Kinetic Analysis of Human Serine/Threonine Protein Phosphatase 2Ca*

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The PPM family of Ser/Thr protein phosphatases have recently been shown to down-regulate the stress response pathways in eukaryotes. Within the stress pathway, key signaling kinases, which are activated by protein phosphorylation, have been proposed as the in vivo substrates of PP2C, the prototypical member of the PPM family. Although it is known that these phosphatases require metal cations for activity, the molecular details of these important reactions have not been established. Therefore, here we report a detailed biochemical study to elucidate the kinetic and chemical mechanism of PP2Ca. Steady-state kinetic and product inhibition studies revealed that PP2Ca employs an ordered sequential mechanism, where the metal cations bind before phosphorylated substrate, and phosphatase is the last product to be released. The metal-dependent activity of PP2C (as reflected in $K_{cat}/K_{m}$, indicated that Fe$^{2+}$ was 1000-fold better than Mg$^{2+}$. The pH rate profiles revealed two ionizations critical for catalytic activity. An enzyme ionization with a $pK_a$ value of 7 must be unprotonated for catalysis, and an enzyme ionization with a $pK_a$ of 9 must be protonated for substrate binding. Brønsted analysis of substrate leaving group $k_{cat}$ indicated that phosphomonoester hydrolysis is rate-limiting at pH 7.0, but not at pH 8.5 where a common step independent of the nature of the substrate and alcohol product limits turnover ($k_{cat}$). Rapid reaction kinetics between phosphomonoester and PP2C yielded exponential “bursts” of product formation, consistent with phosphate release being the slow catalytic step at pH 8.5. Dephosphorylation of synthetic phosphopeptides corresponding to several protein kinases revealed that PP2C displays a strong preference for diphosphorylated peptides in which the phosphorylated residues are in close proximity.

Protein phosphatases (PP)* catalyze the dephosphorylation of proteins containing phosphoserine/phosphothreonine and are divided into two distinct gene families, designated PPP and PPM (1). Although both PP families require divalent cations for activity, the PPM family is often distinguished by its Mg$^{2+}$ and Mn$^{2+}$ dependence. PP2C is the defining member of the PPM family. PP2C homologues have been identified in bacteria, plants, yeast, and mammals and appear to have a conserved role in negatively regulating stress response. PP2C was shown to be a negative regulator of two mitogen-activated protein kinase (MAPK) pathways involved in stress response, the p38 and c-Jun N-terminal kinase pathways. Like other MAPK pathways, these consist of a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) (2). PP2C is phosphorylated on conserved threonine and tyrosine residues by the activated MAPKK. The MAPKK is activated by phosphorylation on conserved threonine and/or serine residues by the MAPKKK. The stress response pathways are activated by proinflammatory cytokines, osmotic shock, oxidative stress, UV irradiation, and heat shock (3, 4). PP2C is thought to directly dephosphorylate and inactivate protein kinases at several levels in the pathway. The PP2C homologue, MP2C, functions as a negative regulator of a stress-activated pathway in plants. By a yeast genetic analysis, the molecular target of MP2C was determined to be the MAPKKK Ste11 (5). MKK6 and SEK 1, both MAPKKs important in stress response signaling pathways, have been identified as PP2C substrates (6). Additional MAPKKs have also been suggested to be PP2C substrates (7). PP2Ca has been shown to dephosphorylate and inactivate the MAPK p38 (6). Genetic studies with PP2C yeast homologues Ptc1, Ptc2, and Ptc3 (8, 9) implicated an essential role for PP2C in down-regulating the stress response. Collectively, these studies demonstrate a major role for PP2C in turning off, or resetting, the stress response pathways. Unlike the PPP family of protein phosphatases, there are no known specific inhibitors of PP2C and no regulatory subunits have been identified. The catalytic domain appears to be sufficient to impart strict substrate specificity.

The crystal structure of PP2Ca bound with Mn$^{2+}$ has been solved (10) and reveals a central β-sandwich surrounded by α-helices. The active site is located at one end of this β-sandwich and is composed of several invariant carboxylates, which serve as metal-coordinating residues. There are two hexa-coordinated metal sites, M1 and M2, which are 4 Å apart and share a water molecule and the carboxylate side chain of Asp-60. Site M1 is coordinated by 3 water molecules and two additional aspartic acids, Asp-239 and Asp-282. Site M2 is coordinated by 4 water molecules and makes only two direct contacts with the protein, at the backbone carbonyl of Gly-61 and at the bridging Asp-60. The presence of bound phosphate in the x-ray structure suggested the importance of an active site Arg-33. This arginine appears to position the phosphate near the water molecule bridged by the two metal ions. Das et al. (10) have proposed that catalysis proceeds by direct attack of an activated water molecule at the phosphorus center of substrate. Except for this crystallographic study, very little mechanistic data are available for this important class of enzymes.

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* The abbreviations used are: PP, protein phosphatase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; PP2Ca, protein phosphatase 2Ca; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B; M1, metal-binding site 1; M2, metal-binding site 2; pNPP, para-nitrophenyl phosphate; para-nitrophenol; ferrozine, 3,5-di(2-pyridyl)-5,6-bis(4-phenylbulfonic acid)-1,2,4-triazine; DTT, dithiothreitol; Bis-Tris, (bis[2-hydroxyethyl]limino-tris[hydroxymethyl]methane; DiFMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; DiFMU, 6,8-difluoro-4-methylumbelliferone; ε, molar extinction coefficient.
Here we describe a detailed kinetic investigation of human PP2Cα, the archetypal member of the PPM family of protein phosphatases. In this study, we explore the metal-dependent activation, identify critical enzyme ionizations, determine the kinetic mechanism, determine the rate-limiting step in catalysis and probe substrate specificity.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were of the highest purity commercially available. Peptides were from the University of Michigan Protein Core facility. The Fe$^{2+}$ chelator, 3-(2-pyridyl)-5,6-bis(4-hydroxyphenyl)benzene-sulfonic acid-1,2,4-triazine (ferrozine), was obtained from Sigma.

**Overexpression and Purification of Human PP2Cα**—The plasmid pCW-PP2Cα was a generous gift from Dr. Patricia T. W. Cohen (Dundee University). The enzyme was purified according to the method described (11) with several modifications. The pCW-PP2Cα plasmid was used to transform competent BL21 DE3 bacteria. The transformed bacteria were grown on 2×YT plates containing 100 µg/ml ampicillin. Overnight 10-ml cultures originating from isolated colonies were used to inoculate 1 liter of 2× YT containing 140 µg/ml ampicillin. When the growth reached an optical density of 0.8 at 600 nm, 100 µg/ml isopropyl-1-thio-β-D-galactopyranoside was added, and the bacteria were grown for an additional 10 h. The cells were harvested by centrifugation at 5,000 × g (15 min), resuspended in 30 ml of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 0.1 mM EGTA, 100 mM NaCl, 2 mM MnCl$_2$, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 0.03% (v/v) Brij-35), and lysed by French press. The cell debris was pelleted by centrifugation at 48,000 × g (20 min), the supernatant decanted, and the protein precipitated between 30% and 50% saturation with (NH$_4$)$_2$SO$_4$. The pellet was resuspended in 8 ml of buffer B (20 mM triethanolamine, pH 7.0, 1 mM EDTA, 2 mM MnCl$_2$, 0.1% (v/v) β-mercaptoethanol, 5% (v/v) glycerol, 0.03% (v/v) Brij-35) and dialyzed against 1 liter of buffer B for at least 2 h with one change in buffer. The dialysate was loaded onto a 50-ml Q-Sepharose (Amersham Pharmacia Biotech) hydrophilic column, which was pre-equilibrated with buffer B. The column was then washed with 250 ml of buffer B, and the enzyme was eluted with a 0–0.4M linear NaCl gradient of 400 ml. PP2Cα elutes at 250 mM NaCl. Fractions containing high phosphate specificity with para-nitrophenyl phosphate (pNPP) were pooled and concentrated to between 5 and 10 ml by ultrafiltration (Centricon 10 concentrator by Amicon). The concentrated solution was dialyzed against 1 liter of buffer containing 25 mM Na$_2$HPO$_4$, pH 7.0, 1 mM (NH$_4$)$_2$SO$_4$, 1 mM DTT, and 2 mM EDTA for at least 1 h with one change of buffer and then loaded onto a 50-ml Phenyl-Sepharose (Amersham Pharmacia Biotech) hydrophobic column. The column was then washed with 250 ml of buffer and eluted with a 1–0 µM linear (NH$_4$)$_2$SO$_4$ gradient of 400 ml. PP2Cα elutes at 200 mM (NH$_4$)$_2$SO$_4$. Fractions with phosphate specificity were analyzed by SDS-polyacrylamide gel electrophoresis to determine the levels of highest purity and accuracy were pooled and concentrated by ultrafiltration to a final concentration of 0.04 to 0.4 mM. PP2Cα was dialyzed against 1 liter of 50 mM Tris-HCl, pH 7.0, 10% (v/v) glycerol, 1 mM DTT, 2 mM EDTA for 2 h with a change after 1 h, followed by a final dialysis in 50 mM Tris-HCl, pH 7.0, 10% (v/v) glycerol, 1 mM DTT for 2 h with one change. The EDTA was included to obtain metal free (apo)PP2C. EDTA is effective in completely removing bound metal cations, as was evident from the apo-PP2C x-ray structure, which revealed a virtually identical structure to that of holoenzyme (10). The enzyme was stored at −20 °C or −80 °C until use.

**Enzyme Kinetics**—All assays were carried out in a reaction buffer containing 0.5 mM Tris, 0.05 mM Bi-Tris, and 0.1 mM acetate at 25 °C. To determine the kinetic parameters $k_{cat}$ and $k_{cat}/K_{m}$, the initial velocities were measured at various substrate concentrations, and the data were fitted to Equation 1. The computer program KinetAsyst (IntelliKinetics, State College, PA) was used for fitting kinetic data to Equations 1-7. Three types of assays were used to monitor PP2C phosphatase activity and are described below.

Because of its relative stability and high specific activity, the divalent cation Mn$^{2+}$ was utilized for the majority of the kinetic studies. The metal ion was largely avoided due to difficulties with oxidation. For the phosphate inhibition and pH dependence studies, the cation Mg$^{2+}$ was used to avoid the precipitation that occurs with Mn$^{2+}$ in the presence of phosphate and at high pH.

**Continuous Assay**—The continuous assay monitoring the dephosphorylation of pNPP was performed using a temperature-controlled Shimadzu Biospec-1601 UV-visible spectrophotometer and monitoring the absorbance change recorded at 410 nm, pH 7.0. Initial linear rates were determined using the molar extinction coefficient (ε) of 9 mM$^{-1}$ cm$^{-1}$ for the product para-nitrophenol (pNP) at pH 7.0.

**End Point Assays**—Two end point assays were also used to measure PP2C activity. The phosphate detection assay was used for measuring the initial linear rates of dephosphorylation of peptides, amino acids, and aryl substrates. The release of phosphate was measured using the colorimetric method described by Broderus et al. (12), which measures the formation of phosphomolybdate complexes at 850 nm. Briefly, reaction mixtures of 600 µl were stopped with 1 ml of 0.5 N HCl containing 30 mg of ascorbic acid, 5 mg of ammonium hydroxide, and 10 mg of SDS. For color development, 1.5 ml containing 30 mg of sodium amonite, 30 mg of sodium citrate, and 1 µl glacial acetic acid were added and the absorbance was read at 850 nm. The other end point assay was developed to determine the rate of pNPP hydrolysis for the pH profile using the molar extinction coefficient of 18,000 M$^{-1}$ cm$^{-1}$ for pNP at basic pH values. This assay was developed after the method described in Ref. 13, which utilizes a stop solution containing 1 N NaOH. To prevent the precipitation of metal which occurs upon the addition of 1 N NaOH to a PP2C reaction mixture, a stop solution containing 0.5 M EDTA, pH 10, was used. A comparison of the continuous assay and the two end point assays revealed that these three assays yielded identical rates.

$$v = \frac{k_{cat}}{1 + K_{m}} (S)$$

(Eq. 1)

**Metal Dependence**—Various concentrations of metal ions were combined with apo-PP2C and allowed to incubate for 2 min. The phosphatase reaction was initiated by the addition of pNPP to a final concentration of 0.6 mM. PP2C activity was measured using the columnometric method. The inhibition constants for Ca$^{2+}$ and Zn$^{2+}$ were determined by adding various amounts of MnCl$_2$ at fixed concentrations of the inhibiting metal ion at saturating levels of pNPP. The data were fitted to Equation 2 to yield the inhibition constant. The inhibition was competitive with respect to Mn$^{2+}$.

$$v = \frac{k_{cat}}{1 + K_{m}} \left(\frac{1}{1 + [I]} + S\right)$$

(Eq. 2)

where $K_{m}$ is the inhibition constant, the inhibitor concentration and S is the Mn$^{2+}$ concentration. This kind of inhibition is known as competitive inhibition.

**Fe$^{2+}$ Detection**—A reaction mixture containing 3 mM Fe$^{2+}$, 4 mM pNPP, and 0.59 µM PP2C was separated into two cuvettes. One was monitored for phosphatase activity using the continuous assay, and the other was used to monitor the Fe$^{2+}$ concentration during the course of the reaction. The Fe$^{2+}$ concentration was determined using the Fe$^{2+}$-specific chelator, ferrozine, which has a characteristic absorbance at 562 nm. The measured Fe$^{2+}$ concentrations and PP2C activity were plotted versus time using the computer program KaleidaGraph (Abelbeck Software).

**Steady-state Kinetics**—The end point assay using the EDTA stop solution was used to measure the pH dependence of PP2C activity. For the construction of the pH profiles, $k_{cat}$ and $k_{cat}/K_{m}$ were obtained at various pH values with pNPP as the variable substrate at saturating levels of Mn$^{2+}$ (10 mM) or Mg$^{2+}$ (40 mM). The pH data for Mn$^{2+}$ and Mg$^{2+}$ were fitted to Equations 3 and 4, respectively, where C is the pH-independent value of either $k_{cat}$ or $k_{cat}/K_{m}$. H is the proton concentration; and $K_{c}$ and $K_{b}$ are the ionization constants of the groups involved in the reaction.

$$v = \frac{C}{1 + KH}$$

(Eq. 3)

$$v = \frac{C}{1 + H/K} + K_{b}$$

(Eq. 4)

The catalytic mechanism of PP2C was determined by performing bisubstrate studies treating the metal cation as a pseudo-substrate. Continuous assays were performed by varying the amounts of pNPP at fixed levels of Mn$^{2+}$ and the data were fitted to Equation 5 where $K_{c}$ and $K_{b}$ are the Michaelis constants for pNPP and Mn$^{2+}$, respectively.

$$v = \frac{[I]_{cat} \cdot A \cdot B / (K_{c} + [I]_{cat} + K_{b} + [I]_{cat} \cdot A + B)}{1 + KH}$$

(Eq. 5)

The P$_{i}$ product inhibition of PP2C was analyzed with Mg$^{2+}$ to avoid the precipitation of Mn$^{2+}$ and P$_{i}$. Continuous assays were performed by varying Mg$^{2+}$ or pNPP in saturating levels of the other substrate at fixed concentrations of the product P$_{i}$. The inhibition of P$_{i}$ with respect to pNPP was competitive, and the data were fitted to Equation 2; in contrast, the inhibition of P$_{i}$ with respect to metal was uncompetitive, and the data were fitted to Equation 6.

$$v = \frac{k_{cat} \cdot S / (K_{m} + S) + (1 + IK_{p})}{1 + IK_{p}}$$

(Eq. 6)

The leaving group dependence of PP2C was analyzed by determining the dephosphorylation rate of several artificial compounds, which differ
by the pKa of the leaving group. Substrates 6,8-difluoro-4-methylumbelliferyl phosphate (DifMUP) (pKa = 4.9), pNPP (7.1), 4-methylumbelliferyl phosphate (7.8), β-naphthyl phosphate (9.38), phenyl phosphate (9.99), phosphoserine (14.1) (Ref. 14), and phosphothreonine in the peptide GIPRYpTEHV (15.0) (Ref. 14) were analyzed using the phosphatase detection assay at pH 7.0 and 8.5. Brønsted values were obtained by linear least-squares fitting of log (kcat) versus pKa of leaving group for the substrates using the computer program KaleidaGraph (Abelbeck Software.)

Pre-steady-state Kinetics—Enzyme and substrate were rapidly mixed at pH 8.5 and 25 °C in a temperature-controlled SF-61 stopped-flow spectrophotometer (Hi-Tech Scientific). The absorbance of pNPP and DifMUP were monitored at 410 nm and 358 nm, respectively. The enzyme was combined with Mn2+ in reaction buffer, pH 8.5, prior to rapid mixing with the substrate also diluted into reaction buffer, pH 8.5. The final concentration of Mn2+ was 10 μM. The data were fitted to Equation 7 using the nonlinear least-squares fitting capability of the kinetics software (KinetAsyst, Hi-Tech Ltd, Salisbury, U.K.) where A is the amplitude of the burst, k is the first-order rate of the burst, B is the slope of the linear portion of the curve, C is the intercept of the line, and t is time.

Absorbance = A · e⁻ᵏᵗ + B · t + C

(Eq. 7)

The amplitude was converted to concentration of pNPP using the ε₉₁₀ of 18 mM⁻¹ cm⁻¹. The correlation between the concentration of pNPP burst and the final enzyme concentration was determined by linear least-squares fitting.

Analysis of Peptide Substrates—The following phosphopeptides were analyzed as PP2C substrates using the phosphate detection assay: GIPRYpTEHV, corresponding to Cdc2 kinase (15); KIGEGtpYPGVYK, corresponding to Cdc2 kinase (15); DDE(Nle)pTGpYVATR, corresponding to JNK180–189 kinase with variations of the native -pTPpY- sequence corresponding to p38 kinase (16); and F(Nle)(Nle)pYPpTVVTR, corresponding to Cdc2 kinase (15); KIGEGpTpYGVVYK, corresponding to Cdc2 kinase (15); DDE(Nle)pTGpYVATR, corresponding to JNK180–189 kinase with variations of the native -pTPpY- sequence corresponding to p38 kinase (16); and F(Nle)(Nle)pYPpTVVTR, corresponding to Cdc2 kinase (15). These ions were found to competitively inhibit the PP2C Mn2+−dependent activity. By varying the Mn2+ concentration at fixed levels of either Ca2+ or Zn2+ , a fit to Equation 2 yielded KI values of 4.45 ± 0.54 mM and 12.0 ± 1.8 μM for Ca2+ and Zn2+, respectively.

To determine the oxidation state of iron required for PP2C activity, the Fe2+− detector ferrozine, was used to monitor the effective concentration of Fe2+ in the phosphatase assay. PP2C activity in a parallel reaction was recorded at specific time points and related to the free Fe2+ concentration (Fig. 2). A direct relationship between loss in Fe2+ cation (due to oxidation to Fe3+) and loss in activity was observed.

pH Profiles—To determine the ionizations important for catalysis and substrate binding, the kcat and kcat/KmNPP values were obtained at various pH values and saturating concentrations of either Mn2+ or Mg2+. The plot of kcat versus pH obtained in Mn2+− revealed one ionization that must be unprotonated for catalysis and has a pKa value of 7.18 ± 0.08 (Fig. 3B). The pH-independent kcat/KmNPP value was 1.290 ± 314 M⁻¹ s⁻¹. Due to precipitation of Mn2+ at basic pH values, data could not be collected at pH values higher than 8.5. However, difficulties with precipitation were not encountered with Mg2+. This allowed us to measure the Mg2+− dependent rates above pH 8.5. The plot of kcat versus pH obtained with Mg2+− revealed an ionization that must be unprotonated for catalysis with a pKa value of 7.42 ± 0.09 (Fig. 4A) and a pH-independent value of 0.11 ± 0.01 s⁻¹. With Mg2+,
the $k_{\text{cat}}/K_{\text{pNPP}}$ pH profile revealed a second critical ionization that must be protonated for activity and has a $pK_a$ value of 8.96 ± 0.12 (Fig. 4B). The pH-independent $k_{\text{cat}}/K_{\text{pNPP}}$ value was 20.3 ± 3.2 $\text{mM}^{-1} \text{s}^{-1}$.

Steady-state Kinetic Analysis—Bisubstrate studies were performed to determine the kinetic mechanism of PP2C and to probe the order of substrate binding. The concentration of $\text{pNPP}$ was varied at fixed concentrations of $\text{Mg}^{2+}$ and the double-reciprocal plot of $1/v$ versus $1/[\text{pNPP}]$ was constructed for each fixed concentration of $\text{Mg}^{2+}$ (Fig. 5). A series of lines that intersect to the left of the vertical axis and above the horizontal axis were obtained, suggesting a sequential mechanism. In a sequential mechanism, both substrates must bind before catalysis can occur. Thus, PP2C forms a ternary complex with metal and the phosphomonoester substrate prior to catalysis.

To determine the order of substrate binding and product release, product inhibition studies were performed. The concentration of $\text{pNPP}$ was varied at fixed levels of $\text{Pi}$ and saturating levels of $\text{Mg}^{2+}$. A series of parallel lines was obtained in the double-reciprocal plot indicating that $\text{Pi}$ behaves as an uncompetitive inhibitor with respect to $\text{Mg}^{2+}$ (Fig. 6B). The $K_{\text{ii}}$ value was determined to be 3.61 ± 0.16 mM.

To determine the nature of the rate-limiting step during
PP2C catalysis, several phosphomonoester substrates with different leaving group $pK_a$ values were analyzed. A Brønsted plot of $\log k_{cat}$ versus $pK_a$ of leaving group was obtained at pH 7.0 and at pH 8.5. At pH 7.0, a $\beta$ of $-0.32 \pm 0.03$ (Fig. 8) was determined, indicating that chemistry is at least partially rate-limiting. A $\beta$ value of $0.03 \pm 0.02$ was calculated at pH 8.5, suggesting that chemistry does not contribute to the rate-limiting step at this pH. The similar $k_{cat}$ values obtained at pH 8.5 for these distinct substrates suggest that they share a common rate-limiting step, which is independent of the nature of the substrate and the released alcohol product. The rates obtained with DiFMUP are identical at pH 7.0 and 8.5, suggesting that the rate-limiting step is the same as both pH values and that chemistry does not contribute to the turnover rate with this substrate. With a leaving group $pK_a$ of 4.6, DiFMUP does not fall on the Brønsted plot at pH 7. Instead, there appears to be a break in the plot when the $pK_a$ of the leaving group is less than 7. The Brønsted analysis indicated that the turnover rate of DiFMUP will not exceed this common slow step, even when chemistry is made very fast with good leaving groups. The point at which the two Brønsted plots cross (Fig. 7) appears to represent a transition in the rate-limiting step as a function of pH and the $pK_a$ of the leaving group. Collectively, these data suggest that the common physical release of $P_i$ may limit the rate of turnover when the rate of chemistry is fast.

Pre-steady-state Kinetics—Product inhibition studies revealed that $pNP$ is released prior to $P_i$. Leaving group dependence studies suggested that at pH 8.5 phosphate release may be the common slow step among the various substrates. If $pNP$ release occurs fast relative to a later step, a pre-steady-state “burst” of product should be detectable at pH 8.5. The product $P_i$ concentrations were as follows: ●, 0.5 mM; ●, 1.0 mM; ▼, 2.0 mM; ■, 5.0 mM; ▲, 10.0 mM.

The rate of enzyme reaction of PP2C with substrate was followed by measuring the formation of $P_i$ and DiFMU from the substrates $pNP$ and DiFMUP, respectively. These experiments revealed an exponential product burst at pH 8.5, followed by a slower linear rate (Fig. 8A). The amplitude of the observed burst was proportional to the enzyme concentration (Fig. 8B). The burst phase yielded first order rate constants between 20–200 s$^{-1}$, while the linear phase corresponded to the steady-state initial velocity. Burst kinetics were not observed with $pNP$ at pH 7.0, consistent with rate-limiting chemistry.

Phosphopeptide Substrate Specificity— Several phosphorylated peptides were analyzed to explore PP2C’s substrate specificity. The $k_{cat}$ and $k_{cat}/K_m$ values were determined for a variety of physiologically relevant peptide substrates and are listed in Table II. The $k_{cat}$ values were determined at pH 7.0 and found to range from 0.05 to 0.35 s$^{-1}$ compared with 1.02 s$^{-1}$ with $pNP$. Given the $pK_a$ for serine/threonine residues, these $k_{cat}$ values are 15–100-fold faster than the Brønsted plot obtained at pH 7.0 would predict, suggesting that properties intrinsic to the peptide are important for increasing the rate of chemistry at physiological pH values. At pH 8.5, the $k_{cat}$ value obtained for the phosphopeptide was similar to the $k_{cat}$ value obtained for the other substrates at this pH, indicating that dephosphorylation of the peptide is restricted by the same rate-limiting step. The $k_{cat}/K_m$ values for the substrates approached a 90-fold greater value than that for $pNP$. Thus, the specificity constants re-
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Discussion

catalytic mechanism of pp2c—bisubstrate steady-state kinetics were combined with product inhibition studies to determine the kinetic mechanism of pp2c. the bisubstrate studies revealed a sequential mechanism where both metal cations and phosphorylated substrate must bind prior to catalysis. the product inhibition studies indicated the order of substrate binding and product release. product p

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the same slow step at both pH 7.0 and 8.5 ($k_{cat} \approx 1.5 \text{ s}^{-1}$). Consistent with the minimal kinetic mechanism proposed in Scheme 1, the physical release of phosphate would be a common step that is independent of the nature of the phosphomonoester and the alcohol product.

To explore the possibility that phosphate release was rate-limiting at pH 8.5, pre-steady-state kinetic experiments were performed to determine if an exponential burst of the alcohol product could be detected prior to the build-up of the steady-state product. Pre-steady-state kinetic experiments were performed to determine if an exponential burst of the alcohol product could be detected prior to the build-up of the steady-state product. The Brønsted plots suggest that at pH 8.5, phosphate release may be the rate-limiting step. These data also indicated that as the leaving group $pK_a$ decreases, chemistry becomes less rate-limiting, or phosphate release becomes more rate-limiting. This suggested that product bursts could be observed at pH 8.5 with substrates that have lower leaving group $pK_a$ values, such as $p$NPP and DiFUMU. The detection of a pre-steady-state burst requires that the release of the chromophoric product ($p$NP and DiFUMU) occurs before the rate-limiting step in the reaction. The product inhibition studies suggested that phosphate release is the last kinetic step. If phosphate release is the rate-limiting step, a burst of product would be detectable upon rapid mixing of enzyme and substrate. The detection of product bursts (Fig. 8) whose amplitude correlates with the enzyme concentration is in excellent agreement with this kinetic model.

Although x-ray studies did not reveal a nucleophilic residue (10), we cannot rule out the possibility that PP2C forms a phosphoenzyme intermediate. Both the pre-steady-state and steady-state data are also fully consistent with this mechanism. In this model, formation of the intermediate would limit turnover at low pH, while at high pH intermediate hydrolysis would be rate-determining. The phosphoenzyme intermediate mechanism would be akin to that utilized by the protein-tyrosine phosphatases (21). The fact that we were unable to trap a phosphoenzyme intermediate with a variety of peptide substrates at pH 8.5 argues against the involvement of a side-chain nucleophile and, as we have proposed, suggests that the slow step at pH 8.5 involves the physical release of phosphate after the rapid attack of the metal bound water molecule.

**Metal Dependence**—The metal ions Mg$^{2+}$ and Mn$^{2+}$ were previously shown to activate putative PP2C enzymes. The results reported here reveal that PP2C is most active with Fe$^{2+}$, as determined by comparing $k_{cat}$ and $k_{cat}/K_m$ values obtained with various metal cations. The metal dependence is of interest since the physiological metals ions of PP2C are not known. A comparison of $k_{cat}/K_m$ values suggests that Fe$^{3+}$ is preferred over all other metal ions analyzed. In fact, the $k_{cat}/K_m$ value for Fe$^{2+}$ was determined to be 1,800-fold greater than the $k_{cat}/K_m$ obtained with Mg$^{2+}$. This was surprising considering that PP2C was initially defined by its Mg$^{2+}$ dependence (22) and that there were no previous reports of PP2C Fe$^{2+}$-dependent activity. It is possible that post-translational modification or effector molecule binding may alter the metal-binding properties of native PP2C from those observed with recombinant enzyme.

The importance of the iron oxidation state appears to be similar to that seen with the di-iron proteins ribonucleotide reductase and purple acid phosphatase. Ribonucleotide reductase has been shown to only bind Fe$^{2+}$, as determined by comparing $k_{cat}$ and $k_{cat}/K_m$ values obtained with various metal cations. The metal dependence of PP2C suggests that both the nature of the metal and its valence state will play a key role in regulating the cellular activity of PP2C.

**Substrate Specificity**—Although artificial substrates yielded $k_{cat}$ values higher than those obtained with several phosphorylated peptides (Table II), the $k_{cat}/K_m$ values clearly demonstrated that phosphopeptides are greatly preferred over the artificial substrates examined. The $k_{cat}/K_m$ values are more useful for analyzing the substrate specificity, since the $k_{cat}/K_m$ constant

| Substrate | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|-----------|----------|-------|--------------|
| $p$NPP    | 1.02 ± 0.01 | 1.18 ± 0.03 | 859.9 ± 15.9 |
| KIEEG(pT)pYGVVYK (Cdc2) | 0.35 ± 0.04 | 0.005 ± 0.001 | 75,000 ± 18,000 |
| KIEEGT(pYGVVYK) (Cdc2) | ~0 | ~0 | ~0 |

**Table II**

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**Table III**

**PP2Ca pH dependence**

Assays were performed in Tris/Bis-Tris/acetate buffer at 25 °C. Rates were monitored by measuring $p$NPP formation at 410 nm. Profiles for Mn$^{2+}$ were determined between pH 5 and 8.5. Precipitation prevented obtaining data at higher pH values. Profiles for Mg$^{2+}$ were determined between pH 5 and 10. $C$ is the pH-independent value of either $k_{cat}$ or $k_{cat}/K_m$.

**Scheme 1**

| Profile | Metal ion | $pK_{a1}$ | $pK_{a2}$ |
|---------|-----------|-----------|-----------|
| $k_{cat}$ | Mn$^{2+}$ | 2.21 ± 0.65 s$^{-1}$ | 7.00 ± 0.09 |
| $k_{cat}/K_m$ | Mn$^{2+}$ | 1.280 ± 314 m$^{-1}$s$^{-1}$ | 7.18 ± 0.08 |
| $k_{cat}$ | Mg$^{2+}$ | 0.11 ± 0.01 s$^{-1}$ | 7.42 ± 0.09 |
| $k_{cat}/K_m$ | Mg$^{2+}$ | 20.3 ± 3.2 m$^{-1}$s$^{-1}$ | 7.23 ± 0.12 |

| Substrate | $K_m$ | $K_{cat}$ | $K_{cat}/K_m$ |
|-----------|-------|-----------|---------------|
| $p$NPP    | 4,800 | 1,500 | 3,200 ± 1,200 |
| $p$NP     | 7.00 ± 0.09 | 7.00 ± 0.09 | 1.18 ± 0.03 | 859.9 ± 15.9 |
| $p$NP     | 0.005 ± 0.001 | 0.005 ± 0.001 | 75,000 ± 18,000 |

**Table III**

**Assays performed in Tris/Bis-Tris/acetate buffer at 25 °C. Rates were monitored by measuring $p$NPP formation at 410 nm. Profiles for Mn$^{2+}$ were determined between pH 5 and 8.5. Precipitation prevented obtaining data at higher pH values. Profiles for Mg$^{2+}$ were determined between pH 5 and 10. $C$ is the pH-independent value of either $k_{cat}$ or $k_{cat}/K_m$.**
reflects both binding and catalysis, whereas \( k_{\text{cat}} \) does not reflect binding affinity. The \( k_{\text{cat}}/K_m \) for the Cdc2 peptide KIGEG(pT) (pY)GVVVK was 85-fold greater than the \( k_{\text{cat}}/K_m \) value for pNPP. The peptide DDENle(pT)G(pY)VATR, corresponding to the activation lip of PP2C’s authentic in vivo substrate p38 (6), was also found to be a far better substrate than pNPP, with an 18-fold greater \( k_{\text{cat}}/K_m \) value. It is interesting to note that diphosphorylated peptides (Table II) appear to be much better substrates, than singly threonine-phosphorylated peptides. This is a significant observation since the proposed substrates MKK6, SEK1, and p38 (6) are diphosphorylated on neighboring residues. Collectively, this may suggest that a large component of PP2C’s substrate specificity lies within the ability to recognize and bind the diphosphorylated “active” form of these physiological substrates. By analogy, the dual-specificity protein-tyrosine phosphatases are believed to specifically recognize and dephosphorylate the diphosphorylated forms of the mitogen-activated protein kinases (13, 27). With the dual-specificity protein-tyrosine phosphatases, both phospho residues can be hydrolyzed, although a few dual-specificity protein-tyrosine phosphatases will only catalyze hydrolysis of phosphotyrosine in the context of the diphosphorylated species (13, 27). Since dual phosphorylation is required to maintain high kinase activity, the actions of single-specificity phosphatases are sufficient to inactivate the mitogen-activated protein kinases. The use of single-specificity phosphatases may indicate an additional level of mitogen-activated protein kinase regulation in which PP2C and a tyrosine-specific phosphatase may act in concert to fully inactivate and reset the stress signaling pathways.

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