The Catalytic Mechanism of Cdc25A Phosphatase

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Abstract

Cdc25 phosphatases are dual specificity phosphatases which dephosphorylate and activate cyclin-dependent kinases, thereby effecting the progression from one phase of the cell cycle to the next. Despite its central role in the cell cycle, relatively little is known about the catalytic mechanism of Cdc25. In order to provide insights into the catalytic mechanism of Cdc25, we have performed a detailed mechanistic analysis of the catalytic domain of human Cdc25A. Our kinetic isotope effect results, Bronsted analysis, and pH dependence studies employing a range of aryl phosphates clearly indicate a dissociative transition state for the Cdc25A reaction that does not involve a general acid for the hydrolysis of substrates with low leaving group pK\textsubscript{a} values (5.45-8.05). Interestingly, our Bronsted analysis and pH dependence studies reveal that Cdc25A employs a different mechanism for the hydrolysis of substrates with high leaving group pK\textsubscript{a} values (8.68-9.99) that appears to require the protonation of glutamic acid 431. Mutation of glutamic acid 431 into glutamine leads to a dramatic drop in the hydrolysis rate for the high leaving group pK\textsubscript{a} substrates and the disappearance of the basic limb of the pH-rate profile for the substrate with a leaving group pK\textsubscript{a} of 8.05, indicating that glutamic acid 431 is essential for the efficient hydrolysis of substrates with high leaving group pK\textsubscript{a}. We suggest that hydrolysis of the high leaving group pK\textsubscript{a} substrates proceeds through an unfavored, but more catalytically active form of Cdc25A, and propose several models illustrating this. Since the activity of Cdc25A towards small molecule substrates is several orders of magnitude lower than towards the
physiological substrate, cyclin/cdk, we suggest that the cyclin/cdk is able to preferentially induce this more catalytically active form of Cdc25A for efficient phosphothreonine and phosphotyrosine dephosphorylation.
INTRODUCTION:

Cdc25 phosphatases play a critical role in cell cycle progression (1-3). An increasing number of studies have shown a positive correlation between Cdc25 activity and cancer (4-7), while others have shown that inhibiting Cdc25 halts cell cycle progression (8-11). Thus, Cdc25 is both an attractive drug target and the subject of much ongoing biomedical research.

Progression from one phase of the cell cycle to the next is largely mediated by the rapid phosphorylation of various cellular targets by cyclin-dependent kinases (cdks) at the phase transitions (12). This, in turn, is triggered by the rapid dephosphorylation of cyclin/cdk complexes on Thr14 and Tyr15 by Cdc25 proteins, which are dual specificity phosphatases (13-16). Humans have three isoforms of Cdc25 termed Cdc25A, Cdc25B, and Cdc25C, and each plays a distinct role in the cell cycle (3).

Despite what is known about Cdc25’s role in the cell cycle, relatively little is known about its catalytic mechanism. Cdc25 is classified as a protein-tyrosine phosphatase (PTPase) because it contains the \textbf{CX}_5\textbf{R} (one-letter amino acid abbreviations) PTPase signature motif, which characterizes the PTPase superfamily. Enzymes of the PTPase superfamily can be divided into 3 classes—tyrosine specific, low molecular weight, and dual-specific phosphatases. The dual specific phosphatases can be further split into the VH1-like phosphatases, MAP-kinase phosphatases, kinase-associated phosphatase (KAP), and Cdc25 phosphatases based upon sequence comparisons. In previous years, much progress has been made in understanding the mechanism of PTPases. The \textbf{CX}_5\textbf{R} motif plays a vital catalytic role in all PTPases. It has been shown that the cysteine residue in this motif functions as a nucleophile, attacking the substrate phosphate atom directly and displacing the leaving group to form a phosphocysteine intermediate.
The arginine in this motif has been shown to be particularly important for transition state stabilization (23-25) and is essential for catalysis (26-28). For Cdc25B the cysteine and arginine in the PTPase signature motif have been shown, by site-directed mutagenesis, to be required for catalysis (29). This suggests that the cysteine and arginine residues in the CX$_5$R motif play the same role in Cdc25 as they do for other PTPases.

Apart from the cysteine and arginine residues in the CX$_5$R motif, it is not known whether other residues participate in catalysis for Cdc25. This is in contrast to other PTPases for which a set of conserved, catalytically important residues has been defined, and functions have been determined for each of these residues (21). For example, a conserved acidic residue, the general acid, has been shown to play an important role in catalysis in all PTPases examined to date, including the Yersinia PTPase (30), PTP1B (31, 32), the low molecular weight PTPases (33-35), VHR (36, 37), and MKP3 (38). The general acid facilitates catalysis by protonating the leaving group oxygen, thereby neutralizing the negative charge that develops as the phosphate ester bond is broken. This stabilizes the transition state and enhances the rate of catalysis--by >1000 fold in the case of the Yersinia PTPase (30).

This information regarding the identities and functions of catalytically important residues in PTPases is very valuable. One group has used this information to guide structure-based inhibitor design for PTPases in which they were able to design selective PTPase inhibitors by targeting both conserved residues important for catalysis and non-conserved residues important for substrate specificity (39). In addition, knowing the identity of the general acid has been used to design more highly potent substrate trapping mutants of PTPases (40, 41), and may aid in the identification of physiological substrates of PTPases.
Though it is potentially valuable information, the identity of the general acid in Cdc25 is still unknown. As it turns out, performing a sequence alignment of Cdc25 with a template based upon the prototypical PTPases described above does not reveal the catalytically important residues of Cdc25 (42). Cdc25 is somewhat of an outlier among the PTPases. This was revealed by homology modelling (43) and X-ray crystallographic studies (44, 45). These studies revealed that Cdc25 is structurally much more similar to the sulfurtransfer enzyme rhodanese than it is to any other PTPases. Because it is so dissimilar to other PTPases, aside from the role of the cysteine and arginine in the \( \text{CX}_5\text{R} \) motif, no obvious conclusions can be drawn about the mechanism of Cdc25 or the identity of Cdc25’s catalytically important residues simply based upon previous studies of PTPases. For this reason, we chose to perform a detailed mechanistic analysis of Cdc25A. The main goals of this work are to provide a description of the transition state of the Cdc25A catalyzed reaction, and to determine the role of general acid catalysis and the identity of the general acid in Cdc25A. In the process, we have revealed some unique features of the Cdc25A-catalyzed reaction.

MATERIALS AND METHODS:

*Expression and Purification of Cdc25A Catalytic Domain and Mutant Proteins.* The catalytic domain of Cdc25A was expressed and purified based upon the procedure described by Fauman (44). A mammalian expression vector containing the full length human Cdc25A was obtained from Dr. Katsumi Yamashita of Kanazawa University, Japan. The region encoding the catalytic domain of Cdc25A (residues 336-523) was amplified by PCR and subcloned into the vector pCR2.1 using the TA cloning kit from Invitrogen, with a 5’ primer that contained an NheI
site followed by an initiating methionine codon and a 3’ primer that contained an EcoRI site followed by a stop codon. The region encoding the catalytic domain was then subcloned into the vector pUC118. The Cdc25A catalytic domain mutants, E431Q, E435Q, F432A, and G437S, were obtained using the Quick Change site-directed mutagenesis kit from Stratagene and the appropriate mutagenic primers obtained from the Oligonucleotide Synthesis Facility of Albert Einstein College of Medicine. The sequence of the wild type and mutant Cdc25As were confirmed by DNA sequencing. Using the NheI and EcoRI sites, the catalytic domain was subcloned into the bacterial expression vector pET21a from Novagen. E. coli BL21DE3 cells were transformed with this vector, grown at 37 °C in 1 L cultures to an O.D. at 600 nm of 0.6-0.8, and induced to express the catalytic domain of Cdc25A by the addition of 0.2 mM IPTG. After induction, the cultures were grown at room temperature for 16h, and then pelleted by centrifugation. Pellets from 1 L of culture were resuspended in 30 ml of ice-cold pH 7.5 25 mM HEPES, 2 mM DTT, 1 mM EDTA (Buffer A). The cells were lysed in a French press at 12,000 psi. The lysate was then centrifuged at 15,000 rpm for 40 minutes and supernatant filtered using a 0.45 micron membrane syringe filter from Pall Gellman Laboratories. The supernatant was then bound at 4°C to 50mL of Poros HS-50 resin (PerSeptive Biosystems) that was equilibrated at 4°C in Buffer A. After 20 minutes, the resin was added to a column and washed with 250 ml of Buffer A. The protein was eluted using a 500 ml, 0-0.5 M linear, sodium chloride in Buffer A gradient at 4°C. The fractions containing Cdc25A were concentrated in a Centriprep YM-10 (10,000 MWCO) concentrator from Amicon. Gel filtration chromatography (G-50) was then performed in Buffer A containing 200 mM sodium chloride, and the protein was concentrated as before. Proteins were judged to be at least 95% pure by SDS-PAGE and the identity of the
Cdc25A wild type catalytic domain was confirmed by N-terminal protein sequencing performed by the Laboratory for Macromolecular Analysis and Proteomics of Albert Einstein College of Medicine. We routinely obtained 30-50 mg of protein per liter of culture. The Cdc25A mutants were purified identically except that E431Q and E435Q required a 0-0.75 M linear sodium chloride gradient for elution during the ion exchange chromatography step. Also, the yields of the E431Q mutant were significantly lower as much of it seemed to reside in the pellet fraction.

**Cdc25A Substrates.** The substrates 4-nitrophenyl phosphate (pNPP), 3-O-methylfluorescein phosphate, 4-methylumbelliferyl phosphate, β-naphthyl acid phosphate, and phenyl phosphate were obtained from Sigma. 6,8-difluoro-4-methylumbelliferyl phosphate was obtained from Molecular Probes. Other aryl phosphates, 2-chloro-4-nitrophenyl phosphate, 4-acetylphenyl phosphate, 4-trifluromethylphenyl phosphate, 4-chlorophenyl phosphate, and 4-fluorophenyl phosphate were synthesized according to Hall and Williams (46). The 2-chloro-4-nitrophenyl phosphate was obtained at 37% purity, with inorganic phosphate as the major impurity judging by the full hydrolysis of the substrate using *Yersinia*-PTPase or Cdc25A catalytic domain, and the inorganic phosphate assay described below. Appropriate corrections were made when preparing solutions of this substrate and the impurities did not interfere with the phosphatase assays. All other substrates were at least 95% pure.

**Steady State Kinetics.** All assays were performed at 30°C. The following buffers were used, pH 5.4-5.8 100 mM succinate, pH 5.8-7.2 50 mM 3,3-dimethyl glutarate, pH 7.2-8.8 TRICINE. In addition each buffer also contained 1 mM EDTA, 1 mM DTT and was adjusted to an ionic strength of 150 mM with sodium chloride. Assay mixtures of 100 µl total were set up in 1.1 ml polypropylene tubes from Marsh Biomedical. Reactions were started by the addition of
an appropriate amount of enzyme (2-10 µM). For the substrates 4-acetylphenyl phosphate, 4-trifluoromethyl phenyl phosphate, 4-chlorophenyl phosphate, 4-fluorophenyl phosphate, 4-methylumbelliferyl phosphate, β-naphthyl phosphate, and phenyl phosphate the reaction mixture was quenched with 50 µl of 10% trichloroacetic acid and the amount of inorganic phosphate released was quantitated based on the method of Black and Jones (47). The only differences were that 100 µl of the ammonium molybdate/ascorbic acid solution was added to each quenched reaction mixture followed 2-5 minutes later by 200 µl of the citrate/arsenite solution. 200 µl of each reaction mixture was then transferred to a 96-well plate, where the absorbance at 700 nm was read using a Spectra Max 340 plate reader from Molecular Devices. As some of these substrates are only soluble to 5-10 mM, sufficient substrate could not be added to saturate Cdc25A. Therefore $k_{cat}/K_m$ values were determined by employing a series of assay solutions containing range of substrate concentrations (0.3-1.7 mM) well below the $K_m$. The $k_{cat}/K_m$ values were obtained from a linear least square fit of the plot of absorbance verses substrate concentration and using the appropriate form of the Michaelis-Menton equation

$$v = k_{cat}[E][S]/K_m \quad [1]$$

where $v$ is the initial rate and $[E]$ is the total enzyme concentration. The substrates 2-chloro-4-nitrophenyl phosphate and pNPP were assayed similarly except that the reaction mixtures were quenched with 100 µl of 2M sodium hydroxide. 150 µl of each mixture was then transferred to a 96-well plate where the absorbance at 405 nm was read in a plate reader. In addition, both of these compounds were sufficiently soluble such that concentrations encompassing the $K_m$ value of Cdc25A could be prepared and the Michaelis-Menton equation [2] was used to determine
both $k_{\text{cat}}$ and $K_m$. The initial rate versus substrate concentration could be fit directly to the equation

$$v = \frac{k_{\text{cat}}[E][S]}{(K_m + [S])}$$

[2]

where $v$ is the initial rate, $[E]$ is the total enzyme concentration and $[S]$ is the initial substrate concentration using KaleidaGraph (Synergy Software).

For the substrates 3-O-methylfluorescein phosphate (OMFP) and 6,8-difluoro-4-methylumbelliferyl phosphate (DIFMUP) continuous assays were performed. In both cases, 1 ml reaction volumes were set up in 1 ml quartz cuvettes. The cuvettes were placed in the temperature-controlled holder of the spectrophotometer or fluorimeter and allowed to equilibrate to 30°C before an appropriate amount of enzyme was added to initiate the reaction. For OMFP, 100 µM of OMFP was used and the absorbance was monitored at 400 nm over the entire reaction time-course in a Perkin Elmer Lambda 14 UV/Vis spectrophotometer. Both the $k_{\text{cat}}$ and $K_m$ values were obtained by a nonlinear least squares fit to equation [3] as described previously (48),

$$t = \frac{[P]}{k_{\text{cat}}[E]} + \left(\frac{K_m}{k_{\text{cat}}[E]}\right) \ln\left(\frac{[S]_0}{([S]_0 - [P])}\right)$$

[3]

where $[S]_0$ is the initial substrate concentration while $[P]$ is the product concentration at time $t$. 

For DIFMUP, 0.5 µM substrate was used and the fluorescence of the product was monitored at 450 nm using an excitation of 380 nm in a Perkin Elmer LS 50 B Luminescence spectrometer. The $k_{\text{cat}}/K_m$ value was obtained by nonlinear least square fit to equation [4],

$$[P] = [S]_0(1-\exp(-k_{\text{cat}}[E]t/K_m))$$

[4]
where \([S]_0\) is the initial substrate concentration while \([P]\) is the product concentration at time \(t\).

**Leaving Group Dependence.** The leaving group dependence studies were performed at pH 7.0. The \(\log(k_{cat}/K_m)\) value was plotted against leaving group \(pK_a\) value for the 1-ringed aryl phosphate substrates. The \(\beta_{lg}\) value was obtained as the slope of the linear least squares fit to the data. For the wild type Cdc25A in which there was a distinct kink in the leaving group dependence, the data from \(pK_a\) 5.45-8.05 was fit separately from the data from \(pK_a\) 8.68-9.99.

**pH Dependence Studies.** The \(k_{cat}/K_m\) values were plotted as a function of pH for several substrates. The second ionization constants (\(pK_{S2}\)) of 5.00 ± 0.01, 5.24 ± 0.01, 5.52 ± 0.01, and 5.72 ± 0.02 were obtained, by titration, for the substrates \(p\)NPP, 4-acetylphenyl phosphate, 4-chlorophenyl phosphate, and phenyl phosphate respectively. To fit the \(k_{cat}/K_m\) verses pH data, one of two equations was used:

\[
k_{cat}/K_m = \frac{(k_{cat}/K_m)_{max}}{1 + H/K_{S2} + H/K_{E1}}
\]  \[5\]

\[
k_{cat}/K_m = \frac{(k_{cat}/K_m)_{max}}{(1 + H/K_{S2})(1 + H/K_{E1} + K_{E2}/H)}
\]  \[6\]

where \((k_{cat}/K_m)_{max}\) is the highest theoretical value of \(k_{cat}/K_m\), \(H\) is the proton concentration, \(K_{S2}\) is the second ionization constant of the substrate, and \(K_{E1}\) and \(K_{E2}\) are the ionization constants of the enzyme. For the wild-type Cdc25A using the substrates 2-chloro-4-nitrophenyl phosphate and \(p\)NPP, and for the E431Q mutant in all cases, equation [5] was used. For the wild-type enzyme using the substrates 4-chlorophenyl phosphate and phenyl phosphate,
equation [6] was used, where $\text{pK}_{\text{E}1}$ was fixed at 5.5 based on the average of the values obtained for $\text{pK}_{\text{E}1}$ for wild-type Cdc25A with 2-chloro-4-nitrophenyl phosphate and $p\text{NPP}$. In all cases, the second ionization constant of the substrate was fixed using the $p\text{K}_\text{a}$ values determined from the titration experiments.

**Kinetic Isotope Effect Determinations.** Bovine alkaline phosphatase, type 1-S from Sigma, was used as received. Ethyl ether was distilled from alumina. Natural abundance $p$-nitrophenyl phosphate, $[^{14}\text{N}]-p$-nitrophenyl phosphate, $[^{15}\text{N}, \text{nonbridge-}^{18}\text{O}_3]-p$-nitrophenyl phosphate, $[^{14}\text{N}]-p$-nitrophenol, and $[^{15}\text{N}, \text{O}]-p$-nitrophenol were synthesized as described previously (49). $[^{14}\text{N}]-p$-nitrophenol, and $[^{15}\text{N}, \text{O}]-p$-nitrophenol were then mixed to reconstitute the natural abundance of $^{15}\text{N}$, and then the mixture was phosphorylated to produce $p$-nitrophenyl phosphate using the same method as referred to above. This mixture was used for determination of $^{18}(\text{V/K})_{\text{bridge}}$. The $[^{14}\text{N}]-p$-nitrophenyl phosphate and $[^{15}\text{N}, \text{nonbridge-}^{18}\text{O}_3]-p$-nitrophenyl phosphate were also mixed to reconstitute the natural abundance of $^{15}\text{N}$. This mixture was used for determination of $^{18}(\text{V/K})_{\text{nonbridge}}$. The isotopic abundance of the mixtures was determined by isotope ratio mass spectrometry.

Isotope effect determinations were made at 30 °C, in either pH 7.2, 500 mM Tricine buffer or pH 6.1, 250 mM BisTris buffer, containing 1 mM DTT. The buffer was degassed by bubbling nitrogen through it for 30 min before initiating experiments. The protocols for carrying out these experiments were the same as those previously described (25). The enzymatic
reactions were performed in triplicate, and allowed to proceed to fractions of reaction ranging from 33 to 65% and stopped by titration to pH 4. The reaction solutions were then extracted three times with an equivalent volume of ethyl ether to quantitatively remove the $p$-nitrophenol product. The aqueous fractions containing unreacted $p$NPP were titrated to pH 9.0 and treated with excess alkaline phosphatase overnight to hydrolyze the remaining $p$NPP. The nitrophenol thus released was isolated as described above. The nitrophenol samples were purified by sublimation and analyzed by isotope ratio mass spectrometry. Isotopic analyses were performed using an ANCA-NT combustion system in tandem with a Europa 20-20 isotope ratio mass spectrometer.

Reactions were begun with 100 micromoles or more of the substrate and sufficient enzyme so that the background hydrolysis rate was negligible compared to the enzymatic reaction. Parallel experiments without enzyme were used to establish background hydrolysis rates under the experimental conditions.

Isotope effects were calculated from the isotopic ratio in the $p$-nitrophenol product at partial reaction ($R_p$), in the residual substrate ($R_s$), and in the starting material ($R_o$). Equations 1 and 2 were used to calculate the observed isotope effect either from $R_p$ and $R_o$ or from $R_s$ and $R_o$ respectively at fraction of reaction $f(50)$. Thus each experiment yields two independent determinations of the isotope effect.

\[
\text{isotope effect} = \frac{\log (1 - f)}{\log (1 - f) \left(\frac{R_p}{R_o}\right)}
\]

[7]

\[
\text{isotope effect} = \frac{\log (1 - f)}{\log [(1 - f) \left(\frac{R_s}{R_o}\right)]}
\]

[8]
RO was determined separately from unreacted substrate by isotope ratio mass spectroscopic analysis and, as a control, from p-nitrophenol isolated after complete hydrolysis of substrate using the isolation and purification procedures used in the isotope effect experiments. The agreement of these two numbers demonstrated that, within experimental error, no isotopic fractionation occurs as a result of the procedures used to isolate and purify the p-nitrophenol.

The $^{18}$O isotope effects were measured by the remote-label method (51), as previously described for the solution reactions of pNPP (49). In these experiments the nitrogen atom in the substrate is used as a reporter for the bridging oxygen atom or the nonbridging oxygen atoms. These experiments yield an observed isotope effect that is the product of the effect due to $^{15}$N and to $^{18}$O substitutions. The observed isotope effects from these experiments were then corrected for the $^{15}$N effect and for incomplete levels of isotopic incorporation in the starting material as previously described (52).

The notation used to express isotope effects is that of Northrop (53) in which a leading superscript denoting the heavier isotope indicates the isotope effect on the following kinetic quantity; for example $^{15}k$ denotes $k_{14}/k_{15}$, the nitrogen-15 isotope effect on the rate constant $k$. Since all isotope effects in this study were measured by the competitive method they are isotope effects on V/K, and thus are designated as $^{15}$(V/K), etc. Figure 1 shows a diagram of the substrate with the nomenclature used to describe the isotope effects at each position.

RESULTS AND DISCUSSION:
The goals of this study were to further define the catalytic mechanism of Cdc25A phosphatase and identify the catalytically important residues, and in particular to identify the general acid. Structural studies of human Cdc25A (44) and Cdc25B (45) phosphatases revealed enzymes with shallow, solvent-exposed active sites with relatively few protein functional groups in positions where they would be likely to participate in catalysis. This is in contrast to other PTPases such as *Yersinia* PTPase (54), PTP1B (55-57), the low molecular weight PTPase (58, 59) and VHR (60), which have surface loops that close around the substrate, bringing a variety of functional groups, including the general acid into the active site, and virtually surrounding the substrate. In Cdc25, the only residues which appear able to participate directly in catalysis are located in the phosphate-binding loop itself, corresponding to the CX5R motif (residues C430-R436 in Cdc25A). For example, it has been suggested that either E431 (44) or E435 (45) may serve as the general acid for Cdc25. From the crystal structures, it appeared that these were the only acidic residues that would be in the vicinity of the bridging oxygen in a phosphate ester substrate. It was also suggested that F432 may play a role in binding aryl phosphate substrates such as phosphotyrosine by participating in a hydrophobic interaction with the phenyl ring of the substrate (44). We, therefore, decided to determine the role of these residues in catalysis through site-directed mutagenesis and kinetic analysis.

*Hallmarks of General Acid Catalysis.* General acid catalysis in PTPases has several distinguishing features which can be revealed experimentally through kinetic analysis. First, general acid catalysis in PTPases is marked by characteristic kinetic isotope effects, which will be discussed below. Second, PTPases with general acids show bell-shaped pH profiles where the maximum catalytic rate usually occurs around pH 5-6 with aryl phosphates as substrates.
Third, PTPases with general acids show relatively flat leaving group dependencies, that is the rate of catalysis is affected minimally by the $pK_a$ of the leaving group. Fourth, removal of the general acid through site-directed mutagenesis results in characteristic changes in the kinetic isotope effects, a flat pH profile with a significant drop in the rate of catalysis, and a significant leaving group dependence where the rate of catalysis drops as the leaving group $pK_a$ rises.

**Mutation of Acidic and Hydrophobic Residues within the Cdc25A Active Site Has Little Effect on pNPP Hydrolysis.** The catalytic domain of Cdc25A (residues 336-523) and several point mutants were constructed, overexpressed in *E. coli*, and purified to >95% purity based on SDS-PAGE. The kinetic parameters of these mutants using pNPP as a substrate are shown in Table I. As shown in Table I, mutation of either acidic residue in the active site loop of Cdc25A has only modest effects on the kinetic parameters. In the case of both E431Q and E435Q, the $K_m$ decreases 2-fold, while the $k_{cat}$ is enhanced slightly. The F432 mutant, likewise, shows only modest differences from the wild type enzyme—a 2-fold drop in both $k_{cat}$ and $K_m$, leaving the $k_{cat}/K_m$ value virtually the same as wild type. These results suggest that neither of these acidic residues nor F432 are required for the hydrolysis of pNPP.

**Addition of a Serine Residue after the Active Site Arginine Has a Detrimental Effect on Cdc25A PTPase Activity.** All PTPases identified to date, except Cdc25, have an active site loop consisting of CX$_5$R(S/T). The serine/threonine hydroxyl hydrogen bonds with the active site cysteine sulfur atom (21, 54, 55, 58, 60, 61). This interaction stabilizes the thiolate anion and assists in the hydrolysis of the phosphocysteine intermediate (21, 32, 62-64). It has been known
for some time that the activity of Cdc25 against pNPP is orders of magnitude below that of most other phosphatases such as *Yersinia* PTPase, PTP1B, or VHR (65). Unlike these enzymes, Cdc25 phosphatases do not contain a hydroxyl in this position, instead containing a glycine. One possibility is that Cdc25’s relatively low pNPP activity results from the absence of this hydroxyl group. To test this possibility, we mutated this glycine into a serine residue in order to determine if this would rescue Cdc25’s pNPP activity. As Table I shows, this mutation resulted in a 5-fold reduction in $k_{\text{cat}}$. So, rather than rescuing Cdc25’s activity, this mutation was actually detrimental, probably due to perturbation of the active site structure.

*Kinetic Isotope Effects.* Another possibility is that Cdc25’s low pNPP activity is due to a lack of a general acid to facilitate catalysis. One of the best ways to probe the transition state of PTPase catalyzed reactions and reveal the existence of a general acid is through the measurement of kinetic isotope effects. In the past, this approach has been highly successful in revealing the existence of general acid catalysis and identifying the residues that function as general acids in PTPases such as *Yersinia* PTPase and PTP1 (31), the low-molecular weight PTPase Stp1 (66), VHR (37), and MKP3 (38). We decided to determine the kinetic isotope effects for the wild-type Cdc25A in order to determine if it in fact does employ general acid catalysis.

Figure 1 shows the structure of pNPP along with the isotope effects that were measured in this study. Table II shows the resulting isotope effects for the wild-type Cdc25A-catalyzed pNPP hydrolysis reaction along with isotope effects for the reactions catalyzed by wild-type VHR and the general acid deficient VHR determined in a previous study (37) for comparison. The kinetic isotope effect data for the VHR-catalyzed reaction are typical of those obtained with the *Yersinia* PTPase, PTP1 and Stp1.
Isotope effects are only expressed for an enzyme-catalyzed reaction when certain conditions are met. Namely, the chemical step must be at least partially rate-limiting and the formation of the enzyme-substrate complex must be freely reversible. As Table II indicates, significant isotope effects were observed for Cdc25A. Because the observed isotope effects are large and similar to those of the uncatalyzed reactions, it suggests that chemistry is at least largely rate-limiting for the hydrolysis of pNPP by Cdc25A.

The observed isotope effects reveal several important details about the mechanism of Cdc25A. The primary $^{18}(V/K)_{\text{bridge}}$ isotope effect reveals the extent of P-O bond cleavage in the transition state (49). The large $^{18}(V/K)_{\text{bridge}}$ of 2.91-3.57% for Cdc25A indicates a substantial extent of bond cleavage between the phosphorus atom and the leaving group in the transition state. This effect is of similar magnitude to that of either the general acid deficient PTPases (31, 38, 66), or the dual-specific phosphatase VHR/D92N, which has a $^{18}(V/K)_{\text{bridge}}$ isotope effect of 2.94%. For PTPases in which a general acid is functioning, the $^{18}(V/K)_{\text{bridge}}$ isotope effect is suppressed due to protonation, as O-H bond formation partially compensates for O-P bond cleavage. This can be seen in the native VHR $^{18}(V/K)_{\text{bridge}}$ isotope effect of 1.18%, which is 2.5-fold lower than that of the general acid deficient VHR/D92N. The high magnitude of the $^{18}(V/K)_{\text{bridge}}$ isotope effect of 3.57% for Cdc25A suggests that Cdc25A does not employ a general acid for pNPP hydrolysis.

The $^{15}(V/K)$ isotope effect is sensitive to the amount of negative charge that is
delocalized into the phenyl ring at the transition state, and therefore reflects the amount of negative charge that develops on the leaving group as the P-O bond is broken (49). For PTPases with functional general acids, this isotope effect is unity within experimental error, as typified by the value of $^{15}\text{(V/K)}$ for wild type VHR (Table II). This is attributed to protonation of the bridging oxygen by the general acid (D92 for VHR) which neutralizes the negative charge on the bridging oxygen as the P-O bond is broken. When the general acids of PTPases are impaired, the $^{15}\text{(V/K)}$ isotope effect becomes more like that of the uncatalyzed hydrolysis of the $p$NPP dianion, which is 1.0028 (0.28%) at 95 °C. For VHR/D92N, $^{15}\text{(V/K)}$ is 0.30%. Strikingly, the $^{15}\text{(V/K)}$ isotope effect is also 0.30% for the wild-type Cdc25A at pH 7.2 and this value is unchanged when the isotope effect is measured at pH 6.1. Together, this implies that Cdc25A does not employ general acid catalysis in the hydrolysis of $p$NPP. This is a very rare finding that has never before been observed for native PTPases.

Additionally, the $^{18}\text{(V/K)}_{\text{nonbridge}}$ isotope effect indicates the degree to which the transition state is associative or dissociative (67). Experimental results for nonbridge isotope effects in phosphoryl transfer reactions generally follow the trend suggested by calculations that predict normal $^{18}_k_{\text{nonbridge}}$ isotope effects for associative transition states, and small, inverse values for dissociative ones. For the hydrolysis of the dianion of $p$NPP, which proceeds by a loose transition state, $^{18}_k_{\text{nonbridge}} = 0.9994$. The tighter transition states of diesters and triesters are characterized by values for $^{18}_k_{\text{nonbridge}}$ in the range of 1.0028 – 1.0254. In all
previous cases examined, when the general acid of a PTPase is mutated, the $^{18}(V/K)_{\text{nonbridge}}$ isotope effect becomes more normal, indicating that a slightly more nucleophilic participation is required to expel the leaving group. Interestingly, for Cdc25A, even though the $^{18}(V/K)_{\text{bridge}}$ and the $^{15}(V/K)$ clearly indicate that there is no general acid catalysis, the value for $^{18}(V/K)_{\text{nonbridge}}$ is even more inverse than that of the solution dianion reaction. This indicates that Cdc25A employs a different strategy for transition state stabilization in the absence of general acid catalysis than do PTPases which have their general acids removed via site-directed mutagenesis.

Taken together, the kinetic isotope effects for Cdc25A reveal three important things about the mechanism of $p$NPP hydrolysis by Cdc25A. First, the presence of substantial isotope effects, some of which are at their maximum expected values, reveals that the chemical step of phosphoryl transfer from substrate to the enzymatic nucleophile is at least largely rate-limiting for V/K. Second, the substantial, normal $^{18}(V/K)_{\text{bridge}}$ and $^{15}(V/K)$ isotope effects reveal that Cdc25A does not employ general acid catalysis for $p$NPP hydrolysis. And third, the $^{18}(V/K)_{\text{bridge}}$ and the inverse $^{18}(V/K)_{\text{nonbridge}}$ isotope effects reveal a highly dissociative transition state for the hydrolysis of $p$NPP by Cdc25A.

*Active Site Preferences of Cdc25A.* Clearly, based on the kinetic isotope effects, Cdc25A does not employ general acid catalysis for the hydrolysis of $p$NPP. Another way to determine the presence or absence of general acid catalysis is by performing a leaving group dependence
study and determining the $\beta_{lg}$ value. A $\beta_{lg}$ value is obtained from the slope of a plot of the logarithm of the hydrolysis rate constant versus the $pK_a$ of the leaving group of the substrate.

The $\beta_{lg}$ value, like the $^{15}(V/K)$ isotope effect, reflects the amount of negative charge delocalized into the phenyl ring at the transition state. The solution dianion reaction for a series of aryl phosphate esters shows a strong leaving group dependence, with a $\beta_{lg}$ value of 1.23 (68). In the uncatalyzed reaction at lower pH where the monoanion is the reactive species, the leaving group is protonated in the transition state, and $\beta_{lg}$ is reduced to 0.27 (68). For PTPases with general acid catalysis, the leaving group dependence is generally similar to that observed for the uncatalyzed monoanion reaction (35, 69-71) or near zero (20, 34, 72-74). We decided to test the ability of Cdc25A to hydrolyze a variety of aryl phosphates with varying leaving group $pK_a$ values and steric properties. We did this in order to both determine the $\beta_{lg}$ value, and to determine the steric preferences of Cdc25A’s active site.

Table III shows the $k_{cat}/K_m$ values of Cdc25A and two of its site-directed mutants for a variety of substrates. The value of $k_{cat}/K_m$ was chosen for comparison, because it is known, from the kinetic isotope effects, to reflect the rate of the first chemical step in catalysis for Cdc25A in pNPP hydrolysis. The $k_{cat}/K_m$ value is also a measure of the substrate specificity. A comparison of some of the $k_{cat}/K_m$ values reveals several important features of the active site preferences and mechanism of Cdc25A. First, Cdc25A shows a strong (73-fold) preference for 3-O-methylfluorescein phosphate (OMFP) over 6,8-difluoro-4-methylumbelliferyl phosphate.
(DIFMUP) even though these two substrates have very similar pK_a values. Cdc25A also shows a more modest preference for 2-ringed substrates over 1-ringed substrates. The $k_{cat}/K_m$ value for β-naphthyl phosphate is 3.5-fold higher than that for 4-chlorophenyl phosphate though these substrates share the same pK_a value, and the $k_{cat}/K_m$ value for 4-methylumbelliferyl phosphate is 2.3-fold higher than that for 4-acetylphenyl phosphate even though these substrates also have similar pK_a values. These results are similar to those obtained for another dual specificity phosphatase, MKP3, for which it was shown that the enzyme has a strong preference for OMFP over pNPP (280-fold) and a more modest preference for β-naphthyl phosphate over pNPP (5-fold) (73). This preference for bulky substrates has been noted for several dual specificity phosphatases (72-77) which may be the result of the shallow active site pockets exhibited by dual specificity PTPases, which allow these enzymes to accommodate phosphothreonine and phosphoserine, as well as phosphotyrosine.

*Leaving Group Dependence of Cdc25A.* Comparison of substrates with similar structures but different pK_a values reveals that the pK_a of the substrate can be an important factor in the rate of Cdc25A catalysis. DIFMUP and 4-methylumbelliferyl phosphate (MUP) differ structurally only by two fluorine atoms, yet their leaving group pK_a values are significantly different, 4.7 for DIFMUP and 7.8 for MUP. This difference leads to a 430-fold enhancement in $k_{cat}/K_m$ for DIFMUP verses MUP. A similar pattern is observed for pNPP and 2-chloro-4-nitrophenyl phosphate. Addition of a chlorine atom at the 2 position to pNPP decreases the pK_a of the leaving group from 7.1 to 5.5 and increases the $k_{cat}/K_m$ value for Cdc25A 16-fold.
Clearly the pK_a of the leaving group can have a dramatic effect on the rate of catalysis for Cdc25A.

We further quantified this leaving group effect by constructing a Bronsted plot with the 1-ringed aryl phosphate substrates. This is shown in Figure 2. As the purpose of a Bronsted plot is to quantify the effect of the electronic properties of the substrates on the rate of catalysis, it is best to minimize the steric differences between the substrates used in such a plot. Since we have shown that the steric properties of substrates alone can have substantial effects on the rate of catalysis, we chose to include only the one-ringed aryl phosphates in the Bronsted plot. The most dramatic feature of the Bronsted plot for Cdc25A is the kink at the pK_a value of 8. In the low pK_a region, from 5.45 to 8.05, the slope is substantially negative, giving a β_lg value of -0.8 ± 0.1. This value indicates that a substantial amount of negative charge develops on the leaving group oxygen at the transition state, and suggests that no general acid catalysis occurs for substrates with leaving group pK_a values between 5.45 and 8.05. Since pNPP has a leaving group pK_a of 7.14, this suggests that general acid catalysis does not occur for the hydrolysis of pNPP by Cdc25A, which is entirely consistent with the isotope effects for pNPP discussed above. Surprisingly, the leaving group dependence from pK_a 8.05-9.99 is flat, giving a β_lg value of around zero (0.08 ± 0.02). A kink in the leaving group dependence is usually attributed to a change in the mechanism or a change in the rate-limiting step. A flat leaving group dependence suggests one of two things for phosphate ester hydrolysis; either protonation of the leaving group occurs at the transition state as in general acid catalysis or cleavage of the phosphate ester bond is not the rate-limiting step. We performed several further studies in an attempt to distinguish
these possibilities.

*The pH Dependence of Cdc25A-Catalyzed Reaction.* To ascertain the possible involvement of a general acid in hydrolyzing these high-leaving group $pK_a$ substrates we performed pH dependence studies with several aryl phosphate substrates with a wide range of leaving group $pK_a$ values. Figure 3A shows the pH dependence of wild-type Cdc25A-catalyzed hydrolysis of the aryl phosphates 2-chloro-4-nitrophenyl phosphate, $pNPP$, 4-acetylphenyl phosphate, 4-chlorophenyl phosphate, and phenyl phosphate. Not surprisingly, $pNPP$ shows relatively flat pH dependence between pH 6 and 8.8, which is characteristic of a lack of general acid catalysis, and consistent with the kinetic isotope effects and the leaving group dependence mentioned above. The substrate 2-chloro-4-nitrophenyl phosphate, which has a leaving group $pK_a$ of 5.45, also shows a flat pH dependence, which is again characteristic of a lack of general acid participation and consistent with the leaving group dependence for substrates in this low leaving group $pK_a$ range. The $k_{cat}/K_m$ values below pH 5.8 could not be accurately measured because of the instability of Cdc25A at these pH values. Table IV lists the pH dependent parameters for the data displayed in Figure 3 along with the equation that was used to fit the data. The second ionization constants of the substrates were fixed at the experimentally determined values. The observed $pK_{E1}$ (5.47-5.57) for the $pNPP$ and 2-chloro-4-nitrophenyl phosphate reactions likely corresponds to the active site cysteine nucleophile, and is similar to that (5.6-6.3) observed for Cdc25B (77).

Interestingly, the substrates 4-chlorophenyl phosphate and phenyl phosphate, which have leaving group $pK_a$’s of 9.38 and 9.99 respectively, show pH optima at 6.2 and steadily
decreasing $k_{cat}/K_m$ values at higher pH values, quite unlike the pH dependencies of the lower pK_a substrates. This behavior is consistent with a Cdc25A residue that must be protonated for optimal catalytic efficiency--possibly a general acid. The pH dependencies for these two substrates are similar to the pH dependencies of other general acid-containing PTPases such as Yersinia PTPase (30), PTP1 (70), or VHR (63). As the pK_E1 values (ionization constants of the active site Cys residue) were largely obscured by the ionizations giving rise to pK_E2, the pK_E1 values were fixed at 5.5 (derived from the pNPP and 2-chloro-4-nitrophenyl phosphate reactions) for these two substrates and equation [6] was used to fit the data. As Table IV indicates, an apparent pK_E2 value of 6.00-6.06 was obtained for these substrates—similar to that obtained for Cdc25B with the physiological substrate (77) and consistent with the pK_a values obtained for the general acid of other PTPases (30, 63, 70).

The pH dependence of 4-acetylphenyl phosphate, which has a leaving group pK_a of 8.05, appears to show a pH dependence intermediate between those of the high pK_a substrates and the low pK_a substrates. The $k_{cat}/K_m$ is highest at pH 5.8 and decreases as the pH is raised but not as drastically as for 4-chlorophenyl phosphate or phenyl phosphate. Both the pH dependence and leaving group dependence of Cdc25A are consistent with a general acid independent mechanism for substrates with leaving group pK_a values below 8, and a general acid catalyzed mechanism for substrates with leaving group pK_a values above 8.

Effect of Altering the Active Site Acidic Residues on the pH Dependence and Leaving-group Dependence Studies. In order to identify the residue that appears to serve as a general acid
for the hydrolysis of the high-leaving group pK_a substrates, we determined the leaving group
dependence and the pH dependence for the two site-directed mutants of Cdc25A, E431Q and
E435Q. As stated before, neither mutant showed deficient pNPP activity, which is not surprising
since all the results discussed previously indicate that Cdc25A does not employ general acid
catalysis for the hydrolysis of pNPP. Table III shows the kinetic parameters obtained with these
mutant enzymes. The E435Q mutant followed the same general trends as the wild-type Cdc25A
in both leaving group dependence and pH dependence. Namely, the leaving group dependence is
substantially negative below pK_a of 8, and flat from 8 to 10, while the pH dependence for pNPP
is flat, and the pH dependence for phenyl phosphate is more bell-shaped (data not shown). We,
therefore, concluded that E435 does not act as a general acid, and focused on the E431Q mutant.

The leaving group dependence of E431Q is shown in Figure 2. The \( \beta_{lg} \) value obtained
using the substrates 2-chloro-4-nitrophenyl phosphate, pNPP, and 4-acetylphenyl phosphate
was -1.3 ± 0.2. This value is even greater than that obtained for the wild-type enzyme and
similar to the nonenzymatic solution reaction (\( \beta_{lg} = -1.23; (68) \)), and is consistent with an
absence of general acid catalysis. Further, the difference in \( k_{cat}/K_m \) values for the substrates
6,8-difluoro-4-methyl umbelliferyl phosphate and 4-methylumbelliferyl phosphate,
corresponds to a \( \beta_{lg} \) value of -1.35. Unfortunately, we were not able to obtain \( k_{cat}/K_m \) values
for the substrates with leaving group pK_a values higher than 8.05 for E431Q (Table III), so we
do not know whether the trend in leaving group dependence would continue over this range. The
signal was simply too low and not reproducible, even at high enzyme concentrations (up to 13
μM) and long incubation times (4 hours). We estimate that the $k_{cat}/K_m$ values for the high $pK_a$ substrates 4-trifluoromethylphenyl phosphate, 4-chlorophenyl phosphate, 4-fluorophenyl phosphate, and phenyl phosphate are well below 0.1 M$^{-1}$s$^{-1}$, and at least 10-fold lower than that of 4-acetylphenyl phosphate. This is a significant difference from both the wild-type enzyme and E435Q, which show virtually the same $k_{cat}/K_m$ values for all of these substrates. We, therefore, conclude that E431 plays an essential role in the efficient hydrolysis of substrates with leaving group $pK_a$ values higher than 8.

We also examined the pH dependence of E431Q using the substrates 2-chloro-4-nitrophenyl phosphate, $p$NPP, and 4-acetylphenyl phosphate as shown in Figure 3B. Just as for the wild-type enzyme, relatively flat pH dependencies were observed for 2-chloro-4-nitrophenyl phosphate and $p$NPP. As Table IV shows, $pK_{E1}$ values of 6.06 ± 0.18 and 6.20 ± 0.08 were obtained for 2-chloro-4-nitrophenyl phosphate and $p$NPP reactions, respectively. However, a flat pH dependence was also observed for the 4-acetylphenyl phosphate reaction with a $pK_{E1}$ value of 6.51 ± 0.14. The $pK_a$ value of the active site Cys residue ($pK_{E1}$ =6.1-6.5) for the E431Q mutant is substantially higher than that ($pK_{E1}$ =5.5) for the wild-type Cdc25A, possibly due to the elimination of the carboxyl group near the active site Cys residue. The flat pH dependence for 4-acetylphenyl phosphate is quite unlike the wild-type enzyme which showed a steady decrease in $k_{cat}/K_m$ value as the pH was raised. Thus, mutating residue 431 from glutamic acid into glutamine abolishes the basic limb of the pH profile for 4-acetylphenyl phosphate. Therefore, we conclude that E431 is responsible for the basic limb of the pH profile.
seen for wild-type Cdc25A with 4-acetylphenyl phosphate (Figure 3A) and that E431 must be protonated for optimal catalysis of this substrate. Glutamic acid 431 most likely plays the same role in the hydrolysis of the higher pKₐ substrates 4-trifluoromethylphenyl phosphate, 4-chlorophenyl phosphate, 4-fluorophenyl phosphate, and phenyl phosphate. Based on the fact that mutation of glutamic acid 431 into glutamine both greatly diminishes Cdc25A’s ability to hydrolyze high pKₐ substrates and eliminates the pH dependence of 4-acetylphenyl phosphate, we conclude that E431 must be protonated in order for Cdc25A to efficiently hydrolyze substrates with leaving group pKₐ values of 8 and higher.

It should be noted that one possible criticism of this study is that the phenomena that we have observed are the result of phosphatase contaminants in our preparations of Cdc25A that copurified with it. It could be argued that the kink in the leaving group dependence for the wild-type enzyme is the result of impurities that hydrolyze the high pKₐ substrates very efficiently. And since these enzymes were purified by ion exchange chromatography, mutation of glutamic acid 431 into glutamine would simply eliminate a negative charge on the enzyme and cause E431Q to be purified without these impurities. Thus, it could be argued, that the differences observed between the wild-type enzyme and E431Q are the result of phosphatase impurities present in the wild-type preparation that are not present in the E431Q preparation. We have taken several approaches to address this issue (data not shown). First, we performed leaving group dependencies on different forms of Cdc25 proteins obtained under much different purification conditions, reasoning that different forms of Cdc25 should copurify with much different impurities. The same leaving group dependence pattern was observed for
Cdc25A/E435Q, GST-Cdc25A, and GST-Cdc25B suggesting that this kink in the leaving group dependence was due to Cdc25A and not impurities. Next, we determined the IC$_{50}$ value of vanadate for Cdc25A using both pNPP and 4-chlorophenyl phosphate as substrates. In each case we obtained the same IC$_{50}$ value of 9 µM, which suggests that both of these substrates are hydrolyzed via the same active site. We also performed iodoacetate inactivation of Cdc25A. Iodoacetate can specifically modify the active site Cys residue in PTPases leading to enzyme inactivation (78). Since Cdc25A has an active site thiolate that is required for activity, iodoacetate should completely inactivate Cdc25A. And since, E. coli is not known to have any thiol dependent phosphatases, iodoacetate should not inactivate any E. coli derived phosphatase impurities. Upon incubation of wild-type Cdc25A with iodoacetate, we observed complete inactivation within 6 minutes suggesting that all phosphatase activity in the Cdc25A preparation was the result of Cdc25A and not impurities. Additionally, we observed the same rate of inactivation whether pNPP or phenyl phosphate was used to measure residual phosphatase activity, which again suggests that these substrates are hydrolyzed via the same active site. Taken together, these results show that the kinetic data presented in this study are the intrinsic properties of Cdc25A and its mutants, and not phosphatase impurities.

_Proposed Models for Cdc25A Catalysis._ The finding that Cdc25A appears to hydrolyze low leaving group pK$_a$ substrates, like pNPP, without the aid of a general acid, and higher pK$_a$ substrates with a general acid is puzzling. We propose several possible kinetic models, shown in Figures 4 and 5 to explain how this could occur and to account for all of the experimental data presented in this study. The $k_{cat}/K_m$ value reflects all steps up to and including the first
irreversible step, which for PTPases is the formation of the phosphoenzyme intermediate. Therefore, in each model, all steps leading up to phosphoenzyme intermediate formation are shown, and “catalysis” refers to this phosphoenzyme intermediate formation. The hydrolysis of the phosphoenzyme intermediate is the same for all substrates and should proceed rapidly after its formation.

In model A (Figure 4) we propose that E431 can act as a general acid, but it is poorly positioned to participate efficiently in hydrolysis of small molecule substrates. For the substrates with low leaving group pKₐ values (2-chloro-4-nitrophenyl phosphate or pNPP), the transition state for phosphoenzyme intermediate formation is reached well before any protonation of the leaving group can occur. The kinetic isotope effects are consistent with this, indicating no protonation of the leaving group, para-nitrophenol, at the transition state. As the transition state can be reached easily for these substrates without protonation of the leaving group, the rate of catalysis does not depend upon the protonation state of the enzyme. Therefore these substrates show flat pH dependencies. The substrates with higher leaving group pKₐ values, 4-chlorophenyl phosphate and phenyl phosphate, are intrinsically much less reactive than 2-chloro-4-nitrophenyl phosphate or pNPP. Therefore the attainment of the transition state will occur much more slowly for the higher leaving group pKₐ substrates, in the absence of a general acid, than for the lower leaving group pKₐ substrates. This is depicted as the set of arrowheads of descending length emanating from the enzyme-substrate complex in which E431 is not protonated in the bottom half of Figure 4. For the higher leaving group pKₐ substrates, the attainment of the transition state is sufficiently slow that protonation of the leaving group occurs
at or before the transition state when the enzyme is in its properly protonated form. Therefore, protonation of the leaving group facilitates catalysis for the substrates 4-acetylphenyl phosphate, 4-chlorophenyl phosphate, and phenyl phosphate, but the rate of hydrolysis of these substrates is still relatively low because the general acid is not optimally positioned when bound with small molecule substrates. As depicted in Figure 4, the rate of general acid independent catalysis is much lower than the rate of general acid-dependent catalysis for the substrates 4-chlorophenyl phosphate and phenyl phosphate. Therefore, the rate of catalysis for these substrates depends upon the proportion of Cdc25A in which E431 is protonated, and they produce bell-shaped pH profiles. For the substrate 4-acetylphenyl phosphate, the rate of general acid-independent catalysis is more comparable to the rate of general acid-dependent catalysis. For this reason, as the pH of the buffer is raised, the $k_{cat}/K_m$ value decreases, but not as dramatically as for 4-chlorophenyl phosphate or phenyl phosphate.

In model B (Figure 5) we propose that E431 is a general acid, but that it is poorly positioned to participate in catalysis in the initial enzyme substrate complex. The enzyme substrate complex can subsequently undergo a relatively slow conformational rearrangement to bring the E431 into proper position to function as a general acid. At the very left of Figure 5, Cdc25A is shown as an equilibrium between its general acid-protonated and deprotonated forms. The enzyme and substrate then come together to form the enzyme substrate complex in which E431 is not properly positioned to function as a general acid. At this point, there are two competing processes; general acid-independent catalysis, and a conformational change that brings E431 into proper position for catalysis. For the low leaving group pK$_a$ substrates, the general acid-independent catalysis is much faster than the conformational change, whereas for
the high $pK_a$ substrates the conformational change is much faster than the general acid-independent catalysis. Once the conformational change occurs that brings E431 into proper position, general acid-catalyzed hydrolysis will presumably occur rapidly, regardless of the identity of the substrate, as long as E431 is protonated. The substrates 2-chloro-4-nitrophenyl phosphate and $p$NPP primarily proceed through the general acid independent route, before the conformational change has had time to occur. As this route does not depend upon the protonation state of E431, the rate of hydrolysis of these substrates shows little pH dependence. As the $pK_a$ of the substrate leaving group is raised, the substrate becomes less and less reactive in the absence of a general acid, which is depicted in Figure 5 as the series of arrows of decreasing size emanating from the enzyme-substrate complex structures in which E431 is deprotonated or poorly positioned. Therefore the substrates 4-chlorophenyl phosphate and phenyl phosphate primarily proceed through the acid catalyzed route, because the rate of conformational change followed by acid catalysis is simply much faster than the rate of hydrolysis in the absence of a general acid for these substrates. The overall rate of hydrolysis for these high leaving group $pK_a$ substrates depends upon both the rate of conformational change and the proportion of Cdc25A molecules that have E431 protonated. As a result, the $k_{cat}/K_m$ values for these substrates decreases as the pH of the buffer is increased.

For the substrate 4-acetylphenyl phosphate, which has a leaving group $pK_a$ that lies between those of $p$NPP and 4-chlorophenyl phosphate, the reaction seems to proceed through a mechanism that lies between those of the high and low $pK_a$ substrates. It is likely that for this substrate, the rate of general acid-independent catalysis is comparable to the rate of
conformational change. As a result, the hydrolysis of this substrate can proceed via the general acid-independent or the acid catalyzed route efficiently. However, as the pH of the buffer is raised less of the protonated form of the enzyme is available and a greater proportion of the hydrolysis takes place through the general acid independent routes. The net result is a significant pH dependence for 4-acetylphenyl phosphate, but one that is substantially flatter than the pH dependence for the higher pKa substrates.

In Model B, we have shown E431 as the general acid—protonating the leaving group directly. It is also possible that E431 is simply a residue that must be protonated in order for Cdc25A to achieve a more active conformation, perhaps through a slow conformational change involving rearrangement of the active site phosphate-binding loop. In this case the reasoning would be essentially identical as Model B, so we have not shown this possibility as a separate model. In both cases, the conformational change must be rate-limiting for substrates with more basic leaving groups, in order to account for the lack of leaving group effect.

The idea that a conformational change occurs during the mechanism was tested by determining the kinetic constants of Cdc25A in 45% glycerol. It has been suggested that glycerol can alter the conformational properties of PTPases, such as MKP3 for which glycerol appears to stabilize the more active conformation (73). In both cases in Model B (shown in Figure 5), we would expect glycerol to have differential effects on different substrates. Indeed we found that 45% glycerol modestly activates Cdc25A towards pNPP (1.5 fold), more strongly activates Cdc25A toward 4-acetylphenyl phosphate (2-fold), and more substantially activates Cdc25A’s activity towards phenyl phosphate (4.2 fold) (Table V). Further, when E431 was mutated, these effects were no longer observed, and in fact glycerol had a slight inhibitory effect
on this mutant. This suggests that glycerol stabilizes the more active form of the enzyme; in mechanism B this corresponds to the form in which the general acid E431 is properly positioned or in which the active site is more properly formed. In terms of mechanism A, perhaps glycerol could serve to position the general acid closer to the substrate and facilitate proton transfer. It has been known for some time, and shown quantitatively (77) that Cdc25 has much higher activity towards cyclin/cdk complexes than for small molecule substrates. In terms of the mechanisms presented here, we suggest that binding of the physiological cyclin/cdk substrate may facilitate the proper positioning of the general acid, and/or stabilizes the active conformation of Cdc25A leading to an enhanced catalytic rate for the dephosphorylation of the physiological substrate. This is analogous to what has been observed in the dual specificity phosphatase MKP3 (22, 73, 74, 79).

**Biological Implications.** Admittedly the three possible mechanisms presented here cannot necessarily be distinguished by the experiments that we have presented. However, we have revealed some novel features of the Cdc25A mechanism, and some general conclusions can be drawn that are applicable regardless of the specific mechanism. The finding that Cdc25A may hydrolyze low leaving group pK_a substrates, like \( pNPP \), via one mechanism and higher leaving group pK_a substrates by another is intriguing. In many studies of PTPases, \( pNPP \) is used as a substrate where it is regarded as a phosphotyrosine mimic. While \( pNPP \) is sterically very much like phosphotyrosine, it is very different electronically. The pK_a of para-nitrophenol is 7.1 while the pK_a of a tyrosine hydroxyl is 10.5. The large negative \( \beta_{1g} \) value (-1.23) for the solution dianion reaction means that \( pNPP \) is, intrinsically, 10,000-fold more reactive to
hydrolysis than phosphotyrosine based on the difference in their pKₐ values. Because of this substantial difference in reactivity, it is possible that the mechanism that Cdc25A employs for pNPP hydrolysis may not reflect the mechanism that Cdc25A employs for the hydrolysis of phosphotyrosine or phosphothreonine in the physiological substrates. The pKₐ of tyrosine is actually much closer to that of phenol (pKₐ=9.99) than it is to para-nitrophenol, therefore phenyl phosphate is a more accurate phosphotyrosine mimic than is pNPP. For this reason, it is likely that the mechanism that Cdc25A employs for the hydrolysis of higher pKₐ substrates such as phenyl phosphate more accurately reflects the mechanism that Cdc25A employs for the hydrolysis of physiological substrates which have the high pKₐ leaving groups tyrosine and threonine.

It has been suggested recently that the general acid of Cdc25B does not actually reside on Cdc25B, but may actually reside on the physiological cyclin/cdk substrate instead (77). This was concluded based on the facts that the small molecule substrates pNPP, and OMFP showed flat pH dependencies, whereas the bell-shaped pH profile was only observed in the hydrolysis of the physiological cyclin/cdk substrate. We, on the other hand, have shown that the pH dependence of Cdc25A for high leaving group pKₐ, small-molecule substrates appears identical to that obtained by Chen et. al. (77) using the cyclin/cdk substrate; indicating that this pH dependent behavior is not a phenomenon particular to the physiological substrate. This suggests that the general acid may in fact reside on Cdc25 itself. Mutation of E431 both drastically reduces the catalytic rate for the high leaving group pKₐ substrates, but not for the lower pKₐ substrates, and
flattens the pH profile for a substrate with intermediate leaving group pK\textsubscript{a} value (4-acetylphenyl phosphate). This suggests that E431 can possibly act as a general acid for the hydrolysis of these higher leaving group pK\textsubscript{a} substrates. Chen et al. (77) found that when the corresponding residue of Cdc25B (E474) was mutated, the rate of hydrolysis of the cyclin/cdk substrate dropped 100-fold while it remained the same for pNPP. This is consistent with our results in which the mutation of E431 to glutamine dramatically decreased the rate of catalysis for the high leaving group pK\textsubscript{a} substrates but not the lower leaving group pK\textsubscript{a} substrates like pNPP. However, Chen et al. (77) found that when E474 was mutated to glutamine, the pH profile for the cdk/cyclin substrate hydrolysis remained bell-shaped. This is not consistent with our results, in which we have shown that mutation of E431 to glutamine flattens the pH dependence of the substrate 4-acetylphenyl phosphate. The source of this discrepancy is currently unknown.

In any case, this study reveals several important features of the mechanism of Cdc25A. First, Cdc25A does not employ a general acid in the hydrolysis of low leaving group pK\textsubscript{a} substrates like pNPP. Second, even in the absence of general acid catalysis, the transition state of pNPP hydrolysis is highly dissociative. Third, Cdc25A shows a strong preference for bulky, multi-ringed substrates over smaller one-ringed substrates. Fourth, E431 plays an essential role in the hydrolysis of high leaving group pK\textsubscript{a} substrates, for which it appears to behave as a general acid. Whether or not E431 is the true general acid in the hydrolysis of the physiological cyclin/cdk substrates remains to be seen. Future experiments will be directed towards this question.
REFERENCES:

1. Coleman, T. R., and Dunphy, W. G. (1994) Curr. Op. Cell. Biol. 6, 877-882
2. Hoffmann, I., and Karsenti, E. (1994) J. Cell. Sci. Suppl. 18, 75-79
3. Nilsson, I., and Hoffmann, I. (2000) Prog. Cell Cycle Res. 4, 107-114
4. Hernandez, S., Hernandez, L., Bea, S., Pinyol, M., Nayach, I., Bellosillo, B., Nadal, A., Ferrer, A., Fernandez, P. L., Montserrat, E., Cardesa, A. and Campo, E. (2000) Int. J. Cancer (Pred. Oncol.) 89, 148-152
5. Cangi, M. G., Cukor, B., Soung, P., Signoretti, S., Moreira, G. Jr., Ranashinge, M., Cudy, B., Pagano, M., and Loda, M. (2000) J. Clin. Invest. 106, 753-761
6. Broggini, M., Buraggi, G., Brenna, A., Riva, L., Codegoni, A. M., Torri, V., Lissoni, A., Mangioni, C., and D’Incalci, M. (2000) Anticancer Res. 20, 4835-4840
7. Hernandez, S., Bessa, X., Bea, S., Hernandez, L., Nadal, A., Mallofre, C., Montana, J., Castells, A., Fernandez, P. L., Cardesa, A., Campo, E. (2001) Lab Invest. 81, 465-473
8. Wu, F. Y. -H., and Sun, T. -P. (1999) Eur. J. Cancer 35, 1388-1393
9. Tamura, K., Rice, R. L., Wipf, P., and Lazo, J. S. (1999) Oncongene 18, 6989-6996
10. Takahashi, M., Dodo, K., Sugimoto, Y., Aoyagi, Y., Yamada, Y., Hashimoto, Y., and Shirai, R. (2000) Bioorg. Med. Chem. Lett. 10, 2571-2574
11. Peng, H., Xie, W., Otterness, D. M., Cogswell, J. P., McConnel, R. T., Carter, H. L., Powis, G., Abraham, R. T., and Zalkow, L. H. (2001) J. Med. Chem. 44, 834-848
12. Morgan, D. O. (1997) Annu. Rev. Cell Dev. Biol. 13, 261-291
13. Dunphy, W. G. and A. Kumagai (1991) Cell 67, 189-196
14. Gautier, J., Solomon, M. J., Booher, R. N., Bazan, J. F., and Kirschner, M. W. (1991) *Cell* **67**, 197-211

15. Millar, J. B., McGowan, C. H., Lanaers, G., Jones, R., and Russel P. (1991) *Embo J* **10**, 4301-4309

16. Strausfeld, U., Labbe, J. C., Fesquet, D., Cavadore, J. C., Picard, A., Sadhu, K., Russel, P., and Doree, M. (1991) *Nature* **351**, 242-245

17. Guan, K. L., and Dixon, J. E. (1991) *J. Biol. Chem.* **266**, 17026-17030

18. Cho, H., Krishnaraj, R., Kitas, E., Bannworth, W., and Walsh, C. T. (1992) *J. Am. Chem Soc.* **114**, 7296-7298

19. Wo, Y. Y., Zhou, M. M., Stevis, P., Davis, J. P., Zhang, Z.-Y., and Van Etten, R. L. (1992) *Biochemistry* **31**, 1712-1721

20. Denu, J. M., Lohse, D. L., Vijayalakshmi, J., Saper, M. A., and Dixon, J. E. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 2493-2498

21. Zhang, Z. -Y. (1998) *Crit. Rev. Biochem. Mol. Biol.* **33**, 1-52

22. Zhao, Y., and Zhang, Z.-Y. (2001) *J. Biol. Chem.* **276**, 32382-32391

23. Zhang, Z.-Y., Wang, Y., Wu, L., Fauman, E., Stuckey, J. A., Schubert, H. L., Saper, M. A., and Dixon, J. E. (1994) *Biochemistry* **33**, 15266-15270

24. Zhang, Y. L., Hollfelder, F., Gordon, S. J., Chen, L., Keng, Y. F., Wu, L., Herschlag, D., and Zhang, Z. Y. (1999) *Biochemistry* **38**, 12111-12123

25. R. H. Hoff, L. Wu, B. Zhou, Z.-Y. Zhang, and A. C. Hengge (1999) *J. Am. Chem. Soc.* **121**, 9514-9521

26. Streuli, M., Krueger, N. X., Thai, T., Tang, M., and Saito, H. (1990) *EMBO J.* **9**, 2399-
27. Johnson, P., Ostergaard, H. L., Wasden C., and Trowbridge, I. S. (1992) *J. Biol. Chem.* **267**, 8035-8041

28. Cirri, P., Chiarugi, P., Camici, G., Manao, G., Raugei, G., Cappugi, G., and Ramponi, G. (1993) *Eur J Biochem* **214**, 647-57

29. Xu, X., and Burke, S. P. (1996) *J. Biol. Chem.* **271**, 5118-5124

30. Zhang, Z.-Y., Wang, Y., and Dixon, J. E. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 1624-1627

31. Hengge, A. C., Sowa, G. A., Wu, L., and Zhang, Z.-Y. (1995) *Biochemistry* **34**, 13982-13987

32. Lohse, D. L., Denu, J. M., Santoro, N., and Dixon, J. E. (1997) *Biochemistry* **36**, 4568-4575

33. Taddei, N., Chiarugi, P., Cirri, P., Fiaschi, T., Stefani, M., and Ramponi, G. (1994) *FEBS Lett.* **350**, 328-332

34. Zhang, Z., Harms, E., and Van Etten, R. L. (1994) *J. Biol. Chem.* **269**, 25947-25950

35. Wu, L., and Zhang, Z.-Y. (1996) *Biochemistry* **35**, 5426-5434

36. Denu, J. M., Zhou, G., Guo, Y., and Dixon, J. E. (1995) *Biochemistry* **34**, 3396-3403

37. Hengge, A. C., Denu, J. M. and Dixon, J. E. (1996) *Biochemistry* **35**, 7084-7092

38. Rigas, J. D., Hoff, R. H., Rice, A. E., Hengge, A. C., and Denu, J. M. (2001) *Biochemistry* **40**, 4398-4406

39. Iversen, L. F., Andersen, H. S., Branner, S., Mortensen, S. B., Peters, G. H., Norris, K., Olsen, O. H., Jeppesen, C. B., Lundt, B. F., Ripka, W., Moller, K. B., and Moller, N. P.
H. (2000) *J. Biol. Chem.* **275**, 10300-10307

40. Flint, A. J., T. Tiganis, T., Barford, D., and Tonks, N. K. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 1680-1685

41. Zhang, Y. L., Yao, Z. J., Sarmiento, M., Wu, L., Burke, T. R. Jr., and Z. -Y. Zhang (2000) *J. Biol. Chem.* **275**, 34205-34212

42. Eckstein, J. W., Beer-Romero, P., and Berdo, I. (1996) *Protein Sci.* **5**, 5-12

43. Hofmann, K., Bucher, P., and Kajava, A. V. (1998) *J. Mol. Biol.* **282**, 195-208

44. Fauman, E. B., Cogswell, J. P., Lovejoy, B., Roque, W. J., Holmes, W., Montana, V. G., Piwnica-Worms, H., Rink, M. J., and Saper, M. A. (1998) *Cell* **93**, 617-625

45. Reynolds, R. A., Yem, A. W., Wolfe, C. L., Deibel, M. R. Jr., Chidester, C. G., and Watenpaugh, K. D. (1999) *J. Mol. Biol.* **293**, 559-568

46. Hall, A. D., and Williams, A. (1986) *Biochemistry* **25**, 4784-4790

47. Black, M. J., and Jones, M. E. (1983) *Anal. Biochem.* **135**, 233-238

48. Zhang, Z. -Y., Maclean, D., Thieme-Sefler, A. M., Roeske, R. M., and J. E. Dixon (1993) *Anal. Biochem.* **211**, 7-15

49. Hengge, A. C., Edens, W. A., and Elsing H. (1994) *J. Am. Chem. Soc.* **116**, 5045-5049

50. Bigeleisen, J. and Wolfsberg, M. (1958) *Adv. Chem. Phys.* **1**, 15-76

51. O’Leary, M. H., and Marlier, J. F. (1979) *J. Am. Chem. Soc.* **101**, 3300-3306

52. Caldwell, S. R.; Raushel, F. M.; Weiss, P. M.; Cleland, W. W. (1991) *Biochemistry* **30**, 7444-7450

53. Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O’Leary, M. H., and Northrop, D. B., eds) pp. 122 University Park Press, Baltimore,
54. Stuckey, J. A., Schubert, H. L., Fauman, E., Zhang, Z.-Y., Dixon, J. E., and Saper, M. A. (1994) *Nature* **370**, 571-575

55. Barford, D., Flint, A. J., and Tonks, N. K. (1994) *Science* **263**, 1397-1404

56. Jia, Z., Barford, D., Flint, A. J., and Tonks, N. K. (1995) *Science* **268**, 1754-1758

57. Sarmiento, M., Puius, Y. A., Vetter, S. W., Keng, Y. F., Wu, L., Zhao, Y., Lawrence, D. S., Almo, S. C., and Zhang, Z.-Y. (2000) *Biochemistry* **39**, 8171-8179

58. Su, X. D., Taddei, N., Stefani, M., Ramponi, G., and Nordlund, P. (1994) *Nature* **370**, 575-578

59. Wang, S., Tabenero, L., Zhang, M., Harms, E., Van Etten, R. L., and Stauffacher, C. V. (2000) *Biochemistry* **39**, 1903-1914

60. Yuvaniyama, J., Denu, J. M., Dixon, J. E., and Saper, M. A. (1996) *Science* **272**, 1328-1331

61. Zhang, M., Van Etten, R. L., and Stauffacher, C. V. (1994) *Biochemistry* **33**, 11097-11105

62. Zhang, Z.-Y., Palfey, B. A., Wu, L., and Zhao, Y. (1995) *Biochemistry* **34**, 16389-16396

63. Denu, J. M., and Dixon, J. E. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 5910-5914

64. Zhao, Y., and Zhang, Z.-Y. (1996) *Biochemistry* **35**, 11797-11804

65. Zhang, Z.-Y., and Dixon, J. E. (1994) *Advances in Enzymology* **68**, 1-36

66. Hengge, A. C., Zhao, Y., Wu, L., and Zhang, Z.-Y. (1997) *Biochemistry* **36**, 7928-7936

67. Cleland, W. W., and Hengge, A. C. (1995) *FASEB J.* **9**, 1585-1594
68. Kirby, A. J., and Varvoglis, A. G. (1967) *J. Am. Chem. Soc.* **89**, 415-423

69. Zhang, Z.-Y. and Van Etten, R. L. (1991) *Biochemistry* **30**, 8954-8959

70. Zhang, Z.-Y. (1995) *J. Biol. Chem.* **270**, 11199-11204

71. Zhao, Y., Wu, L., Noh, S. J., Guan, K.-L., and Zhang, Z.-Y. (1998) *J. Biol. Chem.* **273**, 5484-5492

72. Zhang, Z.-Y., Wu, L., and Chen, L. (1995) *Biochemistry* **34**, 16088-16096

73. Zhou, B., and Zhang, Z.-Y. (1999) *J. Biol. Chem.* **274**, 35526-35534

74. Fjeld, C. C., Rice, A. E., Kim, Y., Gee, K. R., and Denu, J. M. (2000) *J. Biol. Chem.* **275**, 6749-6757

75. Chen, L., Montserat, J., Lawrence, D. S., and Zhang, Z.-Y. (1996) *Biochemistry* **35**, 9349-9354

76. Gottlin, E. B., Xu, X., Epstein, D. M., Burke, S. P., Eckstein, J. W., Ballow, D. P. and Dixon, J. E. (1996) *J. Biol. Chem.* **271**, 27445-27449

77. Chen, W., Wilborn, M., and Rudolph, J. (2000) *Biochemistry* **39**, 10781-10789

78. Zhang, Z.-Y., and Dixon, J. E. (1993) *Biochemistry* **32**, 9340-9345

79. Zhou, B., Wu, L., Shen, K., Zhang, J., Lawrence, D. S., and Zhang, Z.-Y. (2001) *J. Biol. Chem.* **276**, 6506-6515
FIGURE LEGENDS:

Figure 1. The structure of pNPP and the isotope effects determined for Cdc25A in this study. The isotope effect notation used is described under Materials and Methods.

Figure 2. The leaving group dependence of wild-type Cdc25A (filled circles), and of Cdc25A/E431Q (filled triangles). The leaving group dependencies were determined at pH 7.0, 30°C. The lines are based upon the linear least squares fit to the log($k_{cat}/K_m$) verses leaving group pK_a. For wild-type Cdc25A, two separate linear regressions were done; one for the substrates with leaving group pK_a values 5.45-8.05 ($\beta_{lg} = -0.8 \pm 0.1$), and one for the substrates with leaving group pK_a values 8.68-9.99 ($\beta_{lg} = 0.08 \pm 0.02$). For the E431Q mutant, the substrates with leaving group pK_a values 5.45-8.05 gave a $\beta_{lg}$ value of -1.3 ± 0.2, while the substrates with leaving group pK_a values 8.68-9.99 had $k_{cat}/K_m$ values that were too low to be measured accurately.

Figure 3. The pH dependencies for wild-type Cdc25A (A) and the E431Q mutant (B). The substrates 2-chloro-4-nitrophenyl phosphate (circles), pNPP (triangles), 4-acetylphenyl phosphate (diamonds), 4-chlorophenyl phosphate (inverted triangles), and phenyl phosphate (squares) were used. For the E431Q mutant (B), the $k_{cat}/K_m$ values were too low to be measured accurately for the substrates 4-chlorophenyl phosphate and phenyl phosphate. Assays were done at 30°C.
Figure 4. Model A for Cdc25A catalysis. E431 is shown as the general acid, but it is not properly positioned to facilitate catalysis efficiently. Therefore, protonation of the leaving group lags behind the attainment of the transition state for the low leaving group pKₐ substrates, which are intrinsically more reactive than the high leaving group pKₐ substrates. Attainment of the transition state for the high pKₐ substrates is sufficiently slow as to require protonation of the leaving group to occur at or before the transition state.

Figure 5. Model B for Cdc25A catalysis. A slow conformational change can bring E431 into the active site to act as a general acid. The low leaving group pKₐ substrates are sufficiently reactive that they can be hydrolyzed more rapidly than the conformational change can occur, whereas the high leaving group pKₐ substrates are sufficiently unreactive that the conformational change can occur, which brings the general acid into the proper position to facilitate catalysis.
Table I

*Initial kinetic analysis of Cdc25A and several mutants*

| Cdc25A    | $k_{cat}$ (s$^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ (M$^{-1}$s$^{-1}$) |
|-----------|----------------------|------------|----------------------------------|
| Wild Type | 0.157 ± 0.013        | 21 ± 2     | 7.48 ± 0.90                      |
| E431Q     | 0.171 ± 0.005        | 8.6 ± 0.8  | 19.9 ± 1.9                       |
| F432A     | 0.072 ± 0.001        | 12.0 ± 0.5 | 6.0 ± 0.3                        |
| E435Q     | 0.221 ± 0.004        | 10.9 ± 0.5 | 20.2 ± 1                         |
| G437S     | 0.0177 ± 0.0003      | 11.1 ± 0.7 | 1.59 ± 0.12                      |

The assays were performed using pNPP in pH 8.0, 100 mM Tris, 1mM DTT, 1mM EDTA, at 30°C.
Table II

*Kinetic Isotope Effects*

| Enzyme               | $^{15}(V/K)$ ± SE  | $^{18}(V/K)_{\text{bridge}}$ ± SE | $^{18}(V/K)_{\text{nonbridge}}$ ± SE |
|----------------------|--------------------|-----------------------------------|--------------------------------------|
| Cdc25A (pH 7.2)      | 1.0030 ± 0.0002    | 1.0357 ± 0.0049                   | 0.9988 ± 0.003                       |
| Cdc25A (pH 6.1)      | 1.0031 ± 0.0004    | 1.0291 ± 0.0008                   |                                      |
| VHR$^a$              | 0.9999 ± 0.0004    | 1.0118 ± 0.0020                   | 1.0003 ± 0.00      |
| VHR/D92N$^a$         | 1.0030 ± 0.0002    | 1.0294 ± 0.0009                   | 1.0019 ± 0.00      |

Isotope effects for Cdc25A were determined either at pH 7.2, 500 mM Tricine, or pH 6.1, 250 mM BisTris, containing 1 mM DTT, at 30°C, as described under Materials and Methods. $^a$ From (37)
Table III

*Kinetic parameters of Cdc25A, E431Q and E435Q with various small molecule substrates*

\[
k_{\text{cat}}/K_m \ (\text{M}^{-1}\text{s}^{-1})
\]

| Substrate | leaving group pK\text{a} | Cdc25A   | E431Q    | E435Q    |
|-----------|--------------------------|----------|----------|----------|
| OMFP      | 4.6                      | 87,500 ± 900 | 50,000 ± 400 | ND       |
| DIFMUP    | 4.7                      | 1210 ± 1  | 9098 ± 16 | 2000 ± 1 |
| MUP       | 7.8                      | 2.8 ± 0.2 | 2.81 ± 0.05 | 5.97 ± 0.20 |
| β-naph.   | 9.38                     | 4.2 ± 0.2 | <0.1      | 6.72 ± 0.7 |
| 2-Cl-4-NO\text{2} | 5.45                   | 203 ± 16  | 2300 ± 200 | 2000 ± 200 |
| pNPP      | 7.14                     | 12.7 ± 0.6 | 30.9 ± 3.7 | 25.7 ± 1.9 |
| 4-Ac      | 8.05                     | 1.19 ± 0.07 | 0.74 ± 0.05 | 3.27 ± 0.3 |
| 4-CF\text{3} | 8.68                   | 1.0 ± 0.1  | <0.1      | ND       |
| 4-Cl      | 9.38                     | 1.19 ± 0.05 | <0.1      | 2.82 ± 0.3 |
| 4-F       | 9.95                     | 1.4 ± 0.1  | <0.1      | ND       |
| PhPi      | 9.99                     | 1.19 ± 0.05 | <0.1      | 4.42 ± 0.3 |

The assays were performed at pH 7.0, 30 °C as described under Materials and Methods. Listed are the leaving group pK\text{a} values for the substrates. Abbreviations: OMFP, 3-O-methylfluorescein phosphate; DIFMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; MUP, 4-methylumbelliferyl phosphate; β-naph., β-naphthyl phosphate; 2-Cl-4-NO\text{2}, 2-chloro-4-nitrophenyl phosphate; pNPP, 4-nitrophenyl phosphate; 4-Ac, 4-acetylphenyl phosphate; 4-CF\text{3}, 4-trifluoromethylphenyl phosphate; 4-Cl, 4-chlorophenyl phosphate, 4-F, 4-fluorophenyl phosphate; PhPi, phenyl phosphate.
Table IV

**pH dependence parameters of Cdc25A**

| enzyme     | substrate   | pKs2 | pKe1 | pKe2 | (kcat/Km)max | (M⁻¹s⁻¹) |
|------------|-------------|------|------|------|--------------|----------|
| eq.        | (fixed)     |      |      |      |              |          |
| Cdc25A     | 2-Cl-4-NO₂  | 4.6  | 5.47 ± 0.17 | --- | 151 ± 7      | [5]      |
|            | pNPP        | 5.00 | 5.57 ± 0.14 | --- | 17.7 ±       |          |
| 0.5 [5]    |             |      |      |      |              |          |
| Cdc25A     | 4-Cl        | 5.52 |      |      | fixed at 5.5 | 6.06 ±   |
| 0.04 13.8 ± 0.9 [6] | | | | | | |
| Cdc25A     | PhPi        | 5.72 |      |      | fixed at 5.5 | 6.00 ±   |
| 0.08 18 ± 2 [6] | | | | | | |
| E431Q      | 2-Cl-4-NO₂  | 4.6  | 6.06 ± 0.18 | --- | 2300 ± 100   | [5]      |
| [5]        | pNPP        | 5.00 | 6.20 ± 0.10 | --- | 33 ± 1       |          |
| E431Q      | 4-Ac        | 5.24 | 6.50 ± 0.14 | --- | 1.9 ±        |          |

The pH dependence parameters were obtained by fitting the kinetic data to equation [5] or [6] as indicated and described under Materials and Methods. Abbreviations: eq., equation; 2-Cl-4-NO₂, 2-chloro-4-nitrophenyl phosphate; pNPP, 4-nitrophenyl phosphate; 4-Cl, 4-chlorophenyl phosphate; PhPi, phenyl phosphate; 4-Ac, 4-acetylphenyl phosphate
Table V

The effect of glycerol on the activity of Cdc25A

\( k_{\text{cat}}/K_m \) (M\(^{-1}\)s\(^{-1}\))

| Substrate | % glycerol | Cdc25A    | E431Q    |
|-----------|------------|-----------|----------|
| pNPP      | 0          | 10.9 ± 1  | 24.1 ± 3 |
| pNPP      | 45         | 16.4 ± 1  | 15.5 ± 1 |
| 4-Ac      | 0          | 1.04 ± 0.09 | 1.09 ± 0.08 |
| 4-Ac      | 45         | 2.19 ± 0.09 | 0.806 ± 0.05 |
| PhPi      | 0          | 1.19 ± 0.05 | <0.1    |
| PhPi      | 45         | 5.0 ± 0.2  | ND       |

The assays were performed at pH 7.0, 30°C as described under Materials and Methods. Abbreviations: pNPP, 4-nitrophenyl phosphate; 4-Ac, 4-acetylphenyl phosphate; PhPi, phenyl phosphate.
Figure 1:
Figure 2:
Figure 3:

A

$\frac{k_{cat}}{K_m} (M^{-1} s^{-1})$

pH

B

$\frac{k_{cat}}{K_m} (M^{-1} s^{-1})$

pH
Figure 4:

Model A

\[ \text{Cdc25A-PO}_3^{2-} + \text{Ar-OH} \]

\[ \text{H-Cdc25A} + \text{Ar-OP}_3^{2-} \]

\[ \text{Cdc25A} + \text{Ar-OP}_3^{2-} \]

\[ \begin{array}{c}
\text{non-general acid} \\
\text{catalysis for} \\
R=4-\text{Ac, 4-Cl,} \\
\text{and phenyl phosphate} \\
\end{array} \]

\[ \begin{array}{c}
\text{non-general acid} \\
\text{catalysis} \\
R=2-\text{Cl, 4-nitro} \\
R=4-\text{nitro} \\
\end{array} \]

\[ \begin{array}{c}
\text{non-general acid} \\
\text{catalysis} \\
R=2-\text{Cl, 4-nitro} \\
R=4-\text{nitro} \\
R=4-\text{Ac} \\
\text{phenyl phosphate} \\
\end{array} \]
Figure 5:

\[ \text{H-Cdc25A} + \text{Ar-OPO}_3^{2-} \xrightarrow{\text{H}^+} \text{Cdc25A-PO}_3^{2-} + \text{Ar-OH} \]

\[ \text{Cdc25A} + \text{Ar-OPO}_3^{2-} \xrightarrow{\text{general acid catalysis}} \text{Cdc25A-PO}_3^{2-} + \text{Ar-O}^- \]

\[ \text{H-Cdc25A-PO}_3^{2-} \xrightarrow{\text{R=2-Cl,4-nitro}} \text{H-Cdc25A-PO}_3^{2-} \]

\[ \text{H-Cdc25A-PO}_3^{2-} \xrightarrow{\text{R=4-nitro}} \text{H-Cdc25A-PO}_3^{2-} \]

\[ \text{H-Cdc25A-PO}_3^{2-} \xrightarrow{\text{R=4-Ac}} \text{phenyl phosphate} \]

\[ \text{H-Cdc25A-PO}_3^{2-} \xrightarrow{\text{R=4-Cl}} \text{phenyl phosphate} \]
