Kinetic Analysis of the Catalytic Domain of Human Cdc25B*

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The Cdc25 cell cycle regulator is a member of the dual-specificity class of protein-tyrosine phosphatases that hydrolyze phosphotyrosine- and phosphothreonine-containing substrates. To study the mechanism of Cdc25B, we have overexpressed and purified the catalytic domain of human Cdc25B (Xu, X., and Burke, S. P. (1996) J. Biol. Chem. 271, 5118–5124). In the present work, we have analyzed the kinetic properties of the Cdc25B catalytic domain using the artificial substrate 3-O-methylfluorescein phosphate (OMFP). Steady-state kinetic analysis indicated that the $k_{cat}/K_m$ for OMFP hydrolysis is almost 3 orders of magnitude greater than that for p-nitrophenyl phosphate hydrolysis. Like other dual-specificity phosphatases, Cdc25 exhibits a two-step catalytic mechanism, characterized by formation and breakdown of a phosphoenzyme intermediate. Pre-steady-state kinetic analysis of OMFP hydrolysis indicated that formation of the phosphoenzyme intermediate is 20 times faster than subsequent phosphoenzyme breakdown. The resulting burst pattern of product formation allowed us to derive rate constants for enzyme phosphorylation (26 s⁻¹) and dephosphorylation (1.5 s⁻¹) as well as the dissociation constant for OMFP (0.3 mm). Calculations suggest that OMFP binds with higher affinity and reacts faster with Cdc25B than does p-nitrophenyl phosphate. OMFP is a highly efficient substrate for the dual-specificity protein-tyrosine phosphatases VHR and rVH6, but not for two protein-tyrosine phosphatases, PTP1 and YOP. The ability to observe distinct phases of the reaction mechanism during OMFP hydrolysis will facilitate future analysis of critical catalytic residues in Cdc25 and other dual-specificity phosphatases.

The dual-specificity protein-tyrosine phosphatases (DS-PTPs) are enzymes that hydrolyze phosphotyrosine- and phosphothreonine-containing substrates (1). Several DS-PTPases have been shown to be involved in cell cycle control or mitogenic signaling. The Cdc25 protein dephosphorylates cyclin-associated cyclin-dependent kinases and contributes to their activation; three human Cdc25 isoforms (termed A, B, and C) are presumed to be active during different phases of the cell cycle (2). A family of DS-PTPases related to MKP1 (mitogen-activated protein kinase phosphatase) has been found to dephosphorylate mitogen-activated protein kinases that are activated in response to environmental signals (1). However, the natural substrates for many of the DS-PTPases remain unknown.

By sequence alignment, the DS-PTPases are members of a subclass of the protein-tyrosine phosphatase (PTPase) family (3). All PTPases and DS-PTPases studied to date act by a similar kinetic mechanism (4–10); substrate binding is followed by formation of a phosphoenzyme intermediate and release of the first product, dephosphorylated peptide. Subsequently, the enzyme undergoes dephosphorylation and dissociation of the second product, inorganic phosphate. Insight into the mechanism of DS-PTPases has come from analysis of VHR, the simplest and most well characterized enzyme in this class (7–10). The natural substrate for VHR is unknown, but it dephosphorylates mitogen-activated protein kinase peptides that are phosphorylated on threonine and tyrosine within the signature sequence TEY (10). Kinetic analysis of the activity of mutant and wild-type VHR with the artificial substrate p-nitrophenyl phosphate (pNPP) has identified the role of Asp-92 as a general acid in the first step of the reaction and its conjugate carboxylate as a general base in the breakdown of the phosphoenzyme intermediate (Fig. 1) (8). For VHR, phosphoenzyme formation appears to be the rate-limiting step in pNPP hydrolysis under most conditions since no burst is seen in rapid-reaction kinetic experiments (8). Ser-131 has been proposed to donate a proton to stabilize the thiolate leaving group; mutation of this residue to alanine decreases the rate of phosphoenzyme intermediate breakdown and results in a burst pattern of pNPP hydrolysis (9).

Little is known about the mechanism of Cdc25 and how it might differ, either chemically or kinetically, from that of VHR. The two enzymes share very little sequence similarity outside of the active site. It is not apparent from inspection of the primary amino acid sequence which residues in Cdc25 are likely to be analogous to Asp-92 or Ser-131 in VHR. (Mutation of Asp-383 to Asn in GST-Cdc25A, at a position analogous to Asp-92 in VHR, resulted in formation of an inactive enzyme (11).) The natural substrate for Cdc25 is a protein kinase, present in a large macromolecular complex and phosphorylated on adjacent threonyl and tyrosyl residues (2). To date, it has been difficult to obtain sufficient amounts of stoichiometrically phosphorylated natural substrate for kinetic studies. In addition, phosphorylated peptides are poor substrates for Cdc25 in vitro (12). Thus, a good artificial substrate would facilitate analysis of the mechanism of Cdc25-mediated catalysis. Like other PTPases, Cdc25 hydrolyzes pNPP, albeit poorly (12, 13). It has recently been reported that Cdc25 hydrolyzes the fluo-
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FIG. 1. Catalytic mechanism of DS-PTPases. Amino acids are numbered with reference to VHR. In the first step of the reaction, a cysteine thiolate acts as a nucleophile, attacking the substrate phosphorus atom to form the phosphoenzyme intermediate, followed by release of the leaving group (phenolate derivative). The charge on the phenolic oxygen of the leaving group is neutralized by a proton donated by Asp-92. In the second step of the reaction, Asp-92 acts as a general base to promote the breakdown of the phosphoenzyme intermediate, leading to regeneration of the thiolate anion and the release of phosphate (adapted from Ref. 7).

Preparation of OMFP and OMF—OMFP was freshly prepared by sonication in Me$_2$SO and stored in the dark at room temperature for 1 day, after which the solution was discarded because of a slow gain of insolubility at 477 nm and insolubility after freezing and thawing. OMF was similarly prepared, but could be stored at −20°C. OMFP was diluted into reaction mixtures immediately before use since it spontaneously hydrolyzes at alkaline pH with a half-life of ~1 h. The time frames of the experiments described in this paper are significantly shorter than this.

Stability Studies—To determine the stability of the Cdc25B protein at variable pH, OMFP hydrolysis reactions were carried out in a three-component buffer (0.05 M Tris-Cl, 0.05 M Bis-Tris-Cl, and 0.1 mM sodium acetate (TBA buffer)) that maintains constant ionic strength over the pH range 3.6–9.1 (17). The Cdc25B protein was preincubated at 30°C for 1 min at variable pH values and then diluted 250-fold into pH 8.0 buffer containing OMFP at 30°C. OMFP formation was quantified by fluorescence, and fluorescence units were converted to product concentration as described above. Because this experiment showed that the activity of Cdc25B is unstable below pH 8.0, all subsequent experiments were performed at pH 8.2.

Steady-state Kinetics—Reactions with Cdc25B, VHR, and rVH6 were carried out in 0.1 M Tris-Cl, pH 8.2, 0.04 M NaCl, 1 mM dithiothreitol, and 20% glycerol (ionic strength, 0.1). (The activities of VHR and rVH6 were stable under these reaction conditions, although these conditions are not necessarily optimal for their activities.) PTP1 and YOP were assayed in TBA buffer at pH 7.0. Me$_2$SO was maintained at 1% in reaction mixtures that contained OMFP and OMF in order to keep these components soluble. This amount of Me$_2$SO had no significant effect on pNPP hydrolysis and was therefore not included in these reactions. All reactions were carried out at 20°C in a cuvette placed in a temperature-controlled Perkin-Elmer Lambda 6 spectrophotometer, set at a wavelength of 405 nm for the measurement of pNP or 477 nm for the measurement of OMFP. Data were collected continuously over the course of 1 to several minutes with the program UV Data Manager (Perkin-Elmer), and absorbance was converted to product concentration as described below. The data were fit to the Michaelis-Menten equation (Equation 1):

$$V_0 = V_{\text{max}}[S]/(K_m + [S])$$

(Eq. 1)

using the program KaleidaGraph (Synergy Software, Reading, PA). For Cdc25B, $k_{\text{cat}}/K_m$ was obtained by dividing $V_{\text{max}}$ by the concentration of enzyme active sites as determined in the stopped-flow experiments. For other enzymes, the total enzyme concentration was used in this calculation.

Pre-steady-state Kinetics—Experiments were performed by mixing enzyme and substrate at 20°C in a temperature-controlled SF-61 stopped-flow spectrophotometer (Hi-Tech Scientific) equipped with a xenon lamp. The excitation wavelength was 471 nm, and emission was selected using a 530-nm cutoff filter. Data were collected and fit to the equation $Y = a(1 + e^{-rt}) + b$, where $r$ is the program KISS (Kinetic Instruments, Inc., Ann Arbor, MI). For fluorometric measurements, a standard curve was generated by measuring the fluorescence of solutions of OMPF that were reconstituted in assay buffer.

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**Fig. 2. Catalytic domain of human Cdc25B.** A, predicted amino acid sequence of the catalytic domain of Cdc25B produced when a GST-Cdc25B catalytic domain fusion protein was cleaved with thrombin. The first two amino acids in the sequence (Gly-Ser) are derived from the vector. They are followed by amino acids 378–566 of Cdc25B, as previously reported by Galaktionov and Beach (20). Underlined are the active site sequence and two flanking sequences of unknown function that are highly conserved in all Cdc25 proteins and many other DS-PTPases (27). B, SDS-polyacrylamide gel electrophoresis analysis of the purified catalytic domain. Proteins were stained with Coomassie Blue. Lane 1, purified Cdc25B catalytic domain; lane 2, molecular mass markers.

Values for \( K_s \) and \( (k_+ + k_-) \) (see Scheme I below) were obtained by plotting the first-order rate constant for the burst \( (k_{burst}) \) versus substrate concentration and fitting the data to Equation 2:

\[
h_{burst} = \frac{(k_+ + k_-)S}{K_s + [S]} \quad \text{(Eq. 2)}
\]

The sum \( (k_+ + k_-) \) was equal to 27.5 s\(^{-1}\). The rate constant \( k_+ \) was derived by measuring the burst amplitude (B) at variable substrate concentration and fitting the data to Equation 3:

\[
B = \frac{[E][k_0/k_+ + k_-)]^2}{1 + (K_s/[S])^2} \quad \text{(Eq. 3)}
\]

with \(|E|\) equal to 0.043 \( \mu \)M (concentration of active sites, from stoichiometry of the burst at saturating substrate) and solving for \( k_+ \) and \( K_s \). The value of \( k_+ \) was then the difference between 27.5 and \( k_+ \). This method of analysis has been used previously for chymotrypsin (18). Steady-state constants were also derived from the linear portion of the reaction plot by fitting to the Michaelis-Menten equation (Equation 1).

**Product Quantitation by Absorbance**—The p-nitrophenolate ion was quantified using an extinction coefficient of 18,000 \( M^{-1} \) cm\(^{-1}\) at 405 nm, with correction for the ionization of pNPP using the \( pK_a \) value of 7.1 (4). OMFP was quantified using an extinction coefficient of 27,200 \( M^{-1} \) cm\(^{-1}\) at 277 nm in 0.1 M Tris-Cl, pH 8.2, 0.04 M NaCl, 1 mM dithiothreitol, and 20% glycerol or 28,000 \( M^{-1} \) cm\(^{-1}\) in TBA buffer, pH 7.0. For determination of the extinction coefficient of OMFP, 10 \( \mu \)M OMFP (the concentration of which was confirmed by titration) was hydrolyzed to completion, and the absorbance of OMFP was read at 477 nm.

\( pK_a \) of OMFP—Solutions of 5 \( \mu \)M OMFP and 1% MeSO were prepared at variable pH in TBA buffer. (MeSO did not affect the pH of these buffer solutions.) Absorbance (A) of the solutions at 477 nm was measured at room temperature, and the data were fit with KaleidaGraph to the equation \( pK_a = pK + \log(A/26,000) - \log(0.138 - A/26,000) \), where 0.138 was the maximum absorbance of the solution. From this fit, a \( pK_a \) value of 4.6 ± 0.01 was derived.

**RESULTS AND DISCUSSION**

We have purified and characterized by kinetic analysis a homogeneous, stable, and active form of the human Cdc25B protein. In the past, various full-length and truncated Cdc25 proteins have been expressed in and purified from *E. coli* (12–14, 19, 20). In the present study, a GST-Cdc25B catalytic domain fusion protein was expressed in *E. coli*, bound to glutathione-Sepharose beads, and, while immobilized on the beads, cleaved with thrombin. We found that by cleaving the fusion protein while it was immobilized, rather than in solution, proteolytic degradation of the Cdc25B catalytic domain could be minimized. Fig. 2 shows an SDS-polyacrylamide gel electrophoresis analysis of the purified protein and its deduced amino acid sequence. It has been shown previously that the Cdc25B catalytic domain is active and retains the ability to bind physiological substrates (14).

The artificial substrate pNPP has been used frequently in kinetic analyses of phosphatases. The \( K_m \) values of DS-PTPases for pNPP are in the millimolar range. To perform functional studies with Cdc25B, we wanted to use a more reactive substrate than pNPP. Eckstein *et al.* (11) have reported that the \( K_m \) values of Cdc25A and Cdc25C for OMFP are low (36 \( \mu \)M) compared with pNPP. Similarly, we found that OMFP is a dramatically better substrate than pNPP for the Cdc25B catalytic domain (Fig. 3, A and B). The \( K_m \) of the enzyme for OMFP was 37 \( \mu \)M, and the \( k_{cat} \) was 1.2 s\(^{-1}\); in contrast, the corresponding values for pNPP were 8.4 mM and 0.35 s\(^{-1}\). These values represent an 810-fold enhancement of \( k_{cat}/K_m \) for OMFP over pNPP (Table I).
phate. According to this mechanism, $k_{cat}/K_m$ is composed of rate constants for steps leading to formation of $E^P$ (21), as described by Equation 4.

$$k_{cat}/K_m = k_1k_2/(k_{-1} + k_2)$$  \hspace{1cm} (Eq. 4)

The increase in $k_{cat}/K_m$ seen with OMFP might be explained by an increase in the rate constant for the chemical step of intermediate formation ($k_2$). We have determined that the p$K_a$ of OMFP is 4.6 (see “Experimental Procedures”), whereas the p$K_a$ of pNPP is 7.1; thus, OMFP should be a more reactive substrate than pNPP due to its better leaving group, OMF. The increase in $k_{cat}/K_m$ might also be explained by a higher affinity of OMFP for the enzyme. If formation of a phosphoenzyme intermediate from OMFP is sufficiently rapid relative to its breakdown, we would expect to see a burst of product formation upon rapid mixing of enzyme and substrate. This would allow us to determine pre-steady-state rate and binding constants for OMFP.

We measured the activity of the Cdc25B catalytic domain by stopped-flow techniques, measuring the formation of either pNP by absorbance or OMFP by fluorescence. For pNPP, there was no apparent burst of product formation. However, for OMFP, a burst was observed whose amplitude was proportional to the concentration of enzyme (Fig. 4, A and B). The appearance of a burst pattern of product formation indicates that breakdown of the phosphoenzyme intermediate is rate-limiting for OMFP hydrolysis. Burst kinetics of OMFP were further examined by varying the substrate concentration. Analysis of this data enabled determination of the rate constants of the individual chemical steps $k_2$ and $k_3$ as well as the dissociation constant, $K_S$ (18). Plots of $k_{burst}$ (rate constant for the burst at different substrate concentrations) versus [OMFP] (Fig. 5A) and burst amplitude versus [OMFP] (Fig. 5B) yielded these constants, as described under “Experimental Procedures.” A plot of $V_s$ (the steady-state rate, obtained from the linear portion of the reaction curve) versus [OMFP] (Fig. 5C) gave values for $K_m$ and $k_{cat}$ that were similar to those obtained from steady-state analysis, where OMFP formation was measured by absorbance instead of fluorescence. The kinetic and dissociation constants derived from the stopped-flow data are summarized in Table II. For the hydrolysis of OMFP, $k_2$ is ~20 times greater than $k_3$. This result implies that either chemical breakdown of the phosphoenzyme intermediate or product release is rate-limiting in the hydrolysis of OMFP. The $k_{cat}$ for OMFP hydrolysis (1.4 s$^{-1}$) is close to $k_3$ (1.5 s$^{-1}$), confirming this conclusion. Conversely, the lack of a burst for pNPP implies that either substrate binding or phosphoryl transfer is rate-limiting for hydrolysis of this substrate.

![Fig. 4. Effect of enzyme concentration on OMFP hydrolysis by Cdc25B catalytic domain. A, stopped-flow traces of fluorescence generated after aliquots of enzyme were diluted into reaction buffer (at pH 8.2 and 20 °C) and then rapidly mixed in a stopped-flow device with an equal volume OMFP. The final concentration of OMFP was 250 μM; final concentrations of enzyme active sites were 2.7, 1.37, 0.68, and 0.34 μM (top to bottom). B, dependence of burst amplitude on enzyme concentration.](image)

Since $k_2$, the rate constant for intermediate breakdown, must be identical for hydrolysis of either substrate, we can solve for the parameter $k_3$ in pNPP hydrolysis since $k_{cat}{pNPP} = k_2k_3/(k_2 + k_3)$ (21). The $k_{cat}$ for the pNPP reaction (0.35 s$^{-1}$) is close to the calculated $k_3$ (0.46 s$^{-1}$), which implies that the rate-limiting step in pNPP hydrolysis is phosphoenzyme formation. In comparison, the $k_2$ for OMFP hydrolysis is 26 s$^{-1}$. Therefore, the rate of phosphorylation of the enzyme is ~60-fold faster with OMFP than with pNPP. Since $K_{cat}{pNPP} = k_2k_3/(k_2 + k_3)$ (21), the $K_S$ for pNPP can be calculated to be 11 mM. The dissociation constant of the enzyme for OMFP (0.31 mM) is 35-fold lower than that for pNPP. We conclude that better substrate binding and increases in rates of chemical conversion contribute toward making OMFP a superior substrate.

To investigate the general usefulness of OMFP as a protein phosphatase substrate, we also determined steady-state kinetic parameters for two other DS-PTPases (VHR and rVH6) as well as two PTPases, PTP1 and YOP. The DS-PTPases all displayed increased $k_{cat}/K_m$ values with OMFP (Table I). In contrast, the $k_{cat}/K_m$ values of the two PTPases did not increase with OMFP. Comparison of the crystal structures of the DS-PTase VHR (22) and the PTPase YOP (23) reveals that the active site differs in these two classes of enzymes with respect to depth and accessibility to solvent; these differences might play a role in differential substrate binding.

OMFP should prove to be a useful substrate for the kinetic analysis of DS-PTPases due to its relatively low $K_m$ for these enzymes.

| Enzyme Substrate | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$mM$^{-1}$) | Fold increase ($k_{cat}/K_m$) $^a$ |
|------------------|-----------|---------------------|---------------------|-----------------|
| Cdc25B (cat) OMFP | 0.037 | 1.2 | 34 | 810 |
| VHR (native) pNPP | 0.10 | 1.5 | 15 | 556 |
| rVH6 (GST-cat) OMFP | 0.13 | 0.014 | 0.11 | 138 |
| PTPases | | | | |
| PTP1 (native) OMFP | 0.12 | 2.6 | 13 | None |
| YOP (cat) OMFP | 0.92 | 3.4 | 3.7 | 3.4 |

$^a (k_{cat}/K_m{OMFP}/k_{cat}/K_m{pNPP})$.

$^b$ cat, catalytic domain; GST-cat, GST-catalytic domain fusion protein.

**TABLE I**

**Kinetic parameters for DS-PTPases and PTPases from steady-state analysis**

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![Image](image)
enzymes. OMFP has also been used as a substrate for alkaline phosphatase (24) as well as for the phosphatase reaction catalyzed by the (Na\(^+\) + K\(^-\))-ATPase (25), where the \(K_m\) for OMFP is 2 orders of magnitude lower than for pNPP and the \(V_{\text{max}}\) is two times greater. Recently, a wide array of PTPase substrates with low \(K_m\) values have been described (26). Some of these substrates may increase the rate of phosphoenzyme formation sufficiently to allow observation of pre-steady-state events.

Since two phases of OMFP hydrolysis can be observed kinetically with the wild-type Cdc25B catalytic domain, the roles of individual amino acid residues in each phase of the reaction can now be assessed by stopped-flow analysis of mutant enzymes. Other DS-PTPases may also be amenable to the same type of analysis. These studies will provide important information about the similarities and differences in the catalytic mechanism of Cdc25 and other DS-PTPases.

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![Graph A](image1.png)

**Table II**

Kinetic and binding parameters for Cdc25B catalytic domain from stopped-flow analysis

| Substrate | Exponential phase | Linear phase |
|-----------|-------------------|--------------|
|           | \( K_m \) | \( k_{cat} \) | \( k_{cat} \) | \( K_m \) | \( k_{cat} \) |
| OMFP      | 0.031 mM | 26 s\(^{-1}\) | 0.31 mM | 0.034 s\(^{-1}\) | 1.4 |
| pNPP      | 0.46 mM | 1.5 | 11 |

![Graph B](image2.png)

![Graph C](image3.png)

**Fig. 5.** Effect of substrate concentration on OMFP hydrolysis by Cdc25B catalytic domain. The final enzyme active site concentration after mixing was 0.045 mM. A, dependence of burst rate constant (\(k_{\text{burst}}\)) on substrate concentration; B, dependence of burst amplitude on substrate concentration; C, dependence of steady-state rate (\(V_{\text{ss}}\)) on substrate concentration.