Kinetic and Mechanistic Studies of a Cell Cycle Protein Phosphatase Cdc14*

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The Cdc14 family of protein phosphatases is conserved within eukaryotes and antagonizes the action of cyclin-dependent kinases, thereby promoting mitotic exit and cytokinesis. We performed a detailed kinetic and mechanistic study of the Cdc14 phosphatases with both small molecule aryl phosphates and a physiological protein substrate hCdh1. We found that Cdc14 displays a strong preference for two-ringed aryl phosphates over smaller one-ringed or larger, multi-ringed substrates, a finding that may have important implications for inhibitor design. Results from both leaving group and pH dependence of the Cdc14-catalyzed reaction are consistent with a general acid-independent mechanism for substrates with leaving group $pK_a < 7$ and a general acid-dependent mechanism for substrates with leaving group $pK_a > 7$. The use of both low and high leaving group $pK_a$ substrates, in combination with steady-state and pre-steady-state kinetic techniques enabled the isolation and analysis of both the phosphoenzyme (E-P) formation and hydrolysis step. We established the requirement of general acid catalysis for E-P formation in reactions with high leaving group $pK_a$ substrates, and the presence of general base catalysis in E-P hydrolysis. Mutational study of invariant acidic residues in Cdc14 identified Asp$^{50}$ as the general acid during E-P formation and the general base in E-P hydrolysis. We also identified several residues including Asp$^{50}$, Asp$^{129}$, Glu$^{168}$, Glu$^{171}$, and Asp$^{177}$ in the Cdc14 active site cleft that are required for efficient dephosphorylation of hCdh1.

Cdc14 is a protein phosphatase conserved from yeast to man (1, 2). In the budding yeast, Saccharomyces cerevisiae, Cdc14 is essential for cell cycle progression from late anaphase into G1 of the subsequent cell cycle, a process referred to as exit from mitosis (3–5). Cdc14 triggers mitotic exit by antagonizing the cyclin-dependent kinase (CDK)$^1$ activity in late mitosis. For example, Cdc14 dephosphorylates and stabilizes the mitotic CDK inhibitor Sic1, up-regulates Sic1 expression by dephosphorylating the transcription factor Swi5, and induces degradation of mitotic cyclins by dephosphorylating and activating the anaphase promoting complex (APC) regulatory protein Cdh1/Hct1 (6). Biochemical studies also suggest that human Cdc14 homologs can dephosphorylate products of CDKs, such as hCdh1 (7), cyclin E (8), and p53 (9).

Despite their structural similarities, the biological function of Cdc14 phosphatases may vary between species. Thus, in S. cerevisiae, Cdc14 is absolutely required for mitotic exit. In contrast, the Cdc14 homolog Clp1/Fpl1 is not required for mitotic exit in Schizosaccharomyces pombe, but rather is mainly involved in regulating cytokinesis and affects the timing of entry into mitosis (10, 11). In addition, CeCdc14 phosphatase is required for cytokinesis in the Caenorhabditis elegans embryo (12). The functional roles for the two human Cdc14 homologs, hCdc14A and hCdc14B, have not been fully defined, though depletion of hCdc14A by RNAi causes a variety of mitotic defects, including cytokinesis failure (13). It is possible that Cdc14 phosphatases may antagonize CDK activity by dephosphorylation of different substrates and regulate distinct cell-cycle transitions in different species, as CDK activity is important for many different cell cycle processes (14).

Although the role of the Cdc14 phosphatase in cell cycle has been studied extensively, relatively little is known about its catalytic mechanism. Given their critical roles in mitotic exit and cytokinesis, the Cdc14 phosphatases have been implicated as potential therapeutic targets for anticancer drug development. Thus it is important to define Cdc14 active site substrate specificity and identify residues that are essential for Cdc14 catalysis. Interestingly, the Cdc14 phosphatases contain the tyrosine phosphatase (PTP) superfamily. The PTPs are a diverse family of enzymes that includes tyrosine-specific, dual specificity, Cdc25, as well as low molecular weight phosphatases (15). Detailed enzymological studies suggest that the PTPs employ a conserved two-step mechanism (16), shown in Scheme 1, in which E is the enzyme, ArOPO$_4$$^-$$^2$, the substrate, EArOPO$_4$$^-$$^2$, the enzyme-substrate Michaelis complex, E-P, the phospho-enzyme intermediate, and ArOH, the leaving group phenol. In this mechanism, the side chain of the active site Cys residue serves as a nucleophile to accept the phosphoryl group

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§§The abbreviations used are: CDK, cyclin-dependent kinase; Tricine, N-$\beta$-hydroxy-1,1,1,1-tetrahydroxyethylglycine; PTP, protein-tyrosine phosphatase; nNPP, n-nitrophenyl phosphate; KAP, kinase-associated phosphatase; PRL, phosphatase of regenerating liver; PTEN, phosphatase and tensin homolog deleted on chromosome 10; MUP, 4-methylumbelliferyl phosphate; 8-FMUP, 8-fluoro-4-methylumbelliferyl phosphate; 6-FMUP, 6-fluoro-4-methylumbelliferyl phosphate; 5-FMUP, 5-fluoro-4-methylumbelliferyl phosphate; DiFMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; OMFP, 3-O-methylfluorescein phosphate; GST, glutathione S-transferase; NTA, nitrilotriacetic acid; APC, anaphase-promoting complex.
Cdc14 Protein Phosphatases

from the substrate to form E-P. The active site Arg makes bidentate hydrogen bonds with the phosphoryl group in the substrate through its guanidinium group and plays an important role in both substrate binding and transition state stabilization during catalysis. To facilitate substrate turnover, PTPs also utilize an Asp residue, which acts as a general acid by protonating the ester oxygen of the leaving group, thus greatly enhancing the rate of E-P formation. In the second step, E-P hydrolysis occurs by the attack of a nucleophilic water molecule assisted by the same Asp, functioning as a general base, with subsequent release of the free enzyme and inorganic phosphate.

\[
E + \text{H}_2\text{O} \rightarrow E\text{H}_2\text{O} \rightarrow E + \text{OH}^- + \text{P}\text{O}_4^{3-}
\]

Aside from the conserved active site CXXR motif, there are little sequence similarities between Cdc14 and other members of the PTP superfamily. Consequently, it is not known whether other residues also participate in Cdc14 catalysis. This is in contrast to other PTPs for which a number of conserved, catalytically important residues have been identified and characterized (16). In the following, we describe a kinetic investigation of Cdc14 substrate specificity with both small molecule aryl phosphates and a physiological protein substrate hCdh1. We also describe results from site-directed mutagenesis experiments in combination with steady-state and pre-steady-state kinetic analyses that lead to the identification of several catalytically important residues including the general acid/base in Cdc14 catalysis.

**EXPERIMENTAL PROCEDURES**

**Materials—**p-Nitrophenyl phosphate (pNPP) was purchased from Fluka. Phenyl phosphate, 4-methylumbelliferyl phosphate (MUP), β-naphthyl phosphate, and 3-O-methylfluorescein phosphate (OMFP) were obtained from Sigma, 6,8-Difluoro-4-methylumbelliferyl phosphate (DiFUMUP) was purchased from Molecular Probes. 2-Chloro-4-nitrophophate, 4-cyanophenyl phosphate, 4-trifluoromethylphenyl phosphate, and 4-chlorophenyl phosphate were synthesized as described (17). 8-Fluoro-4-methylumbelliferyl phosphate (8-FMUP), 6-Fluoro-4-methylumbelliferyl phosphate (6-FMUP), and 5-Fluoro-4-methylumbelliferyl phosphate (5-FMUP) were prepared as described (18).

**Expression and Purification of Cdc14 and Mutant Proteins**—The cDNA for budding yeast Cdc14 was subcloned into vector pET-15b. The Cdc14 mutants were obtained using the QuikChange site-directed mutagenesis kit from Stratagen. The sequences of the wild-type and mutant Cdc14s were confirmed by DNA sequencing. Wild-type and mutant Cdc14s were transformed in Escherichia coli BL21 (DE3) cells and induced with 0.3 mm isopropryl-1-thio-β-galactopyranosid at room temperature for 20 h. The His₆-tagged proteins were purified by Ni²⁺NTA resin based upon the procedure described by Qiagen. Proteins were judged to be at least 90% pure by SDS-PAGE.

**Steady-state Kinetics**—All assays with small molecule substrates were performed at 30 °C. The following buffers were used: 50 mM succinate (pH 5.0 – 6.0), 50 mM 3,3-dimethyl glutarate (pH 6.0 – 7.2), and 100 mM Tricine (pH 7.2 – 9.0). In addition, each buffer also contained 1 mEq EDTA, 2 mM diethiothreitol and was adjusted to an ionic strength of 150 mM with sodium chloride. To ensure initial rate conditions, the product versus time progress curves were routinely checked for linearity at different substrate and enzyme concentrations. In all cases, the enzyme concentration was at least 100-fold lower than that of the substrate, and the quantity of substrate consumed was less than 10% of the initial value, so that the steady-state assumption was fulfilled. There was no product inhibition under these conditions. For substrates 2-chloro-4-nitrophophate and pNPP, assay mixtures of 200 μl in total volume were set up in 1.1-m polycarbonate tubes from Marsh Biomedical. Reactions were started by the addition of an appropriate amount of Cdc14. The reaction mixtures were quenched with 50 μl of 5 % sodium hydroxide, and the absorbance at 405 nm was read using a plate reader. For substrates 5-FMUP, 8-FMUP, 6-FMUP, MUP, β-naphthyl phosphate, 4-cyanophenyl phosphate, 4-trifluoromethylphenyl phosphate, 4-chlorophenyl phosphate, phenyl phosphate, the reaction mixtures were quenched with 10% trichloroacetic acid, and the amount of inorganic phosphate released was quantitated based on the method of Black and Jones (19). For the substrates 6,8-DiFUMUP, 5-FMUP, 8-FMUP, 6-FMUP, MUP, continuous assays were performed. The reaction (1-mL total volume) was set up in a 1-mL quartz cuvette. The fluorescence of the product was monitored by a PerkinElmer Life Sciences 50B Luminescence spectrometer. When the substrate concentrations were well below the Kₘ, the kₐ/Kₚ values were obtained from a linear least square fit of the plot of initial rate versus substrate concentration using Equation 1,

\[
v = \frac{k_{\text{cat}}[\text{S}]_o[\text{E}]}{[\text{S}]_o + [\text{E}]}(1 - \exp( - k_{\text{cat}}/[\text{E}]))
\]

where v represents the initial rate and [E] is the total enzyme concentration. In continuous assays, the kₐ/Kₚ values were obtained by nonlinear least square fit to Equation 2,

\[
[P] = [\text{S}]_o(1 - \exp( - k_{\text{cat}}/[\text{E}]))
\]

where [S]₀ represents the initial substrate concentration, whereas [P] is the product concentration at time t. When the substrate concentration encompassed the Kₚ, the Michaelis-Menten equation (Equation 3) was used to determine both kₐ and Kₚ. The initial rate versus substrate concentration could be fit directly to the Equation 3,

\[
v = \frac{k_{\text{cat}}[\text{S}]_o}{[\text{S}]_o + [\text{E}]}(1 + [\text{S}]_o/K_{\text{p}})
\]

where v represents the initial rate, [E] is the total enzyme concentration, and [S]₀ is the initial substrate concentration.

**pH Dependence Studies**—The kₐ and kₐ/Kₚ values for the Cdc14-catalyzed hydrolysis of pNPP and DiFUMUP were determined as a function of pH at 30 °C. To fit the kₐ/Kₚ versus pH data for the hydrolysis of pNPP, Equation 4 was used,

\[
k_{\text{cat}}K_{\text{p}} = k_{\text{cat}}[\text{S}]_o/(1 + [\text{S}]_o/K_{\text{p}})(1 + [\text{H}] (1 + K_{\text{BB}}))
\]

where (kₐ/Kₚ)ₚ is the pH-independent value of kₐ/Kₚ, H is the proton concentration, Kₚ is the second ionization constant of the substrate, and Kₚ and Kₚ are the ionization constants of the enzyme (20). To fit the kₐ versus pH data, one of two equations (Equation 5 or 6) was used,

\[
k_{\text{cat}} = k_{\text{cat}}[\text{S}]_o/(1 + [\text{S}]_o/K_{\text{p}})
\]

where (kₐ)ₚ is the pH-independent value of kₐ, H is the proton concentration, and Kₚ and Kₚ are the apparent ionization constants of the enzyme-substrate complex.

**Pre-steady-state kinetic study**—Pre-steady-state kinetic measurements of the wild-type Cdc14 and its D253A mutant-catalyzed hydrolysis of pNPP and DiFUMUP were conducted at pH 7.0 and 30 °C. Experiments were performed by mixing the enzyme and substrate in a temperature-controlled Applied Photophysics SX.18MV stopped-flow spectrophotometer. The excitation wavelength was 390 nm, and the emission wavelength was selected using a 420-nm cutoff filter. The reaction was monitored by the increase in fluorescence of the DiFUMUP product. A standard curve was generated by measuring the fluorescence of solutions of DiFUMUP that were reconstituted in the assay buffer. Data were collected and fit to Equation 7.

\[
y = At + B(1 - e^{-ct}) + C
\]

The burst amplitudes and rate constants were evaluated by the procedure described in Ref. 21. When [S] >> [E], values for Kₚ and (kₐ + kₜ) were obtained by plotting the first-order rate constant for the burst (kₜ), that is b in Equation 7 versus substrate concentration and fitting the data to Equation 8.

\[
k_{\text{burst}} = (k_{\text{a}} + k_{\text{t}})[\text{S}]/(K_{\text{p}} + [\text{S}])
\]

The individual rate constants for the E-P formation (kₜ) and hydrolysis (kₐ) can be determined from the (kₜ + kₐ) value and Equation 9.

\[
A = k_{\text{a}}k_{\text{t}}/(k_{\text{t}} + k_{\text{a}})
\]

**Preparation of Phosphorylated hCdh1 Protein**—A procedure similar to that described previously (7, 22) was used for the preparation of phosphorylated hCdh1. Briefly, a recombinant baculovirus encoding
Continuous Spectrophotometric Assay for Cdc14 and Its Mutants

Using Phosphorylated hCdh1 as a Substrate—Kinetic parameters for the dephosphorylation of the phosphorylated hCdh1 protein were determined using a continuous spectrophotometric assay. This assay incorporates a coupled enzyme system, which uses purine nucleoside phosphorylase and its chromogenic substrate 7-methyl-6-thioguanosine for the quantification of inorganic phosphate produced in the phosphatase reaction (23, 24). The change in absorbance at 360 nm was due to the conversion of 7-methyl-6-thioguanosine to 7-methyl-6-thioguanine in the presence of inorganic phosphate. Quantitation of the inorganic phosphate produced in the phosphatase reaction was determined using the extinction coefficient of 11,200 M⁻¹ cm⁻¹ at 360 nm and pH 7.0. Experiments of wild-type Cdc14 were carried out at 25 °C in a 1.6-ml reaction mixture containing 100 mM Tris at pH 7.0, 100 mM NaCl, 1 mM EDTA, 0.1 mg/ml purine nucleoside phosphorylase (Sigma), and 50 μM 7-methyl-6-thioguanosine. The spectrophotometric measurements were conducted using a PerkinElmer Lambda 14 spectrophotometer equipped with a magnetic stirrer in the cuvette holder. The kcat/Km values for the Cdc14 catalyzed dephosphorylation of hCdh1 were obtained by nonlinear least square fit to Equation 2. Experiments with Cdc14 mutants were carried out at 25 °C in a 200-μl reaction mixture under the same conditions in 96-well plates. The spectrophotometric measurements were obtained using a Molecular Devices Spectro MAX 340 plate reader. The kcat/Km values were obtained from a linear least square fit of the initial rate versus substrate concentration to Equation 1. In these measurements, the phosphorylated hCdh1 concentration was less than 4 μM, and the concentrations of the Cdc14 and its mutants ranged from 6.4 – 64 nM.

RESULTS AND DISCUSSION

Active Site Substrate Specificity—The PTP active site binds and hydrolyzes phosphorylated amino acids such as Tyr(P). Consequently, although the physiological substrates of PTPs are phosphoproteins, members of the PTP superfamily are also capable of hydrolyzing small molecule aryl phosphates. An analysis of the active site substrate specificity of PTPs may furnish critical information with respect to the design of potent active site-directed inhibitors. For example, an investigation of PTP1B active site substrate specificity led to the identification of several potent small molecule aryl phosphate substrates (25, 26), which provided a foundation for the development of the most potent and selective PTP1B inhibitors to date (27, 28).

To begin to probe the active site properties of Cdc14, we explored the ability of Cdc14 to catalyze the hydrolysis of a panel of aromatic phosphates, including 1-, 2-, and multi-ringed substrates. The chemical structures for pNPP, DiFMUP, and OMFP are shown in Fig. 1. All of the experiments described in this study were performed with the full-length recombinant Cdc14 from the budding yeast with an N-terminal His8 tag to enable facile purification. Steady-state parameters for the Cdc14-catalyzed hydrolysis of aryl phosphates were determined at pH 7.0 and 30 °C.

As shown in Table I, the kcat values are similar for all aryl phosphate substrates while the Km values differ in a dramatic fashion. Consequently, the kcat/Km value, which is a measure of substrate specificity, spans several orders of magnitude. A comparison of the kcat/Km values reveals several important features of the active site substrate specificity for Cdc14. First, Cdc14 shows a remarkable (230-fold) preference for DiFMUP over OMFP, even though these two substrates have very similar leaving group pKa values. This is in stark contrast to the Cdc25A dual specificity phosphatase, which exhibits a strong (73-fold) preference for OMFP over DiFMUP (29). Cdc14 also shows a preference for 2-ringed substrates over 1-ringed substrates. The kcat/Km values for 6-FMUP and 5-FMUP are 11- and 9-fold higher than that of pNPP although these substrates share comparable leaving group pKa values. Similarly, the kcat/Km value for β-naphthyl phosphate is 5.3-fold higher than that for 4-chlorophenyl phosphate even though these substrates also have the same leaving group pKa value. Unlike other dual specific phosphatases, such as Cdc25 phosphatases, mitogen-activated protein kinase phosphatases, and VH1-related (VHR), which display a striking preference (~2–3 orders of magnitude) for bulky multi-ringed substrates like OMFP over pNPP (29–34), the kcat/Km for the Cdc14-catalyzed hydrolysis of OMFP is only 8-fold higher than that of pNPP. Interestingly, the tyrosine-specific PTPs exhibit very limited preference for substrates with more than one aromatic ring (20, 25, 31). Collectively, the unique affinity of Cdc14 for 2-ringed aryl phosphate substrates suggest that the active site properties of the Cdc14 phosphatases are distinct from those of other dual specific and tyrosine-specific PTPs. This information regarding the range of functionality that can be readily accommodated by the active site of Cdc14 phosphatases may be useful when designing active site-directed inhibitors for these enzymes.

FIG. 1. The chemical structures of pNPP, DiFMUP, and OMFP.
case of the *Yersinia* PTP (35). The identification of the general acid has enabled the design of more potent substrate trapping mutants of PTPs (45, 46), which should aid in the identification of physiological substrates of PTPs.

Interestingly, evidence suggests that, depending on the substrate, the requirement for general acid catalysis in PTPs may not be universal. For example, it appears that the RNA 5′-triphosphatase Mce1-catalyzed phosphoanhydride hydrolysis does not involve general acid catalysis, which is in accord with the finding that Mce1 lacks a functional group in a position suited for proton donation (47). In addition, no acidic residues in Cdc25 phosphatases or myotubularins were found in a position corresponding to the general acid-containing loop (the WPD loop) observed in most other members of the PTP superfamily. Recent mutational, kinetic and structural studies indicate that acidic residues located in the PTP signature motif may serve as general acids in the reactions catalyzed by the Cdc25 and myotubularin phosphatases (29, 48).

The requirement for general acid/base in Cdc4 catalysis has not been established. As shown in Table I, the $k_{cat}$/value for the Cdc4-catalyzed hydrolysis of pNPP is slightly less than that for DiFMU, but the $k_{cat}/K_m$ value for pNPP is $\sim 1.820$-fold lower than that for DiFMU. The $k_{cat}/K_m$ parameter reflects the overall rate-limiting step of enzyme catalysis, and, in the case of PTPs, previous studies have shown this to be mainly associated with hydrolysis of the E-P intermediate ($k_4$ in Reaction Scheme 1) under acidic to neutral conditions (49). The fact that the Cdc4-catalyzed hydrolysis of pNPP and DiFMU, two substrates that differ markedly in their leaving group $pK_a$ (7.14 for $p$-nitrophenol and 4.7 for DiFMU), display similar $k_{cat}$ values suggests that the rate-determining step is mostly E-P hydrolysis (also see below).

The kinetic parameter $k_{cat}/K_m$ monitors the PTP reaction beginning with binding of the substrate and up to and including the first irreversible step in the kinetic mechanism (Reaction Scheme 1), which is E-P formation accompanied by release of the leaving group. Kinetic isotope effect studies indicate that the chemical step (i.e. phosphoryl transfer from substrate to the enzyme nucleophile) is rate-limiting for the $k_{cat}/K_m$ term (36, 41, 43). In general, the PTP-catalyzed E-P formation exhibits little leaving group dependence due to protonation of the phenolic oxygen by the general acid. In contrast, general acid deficient mutant PTPs show large leaving group dependence on $k_{cat}/K_m$ (37, 40, 50). For Cdc14-catalyzed hydrolysis of pNPP and DiFMU, even when one excludes the contribution from preferential binding of 2-ringed substrates over 1-ringed substrates (10-fold), the $k_{cat}/K_m$ value for DiFMU is still 180-fold higher than that of pNPP. It is rare for a wild-type PTP to display such a large leaving group dependence on $k_{cat}/K_m$. Consequently, it is not clear whether the Cdc14-catalyzed reaction involves general acid catalysis.

In order to further define the catalytic mechanism of Cdc4 and to identify additional catalytically important residues, in particular the general acid/base functionality in PTP catalysis is associated with acidic residues, we primarily focused our attention on the nine invariant acidic residues in the catalytic domain of Cdc4, namely, Asp$^{296}$, Asp$^{299}$, Asp$^{314}$, Glu$^{316}$, Glu$^{317}$, Glu$^{318}$, Asp$^{317}$, Asp$^{323}$, and Glu$^{326}$ (Fig. 2). We made the most conservative site-directed mutations possible (i.e. Glu to Gln and Asp to Asn) in order to remove the ability of these residues to function effectively in general acid-base catalysis while minimizing structural perturbations in the proteins. All of the mutations were verified by DNA sequencing. Recombinant proteins were expressed in *E. coli*, and purified to near homogeneity as judged by SDS-PAGE (data not shown).

While this article was in preparation, the x-ray crystal structure of the catalytic domain of human Cdc14B (residues Pro$^{14}$–His$^{386}$) was published (51). Unlike other members of the PTP superfamily, the structure of hCdc14B reveals a novel arrangement of two structurally equivalent domains arranged in tandem. The N-terminal domain (domain A, residues 44–198 in hCdc14B) may contribute to Cdc14 substrate specificity and shares no sequence similarity with other dual specific phosphatases. The C-terminal domain (domain B, residues 213–386 in hCdc14B) contains the conserved PTP motif. The active site of hCdc14B is located within a long groove between the interface of the A- and B-domain (Fig. 3). Residues of two surface loops of the A-domain, the extended WPD(A) (residues Thr$^{37}$–Asn$^{55}$ in Cdc14) and α5α6α6A (residues Leu$^{117}$–Thr$^{294}$ in Cdc14) loops, form one side of the groove. The WPD (residues Leu$^{249}$–Asp$^{258}$ in Cdc14) and Q-loops (residues Arg$^{317}$–Gly$^{323}$ in Cdc14) of the B-domain create the opposite face of the active site groove, whereas the interdomain linker α-helix (residues Leu$^{184}$–Phe$^{175}$ in Cdc14) is positioned at the entrance to one end of the channel. This structure provides a framework for the interpretation of our mutational data. All of the residues that were subjected to mutagenesis are highlighted in the hCdc14B structure (Fig. 3).

Table II summarizes the kinetic parameters of the wild-type PTPs. The substrates include: 1) multi-ringed aryl phosphate OMFP, 2) 2-ringed aryl phosphates, DiFMU, 8-FMUP, 6-FMUP, 5-FMUP, MUP, and β-Naph (β-naphthyl phosphate), and 3) 1-ringed aryl phosphates, 2-Cl-4-NO$_2$ (2-chloro-4-nitrophenyl phosphate), pNPP, 4-CN (4-cyanophenyl phosphate), 4-CF$_3$ (4-trifluoromethylphenyl phosphate), 4-Cl (4-chlorophenyl phosphate), and PhP (phenyl phosphate).
and the mutant Cdc14s at pH 7.0 and 30 °C, using both pNPP and DiFMUP as substrates. It is apparent that mutants D143N, E168Q, E174Q, and E308Q have $k_{\text{cat}}$ and $K_m$ values very similar to those of the wild-type Cdc14. These results indicate that Asp143, Glu168, Glu174, and Glu308 are not essential for the Cdc14-catalyzed hydrolysis of small molecule aryl phosphates. In contrast, greater than 30-fold decrease in $k_{\text{cat}}$ was observed for D50N and D129N, while the $k_{\text{cat}}/K_m$ values for these two mutants were only decreased 1.2–2.8-fold with pNPP as a substrate. Since Asp50 (in the WPD(A) loop) and Asp129 (in the $\gamma$/H9251$5A/\gamma$9251$6A$ loop) are located within the active site cavity (Fig. 3), it is possible that these two residues may play more important roles in the hydrolysis of the E-P intermediate. Only moderate reduction in $k_{\text{cat}}$ and no change or a slight increase in $k_{\text{cat}}/K_m$ was observed for E171Q and D177N, suggesting that Glu171 and Asp177 are not essential for the Cdc14-catalyzed pNPP hydrolysis.

Asp$^{553}$ is invariant among all Cdc14 phosphatases, and it is also conserved in more related phosphatases including PTEN, PRL, and KAP (Fig. 2). The corresponding Asp92 in PTEN (52) and Asp110 in KAP (53) have been found in a position equivalent to those of the general acid/base residues observed in the WPD loop of other PTPs. In addition, the structure of the hCdc14B/C314S mutant with a Ser(P) peptide reveals that Asp253 in the WPD loop is placed to donate a proton to the O atom of the Ser(P) substrate (Ref. 51 and Fig. 3). Based on the structural data, it seems that Asp253 could function as a general acid/base in the Cdc14-catalyzed reaction. Previous studies have shown that mutations of the general acid in PTPs generally result in two to three orders of magnitude decrease in $k_{\text{cat}}$ and $K_m$ values.
Characterization of Asp253 as a General Acid

As shown in Table II, the $k_{cat}/K_m$ values for the Cdc14-catalyzed hydrolysis of pNPP and DiFMUP decrease 640 to 1200-fold when Asp$^{253}$ is replaced by an Ala. Less dramatic reductions in $k_{cat}$ (50–100-fold) are observed for the D253N mutant. Surprisingly, the $k_{cat}/K_m$ values for D253A and D253N drop only 7- and 26-fold respectively, with pNPP as a substrate, while the $k_{cat}/K_m$ values are virtually the same as those of the wild-type Cdc14 using DiFMUP as a substrate. In order to determine whether Asp$^{253}$ indeed functions as a general acid/base in Cdc14 catalysis, we carried out additional experiments with both the wild-type and Asp$^{253}$ mutant Cdc14 enzymes.

Leaving Group Dependence—The leaving group dependence for the hydrolysis of aryl phosphates catalyzed by Cdc14 was investigated at pH 7.0 and 30 °C using a series of substrates with leaving group $pK_a$ values ranging from 4.7 to 10. Since we have shown that the size of the aryl substrates can have substantial effects on the rate of Cdc14 catalysis (Table I), we analyzed the leaving group effect with the one-ringened and two-ringened substrates separately, in order to exclude steric effects. Fig. 4 shows the Benzenes plots, which relate the logarithm of $k_{cat}/K_m$ to the $pK_a$ values of the leaving group. The slope of the Benzenes plot corresponds to the $\beta_{lg}$ value, which reflects the amount of negative charge developed on the phenolic oxygen at the transition state.

The most dramatic feature of the Benzenes plot with two-ringened substrates for Cdc14 is a break at the $pK_a$ value of 7. A similar break may also exist in the Benzenes plot with one-ringened substrates. In the low $pK_a$ region, from 4.7 to 7, the slope for the two-ringened substrates is substantially negative, giving a $\beta_{lg}$ value of $-1.01 \pm 0.01$. This value is similar to that of the uncatalyzed aryl phosphate dianion reaction ($\beta_{lg} = -1.23$, Ref. 54), which indicates a full negative charge 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 4.7 and 7. Interestingly, the leaving group dependence for two-ringened substrates with leaving group $pK_a$ values from 7.14 to 9.99 is flat, giving a $\beta_{lg}$ value of $-0.12 \pm 0.02$. In addition, the leaving group dependence for one-ringened substrates with leaving group $pK_a$ values from 7.14 to 9.99 is also flat, yielding a $\beta_{lg}$ value of $-0.06 \pm 0.05$. These values are similar to those determined for PTPs with functional general acid catalysis (20, 40, 55) and the uncatalyzed monooxygen reaction ($\beta_{lg} = -0.27$, Ref. 54), in which the leaving group is protonated in the transition state.

The results described above suggest that the Cdc14-catalyzed reaction does not involve a general acid for substrates with low leaving group $pK_a$ values (4.7–7), whereas the hydrolysis of substrates with high leaving group $pK_a$ values (>7) appears to require a general acid. To determine if Asp$^{253}$ fulfills the function of a general acid, we also quantified the leaving group effect for the Cdc14/D253A-catalyzed reaction. Unfortunately, the activities of Cdc14/D253A-catalyzed hydrolysis of one-ringened substrates with leaving group $pK_a$ values >8 are too low to be measurable. However, as shown in Fig. 4, we were able to determine the kinetic parameters for the Cdc14/D253A-catalyzed hydrolysis of 2-ringened substrates with leaving group $pK_a$ values 4.7–7.8, which yielded a $\beta_{lg}$ value of $-1.19 \pm 0.07$. This value indicates that no general acid catalysis occurs for substrates with leaving group $pK_a$ values between 4.7 and 7.8. That the $k_{cat}/K_m$ values for the D253A mutant catalyzed hydrolysis of substrates with leaving group $pK_a$ between 4.7 and 7 are similar to those of the wild-type enzyme is consistent with the notion that the dephosphorylation of substrates with leaving group $pK_a$ below 7 does not require general acid catalysis. Importantly, there was clearly no break at $pK_a = 7$ in the leaving group profile for the D253A mutant as the reaction rates for substrates with leaving group $pK_a$ values higher than 7 continued to drop. The fact that a $\beta_{lg}$ value of $-1.19$ was obtained for the Cdc14/D253A-catalyzed reaction that extends beyond for substrates with leaving group $pK_a$ above 7 and that a further decrease in $k_{cat}/K_m$ values for substrates with leaving group $pK_a$ values >7 suggest that Asp$^{253}$ may indeed act as a general acid in Cdc14-catalyzed hydrolysis of high leaving group $pK_a$ (7.0–9.99) substrates.

**pH Dependence**—To further substantiate that Asp$^{253}$ is the general acid/base in Cdc14, we determined the pH-rate profiles for the hydrolysis of pNPP and DiFMUP by both the wild-type and the D253A mutant Cdc14 phosphatases (Fig. 5). Since the leaving group $pK_a$ for DiFMUP (4.7) is <7, we predicted that the wild-type Cdc14-catalyzed DiFMUP hydrolysis would not exhibit general acid catalysis for the E-P formation step ($k_{cat}/K_m$) but may require general base catalysis for the E-P hydrolysis step ($k_{cat}$). As predicted, DiFMUP shows a flat pH-$k_{cat}/K_m$ profile between pH 5.5 and 8.0 (Fig. 5A), consistent with a lack of general acid and with the leaving group dependence described above. Since no general acid catalysis is involved, removal of the carboxyl group at position 253 has no effect on $k_{cat}/K_m$, and the D253A mutant also displays a flat pH dependence for DiFMUP with $k_{cat}/K_m$ values virtually identical to those of the wild-type enzyme (Fig. 5A).

The pH-$k_{cat}$ profile (Fig. 5B) indicates the presence in the enzyme-substrate complex an ionizable residue that must be deprotonated with a $pK_{dss}$ value of 6.4 ± 0.1 for maximal turnover ($k_{cat}^{max} = 10.5 \pm 0.5$ s$^{-1}$). Because of the ease of expulsion of the leaving group, the rate-limiting step for the Cdc14-catalyzed DiFMUP reaction is most likely E-P hydrolysis ($k_{a}$ in Scheme 1, see below). Consequently, the apparent
Kinetic parameters of wild-type and mutant Cdc14 phosphatases with both pNPP and DiFMUP as substrates

| Cdc14 | pNPP | DiFMUP |
|-------|------|--------|
|       | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) |
| WT    | 4.5 ± 0.2 | 19.6 ± 0.9 | 424 | 8.1 ± 0.2 | 10.5 ± 0.6 | 771,000 |
| D50N  | 0.14 ± 0.03 | 0.40 ± 0.03 | 350 | ND | ND | ND |
| D128N | 0.13 ± 0.01 | 0.57 ± 0.11 | 149 | 0.13 ± 0.01 | 0.79 ± 0.08 | 160,000 |
| D143N | 3.5 ± 0.4 | 9.6 ± 1.0 | 364 | 5.4 ± 0.1 | 6.1 ± 0.3 | 880,000 |
| E168Q | 4.7 ± 0.4 | 12.9 ± 1.5 | 364 | 8.7 ± 0.01 | 16.6 ± 0.01 | 520,000 |
| E171Q | 0.94 ± 0.01 | 2.8 ± 0.3 | 336 | ND | ND | ND |
| E174Q | 4.3 ± 0.4 | 11.2 ± 1.3 | 385 | 8.2 ± 0.4 | 9.6 ± 1.1 | 850,000 |
| D177N | 0.86 ± 0.01 | 1.0 ± 0.1 | 860 | 1.1 ± 0.1 | 0.71 ± 0.08 | 1,500,000 |
| D253A | 0.007 ± 0.001 | 0.11 ± 0.05 | 60 | 0.007 ± 0.001 | 0.0079 ± 0.0014 | 880,000 |
| D253N | 0.09 ± 0.01 | 5.75 ± 1.31 | 16 | 0.08 ± 0.01 | 0.25 ± 0.01 | 320,000 |
| E308Q | 4.0 ± 0.3 | 11.3 ± 1.1 | 353 | 5.7 ± 0.4 | 8.7 ± 3.1 | 650,000 |

\(*\) ND, not determined.

Fig. 4. The leaving group dependence of wild-type Cdc14 (filled circles for one-ringed substrates and filled square for two-ringed substrates), and of Cdc14/D253A (filled triangles for two-ringed substrates). The lines were generated by linear least squares fit to the \( \log(k_{cat}/K_m) \) versus leaving group \( pK_a \). For wild-type Cdc14, two separate linear regressions were done for the two-ringed substrates; one for the substrates with leaving group \( pK_a \) values 4.7–7 (\( \beta_B = -1.01 ± 0.01 \)), and one for the substrates with leaving group \( pK_a \) values 7–9.38 (\( \beta_B = -0.12 ± 0.02 \)). For the D253A mutant, the substrates with leaving group \( pK_a \) values 4.7–7.8 gave a \( \beta_B \) value of \( -1.19 ± 0.07 \), while the substrates with leaving group \( pK_a \) values >8 had \( k_{cat}/K_m \) values that were too low to be measurable.

ionization constant (\( pK_{ES1} = 6.4 \)) corresponds to the general base functionality in E-P. Indeed, the \( k_{cat} \) values for the Cdc14/D253A-catalyzed hydrolysis of DiFMUP (−0.007 s\(^{-1}\)) are more than 3 orders of magnitude slower than \( (k_{cat})_{max} \) of the wild-type enzyme and are insensitive to \( pH \) values (Fig. 5B). Since removal of Asp\(^{253}\) in Dc24 does not affect E-P formation, the large reduction in \( k_{cat} \) for D253A appears primarily due to an impairment of E-P hydrolysis. Thus the rate-limiting step for the D253A-catalyzed DiFMUP reaction is also likely E-P hydrolysis (also see below). Consistent with Asp\(^{253}\) serving as a general base in the Cdc14-catalyzed reaction, mutating residue 253 from Asp into Ala eliminates the acid limb of the pH-\( k_{cat} \) profile for DiFMUP.

Since the leaving group \( pK_a \) for pNPP is 7.14, we predicted that the wild-type Cdc14-catalyzed pNPP hydrolysis would exhibit characteristics of general acid/base catalysis. As expected, the wild-type Cdc14-catalyzed pNPP hydrolysis displays bell-shaped pH dependences for both \( k_{cat} \) and \( k_{cat}/K_m \), as observed with other PTPs using pNPP as a substrate (20, 35, 42). As shown in Fig. 5C, pNPP shows a pH optimum at 5.4 and steadily decreasing \( k_{cat}/K_m \) values at higher pH values, quite unlike the pH dependence of DiFMUP. This behavior is consistent with a Cdc14 residue that must be protonated for optimal catalytic efficiency, possibly a general acid. Because of the instability of Cdc14 at low \( pH \) values, the \( k_{cat}/K_m \) values below \( pH 5.0 \) could not be accurately measured. Consequently, the data were fitted to Equation 4 with the second ionization constant of pNPP (\( pK_{ES2} \)) fixed at the experimentally determined value of 5.1 (29). From this analysis, the \( pK_{ES1} \) (ionization constant of the active site Cys residue) was determined to be 5.5 ± 0.5, the \( pK_{ES2} \) (ionization constant of the general acid) 5.1 ± 0.4, and the \( (k_{cat}/K_m)_{max} (2.3 ± 1.6) \) × 10\(^{5} \) s\(^{-1}\)). These ionization constants are similar to those obtained for other PTPs. Consistent with the involvement of a general acid in the Cdc14-catalyzed hydrolysis of substrates with leaving group \( pK_a \) values >7, the \( k_{cat}/K_m \) values for Cdc14/D253A are dramatically reduced, especially at the optimal \( pH \), and replacement of Asp\(^{253}\) with an Ala abolishes the basic limb of the \( pH \) profile for pNPP (Fig. 5C). The \( pK_{ES1} \) value for Cdc14/D253A could not be measured because of its low activity and instability at \( pH \) below 6. Therefore, we conclude that D253 is responsible for the basic limb of the pH-\( k_{cat}/K_m \) profile seen for wild-type Cdc14 with pNPP and that Asp\(^{253}\) must be protonated for optimal catalysis of this substrate.

As shown in Fig. 5D, the pH versus \( k_{cat} \) profile for the Cdc14-catalyzed pNPP reaction shows a pH optimum at 6.8, and the \( (k_{cat})_{max} \), \( pK_{ES1} \), and \( pK_{ES2} \) values are determined to be 12.9 ± 1.4 s\(^{-1}\), 6.7 ± 0.1, and 6.9 ± 0.1, respectively. It is important to recognize that \( pK_{ES1} \) and \( pK_{ES2} \) are apparent values and may not represent microscopic ionizations of particular groups (56). It is also important to recognize that there are uncertainties in \( pK_a \) values calculated from kinetic data when the differences in \( pK_a \) are less than 0.6 units (57). Nevertheless, the acidic limb of the pH-\( k_{cat} \) profile for pNPP is similar to that of DiFMUP, suggesting that the rate-limiting step likely corresponds to E-P hydrolysis in this pH range and that \( pK_{ES1} \) likely reflects the ionization of the general base function of Asp\(^{253}\). In accordance with this, removal of Asp\(^{253}\) reduces the \( k_{cat} \) by 3 orders of magnitude and eliminates the acidic limb of the pH-\( k_{cat} \) profile (Fig. 5D). Interestingly, unlike the DiFMUP reaction, whose \( k_{cat} \) values level off at a maximum plateau at \( pH > 7 \) (Fig. 5B), the pH-\( k_{cat} \) profile for pNPP shows a steady decrease in \( k_{cat} \) as the \( pH \) is raised (Fig. 5D). This may be caused by the fact that under more basic conditions, Asp\(^{253}\) is deprotonated, so that E-P formation step eventually becomes the rate-limiting step for substrates that require general acid catalysis. Consequently, the apparent \( pK_{ES1} \) (6.9) derived from the basic limb of the pH-\( k_{cat} \) profile for pNPP may not represent an intrinsic ionization constant but reflect a change in the rate-limiting step from E-P hydrolysis at lower \( pH \) values to E-P formation at higher \( pH \) values. Since Asp\(^{253}\) is also in-
volved in E-P formation, its removal also abolishes the basic limb of the pH-$k_{\text{cat}}$ profile for pNPP (Fig. 5D).

We also analyzed the pH dependence of the D253N-catalyzed hydrolysis of DiFMUP and pNPP (data not shown). Similar to D253A, the D253N mutant exhibits flat pH-rate profiles for both $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$. The $k_{\text{cat}}/K_m$ values for the D253N-catalyzed reaction are only slightly lower than those of the D253A mutant. Interestingly, the $k_{\text{cat}}$ for the D253N mutant is ~10-fold higher than D253A (Table II), suggesting that the carboxyamide group in D253N is more efficient than the methyl group in D253A in promoting E-P hydrolysis. Collectively, the results from both leaving group and pH dependence of Cdc14-catalyzed reaction are consistent with a general acid independent mechanism for substrates with leaving group $p$NPP reaction.

Fig. 5. The pH dependence for the hydrolysis of DiFMUP and pNPP by the wild-type (closed squares) and the D253A mutant Cdc14 (closed triangles). A and B depict the pH dependence of $k_{\text{cat}}/K_m$ and $k_{\text{cat}}$ for DiFMUP hydrolysis while C and D show the pH dependence of $k_{\text{cat}}/K_m$ and $k_{\text{cat}}$ for the pNPP reaction.

The presence of a pre-steady-state burst upon the hydrolysis of DiFMUP by Cdc14 is consistent with rate-determining E-P hydrolysis for this substrate. This is in full agreement with the conclusion derived from the steady-state kinetic evidence discussed above. The rate constants of the individual chemical steps $k_2$ and $k_3$ as well as the dissociation constant $K_S$ can be obtained by plots of $k_{\text{burst}}$ (rate constant for the burst phase) versus DiFMUP concentrations (Fig. 6B) as described under “Experimental Procedures” (21). The $k_2$, $k_3$, and $K_S$ values are correspondingly 151 ± 29 s$^{-1}$, 20 ± 5 s$^{-1}$, and 209 ± 66 μM. Thus, for the hydrolysis of DiFMUP, $k_3$ is 7.6-fold greater than $k_2$. Since $k_{\text{cat}} = k_2k_3/k_2 + k_3$, and $K_m = K_sc/k_2 + k_3$, $k_{\text{cat}}/K_m$ can be transformed to $k_3/K_S$. The $k_3/K_S$ value (720,000 m$^{-1}$ s$^{-1}$) determined by the stopped-flow method is similar to the $k_{\text{cat}}/K_m$ value (770,000 m$^{-1}$ s$^{-1}$) obtained by steady-state experiments.

We also measured the Cdc14/D253A-catalyzed DiFMUP hydrolysis by the stopped-flow technique. As shown in Fig. 6A, a burst was also observed in the D253A-catalyzed reaction, indicating E-P hydrolysis as the rate-limiting step. From the substrate concentration dependence of $k_{\text{burst}}$ (Fig. 6B), the $k_2$, $k_3$, and $K_S$ values for the D253A reaction were determined to be 85 ± 10 s$^{-1}$, 0.01 ± 0.001 s$^{-1}$, and 145 ± 33 μM. The $k_3/K_S$ value (586,000 m$^{-1}$ s$^{-1}$) obtained from the stopped-flow experiments is similar to the $k_{\text{cat}}/K_m$ value (880,000 m$^{-1}$ s$^{-1}$) measured by steady-state experiments. Both the $k_2$ and $K_S$ values for D253A are close to those of the wild-type enzyme, indicating that Asp$^{253}$ does not significantly contribute to E-P formation for DiFMUP hydrolysis. The $k_3/K_S$ value for D253A is 2,000-fold lower than that of the wild-type Cdc14, consistent with the notion that Asp$^{253}$ acts as a general base during E-P hydrolysis. Together with the pH-$k_{\text{cat}}$ profile (Fig. 5), this provides the first unambiguous demonstration of the requirement of a general base in E-P hydrolysis of a PTP-catalyzed reaction.

Since $k_3$, the rate constant for E-P hydrolysis, must be identical for dephosphorylation of either substrates, we can approximately solve for the kinetic parameter $k_3$ in the Cdc14-catalyzed pNPP reaction based on $k_{\text{cat}} = k_2k_3/k_2 + k_3$. The calculated $k_3$ for pNPP is 5.8 s$^{-1}$. Therefore, the rate of E-P formation is 26-fold faster with DiFMUP as a substrate than with pNPP. Since $k_2$ and $k_3$ for the pNPP reaction are comparable, both E-P formation and breakdown contributes to the
rate-limiting step in pNPP hydrolysis. This also support the conclusion derived from the pH-
kcat analysis of the pNPP reaction that the \( k_{\text{cat}} \) is rate-limited by both \( k_{1} \) and \( k_{2} \) at pH 7. Since \( k_{\text{cat}}/K_m = k_{2}/K_p \), \( K_p \) for pNPP can be calculated to be 13.8 mM using the estimated \( k_{2} \) value and the experimentally determined \( k_{\text{cat}}/K_m \) for pNPP (420 M\(^{-1}\) s\(^{-1}\)). Thus, the dissociation constant (\( K_d \)) of Cdc14 for DiFMUP (209 \( \mu \)M) is 66-fold lower than that for pNPP, providing further evidence that Cdc14 possesses intrinsically higher affinity for 2-ringed aryl phosphates. This property may relate to the existence of a hydrophobic pocket adjacent to the active site that recognizes the p + 1 Pro residue in Cdc14 substrates. The enhanced substrate binding may be partially responsible for the faster rate of E-P formation with DiFMUP than pNPP.

Activity of Cdc14 Toward Its Physiological Substrate hCdh1—The crystal structure of hCdc14B shows that acidic residues Glu\(^{168}\), Glu\(^{171}\), and Asp\(^{177}\) in the interdomain linker \( \alpha \)-helix (residues Leu\(^{164}\)–Phe\(^{177}\) in Cdc14) cluster at the entrance of the catalytic site, suggesting that these residues may confer specificity for basic residues in the substrates (51). However, substitution of these acidic residues with an Ala reduces the \( k_{\text{cat}}/K_m \) for hydrolysis of a peptide substrate, Ala-Ser(P)-Pro-Arg-Arg-Arg-Arg, by only 2-fold (51). It is not known whether these acidic residues play a role in recognizing protein substrates.

To gain further insight into the molecular basis of Cdc14 substrate specificity, we chose to analyze the Cdc14-catalyzed dephosphorylation of a physiological protein substrate Cdh1. Cdh1 is a substrate-specific co-activator of the APC (58, 59), which functions as an E3 ubiquitin ligase to degrade the mitotic cyclin Clb2 and other substrates during the G1 phase of the cell cycle. Cdh1 is present throughout the cell cycle, but its binding to APC is blocked by Cdk phosphorylation (22, 60). Cdc14 activates APC by dephosphorylating Cdh1, leading to destruction of Cdc28-Clb2 and mitotic exit (4). In addition, human Cdc14A can also dephosphorylate human Cdh1 (hCdh1) to promote the activation of APC\(^{Cdh1} \) in vitro (7).

To follow the Cdc14-catalyzed hCdh1 dephosphorylation, we employed a continuous spectrophotometric enzyme-coupled assay in which the coupling enzyme, purine nucleoside phosphorylase, uses the inorganic phosphate, generated by the action of Cdc14, to convert 7-methyl-6-thioguanosine to 7-methyl-6-thioguanine and ribose-1-phosphate, resulting in an increase in absorbance at 360 nm (23, 24). The reaction was carried out at pH 7 and 25 °C. Fig. 7 shows a typical progress curve of the Cdc14-catalyzed hCdh1 dephosphorylation. To ensure first-order conditions, the reaction was conducted at several hCdh1 concentrations ranging from 0.5 to 4 \( \mu \)M. Identical \( k_{\text{cat}}/K_m \) values for hCdh1 dephosphorylation by the full-length Cdc14 and the catalytic domain (residues 1–374) were determined to be (1.8 ± 0.04) \( \times 10^3 \) and (1.3 ± 0.01) \( \times 10^3 \) M\(^{-1}\) s\(^{-1}\)), respectively. This is consistent with previous observations that C-terminal domain of Cdc14 (residues 375–551) is not required for its phosphatase activity (1). Interestingly, under the same conditions the human Cdc14A catalytic domain (residues 1–379) was able to dephosphorylate hCdh1 with a \( k_{\text{cat}}/K_m \) value of (4.0 ± 0.04) \( \times 10^3 \) M\(^{-1}\) s\(^{-1}\), indicating that hCdh1 is an efficient substrate for both the yeast and human enzymes.

We subsequently evaluated the ability of the Cdc14 mutants to carry out hCdh1 dephosphorylation. As expected, a more than 100-fold decrease in \( k_{\text{cat}}/K_m \) was observed for D253A. In contrast, the \( k_{\text{cat}}/K_m \) values for D143N, E174Q, and E308Q were almost identical to that of the wild-type Cdc14. Thus, Asp\(^{143}\), Glu\(^{174}\), and Glu\(^{308}\) are not essential for hCdh1 dephosphorylation. Compared with wild-type Cdc14, the \( k_{\text{cat}}/K_m \) values of E168Q, E171Q, and D177N were reduced by 2-fold. These results suggest that the acidic cluster in the interdomain linker, Glu\(^{168}\), Glu\(^{171}\), and Asp\(^{177}\), may contribute to Cdc14...
protein substrate recognition. Interestingly, more dramatic decreases in the rate of hCdh1 dephosphorylation were observed for D50N and D129N. The $k_{cat}/K_m$ for the D50N and D129N-catalyzed hCdh1 dephosphorylation was reduced by 5- and 10-fold, respectively, in comparison with that of the wild-type Cdc14. Thus, Asp$^{50}$ and Asp$^{129}$ may play a more important role in Cdc14 substrate recognition.

The residues that we identified as important for hCdh1 dephosphorylation are located in structural elements that surround the Cdc14 catalytic site (Fig. 3). Thus, Asp$^{50}$ and Asp$^{129}$ reside in the WPD(A) (residues Thr$^{37}$–Asn$^{55}$) and a5Mo6A (residues Leu$^{117}$–Thr$^{140}$) surface loops respectively. Asp$^{50}$ is located at the WPD loop (residues Leu$^{249}$–Asp$^{258}$). And Glu$^{168}$, Glu$^{171}$, and Asp$^{177}$ in the interdomain linker $\alpha$-helix (residues Leu$^{164}$–Phe$^{175}$) are positioned at the entrance to one end of the active site groove. Although none of these residues (with the exception of Asp$^{257}$) are found in direct contact with the phosphopeptide Ala-Ser(P)-Pro in the crystal structure of the hCdc14B complex (51), our kinetic data reveal that Asp$^{50}$, Asp$^{129}$, Glu$^{168}$, Glu$^{171}$, and Asp$^{177}$ are important for Cdc14-catalyzed hCdh1 dephosphorylation.

In summary, we have carried out a detailed kinetic and mechanistic study of the Cdc14 phosphatases with both small molecule aryl phosphates and a physiological protein substrate hCdh1. We found that Cdc14 has a unique preference for 2-aminocarboxylic substrates, a finding that may have significant implications for inhibitor design. Steady-state kinetic analysis of the Cdc14-catalyzed reaction showed that Cdc14 hydrolizes low leaving group substrates ($p_K > 7$), like DiFMUP, without the aid of a general acid, and higher $p_K$ substrates ($p_K > 7$) like pNPP, with a general acid. The use of both DiFMUP and pNPP in combination with leaving group dependence, pH dependence, and pre-steady-state kinetic techniques enabled the isolation and analysis of both the E-P formation and hydrolysis step. We were able to establish clearly the requirement of general acid catalysis for the E-P formation step in reactions with high leaving group pK$_a$ substrates, and the presence of general base catalysis in E-P hydrolysis. Mutational study of invariant acidic residues in Cdc14 unambiguously identified Asp$^{50}$, which acts as a general acid during E-P formation and as a general base in E-P hydrolysis. Finally, we provided evidence that Asp$^{50}$, Asp$^{129}$, Glu$^{168}$, Glu$^{171}$, and Asp$^{177}$ are involved in substrate recognition for the physiological protein substrate hCdh1.

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Kinetic and Mechanistic Studies of a Cell Cycle Protein Phosphatase Cdc14
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