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CONSERVATIVE TRYPTOPHAN MUTATIONS IN PROTEIN TYROSINE
PHOSPHATASE PTP1B AND ITS EFFECT ON CATALYTIC RATE AND
CHEMICAL REACTION

by

Teisha B. Richan

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Chemistry

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2017

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ABSTRACT

Conservative Tryptophan Mutations in Protein Tyrosine Phosphatase PTP1B and its
Effect on Catalytic Rate and Chemical Reaction

by

Teisha B. Richan, Master of Science

Utah State University, 2017

Major Professor: Dr. Alvan C. Hengge
Department: Chemistry

Kinases and phosphatases regulate cellular processes by activating or deactivating enzymes. The uncatalyzed hydrolysis of a phosphate-monoester in neutral pH and 25 ° Celsius has a half-life of approximately 1 trillion years. This would seem to make phosphate monoesters too stable for the regulations of cellular processes that would need to respond within seconds to minutes of extracellular stimuli. However, due to the catalytic power of enzymes, the hydrolysis of phosphate-monoesters is reduced to fractions of a second. This makes phosphatases some of the most catalytically efficient enzymes known.

In most PTPs the aspartic acid resides on a flexible protein loop, consisting of about a dozen amino acid residues, called the WPD-loop. PTP catalysis rates span several orders of magnitude. For instance PTP1B (a human PTP) has a $k_{cat} = 30s^{-1}$ whereas YopH PTP has a $k_{cat} = 750s^{-1}$ at pH 5.5 and 25°C. Interestingly, these two enzymes, along with the rest of the PTP family, have crystal structures in which the active sites are

superimposable. This raises the question of why there is such a difference in catalytic rates where the chemistry occurs by the same mechanism in a similar active site.

Recently the rate of the WPD loop motion was correlated to the catalytic rate of the enzymes PTP1B and YopH. The rate of WPD loop motion is perhaps controlled in part by the conserved tryptophan residue on the WPD loop. The indole ring from this residue repositions upon closing within a hydrophobic pocket. Conservative mutations to this tryptophan mutant in YopH rendered the enzyme in a quasi-open position. However conservative mutations in PTP1B do not seem to have the same effect. The conservative mutation W179F in PTP1B was previously constructed and resulted in a decrease in $k_{cat} = 11.4 \text{ s}^{-1}$ at pH 5.5 while maintaining WPD-loop motion. Whereas the W179Y and W179H mutants, presented in this thesis, resulted in a decrease in k_{cat} of 5.04 s^{-1} at pH 5.5 and 0.1 s^{-1} at pH 6.5 respectively. Tryptophan mutants W179Y and W179H maintained general acid catalysis and had the greatest effect on the second catalytic step.

PUBLIC ABSTRACT

Conservative Tryptophan Mutations in Protein Tyrosine Phosphatase PTP1B and
its Effect on Catalytic Rate and Chemical Reaction

Teisha B. Richan

Protein-tyrosine phosphatases (PTPs) catalyze the hydrolysis of phosphorylated tyrosines by a 2-step mechanism involving nucleophilic attack by cysteine and general acid catalysis by aspartic acid. In most PTPs the aspartic acid resides on a flexible protein loop, consisting of about a dozen residues, called the WPD loop. PTP catalysis rates span several orders of magnitude, and differences in WPD loop dynamics have recently been shown to correlate with the rate of enzymatic catalysis. The rate of WPD loop motion could possibly be related to a widely conserved tryptophan residue on the WPD loop. Therefore, point mutants were made in PTP1B (a human PTP) to the conserved tryptophan residue and their effects on catalytic rate and chemical reaction were studied. The results of these studies are presented in this thesis.

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I would like to give a special thanks to my husband Seth for his love and support throughout not only this process but all of our personal growth while in attendance at Utah State University. I absolutely would not have been able to get to this point without him. I am forever grateful to call him my friend, husband, cheerleader, rock, and so much more! Also of course I would like to thank my parents and siblings for their love and support as well. They have been there for babysitting while I took exams, and long phone calls of encouragement throughout this process.

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Teisha B. Richan

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Dedicate this work to my family past, present, and future. My grandparents, parents, and siblings. My daughter Talisia, my husband Seth and all our future children.

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CHAPTER I

BACKGROUND AND SIGNIFICANCE

OVERVIEW

Biology uses phosphorylation to regulate cellular processes. Kinases and phosphatases act as the biological on/off switch via phosphorylation. Kinases phosphorylate tyrosine, serine, or threonine residues while phosphatases dephosphorylate the same respective residues. [1] Phosphate monoesters possess the perfect chemical properties to play this critical regulatory role in biological systems. Phosphate monoesters are stable at physiological pH, they contain a negative charge to keep them inside the cell membrane, and they aren't easily hydrolyzed. [2] This last characteristic of phosphate monoesters is perhaps the most interesting. The uncatalyzed hydrolysis of a phosphate-monoester in neutral pH and 25 ° Celsius has a half-life of approximately 1 trillion years. This would seem to make phosphate monoesters too stable for the regulations of cellular processes that would need to respond within seconds to minutes of extracellular stimuli. However, due to the catalytic power of enzymes, the hydrolysis of phosphate-monoesters is reduced to fractions of a second. This makes phosphatases some of the most catalytically efficient enzymes known.

Phosphatases are a large family of enzymes; within this family is a sub-group of phosphatases called protein tyrosine phosphatases (PTPs). PTPs are responsible for the phosphate hydrolysis reactions from tyrosine side chains. One of the most well-known and studied PTP is PTP1B. PTP1B is a human PTP first isolated from human placenta. [3] It is most commonly known for its negative regulation role in the insulin cellular

response but it has also been linked to obesity. [4] This is also why this particular enzyme is so well known and studied because of the potential it has as a therapeutic target in type two diabetes and cancer.

PTPs MECHANISM AND STRUCTURE

PTPs all have the same mechanism for phosphate-monoester hydrolysis shown in Figure 1.[5] It is a two-step mechanism where hydrolysis occurs via a phosphoenzyme intermediate.[6] The first step is nucleophilic attack from a cysteine thiolate on the phosphate monoester, resulting in the phosphoenzyme intermediate. The second step is an attack of water on the phosphoenzyme intermediate resulting in the release of inorganic phosphate restoring a free enzyme binding site.[5, 7]

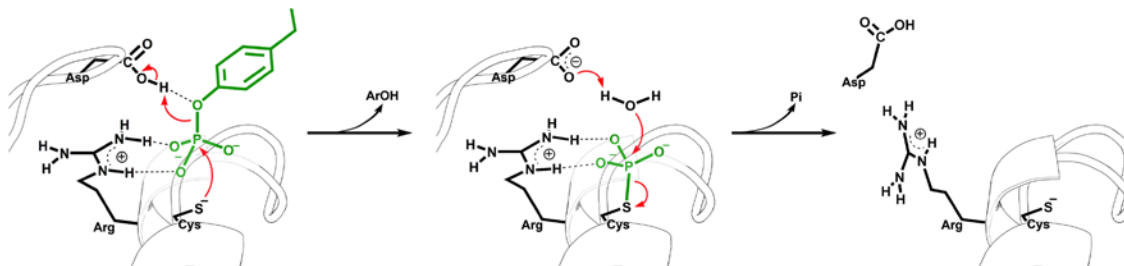


Figure 1: The general mechanism of the PTP-catalyzed reaction. The WPD-loop assumes a catalytically active closed conformation with the general acid in position to protonate the leaving group during formation of the phosphoenzyme intermediate. In the second step this intermediate is hydrolyzed. After the phosphate product is released the WPD-loop open conformation becomes favored.[8]

PTPs also have conserved amino acids that are essential to the stabilization of the transition state of the phosphoester hydrolysis mechanism. Within the PTP binding site there is a conserved sequence C(X)₅R(S/T) termed the “P-loop” which contains the nucleophilic cysteine, and the stabilizing arginine. [6, 9] As seen in Figure 2, the cysteine is deprotonated in the binding pocket along with hydrogen bond stabilization of the transition state via backbone amide protons, and the conserved arginine. [10]

The essential conserved aspartic acid is also seen in Figure 2. This aspartic acid is located on what is called the WPD-loop and sometimes referred to as the general acid loop. This loop gets its name WPD from the three highly conserved amino acids in this region of the enzyme among the PTP family. The tryptophan on this loop slides into a hydrophobic pocket on the back side of the PTP active site during WPD loop motion. The aspartic acid residue on this WPD-loop acts as shown in Figure 1 as the general acid for this mechanism. It protonates the leaving group in the first catalytic step and activates the water in the second hydrolysis catalytic step. [11] The WPD-loop is dynamic in many PTPs. The PTP family is hard to characterize from sequence alone. Two very well studied PTPs, the *Yersinia* PTP, a known virulence factor for the bubonic plague, and PTP1B, the human PTP associated with the insulin cell response, are only approximately 20-30% similar by sequence alone. [6] Despite this sequence disparity, the crystal structures for the enzymes in this family are highly superimposable (see Figure 3). The backbone residues of the P-loop, for instance are superimposable within approximately 0.24 Å. [12, 13]

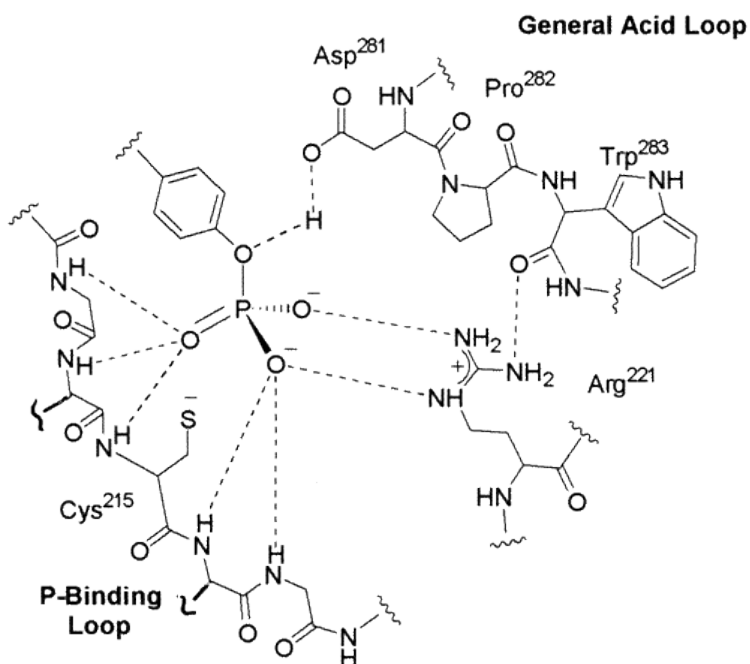


Figure 2: Key binding pocket interactions which involve the P-loop and general acid loop. The P-loop contains the nucleophilic cysteine, and the stabilizing arginine in the active site of PTP. The general acid loop is also known as the WPD loop due to the conserved residues before the general acid. [9]

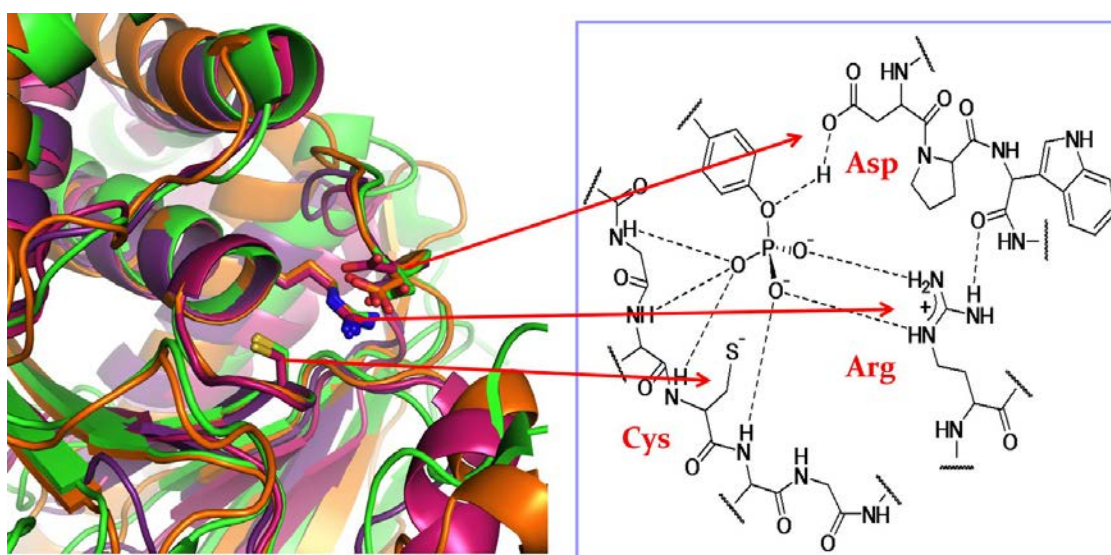


Figure 3: Superimposed active site regions of PTP1B (green), YopH (orange), VHZ (purple), and Sso-PTP (pink). At right, key residues in the P-loop and WPD-loop. Backbone residues in the P-loops align with RMSD of 0.24 Å.

KINETIC ISOTOPE EFFECTS FOR PROTEIN TYROSINE PHOSPHATASES

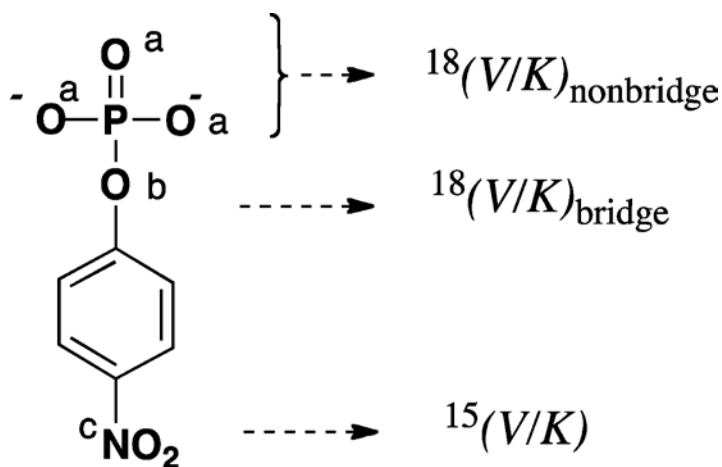
Protein Tyrosine Phosphatases (PTPs) share the same mechanism for catalysis, a superimposable active site and they do not alter the transition state of the reaction. The transition state of PTPs has been extensively studied using kinetic isotope effects (KIEs). The uncatalyzed reaction of phosphoryl hydrolysis has been confirmed to proceed through a concerted reaction with a loose transition state.[14] Due to the cationic arginine residue present in the active site, along with the hydrogen bonding network through the protein backbone which binds the anionic substrate and helps stabilize the transition state, it was an attractive notion that the active site might then be able to stabilize a tight transition state. This notion becomes more attractive when taking into consideration that the nonbridging oxygen atoms of the phosphate monoester substrate would increase in charge during a tight transition state as the nucleophile attacks.[8] KIEs are a perfect method to answer the question of whether PTPs alter the transition state of the phosphoryl hydrolysis along with the question of whether PTPs have a similar transition state across the sub-family of phosphatases. KIE experiments for PTPs are done using isotopically labeled substrates at the positions shown in Figure 4. [1] KIE measurements on reactions catalyzed by PTPs have been done using the competitive method along with remote label method. In the competitive method a mixture of light and heavy isotopically labeled isomers undergoes reaction. Then the isotope effect is calculated from the change in isotopic composition over the course of the reaction. The isotopic composition changes due to the mechanism of the reaction. By labeling the substrate with a different isotope it alters bending and torsional modes of the substrate. To calculate the ^{15}N isotope effect the

ratio of $^{15}\text{N}/^{14}\text{N}$ in the product (R_p) and the residual substrate (R_s) needs to be measured (see Figure 5). Since we know the ratio of the isotopes in the original substrate (R_o) we can then calculate the isotope effect from the equations shown in Equations 1 and 2 below.

Equations 1 and 2: The equations used to calculate the isotope effect with f being the fraction of the reaction of initial substrate used. R_x being the ratio of heavy isotope/light isotope in residual substrate. R_p being the ratio of heavy isotope/light isotope in the product.

$$\text{Isotope effect} = \log(1-f)/\log((1-f)(R_s/R_o)) \quad \text{Equation 1}$$

$$\text{Isotope effect} = \log(1-f)/\log((1-f)(R_p/R_o)) \quad \text{Equation 2}$$



pNPP

Figure 4: Substrate *p*-nitrophenyl phosphate (pNPP) showing the position where isotope effects are measured. a) nonbridge phosphoryl oxygen atoms ($^{18}(\text{V}/\text{K})_{\text{nonbridge}}$), b) bridge oxygen atoms, the position of bond cleavage ($^{18}(\text{V}/\text{K})_{\text{bridge}}$), c) nitrogen atom in leaving group ($^{15}(\text{V}/\text{K})$). [16]

The remote labeling method is employed because to analyze an isotope composition isotope ratio mass spectrometer (IRMS) is used and IRMS can only measure small molecule gases such as N₂, CO, CO₂, and SO₂ (see Figure 6). There is no way to quantitatively measure the converted isotopically labeled oxygen from the labeled substrate, *p*-nitrophenyl phosphate (pNPP), from CO or CO₂ molecule therefore we utilize a remote label, in this case the nitrogen atom, is used to calculate the ¹⁸O isotope effect.[15]

The KIEs for PTPs have been reported for the following: YopH from *Yersinia*; PTP1 from mouse; PTP1B from humans; STP1 from yeast; and VHZ from humans.[17-20] The KIE data from these experiments are summarized in Table 1. The KIE results give information about the transition state of the reaction of phosphoryl transfer within the enzyme active site. The primary isotope effect ¹⁸(V/K)_{bridge} is a result of the degree of cleavage to the P-O bond. If the P-O bond has a late transition state or nearly full bond cleavage then the KIE reaches a maximum value of approximately 1.03. The nitrogen isotope effect, or, ¹⁵(V/K), is a secondary isotope effect. This measures to what extent the negative charge in the leaving group is delocalized into the nitro group. Since the *p*-nitrophenolate anion also exist as a resonance structure to a quinonoid form the nitrogen atom is more tightly bonded when the *p*-nitrophenol leaving group is neutral as compared to when it exists as a phenolate anion. This is due to the fact that N-O bonds are stiffer in regards to vibrational frequencies than N-C bonds. Therefore the ¹⁵(V/K) isotope effect can deduce the state of the leaving group as to whether it is an anion or if the charge has

been fully or partially neutralized by protonation. When the charge has been neutralized this isotope effect results in a KIE close to unity. The other secondary isotope effect present is the $^{18}(\text{V}/\text{K})_{\text{nonbridge}}$. This KIE elucidates whether the transition state resembles a loose transition state or a tight transition state in an associative mechanism. Since the KIE for $^{18}(\text{V}/\text{K})_{\text{nonbridge}}$ is near unity it is indicative of a loose transition state for monoester reactions. All of the KIE data are consistent with a metaphosphate like transition state which is consistent with the uncatalyzed phosphoryl transfer of the reaction in water. [8]

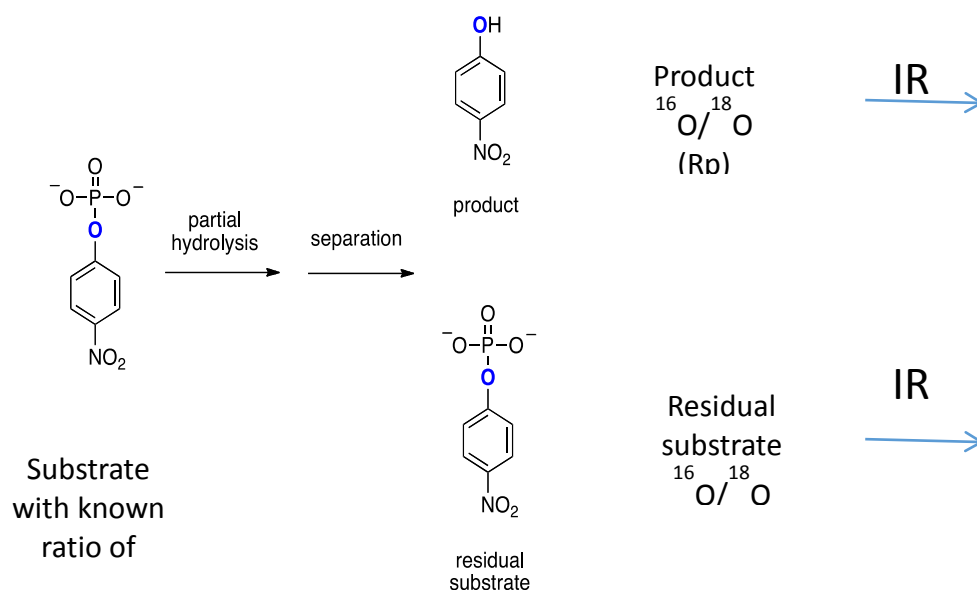


Figure 5: Schematic of how kinetic isotope effects are measured.

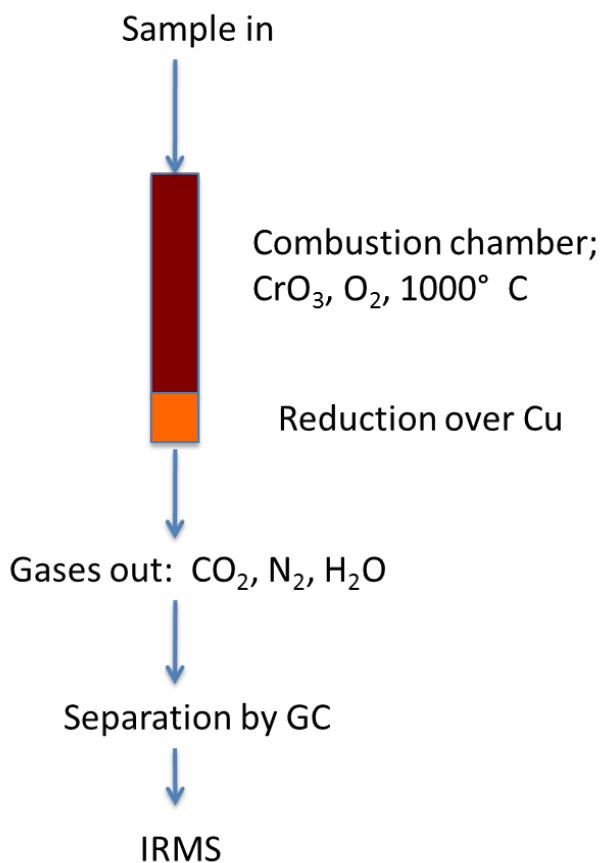


Figure 6: Schematic of isotope ratio mass spectrometer system.

Table 1: Kinetic isotope effects for the reaction of the members of PTP superfamily. Standard errors are in the range 0.0001-0.0008.[8]

Substrate	Enzyme	¹⁵ (V/K)	¹⁸ (V/K) _{bridge}	¹⁸ (V/K) _{bridge}
<i>p</i> NPP	YOP, PTP1, PTP1B	0.9999-1.0004	1.0118-1.0152	0.9998-1.0018
<i>p</i> NPP	Stp1	1.0007	1.0171	1.0007
<i>p</i> NPP	VHZ	1.0013	1.0164	0.9986
<i>m</i> NPP	YopH	0.9996	-	0.9999

DIFFERENCES IN CATALYTIC RATES CORRELATE TO WPD-LOOP MOTION

As previously mentioned, the mechanism for phosphoryl hydrolysis in PTPs is a two-step process. The aspartic acid (D) is highly conserved within the majority of the PTP family. It resides on the WPD-loop and acts as a general acid protonating the leaving group in the first step of the reaction and deprotonating the nucleophile allowing for the attack of water during the second step of phosphate hydrolysis. The WPD-loop, which bears the catalytically relevant aspartic acid, is mobile in many of the PTPs that have been studied. The WPD-loop has been exhibited two distinct conformations in crystal structures: the “open” conformation in which the WPD-loop is extended away from the active site; and a “closed” conformation in which the WPD-loop is over the active site.[21-25] The catalytically active “closed” conformation brings the general acid aspartate residue 8 angstroms closer to the bound substrate which is clearly seen in Figure 7.

The crystal structures of the PTP family’s WPD-loops in the open conformation are highly superimposable with one another, as are the WPD-loops in the closed conformational state. PTPs share many key similarities, but the differences among this family bring up the most interesting chemical and biochemical questions. For instance, the previously mentioned YopH and PTP1B differ in their catalytic rate significantly with YopH having a catalytic rate of 750 s^{-1} making it about 20-fold more active than PTP1B with a catalytic rate of 30 s^{-1} at pH 5.5 and 25°C . [26] With a superimposable active site, the same chemical mechanism, and same transition state for the reaction, the question

remains what is responsible for the extreme rate enhancement in YopH compared to PTP1B? Taking a step back there was another observation made among this family which led to the exploration of these two enzymes. Namely, while many PTPs contain a mobile WPD-loop, some PTPs contain a rigid or non-mobile loop. The PTPs that contain a rigid WPD-loop generally have a lower catalytic rate as seen in Table 2.

Combining this observation with the catalytic power of YopH compared to that of PTP1B, the question emerged whether loop motion might contribute to the rate of catalysis among this family of enzymes. At the present time in literature there exist many arguments for and against the role that dynamics play in an enzyme's catalytic power. YopH and PTP1B lend themselves to be a great case study of the effects motion can potentially play on catalytic rate. The chemistry is rate-limiting among PTPs, the transition states and mechanisms are extremely well characterized, the crystal structures of this family of enzymes are highly superimposable. Yet among all of these similarities YopH and PTP1B have a stark difference in catalytic rates. In a solution phase NMR relaxation dispersion study that focused on the role of the WPD-loop motion in the cleavage step of the reaction the following results were found and are summarized in Table 3.[17, 27] Clearly table 3 illustrates there is a distinct correlation between the rates of loop motion to the catalytic rates of the enzymes. The NMR experiments also suggested a

“...contiguous energy landscape for loop motion and catalytic activity and that loop closure is closely coupled to the protonation of the tyrosine leaving group. Additionally the nature of the occupancy of the active site modulate the WPD loop kinetics and is consistent with the dynamic energy landscape hypothesis of enzyme function.” [17, 27]

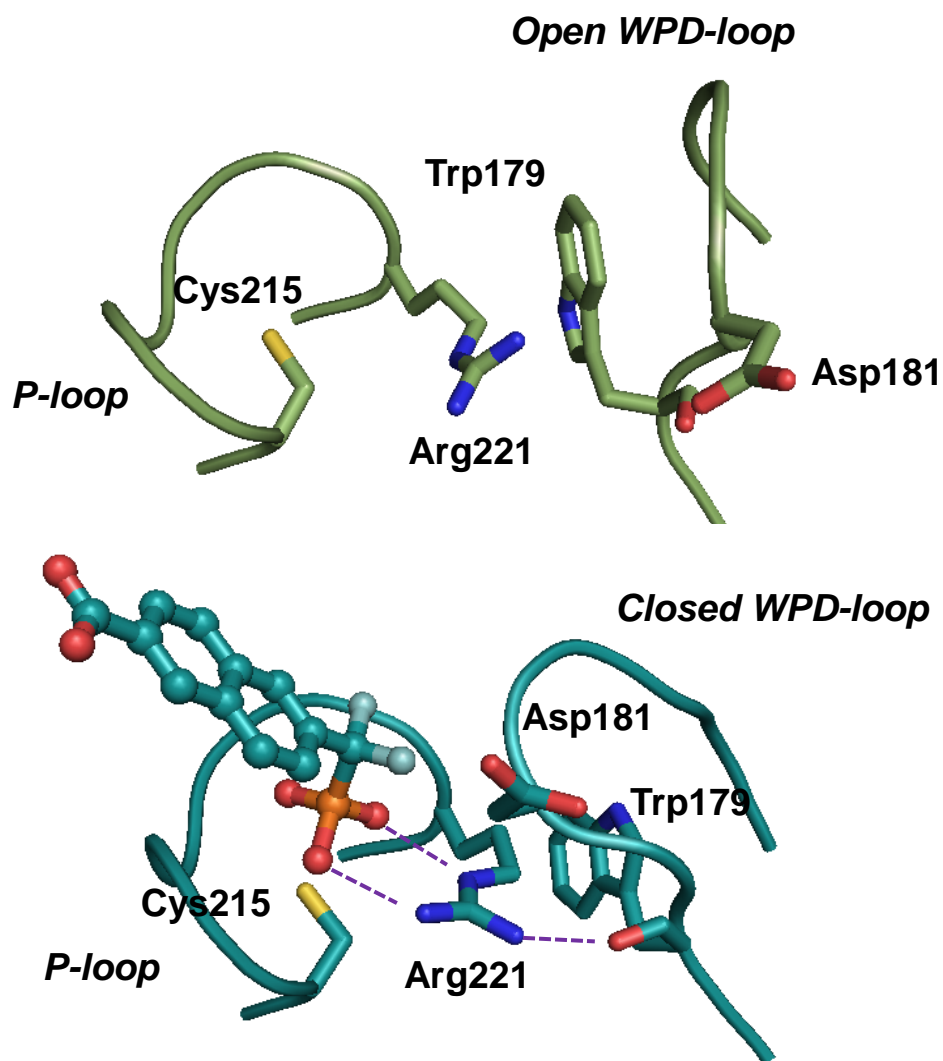


Figure 7 Top: PTP1B (2CMZ) shown in the open WPD-loop conformation with catalytically relevant residues labeled. Bottom: PTP1B (1BZJ) shown in the closed WPD-loop conformation with bound substrate analog (6-(difluoro-phosphono-methyl)-naphthalene-2-carboxylic acid) and catalytically relevant residues labeled.

Table 2 Several PTPs and their catalytic rates along with whether or not they contain a mobile or flexible loop. The k_{cat} values listed below were found using the substrate *p*NPP, at the pH optimum for each enzyme at 25°C.

enzyme	k_{cat} (s^{-1})	<i>flexible loop?</i>
YopH	750	Yes
PTP1B	30	Yes
SsoPTP	3.2	No
VHZ	3.9	No

Table 3: Summary NMR data of WPD loop motions at pH 6.5 and 23 °C. k_{close} (s^{-1}) is the rate of loop closure, k_{open} (s^{-1}) is the rate of loop opening, and $K_{\text{eq (open/close)}}$ is the ratio of open vs closed loop position. [27]

Enzyme	k_{close} (s^{-1})	k_{open} (s^{-1})	$K_{\text{eq (open/close)}}$
PTP1B ligand-free	22 ± 5	890 ± 190	40
PTP1B with peptide bound	30 ± 4	4.5 ± 1	0.15
YopH ligand-free	1240 ± 200	$42,000 \pm 6,000$	34
YopH with peptide bound	1770 ± 240	18 ± 2	0.01

ALTERED CATALYTIC RATES WITH WPD LOOP TRYPTOPHAN MUTANTS IN PTP1B AND YOPH

As previously stated the WPD loop is named from the three highly conserved residues in this loop region of the PTP family. The aspartic acid has previously been mentioned to play a catalytic role in the mechanism of the enzyme. The tryptophan, however, does not play a direct catalytic role, but does participate in the WPD loop motion. The indole side chain slides within a hydrophobic pocket within the enzyme during loop motion. Therefore, in an effort to affect loop motion, and, conversely, the catalytic rate, tryptophan mutants were designed in both YopH and PTP1B. YopH was the first enzyme in which mutations to the conserved tryptophan residue were reported. The conservative W354F mutation was made, as well as W354A. These both resulted in significant decreases in the catalytic rate compared to wildtype YopH. The pH rate profile for PTPs have a characteristic bell curve which is observed in WT YopH and WT PTP1B. The bell curve gives the pK_a of the cysteine nucleophile on the acidic limb and the pK_a of the aspartic acid on the basic limb. These mutants produced a loss of the basic limb in the pH rate profile as seen in Figure 8.[28] This loss of the basic limb is indicative of a loss in general acid catalysis for these mutants. In fact, these previously mentioned mutants both behaved in a similar manner to a D356N mutant, which completely knocks out the general acid/base function.[29]

Analogous mutations in PTP1B proved most interesting as they exhibited different effects on the catalytic rate. While W179A exhibited a large decrease in catalytic ability, the W179F mutation in PTP1B did not have as drastic of an effect on the

catalytic rate of the enzyme as in YopH, as shown in Figure 9. It is also clearly seen that the basic limb for these mutations remains intact indicative of a functional general acid.

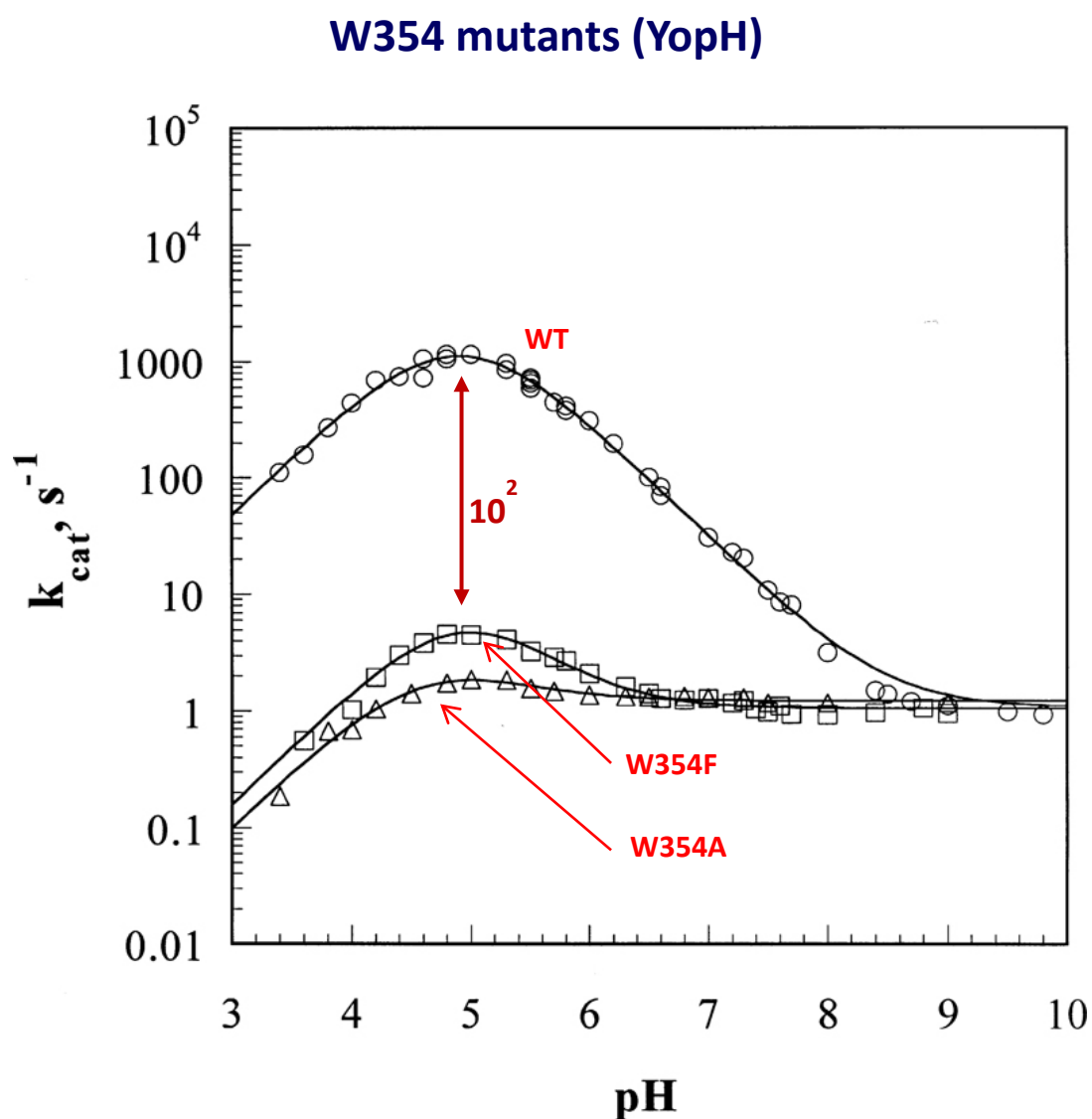


Figure 8. pH rate profiles for the following enzymes: wildtype YopH, YopH W354F, and YopH W354A. The loss of the basic limb is indicative of a loss of general acid catalysis. [29]

W179 mutants (PTP1B)

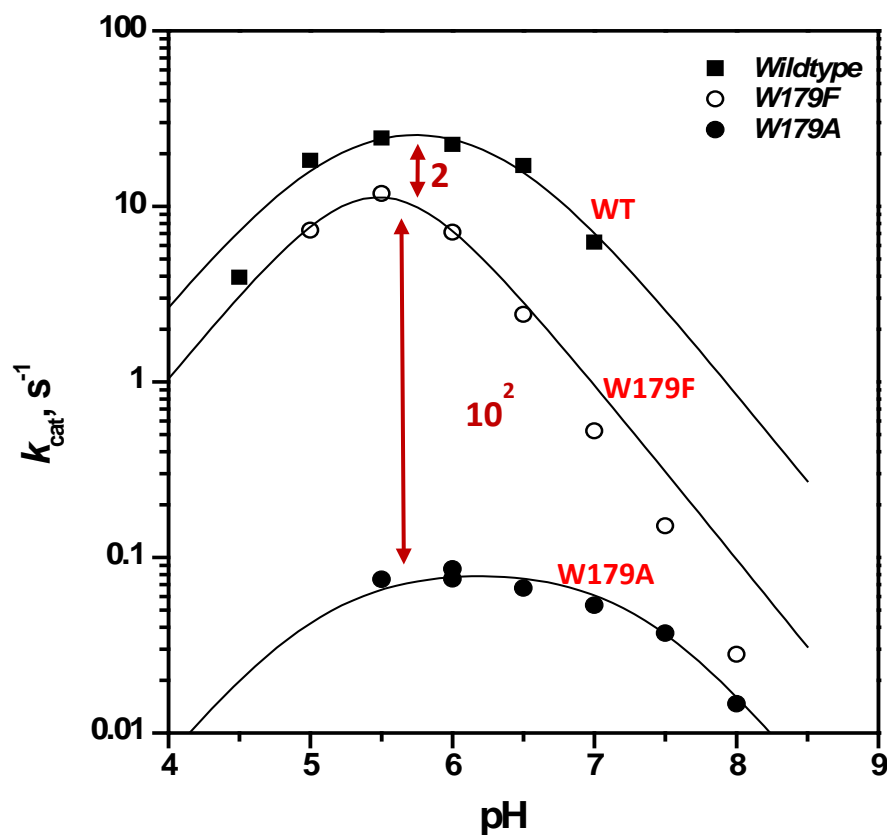


Figure 9. pH rate profiles for wildtype PTP1B and the mutants W179F and W179A. [5]

Along with different effects on the kinetic outcomes from these mutations, there were distinct differences in the structural consequences. The W354F YopH mutation rendered the enzyme's WPD loop in an apparently immobile quasi-open position, resulting in the Asp 356 amino acid residue in a position unproductive for catalysis. [5, 30] In contrast, the corresponding W179F mutation in PTP1B did not limit the enzyme's ability to achieve both normal open and closed WPD loop conformations. In both

wildtype YopH and PTP1B enzymes, the indole ring of the Trp residue resides in an embedded hydrophobic pocket and repositions upon loop closure. The movement of the indole sidechain is different in each enzyme, as seen in Figure 10. In PTP1B WT the Trp side chain slides in the same direction as WPD loop movement, while, in YopH, the side chain slides opposite to the WPD loop movement. [5] However, in the W354F YopH mutation the Phe residues slides in the same direction as WPD loop movement (Figure 9D) which may result from steric hindrance from Pro 355 and Thr 358 resulting in the quasi-open position of the WPD loop. In PTP1B W179F there is no such steric hindrance, which is potentially why this mutation in PTP1B has different kinetic and structural consequences as compared to W354F YopH.

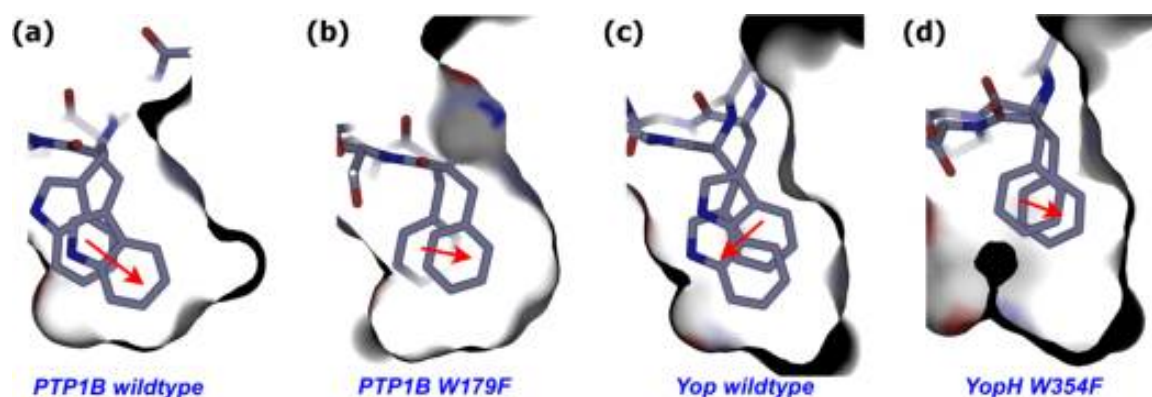


Figure 10: Comparison between PTP1B and YopH structures. Hydrophobic pocket of the conserved tryptophan in the hinge of the WPD-loop, and orientations of the Trp in the native and Phe in the mutant PTPs in open and closed WPD-loop forms. The arrows indicate the movement direction of the Trp or Phe upon WPD-loop closure. These pictures were made from PTP1B and YopH structures superimposed considering the P-loop region, no rotation was applied between each representation. The hydrophobic pockets for all representations are those for the closed or quasi-open (YopH W354F) WPD-loop conformations [30].

Further investigation of conservative mutations to the Trp residue in YopH were made by constructing the mutations W354Y and W354H. The pH rate profiles of these mutants, like that for W354F, all exhibit a loss of the basic limb, indicative of a loss of general acid catalysis. The loss of general acid catalysis was also confirmed using KIE experiments. Also, the crystal structures revealed that the WPD loop in these mutants also adopts a quasi-open position which is catalytically unproductive, as shown in Figure 12. While the WPD loop position is similar for all three mutants, the rationale behind loop closure inhibition is different for each mutant. [3]

In WT YopH, the closed WPD loop conformation is stabilized by a hydrogen bond between the indole N-H and the backbone carbonyl of T358 which none of the mutants can achieve. In W354H the histidine is in a similar position as the five-membered indole ring would be while the WPD loop is in the open position. Due to hydrogen bonding between the N-H of the imidazole ring in W354H and the backbone of P355, this mutant is stabilized in the quasi-open position. Both mutants exhibit a hydrogen bond from the carbonyl backbone of residue 354 to the side chain of P355, which, in WT YopH, is replaced by a hydrogen bond to N-H of R409 (see Figure 13). [3]

The Tyr mutant adopts a quasi-open position due to a steric clash between the hydrogens of the six-membered ring preventing the WPD loop's attaining a fully closed position. There is also a clash between the phenolic oxygen atom and the carbonyl oxygen of T358 in a putative closed WPD loop conformation as seen in Figure 14.[3]

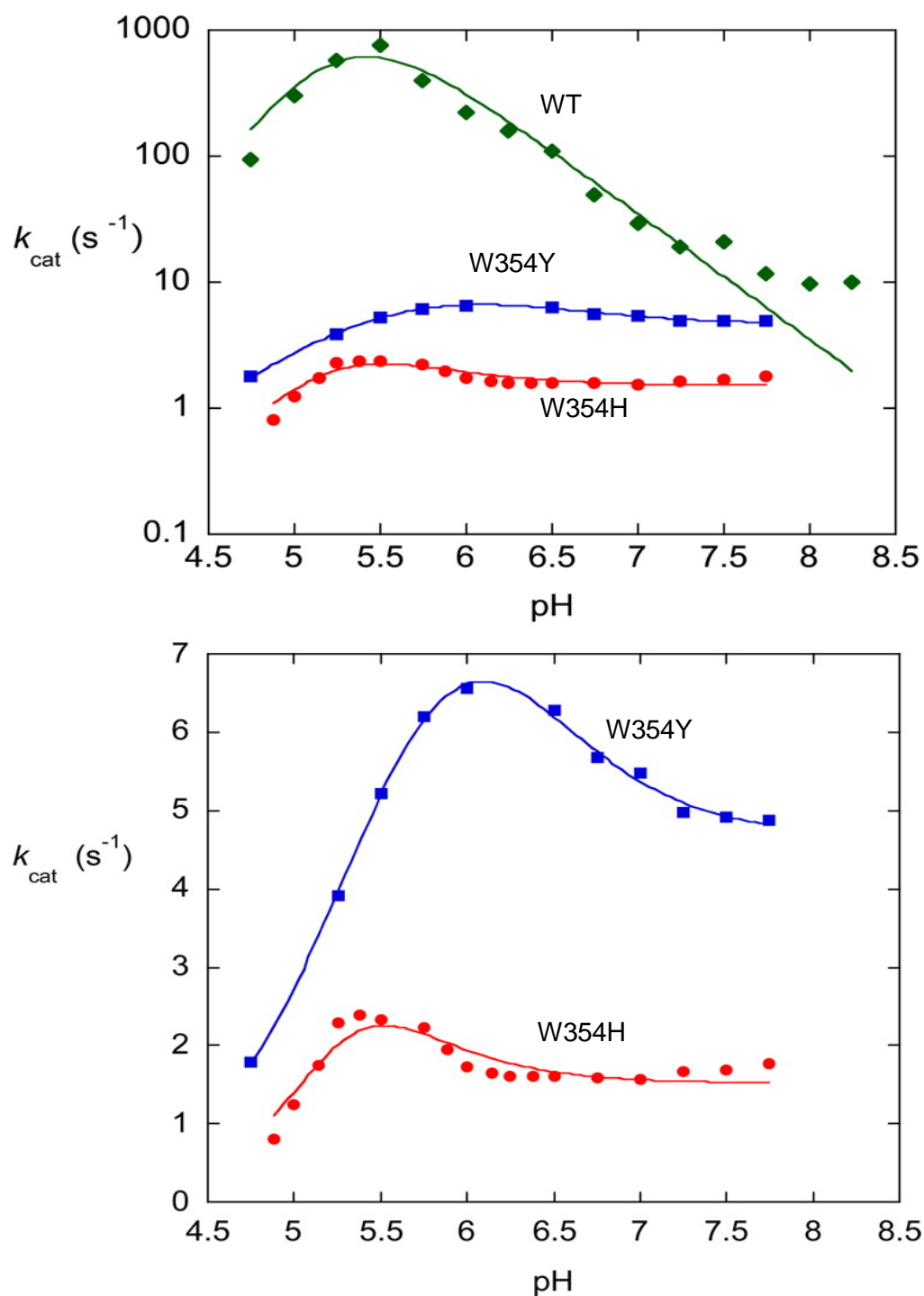


Figure 11: Top: pH rate profiles for the following enzymes: wildtype YopH, W354Y YopH, and W354H YopH. Below: focuses on the W354Y and W354H mutants. [3]

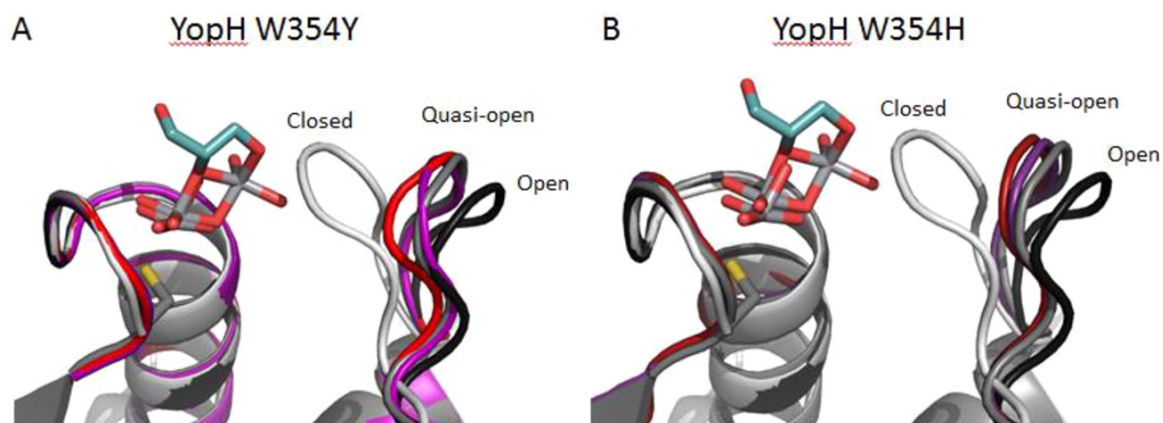


Figure 12: Comparison of WT YopH with mutants W354Y (A) and W354H (B) with vanadate (WT) or divanadate glycerol ester (mutants). The ligand-free, open-loop WT YopH is colored black and the vanadate-bound, closed-loop WT YopH light grey. The W354F mutant is colored dark gray. (A) The ligand-free W354Y structure is colored purple, and the ligand bound colored red. (B) The ligand-free W354H structure is colored purple, and the ligand bound colored red. [3]

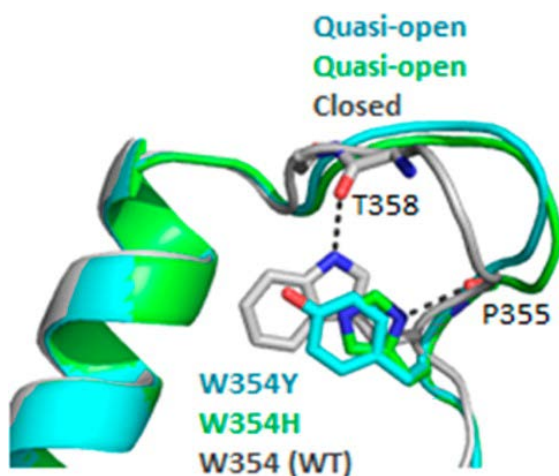


Figure 13: Comparison of the position of the side chains of YopH W354H (green) and W354Y (cyan), with the native YopH W354 (white) in the closed-loop position.[3]

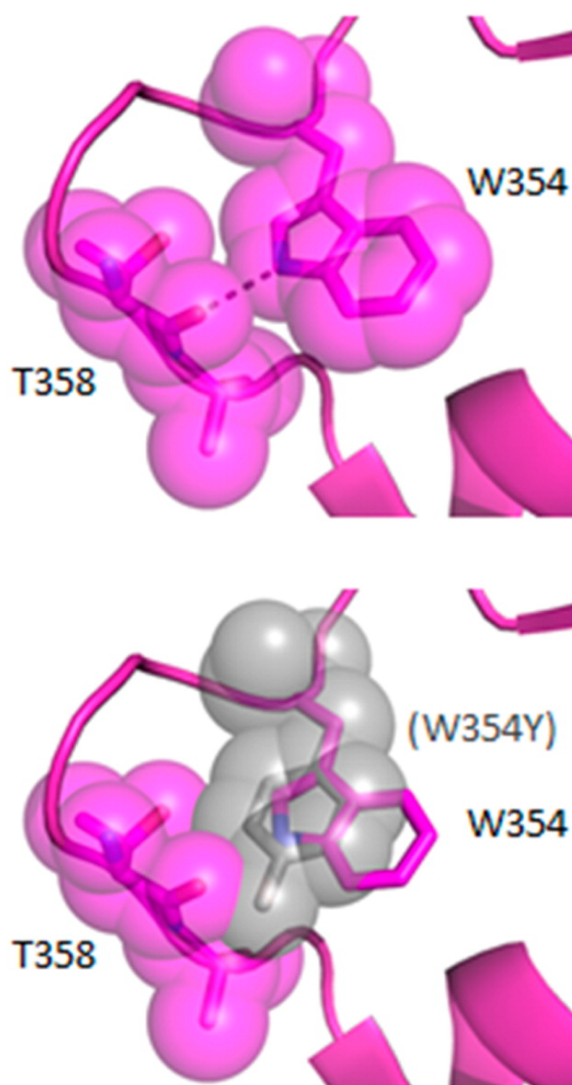


Figure 14: Full loop closure of the W354Y mutant would result in several steric clashes. The top panel shows a space-filling model of the WPD-loop-closed native YopH. The indole N-H of the native enzyme forms a hydrogen bond with the backbone carbonyl of T358. The bottom panel shows the predicted position of the W354Y side chain (gray) in the closed-loop position, assuming the residue backbone and the first two carbons of the side chain occupy the same positions as W354 of the native enzyme (purple).[3]

FURTHER INVESTIGATION OF WPD LOOP MOTION IS NEEDED TO ASCERTAIN ITS POTENTIAL TO BEING COUPLED TO PHOSPHORYL TRANSFER

The motion of the WPD loop has been shown to correlate to the rate of catalysis in YopH and PTP1B enzymes in the PTP family. Further evidence is needed to ascertain how WPD loop motion is coupled to the chemical step of phosphoryl transfer. Further investigation was done with YopH by replacing the Trp residue with conservative mutations which rendered the enzyme in a catalytically unfavorable conformation. In the YopH tryptophan mutants the most energetically favored WPD loop position was a quasi-open position. This quasi-open position left the aspartic acid residue on the WPD loop out of position to perform general acid catalysis. The quasi-open position was a result of steric interactions which also caused a decrease in WPD loop motion. Therefore separating which component, the steric hindrance or decreased WPD loop motion, had the greatest effect in causing the decrease in catalytic rate of the enzyme was inconclusive. In contrast to YopH, since the W179F mutation in PTP1B does not render the WPD loop in an immobile, catalytically disfavored conformation, this enzyme could be used to test the hypothesis that movement of the tryptophan side chain within a hydrophobic pocket regulates loop motion and catalysis.

The work in this thesis builds on the present knowledge of the role that the tryptophan residue plays in the WPD loop motion. Specifically, this examines the effect of the tryptophan residue has on WPD loop motion by attempting to either lock the WPD loop in a catalytically favorable conformation or decrease the rate of motion in the WPD loop that is still catalytically active. This is accomplished by finishing the conservative

mutations that can be made to the tryptophan residue in PTP1B which have already been done in YopH but rendered the enzyme catalytically inactive as previously explained. To this end PTP1B mutations W179H and W179Y were developed and tested. The next chapter contains the methods and materials used to create, purify, and analyze these mutants.

CHAPTER II

METHODS AND MATERIALS

MUTAGENESIS AND CLONING

The plasmid pEt-19b encoding the 37kDa form of the wildtype human PTP1B (amino acid residues 1-321) was provided by Dr. Nicholas K. Tonks as previously reported.[5, 32] Mutagenesis was carried out using the New England Biolabs Q5® Site-Directed Mutagenesis Kit. Primers were designed using NEBasechanger which is also provided by New England Biolabs to work in tandem with the kit. The following primers were purchased through Integrated DNA Technologies. DNA sequencing was used to verify the correct mutation was made during the PCR process. For W179H the following primers were used: Forward: 5'CTATACCACACATCCTGACTTTGGAGTCC 3', Reverse: 5'TGGAAATGTAAGATCTCTCG 3'. For W179Y the following primers were used: Forward: 5'CTATACCACATATCCTGACTTTGGAG 3', Reverse: 5'TGGAAATGTAAGATCTCTCG 3'. The plasmid was then transformed into BL21 DE3 competent *E-coli* cells using heat shock and plated on sterilized agar media plates containing ampicillin.

PROTEIN EXPRESSION AND PURIFICATION

Expression of PTP1B mutants in *E. coli* BL21(DE3) is under control of the T7 promoter. Purification was completed in a similar manner as previously reported with some modifications adapted from information from our collaborators in the Loria lab at Yale.[5, 7] One colony of *E. coli* BL21 (DE3) cells containing the plasmid coding for PTP1B W179H or W179Y was selected for overnight culture (30 mL). The overnight

culture was grown at 37 °C overnight. The overnight culture was then harvested by centrifugation at 4,000 RPM for 10 min then resuspended in 10 mL of LB media containing 100 µg/mL ampicillin. The resuspended culture was then further diluted into 2L growth flasks containing 800 mL of LB media containing 100 µg/mL ampicillin. The cells were grown at 37°C to an optical density of 0.85 at 600nm then induced with 0.6mM IPTG (isopropyl β-D-thiogalactoside) for no more than 12 hours at 25 °C. The cells were then harvested by centrifugation at 8,000 RPM for 20 minutes at 4 °C. Subsequent steps were carried out at 4 °C. The resulting cell pellet was resuspended in 5mL of lysis buffer for every gram of cell pellet. Lysis buffer (pH 6.5) consisted 20 mM Bis-Tris, 1 mM EDTA, 3 mM DTT, 10% glycerol and protease inhibitors (2 mM benzamidine and 2 µg/mL each of aprotinin, leupeptin and pepstatin). The cells were lysed by sonication at 60% power and 60% duty cycle and spun down at 16,000 RPM for 45 minutes. The supernatant was filtered and loaded on to a 5 mL HiTrap™ SP HP column at 1.1 mL/min with the loading buffer (20 mM bis-tris pH 6.5, 1 mM EDTA, 3 mM DTT and 10% glycerol). After all unbound protein was washed off a 5 mL HiTrap™ Q HP column was attached after the SP HP column. The protein was eluted at 1.5mL/min with a 150 mL linear gradient from zero to 0.5 M NaCl in the elution buffer (20 mM bis-tris pH 6.5, 1 mM EDTA, 3 mM DTT, 10% glycerol and 500mM NaCl). The protein fractions were pooled and concentrated to 5mL and loaded on a 320 mL HiLoad 26/60 Superdex™ column using the superdex buffer 10 mM tris pH 7.5, 25 mM NaCl, 0.2 mM EDTA and 3 mM DTT and 10% glycerol. Protein concentration was monitored by UV at 280 nm.

STEADY STATE KINETICS

Michaelis-Menten kinetics measurements were carried out using methods previously reported. [5, 7, 30] All kinetics experiments were carried out at 25°C using *p*-nitrophenyl phosphate (pNPP) and a buffer mix of the components 100mM sodium acetate, 50mM Bis-Tris, and 50mM Tris, which allows for a constant ionic strength over a wide pH range. Reactions were carried out in 96-well plates and initiated by addition of enzyme, with a final reaction volume of 305μL. Reactions were timed and then quenched by addition of 25μL 10M NaOH. The rate of formation of *p*-nitrophenolate was measured by monitoring absorbance at 400nm using the molar extinction coefficient of 18,300 M⁻¹ cm⁻¹. The background absorbance of the substrate caused from non-enzymatic hydrolysis of the substrate was corrected by measuring the respective reaction conditions in the absence of the enzyme. Kinetic parameters were then determined by the initial rate versus concentration of pNPP data fit to the Michaelis-Menten equation.

PRE-STEADY STATE KINETICS

Pre-steady state kinetics were measured using a very similar method to that already reported.[17] Measurements of *p*NPP hydrolysis by PTP1B W179H and W179Y were performed at 3.5 °C using a KinTek stopped-flow spectrophotometer. Release of *p*-nitrophenol was monitored by the increase in absorbance at 410nm in a 100mM succinate buffer at pH 6.0 using an extinction coefficient corrected for this pH which is 1486 M⁻¹ cm⁻¹. The *p*NPP concentration was 20mM and the enzyme concentration varied from 20μM to 50μM. Absorbance spectra of experiment were averaged for each substrate and enzyme concentration. The data was fit to $[p\text{-nitrophenol}] = At + B(1 - e^{-kt})$. At saturating

concentrations of substrate, $k = k_3 + k_5$. The linear steady-state phase $A = k_3k_5 / (k_3 + k_5)$. The magnitude of the burst $B = E_0 [k_3 / (k_3 + k_5)]^2 / (1 + K_M / S_0)^2$.

KINETIC ISOTOPE EFFECTS

The KIEs for these experiments were measured by methods used in previous reports. [1, 3, 5, 7, 17, 27, 30, 33-35] Kinetic isotope effects were measured using the internal competition method, and thus are isotope effects on k_{cat}/K_M which is commonly referred to as V/K . This means that the kinetic isotope effects report on a portion of the overall mechanism up to and including the first irreversible step which is phosphoenzyme intermediate formation. Natural abundance p NPP was used for the measurements of $^{15}(V/K)$. The ^{18}O KIEs were measured by a remote label method which uses the nitrogen atom in p NPP as a reporter for the isotope fractionation in the labeled oxygen positions (see Figure 15) since the labeled oxygen cannot be measured directly. Isotope effect determinations were carried out at 25°Celsius in 100mM sodium acetate, 50mM Bis-Tris, and 50mM Tris at pH 6. The p NPP concentrations were 10mM for the PTP1B W179Y mutant and 20mM for W179H. For PTP1B W179Y the reaction was initiated by addition of enzyme to a final concentration of 0.08 μ M. After reactions reached 40%-60% completion, which took about 3hrs, they were stopped by titrating to pH 3-4 with HCl. The p -nitrophenol was isolated by ether extraction and purified by sublimation. The residual substrate was completely hydrolyzed by alkaline phosphatase a pH 8.5, and subsequently treated in the same way. The $^{15}N/^{14}N$ ratios of the nitrophenol samples were measured by isotope ratio mass spectrometry for the product (Rp) and for the residual substrate (Rs), as well as in the original mixed substrate (Ro). Each experiment gives two

independent determinations of the isotope effect, one from R_s and the other from R_p . A third equation allows the calculation of the isotope effect using R_s and R_p . The isotope effects were calculated using equations 4, 5, and 6. [14]

$$\text{isotope effect} = \log (1 - f) / \log [(1 - f) (R_s / R_o)] \quad (4)$$

$$\text{isotope effect} = \log (1 - f) / \log (1 - f (R_p / R_o)) \quad (5)$$

$$\text{isotope effect} = \log (1 - f) / \log (1 - f (R_p / R_s)) \quad (6)$$

For each isotope effect, the values calculated from R_o and R_p (equation 1) and from R_o and R_s (equation 2) were averaged to give the results reported. The $^{15}\text{(V/K)}$ is given directly from these equations. In the ^{18}O isotope effect experiments the observed KIEs given by the above equations were corrected for the ^{15}N isotope effect and for incomplete levels of isotopic incorporation. For the labeled substrates and the mixtures used for the ^{18}O experiments, the levels of isotopic incorporation were determined by mass spectrometry.

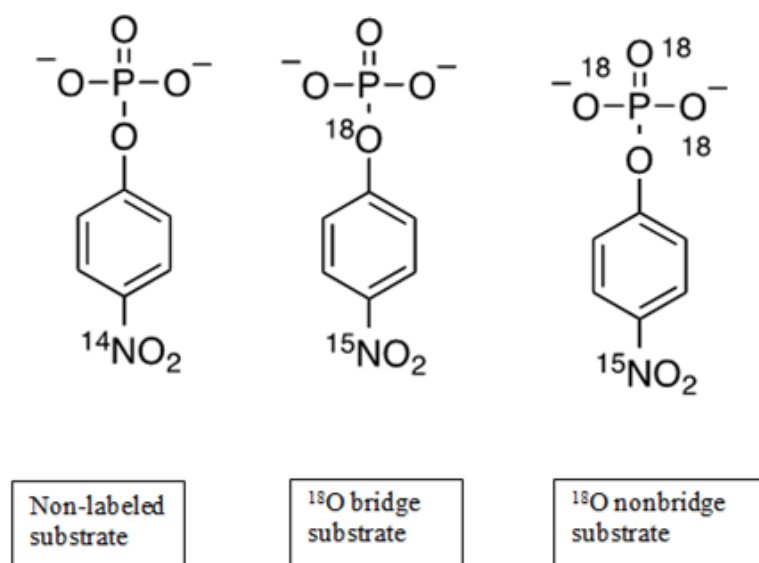


Figure 15: A mixture of these isotopic isomers was used as the substrate to measure the bridge and nonbridge ¹⁸O isotope effect.

CHAPTER III

RESULTS AND DISCUSSION

INTRODUCTION

While there exist many similarities among the PTP family it is the differences among this family which have to the potential to give rise to differences in catalytic rates. Some of the differences among this PTP family of enzyme are as follows. The amino acid sequence isn't highly conserved in the PTP family. Aside from active site residues, namely the WPD loop residues and the CX₅R residues in the p-loop, sequences among the PTP family aren't conserved.

The dynamic WPD loop bears the catalytically relevant aspartic acid necessary for general acid catalysis. The indole ring of the conserved tryptophan slides in a hydrophobic pocket upon WPD loop closure. The rate at which the WPD loop closes has been correlated to catalytic rate. Differences between the hydrophobic pockets of this family of PTP enzymes could contribute to the variation in catalytic rates. To further explain the effect of mutation and build on what is known about the tryptophan residue in PTP1B two additional tryptophan mutants were created. PTP1B W179F was previously created and studied in the Hengge lab by Tiago Brandão. To directly build on this knowledge and further explore the correlation of WPD loop motion to catalytic rate mutants W179H and W179Y were also created and the results from these mutants are presented in this work.

STEADY-STATE RATE KINETICS FOR PTP1B W179Y AND W179H MUTANTS

The W179Y and W179H mutants both exhibit Michaelis-Menten saturation kinetics with the substrate *p*-NPP. The k_{cat} value for the W179Y mutant is $5.04 \pm 0.011 \text{ s}^{-1}$ at 25 °C at pH 5.5 which is about half of the rate exhibited for the previously reported W179F mutant which had a $k_{cat} = 11.4 \text{ s}^{-1}$ at 23 °C at pH 5.5 (see Table 4). W179Y mutant's k_{cat} compares to a PTP1B wildtype k_{cat} of 24.4 s^{-1} at 23 °C at pH 5.5 which is close to approximately a four-fold reduction in rate. However the W179H mutant exhibits a slight shift in pH optimum at pH 6.5 compared to wildtype, W179Y, and W179F which all have pH optimum at pH 5.5. The W179H mutant has a drastically lower k_{cat} value of $0.1 \pm 0.009 \text{ s}^{-1}$ which is a 244-fold decrease in catalytic rate. The K_M values for the W179H and W179Y mutants at pH 6.5 are 4.6 and 3.8 mM at pH 5.5, respectively, higher than the K_M of 1 mM for WT.

Table 4: Steady-state kinetic constants for *p*NPP hydrolysis catalyzed by the wildtype PTP1B and tryptophan 179 mutants.

Construct	K_m (mM)	$k_{cat} \text{ s}^{-1}$	pH	Temperature °C
Wildtype	0.58	24.4	5.5	23
W179Y	3.8	5.04 ± 0.011	5.5	25
W179H	4.6	0.1 ± 0.009	6.5	25
W179F	0.93	11.8	5.5	23
W179A	4.41	0.075	5.5	23

Error reported for mutants W179Y and W179H are standard error. Wildtype, W179F and W179A data taken from [5].

pH RATE PROFILES OF TRYPTOPHAN MUTANTS REVEAL EFFECTS ON GENERAL ACID CATALYSIS

Native PTP enzymes exhibit bell shaped pH rate profiles with the pH optima close to 5.5. [5] The pH rate profiles help to elucidate the effects of the mutation on the enzymatic mechanism. The acidic limb of the pH rate profile is a result of the cysteine 215 residue being deprotonated to produce the active thiolate nucleophile. The basic limb of the pH rate profile is a result of reduced enzymatic activity due to the loss of general acid catalysis by the aspartic acid 181 residue. Our interest is focused more on the retention or loss of general acid catalysis in these tryptophan mutants. Since the tryptophan residue is embedded in the hydrophobic pocket and the indole ring needs to reposition upon WPD-loop closure, the effect of the general acid catalysis among these mutants is key to understanding what potential effects our mutation has on the rate of the reaction. If the mutation results in a loss of general acid catalysis, this would result in significantly reduced k_{cat} due to the enzyme's inability to protonate the leaving group during the reaction. Furthermore, the k_{cat} values would not decrease significantly above the pH optimum due to the change from a bell pH rate profile to a half bell pH rate profile.[5] Conversely, if general acid catalysis is retained then the k_{cat} change shouldn't be as drastic and the pH rate profile should maintain a bell shape.

The W179Y and W179H mutants both exhibit a bell shaped pH rate profiles as seen in Figure 16. As discussed previously this is indicative of retention of general acid catalysis. Both mutations also result in a broadening of the bell shaped curve. For ease of

comparison the wildtype PTP1B pH rate profiles along with the previously reported W179F pH rate profile are included in Figure 16 as well.

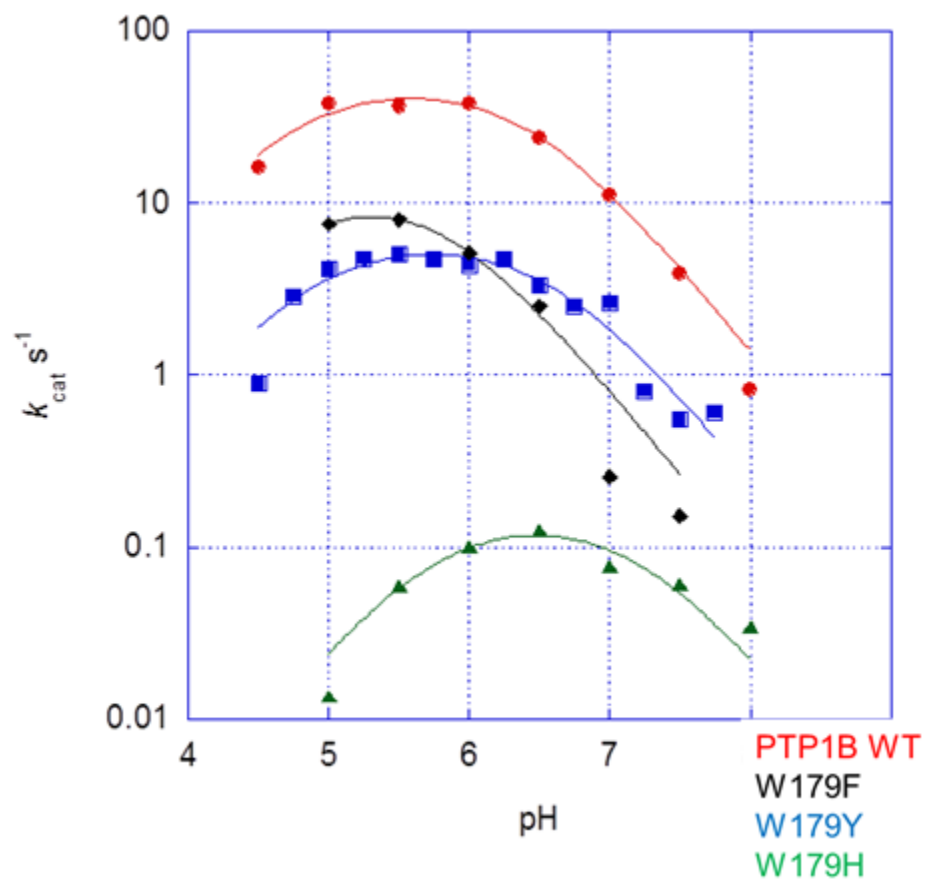


Figure 16: Effects of pH on the hydrolysis of *p*-NPP by tryptophan mutants W179Y, W179H, and W179F compared to PTP1B wildtype. PTP1B WT (red circles), W179F (black exes), W179Y (blue squares) and W179H (green diamonds).

KINETIC ISOTOPE EFFECTS REVEAL TRANSITION STATE EFFECTS OF TRYPTOPHAN MUTATIONS

The expected ranges of the isotope effects in *p*NPP and their interpretation have been discussed extensively in the first chapter of this thesis, and therefore are only summarized here. The KIE for the nitro nitrogen atom, $^{15}(V/K)$, is a result of the negative charge build-up on the nitrophenolate leaving group. When general acid catalysis is retained the potential charge build-up on the nitro group is negated by the protonation of the leaving group resulting in no isotope effect, or an isotope effect of unity. The KIE at the bridge oxygen atom, $^{18}(V/K)_{\text{bridge}}$ is a result of P-O bond fission and is also affected by the general acid catalysis due to the formation of an O-H bond. The normal isotope effect of P-O bond fission is very large with an isotope effect of $^{18}(V/K)_{\text{bridge}} = 1.03$ reflecting a largely broken P-O bond. In wildtype PTPs the $^{18}(V/K)_{\text{bridge}}$ is reduced to 1.0121 due to the protonation of the leaving group by general acid catalysis. Thus $^{18}(V/K)_{\text{bridge}}$ and $^{15}(V/K)$ are good indicators for how general acid catalysis might be compromised by mutagenesis. The KIE for the nonbridging oxygen atoms, $^{18}(V/K)_{\text{nonbridge}}$, is indicative of the hybridization state of the transferring phosphoryl group. A loose transition state exhibits a slightly inverse effect, $^{18}(V/K)_{\text{nonbridge}} = 0.995$, whereas the effect becomes more normal $^{18}(V/K)_{\text{nonbridge}} = 1.025$ for a more associative transition state. [5]

The W179Y and W179H mutations exhibit a $^{15}(V/K)$ similar to that reported for the wildtype and the phenylalanine mutation, which are close to unity. This is indicative of functional general acid catalysis during the reaction thereby carrying out full

neutralization of the leaving group. The $^{18}(\text{V/K})_{\text{bridge}}$ KIE for W179Y is larger than both the W179F and wildtype effects, suggesting a more fully broken P-O bond. The KIE $^{18}(\text{V/K})_{\text{nonbridge}}$ for W179Y is identical for that of W179F which results from a loose transition state. Due to the drastically reduced catalytic rate of the W179H mutant, $^{18}(\text{V/K})_{\text{nonbridge}}$ and $^{18}(\text{V/K})_{\text{bridge}}$ were not feasible to obtain. This was due to two reasons: (1) the high cost of the labeled substrate and (2) the amount of enzyme needed for the reaction to generate enough product to accurately measure the KIE. The KIE data for these tryptophan mutants along with the previously reported W179F mutation and PTP1B wildtype enzymes are summarized in Table 5 below.

Table 5: Isotope effects for the catalyzed reaction of *p*NPP by PTP1B wildtype, W179Y, W179H and W179F mutants.

<i>Construct</i>	$^{15}(\text{V/K})$	$^{18}(\text{V/K})_{\text{bridge}}$	$^{18}(\text{V/K})_{\text{nonbridge}}$	Reference
<i>Wildtype</i>	1.0004(2)	1.0121(9)	1.0018(5)	[7]
<i>W179Y</i>	1.0000(7)	1.0201(7)	1.0027(3)	This work
<i>W179H</i>	0.9996(2)	N/A	N/A	This work
<i>W179F</i>	1.0006(1)	1.0140(9)	1.0027(7)	[5]

Values in parenthesis are the standard errors in the last decimal place.

**PRE-STEADY STATE OR BURST KINETIC EXPERIMENTS REVEAL
TRYPTOPHAN MUTATION HAS GREATEST EFFECT ON THE SECOND
CATALYTIC STEP**

Pre-steady state or burst rate kinetics are exhibited by the YopH and PTP1B wildtype enzymes.[36, 37] The pre-steady state data also suggest that the WPD loop motion is coupled to some other process. For both of the wildtypes enzymes the burst is only resolved at 3.5°C. Increasing the temperature to 5°C and higher, the burst rate becomes too fast to be resolved. This large temperature dependence of YopH and PTP1B burst rate is unexplained, and suggests that the first chemical step may be coupled to protein dynamics or to reorganization of solvent since 4°C is the temperature at which water reaches its maximum viscosity and highest density. [38]

Pre-steady state or burst rate kinetics allow unraveling of the kinetic details of the reaction. Steady state rate kinetics can only give information on the rate determining step of the reaction. Therefore to see the effect the tryptophan mutations have on the first step of the reaction, which is the formation of the phosphoenzyme intermediate, burst kinetics experiments are used see scheme 2. Burst rate kinetics exhibit a biphasic graph in which the “burst phase” is due to the rate of the first step of the catalytic reaction or the fast steps prior to the slow step in a kinetic scheme of the reaction. The linear phase of a burst rate experiment is due to the steady state rate or the slow step of the catalytic reaction. PTP1B W179H and W179Y both exhibit a burst rate which is very similar to the wildtype enzyme (see Table 6). This is evidence that the first step of catalysis, the formation of the phosphoenzyme intermediate, is unaltered by the tryptophan mutation. However, one can clearly see that the second step, as shown previously in steady-state kinetics, is greatly affected by this mutation (see Table 4).

Scheme 2:

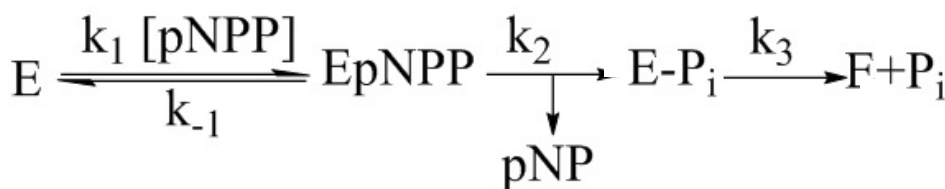


Table 6: Rate constants for PTP1B wildtype, W179Y and W179H calculated from the pre-steady state kinetics experiments obtained using Kintek stopped flow spectrometer and Sigma Plot to analyze data. The reactions were carried out under conditions of [pNPP] = 20mM and [PTP1B Wildtype], [W179Y], and [W179H] = 20μM in 100mM succinate buffer at 3.5°C. Resulting graph is the curve fit for each enzyme averaging at least 6 runs fit to the equation $[pNP] = Ax + B(1 - e^{-kx})$. As shown in scheme 2 k_2 is the rate of the first catalytic step and k_{cat} represents the rate determining step of the reaction or the steady state rate which is synonymous with k_3 from scheme 2.

<i>Enzyme</i>	$k_{cat} (s^{-1})$	$k_2 (s^{-1})$
<i>Wildtype</i>	11.1	399.4
<i>W179Y</i>	1.5	399.6
<i>W179H</i>	0.7	329.8

DISCUSSION

In the wildtype PTP1B the indole ring of the tryptophan residue is embedded in a hydrophobic pocket of the enzyme. The indole ring of the tryptophan residue repositions upon loop closure. In wildtype PTP1B, and the previously reported W179F mutant, this amino acid side chain slides in the same direction as the WPD loop motion. In contrast, the PTP YopH's indole ring slides in the opposite direction as the WPD loop motion. Similarly, tryptophan mutants of YopH have drastically reduced catalytic activity due to

loss of general acid catalysis and rendering the most stable conformation of the WPD loop in a quasi-open position.

Pre-Steady State Kinetics of PTP1B Wildtype, W179Y and W179H

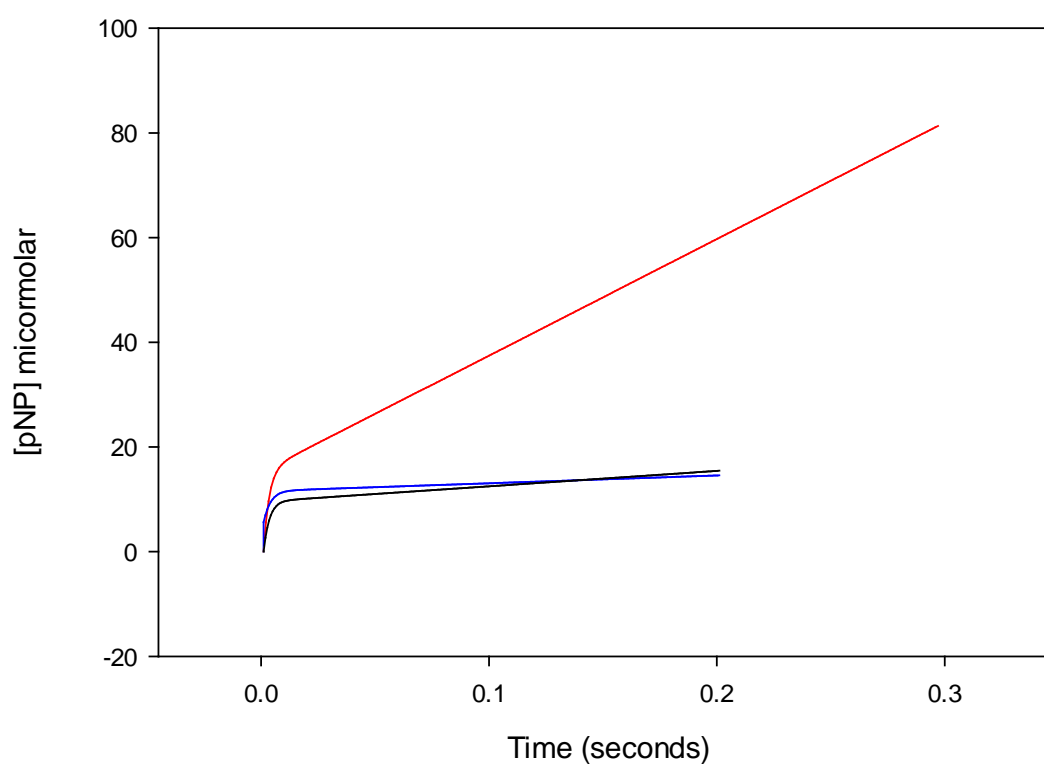


Figure 17: Pre-Steady State Kinetics of PTP1B Wildtype, W179Y and W179H. Obtained using Kintek stopped flow spectrometer and sigma plot to analyze data. The [pNPP] = 20mM and [PTP1B Wildtype], [W179Y], and [W179H] = 20 μ M done in 100mM succinate buffer at 3.5 $^{\circ}$ C. Resulting graph is the curve fit for each enzyme averaging at least 6 runs fit to the equation $[pNP] = Ax + B(1 - e^{-kx})$. Wildtype is shown in red, W179Y is shown in black, and W179H is shown in blue.

Tryptophan mutants in PTP1B, however, do not disrupt general acid catalysis, as confirmed by kinetic isotope effects and pH rate profile data. All three conservative

mutations to the tryptophan residue in PTP1B maintain a loose transition state in which the leaving group is fully neutralized, identical to that of the wildtype mechanism. The tryptophan mutants of PTP1B exhibit a loss in catalytic efficiency which is limited to the second catalytic step, as confirmed by pre-steady state kinetics. To fully elucidate the effects on the second step of catalysis for these mutants a high resolution crystal structure of both apo and ligand bound structures would be valuable. Obtaining protein crystals which diffract has been unsuccessful to date.

The ability of PTP1B tryptophan mutants to maintain catalytic function in contrast to the corresponding mutations in YopH has been attributed to the differences in the hydrophobic pocket in which the residue slides. PTP1B lacks a leucine residue which is present in YopH and could be the reason why PTP1B tolerates mutation to the tryptophan residue which slide in the hydrophobic pocket.

Also, many of the vertebrate PTPs contain two highly conserved proline residues, one on each side of the WPD loop sequence. Pro180 in PTP1B is necessary for maintaining the loop's secondary structure while Pro185 aids in the tryptophan repositioning in the wildtype enzyme. This Pro185 residue also has a polar interaction with the backbone carbonyl group of Gly 183 and the Trp 179 during the course of loop closure.

CONCLUSION

PTPs share the same chemical mechanism for catalysis and superimposable active sites, yet their catalytic rates vary by orders of magnitude. Correlation between WPD

loop motion and rate of catalysis has been strongly supported by previous studies. In this project, a further investigation of the interactions between the hydrophobic pocket and the side chain of the conserved tryptophan residue in PTP1B was carried out. The results show that this mutation largely only affects the second catalytic step in catalysis. They further confirm PTP1B's greater plasticity in the hydrophobic pocket compared to that of the virulence factor PTP YopH. Lastly, mutation to the tryptophan residue in PTP1B does not alter the mechanism and maintains general acid catalysis.

BIOLOGICAL SIGNIFICANCE

Phosphatases are some of the most efficient catalysts known. Understanding how enzymes in general obtain their high catalytic efficiencies has been studied by scientists for years. Whether or not dynamics contribute to the catalytic efficiency of enzymes remains a matter of debate among scientists. The continuation of this work could lend itself as a good case study of the interplay between protein dynamics and enzyme catalysis. Furthermore, if dynamics do in fact play a role in PTP1B's catalytic function, this could be extended to other PTPs as well as other enzymes in which motion seems to be important for catalysis.

FUTURE WORK

The next step in advancing this work as previously mentioned is to obtain protein crystal structures of the apo and ligand bound form of these mutants. The W179Y mutation has the highest probability of maintaining full WPD loop motion. This is due to the results in not only the KIE experiments but also the pH rate profile with its broad

characteristic bell shaped curve. Upon verification of two distinct WPD loop conformations similar to that of wildtype, NMR studies might also be useful with this mutant to see if the mutation alters WPD loop motion rate compared to that of wildtype PTP1B. The W179H mutation has the greatest potential for the WPD loop motion to be limited but yet able to maintain a conformation that is catalytically active since this mutant retains general acid catalysis as verified by pH rate profile and KIE experiments. The state of WPD loop motion again could be verified with this mutant by obtaining apo and ligand bound crystal structures of this mutant.

SUMMARY

The PTP family of enzymes share many defining properties: the same catalytic mechanism, superimposable active sites, and conserved active site residues, and indistinguishable transition states for the chemical step. Despite these similarities catalytic rates among PTPs vary by orders of magnitude. This variance has recently been correlated to the WPD loop dynamics within the enzymes PTP1B and YopH. My work broadens the present knowledge by examining the effect of mutations to the conserved tryptophan residue, which may control the rate at which the WPD loop moves.

Mutation to the conserved tryptophan in PTP1B produced a decrease in rate, with the greatest decrease coming from the mutation W179H. This mutant carries out catalysis of pNPP with a $k_{\text{cat}} = 0.1 \text{ s}^{-1}$ at pH 6.5 and 25°C, while W179Y has a $k_{\text{cat}} = 5.04 \text{ s}^{-1}$ at pH 5.5 and 25°C. Both mutants exhibit reduced catalytic rates while maintaining general acid catalysis along with retained mechanism of reaction. The reduction in catalytic rate has been shown to largely effect the second catalytic step, that of phosphoenzyme

intermediate hydrolysis. Whether or not the previous tryptophan mutants in PTP1B had similar effects is not yet known. Further experiments are needed to elucidate how the second catalytic step is altered in these mutants.

One very useful piece of information that has yet to be achieved is a high resolution crystal structure of these mutants. Obtaining these crystal structures would give greater insight into not only if WPD loop motion is altered in these enzymes but also positioning of the catalytically relevant amino acids within the active site of the enzyme. By obtaining this information we might be able to rationalize the decrease in rate in the tryptophan mutants.

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