis by nonlinear least-squares fits to the equations that reflect ping-pong, rapid equilibrium ordered, and random/steady-state ordered mechanisms (eq 1–eq 3). Statistically the data fit the equation reflecting the rapid equilibrium random/steady-state ordered mechanism the best and the parameters analyzed for 0.2 mM and 2.5 mM Mg^{2+} are summarized in Table 1. To examine the data carefully and judge among the mechanisms, we also used the method of replots as described previously (8). The starting point in this data analysis was to calculate

apparent values of $(k_{\text{cat}})_X$ and $(k_{\text{cat}}/K_m)_X$ ($X = \text{H1P}$ or ATP) by nonlinear least-squares fits of each individual plot of v_0 vs $[X]$ in the primary data sets to the simple Michaelis-Menten equation. The next step in this analysis was to construct the replots of the dependencies of apparent values of $(k_{\text{cat}})_A$ and $(k_{\text{cat}}/K_m)_A$ on $[B]$ and the dependencies of apparent values of $(k_{\text{cat}})_B$ and $(k_{\text{cat}}/K_m)_B$ on $[A]$ and examine their shape. Replots will show a specific, mechanism-based pattern. For example, for the random mechanism of eq 3, apparent values of $(k_{\text{cat}})_A$ and $(k_{\text{cat}}/K_m)_A$ will be hyperbolically dependent on $[B]$ according to the equations given in eq 4 and eq 5. Likewise, apparent values of $(k_{\text{cat}})_B$ and $(k_{\text{cat}}/K_m)_B$ will be hyperbolically dependent on $[A]$.

Replots of apparent values of $(k_{\text{cat}})_{\text{H1P}}$ and $(k_{\text{cat}}/K_m)_{\text{H1P}}$ vs $[\text{MgATP}]$ and apparent values of $(k_{\text{cat}})_{\text{MgATP}}$ and $(k_{\text{cat}}/K_m)_{\text{MgATP}}$ vs $[\text{H1P}]$ through the whole range of free $[\text{Mg}^{2+}]$ all revealed hyperbolic dependencies on substrate concentration, suggesting that the reaction follows either a random or a steady-state ordered mechanism. An example of these replots at 0.2 mM Mg^{2+} was shown in Figure 1B and 1C. A sigmoid kinetic behavior was observed in Figure 1B and data was fitted to an equation reflecting sigmoidicity: $v = v_c/(1 + (K_S/[S])^n)$, yielding a hill coefficient of 1.8 ± 0.1 . To distinguish between the two mechanisms, we conducted substrate analogue and product inhibition studies (see below). Next we examined the effects of Mg^{2+} on the steady-state kinetic parameters and the results are shown in Figure 2. The zero free Mg^{2+} cannot be achieved in the presence of MgATP , therefore, the effect of zero free Mg^{2+} can only be extrapolated from higher Mg^{2+} concentrations. Increasing free Mg^{2+} concentration resulted in an increase in k_{cat} with the maximal k_{cat} being achieved at 5 mM Mg^{2+} (Figure 2A). Increasing free Mg^{2+} concentration also resulted in an increase in $k_{\text{cat}}/K_{\text{H1P}}$ (Figure 2B), which is mainly due to the increase in k_{cat} at high Mg^{2+} concentration. The values of K_{H1P} remain almost the same through the entire Mg^{2+} concentration range, $12 \pm 0.7 \mu\text{M}$ at 0.2 mM free Mg^{2+} , $11 \pm 0.8 \mu\text{M}$ at 1.3 mM free Mg^{2+} , and $7 \pm 0.5 \mu\text{M}$ at 2.5 mM free Mg^{2+} . However, the values of K_{MgATP} decrease with increasing free Mg^{2+} concentration, $60 \pm 20 \mu\text{M}$ at 0.2 mM free Mg^{2+} and $6.1 \pm 1.5 \mu\text{M}$ at 2.5 mM free Mg^{2+} . The much lower sensitivity at low Mg^{2+} concentration contributed to the large error for K_{MgATP} . The increases in both k_{cat} and MgATP binding affinity lead to an increase in $k_{\text{cat}}/K_{\text{MgATP}}$ (Figure 2C). The data in Figures 2A and 2B were fitted to an expression of enzyme activation that requires binding of one activator to achieve full activity: $v = v_c/(1 + (K_A/[A])^n)$, yielding dissociation constants for Mg^{2+} of 0.6 ± 0.07 and 0.6 ± 0.2 mM under the conditions of k_{cat} , $k_{\text{cat}}/K_{\text{H1P}}$. A sigmoid kinetic behavior was observed under the condition of $k_{\text{cat}}/K_{\text{MgATP}}$ (Figure 2C) and data was fitted to an equation reflecting sigmoidicity: $v = v_c/(1 + (K_A/[A])^n)$, yielding dissociation constant for Mg^{2+} of 1.7 ± 0.1 mM and a hill coefficient of 2.1 ± 0.3 .

To ensure that the additional magnesium requirement is not a result of a nonspecific ionic effect, we measured cdk5/p25 activity at different NaCl concentrations. The activity of cdk5 remained the same at NaCl concentrations less than 80 mM, but decreased at high concentrations of NaCl (data not shown).

Inhibition Studies with Substrate Analogues and Product ADP

To determine the kinetic mechanism of cdk5/p25-catalyzed H1P phosphorylation at low Mg^{2+} , inhibition studies were conducted with nucleotide analogue AMP, product ADP, and peptide substrate analogue H1P^{Ala} (PKAPKKAKKL) at 0.2 mM MgCl_2 . We first determined at several concentrations of inhibitor, (i) the dependence of v_0 on $[\text{H1P}]$ at a single $[\text{ATP}]$; (ii) the dependence of v_0 on $[\text{ATP}]$ at a single $[\text{H1P}]$. Examples of $[\text{ATP}]$ -dependence of initial velocity for AMP and H1P^{Ala} inhibition are shown in Figures 3A and 3D. Next we analyzed for (i) the dependence of v_0 on $[\text{H1P}]$ at each $[\text{I}]$ and for (ii) the dependence of v_0 on $[\text{ATP}]$ at each $[\text{I}]$, by nonlinear least-squares fit to the simple Michaelis-Menton equation to calculate apparent values of $(k_{\text{cat}})_X$ and $(k_{\text{cat}}/K_m)_X$ ($X = \text{ATP}$

or H1P). Finally in this analysis we constructed the replots of apparent values of $(k_{\text{cat}})_X$ vs $[I]$ and apparent values of $(k_{\text{cat}}/K_m)_X$ vs $[I]$. Examples of these replots for AMP as an inhibitor are shown in Figures 3B and 3C and examples for H1P^{Ala} are shown in Figures 3E and 3F. We found that when ATP was the variable substrate: (i) for AMP or ADP inhibition, $(k_{\text{cat}})_{\text{ATP}}$ is independent of AMP or ADP and $(k_{\text{cat}}/K_m)_{\text{ATP}}$ titrates with AMP or ADP according to the simple inhibition expression of general form: $v_{\text{inhib}} = v_{\text{control}}/(1 + [I]/K_{i,\text{app}})$; (ii) for H1P^A inhibition, $(k_{\text{cat}})_{\text{ATP}}$ is independent of H1P^{Ala} and $(k_{\text{cat}}/K_m)_{\text{ATP}}$ titrates with H1P^{Ala}. When H1P was the variable substrate: (i) for AMP or ADP inhibition, both $(k_{\text{cat}})_{\text{H1P}}$ and $(k_{\text{cat}}/K_m)_{\text{H1P}}$ titrate with AMP or ADP; (ii) for H1P^{Ala} inhibition, $(k_{\text{cat}})_{\text{H1P}}$ is independent of H1P^{Ala} and $(k_{\text{cat}}/K_m)_{\text{H1P}}$ titrates with H1P^{Ala}. This suggests that H1P^{Ala} is competitive with both ATP and H1P; AMP or ADP is competitive with ATP and noncompetitive with H1P.

These patterns rule out a steady-state ordered mechanism, but are different from the ones well known for a rapid equilibrium random mechanism in which it has been assumed that the competitive inhibitors act as dead-end inhibitors and bind to the same enzyme form as the substrates (15). The patterns seen in this study clearly suggest that the inhibitors interact with the enzyme in a different mode. To examine these results and estimate the values of dissociation constants, we used the method previously described in detail (8), where the general mechanism of Scheme I, in which inhibitor can bind to all four forms of enzyme, was used to derive the rate equation (eq 6) under rapid equilibrium condition.

$$v = \frac{\frac{k_{\text{cat}}[E][A]}{1 + \frac{\alpha K_B}{[B]} + \frac{\alpha K_B}{[B]} \frac{[I]}{K_{i,ea}} + \frac{[I]}{K_{i,eab}}}}{K_A \left\{ \frac{\alpha + \frac{\alpha K_B}{[B]} + \frac{\alpha K_B}{[B]} \frac{[I]}{K_{i,e}} + \frac{[I]}{K_{i,eab}}}{1 + \frac{\alpha K_B}{[B]} + \frac{\alpha K_B}{[B]} \frac{[I]}{K_{i,ea}} + \frac{[I]}{K_{i,eab}}} \right\} + [A]} \quad (6)$$

By inspecting eq 7–eq 10 which describe the dependencies of apparent values $(k_{\text{cat}})_X$ and $(k_{\text{cat}}/K_m)_X$ ($X = \text{ATP}$, or PLK-peptide) on inhibitor, we can immediately see how inhibitors interact with the enzyme.

$$(k_{\text{cat}})_{\text{ATP}} = \frac{k_{\text{cat}}[E]}{1 + \frac{\alpha K_B}{[B]} + \frac{\alpha K_B}{[B]} \frac{[I]}{K_{i,ea}} + \frac{[I]}{K_{i,eab}}} \quad (7)$$

$$(k_{\text{cat}}/K_m)_{\text{ATP}} = \frac{k_{\text{cat}}[E]/\alpha K_A}{1 + \frac{K_B}{[B]} + \frac{K_B}{[B]} \frac{[I]}{K_{i,e}} + \frac{[I]}{\alpha K_{i,eb}}} \quad (8)$$

In the case of H1P^{Ala} inhibition where ATP is the variable substrate, $(k_{\text{cat}})_{\text{ATP}}$ is independent of H1P^{Ala}, which suggests that both $K_{i,ea}$ and $K_{i,eab}$ are very large and H1P^{Ala} cannot bind to either E:A or E:A:B complex. On the other hand, $(k_{\text{cat}}/K_m)_{\text{ATP}}$ titrates with H1P^{Ala} suggesting that H1P^{Ala} can bind to E, E:B or both. It is clear that at this point, we cannot calculate independent estimates of $K_{i,e}$ and $K_{i,eb}$.

We can however, obtain this information from inspecting eq 9 and eq 10. In the case where PLK-peptide is the variable substrate, $(k_{\text{cat}})_{\text{PLK}}$ is independent of H1P^{Ala} which suggests that both $K_{i,eb}$ and $K_{i,eab}$ are very large and H1P^{Ala} cannot bind to either E:B or E:A:B complex; $(k_{\text{cat}}/K_m)_{\text{PLK}}$ titrates with H1P^{Ala}, suggesting that H1P^{Ala} can bind to E or E:A.

$$(k_{\text{cat}})_{\text{PLK}} = \frac{k_{\text{cat}}[E]}{1 + \frac{\alpha K_A}{[A]} + \frac{\alpha K_A}{[A]} \frac{[I]}{K_{i,\text{eb}}} + \frac{[I]}{K_{i,\text{eab}}}} \quad (9)$$

$$(k_{\text{cat}}/K_m)_{\text{PLK}} = \frac{k_{\text{cat}}[E]/\alpha K_B}{1 + \frac{K_A}{[A]} + \frac{K_A}{[A]} \frac{[I]}{K_{i,\text{e}}} + \frac{[I]}{\alpha K_{i,\text{ea}}}} \quad (10)$$

Using this method, we can identify the enzyme forms that inhibitors bind and estimate the dissociation constants as follows: (i) H1P^{Ala} binds only to free enzyme and $K_{i,\text{e}}$ was estimated directly from eq 8 or eq 10 with our previous knowledge of k_{cat} , K_A , K_B , and α ; (ii) AMP behaves as a dead-end inhibitor and binds to both E and E:B complex. $K_{i,\text{eb}}$ was estimated directly from eq 9, $K_{i,\text{e}}$ directly from eq 10, and $K_{i,\text{e}}$ also from use of $K_{i,\text{eb}}$ and eq 8. The mechanism of inhibition and dissociation constants for H1P^{Ala}, AMP, and ADP at low Mg^{2+} concentration are summarized in Table 2.

pH-Dependence of Steady-State Kinetic Parameters of H1P Phosphorylation

The pH stability of cdk5/p25 was determined by incubating enzyme at the desired pH and assaying aliquots at pH 7. Enzyme is stable from pH 6 to 9. Small activity loss occurs at pH 5.5 and 10.0. The phosphorylation of H1P was monitored in a pH range of 5.5–10.0 under conditions of variable H1P (0–480 μM) and fixed ATP (800 μM) at high Mg^{2+} concentration (5 mM). Plots of v_0 versus H1P concentration were used to determine k_{cat} and $k_{\text{cat}}/K_{\text{H1P}}$. The pH profile of k_{cat} showed a bell-shaped curve (Figure 4) and was fitted to equation 11 yielding two apparent pK_a 's of 6.1 and 10.0, respectively. The involvement of an alkaline residue in the catalysis is questionable because of activity loss at higher pH due to protein denaturation. Also, an alkaline group with pK_a of 8.3 was revealed in the ATPase reaction (see below). It is possible that the activity loss at higher pH could simply be due to protein denaturation rather than the titration of an alkaline residue. The $k_{\text{cat}}/K_{\text{H1P}}$ exhibited a similar pH dependence as k_{cat} owing to the pH insensitivity of K_{H1P} (data not shown).

$$y = \frac{C}{1 + 10^{-\text{pH}}/10^{-\text{pKa1}} + 10^{-\text{pKa2}}/10^{-\text{pH}}} \quad (11)$$

As mentioned in the previous study, cdk5/p25 possesses an ATPase activity with a k_{cat} 15-fold less than that for the kinase activity. Since transfer to water is slow, the phosphoryl transfer rate is almost certain to be rate-determining. A pH study was carried out for the cdk5/p25-catalyzed ATP hydrolysis. The K_m for ATP was found to be 20, 11, 8, and 10 μM at pH values of 6, 7, 8, and 9, respectively. The pH profile of k_{cat} for ATP hydrolysis showed a bell-shaped curve and was fit to equation 11 yielding two apparent pK_a 's of 6.3 and 8.3 as shown in Figure 5A. The bell-shaped curve was also found in the pH profile of $k_{\text{cat}}/K_{\text{ATP}}$ without perturbation of two pK_a 's by fitting the data to equation 11 as shown in 5B. Buffer crossover experiments revealed no dependence on the buffer salt. Comparison of the pH profiles of k_{cat} of the kinase and ATPase reactions revealed similar pK_a 's on the acid side, while the alkaline pK_a 's are quite different. It is possible that the group giving rise to the alkaline pK_a in the ATPase reaction is no longer involved in catalysis.

Proton Inventory Study

The proton inventory study was carried out for k_{cat} (initial velocity was measured at saturating concentrations of both ATP and H1P) at low (0.2 mM) and high (5 mM) Mg^{2+} concentrations in the mixture of H_2O and D_2O at pH 7.4 and pD equivalent. The dependence of the ratio of k_{cat} (k_{n}/k_0) in the presence and absence of varying atom fractions of D_2O (n) on n at high and low Mg^{2+} concentration is presented in Figure 6. At high Mg^{2+} concentration, k_{n}/k_0 decreases linearly with n . The solvent deuterium isotope effect on k_{cat} can be expressed by the linear form of the Gross-Butler equation (16) with only one exchangeable hydrogen site:

$$k_{\text{n}}/k_0 = (1 - n + n\phi^{\text{T}}) / (1 - n + n\phi^{\text{R}}) \quad (12)$$

where ϕ^{R} and ϕ^{T} are fractionation factors for the exchangeable hydrogen in the reactant and transition state, respectively. The fitting of data to the equation gives the following estimates: $\phi^{\text{R}} = 1$ and $\phi^{\text{T}} = 0.47$. Given this, we can assign the estimate to SKIE for k_{cat} , $^{\text{D}}k_{\text{cat}} = \phi^{\text{R}} / \phi^{\text{T}} = 2.0 \pm 0.1$ determined from three independent experiments. To make certain that changes in side chain pKa's in light and heavy water are not causing a dramatic difference to SKIE, the proton inventory study for k_{cat} was repeated at low (0.2 mM) and high (5 mM) Mg^{2+} concentrations in the mixture of H_2O and D_2O at pH 8.0 and pD equivalent. No dramatic differences on SKIE were observed and SKIE of 1.8 ± 0.1 and 1.0 ± 0.1 were determined from three independent experiments at high and low Mg^{2+} . At low Mg^{2+} concentration, a linear proton inventory and SKIE value of 1.1 ± 0.1 were observed for k_{cat} (Figure 6). The proton inventory study was also carried out at high and low Mg^{2+} concentrations on $k_{\text{cat}}/K_{\text{H1P}}$ (initial velocity was measured at a saturating concentration of ATP and a concentration of H1P 10-fold less than K_{H1P}) and $k_{\text{cat}}/K_{\text{ATP}}$ (initial velocity was measured at a saturating concentration of H1P and a concentration of ATP 10-fold less than K_{ATP}) in three independent experiments. At both high and low Mg^{2+} concentrations, linear proton inventory and SKIE values close to 1 were found for these parameters and summarized in Table 3.

Influence of D_2O on Inhibitor Binding

The dissociation constant for the ATP competitive inhibitor AMP was measured in pure H_2O and 85% D_2O under conditions of 10 μM ATP and saturating H1P concentration. The dependence of v_0 on $[\text{I}]$ was analyzed by nonlinear least-squares fit to the expression $v_{\text{inhi}} = v_{\text{control}} / [1 + ([\text{I}]/K_{\text{i,app}})]$ to determine $K_{\text{i,app}}$.

DISCUSSION

Many kinases have been reported to bind to a second metal ion in addition to the one required to form the nucleotide-metal complex, though the role of the second metal varies in each case. For example, a second metal ion activates Csk and Src by increasing the k_{cat} without affecting the K_{ATP} (16). However, it activates IRK and v-Fps by decreasing the K_{ATP} without affecting the k_{cat} (17–18). More interestingly, the second Mg^{2+} binding in the active site of cAMP-dependent protein kinase (PKA) inhibits the kinase activity by decreasing its k_{cat} (19). In this study we report the effect of the second Mg^{2+} on the cdk5/p25-catalyzed H1P phosphorylation.

Kinetic Mechanism

The initial velocity study of the cdk5/p25-catalyzed H1P phosphorylation at different concentrations of Mg^{2+} revealed that a second Mg^{2+} is required to bind to cdk5/p25 for its catalytic activity. It activates cdk5/p25 by demonstrating an increase in k_{cat} . The binding of

the second Mg^{2+} facilitates ATP binding by demonstrating a dramatic decrease in K_{ATP} . Likewise, the binding of ATP also favors the binding of the second Mg^{2+} ; the saturation of ATP in the active site decreases the dissociation constant of the second Mg^{2+} by 3-fold. More surprisingly, sigmoid kinetic behavior was observed on the binding of ATP at low concentration of Mg^{2+} and on the binding of Mg^{2+} at low concentration of ATP as demonstrated by a hill coefficient of about 2 in each case. At either low Mg^{2+} or low ATP concentration, the two subunits of the cdk5/p25 dimer interact cooperatively and permit much more sensitive response to the ligand concentration (either ATP or Mg^{2+}). On the other hand, the presence of the second Mg^{2+} makes this type of cooperative interactions unnecessary by inducing a conformational change which favors ATP binding without limiting the access of either substrate. A rapid equilibrium random mechanism in the absence of the second Mg^{2+} was revealed by substrate analogue and product inhibition studies, which is consistent with a random mechanism in the presence of the second Mg^{2+} as reported in a previous study (8).

The discrete mechanism of the second Mg^{2+} coordinated in its interaction with cdk5/p25 is kinetically complex. It might bind to a site other than the active site of the enzyme and induce a conformational change. This hypothesis is supported by the crystal structure of cdk2, which is a member of the cyclin-dependent kinase family and shares 60% sequence identity with cdk5. In the co-crystal structure of cdk2-cyclinA3 with both a peptide substrate and an ATP analogue, there is only one bound Mg^{2+} chelating the α - and γ -phosphates in the active site (20). It is also possible that the second metal was not observed in the crystalline state due to the experimental conditions (the crystal was grown in the presence of 5 mM Mg^{2+}), and actually it is coordinated within the active site of the enzyme and participates in catalysis directly. This hypothesis is supported by the mutual effects of free Mg^{2+} and ATP observed on the binding of the other: the K_{ATP} was lowered by 10-fold at saturating Mg^{2+} , and likewise the K_{d} of free Mg^{2+} was decreased significantly at saturating ATP. Many kinases require a second metal ion for the catalytic activity, such as phosphoenolpyruvate carboxykinase (PEPCK), protein tyrosine kinase, and LRRK2 (21–23). Evidence from these kinases also supports the second hypothesis. For example, crystallographic structure of PEPCK revealed the coexistence of Mg^{2+} and Mn^{2+} in the active site at two distinct locations (22). The two metal ions make contacts with ATP and different amino acid residues, with Mg^{2+} chelating both the β - and γ -phosphate of ATP. We propose that the second Mg^{2+} directly binds to the active site of cdk5/p25 and contacts with β - or γ -phosphate of ATP through either direct or water-mediated interactions.

The fact that Mg^{2+} modulates the activity of cdk5/p25 may have physiological consequences worth noting. The activity of cdk5/p25 could be modulated *in vivo* by changes in free cytoplasmic Mg^{2+} concentration.

Proton Transfer

Based on the chemical principle of phosphoryl transfer, the hydroxyl proton must be removed to form products. Therefore, the study of phosphoryl transfer catalyzed by kinases is inevitably linked to the study of its proton transfer mechanism. In order to further our understanding of the role of the second Mg^{2+} in the H1P phosphorylation, a proton inventory study was conducted at both high and low Mg^{2+} concentrations. The use of the shape of the proton inventory and the size of the solvent isotope effect to determine the number of hydrogenic sites and diagnose the mechanism have been well discussed by Schowen and Venkatasubban (25–26).

A. Proton Transfer and Rate-Determining Step at High Mg^{2+} Concentration—A normal SKIE (2.0) was observed on k_{cat} at high Mg^{2+} concentration. In order to interpret the

isotope effect on cdk5/p25 catalyzed H1P phosphorylation, it is important to know whether the larger intrinsic viscosity of D₂O or an equilibrium isotope effect on a catalytic residue or on buffer pK_a provided the sources for the observed SKIE. First, the observed SKIE is much larger than the predicted influence of D₂O due to viscosity. Since D₂O is 20% more viscous than H₂O, the effects of increased viscosity would account for only 5% rather than the observed 50% change in turnover rate. Second, the lack of SKIE on kinetic parameters at low Mg²⁺ (see below) ruled out the possibility that the observed SKIE is due to an equilibrium isotope effect. With these as foundations, proton inventory can be interpreted. The linear proton inventory with normal SKIE (2.0) observed with k_{cat} at high Mg²⁺ concentration suggests that (i) a single protonic interaction in the catalytic transition state is responsible for the solvent isotope effect—that is, one-proton catalysis, and binding at this protonic site is looser in the transition state than in the reactant state, and (ii) phosphoryl transfer is the rate-limiting step of the process governed by k_{cat} . In an independent study to measure the pre-steady-state kinetics using a rapid quench flow technique, the phosphorylation of H1P exhibited no burst kinetics for k_{cat} which is consistent with a slow phosphoryl transfer and a rapid product release mechanism (data not shown).

Since the enzyme follows a rapid equilibrium random mechanism at high Mg²⁺ concentration, it is reasonable to assume that the substrate is in rapid exchange with the enzyme. As a result, SKIE is anticipated in the parameter $k_{\text{cat}}/K_{\text{H1P}}$ or $k_{\text{cat}}/K_{\text{ATP}}$. Surprisingly, no SKIE was observed in either. The lack of SKIE on $k_{\text{cat}}/K_{\text{H1P}}$ or $k_{\text{cat}}/K_{\text{ATP}}$ may suggest that during the process governed by $k_{\text{cat}}/K_{\text{m}}$, the enzyme-substrate complex E:A or E:S undergoes a slow deuterium-insensitive step preceding the phosphoryl transfer step, but the inhibition data suggest another solution. The dissociation constant of AMP from E:H1P:AMP was found to be lower in 85% D₂O compared with water by approximately 1.5-fold (data not shown). The solvent isotope effect on the dissociation constant originates from solvent reorganization that accompanies substrate or inhibitor binding and generally ranges from 1.2 to 1.8 (27). Based on a predicted SKIE of 1.6 for k_{cat} at 85% D₂O, the equilibrium isotope effect on substrate binding offsets SKIE so that no SKIE is observed for $k_{\text{cat}}/K_{\text{ATP}}$ or $k_{\text{cat}}/K_{\text{H1P}}$. Scheme II depicts the random mechanism for cdk5/p25-catalyzed H1P phosphorylation at high Mg²⁺ concentration, where the binary substrate complex E:S or EA binds to the second substrate to form the active central complex E:ATP:S controlled by the association rate constant k_2 and dissociation rate constant k_{-2} . The catalytic step, k_3 , describes the unimolecular rate constant for the chemical transfer as well as any conformational change associated with this step. Finally, the release of product and the conformational change associated with it is combined in k_4 . Rate equations for k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ of Scheme II were derived by using Cleland's methods of net rate constants (28):

$$k_{\text{cat}} = k_3 k_4 / (k_3 + k_4) \quad (13)$$

$$k_{\text{cat}} / K_{\text{m}} = k_2 k_3 / (k_{-2} + k_3) \quad (14)$$

Due to the relatively rapid process of product release ($k_4 > k_3$), k_{cat} becomes approximately equal to k_3 . Based on the assumption that substrate is in rapid exchange with the enzyme (i.e. $k_{-2} > k_3$), $k_{\text{cat}}/K_{\text{m}}$ could be simplified into the expression as in equation 15.

$$k_{\text{cat}} / K_{\text{m}} = k_2 k_3 / k_{-2} \quad (15)$$

We commonly refer to the K_m as an apparent affinity since it does not provide a direct measure of the real affinity of the substrate and the kinase (K_d). Adams and colleagues reported that the K_m values obtained in the steady-state kinetic analyses may contain terms related to steps occurring in the central ternary complex. They further explained how fast and favorable phosphoryl transfer can overcome weak interactions between the enzyme and substrate and further increase apparent affinity in the thermodynamically coupled systems (24). Considering this, we examined K_m for cdk5/p25-catalyzed H1P phosphorylation at high Mg^{2+} . For substrate recognition, K_m includes all the rate constants in the reaction scheme II as shown below:

$$K_m = (k_{-2} + k_3)k_4 / k_2(k_3 + k_4) \quad (16)$$

Since $k_{-2} > k_3 < k_4$, the complex expression of K_m can be simplified into the approximation given by equation 17:

$$K_m = k_{-2} / k_2 \quad (17)$$

That is exactly the same expression of the real substrate affinity K_d . Like the case observed by Adams, we found that the slow phosphoryl transfer of H1P phosphorylation at high Mg^{2+} concentration ($k_{-2} > k_3 < k_4$) allows a direct measurement of K_d from K_m .

B. Proton Transfer and Rate-limiting Step at Low Mg^{2+} Concentration—No SKIE was detected on k_{cat} , k_{cat}/K_{ATP} , or k_{cat}/K_{H1P} at low Mg^{2+} concentration. The lack of SKIE on k_{cat} should be interpreted cautiously and three mechanisms are possible. First, if the phosphoryl transfer step in the transition state is preceded or followed by a slow conformational change, small or no SKIE would be detected for k_{cat} . Second, if the product release step represented as k_4 in Scheme II is relatively slow ($k_3 > k_4$), then k_{cat} is approximately equal to k_4 and the observed SKIE would be greatly diminished. Third, if the hydroxyl proton was abstracted after the transfer of the γ -phosphate, little or no SKIE would be observed. No special driving force is required for this proton transfer since the pK_a 's of the O-phosphono-threonine is very low (29). This mechanism is not likely, though, due to the SKIE detected on k_{cat} at high Mg^{2+} concentration (see the discussion below). The lack of SKIE on k_{cat}/K_{ATP} or k_{cat}/K_{H1P} could be interpreted as a slow conformational change associated with the chemical transfer step.

Is there a general base in the catalysis?

The observation of large kinetic SKIE on cdk5/p25-catalyzed H1P phosphorylation suggests that the hydroxyl proton was abstracted by a general base in the active site. Indeed, there is structural evidence from the X-ray analysis of members of the cdk family that a conserved aspartate (Asp-127 in cdk2, Asp-126 in cdk5) is within hydrogen-bonding distance of the hydroxyl and, therefore, may serve as a general-base catalyst. The dependence of maximum rate on pH provides additional evidence for a general-base catalyst. The pH-profile of k_{cat} suggests that a group with a pK_a of 6.1 must be unprotonated for activity. The peptide has no pK values in this pH range, and the pK_a obtained for protonation of the γ -phosphate of ATP is 4.6 (30) so that the decrease in rate with changes in pH must reflect the titration of enzyme residues. The group with a pK_a of 6.1 is most likely the general base.

An ATPase reaction that is intrinsic to the protein kinase provides an opportunity to estimate pK_a 's from different enzyme forms. The pH-profiles of k_{cat} and k_{cat}/K_{ATP} of ATPase are bell-shaped (Figure 4). The pK_a of the acidic limb is identical with that obtained from the kinase reaction within experimental error. In addition, since the pK_a of the catalytic base is

essentially the same when determined from the ATPase reaction under the condition representing free enzyme (6.5) and from the kinase reaction under the condition representing E:ATP:H1P (6.1), the presence of H1P and ATP in the active site does not affect the pK_a of the base.

The potential role of Asp-126 as a general-base raises an interesting question regarding the role of the conserved aspartate in protein kinases. On the basis of available crystal structures, it is very likely that an aspartate residue within the hydrogen-bonding distance to the hydroxyl of the substrate is conserved in all protein kinases. This aspartate is clearly important for efficient catalysis, since replacement of it by alanine in PKA and PhK decreased k_{cat} by 2–3 orders of magnitude. The role of this conserved aspartate has drawn considerable attention. Although a general-acid-base role has been given special consideration in the literature (29,31–33), many studies do not provide convincing evidence for a general-base catalyst. Instead, they support a positioning function of the conserved aspartate by attaining appropriate attack geometry between the hydroxyl group and γ phosphate of ATP (29). Alternatively, the aspartate could facilitate dissociation by repelling the phosphoproduct (34). Whether this carboxyl group serves the same function in other protein kinases is still unclear and further research is needed.

ABBREVIATIONS

AD	Alzheimer's disease
Cdk5/p25	cyclin-dependent kinase5
H1P	histone H1-derived peptide PKTPKKAKKL
H1P ^{Ala}	PKAPKKAKKL
H1P ^{DaP}	PKDapPKKAKKL

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Figure 1.

Steady-state kinetic experiment for cdk5/p25-catalyzed H1P phosphorylation at free Mg^{2+} of 0.2 mM. Panel A: Dependence of initial velocity on H1P concentration at MgATP concentration of 331 ($\bullet$), 166 ($\circ$), 83 ($\blacktriangledown$), 41.5 (∇), and 21 ($\blacksquare$) μM . Each data point is the average of duplicate measurements. The data were globally fit to the equation reflecting rapid equilibrium random/steady-state mechanism. Panels B and C: MgATP concentration dependencies of $(k_{\text{cat}})_{\text{H1P}}$ and $(k_{\text{cat}}/K_{\text{m}})_{\text{H1P}}$. Apparent values $(k_{\text{cat}})_{\text{H1P}}$ and $(k_{\text{cat}}/K_{\text{m}})_{\text{H1P}}$ were calculated by fitting the individual plot of v_0 vs [H1P] of panel A to the simple Michaelis-Menten equation.

Figure 2. Free Mg^{2+} effect on steady-state parameters. (A) Dependences of k_{cat} (B) k_{cat}/K_{H1P} and (C) k_{cat}/K_{ATP} on concentrations of free Mg^{2+} .

Figure 3.

Inhibition of the cdk5/p25-catalyzed H1P phosphorylation by substrate analogues H1P^{Ala} and AMP at 0.2 mM Mg²⁺ concentration. Panels A and B: Inhibition by H1P^{Ala}, (A) the dependence of $K_{i,\text{app}}$ on H1P, (B) the dependence of $K_{i,\text{app}}$ on ATP. Panels C and D: Inhibition by AMP, (C) the dependence of $K_{i,\text{app}}$ on ATP, (B) the dependence of $K_{i,\text{app}}$ on H1P.

Figure 4. pH dependence of k_{cat} for the cdk5/p25-catalyzed H1P phosphorylation. The experiments were conducted under conditions of variable H1P (0–480 μ M) and fixed ATP (800 μ M) at 5 mM Mg^{2+} . The data was fit to equation 11.

Figure 5. pH profiles of the ATPase reaction of cdk5/p25. (A) pH-dependence of k_{cat} and (B) pH-dependence of k_{cat}/K_{ATP} . The experiments were conducted under conditions of variable ATP at 5 mM Mg²⁺. The data was fit to equation 11.

Figure 6.

Proton inventory of k_{cat} for the cdk5/p25-catalyzed H1P phosphorylation at 5 mM Mg^{2+} (○) and 0.2 mM Mg^{2+} (●). k_{cat}^n / k_{cat}^0 is the ratio of k_{cat} in the presence and absence of varying atom fractions of deuterium (n). Initial velocities were determined at saturating concentrations of both ATP and H1P.

Scheme I.

Rapid equilibrium random mechanism of cdk5/p25-catalyzed phosphorylation. A represents ATP, and B represents H1P.

Scheme II.

Table 1

Initial Velocity Analysis for Cdk5/p25-Catalyzed H1P Phosphorylation at Low and High Free Mg^{2+} Concentrations^a

	0.2 mM Mg^{2+}	2.5 mM Mg^{2+}
k_{cat} , s^{-1}	0.77 ± 0.04	3.6 ± 0.2
K_{H1P} , μM	12 ± 0.7	7 ± 0.5
K_{ATP} , μM	60 ± 20	6.1 ± 1.5
α	16 ± 2	12 ± 1.7

^aThe parameter estimates were calculated by globally fitting the data into the equation reflecting rapid equilibrium random/steady-state order mechanism. Each parameter estimate is the average of two independent experiments. The error limit is the deviation from the mean.

Table 2

Inhibition of Cdk5/p25 by Substrate Analogues at 0.2 mM Mg^{2+}

inhibitor	substrate		mechanism	inhibn const (mM)	
	fixed	variable		$K_{i,e}$	$K_{i,es}$
AMP	ATP	HIP	NC	2.7 ± 0.3	1.4 ± 0.5
	HIP	ATP	C	2.5 ± 0.7	
ADP	ATP	HIP	NC	0.6 ± 0.1	0.3 ± 0.1
	HIP	ATP	C	0.3 ± 0.1	
HIP ^{Δla}	ATP	HIP	C	0.2 ± 0.1	
	HIP	ATP	C	0.6 ± 0.3	

Table 3

SKIE Parameters for the Cdk5/p25-Catalyzed H1P Phosphorylation at Low and High Free Mg^{2+} Concentrations

	0.2 mM Mg^{2+}	2.5 mM Mg^{2+}
$^D(k_{\text{cat}})$	1.1 ± 0.1	2.0 ± 0.1
$^D(k_{\text{cat}}/K_A)$	1.0 ± 0.1	1.0 ± 0.1
$^D(k_{\text{cat}}/K_B)$	1.0 ± 0.1	1.0 ± 0.1

^aThe dependence of the ratio of each kinetic parameter on varying atom fractions of D_2O (n) was fit to the Gross-Bulter equation $k_n/k_0 = (1 - n + n\phi^T)/(1 - n + n\phi^R)$. SKIE was estimated as the ratio of ϕ^R/ϕ^T . Each parameter estimate is the average of three independent experiments. The error limit is the deviation from the mean.