Kinetic Studies of Cdk5/p25 Kinase: Phosphorylation of Tau and Complex Inhibition by Two Prototype Inhibitors

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ABSTRACT: Cdk5/p25 is a member of the family of cyclin-dependent, Ser/Thr kinases and is thought to play a causal role in Alzheimer's disease (AD) due to its ability to phosphorylate the protein tau, and thus promote the latter's aggregation into intraneuronal tangles. Given this, we and others are seeking inhibitors of cdk5/p25 as possible disease-modifying therapeutics for AD. In this paper, we first report the kinetic mechanism for the cdk5/p25-catalyzed phosphorylation of tau and histone H-1-derived peptide (H1P). These studies served as a necessary kinetic backdrop for investigations of the mechanism of inhibition by prototype inhibitors N4-(6-aminopyrimidin-4-yl)-sulfanilamide (APS) and 1-(5-cyclobutyl-thiazol-2-yl)-3-isoquinolin-5-yl-urea (CTIU). We found that the cdk5/p25-catalyzed phosphorylation of tau follows a rapid equilibrium, random kinetic mechanism, as evidenced by initial velocity analysis indicating sequential addition of tau and ATP, and studies of the mechanism of inhibition by substrate analogue AMP, product ADP, and analogues of peptide substrate H1P. Identical mechanistic conclusions were drawn when H1P was the phosphoryl acceptor. Subsequent studies of inhibition by APS and CTIU revealed that both compounds can bind to all four steady-state forms of the enzyme, to form the complexes E:I, E:I:tau, E:I:ATP, and E:I:tau:ATP. These results contrast with reported claims that APS and CTIU are competitive inhibitors of the binding of ATP.

Neurofibrillary tangles are one of the pathological hallmarks of Alzheimer's disease (AD[1]) (*1, 2*). A major component of NFTs is hyperphosphorylated forms of tau (*3*), a protein whose normal physiological role is thought to be involved in the structural integrity of microtubles. While a number of kinases are able to phosphorylate tau, cdk5/p25 has been implicated as one of the principle AD-associated kinases (*1, 4*).

Cdk5/p25 is a proline-directed Ser/Thr kinase and a member of the broad family of cyclin-dependent kinases that are involved in cell cycle regulation. Unlike other family members that require association with a cyclin for activity, cdk5 associates with the activator protein p35 (*5–7*). Neuronal-specific processing of p35 to p25 by calpain deregulates cdk5 and allows the resultant cdk5/p25 complex to hyperphosphorylate tau (*6–10*). In the AD-brain, the enzyme phosphorylates tau residues S203, T205, T212, T217, S235, S396 and S404 (*1, 2, 5, 11*).

Given the likely role of cdk5/p25 in the pathogenesis of AD, identifying inhibitors of this enzyme has become a priority in the search for drugs to treat AD. Although a detailed understanding of the mechanism of cdk5/p25 is critical for the design of inhibitors, such information is quite limited. While a report exists describing the kinetic mechanism of cdk5/p25 with a peptidic substrate (*12*), no information exists addressing mechanistic issues related to protein substrates. To date, the action of the enzyme toward its physiological protein substrate tau remains unexplored. As part of a program to find inhibitors of cdk5/p25, we are studying the mechanism of the cdk5/p25-catalyzed phosphorylation of tau.

In this paper, we report studies aimed at elucidating the kinetic mechanism of cdk5/p25. While our main focus is understanding phosphorylation of tau, we also investigated the kinetics of phosphorylation of a peptide substrate H1P. We found that the enzyme follows a rapid equilibrium, random mechanism in which either ATP or the phosphoryl acceptor can bind first to the enzyme, and that binding of the first substrate decreases the binding affinity of the second substrate.

We also investigated the mechanism of inhibition of two prototype literature compounds, APS (*12*) and CTIU (*13*). We found that both compounds can bind to all four forms of the enzyme that are present in the steady-state, to form the complexes E:I, E:I:tau, E:I:ATP, and E:I:tau:ATP. These results contrast with reported claims that APS and CTIU are competitive inhibitors of the binding of ATP (*12, 13*).

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[1] Abbreviations: AD, Alzheimer's disease; NFTs, neurofibrillary tangles; cdk5, cyclin-dependent kinase5; H1P, histone H1-derived peptide PKTPKKAKKL; H1P^{Val}, PKVPKKAKKL; H1P^{Ala}, PKAPKKAKKL; H1P^{Glu}, PKEPKKAKKL; H1P^{Dap}, PKDapPKKAKKL; H1P^{PO4}, PKT(PO$_4$)PKKAKKL; APS, N4-(6-aminopyrimidin-4-yl)-sulfanilamide (Pharmacia's PNU 112455A); CTIU, 1-(5-cyclobutyl-thiazol-2-yl)-3-isoquinolin-5-yl-urea.

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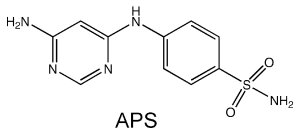
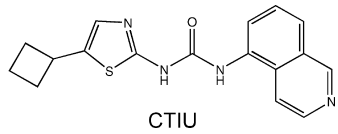




MATERIALS AND METHODS

Materials. ATP, ADP, AMP, phosphoenolpyruvate, magnesium chloride, NADH, 2-(*N*-morpholino)propanesulfonic acid (MOPS), trichloroacetic acid (TCA), bovine serum albumin, pyruvate kinase (type II, rabbit muscle), and lactate dehydrogenase (type II, 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). Peptides $H1P^{Val}$, $H1P^{Ala}$, $H1P^{Glu}$, and phosphopeptide $H1P^{PO4}$ were from the University of California (Los Angeles, CA), and $H1P^{Dap}$ was purchased from New England Peptide (Gardner, MA). [γ-^{33}P]-ATP was from Perkin-Elmer (Boston, MA). APS was purchased from Aldrich and CTIU was synthesized according a literature method (*13*); the proton NMR of the synthesized material is consistent with its structure. Cdk5/p25 was generously provided by Dr. Andrea Musacchio of the European Institute of Oncology in Milan, Italy.

Expression and Purification of Human Tau. Tau encoded by the longest human tau cDNA used in this study was purified from extracts of *Escherichia coli* as described (*14, 15*). The cell pellets were collected and resuspended in suspension buffer (containing 20 mM MOPS, 1 mM EGTA, 2 mM Mg^{2+}, 1 mM PMSF, and 2.5 μg/mL DNase, pH 6.2) after induction of protein expression with isopropyl β-D-thiogalactopyranoside. The cells were lysed with a Microfluidizer (Technika, Chester, CT). The cell lysate was boiled for 10 min in a water bath, and the protein aggregates were removed by centrifugation. The supernatant was loaded onto a MonoS HiTrap column (Amersham Biosciences, Piscataway, NJ) pre-equilibrated with buffer containing 20 mM MOPS, 160 mM NaCl, 1 mM EGTA, and 2 mM Mg^{2+}, pH 6.2. The proteins were eluted with elution buffer having the following composition: 20 mM MOPS, 400 mM NaCl, 1 mM EGTA, and 2 mM Mg^{2+}, pH 6.2. The tau protein was collected and the concentration was measured by a BCA protein assay (Pierce, Rockford, IL), with bovine serum albumin (BSA) as a standard.

Kinetic Analysis of Cdk5/p25-Catalyzed Tau Phosphorylation. The kinase assay for phosphorylation of tau was conducted in buffer containing 20 mM MOPS (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, BSA 0.5 mg/mL, 1 mM EGTA, tau, ATP, and [γ-^{33}P]-ATP. Tau and ATP were used at various concentrations as indicated in the Results section, and the ratio of ATP to [γ-^{33}P]-ATP was kept constant at all ATP concentrations (200 μM ATP/2.5 μCi [γ-^{33}P]-ATP). The reactions were conducted in duplicate, initiated by the addition of 7.8 nM cdk5/p25, and incubated at 30 °C for 45 min. The reaction was stopped by the addition of an equal volume of 2.4 M trichloroacetic acid (TCA), and the mixture was transferred to a multiscreen HV filtration plate (Millipore, Billerica, MA) and washed six times with 1.2 M TCA. The plate was dried and punched, and the samples were counted with a scintillation counter. Background reaction was conducted in the absence of tau. In all cases, reaction progress curves for production of phospho-protein were linear over at last 45 min, and allowed calculation of initial velocities.

Kinetic Analysis of H1P Phosphorylation by Cdk5/p25. ADP production that results from the phosphorylation of H1P by ATP was measured spectrophotometrically using a coupled enzyme assay. This assay uses pyruvate kinase and lactate dehydrogenase to couple the production of ADP with the oxidation of NADH (*16*). The kinase assay was conducted in buffer containing 20 mM MOPS (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 2 mM PEP, 0.2 mM NADH, BSA 0.5 mg/mL, pyruvate kinase 5 IU/mL, lactate dehydrogenase 5 IU/mL, ATP, and H1P. The reactions were conducted in duplicate and initiated by the addition of 6.4 nM cdk5/p25. Reaction progress was monitored by the decrease in absorbance at 340 nm with a microplate spectrophotometer, which allows multiple reactions to be followed simultaneously. Background reaction in the absence of H1P was performed.

ATP Hydrolysis Assay. ATP hydrolysis by cdk5/p25 was measured with the phosphate assay kit according to the manufacturer's instruction (Molecular Probes).

Data Analysis: Basic Equations. 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:

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

Rapid equilibrium ordered:

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

Rapid equilibrium random/steady-state ordered:

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

See Segel for definitions of mechanisms, substrate dissociation constants, and α (*17*).

Data Analysis: Method of Replots. Throughout this paper when referring to replots, we will use the following system of nomenclature: For substrates A and B, $(k_{cat})_A$ and $(k_{cat}/K_m)_A$ refer to values of k_{cat} and k_{cat}/K_m that are determined from the dependence of initial velocity on [A] at one of several fixed concentrations of B; and $(k_{cat})_B$ and $(k_{cat}/K_m)_B$ refer to values of k_{cat} and k_{cat}/K_m that are determined from the dependence of initial velocity on [B] at one of several fixed concentrations of A.

Replots of the dependence of $(k_{cat})_A$ on [B] and the dependence of $(k_{cat}/K_m)_A$ on [B] will exhibit a specific, mechanism-based pattern. For example, for the rapid equilibrium random mechanism of eq 3, the dependence of $(k_{cat})_A$ on [B] and the dependence of $(k_{cat}/K_m)_A$ on [B] are given in eqs 4 and 5, respectively:

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

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

Thus, a nonlinear least-squares of the dependence of $(k_{cat})_A$ on [B] according to eq 4 will provide estimates of k_{cat} and

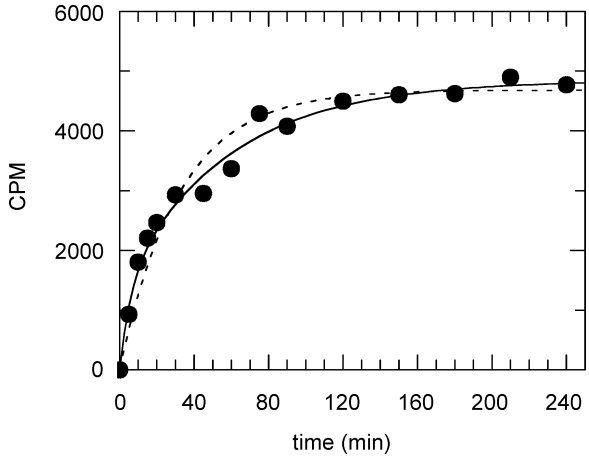


FIGURE 1: Progress curve for cdk5/p25-catalyzed phosphorylation of tau. The time course of reaction of 0.1 μM tau with 200 μM ATP, 2.5 μC [γ-^{33}P]-ATP and 0.06 μM cdk5/p25 was followed with the filter assay for the quantification of phosphorylated tau. The dashed and solid lines correspond to fits of the data to first-order and biphasic rate expressions (see text for details).

αK_B, while nonlinear least-squares of the dependence of $(k_{cat}/K_m)_A$ on [B] according to eq 5 will provide estimates of k_{cat}/K_m and K_B. Similar analyses are also done for the dependence of $(k_{cat})_B$ on [A] and the dependence of $(k_{cat}/K_m)_B$ on [A].

Molecular Modeling of ATP and APS bound to Cdk2. The structures shown in Figure 5 were obtained by loading in and superposing pairs of protein structures (downloaded from the Protein Databank) in the Swiss-Pub Viewer. After loading in the structures, coloring "by layer", and removing the side chains from the selected molecules, all non-α-carbons were removed from the structures in order to create the simplest graphical representation of the molecules. To improve the appearance of the molecules, the display options "OpenGL Rendering" and "Render in Solid 3D" were selected. With the molecules in their backbone representations, an amino acid alignment between the strands was achieved using an iterative fit method which makes incremental improvements in the fit until the rms deviation between corresponding α-carbons is minimized.

RESULTS

Cdk5/p25-Catalyzed Phosphorylation of Tau: Full Reaction Progress Curve. Figure 1 contains a full reaction progress curve for the incorporation of radiolabeled phosphate into tau. This experiment was done with [ATP] = 200 μM $\gg K_{ATP}$ and [tau] = 0.1 μM $\ll \alpha K_{tau}$ (see below for estimates of K_{ATP} and αK_{tau}). Thus, under these conditions, the reactant state is the binary complex E:ATP and the calculated observed rate constant is related to $(k_c/\alpha K_{tau})$[E].

Regardless of the mechanism which best describes this data (see below), the total change in CPM corresponds to a total phosphate incorporation of about 0.6 μM. Since the tau concentration was 0.1 μM, this level of phosphate incorporation suggests that there are six phosphorylation sites on each molecule of tau.

When the data of Figure 1 are fit to a first-order rate law, $y = y_{final}[1 - \exp(-k_{obs}t)]$, the following parameters are calculated: $k_{obs} = (3.1 \pm 0.3) \times 10^{-2}$ min^{-1} and $y_{final} = (4.7 \pm 0.1) \times 10^3$ CPM ($\chi^2 = 17 \times 10^5$ CPM^2). However, the theoretical line drawn using these parameters (dashed line of Figure 1) does not describe the data well.

The systematic departure of data points from this line suggests that the data may best be described by a biphasic or some other high-order rate law. In fact, the data of Figure 1 can be described well (see solid line of Figure 1) by the expression of eq 6 for a biphasic reaction.

$$y = y_1[1 - \exp(-k_{obs,1}t)] + y_2[1 - \exp(-k_{obs,2}t)] \quad (6)$$

Best fit values are $k_{obs,1} = (17 \pm 6) \times 10^{-2}$ min^{-1}, $y_1 = (1.3 \pm 0.5) \times 10^3$ CPM, $k_{obs,2} = (1.8 \pm 0.4) \times 10^{-2}$ min^{-1}, $y_2 = (3.5 \pm 0.5) \times 10^3$ CPM ($\chi^2 = 5.2 \times 10^5$ CPM^2). To compare the two fits of the data, an F-test was conducted with the outcome that there is a 0.001 probability that a simple, first-order rate law describes the data as well as does the expression of eq 6. Equation 6 describes a mechanism in which product is generated through an intermediate that forms and then decomposes through irreversible reaction steps.

Cdk5/p25-Catalyzed Phosphorylation of Tau: Initial Velocity Studies. To determine the kinetic mechanism for the cdk5/p25-catalyzed phosphorylation of tau, initial velocities were measured as a function of tau concentration, at several fixed concentrations of ATP (Figure 2A). The complete data set was subjected to global analysis by nonlinear least-squares fits to the three standard mechanisms, using eqs 1–3. χ^2 values for ordered, ping-ping, and random mechanisms are 54×10^7, 6.6×10^7, and 4.9×10^7, respectively. While statistically the random mechanism fits the data the best (especially when judged by an F-test probability-value of 0.014, random relative to ping-pong, compared to a probability-value of 10^{-12}, random relative to ordered), examination of the theoretical lines through the data reveal no significant differences between random and ping-pong mechanisms (see Figure 2A). This is of course a general problem with global fits of data sets; i.e., it is often difficult to visually judge goodness of fit, especially at low concentrations of substrate. To examine the data more carefully and judge among mechanisms, we employed the method of replots. This is similar to the method used by Gao and Harris in a recent paper to successfully elucidate the kinetic mechanism PDK1 kinase (*18*).

For a ping-pong mechanism, $(k_{cat})_A$ will be hyperbolically dependent on [B] and $(k_{cat}/K_m)_A$ will be independent of [B]; likewise, $(k_{cat})_B$ will be hyperbolically dependent on [A] and $(k_{cat}/K_m)_B$ will be independent of [A]. In contrast, for a rapid equilibrium ordered mechanism, $(k_{cat})_A$ will be hyperbolically dependent on [B] and $(k_{cat}/K_m)_A$ will be linearly dependent on [B]; $(k_{cat})_B$ will be independent of [A] and $(k_{cat}/K_m)_B$ will be hyperbolically dependent on [A]. Finally, for a rapid equilibrium random mechanism $(k_{cat})_X$ and $(k_{cat}/K_m)_X$ (X = A or B) are hyperbolically dependent on the concentration of the other substrate. It should be noted that while these three mechanisms (i.e., ping-pong, rapid equilibrium ordered, and rapid equilibrium random) can be distinguished by the method of replots, there remains ambiguity in distinguishing rapid equilibrium random from steady-state ordered since their rate laws are mathematically equivalent (*17*). However, they can be distinguished through the use of product inhibition studies (see below).

The starting point for our analysis of the data of Figure 2A by the methods of replots was an examination of the primary data sets (i.e., v_o vs [ATP] at fixed [tau] and v_o vs [tau] at fixed [ATP]) to confirm that they all adhered to the

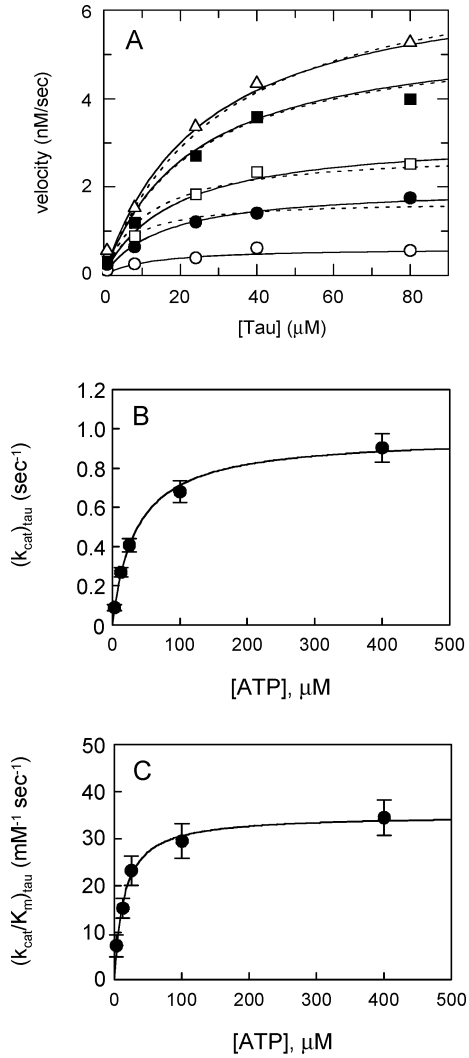


FIGURE 2: Steady-state kinetic experiment for cdk5/p25-catalyzed phosphorylation of tau. Panel A: Dependence of initial velocities on [tau] at [ATP] = 400 (△), 100 (■), 25 (□), 12.5 (●), 3.1 μM (○). Each data point is the average of duplicate determinations. The data set was globally fit to equations for reflecting rapid equilibrium random (solid line) and ping-pong mechanisms (dotted line). Panels B and C: ATP concentration dependencies of $(k_{cat})_{tau}$ and $(k_{cat}/K_m)_{tau}$ values derived from analysis of the data of panel A.

simple Michaelis−Menten equation. This allowed us to calculate, by nonlinear least-squares fits of each individual plot of v_o vs [substrate], apparent values of $(k_{cat})_X$ and $(k_{cat}/K_m)_X$ (X = tau or ATP) that are dependent on the fixed concentration of second substrate.

The next step in this analysis was to construct the replots and examine their shape. Replots of $(k_{cat})_{tau}$ and $(k_{cat}/K_m)_{tau}$ vs [ATP] (Figures 2B and 2C) and replots of $(k_{cat})_{ATP}$ and $(k_{cat}/K_m)_{ATP}$ vs [tau] (data not shown) are hyperbolic and unequivocally show that this reaction follows either a rapid equilibrium random or a steady-state ordered mechanism, and allows us to rule out the ping-pong mechanism that stood as an alternative mechanism from the global fit. The data sets can be fit to the expressions of eqs 4 and 5 to give the following estimates: $k_{cat} = 0.95$ s^{-1}, $K_{ATP} = 14$ μM, $K_{tau} = 8.4$ μM, and $\alpha = 2.8$. These values are similar to values obtained by a global fit to eq 3: $k_{cat} = 1.1 \pm 0.1$ s^{-1}, $K_{ATP} = 14 \pm 2.1$ μM, $K_{tau} = 11 \pm 2.8$ μM, and $\alpha = 2.5 \pm 0.4$ (error limits are from the nonlinear least-squares fit). In Table 1, we summarize the averaged results of this and a second, independent experiment.

Table 1: Initial Velocity Analysis for Cdk/p25-Catalyzed Phosphorylation Reactions[a]

	tau	H1P
k_c (s^{-1})	1.2 ± 0.1	5 ± 1
K_A (μM)	13 ± 1	6 ± 1
K_S (μM)	10 ± 2	7 ± 2
α	2.8 ± 0.3	12 ± 1

[a] Each parameter estimate is the average of two independent experiments. The error limit is the deviation from the mean.

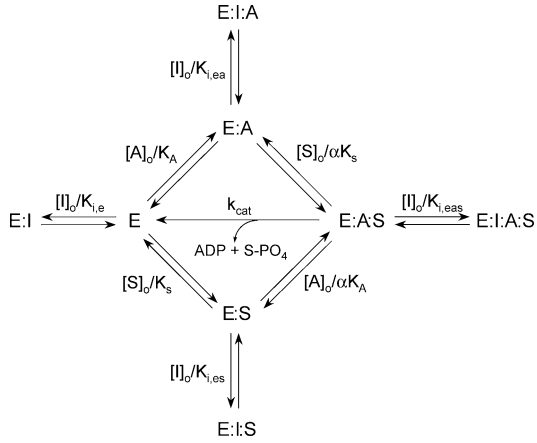
Cdk5/p25-Catalyzed Phosphorylation of H1P: Initial Velocity Studies. To determine the kinetic mechanism of the cdk5/p25-catalyzed phosphorylation of H1P, initial velocities were measured over a range of concentrations of H1P at several fixed concentrations of ATP (data not shown). The data set was globally fit to equations that reflect ping-pong, rapid equilibrium ordered, and rapid equilibrium random/steady-state ordered mechanisms. The rapid equilibrium random (or steady-state ordered) mechanism fits the data significantly better than either the ordered or ping-pong mechanisms as evidenced by very low F-test probability-values and parameter estimates: $k_{cat} = 4.4 \pm 0.5$ s^{-1}, $K_{ATP} = 4.5 \pm 0.1$ μM, $K_{H1P} = 10.5 \pm 2.0$ μM, and cofactor $\alpha = 13 \pm 1.5$. Visual inspection of the fits of the data to all three mechanisms as well as the results of the method of replots confirm this conclusion (data not shown). This experiment was repeated and the parameters were calculated for both experiments by the method of replots. Averaged values for the two experiments are summarized Table 1.

Inhibition Studies with Substrate Analogues. Studies of the inhibition of cdk5/p25-catalyzed phosphorylation of both tau and H1P were conducted with nucleotide analogues AMP and ADP, and peptide substrate analogues $H1P^{Val}$, $H1P^{Ala}$, $H1P^{Dap}$, $H1P^{Glu}$, and $H1P^{PO4}$. Our initial exploratory experiments at [ATP] = 200 μM $\gg K_{ATP}$ and [S] $\sim \alpha K_s$ (S = tau, H1P) reveal that (i) AMP and ADP are well-behaved and sufficiently potent[2] inhibitors of both the tau and H1P reactions to be used in mechanistic studies, (ii) in the tau reaction, only the peptide $H1P^{Dap}$ was well-behaved and sufficiently potent to be used in further studies, and (iii) in the H1P reaction, $H1P^{Ala}$, $H1P^{Dap}$, $H1P^{PO4}$ qualified for further studies.

With these preliminary results in hand, we decided to determine the mechanism of inhibition for AMP, ADP, $H1P^{PO4}$, $H1P^{Ala}$, and $H1P^{Dap}$. Now, a comprehensive analysis of the inhibition of cdk5/p25, or any two-substrate reaction, is a complicated task since these systems all have three independent variables, i.e., A, B, and I. To determine the mechanism of inhibition of these substrate analogues, as well as APS and CTIU (see below), we chose to use the methods of replots. As an example of this method, we analyze inhibition by AMP (data not shown, but will be for inhibitor APS, see below).

[2] "Well-behaved" means that the dependence of initial velocity on inhibitor concentration can be described by the expression $v_{inhib} = v_{control}/[1 + ([I]/K_{i,app})]$. Thus, in all these experiments, (i) the best-fit value of $v_{control}$ equals the velocity at [I] = 0, (ii) there is no hint of partial inhibition, and (iii) there is no need to include high-order terms in the denominator to achieve a good fit to the data. "Sufficiently potent" means that the $K_{i,app}$ is low enough relative to the solubility of the compound to allow a complete kinetic analysis.

Scheme 1[a]



[a] A = ATP; S = tau or H1P.

To determine AMP's mechanism of inhibition, we first determined, at several concentrations of this inhibitor, (i) the dependence of v_o on [tau] at single [ATP] and (ii) the dependence of v_o on [ATP] at a single [tau]. Next, for (i), we analyzed the dependence of v_o on [tau] at each [AMP] to generate [AMP]-dependent values $(V_{max})_{tau}$ and $(V_{max}/K_m)_{tau}$. Replots were then constructed of $(V_{max})_{tau}$ vs [AMP] and $(V_{max}/K_m)_{tau}$ vs [AMP]. Likewise for (ii), we analyzed the dependence of v_o on [ATP] at each [AMP] to generate [AMP]-dependent values $(V_{max})_{ATP}$ and $(V_{max}/K_m)_{ATP}$. Replots were then constructed of $(V_{max})_{ATP}$ vs [AMP] and $(V_{max}/K_m)_{ATP}$ vs [AMP].

Examination of these replots revealed the mechanism of inhibition. We found that $(V_{max})_{ATP}$ is independent of [AMP], but $(V_{max}/K_m)_{ATP}$ depends on [AMP] according to a simple, inhibition expression of general form: $v_{inhib} = v_{control}/(1 + [I]/K_{i,app})$. This pattern indicates that AMP is competitive with ATP. In contrast, both $(V_{max}/K_m)_{tau}$ and $(V_{max})_{tau}$ depend on [AMP] according to $v_{inhib} = v_{control}/(1 + [I]/K_{i,app})$. Thus, AMP is noncompetitive with tau.

To quantify these results and estimate values of various inhibition constants, we start with the general mechanism of Scheme 1, in which inhibitor can bind to all four forms of enzyme.[3] The rate expression of eq 7 describes this mechanism under rapid equilibrium conditions with variable substrate A (i.e., ATP) and fixed substrate S (i.e., tau in this case).

$$v_s = \frac{\dfrac{V_{max}}{1 + \dfrac{\alpha K_S}{[S]_o} + \dfrac{\alpha K_S}{[S]_o}\dfrac{[I]_o}{K_{i,ea}} + \dfrac{[I]_o}{K_{i,eas}}}[A]_o}{K_A\left\{\dfrac{\alpha + \dfrac{\alpha K_S}{[S]_o} + \dfrac{\alpha K_S}{[S]_o}\dfrac{[I]_o}{K_{i,e}} + \dfrac{[I]_o}{K_{i,es}}}{1 + \dfrac{\alpha K_S}{[S]_o} + \dfrac{\alpha K_S}{[S]_o}\dfrac{[I]_o}{K_{i,ea}} + \dfrac{[I]_o}{K_{i,eas}}}\right\} + [A]_o} \quad (7)$$

If we make the reasonable assumption that inhibitor AMP (or other substrate analogue inhibitors) cannot bind to the ternary complex E:A:S, then eq 7 simplifies to

$$v_s = \frac{\dfrac{V_{max}}{1 + \dfrac{\alpha K_s}{[S]_o} + \dfrac{\alpha K_s}{[S]_o}\dfrac{[I]_o}{K_{i,ea}}}[A]_o}{K_A\left\{\dfrac{\alpha + \dfrac{\alpha K_s}{[S]_o} + \dfrac{\alpha K_s}{[S]_o}\dfrac{[I]_o}{K_{i,e}} + \dfrac{[I]_o}{K_{i,es}}}{1 + \dfrac{\alpha K_s}{[S]_o} + \dfrac{\alpha K_s}{[S]_o}\dfrac{[I]_o}{K_{i,ea}}}\right\} + [A]_o} \quad (8)$$

Given this, we can immediately see how the apparent values $(V_{max})_{ATP}$ and $(V_{max}/K_m)_{ATP}$ depend on inhibitor and substrate S:

$$(V_{max})_{ATP} = \frac{V_{max}}{1 + \dfrac{\alpha K_s}{[S]_o} + \dfrac{\alpha K_s}{[S]_o}\dfrac{[I]_o}{K_{i,ea}}} \quad (9)$$

$$(V_{max}/K_m)_{ATP} = \frac{(V_{max}/\alpha K_A)}{1 + \dfrac{K_s}{[S]_o} + \dfrac{K_s}{[S]_o}\dfrac{[I]_o}{K_{i,e}} + \dfrac{[I]_o}{\alpha K_{i,es}}} \quad (10)$$

In the present case in which the inhibitor is AMP, we found that $(V_{max})_{ATP}$ is independent of AMP concentration, which means that $K_{i,ea}$ is very large and AMP cannot bind to the binary complex E:AMP. On the other hand, we found that $(V_{max}/K_m)_{ATP}$ titrates with AMP, meaning that AMP can bind to E or E:S or both. It is clear from inspection of eq 10 that from this single experiment we cannot tell which of these situations holds and thus we are unable, at this point, to calculate independent estimates of $K_{i,e}$ and $K_{i,es}$.

However, we can obtain this information from an analysis of the data when tau is the variable substrate. The symmetry of random mechanisms allows us to write, by simple inspection, the following equations.

$$(V_{max})_S = \frac{V_{max}}{1 + \dfrac{\alpha K_A}{[A]_o} + \dfrac{\alpha K_A}{[A]_o}\dfrac{[I]_o}{K_{i,es}}} \quad (11)$$

$$(V_{max}/K_m)_S = \frac{(V_{max}/\alpha K_S)}{1 + \dfrac{K_A}{[A]_o} + \dfrac{K_A}{[A]_o}\dfrac{[I]_o}{K_{i,e}} + \dfrac{[I]_o}{\alpha K_{i,ea}}} \quad (12)$$

But since, as we just saw above, $\alpha K_{i,ea}$ is very large, we can simplify eq 12 to

$$(V_{max}/K_m)_S = \frac{(V_{max}/\alpha K_S)}{1 + \dfrac{K_A}{[A]_o} + \dfrac{K_A}{[A]_o}\dfrac{[I]_o}{K_{i,e}}} \quad (13)$$

We can now estimate all inhibition constants as follows: (i) $K_{i,es}$ directly from eq 11, (ii) $K_{i,e}$ from use of $K_{i,es}$ and eq 10, and (iii) $K_{i,e}$ directly from eq 13. The parameter estimates of this example, using phosphoryl acceptor tau and inhibitor AMP, are summarized in the first two lines of Table 2.

Finally, using this general method, the mechanism of inhibition and estimation of inhibition dissociation constants were determined for ADP and the three peptide analogues of H1P. These data are all summarized in Table 2.

Inhibition of Cdk5/p25 by APS. In 2001, Pharmacia reported that PNU-112455A (APS) is a competitive inhibitor of the binding of ATP to cdk5/p25 and a noncompetitive inhibitor of the binding of H1P (*12*). This compound was

[3] The need to consider a mechanism of this complexity will become apparent below when we analyze inhibition of cdk5/p25 by APS and CTIU.

Table 2: Inhibition of Cdk5 by Substrate Analogues

	substrate			inhibn const (mM)		
inhibitor	fixed	variable	mechanism	$K_{i,e}$	$K_{i,ea}$	$K_{i,es}$
AMP	tau	ATP	C	(0.32)[a]		
	ATP	tau	NC	0.20		0.33
	H1P	ATP	C	(0.044)		
	ATP	H1P	NC	0.055		0.32
ADP	tau	ATP	C	(0.038)		
	ATP	tau	NC	0.022		0.045
	H1P	ATP	C	(0.13)		
	ATP	H1P	NC	0.14		0.17
$H1P^{PO4}$	tau	ATP				
	ATP	tau				
	H1P	ATP	NC	1.0	12	
	ATP	H1P	C	(1.2)		
$H1P^{Ala}$	tau	ATP				
	ATP	tau				
	H1P	ATP	NC	1.9	5.7	
	ATP	H1P	C	(2.3)		
$H1P^{Dap}$	tau	ATP	NC	8.0	8.8	
	ATP	tau	C	(7.2)		
	H1P	ATP				
	ATP	H1P				

[a] The values in parentheses derive from situations where the inhibitor is an analogue of the variable substrate and were calculated from eq 10. As discussed in the Results section, the dissociation constant estimated from eq 10 is a function of both $K_{i,e}$ and $K_{i,es}$, where s is the fixed substrate. This dissociation constant equal $K_{i,e}$ only if the variable substrate cannot bind to the complex of enzyme and fixed substrate. Thus, better estimates of $K_{i,e}$ come from the analysis in which the inhibitor is an analogue of the fixed substrate.

originally identified through a screen of their compound collection for inhibitors of cdk5/p25. More recently, Pfizer reported the cdk5/p25 inhibitor CTIU, a compound synthesized as part of a medicinal chemistry program based on a structurally related screening hit (*13*). Like APS, CTIU was also reported to be competitive with ATP.

Both compounds appeared to be ideal for use as control inhibitors for our own screen to identify inhibitors of cdk5/p25. Of course, before we used either for this purpose we needed to confirm the mechanism of inhibition, not only when H1P is used as phosphoryl acceptor but also when tau is the phosphoryl acceptor. As an example of our method, we present below inhibition by APS of the cdk/p25-catalyzed phosphorylation of tau.

These studies were done in a manner identical to that outlined above for inhibitor AMP, starting with primary plots of v_o/[E] as a function of [ATP] at various [APS] (Figure 3A) and v_o/[E] as a function of [tau] at various [APS] (Figure 3D). The replots of this data not only show that $(k_{cat})_{tau}$, $(k_{cat}/K_m)_{tau}$, and $(k_{cat}/K_m)_{ATP}$ titrate with APS, as expected from the results of the Pharmacia group, but also the unexpected result that $(k_{cat})_{ATP}$ titrates with APS. Thus, from our results it appears that APS is noncompetitive not only with tau but also with ATP and therefore APS is able to bind to all four forms of the enzyme.

To determine all four inhibitor dissociation constants for the mechanism of Scheme 1, one must determine the dependence of initial velocity on all three independent

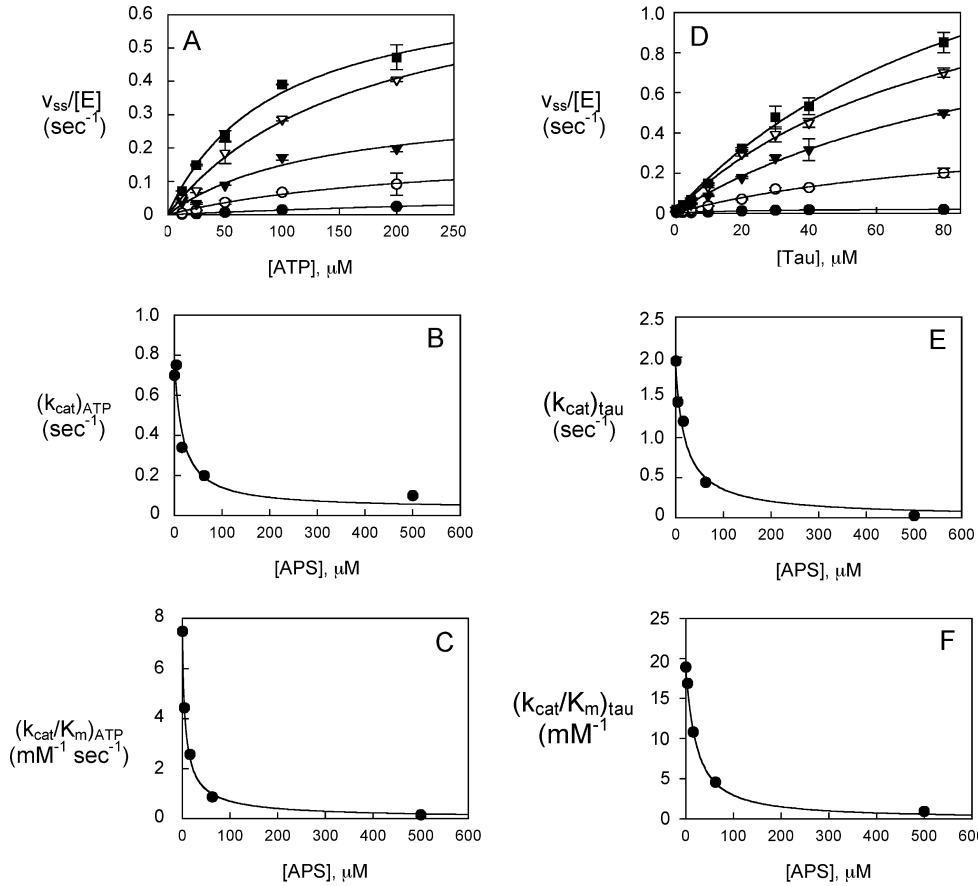


FIGURE 3: Inhibition of the cdk5/p25-catalyzed phosphorylation of tau by APS. Panel A: Plot of v_{ss}/[E] vs [ATP] at [APS] = 0 (■), 4 (▽), 16 (▼), 63 (○), and 500 μM (●), all at a fixed tau concentration of 80 μM. Panels B and C: APS concentration dependencies of $(k_{cat})_{ATP}$ and $(k_{cat}/K_m)_{ATP}$ values derived from analysis of the data of panel A. Panel D: Plot of v_{ss}/[E] vs [tau] at [APS] = 0 (■), 4 (▽), 16 (▼), 63 (○), and 500 μM (●), all at a fixed ATP concentration of 200 μM. Panels E and F: APS concentration dependencies of $(k_{cat})_{tau}$ and $(k_{cat}/K_m)_{tau}$ values derived from analysis of the data of panel D.

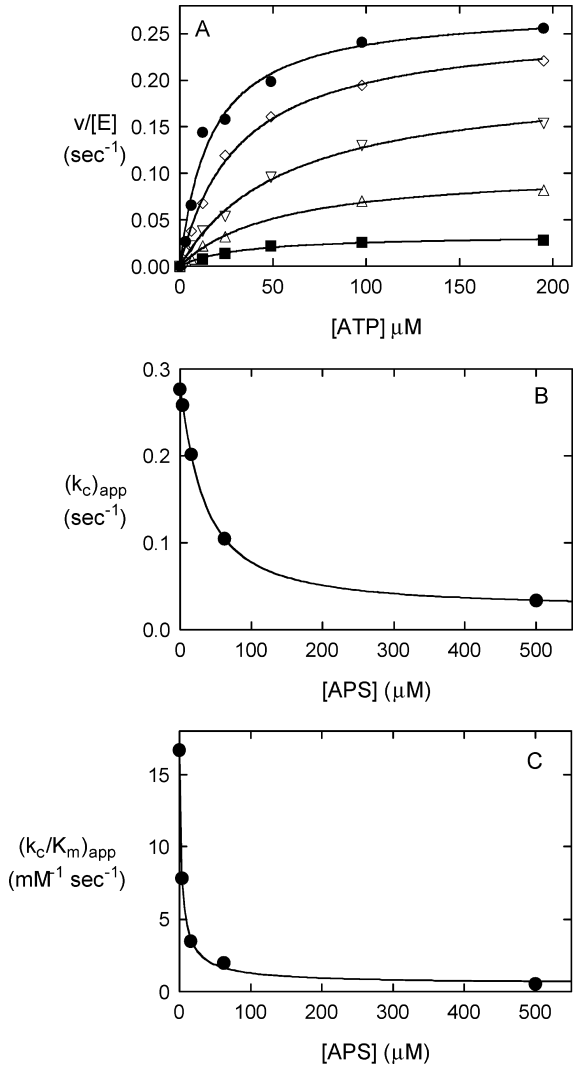


FIGURE 4: Inhibition of the cdk5/p25-catalyzed hydrolysis of ATP by APS. Panel A: ATP concentration dependencies at [APS] = 0 (●), 4 (◇), 16 (▽), 63 (△), and 500 μM (■). (B) Replot of $(k_{cat})_{app}$ vs [APS], and (C) replot of $(k_{cat}/K_m)_{app}$ vs [APS]. Panels B and C: APS concentration dependencies of $(k_{cat})_{app}$ and $(k_{cat}/K_m)_{app}$ values derived from analysis of the data of panel A.

variables: [ATP], [S], and [I]. However, one can avoid such a heroic undertaking if one can acquire estimates of one or more of these dissociation constants by some independent means. We chose to take this latter experimental approach and took advantage of the fact that cdk5/p25 is able to catalyze the hydrolysis of ATP (k_{cat} = 0.28 s^{-1}, K_m = 17 μM; see Figure 4A). Figure 4A shows the [ATP]-dependence of initial velocity for ATP hydrolysis at several fixed concentrations of [APS]. It is evident from inspection of the replots that both $(k_{cat})_{app}$ (Figure 4B) and $(k_{cat}/K_m)_{app}$ (Figure 4C) titrate with APS, yielding best fit values of K_i = 3.6 μM and βK_i = 94 μM, respectively. Global fit of the data of Figure 4A to eq 14 gave the values $k_{cat} = 0.27 \pm 0.01$, $K_m = 17 \pm 2$ μM, $K_i = 4.3 \pm 0.8$ μM, and $\beta K_i = 56 \pm 18$ μM. Thus, APS is a noncompetitive inhibitor of the ATPase activity of cdk5/p25 and can bind both to free enzyme and to the binary complex of enzyme and ATP. Note that K_i and βK_i are equivalent to $K_{i,e}$ and $K_{i,ea}$ of Scheme 1, respectively.

$$v_o/[E] = \frac{\dfrac{k_{cat}}{1+\dfrac{[I]}{\beta K_i}}[ATP]}{K_m\left\{\dfrac{1+\dfrac{[I]}{K_i}}{1+\dfrac{[I]}{\beta K_i}}\right\}+[ATP]} \quad (14)$$

Returning to the problem of estimating all four inhibitor dissociation constants of Scheme 1 for the inhibition of the cdk5/p25-catalyzed phosphorylation of tau, we can see that with this new knowledge of $K_{i,e}$ and $K_{i,ea}$, and our previous knowledge of k_{cat}, K_A, K_{tau}, and α, we can calculate $K_{i,eas}$ by

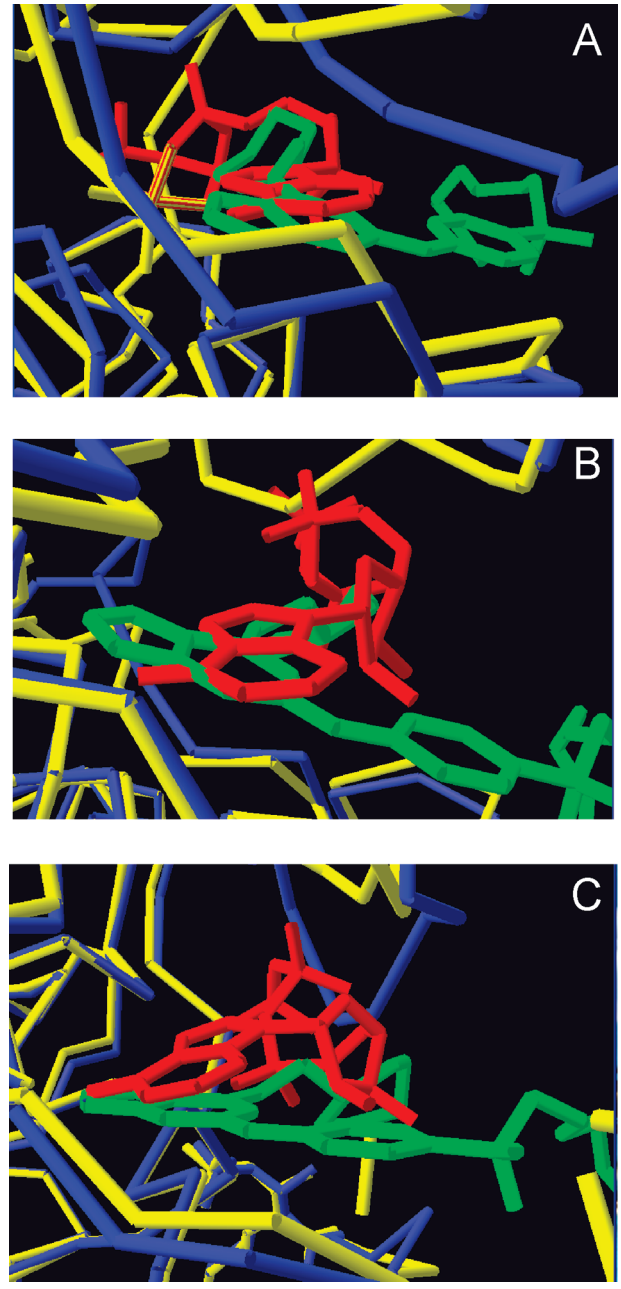


FIGURE 5: Superposition of structures of cdk2 bound to APS and ATP. Panel A: Superposition of the structures of cdk2:ATP and cdk2:APS. Panel B: Superposition of the structures of cyclinA/cdk2:ATP and cyclinA/cdk2:APS. Panel C: Superposition of the structures of cyclinA/phospho-cdk2:ATP and cyclinA/phospho-cdk2:APS.

Table 3: APS and CTIU Inhibitor Dissociation Constants for the Cdk5/p25-catalyzed Phosphorylation of tau and H1P According to the Mechanism of Scheme I

	tau		H1P	
K_i (μM)	APS	CTIU	APS	CTIU
$K_{i,e}$	3.6	0.04	3.6	0.04
$K_{i,es}$	8.8	0.2	1.4	0.04
$K_{i,ea}$	39	1.7	120	4.9
$K_{i,eas}$	26	1.8	150	1.5

nonlinear least-squares of the data of Figure 3B to eq 15, and $K_{i,eas}$ by nonlinear least-squares of the data of Figure 3C to eq 16.

$$(k_{cat})_{ATP} = \frac{k_{cat}}{1 + \frac{\alpha K_{tau}}{[tau]_o} + \frac{\alpha K_{tau}}{[tau]_o}\frac{[I]_o}{K_{i,ea}} + \frac{[I]_o}{K_{i,eas}}} \quad (15)$$

$$(k_{cat}/K_m)_{ATP} = \frac{k_{cat}/\alpha K_A}{1 + \frac{K_{tau}}{[tau]_o} + \frac{K_{tau}}{[tau]_o}\frac{[I]_o}{K_{i,e}} + \frac{[I]_o}{\alpha K_{i,es}}} \quad (16)$$

The same strategy was used when H1P was phosphoryl acceptor. Inhibition constants for reaction of both substrates, as well as for inhibition by CTIU, are summarized in Table 3.

Molecular Modeling of ATP and APS Bound to Various Forms of Cdk2. The three panels of Figure 5 summarize superposition studies of ATP and APS bound to three forms of cdk2. Panel A is the superposition of cdk2:ATP and cdk2:APS; panel B, the superposition of cyclinA/cdk2:ATP and cyclinA/cdk2:APS; and panel C, cyclinA/phospho-cdk2:ATP and cyclinA/phospho-cdk2:APS.

DISCUSSION

The overarching goal of these studies was to establish a background of kinetic data that would be useful in designing high-throughput screening assays of the cdk5/p25-catalyzed phosphorylation of tau and in the interpretation of kinetic experiments with inhibitors that would be discovered in the course of these HTS campaigns. Specifically, we report results of studies designed to elucidate the kinetic mechanism of (i) the cdk5/p25-catalyzed phosphorylation of tau and a peptide substrate and (ii) the inhibition of cdk5/p25 by two literature prototype inhibitors, APS and CTIU.

Kinetic Mechanism of Cdk5/p25. There appears to be no consensus kinetic mechanism for protein kinases (*19*). While there are examples of kinases that follow an ordered mechanism, most follow a rapid equilibrium mechanism; examples include c-Src, Csk, Phk, InRK, and human placental insulin receptor/kinase (*20–25*).

In experiments conducted to elucidate the kinetic mechanism of protein kinases, investigators have more often than not chosen peptides as the phosphoryl acceptor, this choice being motivated by ease of assay design and data collection, and the ability to design peptide substrates with a single phosphorylation site, which can simplify kinetic analysis. Despite these advantages, peptide substrates should be used with the understanding that any mechanistic conclusions based on their phosphorylation may not extend to protein substrates (*19*). For example, the ordered kinetic mechanism for MAP kinase p38α changes from one in which ATP binds prior to binding of a peptide phosphoryl acceptor (*26*) to one in which the protein ATF2 binds before ATP (*27*).

For cdk5/p25, the only study of kinetic mechanism that has been reported is one involving the peptide H1P as substrate, and for this a random mechanism was reported (*12*). In our studies of this enzyme, we sought to first reproduce these results and then to go on to probe the kinetic mechanism using tau, a protein of relevance to human disease. Our kinetic data with H1P, as well as tau, suggest a random rapid equilibrium mechanism. But of course, the initial velocity patterns are identical for this mechanism and a steady-state ordered mechanism. To rule out the latter, we needed to conduct inhibition studies with products and/or substrate analogues (*17, 28, 29*).

The results of these studies are summarized in Table 2. For cdk5/p25-catalyzed phosphorylation with tau, we see that when ATP is the variable substrate, AMP and product ADP are competitive inhibitors, while substrate analogue $H1P^{Dap}$ is a noncompetitive inhibitor. And when tau is the variable substrate, AMP and ADP are noncompetitive inhibitors, while $H1P^{Dap}$ is a competitive inhibitor. Precisely the same pattern was seen with H1P as substrate. When ATP is the variable substrate, AMP and APD are competitive, and product $H1P^{PO4}$ and $H1P^{Ala}$ are noncompetitive; when H1P is the variable substrate, AMP and APD are noncompetitive, and $H1P^{PO4}$ and $H1P^{Ala}$ are competitive. These patterns rule out a steady-state ordered mechanism and indicate a rapid equilibrium random mechanism for phosphorylation of both tau and H1P.

Structural Requirements for Binding of H1P Analogues to Cdk5/p25. In the course of investigating analogues of H1P that might be suitable for the studies discussed above, we acquired inhibition data for several analogues that speak directly to the question of binding selectivity of cdk5/p25 for peptides (Table 2). Of the three analogues of H1P that were studied using H1P as substrate (i.e., $H1P^{Val}$, $H1P^{Ala}$, $H1P^{Glu}$), only $H1P^{Ala}$ displayed any significant inhibitory activity with a K_i value of 2.3 mM. When compared to the K_{H1P} value of 0.007 mM (Table 1), these results indicate a critical role for the Thr in binding interactions with cdk5/p25. Similar findings were reported when hydroxyls were removed in the substrates of other kinases. For example, in a study of cAMP-dependent protein kinase, it was reported that replacement of Ser by Ala decreased binding affinity by 100-fold (*30*). However, massive losses of binding affinity as seen with cdk5/p25 and cAMP-dependent protein kinase are not always the case. The dissociation constant for the complex of $pp60^{c\text{-}src}$ tyrosine kinase and substrate $(FGE)_3\mathbf{Y}(GEF)_2GD$ is only 6-fold less than the dissociation constant for the complex of enzyme and inhibitor $(FGE)_3\mathbf{F}(GEF)_2GD$ (*23*).

It is of some interest to understand the 200-fold decrease in binding affinity that accompanies changing H1P (K_m value of 7 μM, Table 1) into $H1P^{Ala}$. One possible explanation for this dramatic difference centers around the fact that $H1P^{Ala}$ does not contain a hydroxyl group that can participate in hydrogen-bonding interactions with the enzyme. $H1P^{Dap}$, with the amine of 2,3-diaminopropionic acid replacing the hydroxyl of Thr, was designed to test this hypothesis. We reasoned that the amine might mimic the hydrogen bonding capabilities of the hydroxyl group and would thereby recover some of the inhibitor

potency that was lost with $H1P^{Ala}$. Indeed, some inhibitory potency was recovered; $H1P^{Dap}$ is 3-fold more potent than $H1P^{Ala}$ as an inhibitor of cdk5/p25 (Table 2). However, this result combined with those of Table 2 and the literature examples cited above suggests that losses in binding affinity that accompany hydroxyl substitution in kinase substrates are not simply the result of the loss of the hydroxyl moiety, but likely also involve subtle conformational aspects of the substrate that are present in the substrate but are lacking in substrate analogues. Thus, the lower potency of the valine and glutamate peptides compared with the alanine peptide might be attributed to the structural aspects of their side chains with the branch structure of valine's side chain and the extended chain of glutamate not fitting into the binding site on the enzyme and, therefore, hindering the binding of these peptides to the enzyme.

Kinetic Consequences of the Binding of Proteins with Multiple Phosphorylation Sites to Cdk5/p25. Many enzymes, including phosphatases, proteases, transglutaminases, and of course kinases, catalyze nonprocessive modification at multiple sites on their protein substrates. For reaction of an enzyme with a protein that contains multiple reactive sites, the kinetic situation is more complex than the reaction of a peptidic substrate that has only one reactive site. During reaction of an enzyme with a polymeric substrate that has n reactive sites, the enzyme will bind to this substrate to form n different Michaelis complexes, and go on to produce n unique products. The apparent steady-state kinetic parameters for such a reaction will be functions of the microscopic kinetic parameters for reaction of each individual pathway (*31*). Despite the kinetic complexity, initial velocity analyses of these reactions still adhere to Michaelis−Menten kinetics, but now the steady-state kinetic parameters are averages of the microscopic parameters of the individual n reactions (*31*). Such a kinetic situation holds in the present case for reaction of cdk5/p25 with tau, where now k_c, K_m, and k_c/K_m derived from initial velocity analysis reflect the six parallel reaction pathways for phosphorylation of the six reactive hydroxyl moieties of tau with which cdk5/p25 reacts.

Note however that this simplification holds only for initial velocity measurements. If the reaction is allowed to progress toward completion, monophosphorylated tau will itself become a substrate for phosphorylation at one of the five remaining reactive Ser or Thr residues. The number of products-turned-substrates increases rapidly, likely resulting in complex reaction progress curves. This is seen in the multiphasic progress curve of Figure 1. The type of rate law that describes this curve is consistent with a mechanism involving successive intermediates, each with a unique set of kinetic parameters for reaction with cdk5/p25, that ultimately leads to phosphorylation on all six available sites.

Formation of a Nonproductive Complex between Cdk5/p25 and Tau. Interestingly, K_{ATP} for reaction of cdk5/p25 with tau and H1P differ 2-fold, equaling 13 and 6 μM, respectively (Table 1). Also, values of $K_{i,e}$ for inhibition by ADP and AMP are also dependent on the identity of phosphoryl acceptor (see Table 2), with $K_{ADP,e}$ equaling 140 and 22 μM, respectively, for tau and H1P, and with $K_{AMP,e}$ equaling 55 and 200 μM, respectively, for tau and H1P. Since K_{ATP} and $K_{i,e}$ are true dissociation constants for binary complex of enzyme and ATP, ADP, or AMP, their magnitude should be independent of the identity of the phosphoryl acceptor. Combined, these results suggest departure from a simple random mechanism. A mechanism that accounts for these results is one which includes nonproductive binding of phosphoryl acceptor to free enzyme to form a binary complex to which adenosine nucleotides can bind. Thus, the measured values are not simple dissociation constants for binary E:nucleotide complexes, but rather are complex terms that include contribution from an off-reaction pathway introduced by the nonproductive binding of phosphoryl acceptor. It would not be unexpected that the magnitudes of K_{ATP} and $K_{i,e}$ would depend on the identity of the phosphoryl acceptor.

Structural and Kinetic Analysis of the Binding of APS to Cdk5/p25. In this work, we report kinetic data suggesting that APS can bind to all four substrate-bound forms of cdk5/p25 (see Scheme 1). This is at odds with an earlier study (*12*) reporting that APS is competitive with the binding of ATP, both cdk5/p25 and its close structural homologue cdk2.[4] In support of this conclusion, the authors of this paper also reported a structural study in which they superimposed the structures of cdk2:ATP and cdk2:APS, showing that ATP and APS cannot simultaneously bind to the active site of cdk2. We were puzzled by this discrepancy and, in the hopes of shedding light on the situation, conducted the structure superpositions shown in Figure 5.

When we superimposed the structures of cdk2:ATP and cdk2:APS we saw, like the Pharmacia group, that ATP and APS could not simultaneously bind to the active site of cdk2 (see Figure 5A). This is of course consistent with a competitive mechanism of inhibition by APS. However, the superposition of Figure 5A, again like that of the Pharmacia group, was conducted with the structures based simply on cdk2. Since cdk2 must bind cyclin A to be an active kinase, it was of some interest to conduct superposition studies with structures derived from the complex cyclinA/cdk2, where cyclin A induces a conformation change in cdk2 to render it an active kinase. When this superposition was done, we found that ATP and APS could nearly both fit into the active site of cdk2 (Figure 5B). Of course, the form of cdk2 which is physiologically relevant is phospo-cdk2 bound to cyclin A. Phosphorylation of cdk2 induces yet another conformational change, making cyclinA/phospho-cdk2 more active than cyclinA/cdk2. As shown in Figure 5C, superposition of cyclinA/phospho-cdk2:ATP and cyclinA/phospho-cdk2:APS clearly shows simultaneous binding of ATP and APS to the active site of cdk2.

These observations of the binding of ATP and APS to the three forms of cdk2 are relevant to results reported herein. Cdk5 does not bind a cyclin nor is it phosphorylated, but it does bind activator protein p25 to form cdk5/p25. Binding of p25 to cdk5 induces a conformational change that renders cdk5/p25 catalytically competent. We propose, by analogy to phosphorylated and fully active cyclinA/phospho-cdk2 which can simultaneously bind

[4] In the published work, the investigators do not report studies that would have allowed them to say if APS binds to E or E:H1P or both.

ATP and APS, that the fully active complex cdk5/p25 can also simultaneously bind ATP and APS. Such binding predicts the sort of complex mechanism of inhibition that we observe for APS.

This structural analysis does not explain the kinetic data from the Pharmacia group that suggests an ATP-competitive mechanism for APS. On examination of these kinetic data, one finds that only two nonzero concentrations of APS were used: 5 and 20 μM. While one could argue that an accurate assessment of inhibitor mechanism is impossible with only two concentrations of inhibitor, it is beyond question that the higher inhibitor concentration of only 20 μM does not allow detection of an E:A:I complex with a $K_{i,ea}$ of 120 μM (see Table 3; S = H1P). We were able to detect this complex because we used a much broader inhibitor concentration range: 4, 16, 63, and 500 μM.

CONCLUSIONS

The cdk5/p25-catalyzed phosphorylation of tau follows a random order of addition of substrates with $k_c = 1\ s^{-1}$, $K_{ATP} = 13\ \mu M$, $K_{tau} = 10\ \mu M$, and $\alpha = 3$. With knowledge of the kinetic mechanism and dissociation constants, we are able to choose values of initial substrate concentrations for high-throughput screening assays that allow identification of inhibitors that bind to one or more of the four steady-state enzyme species E, E:ATP, E:tau, and E:ATP:tau. Without this knowledge, it is impossible to choose substrate concentrations for multisubstrate enzymes in an informed manner.

Values of k_c for phosphorylation of tau and H1P are 1 and 5 s^{-1}, respectively, and values of $k_c/\alpha K_S$ are 7 and 60 $mM^{-1}\ s^{-1}$. The lack of kinetic advantage of tau over H1P—indeed H1P has an advantage over tau—suggests that when tau binds to cdk5/p25 it establishes no catalytically important interactions with the enzyme over and above those that the peptide substrate establishes. Therefore, one should not anticipate finding different sorts of inhibitors in screens using tau as phosphoryl acceptor instead of H1P. This of course could have been otherwise, and for this reason a complete kinetic analysis is needed for both substrates.

Cdk5/p25 offers diverse binding modes to inhibitors that are structurally unrelated to its substrates. While this is good news for those who seek to identify such inhibitors through campaigns of high-throughput screening, it is also a reminder that to detect these binding modes and to estimate their inhibitor dissociation constants, it is imperative that one conduct thorough kinetic analyses using inhibitor and substrate concentration ranges that are sufficiently broad. With such analyses and a complete knowledge of the mechanism of inhibition, structural variation of an inhibitor can be conducted with a view toward optimizing only those binding modes that one chooses to optimize.

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