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Form and Function in Protein Dephosphorylation

Minireview

John M. Denu*‡, Jeanne A. Stuckey*, Mark A. Saper*† and Jack E. Dixon*
*Department of Biological Chemistry
†Biophysics Research Division
University of Michigan
Ann Arbor, Michigan 48109–0606
‡Present address:
Oregon Health Sciences University Medical School
Department of Biochemistry and Molecular Biology L224
Portland, Oregon 92701–3098

The phosphate ester is an extremely important chemical bond within the living cell, serving as an energy source, as a means of joining RNA/DNA molecules, and as an efficient mechanism to regulate the activity of enzymes and proteins by modifying amino acid side-chains. Protein phosphorylation is a highly regulated process by which information can be shuttled from the cell surface to the nucleus. There are two classes of enzymes that regulate signalling through the phosphorylation and dephosphorylation of proteins, namely protein kinases and protein phosphatases. This review will focus on the structure and function of the protein phosphatases. Protein phosphatases are generally divided into two main groups based on substrate specificity. Protein Phosphatases (PPs) specifically hydrolyze serine/threonine phosphoesters and Protein Tyrosine Phosphatases (PTPs) are phosphotyrosine-specific. A sub-family of PTPs, dual specificity phosphatases or dual specificity PTPs, are capable of efficient hydrolysis of both phosphotyrosine and phosphoserine/threonine. Recently solved X-ray structures of both PTPs and PPs have provided a wealth of new knowledge on the structure and catalytic mechanism of both enzyme families. Although both PPs and PTPs catalyze phosphoester hydrolysis, they utilize completely different structures and distinct catalytic mechanisms. These differences among the phosphatases are in stark contrast to the serine/threonine and tyrosine protein kinases which are all predicted to have a common structure (reviewed by Johnson et al., 1996).

Structure of Serine/Threonine Protein Phosphatases

The PPs comprise a large family of metallo-protein phosphatases whose functions within the cell are extremely diverse and highly regulated (reviewed by Shenolikar, 1994). Amino acid sequences located outside of the catalytic domain serve as binding sites for proteins that regulate activity. Amino acid sequence comparisons within the catalytic domains revealed that two main gene families exist. Calcinuerin (PP2B), PP2A and PP1 are members of the same gene family, whereas PP2C shares no sequence homology to either PP2B, PP2A or PP1 and thus represents a distinct gene family. Calcinuerin is critical for T-cell activation and is the target for many immunosuppressants. PP1 is involved in regulating glycogen metabolism in response to insulin and adrenaline, smooth muscle contraction and protein synthesis, while PP2C is involved in the osmoregulation of fission yeast (Shiozaki and Russell, 1995). PP2A has been implicated in the regulation of cell cycle progression, DNA replication, transcription, and translation. The X-ray structures of calcinuerin (Griffith et al., 1995; Kissinger et al., 1995) and PP1 (Goldberg et al., 1995; Egloff et al., 1995) were recently solved. These structures revealed a common motif of a central β-α-β-α-β scaffold containing a dinuclear metal ion center (Figure 1) located at the active site. The metals are coordinated by three histidines (green), two aspartic acids (yellow) and an asparagine (magenta) which are mainly found on loops between secondary structural elements of the β-α-β-α-β motif. These loops also contain important catalytic residues (proposed residues are highlighted in color, Figure 1). The metals appear to be important for both catalytic activity and for maintaining the structural integrity of the protein. Since it was observed that several different metals (Fe, Zn, Mn) can be accommodated at these sites (Griffith et al., 1995; Egloff et al., 1995; Kissinger et al., 1995), the possibility exists that both the identity and the valence state of the metal may regulate enzymatic activity. Sequence homology to other proteins and X-ray structural studies of purple acid phosphatase (Strater et al., 1995) have uncovered that the PPs belong to an even larger structural family of metallo-phosphoesterases (Goldberg et al. 1995). These catalysts range in diversity from bacterial exonucleases to RNA debranching enzymes. Clearly nature has devised an efficient phosphoesterase and used this strategy in a variety of physiological processes.

Structure of Protein Tyrosine Phosphatases

PTPs play important roles in the regulation of signal transduction pathways (reviewed by Tonks and Neel, 1996). The physiological effects on signaling can be positive as with SHP-2 in receptor activation of Ras, or negative as in case of SHP-1 in B lymphocyte signaling. The dual specificity phosphatase $p80^{CDC25}$ is necessary for activating the cell cycle kinase $p34^{CDC2}$ by dephosphorylating adjacent tyrosine and threonine residues. Mitogen-activated protein kinase phosphatase-1 (MKP1) is implicated as a negative regulator of mitogen-activated protein kinase by direct dephosphorylation of threonine-183 and tyrosine-185.

The PTPs are characterized by the active-site sequence motif HCxxGxxRS(T) within the catalytic domain of approximately 200–300 residues. Unlike the PPs, the PTPs do not require metal ions for catalysis. Outside of the catalytic domain, the amino acid sequences vary greatly. Additional domains may include SH2 domains (as in SHP-1,-2) for intracellular PTPs and extracellular fibronectin-type or immunoglobulin repeats for receptor-like PTPs. Interestingly, most of the receptor-like PTPs contain a second intracellular PTP domain that displays little or no catalytic activity, suggesting that this domain has a role other than catalysis. Within the catalytic domain, the PTPs share >30% sequence identity. In contrast, if one compares the sequence identity

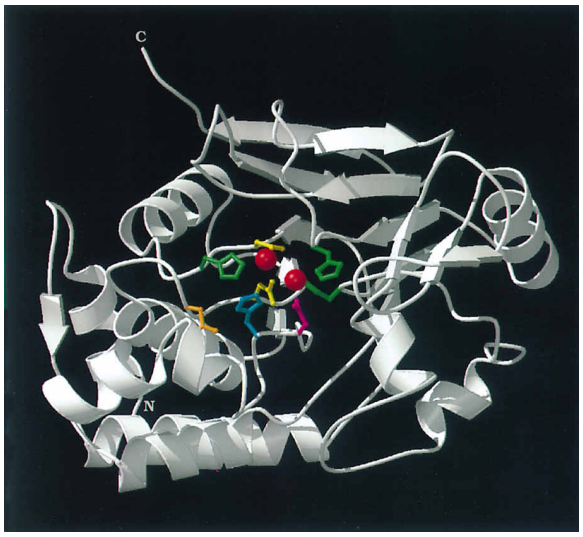


Figure 1. Active Site and Metal Coordination in PP1

The backbone of PP1 is represented as a gray ribbon diagram where arrows depict β-strands and coils represent α-helices. Invariant residues found in PPs are shown as ball-and-stick drawings. The two metal ions are shown as red spheres. Amino acids involved in metal coordination are histidines (green), aspartic acids (yellow), and asparagine (magenta). A catalytically important histidine (blue) and aspartic acid (orange) are also shown. The histidine may serve as general acid by donating a proton to the leaving group. Coordinates were kindly provided by Drs. Jonathan Goldberg and John Kuriyan. The picture was created using Molscript and Raster3D.

between the PTPs and the dual specificity phosphatases, there is less than 5% sequence identity. This low sequence identity is only slightly better than that predicted by random chance for two completely unrelated proteins. Despite this limited identity, the recently solved X-ray structure (Yuvaniyama, et al., 1996) of the human dual specificity phosphatase VHR (Vaccinia H1-related) reveals that the overall structural fold (Figure 2A) is amazingly similar to the structures of PTP1B (Barford et al., 1994; Figure 2B) and the Yersinia PTP (Stuckey et al., 1994). Direct comparison of VHR with PTP1B and with the Yersinia PTP provides insight into what structural elements constitute the minimum PTP catalytic domain as well as suggesting what regions define substrate specificity. The PTPs consist of a single α+β type domain where the four parallel β-strands forming the core are sandwiched by α-helices. Amino acids within the PTP signature motif HCxxGxxRS(T) (shown in red, Figure 2A,B) are found between the β-strand that ends with HC and the α-helix which starts with RS of the signature motif. These amino acids form the phosphate binding pocket for substrate. At the base of the active-site cleft sits the cysteine catalytic nucleophile (Shown in green, Figure 2A,B). The arginine (shown in blue, Figure 2A,B) guanidinium group points in toward the active site to assist in phosphate binding and catalysis. Located roughly 30–40 residues amino-terminal of the cysteine nucleophile is a catalytically important aspartic acid (shown in gold, Figure 2A,B) located on a flexible loop (shown in yellow, Figure 2A,B) which undergoes a dramatic conformational change when substrate is bound to enzyme.

The major structural differences in the catalytic domain of the PTPs and the dual-specificity PTPs occur at insertion loops between secondary structural elements and may play an important role in substrate specificity and subcellular targeting. Jia et al. (1995) have shown that amino acids $YRDV_{(46–49)}$ located between the first α-helix and the first β-strand (loop region shown in magenta, Figure 2B) in PTP1B make specific contacts to the peptide backbone and the tyrosine ring of a phosphorylated peptide. This region also contributes to the overall depth of the active-site pocket. In VHR there is no sequence homology within this region; instead, the loop connecting α1 and β1 is 75% shorter than PTP1B (shown in magenta, Figure 2A). As a result, the lack of an extensive loop structure decreases the overall depth of the pocket. It has therefore been suggested that a determinant of phospho-tyrosine specificity or dual specificity of PTPs lies within the depth of the pocket. The shallower pocket (6Å) of VHR can accommodate both phosphotyrosine and phosphoserine/threonine, whereas with PTP1B and the Yersinia PTP, only the longer phosphotyrosine moiety can reach the cysteine nucleophile located at the base of the active-site cleft (9Å deep) (Yuvaniyama, et al., 1996). It is attractive to suggest that the region between a1 and b1 may play a major role in substrate specificity and recognition for all PTPs; however, this remains to be tested.

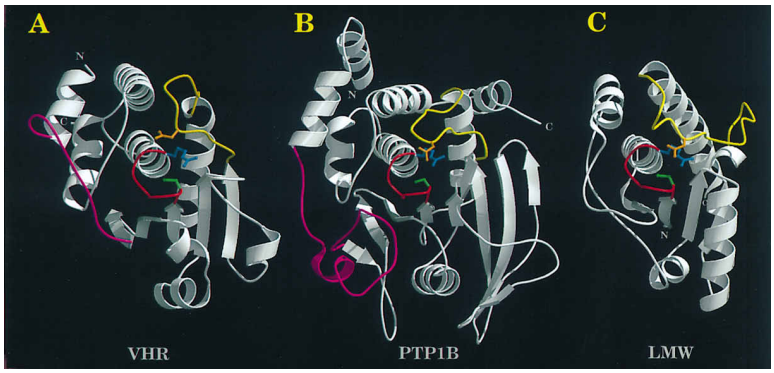


Figure 2. Catalytic Domain of Protein Tyrosine Phosphatases and a Low Molecular Weight Phosphatase

Backbone representations of (A) VHR, (B) PTP1B, and (C) LMW (Low Molecular Weight Phosphatase) in the same orientation are shown as gray ribbons, where arrows and coils represent β-strands and α-helices, respectively. The VHR and LMW structures were aligned to PTP1B by overlapping the β-strand and α-helix that flanks the active site motif. The catalytic cysteine (green) and arginine (blue) flank the active site motif (red). The catalytic aspartic acid (gold) lies on a flexible loop shown in yellow. Substrate recognition involves residues present on the variable loop (magenta). Coordinates for PTP1B were kindly provided by Dr. David Barford. The LMW coordinates were obtained from the Brookhaven Protein Data Bank, 1PHR. Models were drawn with Molscript and Raster3D.

Recently the X-ray stucture of the membrane-proximal catalytic domain (D1) of a receptor-like PTP (murine RPTPα) was recently described by Bilwes et al. (1996). Structural differences from the PTP1B fold were observed, with the most significant deviatation seen at the amino-terminal helix-turn-helix region of RPTPα. The turn of this segment was found inserted into the active site of a dyad-related D1 monomer. The authors propose that this dimerization is a physiological mechanism for downregulating the activity of receptor-like PTPs. These provocative results provide the basis for further biochemical studies into the importance of dimerization for all receptor-like PTPs.

The catalytic strategy used by the PTPs appears to have been employed by an additional class of phosphatases, referred to as the low molecular weight phosphatases. The physiological function of this ubiquitous enzyme remains unclear. These enzymes share the cysteine and arginine catalytic residues from the PTP signature motif HCxxGxxRS(T), but share no other sequence homology (Zhang et al., 1994). Although the X-ray structure (Su et al., 1994; Zhang et al., 1994) of the bovine low molecular weight phosphatase revealed that this stretch of amino acids also forms a similar active-site architecture (Figure 2C), the topology of the structure is completely different between PTPs and low molecular weight phosphatases. This has lead to the suggestion that the low molecular weight phosphatases and PTPs represent examples of convergent evolution. Similar to PTPs, an aspartic acid (shown in gold, Figure 2) functions as a general acid in the cleavage of the phosphate ester bond. The use of a cysteine, arginine and aspartic acid residue suggests a similar mechanism is employed by the two families of phosphatases.

Molecular Reaction Mechanism

Serine/Threonine Protein Phosphatases

Catalysis by the PPs (members of the PP1 and PP2A/B family) has been proposed to proceed by direct attack of an activated water molecule at the phosphorus center of the substrate, without phosphoryl transfer to the enzyme (Egloff et al., 1995). However, only limited data is available concerning the catalytic mechanism of PPs. The structurally related purple acid phosphatase has been shown to proceed through inversion of conformation of the phosphate moiety, strongly suggesting that no phosphoenzyme intermediate is formed during catalysis (Mueller, et al., 1993). Nucleophilic attack of water at the phosphorus atom of substrate is thought to occur via an activated water molecule that is bridged between the two metal ions. Expulsion of the leaving group may be enhanced by protonation from a nearby histidine (shown in blue, Figure 1) acting as a general acid. An aspartic acid (shown in orange, Figure 1) interacts with this histidine and may assist in orientation or may help polarize the histidine for optimal proton transfer. Although several amino acids have been implicated in either metal binding or catalysis (Figure 1), further studies are needed to substantiate the proposed reaction mechanism and probe the function of specific amino acids.

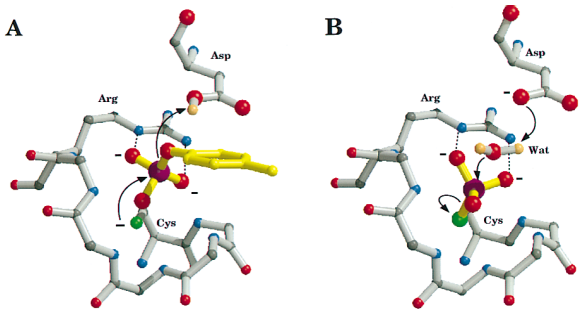


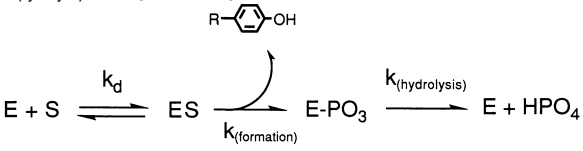
Figure 3. Catalytic Mechanism of Protein Tyrosine Phosphatases: Enzyme–Substrate Complex and Phosphoenzyme Intermediate

(A) A model of the enzyme-substrate complex of PTP derived from two X-ray crystallographic models: Cys-ser mutant of PTP1B complexed with phosphotyrosine (Jia et al., 1995), and Yersinia PTP complexed with vanadate (Denu et al, 1996). The backbone atoms of the active site loop from cysteine to arginine are shown as a ball-and-stick model. The phospho-tyrosine substrate (shown in yellow) is bound to the center of the active-site motif with the protonated general acid (Asp) lying within hydrogen bonding distance of the scissile oxygen of the substrate. The dianion of the phosphoryl group is coordinated by the nitrogens of the arginine side chain and by the amide groups of the active site motif. The thiolate anion of cysteine is poised for nucleophilic attack. Individuals atoms are represented as spheres with the following colors: Cα and carbonyl carbons, gray; oxygens, red; nitrogens, blue; phosphorus, magenta; sulfur, green; and hydrogens, orange (not found in original crystal structures). Dashed lines represent hydrogen bonds.

(B) A model of the phosphoenzyme intermediate of PTP derived in a similar manner as (A) with the same atom colors as (A). The phosphorus is covalently bound to the Sγ of the cysteine. A water molecule (Wat) is in a position such that aspartic acid can abstract the proton. Figures were drawn using Molscript and Raster3D.

Protein Tyrosine Phosphatases

In contrast to the catalytic mechanism employed by PPs, the PTPs proceed through a phosphoenzyme intermediate. The enzymatic reaction involves phosphoryl-cysteine intermediate formation after nucleophilic attack of the phosphorus atom of the substrate by the thiolate anion of cysteine (Denu et al., 1996). The reaction can be represented as a two-step chemical process: phosphoryl transfer to the enzyme accompanied by rapid release of dephosphorylated product (denoted by rate constant $k_{(formation)}$ in Equation 1); and hydrolysis of the thiol-phosphate intermediate concomitant with rapid release of phosphate (denoted by rate constant $k_{(hydrolysis)}$ in Equation 1).



(1)

To form the catalytically competent complex ES, the enzyme binds and reacts with the dianion of phosphate–containing substrate (Figure 3A). On the enzyme an aspartic acid must be protonated and the nucleophilic cysteine must be unprotonated (thiolate anion) for phosphoryl transfer to the enzyme (Denu et al., 1996; Zhang, 1995). In the Michaelis complex (Jia et al., 1995), the three nonbridging oxygens of the phosphoryl group are

coordinated by bidentate hydrogen bonds to the guanidinium group of arginine and by the backbone amide N-H groups of the active-site loop (Figure 3A) . Situated directly underneath the phosphoryl group and at the base of the active-site cleft, is the nucleophilic cysteine thiolate anion (shown in green, Figure 3A). The substrate (shown in yellow, Figure 3A) is positioned such that attack of the thiolate is directly in line with the P-O bond and ideally situated for efficient expulsion of the leaving group. To further enhance leaving group expulsion, near the top of the cleft the aspartic acid is positioned to act as a general acid by protonating the leaving group phenolic oxygen. The aspartic acid is found on a separate loop (shown in yellow, Figure 2) that was shown to be flexible for both Yersinia PTP (Stuckey et al., 1994) and PTP1B (Jia et al., 1995). When the enzyme is complexed with phosphorylated substrates, this loop folds over the active site, bringing the aspartic acid into position for leaving group protonation. In the open conformation, the loop is flipped away from the active site and the aspartic acid is approximately 8–12 Å removed from its location in the Michaelis complex (Stuckey et al., 1994). It is not yet known whether the analogous loop in VHR is capable of such dramatic movement. In the sulfate-bound complex of VHR, the loop does not cover the active site to the same extent as in the other two PTP crystal structures (Yuvaniyama et al., 1996).

To explore the transition-state structure for catalysis, heavy atom kinetic isotope effects have been measured with PTP1, Yersinia PTP and VHR (Hengge et al., 1996). The kinetic isotope effects indicate that P-O bond cleavage is far advanced and there is little bond formation to the nucleophile cysteine during the transition state. It was also demonstrated that proton transfer from aspartic acid to the bridging oxygen is concomitant with P-O cleavage, such that no charge is developed on the leaving group. Consistent with concomitant proton transfer, leaving group pK_a values have little effect on the rate of phosphoryl transfer (Zhang, 1995; Denu et al., 1996). When aspartic acid is replaced by asparagine, phosphopeptides and phosphotyrosine are 100-fold worse than substrates with good leaving groups such as p-nitrophenylphosphate, a commonly employed artificial substrate. Thus, with physiological substrates, proton donation by aspartic acid is more critical for efficient catalysis than with labile artificial substrates.

Once the phosphoenzyme intermediate is formed, it must undergo hydrolysis by water to result in the turnover of the enzyme (Figure 3B). Activation of the water molecule by a general base would be expected to facilitate hydrolysis. Mutagenic studies with VHR have shown that the conserved aspartic acid enhances intermediate hydrolysis (Denu et al., 1996), suggesting that the aspartic acid may function as the general base by proton abstraction from a water molecule. Further support for this idea comes from the X-ray structure of Yersinia PTP solved with the inhibitor vanadate covalently bound to cysteine (Denu et al., 1996). With trigonal bipyramidal geometry, the vanadate mimics the transition for both chemical steps and underscores the importance of conserved arginine and aspartic acid residues. Similar to the ES complex, two of the equatorial oxygens of vanadate are hydrogen bonded to arginine in a bidendate fashion. The aspartic acid makes a hydrogen bond of 2.8 Å to the apical oxygen of vanadate, consistent with the role as general acid/base.

The recently solved structures of protein phosphatases have greatly advanced our knowledge of the molecular mechanism of catalysis and substrate specificity. Understanding the molecular events of these essential reactions will lead to a better understanding of the specific physiological functions of the protein phosphatases. Also, a detailed description of the inherent differences between and among PTPs and PPs will undoubtedly augment the search for and design of specific phosphatase inhibitors.

Because of reference limitations imposed in minireviews, we wish to acknowledge the important contributions made by other investigators whose work we were unable to cite. Reference to this work can be found within the cited publications of many of the selected reading articles.

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