Effects of Regulatory Subunits on the Kinetics of Protein Phosphatase 2A[†]

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ABSTRACT: Both the scaffold (A) and the regulatory (R) subunits of protein phosphatase 2A regulate enzyme activity and specificity. Heterotrimeric enzymes containing different R-subunits differ in their specific activities for substrates. Kinetic parameters for the dephosphorylation of a phosphopeptide by different oligomeric forms of PP2A were determined to begin to elucidate the molecular basis of regulatory subunit effects on phosphatase activity. Using steady state kinetics and the pH dependence of kinetic parameters, we have explored the effect of the A- and R-subunits on the kinetic and chemical mechanism of PP2A. The regulatory subunits affected a broad range of kinetic parameters. The C-subunit and AC dimer were qualitatively similar with respect to the product inhibition patterns and the pH dependence of kinetic parameters. However, a 22-fold decrease in rate and a 4.7-fold decrease in K_m can be attributed to the presence of the A-subunit. The presence of the R2α (Bα or PR55α) subunit caused an additional decrease in K_m and changed the kinetic mechanism of peptide dephosphorylation. The R2α-subunit also caused significant changes in the pH dependence of kinetic parameters as compared to the free C subunit or AC heterodimer. The data support an important role for the regulatory subunits in determining both the affinity of PP2A heterotrimers for peptide substrates and the mechanism by which they are dephosphorylated.

Protein phosphatase 2A (PP2A)[1] is an ubiquitously expressed protein serine/threonine phosphatase implicated in many cellular signaling pathways. The catalytic subunit of PP2A contains an amino acid sequence motif shared by enzymes that hydrolyze phosphoester bonds. This phosphoesterase motif encodes a beta sandwich (*β*−α−*β*−α−*β*) protein structure that binds two metal ions in the active site (*1*). The catalytic subunit of PP2A shares sequence similarity with the catalytic subunits of other members of the protein serine/threonine phosphatase family, including calcineurin and protein phosphatase 1 (PP1).

Native PP2A exists primarily as a heterotrimer composed of a 36-kDa catalytic subunit (C), a 64-kD scaffolding subunit (A), and one of a variety of regulatory subunits (B or R). The regulatory subunits are the most variable component of the holoenzyme and regulate substrate preference (*2*) and subcellular localization of the complex (*3*). Three gene families[2] termed R2 (B or PR55), R5 (B′ or B56), and R3 (B" or PR72) (*4*) encode multiple isoforms and splice variants of PP2A regulatory subunits. The R subunits within each gene family share significant amino acid sequence similarity, but no sequence similarity exists between the three gene families. The catalytic and regulatory subunits both interact directly with the A-subunit (*5*). Cross-linking studies indicate that additional contacts are made between the regulatory and catalytic subunits in the heterotrimeric complex (*6*). The C-subunit binds to the A-subunit with a K_d in the picomolar range (*6*, *7*), while the R-subunits bind to the AC dimeric complex. In vitro, the regulatory subunits do not form stable complexes with either the C- or A-subunits alone (unpublished observation).

Native PP2A can be isolated as both heterotrimers and AC dimers (*8*). The AC dimer may result from the dissociation of R-subunits from the AC core during purification (*9*). The monomeric C-subunit can be purified as a highly active enzyme from the AC core dimer by ethanol precipitation (*10*). The PP2A C-subunit is not present as a free protein in vivo, but it may form complexes with proteins other than the A- and R-subunits, such as the B-cell receptor associated α4 protein (*11*).

The regulatory subunits of PP2A affect enzyme activity. Binding of the A-subunit to the C-subunit results in a reduction in the specific activity of the enzyme due to formation of the AC dimer (*6*, *7*). PP2A heterotrimers containing different R-subunits have different specific activities toward exogenous substrates (*2*). The R-subunits also play a critical role in targeting PP2A to other macromolecules, including important substrates, through formation of stable complexes (*12*−*16*). Neither the molecular basis of substrate targeting by R-subunits nor the mechanisms by which they regulate PP2A activity have been investigated.

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[1] Abbreviations: PIPES, 1,4-piperazine-diethane sulfonic acid; TAPS, *N*-tris[hydroxymethyl]methyl-4-aminobutanesulfonic acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid.

[2] The nomenclature for the PP2A regulatory subunits used in this paper corresponds to the names assigned to the human genes. In this nomenclature, the R2 subunit (gene name PPP2R2) family corresponds to the B/PR55 family; the R3 subunit (gene name PPP2R3) corresponds to the PR72 family; and the R5 subunit (gene name PPP2R5) corresponds to the B′/B56 family. Greek letters denote individual genes within each regulatory subunit family.

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We wanted to determine the mechanistic basis for the effects of regulatory subunits on PP2A activity. Using steady-state kinetics and the pH dependence of kinetic parameters, we compared different complexes of PP2A to explore the effect of the A- and R-subunits on the kinetic and chemical mechanism of PP2A. The monomeric C-subunit, the dimeric AC complex, and a heterotrimer containing the R2-subunit were characterized with respect to product inhibition patterns and pH profiles using a phosphorylated synthetic peptide as substrate. The data show that the A-subunit causes a substantial increase in the affinity of PP2A for the peptide substrate and a decrease in V_{max}. The R2α regulatory subunit causes a further increase in affinity and changes the kinetic mechanism of peptide dephosphorylation. These observations support a role for both the A-subunit and the regulatory subunits in the interaction of PP2A with its substrates.

MATERIALS AND METHODS

Protein Purification. Heterotrimeric and dimeric PP2A were purified from bovine brain as described previously (*9*). Free C-subunit was isolated from higher order complexes by ethanol precipitation (*10*). Recombinant AC-R2α was expressed in SF9 cells and purified as previously described (*2*). All enzyme preparations were at least 95% pure as determined by SDS−PAGE gels stained with Coomassie blue.

Phosphorylation of Myosin Light Chain Peptide. A synthetic peptide based on amino acids 6−23 (KAKTTKKRPQRATSNVFS) that surround the single phosphorylation site (underlined) of chicken smooth muscle myosin light chain (*17*) was phosphorylated in vitro to a specific activity of 250 cpm/pmol by rabbit skeletal muscle myosin light chain kinase as described previously (*9*). The ^{32}P-phosphopeptide was isolated from free ATP by ion exchange though AG1 × 8 resin (BioRad) and concentrated in a vacuum concentrator as described previously (*18*). The ^{32}P-phosphopeptide was resuspended in a total volume of 0.2−0.3 mL, and column debris was removed by centrifugation at 14 000 rpm for 15 min. The peptide was then desalted through Biogel P2 resin (BioRad) and concentrated in a vacuum concentrator. The peptide was resuspended in water, the pH was adjusted to 7.0, and the substrate was quantitated as described previously (*18*). The phosphopeptide contained at least 0.92 mole of phosphate/mole of peptide, and the ^{32}P incorporation was 92% or higher.

Phosphatase Assays. All assays were performed at 30 °C, for variable amounts of time, in a volume of 50 μL. Assays using ^{32}P-labeled substrate were performed using a modification of a previous procedure (*18*). The assays were started by the addition of enzyme. The reaction was stopped by addition of 450 μL of ice-cold 75 mM phosphoric acid, and this mixture was added to 1 mL of AG50 × 8 (BioRad) resin carefully aliquoted into 1.5-mL microcentrifuge tubes. The tubes were mixed vigorously by shaking for 10 s then spun briefly in a microcentrifuge to pellet the resin. Two hundred fifty microliters of the supernatant was removed, placed into 7-mL scintillation vials with scintillant, and counted. The rate of the reaction was calculated from the measured release of $^{32}PO_4$ minus the cpm from a background control assay containing the same concentration of substrate but no enzyme.

Assays for the pH dependence of kinetic parameters for the C-subunit were performed with the same peptide as above, synthesized with unlabeled phosphoserine at position 19. Assays for Lineweaver−Burke plots were performed in 96-well plates, in a volume of 50 μL, and stopped with 100 μL of Biomol Green Reagent (Biomol). The rate of hydrolysis was determined from the absorbance at 630 nm of timed assays and compared to a standard curve generated with inorganic phosphate as described by the manufacturer.

pH Studies. All forms of PP2A appeared to be stable at the pH extremes, with no loss of activity observed when the enzymes were incubated on ice in buffer at pH 5.6 or pH 8.5 for up to 30 min, and then assayed for activity at pH 7.0. The pH did not change during the course of enzyme assays. The pH of each assay mixture was determined before assays were started. Assays were performed in PIPES buffer between pH 5.6 to pH 6.5, in MOPS buffer between pH 6.5 to pH 7.5, and TAPS buffer between pH 7.5 to pH 9.0. No differences in rate or K_m were observed between overlapping points at the same pH in the different buffers.

The pH dependence of pK_{is} profiles were performed with a peptide whose sequence was identical to the substrate peptide except for a single amino acid change. Serine 19 was changed to alanine giving rise to S19A peptide. The substrate concentration was held at K_m in the presence of differing concentrations of the peptide inhibitor, and the pH was varied. The data were plotted as inverse rate vs concentration of inhibitor, and the K_{is} was determined as the negative ordinate intercept divided by two.

Data Analysis. Data were fitted using the appropriate rate equations and computer programs developed by Cleland (*19*). Substrate saturation curves obtained from the pH dependence for kinetic parameters were fitted using eq 1. Data for competitive and noncompetitive inhibition were fitted using eqs 2 and 3, respectively. Data from pH curves where the slope was +1 and/or −1 were fitted using eq 4.

$$v = VA/(K_a + A) \quad (1)$$

$$v = VA/(K_a[1 + I/K_{is}] + A) \quad (2)$$

$$v = VA/(K_a[1 + I/K_{is}] + A[1 + I/K_{ii}]) \quad (3)$$

$$\log y = \log [C/(1 + H/K_1 + K_2/H)] \quad (4)$$

RESULTS

Product Inhibition with Hydroxy-Peptide. Product inhibition studies were used to obtain information about the kinetic mechanism of different PP2A enzymes, as well as the affinity of each of the enzymes for different parts of the phosphopeptide substrate. The results of product inhibition with myosin light chain hydroxy-peptide are summarized in Table 1. All forms of PP2A displayed competitive patterns of inhibition with hydroxy-peptide versus the phospho-peptide substrate. The K_{is} for hydroxy-peptide with the AC core enzyme and bovine brain AC-R2 enzymes were near the K_m for the phosphopeptide (shown in Table 2). The data show that the AC dimer and AC-R2 holoenzyme have much greater affinity for the peptide moiety than the free C-subunit. There was a 4.7-fold decrease in K_m and a 30-fold decrease in K_{is} for hydroxypeptide with AC as compared to the

Table 1: Comparison of Hydroxy-Peptide Inhibition Patterns and Constants[a]

enzyme	inhibition pattern	K_{is}[b] (μM)
C-subunit	competitive	1074 ± 200
AC dimer	competitive	32.1 ± 8.2
AC-R2	competitive	17 ± 3.8

[a] Values are averages of at least three independent determinations. [b] K_{is} is the slope inhibition constant.

Table 2: Comparison of Kinetic Constants

enzyme	K_m (μM)	V/E_T (s^{-1})	$V/K\ E_T$ ($M^{-1}\ s^{-1}$)
C-subunit	105.7 ± 8.5	29.7 ± 1.3	2.8×10^5
AC dimer	22.3 ± 5.0	1.3 ± 0.2	6.0×10^4
AC-R2	10.8 ± 2.4	1.2 ± 0.1	1.1×10^5

C-subunit. There was a 10-fold decrease in K_m and a 50-fold decrease in K_{is} for hydroxypeptide with AC-R2 as compared to the C-subunit. Peptide inhibition curves were also obtained for recombinant AC-R2α (not shown). The recombinant enzyme had a K_m and K_{is} for hydroxy-peptide identical to that of the native AC-R2 purified from bovine brain. The hydroxy-peptide inhibition patterns at pH 6.5 and pH 8.0 for all three enzymes showed that inhibition patterns remained competitive over the entire pH range. Curves showing the fitted data for each enzyme are plotted in Figure 1.

Considering the relatively high affinity of the peptide for the AC and AC-R2 enzymes, we examined the ability of L-serine to inhibit phosphatase activity. No inhibition of activity was observed in the presence of 25 mM serine indicating that a single hydroxylated amino acid binds poorly, if at all, to the active site of any form of PP2A.

Product Inhibition with Phosphate. Phosphate was also used as a product inhibitor with myosin light chain phosphopeptide as the substrate. The results are summarized in Table 3. A competitive pattern of inhibition was observed under all pH conditions for phosphate inhibition of the C-subunit (Figure 2A). The data for the phosphate inhibition with the AC dimer was competitive at pH 7 (Figure 2B). At pH 8.0, however, the pattern clearly became noncompetitive for AC with an intercept inhibition constant of 8.24 mM and a slope inhibition constant of 2.59 mM (Figure 3) showing that the kinetic mechanism for AC is pH-dependent. The rates of the reactions were determined by subtracting out the cpm from a similar assay containing the same concentration of substrate but no enzyme. Background curves were linear, and the amount of background cpm obtained was unaffected by the concentrations of phosphate or hydroxy-peptide in the product inhibition studies. Since the background was subtracted out, errors in the background determination affected slow assays containing high concentrations of inhibitor more than fast ones, as can be seen in Figures 2 and 3 at the highest phosphate concentrations.

Phosphate inhibition patterns for bovine brain AC-R2 were noncompetitive under all pH conditions (Figure 2C). The noncompetitive pattern indicates that phosphate binds to both the free enzyme and the enzyme/hydroxy-peptide complex and that the kinetic mechanism changes in the heterotrimer relative to the AC dimer. Ordered product release occurs with the heterotrimer, with phosphate released before hydroxy-peptide most likely due to a slower dissociation of

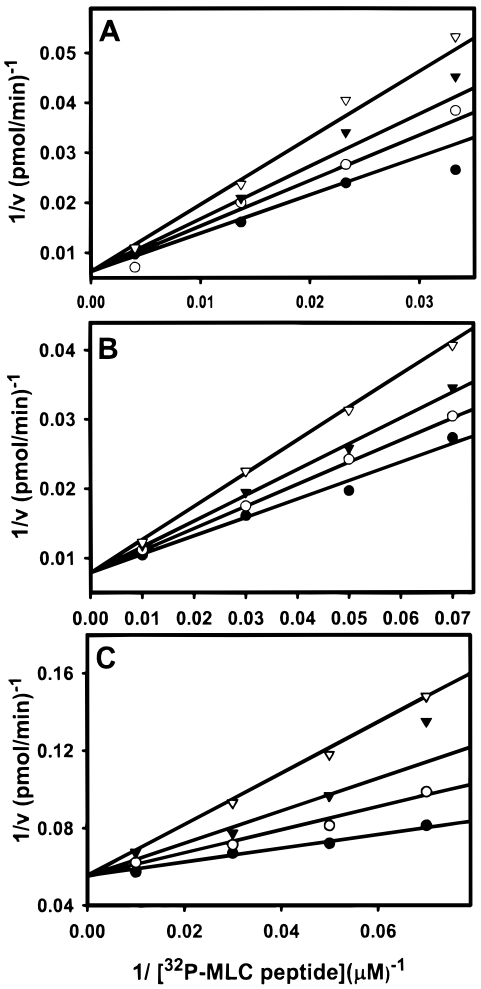


FIGURE 1: Inhibition of PP2A enzyme forms with hydroxy-peptide. In panel A, the concentration of phospho-peptide substrate was varied at 30, 42.8, 73.2, and 250 μM in the presence of 0 (filled circles), 250 (open circles), 500 (filled triangles), or 1000 (open triangles) μM hydroxy peptide, in a reaction with C-subunit. The concentration of phospho-peptide was varied at 14, 20, 33.3, and 100 μM in the presence of 0 (filled circles), 15 (open circles), 30 (filled triangles), or 60 (open triangles) μM hydroxy-peptide in a reaction with AC dimer (B) or with AC-R2 (C). Data for all three curves was fitted for competitive inhibition.

Table 3: Comparison of Phosphate Inhibition Patterns and Constants[a]

enzyme	inhibition pattern	K_{is}[b] (mM)	K_{ii}[c] (mM)
C-subunit	competitive	4.6 ± 0.6	
AC dimer	competitive	12.5 ± 2.0	
AC-R2	noncompetitive	6.9 ± 2.4	12.0 ± 5.6

[a] Values are averages of at least three independent determinations. [b] K_{is} is the slope inhibition constant. [c] K_{ii} is the intercept inhibition constant

hydroxy-peptide from the heterotrimer than from the other PP2A enzyme forms.

pH Dependence of Kinetic Parameters. Previous studies have shown that R-subunits play an important role in substrate interaction (*2*). Significant amounts of R2α, R2*β*, and R2*γ* are present in bovine brain (*20*). Hence, native heterotrimers purified from bovine brain are likely to be a mixture of isoforms containing different R2 subunits. To eliminate contributions from other isoforms, recombinant AC-R2α was used for the pH studies. The pH profiles for

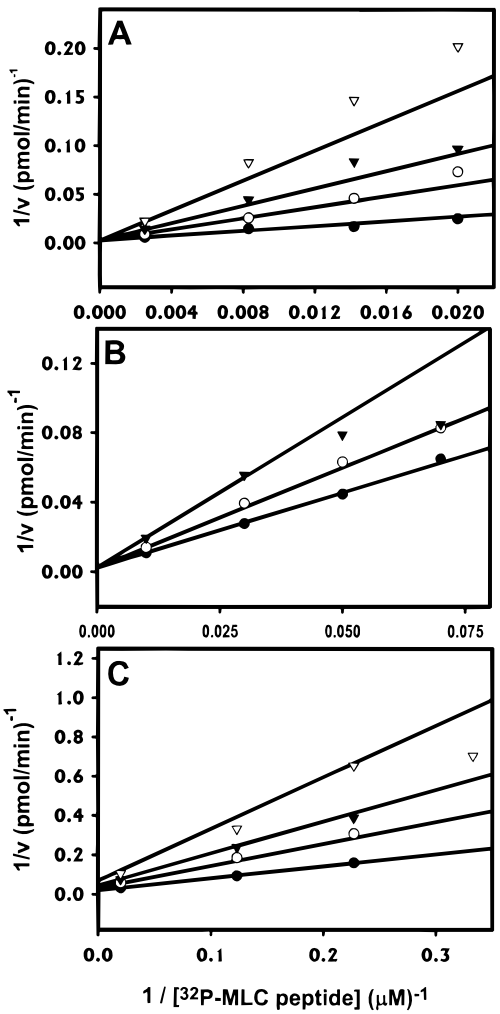


FIGURE 2: Inhibition of PP2A enzyme forms with inorganic phosphate. In panel A, the concentration of phospho-peptide substrate was varied at 50, 70.6, 120, and 400 μM in the presence of 0 (filled circles), 5 (open circles), 10 (filled triangles), or 20 (open triangles) mM inorganic phosphate, in a reaction with C-subunit. In panel B, the concentration of phospho-peptide was varied at 14, 20, 33.3, and 100 μM in the presence of 6 (filled circles), 12 (open circles), or 24 (filled triangles) mM inorganic phosphate in a reaction with the AC dimer. In panel C, the concentration of phospho-peptide substrate was varied at 3, 4.4, 8.12, and 50 μM in the presence of 0 (filled circles), 6 (open circles), 12 (filled triangles), or 24 (open triangles) mM inorganic phosphate in a reaction with AC-R2. Data for panels A and B are fitted to the equation for competitive inhibition, while data for panel C is fitted to the equation for noncompetitive inhibition. Data shown represent one of three independent determinations. Despite the apparent nonlinearity of closed triangles shown in panel B, there was no evidence that phosphate inhibition becomes nonlinear.

the three forms of PP2A are shown in Figure 4 and the p*K*'s are compared in Table 4. The values for V/E_T and V/KE_T are listed in Table 1.

All forms of PP2A had a p*K* on the acid side of the V/E_T pH profile with a limiting slope of +1. The value of the p*K* observed with the C-subunit (5.8) is perturbed to a more basic value in the AC dimer (6.37) and AC-R2α (6.27). The catalytic subunit is sufficient to catalyze the reaction in the absence of either regulatory subunit, hence the p*K* observed in the V/E_T profile is due to a residue in the C-subunit. An additional p*K* of 8.1 in the log V/E_T profile for AC-R2α with a limiting slope of −1 (Figure 4C) was absent in the profiles for the C-subunit and AC dimer. A single p*K* of 5.8

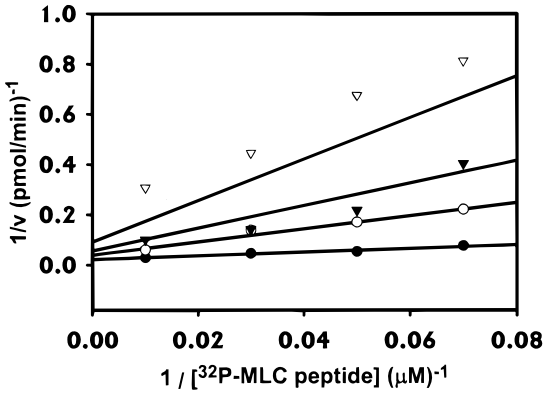


FIGURE 3: Inhibition of the AC dimer with phosphate at pH 8.0. The concentration of phospho-peptide was varied at 14, 20, 33.3, and 100 μM in the presence of 0 (filled circles), 6 (open circles), 12 (filled triangles), or 24 (open triangles) mM inorganic phosphate in a reaction with the AC dimer and fitted to the equation for noncompetitive inhibition.

with a limiting slope of +1 was observed in the V/KE_T profile for AC-R2α (Figure 4C). Because of an apparent decrease in K_m proportional to the decrease in V/E_T, no p*K*'s were observed in the V/KE_T profile for the C-subunit and AC dimer, despite the p*K* in the V/E_T profiles for both enzymes.

pH Dependence of K_{is}. A dead end inhibitor can mimic substrate binding to enzyme but is unable to go through any catalytic steps. The pH dependence of K_{is} of a dead end competitive inhibitor can be used to determine binding p*K*'s for substrates (*21*). The pH dependence of K_{is} for a peptide based on the sequence of the substrate peptide but containing a single amino acid change of serine 19 to alanine was determined. The profiles for the AC dimer and AC-R2α enzymes were compared to determine the effects of pH on peptide binding to these enzymes. The data are shown in Figure 5. No p*K*'s were observed over the pH range of 5.6−8.6 for either the AC dimer or AC-R2α enzymes. The pH independence of pK_{is} for the peptide indicates that the p*K*s observed on the V/E_T profiles are most likely attributable to an interaction between phosphate and residues on the enzyme. The pH independence of pK_{is} for S19A peptide also indicates that the p*K* of 5.8 observed on V/KE_T for AC-R2α may also be due to an interaction between the enzyme and the phosphate group.

DISCUSSION

An important aspect of signaling fidelity is the ability to target kinase and phosphatase activity through substrate recognition. A comparison of the binding constants for substrates and inhibitors for the different PP2A enzymes provides information about how the regulatory subunits affect substrate and product interactions. Hydroxy-peptide was able to compete with substrate for the active site on the C-subunit indicating that the catalytic subunit has a low affinity recognition site for the hydroxy-peptide. The presence of the A- and R-subunits in the oligomeric enzymes greatly improved binding to the hydroxy-peptide. The R-subunits are known to be important for substrate selectivity (*2*). Our data indicate that a major mechanism involved in the actions of the R-subunits is to increase affinity for the peptide moiety. The A-subunit had been thought to function primarily

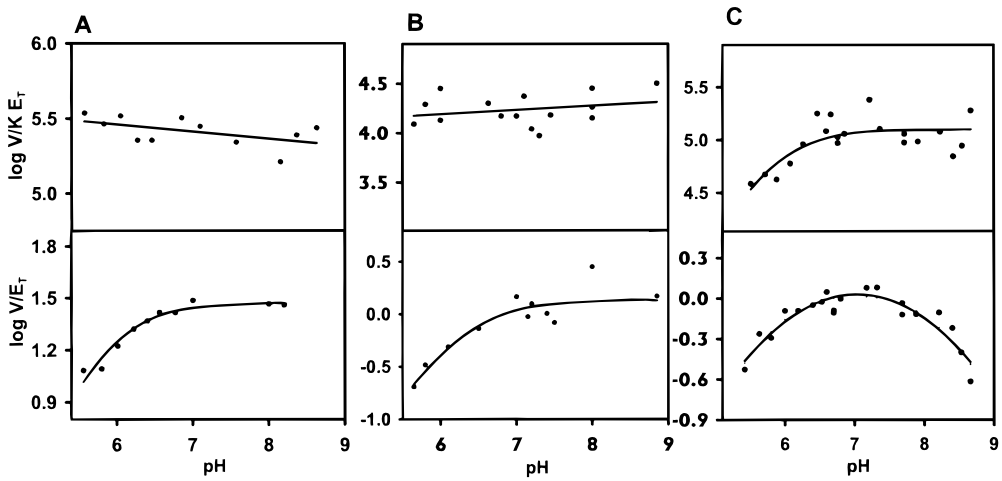


FIGURE 4: pH dependence of kinetic parameters. The pH dependence for V/KE_T (top panel) and V/E_T (lower panel) for the C-subunit (A), AC dimer (B), and AC-R2α with phospho-peptide.

Table 4: Comparison of p*K*s for the pH Dependence on V/E_T and V/KE_T

enzyme	pK_1 V/E_T	pK_2 V/E_T	p*K* $V/K\ E_T$
C subunit	5.8 ± 0.1		
AC dimer	6.4 ± 0.2		
AC-R2α	6.3 ± 0.1	8.0 ± 0.1	5.8 ± 0.2

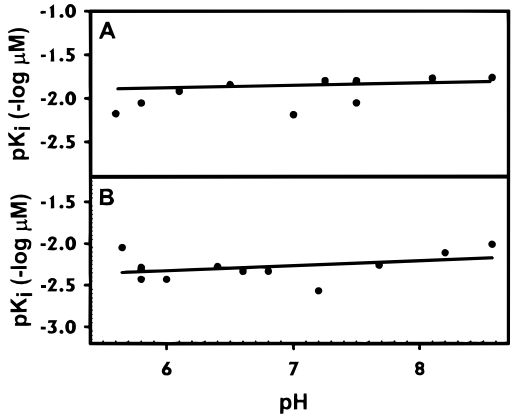


FIGURE 5: pH dependence of K_{is} for the S19A peptide inhibitor. Data for the AC dimer is shown in panel A, while data for the AC-R2α enzyme is shown in panel B. Data were fitted as a first-order linear regression.

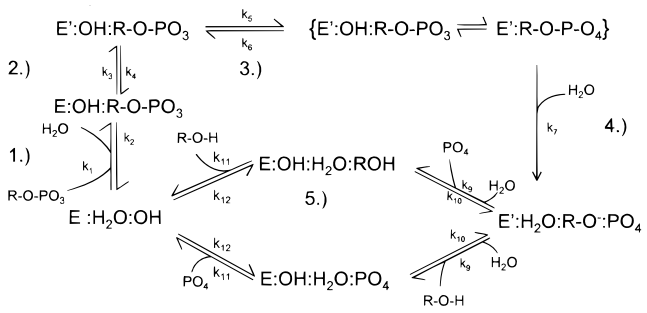
as a scaffold, bringing the R- and C-subunits together. The improved peptide binding exhibited by the AC dimer was surprising and argues that the A-subunit also plays a role in substrate interaction. This might be achieved through a conformational change in the C-subunit or a through a direct interaction between the A-subunit and the peptide substrate. The phosphate inhibition patterns for AC-R2 were noncompetitive (and independent of pH) indicating that this enzyme has slower hydroxy-peptide release due to a tighter interaction with the peptide moiety. The pK_{is} profiles for the PP2A enzyme forms showed a pH independence, despite the presence of multiple ionizable residues in the peptide. The ionization of functional groups with p*K*'s below 5 and above 9 could contribute ionic interactions between the substrate and the enzyme that would not be titrated over the pH range examined. Alternatively, binding of the peptide moiety might be dependent on nonionizable groups such as hydroxyls or amides. Considering the broad spectrum of substrates dephosphorylated by PP2A, it seems possible that substrates could be recognized by interactions between residues on PP2A and residues in the peptide backbone rather than specific side chain residues.

The catalytic mechanism of the dinuclear metal center phosphatases is not well understood. The active site metals are proposed to increase the nucleophilicity of a metal bound water molecule and to make the phosphorus atom a better electrophile by withdrawing electrons from the phosphate oxygens (*22*). Unlike the protein tyrosine phosphatases, there is no evidence that the dinuclear center phosphatases form a covalent enzyme−substrate intermediate during the hydrolysis reaction (*23*, *24*). Rather, the phosphate group is transferred directly to water with a net inversion of configuration of the oxygens around the phosphate center (*25*). A general acid then protonates the alkoxide leaving group (*22*). Ligand replacement by water has been suggested to displace inorganic phosphate from the active site (*26*). Isotope exchange studies indicate that the attacking water molecule is liganded to an active site metal and that an ^{18}O label in the bridging ester-oxygen is retained in the hydroxyl leaving group (*27*).

Crystal structures have been solved for calcineurin as a phosphate/enzyme binary complex (*28*, *29*) and for PP1 as an enzyme/tungstate binary complex (*30*). In the binary structures, two of the phosphate oxygens that are not bound to metals are hydrogen bonded to a conserved histidine and a conserved arginine (*28*, *30*), respectively. It is unclear which of the phosphate oxygens represents the position of the phosphate ester bond. Either the conserved histidine (*22*) or the conserved arginine (*26*) could act as the general acid. Water could also serve in this capacity (*31*).

The acid side of the V/E_T and V/KE_T pH profiles for the C-subunit and AC dimer of PP2A are qualitatively similar to those determined for calcineurin (*32*) and the λ phosphatase (*31*). Common to all the profiles was a p*K* with a limiting slope of +1 on the V/E_T profile indicating the presence of a proton acceptor important for catalysis. The acidic p*K* has been attributed to protonation of a metal-bound hydroxyl group (*24*). However, the p*K* might also be due to protonation of a general base important for accepting a proton from a metal-bound water molecule or for positioning a water molecule to displace phosphate from the metal center.

Scheme 1



Several points along enzyme-catalyzed reactions can be rate-limiting. The binding and release of substrates and products, catalytic steps, and enzyme isomerizations are all potential rate-limiting steps. Although the slowest PP2A enzyme was AC-R2, most of the rate decrease can be attributed to the A-subunit. Primary and secondary isotope effects indicate that the catalytic steps are partially rate-limiting for the related phosphatases calcineurin (*33*) and λ phage phosphatase (*31*), and this is likely to be true in general for this class of enzymes. The ionization state of the phosphate group may also be important for substrate binding and catalysis (*31*, *33*).

Phosphorylation is not energetically feasible without coupling it to a thermodynamically favorable reaction. For example, the phosphorylation catalyzed by protein kinases is driven forward by coupling the reaction to ATP hydrolysis. Therefore, it is reasonable to assume that the hydrolysis reaction catalyzed by the dinuclear center phosphatases is irreversible, since there is no thermodynamically coupled reaction to drive the reverse phosphorylation reaction. If the reaction is irreversible, then V/KE_T reflects only the steps up to hydrolysis, and the p*K* observed on the V/E_T profile could reflect a product release step that is dependent on a general base. A general base-dependent product release step has been shown to be rate-limiting for kynureninase where the rate-limiting step occurs after an irreversible step (*34*). Ligand replacement by water to conserve the coordinate geometry about the metals has been proposed previously for PAP (*26*) and could involve a general base. One water molecule would be needed to replace the nucleophile, while a second water molecule would be needed to displace the phosphate oxygens from the metal ligand sphere. In our study, the pK_{is} profiles for the competitive dead end S19A peptide inhibitor was pH-independent for the AC dimer and AC-R2α enzymes. The compensatory effect observed on the acid side of the V/KE_T pH profiles for the C-subunit and AC dimer and on the base side of the profiles for AC-R2α are probably due to an interaction between residues in the active site and the phosphate group of the substrate.

We propose a mechanism, illustrated in Scheme 1, utilizing the ideas presented above. An enzyme isomerization step (step 2) is included in consideration of the rate decrease observed between the C-subunit and AC dimer. An enzyme isomerization step has been proposed for calcineurin (*33*) and could be partially rate-limiting for this enzyme. Since hydrolysis is considered irreversible, k_8 has been omitted from the scheme as this rate is essentially zero (step 4). Isotope effects with calcineurin (*33*) and lambda phosphatase (*31*) indicate that protonation of the alkoxy leaving group lags behind the nucleophilic attack by water on the phosphate center. We suggest that the alkoxide may transiently associate with the metal center before protonation (step 4). In step 4 and 5 in Scheme 1, a water molecule replaces the nucleophilic water and phosphate, respectively. Displacement of the phosphate by water may be dependent on a general base important in positioning a water molecule to displace the phosphate oxygens from the ligand sphere of the metals or to replace the nucleophile. Water might act directly as the general acid to donate a proton to the alkoxide leaving group. However, donation of a proton from a general acid to the alkoxide ion is more consistent with our data. Although no p*K* was observed on the base side of the V/E_T profile for the C-subunit or AC dimer, one does appear in the AC-R2α profile. More information is required to accurately interpret this result. One possible explanation is that the appearance of the general acid p*K* could be due to a perturbation of the general acid p*K* by the R2 subunit such that the p*K* is now observed within the pH range of the collected data. Protonation of the alkoxide would occur prior to product release, shown as step 5. The upper pathway, with phosphate released first predominates in the heterotrimer, but both the upper and lower product release pathways may occur for the C-subunit and AC dimer.

PP2A is not one enzyme but a family of oligomers built on a common AC core. The variable R-subunits act as exchangeable accessories, each one conferring unique properties on the enzyme. While the studies with the C-subunit and AC dimer provide information about mechanism and how the scaffold and regulatory subunits change PP2A activity, the heterotrimer is considered the physiologically relevant form of PP2A. A major mechanism for regulating PP2A activity involves substrate targeting by the regulatory subunits. R2α alters the kinetic mechanism of PP2A possibly due to an enhancement of the interaction with the peptide substrate. The decrease in the K_m and K_{is} with little decrease in overall rate as compared to the AC dimer reinforces the notion that the R-subunits act in an isoform-specific manner to enhance dephosphorylation of specific phosphoprotein targets. PP2A may also be regulated by colocalization with other macromolecules. The R-subunits are known to be important for the formation of many of the complexes formed between PP2A and other proteins. The R2 family members are important to co-localize PP2A with tau and CaMKIV (*12*, *16*), R3 family members with p107 and Cdc6 (*13*, *14*), and R5 family members with the adenomatous polyposis coli (APC) protein (*15*). The protein bound to PP2A in the complex may itself be a target of PP2A activity. Alternatively, the bound protein may direct PP2A to substrates on much larger signaling complexes. The heterotrimer may be optimized for activity in a macromolecular protein environment. Future studies to characterize enzyme activity co-localized with other macromolecules will examine this aspect.

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