Catalytic Mechanism of Cdc25[†]

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ABSTRACT: Cdc25 is a dual-specificity phosphatase that catalyzes the activation of the cyclin-dependent kinases, thus causing initiation and progression of successive phases of the cell cycle. Although it is not significantly homologous in sequence or structure to other dual-specificity phosphatases, Cdc25 belongs to the class of well-studied cysteine phosphatases as it contains their active site signature motif. Like other dual-specificity phosphatases, Cdc25 contains an active site cysteine whose pK_a of 5.9 can be measured in pH-dependent kinetics using both small molecule and protein substrates such as Cdk2-pTpY/CycA. We have previously shown that the catalytic acid expected in phosphatases of this family and apparent in kinetics with the natural protein substrate does not appear to lie within the known structure of Cdc25 [Chen, W., et al. (2000) *Biochemistry 39*, 10781]. Here we provide experimental evidence for a novel mechanism wherein Cdc25 uses as its substrate a monoprotonated phosphate in contrast to the more typical bisanionic phosphate. Our pH-dependent studies, including one-turnover kinetics, solvent kinetic isotope effects, equilibrium perturbation, substrate depletion, and viscosity measurements, show that the monoprotonated phosphate of the protein substrate Cdk2-pTpY/CycA provides the critical proton to the leaving group. Additionally, we provide evidence that Glu474 on the Cdc25 enzyme serves an important role as a base in the transfer of the proton from the phosphate to the leaving group. Because of its greater intrinsic reactivity, the use of a monoprotonated phosphate as a phosphatase substrate is a chemically attractive solution and suggests the possibility of designing inhibitors specific for the Cdc25 dual-specificity phosphatase, an important anticancer target.

Cdc25 phosphatases are critical components of the regulatory machinery of the eukaryotic cell cycle (*1*). They exert their effect by dephosphorylating and thus activating the Cdk[1]−cyclin complexes, the regulatory kinases whose phosphorylation activity of numerous cellular targets leads directly to cell cycle progression. The important role of the Cdc25 phosphatases in cell cycle regulation is becoming increasingly apparent as more and more studies show increased levels of expression of the Cdc25A and Cdc25B homologues associated with cancer (*2*−*10*). Additionally, specific phosphatase inhibitors of Cdc25 have been shown to block cell cycle progression (*11*−*14*). Therefore, the biochemistry of the Cdc25 phosphatases continues to be intensively investigated, and Cdc25 is a viable pharmaceutical target for anticancer therapy.

On the basis of the active site motif HCX_5R, Cdc25 has been classified as a dual-specificity phosphatase (DSP) (for a review of protein phosphatases, see ref *15*). In this motif, H is a highly conserved histidine residue, C is the catalytic cysteine, the five X residues form a loop whose backbone amides hydrogen bond to the phosphate of the substrate, and R is a highly conserved arginine that is required for binding and transition-state stabilization of the phosphate. The DSPs have the ability to hydrolyze phosphoserine/threonine as well as phosphotyrosine residues and belong to the superfamily of protein tyrosine phosphatases (PTPases). The PTPases all contain the HCX_5R motif and proceed via a two-step mechanism. In the first step, the bisanionic phosphate moiety of the substrate, cradled into the active site, is attacked by the thiolate of the catalytic cysteine which forms a covalent phosphocysteine intermediate. In the second step, the catalytic cycle is completed by the hydrolysis of the phosphocysteine intermediate. In addition to the catalytic cysteine, the other key residue required in this mechanism is the catalytic acid that donates a proton to the oxygen of the leaving group (threonyl or tyrosyl). The catalytic acid is generally an aspartic acid that is located on a separate loop near the top of the active site cleft. The catalytic acid in most PTPases can easily be identified by sequence homology, structural studies, bell-shaped pH-dependent kinetics, and/or mutagenesis. However, this has not held true for the Cdc25 phosphatases.

When it was first realized that the Cdc25 proteins had intrinsic phosphatase activity, their dissimilarity to other phosphatases based on primary sequence comparisons was noted along with their apparently low phosphatase activity (*16*). It appeared that the only important sequence feature conserved between Cdc25 and other PTPases was the HCX_5R loop, and there appeared to be no sequence corresponding to the catalytic acid loop. The first attempt to define the

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[1] Abbreviations: PTPase, protein tyrosine phosphatase; DSP, dual-specificity phosphatase; Cdk, cyclin-dependent kinase; Cyc, cyclin; mFP, 3-*O*-methylfluorescein phosphate; pNPP, *p*-nitrophenyl phosphate; IAA, iodoacetic acid; pT, phosphothreonine; pY, phosphotyrosine; WT, r25B2 catalytic domain of Cdc25B comprising residues 377−566; E474Q, same construct of Cdc25B as the WT containing the E474Q mutation; SKIE, solvent kinetic isotope effect.

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catalytic acid was performed using a combination of alignments of catalytic residues and secondary structure motifs (*17*). Mutagenesis of the proposed residue (Asp383 in Cdc25A) yielded an inactive protein, where it was later shown that Asp383 played a structural, and not a catalytic, role (*18*). The crystal structures of the catalytic domains of Cdc25A (*18*) and Cdc25B (*19*) showed that the Cdc25s displayed a topology identical to that of the sulfur transfer protein rhodanese and completely dissimilar to those of other known PTPases or DSPs. Although the HCX_5R motif did appear to form the active site loop, with Cys473 (in Cdc25B) being the catalytic cysteine, there was no loop overhanging the active site that contained the putative catalytic acid. Two different potential catalytic acids were suggested on the basis of the proximity to the active site, namely, Glu474 and Glu478 (in Cdc25B), two of the X residues within the active site motif. These suggestions led to our efforts to test these two residues as well as other potential residues within the unstructured C-terminal region by mutagenesis and pH-dependent assays (*20*). These studies were greatly facilitated by the availability of the protein substrate (*21*), which, in contrast to the small molecule artificial substrates *p*-nitrophenyl phosphate (pNPP) and 3-*O*-methylfluorescein phosphate (mFP), shows bell-shaped pH-dependent kinetics similar to those of other PTPases and DSPs. However, on the basis of the retention of the bell-shaped pH-dependent behavior in all the mutated proteins, the catalytic acid was not found by this procedure. Given that there are no other ionizable residues in the region of the active site, we thus speculated that the catalytic acid may actually reside on the protein substrate.

In this paper, we delve further into the detailed catalytic mechanism of Cdc25B using the Cdk2-pTpY/CycA substrate to define the detailed chemical and kinetic mechanism of Cdc25B. Our pH-dependent studies include one-turnover experiments, K_m measurements, viscosity dependence, solvent kinetic isotope effects, equilibrium perturbation, and substrate depletion, all of which support a novel mechanism for the Cdc25B phosphatase wherein the proton required by the leaving group is provided by the protein substrate via its monoprotonated, not bisanionic, phosphate (Figure 9). Additionally, we are able to explain the reduction in k_{cat}/K_m seen previously for the E474Q mutation by providing evidence that Glu474 acting as a catalytic base plays an important role in proton transfer from the phosphate to the leaving group.

EXPERIMENTAL PROCEDURES

Reagents. *p*-Nitrophenyl phosphate (pNPP), 3-*O*-methylfluorescein phosphate (mFP), iodoacetic acid (IAA), and α-naphthyl phosphate were purchased from Sigma. G-50 Sephadex and [γ-^{32}P]ATP were obtained from Amersham-Pharmacia. Ni−NTA−agarose beads were purchased from Qiagen. The anti-Cdk2 antibody was obtained from Upstate Biotechnology, and the anti-Cdc25 antibody was obtained from Santa Cruz. All other reagents were of the highest grade commercially available.

Proteins. The catalytic domain construct WT (=Δ25B2, residues 377−566) and the protein substrate Cdk2-pTpY/CycA have been described previously (*20*). The E474Q mutation, originally in the Δ25B1 construct, was incorporated into the r25B2 construct by site-directed mutagenesis using the primers described previously (*20*). The E474A mutation was generated using analogous primers, substituting the nucleotides CAA of the Gln mutation with GCA for the Ala mutation. Expression and purification of E474Q and E474A in the r25B2 construct were performed as for the WT protein.

Assays. All phosphatase reactions using pNPP, mFP, and Cdk2-pTpY/CycA were performed as previously described (*20*). All k_{cat}/K_m reaction rate determinations were performed using at least two enzyme concentrations for reproducibility, each consisting of at least five time points to ensure linearity. The viscosity experiments required vigorous mixing by repipetting. The relative viscosities were deduced from sucrose concentrations as reported elsewhere (*22*, *23*). The SKIE experiments were performed in 95% D_2O as follows. The Cdk2-pTpY/CycA substrate was prepared daily in H_2O buffers as usual and subsequently concentrated to 5−10 μM by centrifugation for 30 min in an Ultrafree device from Millipore. Buffers were prepared in H_2O and D_2O in parallel, and the pH for the D_2O buffers was corrected according to the formula pD = meter reading + 0.4. Reaction mixtures containing Cdk2-pTpY/CycA were set up using buffers and BSA prepared in either H_2O or D_2O. Cdc25B was prediluted in the appropriate solvent and used to initiate the reactions. Transient-state kinetics were performed in a QFM-400 rapid quench apparatus from BioLogic. The volume of the reactants was 20 μL for each enzyme and the labeled Cdk2-pTpY/CycA substrate. The quench solution was 20 μL of 1 M HCl. The samples (15−30 different time points between 50 and 2500 ms and 0.5 and 12 min for WT and E474Q, respectively) were subsequently subjected to TCA precipitation and centrifugation as for the steady-state experiments. The equilibrium perturbation experiment was performed by preincubating Cdc25B and Cdk2-pTpY/CycA for 5 s (10 half-times) at varying pHs. The samples were then diluted 8−10-fold in 2× buffer at pH 6, and the amount of product formed was monitored as a function of time. The activity of α-naphthyl phosphate was measured by UV−vis spectroscopy at 324 nm using an extinction coefficient of 1480 M^{-1} cm^{-1}. The concentration of the enzyme was 1 μM, and the concentration of the substrate was varied from 100 to 500 μM. The inhibition studies of inorganic phosphate and α-naphthyl phosphate were performed by generating IC_{50} curves versus the substrate mFP. The mFP concentration was 25 μM; the enzyme was at 5 nM, and the inhibitors were at eight different concentrations in the range of 1−50 mM.

IAA Inactivation. IAA inactivation experiments were performed by directly monitoring the time-dependent loss of activity using mFP as a substrate at varying IAA concentrations (2−20 mM). The pH was varied using the three-component buffer as for the pH-dependent substrate kinetics with the exception that DTT was omitted. The data were fitted to eq 4 and corrected for non-active site-directed inactivation by subtracting the rate of inactivation seen in the presence of tungstate (100 μM), a competitive inhibitor of Cdc25B.

Substrate Depletion. One equivalent of Cdc25B was incubated with 1 equiv of Cdk2-pTpY/CycA at varying pHs. (The Cdk2 protein has an N-terminal six-His tag.) Half the sample was immediately quenched in Laemmli buffer. The second half of the sample was incubated with Ni−agarose beads for 5 min. The supernatant containing unbound species

was quenched with Laemmli buffer. Following SDS−PAGE and Western blotting, the samples were developed using an anti-Cdk2 antibody and an anti-Cdc25B antibody. The secondary antibodies, anti-mouse and anti-rabbit, respectively, were coupled to HRP, and the proteins were detected using chemiluminescence and autoradiography.

Equation Fitting. IC_{50} curves were fitted to eq 1 using Excel (Microsoft)

$$\% \text{ activity} = \frac{100\%}{1 + \left(\frac{[I]}{IC_{50}}\right)^{n_H}} \quad (1)$$

where [I] is the concentration of inhibitor and n_H is the Hill slope. The pH-dependent kinetics of Cdc25B were fitted to eq 2 or 3 using IGOR software (Wavemetrics).

$$v = \frac{C}{1 + \frac{[H]}{K_a}} \quad (2a)$$

$$v = \frac{C}{1 + \frac{K_b}{[H]}} \quad (2b)$$

$$v = \frac{C}{1 + \frac{[H]}{K_a} + \frac{K_b}{[H]}} \quad (3)$$

In eqs 2 and 3, C is the pH-independent value of k_{cat}, k_{cat}/K_m, or [S], [H] is the proton concentration, and K_a and K_b are independent ionization constants. The transient kinetics monitoring the formation of inorganic phosphate (P_i) were fitted to a first-order exponential using eq 4

$$\text{amount of } P_i = A(1 - e^{-kt}) \quad (4)$$

where A is the amplitude and k is the pseudo-first-order rate constant.

RESULTS

The E474Q mutant, previously prepared in the context of the Δ25B1 construct (the catalytic domain of Cdc25B lacking the last 17 residues) (*20*), was prepared in the context of the Δ25B2 construct consisting of the full catalytic domain that includes the 17 C-terminal residues. This mutant, designated E474Q hereafter, was compared to the Δ25B2 protein, hereafter designated as WT, using the substrates pNPP, mFP, and Cdk2-pTpY/CycA. As expected and as noted previously using the truncated proteins, there were no significant differences between the WT and E474Q mutant using the artificial substrates pNPP and mFP (data not shown). Also as expected, the E474Q mutant was found to be 200-fold less active than the WT using the Cdk2-pTpY/CycA substrate, despite having an unchanged pH−rate profile [Figure 1 (○ vs ●)]. The data were fitted to eq 3 without fixing either of the two pK_as. The fitted pK_as for Cys473 for both the WT and E474Q were found to be 5.9 ± 0.3, in good agreement with the pK_as determined previously using pNPP and mFP (*20*) and in good agreement with the pH-dependent inactivation of Cdc25B by the thiol modification

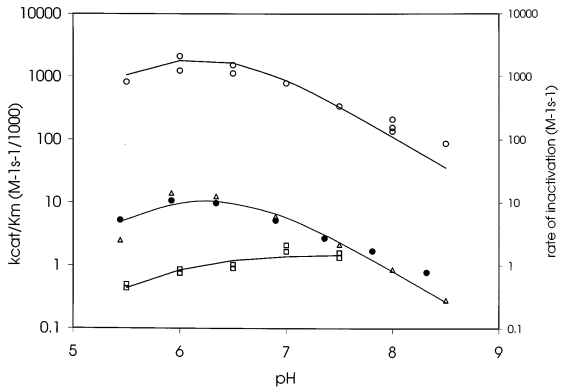


FIGURE 1: Shown using the left axis are the pH-dependent k_{cat}/K_m values of the reaction of the WT (○), E474Q (●), and E474A (△) with Cdk2-pTpY/CycA. The concentration of the enzyme was 0.87 nM for the WT and 61 nM for E474Q and E474A. The concentration of the substrate was 321 nM. The lines are generated by fitting the data to eq 3. Shown using the right axis is the pH-dependent inactivation of the WT by iodoacetic acid (□). The line was generated by fitting the data to eq 2a.

reagent iodoacetic acid (IAA) [Figure 1 (□)]. The fitted pK_as for the upper branch of the pH−rate profile, the phosphate, for both the WT and E474Q were found to be 6.5 ± 0.2 and 6.6 ± 0.2, respectively. These fits are visually and mathematically indistinguishable in comparison to fitting with three ionizations (two acidic and one basic) as previously reported (*20*). These fits do require a lowering of the pH-independent rate constant by factors of 2−3. Thus, values of (3.8 ± 0.5) × 10^6 and (1.9 ± 0.3) × 10^4 M^{-1} s^{-1} are found for the WT and E474Q, respectively. These results are in agreement with those found previously for the truncated E474Q construct[2] (*20*).

Deamidation of Gln and Asn Residues Has Been Observed in Many Proteins, both in Vitro and in Vivo (*24*). Rates of deamidation of individual amide residues vary widely from days to many years depending on the primary sequence and the three-dimensional structure as well as solution properties such as pH, temperature, ionic strength, and buffer ions. Given that the pH−rate profile for the E474Q mutant was identical to that of the WT and 200-fold slower overall [Figure 1 (●)], we considered the possibility that this was the result of the activity of 0.5% of the desired mutant deamidating back to the WT protein. By preparing the E474A mutant and observing essentially identical results [Figure 1 (△)], we have ruled out this possible artifact.

We attempted to determine a K_m for the Cdk2-pTpY/CycA substrate for the WT and E474Q enzymes at pH 6.0 and 8.5. The phosphorylated protein substrate denatures during concentration, which is why all previously reported experiments are k_{cat}/K_m experiments. We were able to achieve a concentration of 5−10 μM without perturbing the substrate, but were unable to observe significant saturation at pH 6.0 for either the WT or E474Q (data not shown). The assays at pH 8.5 showed similar nonsaturating behavior. To avoid further complications, all subsequent experiments were performed at 100−900 nM substrate.

The reaction of Cdc25B with Cdk2-pTpY/CycA was assessed under single-turnover conditions where there is a

[2] Note, the pH-independent rate constant reported in Table 4 of ref *20* was mistakenly reported as 42 000 ± 8400 M^{-1} s^{-1}. In agreement with Figure 4B and the actual result, it should be 4200 ± 840 M^{-1} s^{-1}.

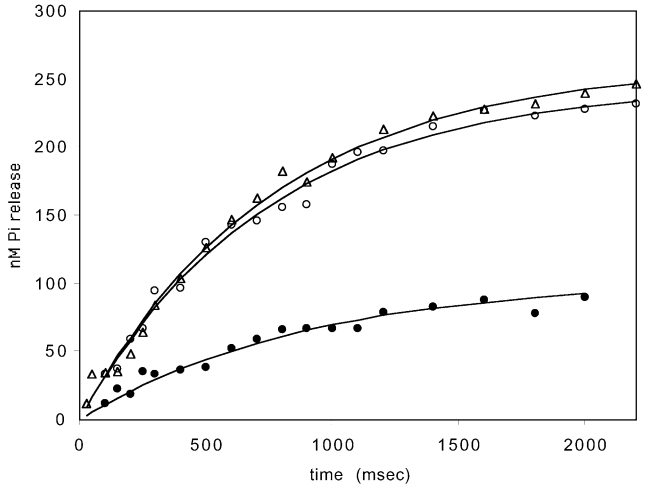


FIGURE 2: Single-turnover experiments of Cdk2-pTpY/CycA with the WT enzyme at pH 6.0. The concentration of the substrate was 300 (○ and △) or 150 nM (●), and the concentration of the enzyme was 10 (○ and ●) or 5 μM (△). The lines are generated by fitting the data to eq 4. The experiment shows that doubling the enzyme changes neither the rate *k* nor the amplitude *A*, whereas using half the substrate changes the amplitude *A* but not the rate *k*.

large excess of enzyme over substrate. As shown in Figure 2, at pH 6.0, the reaction can be fitted to a first-order exponential (eq 4). Both the amplitude *A* and the pseudo-first-order rate constant are independent of enzyme concentration. The fitted amplitude is approximately 1 equiv of substrate, as expected, whereas the rate constant is 1.2 ± 0.2 s^{-1}. This observation suggests that the rate-determining step in this reaction is formation of the phospho−enzyme intermediate, given that we have previously shown the rate of hydrolysis of the phospho−enzyme intermediate to be 1.8 s^{-1} (*20*). This result is consistent with the lack of an observed burst of product formation under reaction conditions at a high enzyme concentration (300 nM) and high substrate concentrations (1−5 μM) (data not shown). In contrast, varying substrate concentrations yield changes in the amplitude, but again no changes in the apparent first-order rate constant of 1.2 s^{-1} (Figure 2). Using the data derived from these single-turnover experiments at pH values between 5.5 and 8.5, there is no apparent pH dependence for the rate constant *k* for either the WT or E474Q (Figure 3). The apparent rate constant for the WT is 1.2 ± 0.2 s^{-1}, and the apparent rate constant for E474Q is 0.2 min^{-1}. Thus, E474Q is 300-fold slower than the WT in this assay, in good agreement with the 200-fold difference observed in the k_{cat}/K_m experiment. This implies that the chemical step or the rate of proton transfer of the enzyme-catalyzed reaction is not pH-dependent for either the WT or E474Q (see the Discussion). However, there is a pH dependence for the total amplitude *A* for both the WT and E474Q (Figure 3). The pH dependence of the amplitude *A* was fitted to eq 2b and yielded pK_as of 7.4 ± 0.2 for the WT and 7.6 ± 0.2 for E474Q.

The dissociation of the bisanionic enzyme−substrate complex to yield a functioning substrate was followed using a perturbation-of-equilibrium experiment. Following the preincubation of Cdc25B with Cdk2-pTpY/CycA at varying pHs and subsequent dilution into buffer at pH 6, the amount of product formed as a ratio of total substrate was monitored (Figure 4). Prior to dilution (after 10 half-lives), 1 equiv of

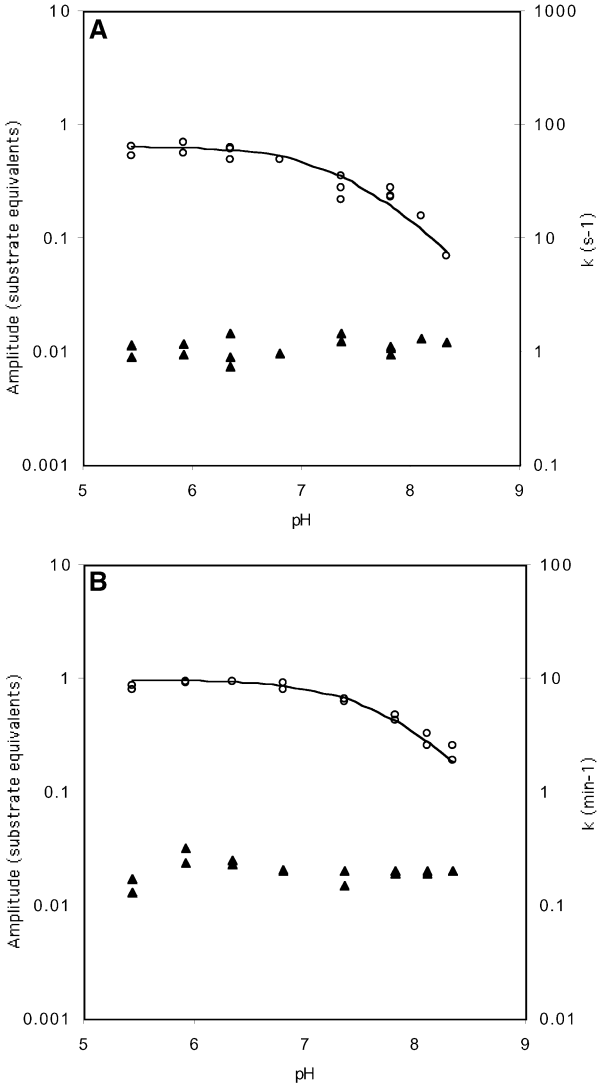


FIGURE 3: pH-dependent behavior of the amplitude (○) and the first-order rate constant (▲) for the WT (A) and E474Q (B) reacting with Cdk2-pTpY/CycA under single-turnover conditions. The experimental conditions are as described in the legend of Figure 2 with the added variation of pH. The lines were generated by fitting the data to eq 2b.

substrate is converted to product at pH 6, with less conversion at progressively higher pHs, consistent with the amplitude dependence seen in Figure 3. Following dilution, all of the substrate was converted to product, consistent with the dissociation of the bisanionic substrate and the subsequent reaction of the monoprotonated substrate. The formation of >1 equiv arises from the reaction of the second phosphate (the phosphotyrosine) on the Cdk2-pTpY/CycA substrate.

The formation of a tight-binding nonproductive enzyme−substrate complex at high pH was shown using a substrate depletion experiment (Figure 5). Equimolar amounts of Cdk2-pTpY/CycA were incubated with Cdc25B at varying pHs. Most of the Cdk2-pTpY/CycA substrate was then removed from this solution following incubation with Ni−agarose beads and brief centrifugation. At low pH values, the amount of Cdc25B in the remaining supernatant is essentially identical to the amount originally present. In contrast, at elevated pHs, pH 9 in particular, there is significant depletion of the Cdc25B from the supernatant, indicating formation of a tight-binding enzyme−substrate

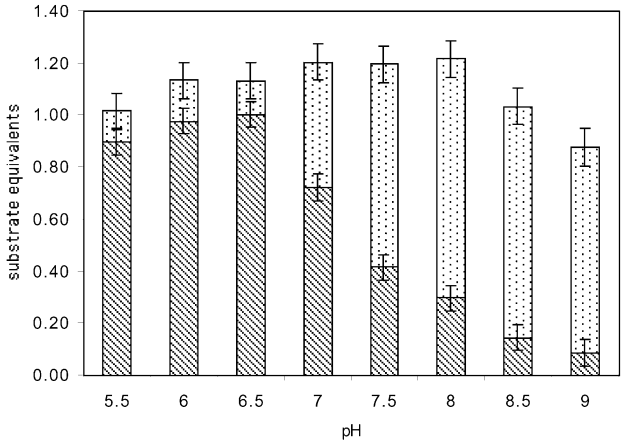


FIGURE 4: Equilibrium perturbation experiment. Excess enzyme (10 μM) was incubated with Cdk2-pTpY/CycA (1 μM) for 5 s (10 half-lives). A sample was removed and subjected to TCA precipitation for purposes of product determination prior to perturbation (cross hatched). Within 10 s, the solution was diluted 8-fold to a final pH of ~6. Subsequently, aliquots were removed and quantitated for phosphate release (dotted). All samples were assessed in duplicate, and the results are the averages of two separate experiments.

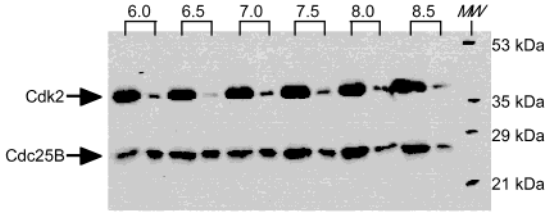


FIGURE 5: Western blot of the substrate depletion experiment. Equimolar mixtures of Cdc25B and Cdk2-pTpY/CycA were incubated at varying pHs as indicated and subjected to substrate depletion using Ni−agarose affinity chromatography. Shown in alternating wells are the initial amounts (first well) of Cdk2 and Cdc25B compared to the amounts remaining following substrate depletion (second well). The molecular mass (MW) markers are also indicated. Note the reduction in the level of Cdc25B in the depleted samples in comparison to the initial amounts at elevated pH (7−8.5).

complex. The lack of complete depletion of Cdc25B is attributed to the turnover of substrate (half-life is 0.5 s), the finite off-rate of the substrate, and the duration (~6 min) of the substrate depletion experiment.

The pH-dependent activity and inhibition kinetics of α-naphthyl phosphate were measured under k_{cat}/K_m conditions in a pH-dependent manner (Figure 6). The activity of α-naphthyl phosphate was monitored by UV−vis absorbance at 324 nm. The inhibition kinetics were measured by performing IC_{50} experiments using the substrate mFP to monitor the reaction. The activity of α-naphthyl phosphate displayed a pH dependence very similar to that seen for the protein substrate (apparent pK_a = 6.4 ± 0.2). However, the IC_{50}s did not show any pH dependence. Although the E474Q mutant shows no measurable activity with α-naphthyl phosphate, this compound did inhibit E474Q with a similar K_i in a pH-independent manner. Additionally, inorganic phosphate inhibits the WT and E474Q with a K_i of 18.7 ± 3.5 mM, also in a pH-independent manner (data not shown). Interestingly, although both α-naphthyl phosphate and inorganic phosphate have pK_as in the range of the assay (6.0−6.9), they display no pH dependence in their inhibition of Cdc25B.

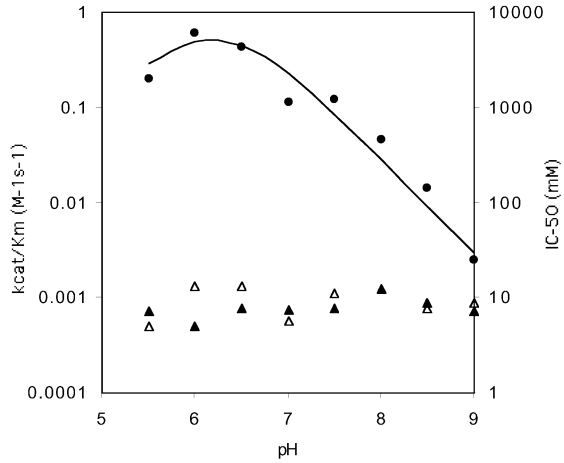


FIGURE 6: pH-dependent activity and inhibition by α-naphthyl phosphate. The activity of α-naphthyl phosphate was monitored under k_{cat}/K_m conditions for the WT enzyme (●), and the data were fitted to eq 3. There was no detectable activity with the E474Q enzyme. The IC_{50}s for α-naphthyl phosphate were determined vs the substrate mFP for both WT (▲) and E474Q (△) enzymes.

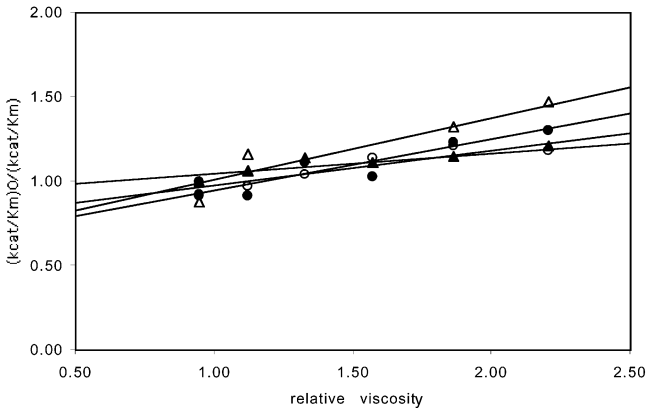


FIGURE 7: Viscosity dependence of the WT and E474Q plotted as the ratio of the observed rate at a given viscosity to the rate observed in the absence of sucrose. The experiments with the WT at pH 6.0 (●) and 8.5 (○) are contrasted with those of E474Q at pH 6.0 (▲) and 8.5 (△). The concentration of the WT was 0.42 and 11 nM at pH 6.0 and 8.5, respectively. The concentration of E474Q was 31 and 775 nM at pH 6.0 and 8.5, respectively. The concentration of Cdk2-pTpY/CycA was 480 nM for all assays.

The viscosity dependence of the reaction of Cdc25B with Cdk2-pTpY/CycA was determined using increasing concentrations of sucrose at both pH 6 and 8.5. The data from the k_{cat}/K_m experiments are plotted as a ratio of the observed rate compared to that of the sucrose-free reaction. As shown in Figure 7, there is little dependence on viscosity for either the WT or E474Q enzyme at either pH.

The solvent kinetic isotope effects (SKIEs) in 90% D_2O for the k_{cat}/K_m of Cdc25B reacting with Cdk2-pTpY/CycA were determined as a function of pH (pD) (Figure 7). Data were fitted to eq 3. For the WT, the pK_a for Cys473 in H_2O was found to be 5.9 ± 0.2, whereas the pK_a for the phosphate was found to be 6.6 ± 0.3, in good agreement with the results of the experiment shown in Figure 1. When the WT enzyme was assayed in 90% D_2O, the pK_as for the cysteine and the phosphate moved to higher values (6.0 ± 0.2 and 6.9 ± 0.2, respectively). Noticeably, the pH-independent rate constant increased by a factor of 2.2, from (3.5 ± 0.5) × 10^6 to (7.7 ± 0.7) × 10^6 M^{-1} s^{-1}. For E474Q, the pK_a for Cys473 in H_2O was found to be 5.7 ± 0.3, whereas the pK_a for the

phosphate was found to be 6.4 ± 0.2, again in good agreement with the results of the experiment shown in Figure 1. When E474Q was assayed in 90% D_2O, the pK_as for the cysteine and the phosphate again moved to higher values (6.0 ± 0.3 and 6.9 ± 0.2, respectively). In contrast to the WT, however, the E474Q enzyme did not show a corresponding increase in the pH-independent rate constant [(2.1 ± 0.2) × 10^4 vs (2.3 ± 0.2) × 10^4 M^{-1} s^{-1}]. Control experiments using the artificial substrates pNPP and mFP do not show a SKIE for either k_{cat} or k_{cat}/K_m at pH 6.0 or 8.5 (data not shown).

DISCUSSION

The goal of the work described in this paper is to understand the detailed chemical and kinetic mechanism of Cdc25B, particularly with regard to identifying the putative catalytic acid and understanding the role of Glu474 in catalysis. The reaction catalyzed by Cdc25 is a simple one, namely, the transfer of a phosphate from a phosphoamino acid to water. On the basis of the precedent from other PTPases and DSPs and the crystal structure of Cdc25B, the bisanionic phosphate of the substrate is proposed to bind in the loop of amide backbones and the bidentate arginine created by the HCX_5R motif. The reaction is proposed to proceed via a phospho−cysteine intermediate (Figure 9). This mechanism is supported by mutagenesis studies that show replacement of the catalytic Cys (Cys473 in Cdc25B) with Ser yields a protein with no phosphatase activity (*25*). It is also supported by pH-dependent kinetics that show the apparent pH dependence of the cysteine using two different substrates in both steady-state kinetics and rapid quench kinetics (*20*), by the pH-dependent inactivation of Cdc25B by iodoacetic acid (Figure 1), and by the crystal structures of the catalytic domain (*18*, *19*). On the basis of the precedent from other PTPases and DSPs and on the basis of the bell-shaped pH dependence with its protein substrate Cdk2-pTpY/CycA, the reaction of Cdc25B has also been presumed to require a catalytic acid for the protonation of the leaving group. So far, attempts to identify the catalytic acid definitively have been unsuccessful (*20*). On the basis of the lack of direct evidence that Cdc25 uses the bisanionic substrate and the apparent nonexistence of the catalytic acid, we have re-evaluated the applicability of this commonly accepted mechanism for Cdc25.

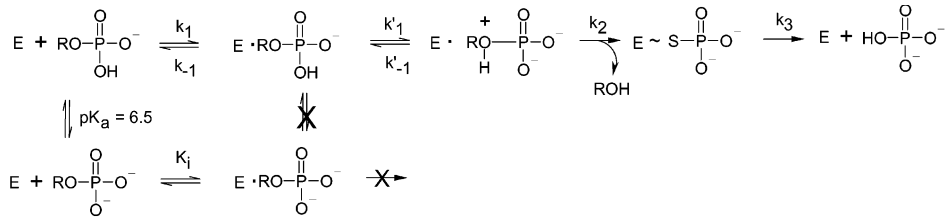
Theoretically, substitution reactions at tetrahedral phosphate esters can occur by three limiting mechanisms, associative (S_N2-like), dissociative (S_N1-like), and concerted. Nonenzymatic phosphate ester hydrolysis in solution is largely a dissociative process with a metaphosphate-like transition state (Figure 9) (*26*, *27*). Kinetic measurements using specifically isotopically substituted pNPP substrates have demonstrated that a dissociative transition state is also found experimentally for many protein phosphatases, including the *Yersinia* PTPase (*28*), the *Vaccinia* dual-specificity phosphatase VHR (*29*), and the MAP kinase phosphatase (*30*). Most recently, a dissociative-type mechanism for the substrate pNPP has also been found for Cdc25A (*31*). However, pNPP is a poor substrate for Cdc25 with an unnaturally low pK_a for the leaving group (pK_a = 7.14) that may proceed through a different mechanism when better substrates are used. Indeed, Zhang and co-workers have proposed more than one mode of catalysis for Cdc25, which depends on the pK_a of the leaving group and the conformation of their proposed catalytic acid Glu431 (Glu474 in Cdc25B). A change in mechanism for differing substrates may be especially relevant when the substrate is 6 orders of magnitude more reactive, in particular, the protein substrate Cdk2-pTpY/CycA (*21*).

However, a dissociative mechanism of phosphate ester hydrolysis does not a priori require a bisanionic phosphate as a substrate (*32*). The hydrolysis of monoprotonated phosphate esters can also proceed via a dissociative mechanism, wherein the proton on the phosphate is transferred to the leaving group either prior to or during formation of the metaphosphate-like transition state (Figure 9). As a matter of fact, the reactions of monoprotonated phosphates with pK_as of the leaving group of >6.5 have been shown to be 10−100-fold faster than for the corresponding bisanionic substrates (*32*). That is, the monoprotonated phosphate ester is intrinsically more reactive than the bisanionic phosphate ester. Although the rate of proton transfer from the phosphate to the leaving group can contribute to the rate-determining step of the reaction, it does not necessarily do so. This seems to be especially true when proton transfer precedes phosphate ester hydrolysis, as is expected for substrates with elevated leaving group pK_as (*32*). Thus, a mechanism utilizing a monoprotonated phosphate with proton transfer to the leaving group preceding phosphate ester hydrolysis is a viable alternative mechanism for Cdc25 (Figure 9). This alternative mechanism is especially relevant for the threonyl and tyrosyl leaving groups of the protein substrate Cdk2-pTpY/CycA, the leaving groups of which have pK_as of ∼14 and ∼10, respectively.

In this paper, we have accumulated evidence which shows that the Cdc25B-catalyzed phosphorolysis of protein substrates proceeds via a mechanism using a monoprotonated phosphate with an apparent pK_a of 6.5. This mechanism provides an explanation for the pH dependence observed in k_{cat}/K_m experiments using Cdk2-pTpY/CycA representing the ionization state of the free enzyme and the free substrate. Thus, we propose that the rate of the reaction at high pH values is slower than at low pH values due to pH-dependent substrate depletion and not due to the loss of a proton on the enzyme. Note, this working model for the chemical mechanism of Cdc25 is significantly different from that proposed for other PTPases or DSPs and has important consequences for inhibitor design (Figure 9). The experimental results presented below provide support for this model.

The first litmus test that this new mechanism must pass is that our previously published pH dependence of Cdc25B's activity with Cdk2-pTpY/CycA that was fitted to three ionizations (two bases and one acid) should fit to two ionizations (one acid and one base) (*20*). The original fits included as relevant ionizations the catalytic cysteine as a thiolate, the bisanionic phosphate, and the putative catalytic acid. In these fits, we had fixed the pK_a of the catalytic cysteine at 5.9 based on the pH dependence of the Cdc25 reaction with pNPP and mFP. Additionally, we had fixed the pK_a of the bisanionic phosphate at 6.0 based on the expected pK_a of phosphothreonine. We have now refitted the data to two ionizations (one acidic and one basic) without fixing any of the pK_as (Figure 1). This procedure yields an apparent pK_a of 5.9 ± 0.3 for the catalytic cysteine and an

Scheme 1



apparent pK_a of 6.5 ± 0.2 for the right-hand branch of the pH profile (Figure 1). This value is in reasonable agreement with the expected pK_a of 6.0 for the phosphothreonyl substrate that we are assaying in the reaction using Cdk2-pTpY/CycA. The apparent perturbation of the expected pK_a of the phosphothreonine from 6.0 to 6.5 may be due to the neighboring phosphate on Tyr15 or other amino acids located near the phosphothreonine residue in the Cdk2-pTpY/CycA complex. The curves newly fitted to two ionizations fit equally as well as the published curves using three ionizations. This is mainly because of the sparse amount of data we are able to obtain below pH 5.5 due to the instability of Cdc25 under acidic conditions. Cdc25, unlike PTP1b and the *Yersinia* phosphatase, cannot be assayed in the pH range of 4−5, the source of the data that strongly support a bisanionic phosphate as a substrate for these other phosphatases (*33*, *34*). Thus, the pH dependence of the protein substrate for Cdc25B is consistent with the new model where the origin of the pK_a of 6.5 in the k_{cat}/K_m profile is generated by the phosphate on the substrate (Figure 9). However, this observation alone does not distinguish between a bisanionic or a monoprotonated substrate, and thus, we have pursued this question further using steady-state and single-turnover kinetic experiments.

If the pH dependence of the k_{cat}/K_m is due to pH-dependent substrate titration, one expects to observe a pH-dependent K_m and a pH-independent k_{cat}. That is, the acidic branch of a bell-shaped profile could theoretically arise if the apparent K_m for the substrate is *elevated* at high pH versus that at low pH. To test this possibility, we attempted to measure the K_m for Cdk2-pTpY/CycA at pH 6.0 and 8.5 for both WT and E474Q enzymes. At pH 6.0, there is incomplete saturation at 5 μM, the highest achievable concentration (data not shown). This would suggest an even higher and also undeterminable K_m at pH 8.5 if the k_{cat}/K_m effect is due to a difference in the apparent K_m. Thus, these K_m measurements are not informative with regard to the novel mechanism using a monoprotonated substrate.

To address our proposed mechanism more directly, we monitored the reaction of Cdc25B with Cdk2-pTpY/CycA using transient-state kinetics. In a single-turnover experiment at concentrations of enzyme in significant excess over substrate, the depletion of substrate (formation of product) will follow pseudo-first-order kinetics (*35*). If the rate of substrate binding is limiting, then this exponential is defined by the pseudo-first-order rate constant k_1[E] and is dependent on enzyme concentration (Scheme 1). If instead the rate of the chemical reaction is limiting, then the exponential is defined by the first-order rate constant k_2 and is independent of enzyme concentration. (For simplicity, we assume a simple model for the WT enzyme where k_1' is kinetically silent; e.g., $k_1' \gg k_2$.) Either way, the observed rate will be independent of substrate concentration, whereas the amplitude of the observed reaction will be dependent on substrate concentration. As shown in Figure 2, this is exactly what we observe for the single-turnover experiment of Cdc25B with Cdk2-pTpY/CycA. At pH 6.0, a doubling of the enzyme concentration yields the same apparent amplitude and the same apparent rate constant. On the other hand, using half the amount of substrate only leads to half the amount of product formed, again with the same apparent rate constant. In next performing these experiments in a pH-dependent manner, we observe the apparent rate constant for the exponential fit is not pH-dependent, whereas the amplitude is pH-dependent (Figure 3). The pH dependence of the amplitude was fitted to eq 2 to yield a pK_a of 7.5 ± 0.2. Although this is 1 log unit higher than the apparent pK_a in the k_{cat}/K_m experiment (see below), these data strongly suggest that the rate of chemistry (k_2) and the concentration of enzyme are independent of pH. As a consequence, the concentration of the substrate must be dependent on pH. Thus, performing assays at high pH under k_{cat} (single-turnover) conditions depletes the concentration of the available substrate, effectively lowering the observed amplitude of product formation but not affecting the rate. Likewise, performing assays at high pH under k_{cat}/K_m conditions depletes the concentration of the available substrate, effectively slowing the observed rate and yielding the pH−rate profile shown in Figure 1. Therefore, the pH dependence of this one-turnover experiment supports our proposed novel mechanism involving a monoprotonated phosphate on the Cdk2-pTpY/CycA substrate.

These transient-state kinetics raise an important issue. If the monoprotonated substrate is siphoned off by reacting it with Cdc25B, one might expect a perturbation in the equilibrium between the monoprotonated and bisanionic substrate, either bound to the enzyme or free in solution (Scheme 1). Additionally, as the re-establishment of equilibrium involves a simple proton transfer from water, one would expect this reaction to be much faster than any other step in the enzymatic process. Thus, rapid proton transfer should effectively replenish the depleted monoprotonated substrate essentially instantaneously at elevated pHs and yield equivalent amounts of consumed substrate at all pHs. However, given that we do observe pH dependence of the amplitudes of the formed product (Figure 3), we propose that the bisanionic substrate can bind to Cdc25B in a tight nonproductive complex that is not able to accept a proton from solution (Scheme 1). This type of tight-binding and nonproductive complex is reminiscent of the so-called substrate trapping mutants that have been generated for many cysteine phosphatases. Mutation of the catalytic cysteine to a serine or an alanine or mutation of the catalytic acid to an alanine has yielded tight enzyme−substrate complexes that are useful for the identification of native protein substrates [e.g., PTP1b (*36*, *37*)], identification of cellular functionality

[e.g., PTEN (*38*)], and cocrystallization of enzyme−substrate complexes [e.g., VHR (*39*)]. Cysteine to serine mutants of the Cdc25s have been used previously to capture protein substrates (*25*). It now appears that Cdc25B can form such a substrate-trapping mode at elevated pHs (see further evidence below). Slow reaction from such a nonproductive complex to yield product or slow release of substrate followed by protonation and subsequent reaction with Cdc25B can lead to formation of product. This may be the origin of a systematic deviation of the data which yields an observed pK_a of 7.5 instead of the expected value of 6.5. That is to say, at any given pH, there is a greater amount of product formed (larger amplitude) than expected if the reaction were to proceed solely by the mechanism in the top line of Scheme 1. However, the rate of catalysis from the bisanionic complex is sufficiently slow and/or the binding of the bisanionic substrate is sufficiently tight under the reaction conditions to allow the pH dependence of the amplitudes to be observed in Figure 3.

Given that the proposal of a tight-binding, nonproductive enzyme−substrate complex at high pH is essential to the interpretation of our results and our novel mechanism, we have performed a series of experiments to test this claim further. First, we have ruled out a nonreversible pH-dependent perturbation of the Cdk2-pTpY/CycA substrate by preincubating high concentrations of the substrate (4 μM) at pH values between 6 and 9 followed by dilution (10-fold) and assay at pH 6. These results, under both steady-state and one-turnover conditions, yielded identical rates that were independent of the pH of the preincubation (data not shown). Second, we have performed an experiment using a perturbation from equilibrium method that demonstrates that the nonproductive complex is not irreversible and can be rescued. By preincubating Cdc25B and Cdk2-pTpY/CycA at varying pHs, and then diluting the samples to pH 6, we can demonstrate that all of the substrate is now catalytically competent to form product (Figure 4). Thus, the nonproductive complex formed at elevated pHs is not stable at low pHs, and the dissociation rate must be relatively rapid as stoichiometric formation of product is observed as fast as we are able to measure (<20 s). We do not believe that protonation of the bisanionic nonproductive complex can take place without dissociation, or else such a protonation would also occur in our single-turnover experiments, yielding equivalent product formation at all pHs.

We have tested the concept of a substrate-trapping mode for Cdc25B at elevated pHs using a substrate depletion experiment. Incubation of Cdc25B with Cdk2-pTpY/CycA followed by substrate depletion using the six-His tag on Cdk2 yielded noticeable reduction of Cdc25B at high pHs (Figure 5). Given the half-life of the Cdc25B reaction (0.5 s) and the expected finite lifetime of the enzyme−substrate complex, we consider the depletion of any WT enzyme by a fully active substrate an amazing observation and direct evidence for the existence of the complex.

Additional strong support of nonproductive binding of the bisanionic substrate to Cdc25 comes from another series of experiments. First, inhibition studies using inorganic phosphate and competing substrates such as α-naphthyl phosphate are pH-independent across the pH range of 5.5−9.0 (Figure 6). Although the binding of these inhibitors or competing substrates is weak (millimolar), their pH-independent behavior as inhibitors is observed in the range where ionizations of these compounds take place (pK_as = 6−7). The other experiment in support of nonproductive binding of substrate comes from inhibition studies using the unphosphorylated Cdk2−CycA complex. At 900 nM Cdk2-pTpY/CycA, addition of 75 μM Cdk2−CycA complex yields 50% inhibition (data not shown). This suggests some affinity of the unphosphorylated Cdk2−CycA complex for the phosphatase. Thus, although we have been unable to measure the K_i for the bisanionic phosphate form of Cdk2-pTpY/CycA directly, we believe that it is in a range relevant to the conditions of our experiments.

Having obtained significant evidence that Cdc25B utilizes a monoprotonated substrate, we next turned to the question of other catalytically important residues. When the substrate contains a monoprotonated phosphate, there is no longer a requirement for a catalytic acid as found for other cysteine phosphatases (Figure 9). In contrast, it is a catalytic base that may be expected to play an important role, either directly or through a bridging water molecule. We thus turned to more steady-state and transient kinetic experiments to address the potential role of Glu474 as a catalytic base in the Cdc25B reaction with Cdk2-pTpY/CycA. We have previously shown that E474 is an important residue as the E474Q mutant has 200-fold less activity, albeit with the same pH dependence as the wild-type enzyme (*20*). We have confirmed this result, in terms of both pH dependence and 200-fold reduction in activity, in the context of the full-length catalytic domain (Figure 1). In the traditional mechanism of PTPases and DSPs, this implies that Glu474 cannot be the catalytic acid, as the pH−rate profile should become pH-independent between pH 6 and 9. In our novel mechanism using the monoprotonated phosphate as a substrate, there is no longer a role for a traditional catalytic acid. We propose that Glu474, as a catalytically important residue located within the active site, can either directly promote the transfer (pathway b, Figure 9) or simply stabilize the proton on the leaving group prior to formation of the transition state (pathway a, Figure 9). As described below, we have obtained evidence that supports the idea that Glu474 is the catalytic base in the formation of the transition state in the Cdc25-catalyzed reaction.

We first examined the role of substrate concentration as determined by pH in a transient kinetic experiment in the presence of excess E474Q over substrate. Just as for the WT, the observed rate of the reaction was independent of the enzyme concentration at all pHs (Figure 3). This again implies that enzyme−substrate association is not the rate-determining step. One can propose that the E474Q mutant has a new rate-determining step, namely, the rate of proton transfer (k_1') (Scheme 1). A mechanism with k_1' as the new rate-determining step is expected to follow the same single-turnover kinetics as the WT enzyme, where k_2 is the rate-determining step. Thus, for E474Q, as for the WT, the experiments performed at low enzyme concentrations yield pH-dependent k_{cat}/K_m values (Figure 1) and the observed rates of reaction at high enzyme concentrations become independent of pH (Figure 3). These results are consistent with our model that the observed pH dependence in the k_{cat}/K_m experiments arises from substrate depletion.

We next returned to steady-state k_{cat}/K_m experiments to examine further the role of binding and/or proton transfer

in the rate-determining step of the reaction of E474Q with Cdk2-pTpY/CycA. We first confirmed our single-turnover results using viscosity dependence to show that enzyme−substrate association was not rate-determining for either the WT or E474Q. Many enzymes that use proteins as substrates have been shown to exhibit slow association rates (*40*), and thus, it was important to provide additional support to the single-turnover results shown above. Using sucrose as a microviscogen, we observed only a small and essentially identical dependence on sucrose at both pH 6.0 and 8.5 for both the WT and E474Q (Figure 7). We do not consider these effects significant given that the rate of the reaction, which is quite sensitive to ionic strength and buffer selection (data not shown), is maximally only 30% depressed at the highest concentration of sucrose corresponding to 0.9 M.

Since enzyme−substrate association does not appear to be rate-determining, we next attempted to address the question of proton transfer in the rate-determining step of Cdc25 with its Cdk2-pTpY/CycA substrate. Many enzymes that transfer a solvent exchangeable proton as part of the rate-determining step will show a solvent kinetic isotope effect (SKIE) between 1.7- and 4-fold (*41*). Given that we propose a proton transfer from the phosphate to the leaving group, we considered it possible that we would observe a normal SKIE, particularly for the E474Q mutant lacking the proposed catalytic base. In general, SKIEs can be complicated by the fact that the pK_as of any critical ionizations on either the enzyme or the substrate will shift in a pH-dependent manner. Also, given that one has replaced all the exchangeable protons on both the enzyme and the substrate with deuterium, normal SKIEs (i.e., slower rates of reaction in D_2O vs H_2O) must be approached with caution as they can arise from numerous other nonspecific causes that tend to slow rates of reaction. Instead of observing a normal SKIE, however, we have observed an inverse SKIE for the WT enzyme (Figure 8A). The increased rate in D_2O was accompanied by the expected shift in apparent pK_as by ~0.3 for the Cys473 and ~0.5 for the phosphate. These data suggest that proton transfer is not part of the rate-determining step in the reaction with the WT, but rather that the ionization of the catalytic Cys473 plays a critical role. Inverse SKIEs are rarely found and are only associated with cysteine residues, metal ion sites, or low-energy hydrogen bonds (*41*). Cysteine is the only amino acid with an inverse fractionation factor in the ground state that will generate an inverse SKIE. Given the presence of the catalytic Cys473, it seems reasonable to suggest that the observed inverse SKIE arises from this residue. When we repeat the SKIE experiment with the E474Q mutant, we still observe the expected perturbation of the pH dependence to higher values, but there is no longer a detectable absolute inverse SKIE (Figure 8B). Given the similarity in the kinetics of all the experiments described so far between the WT and the E474Q mutant, the simplest explanation for this observation is that a normal SKIE now obscures the inverse SKIE observed for the WT enzyme. That is, proton transfer (k_1') in the E474Q mutant now becomes part of the rate-determining step, thereby obscuring the inverse SKIE caused by the catalytic cysteine (Scheme 1 and Figure 9). This experiment thus provides evidence that Glu474 is involved in the proton transfer step from the monoprotonated phosphate to the leaving group.

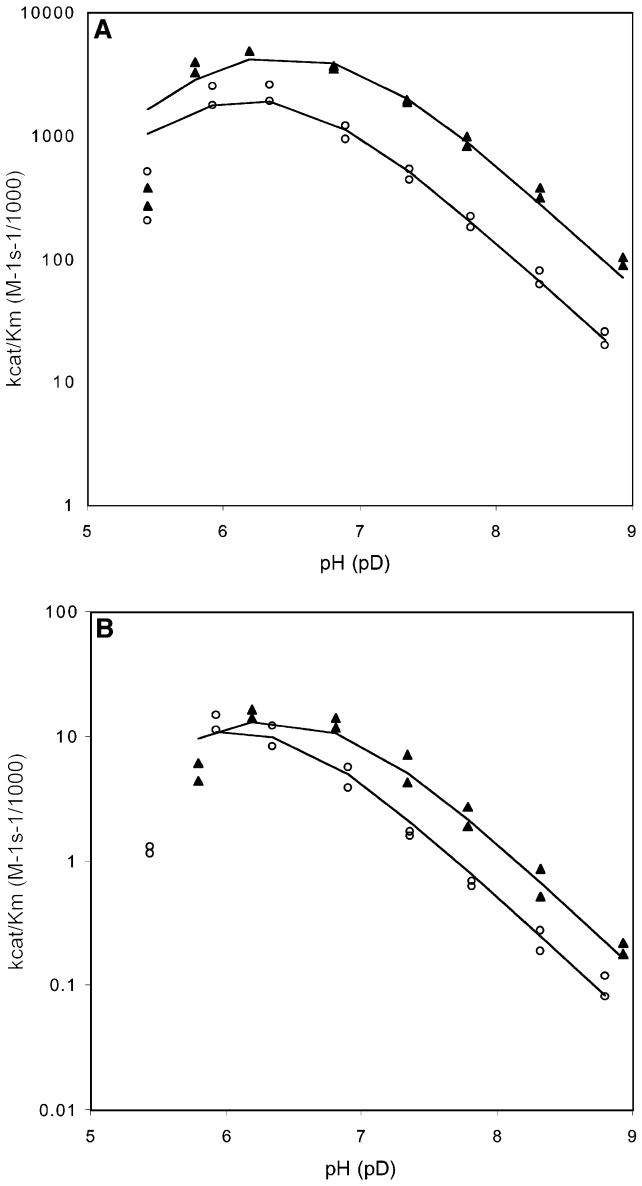


FIGURE 8: SKIE for the k_{cat}/K_m of the WT (A) and E474Q (B) plotted as a function of pH. The results of the experiments in H_2O (○) are contrasted with the results of the experiments containing 95% D_2O (▲). The concentration of WT and E474Q enzymes was 0.3−3 nM and 0.13−0.5 μM, respectively. The concentration of Cdk2-pTpY/CycA was 321 nM. The fitted lines are generated by fitting the data to eq 3.

Recently, Zhang and co-workers have described the catalytic mechanism of Cdc25A using solely artificial substrates (*31*). Using ^{18}O and ^{15}N isotope effects with pNPP as the substrate, they conclude that the Cdc25A-catalyzed reaction has a dissociative transition state, like all other cysteine phosphatases tested thus far. By comparing reactions where the pK_a of the leaving group on the substrate is low (e.g., pNPP and mFP) to ones where the pK_a of the leaving group on the substrate is high (e.g., α-naphthyl phosphate), they conclude that the reaction can proceed with or without acid catalysis. Using mutagenesis, they suggest that Glu431 (Glu474 in Cdc25B) plays the role of the traditional catalytic acid of dual-specificity phosphatases for substrates whose leaving group pK_as are high (>8.05). Unfortunately, all but one of these artificial substrates have no measurable activity with this mutation, in agreement with our observation that

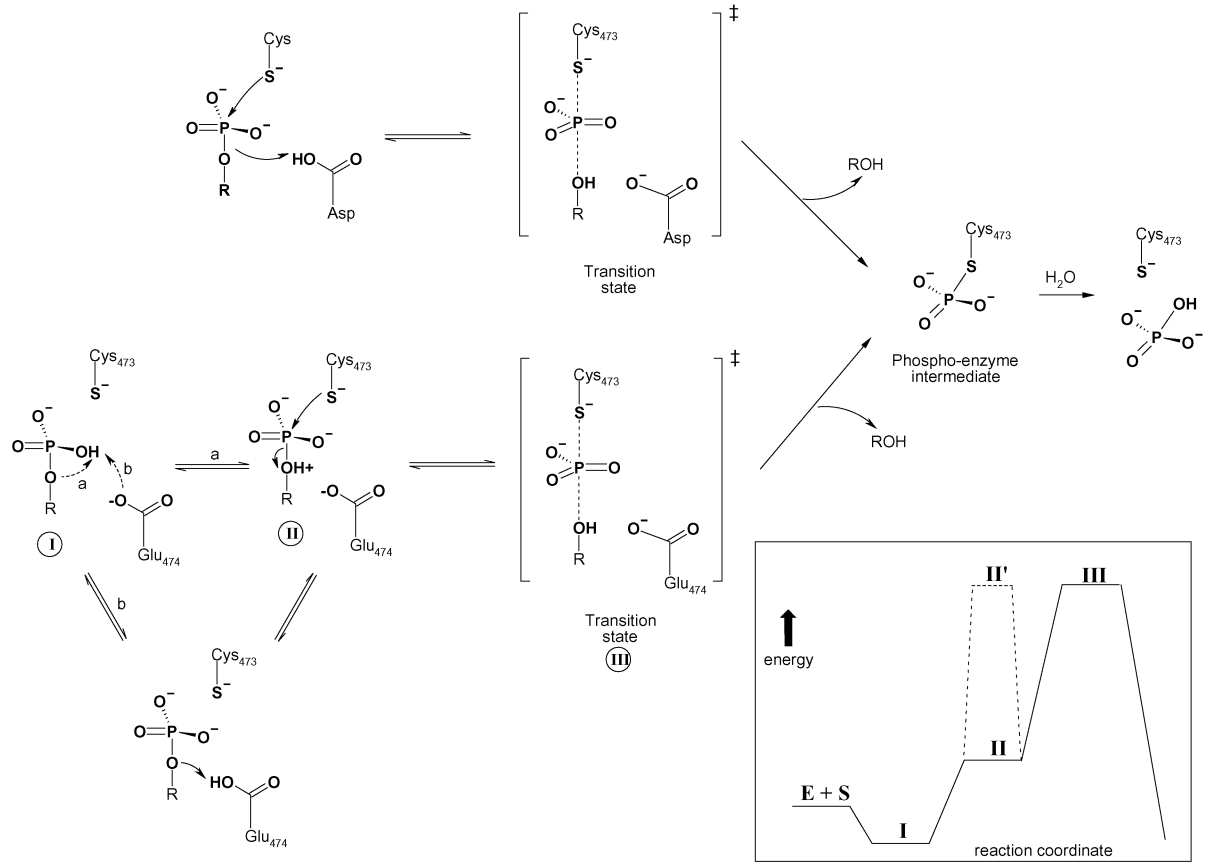


FIGURE 9: Reaction mechanisms for Cdc25. The dashed lines in the structures indicate bonds with low bond order. The top reaction shows the classic mechanism using a bisanionic substrate leading to a meta-phosphate-like transition state with a leaving group that was protonated by the enzyme. The bottom reaction shows the novel mechanism using a monoprotonated substrate, leading to the same meta-phosphate-like transition state where the leaving group is protonated by the substrate. Pathway a in this mechanism involves spontaneous proton transfer with stabilization of the protonated leaving group by Glu474. Pathway b proceeds by direct involvement of Glu474 in catalyzing the transfer of the proton from the phosphate to the leaving group. At present, we cannot distinguish between pathways a and b. The inset shows a reaction coordinate that is useful in understanding the reaction mechanism of the monoprotonated substrate in conjunction with Scheme 1. The dotted line indicates the putative effect of the E474Q mutation on the reaction kinetics with Cdk2-pTpY/CycA. The relative level of the II state energetically compared to I and III is not known.

the E474Q mutant has no activity using α-naphthyl phosphate. The most likely explanation for this lack of activity in the context of our proposed mechanism is that the E431Q mutant cannot catalyze proton transfer from the phosphate to the leaving group. Therefore, to remain in agreement with our data using the Cdk2-pTpY/CycA substrate, these artificial substrates should have 200-fold less activity and an unchanged pH−rate profile. Such a low activity is below the detection limit for these artificial substrates ($k_{cat}/K_m = 1\ M^{-1}\ s^{-1}$), and thus, finding no activity for the E431Q mutants is not surprising and points out one of the limitations of these substrates. Notably, the one substrate that does retain some activity with this mutant (4-acetylphenyl phosphate) has a borderline pK_a for its leaving group of 8.05 and an unusual pH dependence, and possibly undergoes a non-acid-catalyzed reaction with the E431Q mutant. Overall, our work using a more relevant and more reactive substrate has allowed us to propose and provide evidence for a novel mechanism for the Cdc25 phosphatases.

In conclusion, our experiments involving the basic mechanism of the Cdc25B phosphatase allow us to derive important insights into the mechanism of Cdc25 phosphatases. Cdc25 apparently uses a mechanism substantially different from those of other phosphatases in its preference for a monoprotonated substrate and the use of a catalytic base instead of a catalytic acid. Using single-turnover kinetics, we have provided strong evidence that the substrate provides the proton to the leaving group. Whereas having the phosphate as the source of this proton is not the only possible explanation for our data, we believe this to be the simplest and most likely explanation. Alternatively, an amino acid located on the protein substrate could provide the proton to the leaving group of a bisanionic substrate, in a manner analogous to that of the catalytic Asp located on the enzyme for other PTPases. The primary difficulty with this model is that the Cdk-pTpY/CycA substrate is bisphosphorylated and Cdc25 removes both of these phosphates. Thus, such a mechanism would require the proper orientation of a catalytic acid residue on the protein substrate for two different leaving groups, one for Thr14 and one for Tyr15.

A possible reason for Cdc25's use of a bisanionic instead of a monoanionic substrate lies in its strong preference for phosphothreonine (pK_a of the leaving group = 14) versus phosphotyrosine (pK_a of the leaving group = 10). In this respect, Cdc25 differs from other other well-characterized DSPs. For example, VHR appears to greatly prefer aromatic phosphates over threonyl phosphates (*42*). Thus, given the higher intrinsic reactivity of monoprotonated phosphate esters in comparison to bisanionic phosphate esters, especially when the pK_a of the leaving group is high, our results are not as

unexpected as they first appear. Note that phosphatases that work primarily on phosphoserine/threonine residues proceed by metal ion-assisted catalysis. Also, when this novel mechanism for Cdc25 is being considered, it is important to remember the lack of overall sequence and structural homology between Cdc25 and other cysteine phosphatases. In addition to lacking a residue corresponding to the catalytic acid, Cdc25 is the only catalytically active member of the HCX_5R family that lacks the serine/threonine residue immediately following the arginine of the motif. The hydroxyl group of this residue has been shown to be important for the catalytic activity in a number of phosphatases, playing a role in activation of the catalytic cysteine (*43*, *44*) or in promoting hydrolysis of the phospho−enzyme intermediate (*45*). Upon examination of the crystal structures of Cdc25A and Cdc25B, it is unclear what residues perform these tasks in the Cdc25s. The lack of a catalytic acid in Cdc25, which has been shown to be important for activation of a water molecule in the hydrolysis of the phospho−enzyme intermediate (*46*), also raises the question of how the second half-reaction is catalyzed by Cdc25. Most importantly, our novel mechanism is useful in the goal of creating specific and potent inhibitors of Cdc25 for purposes of anticancer therapy. Given Cdc25's use of the monoprotonated substrate and the apparently high affinity for a bisanionic substrate in a nonproductive complex, perhaps it will be possible to design mechanism-based inhibitors that interfere specifically with Cdc25 while leaving other phosphatases that turn over bisanionic substrates untouched.

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NOTE ADDED AFTER ASAP POSTING

This article was inadvertently released ASAP on 11/12/02 with a paragraph describing Supporting Information. There is no Supporting Information for this paper. The corrected version was posted 12/03/02.

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