# Chemical and Mechanistic Approaches to the Study of Protein Tyrosine Phosphatases

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**ABSTRACT**

Protein tyrosine phosphatases (PTPs) are signaling enzymes that control a diverse array of cellular processes. Site-directed mutagenesis, combined with detailed kinetic and mechanistic studies of *Yersinia* PTP, have contributed greatly to the understanding of the chemical mechanism for PTP catalysis, the nature of the enzymatic transition state, and the means by which the transition state is stabilized. Significant progress has been made in developing specific small-molecule inhibitors as tools to dissect the functional roles of PTP both in normal physiology and pathological conditions. Despite the conserved structural and catalytic properties, recent results show that there are sufficient differences in the PTPs so that potent and selective bidentate inhibitors that simultaneously bind both the active site and a unique adjacent site can be obtained.

## Introduction

Protein tyrosine phosphatases (PTPs) are a large and structurally diverse family of signaling enzymes that, together with protein tyrosine kinases, modulate the cellular level of tyrosine phosphorylation (Figure 1).[1,2] An appropriate level of tyrosine phosphorylation is essential for regulating cell growth, differentiation, metabolism, progression through the cell cycle, cell−cell communication, cell migration, gene transcription, ion channel activity, the immune response, and apoptosis/survival decisions. As observed with protein tyrosine kinases, defective or inappropriate regulation of PTP activity leads to aberrant tyrosine phosphorylation, which contributes to the development of many human diseases, including cancers and diabetes.[3] The hallmark that defines the PTP superfamily is the active site sequence **C**(X)$_5$**R**(S/T), also known as the PTP signature motif, in the catalytic domain. On the basis of this unique structural feature, it is estimated that more than 100 PTPs are encoded in the human genome. The PTP superfamily can be divided into at least four subfamilies: (1) classical pTyr specific PTPs, (2) dual-specificity phosphatases (DSPs), (3) Cdc25 phosphatases, and (4) low molecular weight (LMW) PTPs (Figure 2). Aside from the PTP signature motif, these four subfamilies share little sequence similarity to one another. However, they are considered part of the same superfamily because of the conserved structural features in the active site and their common catalytic mechanism.

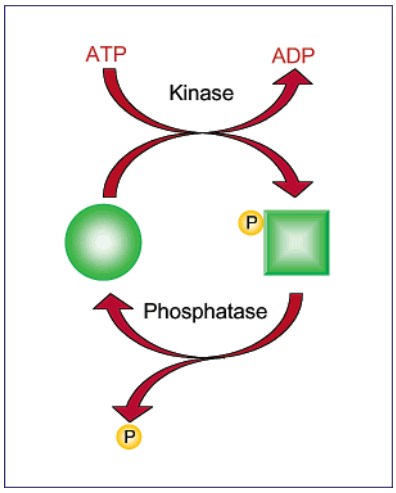


**FIGURE 1.** The protein phosphorylation state is regulated by the relative activity of protein kinases and phosphatases. The addition and removal of the phosphoryl group can lead to structural and therefore functional change of a protein molecule.

The classical PTPs were originally discovered on the basis of their ability to dephosphorylate pTyr-containing proteins. According to their subcellular localization, tyrosine specific PTPs can be further divided into intracellular and receptor-like PTPs. The VH1- and Cdc14-like DSPs are distinguished by their ability to hydrolyze pSer/pThr as well as pTyr residues. Also included in the DSP subfamily are PTEN and myotubularin, which hydrolyze phosphoinositides, and Mce1 and BVP, which have mRNA 5′-triphosphatases activity. Although Cdc25 phosphatases exhibit dual specificity toward protein substrates, they are classified as a separate subfamily because they are more distantly related to other members of the PTP superfamily at both primary and tertiary structural levels. The LMW PTPs share no significant sequence homology with other members of the PTP superfamily beyond the PTP signature motif.

By catalyzing removal of a phosphoryl group from a substrate, PTPs can act both as "on" and "off" switches for signal transduction. For example, mice lacking functional PTP1B exhibit increased sensitivity toward insulin and are resistant to obesity, suggesting that PTP1B is a major negative regulator of both insulin and leptin signaling. PTEN, a tumor suppressor, is mutated in several major types of neoplasia, including brain, breast, and prostate cancers. On the other hand, CD45, through its capacity to dephosphorylate and activate *src* family tyrosine kinases, is essential for signaling in stimulated T and B cells. Cdc25 phosphatases dephosphorylate and activate cyclin-dependent kinases and are required for cell cycle progression.

Zhong-Yin Zhang was born in Yantai, China. He received his B.S. in Chemistry from Nankai University in 1984 and his Ph.D. in Biochemistry from Purdue University in 1990. After a brief stint at the Upjohn Company, he carried out postdoctoral work in the laboratory of Jack Dixon at the University of Michigan. He joined the faculty of the Albert Einstein College of Medicine in 1994, where he is a Professor of Molecular Pharmacology and Biochemistry. His major research interests are in the area of protein tyrosine phosphatases: mechanism of catalysis and regulation, molecular basis of substrate specificity, and development and use of small molecule inhibitors to dissect PTP function.

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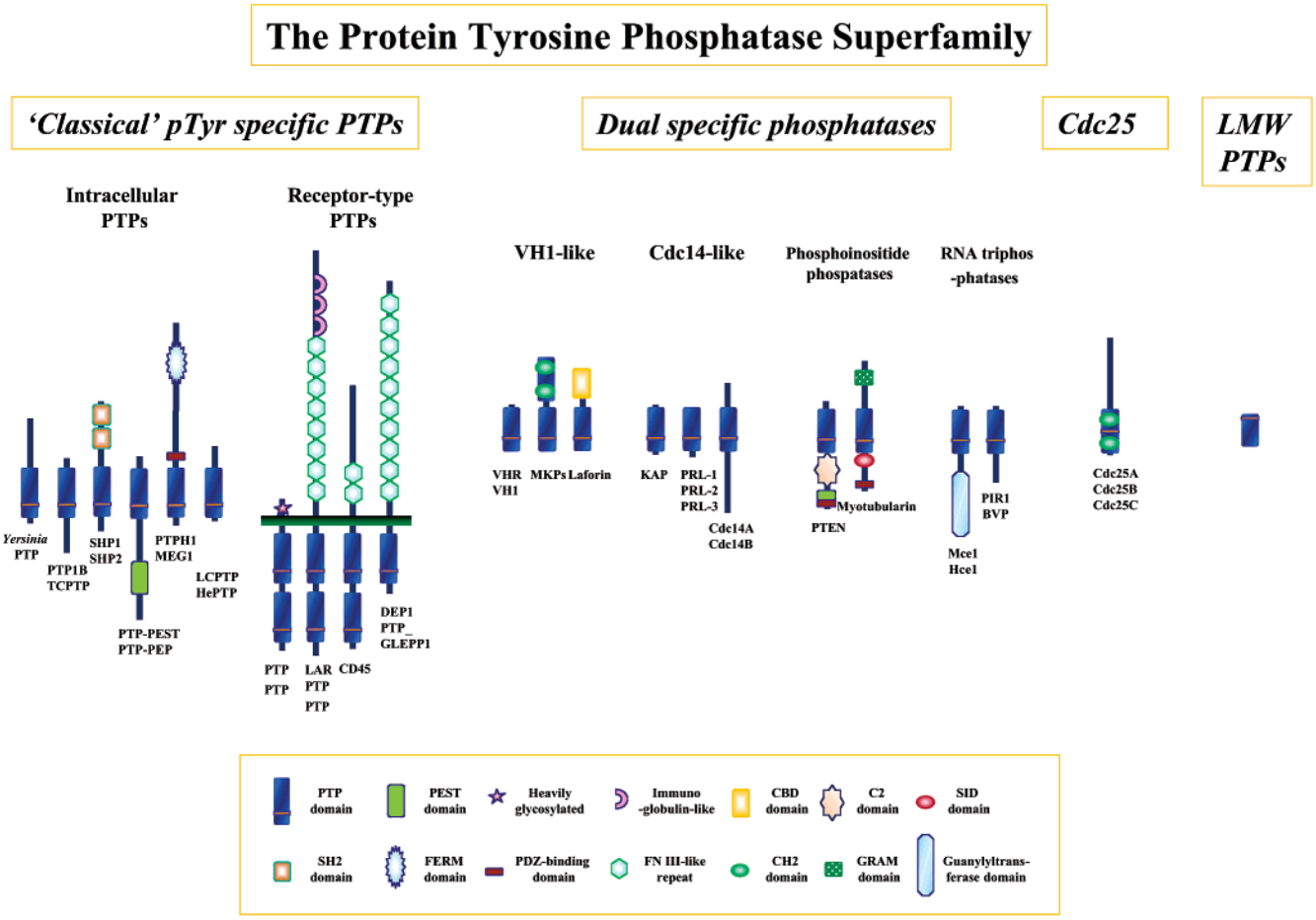



**FIGURE 2.** Classification and general structural features of the PTP superfamily.

To further understand the role of PTPs in signal transduction, it is essential to have a detailed understanding of how they catalyze substrate dephosphorylation. Understanding the molecular basis for PTP catalysis will promote development of new experimental approaches (such as the creation of PTPs with altered catalytic and regulatory properties and the design and development of specific PTP inhibitors) that will reveal mechanisms by which these enzymes control critical cell functions. In this Account, I discuss current understanding of the catalytic mechanism shared by the PTPs, the nature of the enzymatic transition state, and the means by which it is stabilized by the PTPs, based primarily on mechanistic studies with the *Yersinia* PTP. I then highlight recent progress in acquiring potent and selective PTP inhibitors and their use as chemical tools to define the roles of PTPs in signal transduction.

## Mechanism of PTP Catalysis

The PTPs are intriguing enzymes; unlike alkaline and Ser/Thr phosphatases, they require no metal ions and catalyze phosphate hydrolysis via a covalent phosphocysteine intermediate.[4−6] Thus, the PTP reaction is nucleophilic in nature and is composed of at least two chemical steps: the formation ($k_2$) and breakdown ($k_3$) of the phosphoenzyme intermediate (E−P) (Scheme 1). Site-directed mutagenesis experiments reveal that the active site nucleophile corresponds to the invariant Cys residue (e.g., Cys403 in the *Yersinia* PTP) in the PTP signature motif. Substitutions of the Cys residue completely abrogate PTP activity and eliminate PTP's ability to form E−P.[5]

**Scheme 1**

$$E + ArOPO_3^{2-} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot ArOPO_3^{2-} \xrightarrow{k_2} E\text{-}P \xrightarrow{k_3} E + HOPO_3^{2-}$$

$$E \cdot ArOPO_3^{2-} \xrightarrow{k_2} ArOH$$

The *Yersinia* PTP is essential for virulence of the bacteria responsible for the plague, and it is the most active PTP known to date. This has made it a model system for detailed mechanistic studies to define residues important in PTP catalysis. A systematic mutagenesis analysis of conserved acidic residues in *Yersinia* PTP revealed that Asp356 must be protonated for optimal PTP activity and substitution of Asp356 to Asn reduced the catalytic activity by 3 orders of magnitude.[7] The results suggest that Asp356 acts as a general acid during E−P formation ($k_2$), probably by protonating the leaving group. Consistent with the assignment of the invariant Asp residue as a general acid in the E−P formation step, removal of the general acid resulted in characteristic changes in the kinetic isotope effects,[8] a flat pH-rate profile with a significant drop in the rate of catalysis,[7] and a significant leaving group dependence where the rate of catalysis drops as the leaving group p$K_a$ rises.[9] Further pre-steady-state and steady-state kinetic analysis of wild-type

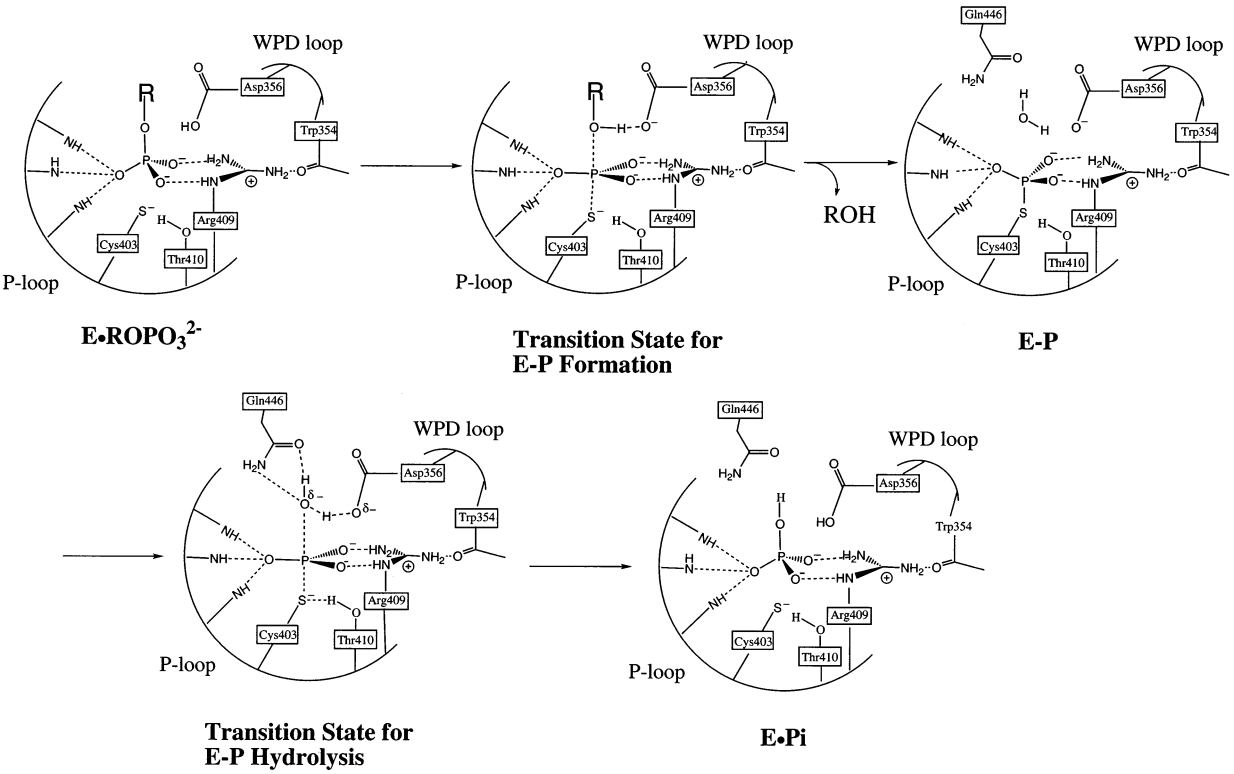



**FIGURE 3.** Chemical mechanism and transition state of the *Yersinia* PTP reaction.

and mutant PTPs indicated that the same invariant Asp residue acts as a general base to activate the nucleophilic water in the E−P hydrolysis step ($k_3$).[10] To probe the role of the invariant Arg in the PTP signature motif (Arg409 in the *Yersinia* PTP), it was changed to Lys and Ala.[11] A 8200-fold decrease in $k_{cat}$ and a 26-fold increase in $K_m$ were observed for the R409A mutant. Interestingly, the R409K mutant displayed a $k_{cat}$ value identical to that of R409A, and the apparent $K_m$ value for *p*NPP was only 1.9-fold higher than that of the wild-type enzyme. These results suggest that although the active site Arg plays a role in substrate binding, it is more important for catalysis. A conserved Ser or Thr (Thr410 in *Yersinia* PTP) is often found in the PTP signature motif immediately after the invariant Arg residue. Mutagenesis experiments indicate that the main function for the conserved hydroxyl group is to facilitate E−P hydrolysis.[12] Kinetic analyses of the invariant Gln446 mutants suggest that it plays a role for the precise placement of the nucleophilic water during E−P hydrolysis.[13] A chemical mechanism for *Yersinia* PTP is depicted in Figure 3. Despite variation in the primary structures and differences in the active site substrate specificities, it appears that this mechanism is applicable to all PTPs.[14] The proposed mechanism is supported by structural data.[15,16] Although sequences flanking the PTP motif among members of different subfamilies lack general similarity, the three-dimensional structures of PTP catalytic domains share strikingly similar features. The PTPs are $\alpha + \beta$ proteins with tertiary folds composed of a highly twisted mixed $\beta$-sheet flanked by $\alpha$-helices on both sides (Figure 4). The PTP active site is located within a crevice on the protein surface. At the bottom of the active site is the phosphate-binding loop (P-loop) formed by the

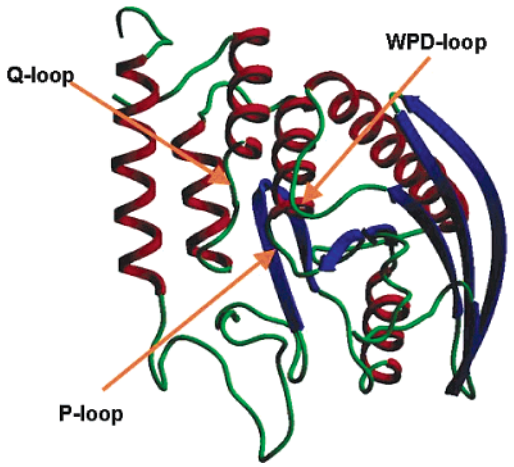



**FIGURE 4.** Ribbon diagram of the *Yersinia* PTP tertiary structure. The active site P-loop contains Cys403, Arg409, and Thr410. Adjacent to the P-loop is the movable WPD-loop, which harbors the invariant Asp356. The Q-loop contains the conserved Gln446.

PTP signature motif. The S$\gamma$ atom of the active site Cys is poised 3.1−3.6 Å from the phosphorus atom of pTyr such that the S$\gamma$ of Cys403, the phosphorus of pTyr, and the phenolic oxygen in tyrosine are approximately collinear (Figure 5). This is consistent with the active site Cys acting as a nucleophile in the catalytic mechanism. The side chain of Arg409 forms a bidentate hydrogen bond with two of the nonbridge phosphoryl oxygens in the substrate. The hydroxyl group of the conserved Thr410 is approximately 3 Å to the S$\gamma$ of Cys403, making a reasonable good $S^{-}\cdots HO$ hydrogen bond.

Asp356 is found on a flexible loop, which undergoes a major conformational change upon substrate binding.[15]

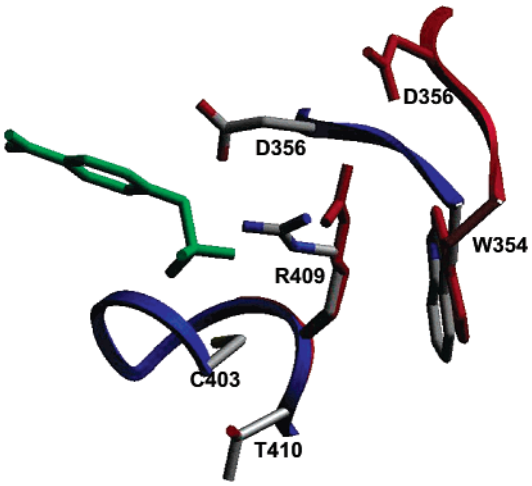



**FIGURE 5.** Conformational change in the WPD loop that occurs upon ligand binding in the *Yersinia* PTP. Highlighted in the figure are Cys403, Arg409, and Thr410 in the P-loop, Asp356, and Trp354 in the WPD-loop, and the substrate pTyr (shown in green), which is manually docked into the active site. The unliganded structure is shown in red, and the pTyr-bound structure is shown in blue.

In the unliganded *Yersinia* PTP structure, Asp356 is greater than 10 Å from the active site (Figure 5). In the ligand bound structure, the WPD loop (residues 351−360 in *Yersinia* PTP) has moved like a "flap" to cover the active site, placing the side chain of Asp356 in close proximity to the scissile oxygen of a substrate. These observations are consistent with the role of Asp356 to donate a proton to the leaving group during the E−P formation step. In addition, the crystal structure of *Yersinia* PTP complexed with vanadate shows that the apical oxygen, which is structurally homologous to the scissile oxygen of the substrate or the attacking water during E−P hydrolysis, makes a hydrogen bond with the site chain of Asp356.[17] This is consistent with Asp356's role as a general acid/base in catalysis. The apical oxygen also forms a hydrogen bond with Gln446 in the Q-loop, which supports its role in E−P hydrolysis.

The interactions between Arg409 and Trp354 are important for WPD loop motion and general acid catalysis (Figure 5). Mutation of the hinge residue Trp354 to Ala completely disables general acid catalysis.[9] In addition, a major decrease in the affinity for oxyanions was observed for the mutant, which is consistent with Trp354 playing a role in aligning Arg409 for oxyanion binding. Interestingly, general acid catalysis is also rendered inoperative in R409K.[18] These results indicate that general acid catalysis in PTPs is coupled to the correct positioning of the WPD loop, which is determined by proper interactions between the active site Arg and the conserved Trp at the hinge position. The dynamic flexibility and coupling of the

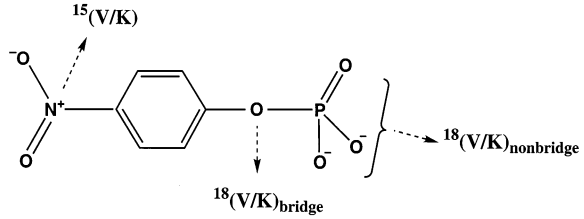



**FIGURE 6.** The structure of *p*NPP and the kinetic isotope effects measured for the *Yersinia* PTPs.

phosphate binding site and the general acid containing WPD-loop in PTPs is further supported by studies using resonance Raman and fluorescence spectroscopic techniques and deuterium/hydrogen exchange monitored by mass spectrometry.[19−21]

## Transition States of the PTP Reaction

Phosphoryl transfer reactions can, in principle, occur via one of two limiting mechanisms analogous to those for substitution at tetrahedral carbon: $SN_1$- or $SN_2$-like. The wealth of information obtained for solution reactions over decades of study suggests that phosphate monoesters are cleaved through a concerted mechanism with a dissociative transition state (Scheme 2), in which bond formation to the incoming nucleophile is minimal and bond breaking between the phosphorus and the leaving group is substantial.[22−25] Hydrolysis of phosphate monoesters is a thermodynamically favorable process, but in the absence of enzymes, phosphate monoesters are almost kinetically inert at physiological conditions. However, the rate of pTyr hydrolysis can be accelerated $10^{14}$-fold by the *Yersinia* PTP. There is considerable interest in the means by which PTPs catalyze this reaction. An important issue in understanding enzymatic catalysis is whether the transition state for an enzymatic reaction is altered from that for the uncatalyzed reaction in solution. Do PTPs catalyze phosphoryl transfers via a dissociative or an associative transition state? Different strategies may be utilized by a PTP to accelerate a reaction depending on the nature of the transition state. Several physical organic chemistry techniques, including kinetic isotope effects, thio effects, and linear free energy correlation, have been employed to determine and characterize the transition state of the PTP-catalyzed reaction.

To determine the transition state structure for the E−P formation step, isotope effects on three sites in *p*NPP (Figure 6) were measured on V/K.[8,18,26] Table 1 summarizes the isotope effects for the *Yersinia* PTP-catalyzed *p*NPP reaction. For comparison, isotope effects for the solution reactions are listed in Table 2. The fact that isotope effects on the PTP reactions are similar to those of the solution reactions suggests that chemistry, i.e. P−O bond cleavage ($k_2$), is rate-limiting for the V/K portion of the mechanism

**Scheme 2**

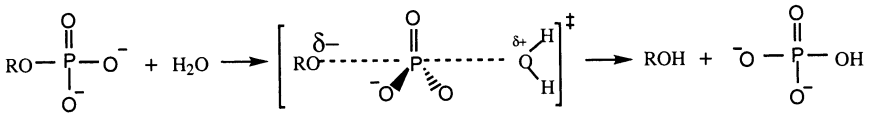


**Table 1. Kinetic Isotope Effects for Reactions of *Yersinia* PTPs with *p*NPP[8,18,26]**

| *Yersinia* PTP | $^{15}$(V/K) | $^{18}$(V/K)$_{bridge}$ | $^{18}$(V/K)$_{nonbridge}$ |
|---|---|---|---|
| Wild Type | 0.9999(3) | 1.0160(15) | 1.0001(13) |
| D356N | 1.0024(5) | 1.0275(16) | 1.0022(5) |
| D356A | 1.0022(3) | 1.0274(8) | 1.0007(5) |
| W354F | 1.0013(2) | 1.0240(10) | 1.0015(8) |
| W454A | 1.0021(2) | 1.0310(5) | 1.0038(5) |
| R409K | 1.0020(5) | 1.0273(3) | 1.0049(7) |
| R409A | 1.0012(3) | 1.0200(5) | 0.9990(7) |
| D356N/R409K | 1.0022(1) | 1.0317(3) | 1.0045(2) |
| D356N/R409A | 1.0024(4) | 1.0340(11) | 1.0027(5) |
| D356A/R409K | 1.0023(1) | 1.0322(9) | 1.0045(2) |
| D356A/R409A | 1.0025(1) | 1.0310(11) | 1.0030(2) |

**Table 2. Kinetic Isotope Effects for Uncatalyzed Solution Hydrolysis of *p*NPP[8]**

| *p*NPP | $^{15}$(V/K) | $^{18}$(V/K)$_{bridge}$ | $^{18}$(V/K)$_{nonbridge}$ |
|---|---|---|---|
| monoanion | 1.0005(2) | 1.0106(3) | 1.0224(5) |
| dianion | 1.0034(2) | 1.0230(5) | 0.9993(7) |

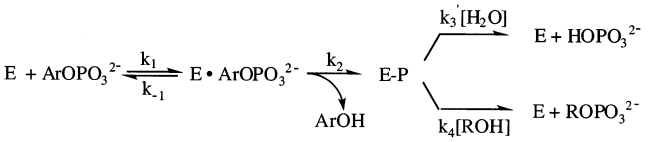
and that substrate binding and WPD-loop motion are both rapidly reversible and do not introduce a commitment toward catalysis.

The $^{18}$(V/K)$_{nonbridge}$ isotope effect is a predictable tool in assessing the dissociative versus associative character of a transferring phosphoryl group in the transition state because its value is negligible or slightly inverse for dissociative transition states typical of monoesters, small but normal (typically from 1.003 to 1.006) for diesters of *p*-nitrophenol where the transition state is somewhat associative, larger and normal (1.006 to 1.025) for triesters where the reaction is more associative. The near unity value for the *Yersinia* PTP is similar to those of solution reactions of dianion and is consistent with the known metaphosphate-like nature of the phosphoryl group in a dissociative transition state.

The secondary $^{15}$N isotope effect in the nitrogen atom of the leaving group, $^{15}$(V/K), is sensitive to the amount of negative charge delocalized into the aromatic *p*-nitrophenol ring and gives a measure of the charge that is developed on the phenolic oxygen in the transition state. The $^{15}$(V/K) effect for the *Yersinia* PTP is unity, indicating complete charge neutralization due to proton transfer from the general acid in the transition state. When the general acid is deleted (D356N and D356A) or impaired (W454A and R409K), the $^{15}$(V/K) values are similar to those of the dianion reactions, consistent with charge delocalization into the ring in the transition state.

The magnitude of the primary $^{18}$O isotope effect at the bridge position contains information about the degree of P−O bond cleavage in the transition state. The $^{18}$(V/K)$_{bridge}$ effect for the monoanion is smaller than that in the solution reactions of dianion which is due to the fact that the cleavage of the P−O bond is partially compensated for by the transfer of the proton to the phenolic oxygen. The $^{18}$(V/K)$_{bridge}$ isotope effects of the PTPs are slightly larger than that of the monoanion reaction and indicate that the degree of P−O bond cleavage in the transition state is advanced. When the general acid is removed, the $^{18}$(V/K)$_{bridge}$ isotope effects increase in magnitude and are similar to those of the dianion reactions, confirming the high degree of P−O bond cleavage.

**Scheme 3**




Collectively, these studies provide detailed comparison of the transition state of a phosphoryl transfer enzyme with that of solution reactions. The PTP transition state is highly dissociative and similar to that of the uncatalyzed reaction. Thus, the transition state of the solution reaction is essentially the one stabilized by the PTPs leading to catalysis. A dissociative transition state for the E−P formation step is depicted in Figure 3, in which P−O bond to the leaving group is largely broken, proton transfer to the leaving group oxygen is correspondingly advanced such that the departing phenol has no charge, and the central phosphoryl group resembles metaphosphate in structure.

To probe the transition state of the E−P hydrolysis step, Brønsted correlation was applied to study the effect of changing nucleophile p$K_a$ on the second-order rate constants for the reaction of $\beta$-substituted ethanols with E−P.[27,28] This is based on the fact that E−P can partition with water to give the hydrolysis product (inorganic phosphate) and with alcohols to produce alkyl phosphates. Scheme 3 illustrates the partitioning of E−P in the presence of alcohol ROH, in which $k_3$ (= $k_3'$[$H_2O$]) is the rate of hydrolysis while $k_4$[ROH] is the rate of E−P reacting with ROH to form the phosphorylated alcohol ($ROPO_3^{2-}$). The $\beta_{nu}$ parameter obtained from the Brønsted correlation can be viewed as an empirical index of the fraction of charge transferred to the nucleophile and correspondingly reflects the degree of bond formation between the nucleophile and the phosphorus in the transition state. The $\beta_{nu}$ values for the LMW PTP and the *Yersinia* PTP-catalyzed reaction are 0.14 and 0.15, respectively, which fall in the range of 0.1−0.2 observed for solution reactions of monoesters. The small dependence of the second-order rate constant ($k_4$) on basicity for reactions of alcohols with E−P suggests that the transition state for the dephosphorylation of E−P is also highly dissociative, similar to the solution reactions (Figure 3). Furthermore, the fact that there is dependence of phosphoryl transfer on the nucleophile basicity also indicates that the entering nucleophile is a required participant in E−P decomposition and argues against the existence of a free metaphosphate intermediate in the PTP-catalyzed reaction.

## How Are the Transition States Stabilized by the PTPs?

It is clear from the evidence presented above that PTPs do not alter the transition state for phosphate hydrolysis and that PTPs achieve their remarkable catalytic efficiency by stabilizing the transition state of the solution reaction. How do PTPs stabilize a dissociative transition state? An important catalytic strategy employed by the PTPs for transition stabilization is to neutralize the buildup of

negative charge on leaving group. Thus, the transition state for E−P formation is stabilized by the general acid Asp, which facilitates the departure of the leaving phenoxide.[8] It is interesting to note that, in general, substitutions at the conserved Asp have a more profound effect on the step leading to E−P formation, as compared to its decomposition.[10] This is consistent with a dissociative transition state: a greater help is needed to facilitate the departure of the leaving group in the E−P formation step (where the Asp acts as a general acid) than to activate the nucleophilic water in the E−P hydrolysis step (where the Asp acts as a general base). Since the transition state for E−P hydrolysis step is also dissociative, charge stabilization on the leaving thiolate is also important since P−S bond breaking is substantial in the transition state. Indeed, a larger decrease in the rate of E−P hydrolysis as compared to that of E−P formation is observed when the conserved hydroxyl group in the P-loop (Thr410) is eliminated.[12,27]

It must be pointed out that leaving group stabilization is not the only strategy that PTPs utilize to lower the transition state energy. The general acid Asp only contributes a factor of $10^3$, while the conserved Ser/Thr in the P-loop contribute a factor of $10^2$ in rate enhancement. Another important strategy used by the PTPs is preferential (stronger) interaction between the active site Arg and the phosphoryl moiety in the transition state than in the ground state by precise geometric complementarity. Positively charged Arg residue(s) are uniformly present in the active site of phosphoryl transfer enzymes. Because charge density in nonbridge phosphoryl oxygens increases in an associative transition state and decrease in a dissociative one, it was suggested that interactions between an Arg with phosphoryl oxygens would inhibit the dissociative pathway but promote the associative process. Does positively charged active site Arg alter the transition state?

To examine the effect of positive charge on the transition state free of effect on general acid catalysis, double mutants were made in which general acid catalysis was removed by mutation of Asp356 to either Asn or Ala in addition to the mutation to Arg409.[26] Kinetic analysis suggests that the side chain of Arg409 contributes 3.4 kcal/mol to transition state stabilization through interactions with the phosphoryl oxygens. Comparisons of the isotope effects for reactions of the double mutants with data from general acid single mutants show that mutation of Arg to either Lys or to Ala does not significantly affect the transition state for phosphoryl transfer (Table 1). Thus, Arg409 functions to stabilize the transition state but does not alter it from its structure in the uncatalyzed reaction.

How can Arg stabilize a dissociative transition state? The ability of Lys to effectively substitute for Arg in substrate binding but not in catalysis suggests that PTPs likely employ the unique structural properties of the guanidinium side chain of Arg to preferentially stabilize the transition state. As the reaction proceeds from the ground state tetrahedral orthophosphate to the metaphosphate-like transition state (Figure 3), the coplanar geometry of the guanidinium group is ideally positioned to stabilize the $sp^2$ hybridization of the $PO_3$ oxygens. Molecular modeling suggests that hydrogen bonding distances between the phosphoryl oxygens and the side chain of the invariant Arg and the main chain amides of the P-loop are shortened by 0.05−0.1 Å from the ground state to the transition state.[29] It is possible that the decrease in charge density on the phosphoryl oxygens in a dissociative transition state is more than offset by the optimal geometric alignment of the guanidinium group and the phosphoryl oxygens. The importance of this geometric alignment for transition state stabilization is supported by the observed large thio effect in the PTP reaction, which results from the inability to achieve precise transition state complementarity in the PTP active site with the larger sulfur substitution.[28]

## PTP Inhibitor Development

Despite the large number of PTPs identified to date and the involvement of PTPs in human diseases, a detailed understanding of the role played by PTPs in normal physiology and in pathogenic conditions has been hampered by the absence of PTP-specific inhibitors. Such compounds could serve as useful tools for determining the physiological functions of PTPs and may constitute valuable therapeutics in the treatment of human diseases. However, given the highly conserved nature of PTP active sites, it has been questioned whether selectivity in PTP inhibition can be achieved.

Recent work indicates that it is feasible to acquire potent and highly selective PTP inhibitory agents. Biochemical studies reveal that pTyr alone is not sufficient for high affinity binding to PTPs and that amino acids flanking the pTyr furnish additional interactions for efficient and high affinity PTP recognition.[30−34] These results indicate that there are subpockets adjacent to the PTP active site that can also be targeted for inhibitor development. These studies, together with the discovery of a second aryl phosphate-binding site adjacent to the active site in PTP1B,[35] provide a molecular basis for addressing and manipulating PTP inhibitor potency and specificity and suggest a novel paradigm for the design of potent and specific PTP inhibitors; namely bidentate ligands that bind to both the active site and a unique adjacent peripheral site. Consequently, unique PTP sub-pockets that border the active site may be targeted to enhance inhibitor affinity and selectivity (Figure 7). The rationale for the enhanced affinity of bidentate inhibitors is based on the principle of additivity of free energy of binding. The interaction of an inhibitor with two independent sites (e.g., pTyr site and a unique peripheral site) in one PTP would be expected to confer exquisite specificity, since other PTPs may not possess an identical second site interaction.

A combinatorial library/high-throughput screening approach was employed to identify bidentate inhibitors capable of simultaneously occupying both the active site and a unique peripheral site in PTP1B.[36] This led to the identification of compound **1** (Figure 8), which displays a

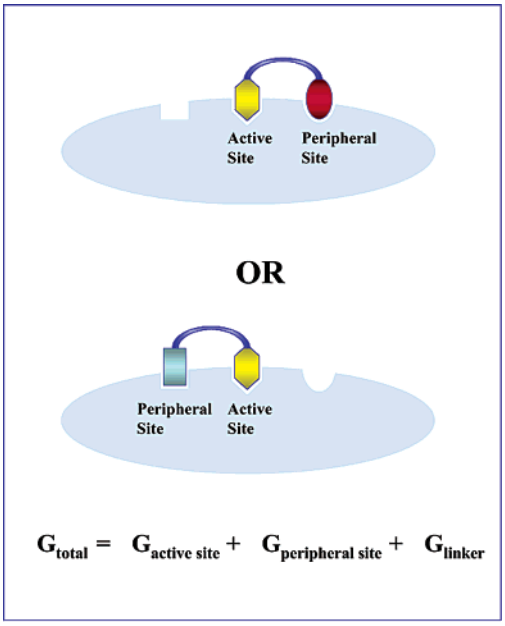



**FIGURE 7.** A strategy for acquiring potent and selective PTP inhibitors. On the basis of the principle of additivity of free energy of binding, high affinity ligands can be obtained by linking two functional groups that bind to the active site (pTyr-binding site) and a peripheral site. Specificity arises from the fact that the peripheral site is not conserved and from the fact that the tethered ligand has to bind both sites simultaneously.

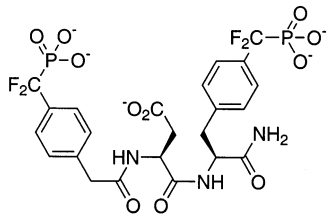


**FIGURE 8.** Structure of compound **1**.

$K_i$ value of 2.4 nM for PTP1B and exhibits several orders of magnitude selectivity in favor of PTP1B against a panel of PTPs. Compound **1** is the most potent and selective PTP1B inhibitor identified to date. Further studies suggest that while the nonhydrolyzable pTyr mimic phosphonodifluoromethyl phenylalanine is bound to PTP1B active site, the distal 4-phosphonodifluoromethyl phenylacetic acid in **1** occupies a distinct area involving residues Lys36, Lys41, Arg47, Asp48, Ser50, and Lys116.[37] The results show that although many of the residues in contact with compound **1** are not unique to PTP1B, the combinations of all contact residues differ between PTP isozymes, which suggest that the binding surface defined by these residues in individual PTPs determines inhibitor selectivity. Collectively, the results serve as a proof-of-concept in PTP inhibitor development and establish the feasibility of acquiring potent, yet highly selective, PTP inhibitory agents.

Several cell permeable analogues of **1** have been prepared and evaluated in a number of insulin sensitive cell lines. Consistent with results from PTP1B$^{-/-}$ mice,[38] PTP1B specific inhibitors can work synergistically with insulin to increase the phosphorylation level in both insulin receptor and insulin receptor substrate 1 (unpublished observations). In addition, the compounds can also increase the insulin-stimulated activation of Akt and augment the insulin stimulated glucose uptake. Together, these results establish that compound **1** is also an effective PTP1B inhibitor in vivo. Since PTP1B is known to be a participant in many cellular processes, PTP1B inhibitors, such as **1**, should prove useful in dissecting the precise roles played by PTP1B in signal transduction pathways. They could also furnish a molecular foundation upon which therapeutically useful agents can be based.

## Conclusion and Future Prospects

Until now, our understanding of PTP catalysis has been based primarily on studies with small molecule aryl phosphates, such as *p*NPP. The use of artificial substrates has allowed a full characterization of the transition state of the PTP-catalyzed reaction and identification of catalytically essential amino acid residues. Future mechanistic investigations will focus on physiological substrates. Recent studies with physiological substrates show that PTPs display exquisite specificity in their substrate recognition, which involve extensive protein−protein contacts in addition to interactions that engage the phosphoamino acid residue.[39−42] Detailed knowledge of the molecular basis of PTP substrate specificity and regulation will be important for understanding the specific functional role of PTPs in cellular signaling. Rapid identification and characterization of PTP substrates with high-affinity substrate-trapping mutants developed from mechanistic studies[43] should help place and integrate individual PTPs into the ever growing web of signaling network. Finally, potent and selective cell permeant PTP inhibitors should find increasing use in dissecting the roles of PTPs both in normal cellular physiology and in pathogenic conditions. In principle, an identical approach applied to PTP1B (i.e., to create bidentate inhibitors that bind both the active site and a unique adjacent peripheral site) could also be used to produce unique low molecular weight inhibitors that are specific for other PTP isoenzymes.

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