Insights into the Importance of WPD-Loop Sequence for Activity and Structure in Protein Tyrosine Phosphatases

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Abstract

Protein tyrosine phosphatases (PTPs) possess a mobile, conserved catalytic loop, the WPD-loop, which brings an aspartic acid into the active site where it acts as an acid/base catalyst. Prior experimental and computational studies, focused on the human enzyme PTP1B and the PTP from *Yersinia pestis*, YopH, suggested that loop conformational dynamics are important in regulating both catalysis and evolvability. Also, work on Chimeras of YopH bearing parts of the WPD-loop sequence from PTP1B demonstrated unusual structural perturbations and reduced activity. In the present study, we have generated a chimeric protein in which the WPD-loop of YopH is transposed into PTP1B, and eight chimeras that systematically restored the loop sequence back to native PTP1B. Of these, four chimeras were soluble and were subjected to detailed biochemical and structural characterization, and a computational analysis of their WPD-loop dynamics in catalysis. These chimeras maintain backbone structural integrity, with somewhat slower rates than either wild-type parent, despite unaltered chemical mechanisms and transition states. The chimeric proteins' WPD-loops differ significantly in their relative stability and rigidity. In particular, the open WPD-loops sample multiple metastable and interconverting conformations. The time required for interconversion, coupled with electrostatic effects revealed by simulations, likely accounts for the activity differences between chimeras, and relative to the native enzymes. These differences in loop dynamics affect both the pH dependency of catalysis and turnover rate. Our results further the understanding of connections between enzyme activity and the dynamics of catalytically important groups, particularly the effects of non-catalytic residues on key conformational equilibria.

Keywords: Protein Tyrosine Phosphatases • PTP1B • YopH • Conformational Plasticity • Loop Dynamics

Introduction

Reversible phosphorylation is a common post-translational modification seen in over 30% of eukaryotic proteins.¹⁻⁵ Phosphatases work in tandem with kinases to regulate this process in a broad spectrum of organisms.³ Among the protein phosphatase families, protein tyrosine phosphatases (PTPs) are defined by a signature motif in the active site, the P-loop (HCX₅R), including a cysteine residue required for catalysis. Among the subclass of classical pTyr-specific PTPs, protein tyrosine phosphatase 1B (PTP1B) and *Yersinia* outer protein H (YopH) are the most studied. PTP1B is a human PTP whose best-known biological role is that of a negative regulator in the insulin signaling pathway,^{3, 4, 6-15} while YopH is the virulence factor of *Yersinia pestis*, responsible for the bubonic plague.^{8, 9}

PTPs catalyze dephosphorylation through a two-step mechanism that involves an essential aspartic acid, and a nucleophilic cysteine, and an arginine, both found in the P-loop. In addition to the P-loop, another conserved structural motif among the “classical” PTPs is a mobile general acid loop consisting of about a dozen residues, called the WPD-loop, defined by the conserved residues tryptophan, proline, and aspartate found near the center of the loop. Upon substrate binding, this loop moves approximately 8 Å closer to the P-loop¹⁶⁻¹⁸, bringing the aspartic acid into position to protonate the leaving group in the first step of catalysis (**Figure 1**). In this step a cysteine residue functions as a nucleophile to attack the phosphate ester while the aspartic acid protonates the aryl leaving group. This step is followed by the hydrolysis of the cysteinyl-phosphate intermediate, using a water molecule that is activated by the same aspartate. Positioning of the nucleophilic water is assisted by a glutamine residue on the Q-loop, another conserved protein element in all classical PTPs. Another loop common to the classical PTPs is the E-loop, which contains a

conserved glutamate residue that is usually found in a hydrogen bonding interaction with the conserved P-loop arginine.¹⁹

Figure 1. PTPs utilize a two-step mechanism, involving the P-loop (shown in green) and the WPD-loop (shown in orange). The WPD-loop exhibits two distinct conformations: an open, non-catalytic conformation, and a closed, catalytically active one, shown here complexed with the transition state analog vanadate. The WPD-loop closes toward the P-loop which brings the conserved aspartic acid into position to protonate the leaving group, followed by a subsequent step where the same residue acts as a general base to activate a water molecule in the hydrolysis of the phosphocysteine intermediate. The vanadate ion is shown in spheres and key catalytic side chains are shown in sticks. The WPD-loop is shown in orange, and the P-loop is shown in green. This figure is adapted from ref. ²⁰. Copyright 2021 American Chemical Society.

PTPs share the same catalytic residues, active site structure, overall mechanism, and transition state for the chemical steps. Among the “classical” PTPs with a mobile WPD-loop, the backbones for the open and closed conformations superimpose well. Despite these mechanistic and structural similarities, their catalytic rates vary over several orders of magnitude and they exhibit different pH-rate dependencies. For example, at 25 °C, YopH and PTP1B exhibit k_{cat} values of 720 s⁻¹, and 52 s⁻¹, respectively, at their pH optima.^{21, 22} While these enzymes have a similar pH optimum, PTP1B has a broader pH-rate profile²³ than YopH.²⁴

NMR dynamics studies demonstrate a correlation between the respective rates of WPD-loop closure and catalysis in YopH and PTP1B, but the underlying causes for their different dynamics are unknown.²⁵ Because only the WPD sequence is highly conserved within the ~12-residues comprising this loop in the PTP family, the residues within the mobile loops from different enzymes have different hydrogen bond networks. To explore the role of these non-conserved residues in the WPD-loop on catalytic rate and pH dependency, we have created and characterized loop-swapped chimeras. In a previous report, we described chimeras in which the WPD-loop of PTP1B was exchanged into YopH, which resulted in unexpected and counterintuitive kinetic and structural results.²⁶ This study reports the characterization of reverse chimeras, based on the transposition of the YopH loop into PTP1B. Chimera enzymatic functions were examined by steady-state kinetics and kinetic isotope effects; enzyme structures and dynamic behavior were characterized by X-ray crystallography and molecular dynamics simulations. PTP1B is shown to be more tolerant of chimera substitutions than YopH, and these chimeras do not exhibit the structural disruptions found in the YopH-based chimeras.²⁶ The combined results show how the non-catalytic amino acids within this mobile element of classical PTPs affect catalysis, WPD-loop mobility, and the equilibrium between open and closed states. This, in turn, more generally

highlights the potential for targeting non-catalytic residues as hotspots for manipulating the conformational equilibria of catalytic loops and thus regulating enzyme activity.²⁷⁻³⁰

Materials and Methods

Chemicals

Dithiothreitol (DTT) and ampicillin (AMP) were purchased from GoldBio. Restriction enzymes were purchased from Integrated DNA Technologies. Protease-inhibitor tablets were purchased from Sigma-Aldrich. All other buffers and reagents were purchased from Sigma-Aldrich or Fisher. The substrate *p*-nitrophenyl phosphate (*p*NPP) was synthesized using published methods.²² Crystallography screens, trays, and coverslips were purchased from Hampton Research.

Mutagenesis and Expression

The plasmid pEt-19b encoding the 37 kDa form of wild-type human protein PTP1B (residues 1 to 321) was provided by Dr. N. K. Tonks. The first chimera, designated Chimera 0, was made by substituting loop residues of wild-type PTP1B *via* the Q5-SDM kit (New England Biolabs), replacing with residues HVGNWPDQTAVS from the YopH WPD-loop in the corresponding region (**Table 1**). Chimera 0 was subsequently used as the DNA template for the subsequent chimeras. Chimeras 1 to 7 were created using polymerase chain reaction (PCR) with primers encoding residues before the mutation and the target mutation itself. The chimera DNA was then cleaved using restriction enzyme DpnI and ligated into the pEt-19b vector using T4 ligase. Each subsequent chimera was made using the previous chimera DNA as a template. The primers used are listed in **Table S1**.

Table 1. Loop-swapped chimeras made with the WPD-loop of YopH incorporated into PTP1B.^a

Enzyme	WPD-Loop Sequence	Solubility	Activity
PTP1B	¹⁷⁵HYTTWPD^{FGVPE}₁₈₆	Soluble	Active
YopH	³⁴⁶HVGNWPD^{QTAVS}₃₅₇	Soluble	Active
Chimera 0	HVGNWPDQTAVS	Insoluble	/
Chimera 1	HYGNWPDQTAVS	Soluble	Inactive
Chimera 2	HYGNWPDQTAVE	Insoluble	/
Chimera 3	HYGNWPDQTAPE	Soluble	Active
Chimera 4	HYTNWPDQTAPE	Soluble	Active
Chimera 5	HYTTWPDQTAPE	Insoluble	/
Chimera 6	HYTTWPDQTVPE	Insoluble	/
Chimera 7	HYTTWPDQGVPE	Soluble	Active

^a PTP1B residues are shown in red, YopH residues are shown in blue. Residues common to both are shown in black. The designation “/” in the Activity column indicates an insoluble chimera for which activity could not be assessed.

The DNA was transformed into BL21-DE3 cells and grown overnight at 37°C on an LB culture plate containing 100 µg/mL ampicillin. One colony was selected and placed into 10 mL SOC media containing 100 µg/mL ampicillin and grown overnight. The following morning, 1 L LB media containing 100 µg/mL ampicillin was inoculated with the 10 mL overnight growth and shaken at 170 rpm at 37°C until the OD₆₀₀ reached 0.6-0.8 Abs. After reaching the optimal OD, the 1 L growth was induced by 0.1 mM isopropyl β-D-thiogalactoside (IPTG) and shaken at 170

rpm at room temperature overnight. The cells were harvested by centrifugation at 12000g for 30 minutes at 4°C and stored at -80 °C.

Protein Purification

All chimeras were expressed and purified as follows, based on the WT PTP1B protocol.³¹ The cells were thawed on ice and resuspended in 10X their equivalent volume of a lysis buffer, consisting of 50 mM imidazole pH 7.5, 1 mM EDTA, 3 mM DTT, and 10% glycerol with one crushed protease-inhibitor tablet for every 50 mL of solution. The cells were lysed by sonication at 60% power for 10 pulses then mixed on ice for 1 minute and repeated 4-6 times until completely lysed. The cell lysate was centrifuged at 4°C at 29000 g for 30 minutes. The supernatant was filtered with a 0.45-micron syringe filter.

The filtrate was then purified via a 5 mL HiTrap™ Q HP column attached above a 5 mL HiTrap™ SP HP column using an FPLC filtration system. Both columns were equilibrated with lysis buffer. The cell lysate was loaded onto the columns at 1.5 mL/min, and the columns were washed with lysis buffer until the absorbance at 280 nm baselined. Elution was processed using a 100% gradient with elution buffer containing 500 mM NaCl, 50 mM imidazole pH 7.5, 1 mM EDTA, 3 mM DTT, and 10% glycerol. Eluted fractions exhibiting absorbance at 280 nm were collected and tested for phosphatase activity by addition of a few microliters of each fraction to a solution of *p*-nitrophenylphosphate (*p*NPP), where an absorption increase at 400 nM indicated release of *p*-nitrophenol. Fractions that showed activity were assayed for purity on a 15% SDS-PAGE gel.

The pooled fractions were loaded onto a desalting column and buffer exchanged into S-loading buffer (50 mM Bis-Tris pH 6.5, 1 mM EDTA, 3 mM DTT, and 10% glycerol). This solution was

then loaded at 1.5 mL/min onto the equilibrated HiTrap™ SP HP column . The column was washed with the loading buffer until Abs_{280nm} baselined, then eluted with S-elution buffer containing 500 mM NaCl, 50 mM Bis-Tris pH 6.5, 1 mM EDTA, 3 mM DTT, and 10% glycerol. Fractions with absorbance at 280 nm were collected and assayed for activity with *p*NPP, the fractions that showed activity were checked for purity on a 15% SDS-PAGE gel.

The active fractions were pooled (ranging from 30-40 mL) and concentrated to <12 mL, loaded onto a pre-equilibrated HiLoad 26/60 Superdex 200 prepgrade column (GE) and purified using 10 mM Tris buffer pH 7.5, with 25 mM NaCl, 0.2 mM EDTA, and 3 mM DTT. Fractions were assayed with *p*NPP for activity and for purity on a 15% SDS-PAGE gel. Pure protein was concentrated to 10-35 mg/mL, and either immediately used for crystallization experiments or diluted with 10% glycerol and frozen with liquid nitrogen and stored at -80°C in aliquots.

X-ray Crystallography

Crystals for Chimera 3 and Chimera 4 were grown by hanging drop vapor diffusion at 4 °C using 10-15 mg/mL protein and a precipitant solution of 0.1 M tris hydrochloride pH 6.5-8.5, 0.2 M magnesium acetate tetrahydrate, and 20-25 % PEG 8000 at a 2:2:0.5 protein:well:20% benzamidine hydrochloride drop ratio. Both the tungstate and vanadate bound structures were obtained by adding either 3.9 mM sodium tungstate (Na₂WO₄) or 1 mM sodium metavanadate (Na₃VO₄) to the protein for co-crystallization. Crystals grew in 24 hours and were transferred to a cryo-protectant solution containing mother liquor, 20% benzamidine hydrochloride, and 50% sucrose before flash freezing in liquid nitrogen.

Crystals for Chimera 7 were grown by sitting drop vapor diffusion at 4 °C using 12 mg/mL protein. The crystallization drop was prepared by mixing 2 µL of protein solution, 0.5 µL sucrose

30 % (w/v) and 3 μ L of precipitant solution (0.1 M Hepes pH 7.5, 0.2 M magnesium acetate and 15-20 % polyethylene glycol 8000). Single crystals were visible after three days. Cryoprotection was performed by transferring crystals stepwise into stabilization solution with increasing glycerol amounts to a final concentration of 15% and the respective initial concentrations of ligands present in the protein and precipitant solutions, and then flash-cooled in liquid nitrogen.

Diffraction data for Chimeras 3 and 4 were collected on the Stanford Synchrotron Radiation Lightsource (SSRL) beamline 9-2, and diffraction data for Chimera 7 were collected on a home source (Rigaku Micromax 007/Raxis IV++) (**Table S2**). Molecular replacement was performed with Phaser-MR³² as implemented in Phenix³³ using WT PTP1B (PDB ID: 3I80)²¹ as a search model for Chimera 3 and 4 structures, respectively. Data for Chimera 7 bound with Hepes structures were indexed and processed using DENZO and SCALEPACK in the HKL2000 program suite, and molecular replacement was done with Phaser as provided in the CCP4 program suite³⁴ using WT PTP1B (PDB ID 3I80²¹) with active-site water molecules manually removed. Phenix.refine was used for refinement.³⁵ Model building was performed using Coot.³⁶ All figures of the enzyme structures and structural alignments therein were made using Pymol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.).

Steady-State Kinetics

Steady-state kinetic parameters were measured at 25°C. Concentrated protein aliquots were thawed on ice and diluted with a buffer base mix (BBM) containing 50 mM sodium acetate, 100mM tris, and 100 mM bis-tris from pH 4.35-7.5. This buffer system maintains constant ionic strength throughout the pH range examined. A 50 mM solution of the dicyclohexylammonium salt of *p*NPP was prepared in the buffer base mix. Reactions were run on 96-well plates, diluted enzymes were added to reactions with substrate concentrations from 0.76-22.73 mM and allowed

to proceed for 10 minutes. The reactions were quenched using 50 μL of 10 M NaOH, and the amount of the product *p*-nitrophenol was assayed from the absorption at 400 nm using the molar extinction coefficient of 18,300 $\text{M}^{-1}\text{cm}^{-1}$. Reaction blanks were made using identical conditions replacing the enzyme with buffer, to correct for non-enzymatic hydrolysis of the substrate. The amount of product released and elapsed time were used to calculate the initial rates. The data were fitted to the Michaelis-Menten equation to obtain steady-state kinetic parameters. Kinetic data were obtained on Chimeras 3, 4, and 7 as a function of pH to obtain pH rate profiles. The bell-shaped pH rate profiles were fitted to Eq. 1 and 2 for two ionizable residues, one protonated and the other deprotonated. In Eq. 2, K_{S2} was set to the second ionization constant of the substrate *p*NPP ($pK_a = 4.96$).³⁷

$$k_{cat} = \frac{k_{cat}^{lim}}{\left(1 + \frac{[H^+]}{K_{E1}} + \frac{K_{E2}}{[H^+]}\right)} \quad (1)$$

$$\frac{k_{cat}}{K_m} = \frac{\left(\frac{k_{cat}}{K_m}\right)^{lim}}{\left(\left(1 + \frac{[H^+]}{K_{S2}}\right)\left(1 + \frac{[H^+]}{K_{E1}} + \frac{K_{E2}}{[H^+]}\right)\right)} \quad (2)$$

Magnesium inhibition assays were carried out with *p*NPP as the substrate using the same methods, using enzyme that was incubated with 0-4.55 mM magnesium acetate on ice for 5 minutes prior to the assay.

Kinetic Isotope Effects (KIEs)

The ^{18}O KIE in the scissile P-O bond and the ^{15}N KIE in the leaving group were measured using the competitive method and isotope ratio mass spectrometry.^{21, 22} **Figure S1** shows the positions in the substrate where KIEs were measured and the isotopic isomers used.

KIEs measured by the competitive method are isotope effects on V/K , the part of the mechanism up to the first irreversible step. For PTPs, this is the first chemical step shown in **Figure 1**, cleavage of the *p*NPP substrate.^{22, 24} Concentrated protein aliquots were thawed on ice and diluted to 9.26 and 17.45 μ M for Chimera 3 and Chimera 4 respectively in reaction with the buffer base mix. Natural abundance *p*NPP was used for measurements of $^{15}(V/K)$. ^{15}N , ^{18}O -labeled *p*NPP used for measurement of ^{18}O isotope effects by the remote label method were synthesized using previously published methods.³⁹ Isotope effect determinations were carried out in triplicate, at 25 °C in 100 mM Bis-tris at pH 5.5, by adding 200 μ L enzyme and 108 micromoles *p*NPP in 5 mL of buffer and allowed to react until approximately 50% completion, approximately 2-3 hours. At this point, the enzymatic reactions were stopped by titration to pH 4 with HCl. The *p*-nitrophenol product was extracted by ether and purified by sublimation. The residual substrate remaining in the aqueous layer was completely hydrolyzed using bovine alkaline phosphatase at pH 9, and the *p*-nitrophenol released was isolated in the same way. The KIEs were calculated from the nitrogen isotopic ratios in the *p*-nitrophenol product at partial reaction (R_p), in the residual substrate (R_s), and the starting reactant (R_o). Eq. 3 was used to calculate the observed KIE from R_s and R_o at fraction of reaction f , and Eq. 4 from R_p and R_o . These were the same within experimental error and averaged to give the final results.

$$KIE = \frac{\log(1-f)}{\log\left[(1-f)\left(\frac{R_s}{R_o}\right)\right]} \quad (3)$$

$$KIE = \frac{\log(1-f)}{\log\left[1-f\left(\frac{R_p}{R_o}\right)\right]} \quad (4)$$

Molecular Dynamics Simulations and Analysis

Molecular dynamics (MD) simulations of wild-type (WT) PTP1B as well as the soluble proteins Chimeras 1, 3, 4 and 7, were performed using the Amber18⁴⁰ simulation package. All

simulations were performed in the phosphoenzyme intermediate state (which represents the reactive species for the second chemical step, **Figure 1**) and were performed for each enzyme starting from both the WPD-loop closed and open conformational states, using the PDB⁴¹ structures outlined in **Table S3** for each system and state. Simulation starting structures of WT PTP1B were prepared as described previously,⁴² and Chimeras 1, 3, 4 and 7 were also prepared using the available crystal structures from both this and a previous study⁴³ where possible. For simulations of systems with no available crystal structure, the most closely related (in terms of WPD-loop sequence) crystal structure was modified (through PyMOL mutagenesis) to generate the starting structure (no more than three mutations were required for any system, see **Table S3** for further details).

All simulations were performed under periodic boundary conditions with octahedral water boxes using the ff14SB⁴⁴ force field and TIP3P⁴⁵ water model alongside our previously developed parameters to describe the phosphorylated cysteine.⁴² Following the equilibration of each system in the NPT ensemble (298 K, 1 atm, see the **Supporting Information**), production MD simulations of each enzyme in both WPD-loop conformational states were performed for twenty replicas of 100 ns each (**Figures S2 and S3**).

MD simulation analysis were performed using CPPTRAJ.⁴⁶ The WPD-loop (for C α RMSD calculations) was defined as residues 176-190 of PTP1B. Hydrogen bonds were defined as being present if the donor-acceptor distance was ≤ 3.5 Å and if the donor-hydrogen-acceptor angle was $180 \pm 45^\circ$. The R⁴⁷ software package was used to perform the two-sample *t*-tests on the C α RMSF calculations, using the twenty replicas performed per complex and a *p*-value significance cut-off of less than 0.05.

Results

Chimera Construction and Expression

Table 1 shows which residues were initially swapped from YopH and their mutation back to native PTP1B residues in subsequent chimeras; the active site hydrogen bonding patterns in wild-type PTP1B are shown in **Figure 2**. In the initial chimera construct, designated Chimera 0, eleven residues in the PTP1B WPD-loop region were swapped for the corresponding residues from YopH. This protein was insoluble, potentially due to unfavorable interactions involving the hydrophobic sidechain of Val176. The corresponding residue in native PTP1B is Tyr176, whose hydroxyl group forms a hydrogen bond with the sidechain of Ser190. An analogous insolubility outcome from swapping this residue was noted in a YopH chimera, as previously reported.²⁶ Accordingly, Val176 in the chimera construct was restored to Tyr as in native in PTP1B. This protein, Chimera 1, was soluble but inactive. Some of the subsequent chimeras were insoluble, for reasons that are not evident, and were not characterized. Chimeras 3, 4 and 7 were soluble, catalytically active, and were kinetically and structurally characterized. The final Chimera 7 carries a single amino acid substitution F182Q. This residue is adjacent to the general acid; previous work has hypothesized that the reorientation of the peptide bond joining these residues affects the energy barrier for loop movement.^{48, 49} YopH is unusual in having a glutamine in the Asp + 1 position while most members of the classical PTP family have a conserved histidine in this position (**Figure S4**).

Figure 2. Active site hydrogen bonding differences between (A) ligand-free (PDB ID: 2CM2⁵⁰) and (B) ligand-bound (PDB ID: 3I80²¹) WT PTP1B. The WPD-loop is shown in orange, and the phosphate-binding P-loop is shown in green. The sidechain conformation of E186 changes between the open and closed forms of the WPD-loop.

Kinetic Characterization

Chimeras 3, 4 and 7 are slower than either of the parent enzymes, but exhibit the bell-shaped pH-rate profile characteristic of the PTP family⁵¹ (**Figure 3**). In Table 2 the pH optima and kinetic constants for the chimeras are compared with their parent enzymes, with the most active YopH-based chimera from a previous investigation²⁶, and several other native PTPs. The Chimera 3 and 4 turnover rates (k_{cat}) differ by approximately 10-fold despite their sequences differing by a single residue, in position 177 (**Table 1**). The identity of the residue at this position was recently shown to affect the conformational dynamics of native PTP1B, causing a population shift towards a catalytically-active closed conformation of the WPD loop even in the unliganded form of the enzyme⁵². Chimera 7 (the F182Q point mutant) exhibits a rate about an order of magnitude slower than WT PTP1B in k_{cat} , with no shift in the pH optimum, but a broader maximum than the native enzyme.

Figure 3. pH rate profiles for WT PTP1B, WT YopH, and Chimeras 3, 4 and 7. Steady-state kinetics for the chimeras show restoration of mechanism and imply maintained general acid catalysis from functional WPD-loop closure. Note the logarithmic scale on the y-axis.

Table 2. Kinetic data for native PTPs and Chimeras with *p*NPP at 25 °C.^a

Enzyme	pH Optimum	$k_{\text{cat}}^{\text{lim}}$ (s ⁻¹)	K_M (mM)	$k_{\text{cat}}/K_M^{\text{lim}}$ (mM ⁻¹ s ⁻¹)	Reference
PTP1B	5.5	24.4 ± 0.4	0.58 ± 0.01	37.7 ± 1.9	²¹
YopH	5.5	720 ± 25	0.98 ± 0.11	735	²²
YopH Chimera 3	5.5	5.10	5.69	0.90	²⁶
Chimera 3	6.0	1.8 ± 0.1	6.9 ± 2.0	0.26 ± 0.02	This work
Chimera 4	5.5	15.4 ± 0.8	8.7 ± 3.2	5.54 ± 0.77	This work
Chimera 7	5.5	2.5 ± 0.4	0.39 ± 0.02	11.4 ± 4.0	This work

^a The k_{cat} and K_M numbers are the limiting values obtained from Eqs. 1 and 2. K_M values are obtained at the pH optimum for each enzyme.

The kinetic pK_a values obtained from fits of the pH-rate data using Eq. 1 reflect the nucleophilic cysteine, Cys215 (pK_{a1}), and the general acid, Asp181 (pK_{a2}). The values differ from those of the WT parent PTP1B and between the Chimeras (**Table S4**). Visual inspection of the pH-rate profiles shows the broader ones of Chimera 3 and 7 more closely resemble that of native PTP1B, while the narrower profile of Chimera 4 more resembles YopH.

Magnesium Inhibition

Magnesium is reported to have a modest activating effect on native PTP1B.⁵³ Steady-state kinetic data showed an inhibitory effect of Mg^{2+} on Chimera 4 (**Figure S5**). The substrate concentration versus velocity data were fitted to the competitive, uncompetitive, and mixed inhibition models. The mixed inhibition model gave the best fit results and was used to obtain the K_i value of 4.8 ± 0.2 mM.

Kinetic Isotope Effects

The KIEs (**Table 3**) verify that general acid catalysis is operational in each of the active chimeras. The near-unity ^{15}N KIEs for Chimera 3 and 7 show the leaving group is fully neutralized in the transition state, as in the native enzymatic reaction. Chimera 4 shows the presence of a partial charge on the leaving group in the transition state of the first step of catalysis, but the value is within the range of data from other native PTPs. Removal of the general acid by mutation from Asp to Asn results in much higher KIEs in both labeled positions. The KIEs for the chimeras are within the bounds of past results from WT PTPs⁵⁴, indicating a functional general acid and conventional mechanism.

Table 3. Kinetic isotope effects the active chimeras together with those of native YopH and PTP1B. The range of KIEs is provided for a broader set of enzymes in the PTP superfamily including dual-specificity phosphatases; the specific enzymes are given in the reference indicated.^a

Enzyme	¹⁵ (<i>V/K</i>)	¹⁸ (<i>V/K</i>) _{bridge}	Reference
PTP1B	1.0004(2)	1.0121(9)	21
YopH	0.9999(3)	1.0152(6)	22
Range of WT PTPs	0.9999 – 1.0013	1.0118 – 1.0152	54
D to N mutants	1.0024 – 1.0030	1.0275 – 1.0297	54
Chimera 3	1.0001 (5)	1.0138 (8)	This work.
Chimera 4	1.0011 (6)	1.0129 (25)	This work.
Chimera 7	1.0004 (1)	1.0146 (5)	This work.

^a The values in parenthesis are the standard errors in the last decimal place. The D to N mutant entries show how the isotope effects are affected when general acid catalysis is lost.

X-Ray Structural Characterization

Figure 4 shows the P-loops and WPD-loops of Chimeras 3, 4 and 7 alongside those of native PTP1B and YopH. In their vanadate complexes, the WPD-loops of Chimeras 3 and 4 are in the closed conformation and are highly superimposable with WT PTP1B. Unlike the extended α -helix and unnatural loop position observed in chimeras of YopH²⁶, Chimeras 3, 4 and 7 maintained structural integrity, further confirming that PTP1B is more tolerant to mutations than YopH⁵⁵.

Figure 4. WT PTP1B and YopH structures are shown in panels A, C, and E, while Chimera 3, 4, and 7 structures are shown in panels B, D, and F. **(A)** PTP1B and YopH have superimposable active site secondary structures, however, YopH lacks the loop structure containing K116 in PTP1B. **(B)** Chimeras 3, 4, and 7 have retained structural integrity in the active site; structures of the free forms of Chimera 3 and 7 indicate a shift to a more thermodynamically favorable closed conformation of the WPD loop (as discussed below, Chimera 7 crystallized with a molecule of HEPES buffer in the active site) **(C, D)** VO_4 bound WT and structures revealed second step transition state analogs with a trigonal bipyramidal geometry in which the active site cysteine is one of the apical ligands. **(E, F)** WO_4 -bound Chimeras 3 and 4 showed unconventional sidechain conformations of Asp181. The sidechain of K116 and the presence of a

magnesium ion is suspected to direct the Asp carboxylate away from the active site in these two structures. WT PTP1B is colored in orange, WT YopH in dark green, Chimera 3 in blue, Chimera 4 in yellow, and Chimera 7 in light green.

Ligand-free Structures

The ligand-free structure of Chimera 3 shows the WPD-loop in the closed conformation, while the Chimera 4 structure shows its WPD-loop in the fully open position, as in WT PTP1B. Although ligand-free structures of PTPs typically show the WPD-loop in the open conformation, there is a previous X-ray structure of WT PTP1B with the loop closed along with discontinuous electron density indicating a partial occupancy of the loop open conformation.⁵⁶ However, compared to that ligand-free loop-closed structure, there is no evidence for partial occupancy of an open conformation in the electron density of the Chimera 3 structure. The sidechain conformation of Asp181 is oriented away from the P-loop, stabilized by hydrogen bonding with the sidechain of Lys116 (2.9 Å), as observed in the tungstate complex. Chimera 3 has the T to G substitution at residue 177, and a previous study of the T177G variant of PTP1B found this single substitution results in a preference for the WPD-loop closed conformation.²⁰

The crystal structure of Chimera 7 showed the presence of a HEPES molecule from the crystallization buffer at the active site (**Figure S6**). The sulfonyl group makes hydrogen bonds to P-loop residues and to Arg221, analogous to those of a phosphate ester substrate, and the WPD-loop is in the closed conformation. The alkyl ring of the HEPES molecule is in a position that would clash with the side chain of F182 in native PTP1B. Other details of HEPES interactions are shown and discussed in the **Supporting Information**.

Vanadate-Bound Structures

Vanadate is notable for its ability to adopt a trigonal bipyramidal geometry, providing a structural transition state analog for phosphoryl transfer. The vanadate-bound crystal structures show an apical interaction with the sulfur of cysteine and a trigonal bipyramidal geometry, an analog of the transition state for the second step shown in **Figure 1**. The active sites in the vanadate-bound complexes of Chimeras 3 and 4 are highly superimposable, with an RMSD of 0.07 Å when aligning 8 P-loop residues, H214-R221. The vanadium-sulfur distance is 2.6 Å and the apical V-O distance is 1.8 Å. These compare well to the corresponding distances in the vanadate structure of WT PTP1B, where the same TS analog has a V-S distance of 2.5 Å and V-O (apical) distance of 2.1 Å.²¹

A published structure of PTP1B complexed with the vanadate ester of the peptide DADEYL shows a difference in the orientation of residue Gln262 in the two chemical steps of catalysis.²¹ In the first cleavage step, the Gln262 sidechain points away from the P-loop, while the Tyr in the DADEYL peptide is covalently bound to the central vanadium atom. In the transition state analog of the second hydrolysis step, the leaving group peptide is absent, allowing the Gln262 sidechain to orient towards the vanadate and form a hydrogen bond with the apical oxygen, consistent with the accepted role of this residue in positioning the nucleophilic water in the second step (TSA1, PDB ID: 3I7Z, TSA2, PDB ID: 3I80).²¹ In the vanadate-bound structures of Chimeras 3 and 4, Gln262 is similarly oriented and hydrogen bonds to the apical oxygen of vanadate, with the distance of 2.6 Å.

Tungstate-Bound Structures

Conventionally, the WPD-loop Asp sidechain in ligand-bound structures of both WT PTP1B and YopH is oriented towards the P-loop, where it protonates the leaving group oxygen of the bound substrate. However, in the tungstate-bound chimera structures, this side chain is pointing away from the active site, despite the lack of any significant change in the conformation of the WPD-loop backbone. This atypical aspartate sidechain conformation is facilitated by hydrogen bonding with Lys116 and Gln182, and by coordination with a magnesium ion from the crystallization solution. This conformation explains the inhibitory effect of magnesium.

Molecular Dynamics Simulations

Analysis of the conformational stability of the open and closed states of the WPD-loop (**Figure 1**) shows that the closed conformation has similar stability (over these timescales at least) for WT PTP1B and for all chimeras studied. Specifically, the root mean square deviation (RMSD) of the WPD-loop C α -atoms are <1.5 Å for the majority of simulation time for all structures (top panel of **Figure 5A**). In contrast, simulations of the open WPD-loop showed large differences in the relative stability of the open WPD-loop conformation across the PTPs. Both WT PTP1B and Chimera 7 (F182Q), have a (relatively) highly stable open WPD-loop conformation, whilst Chimeras 1, 3 and 4 are all notably less stable (bottom panel of **Figure 5A**).

Figure 5. (A) Histograms of the WPD-loop C_{α} RMSD for simulations of the WT PTP1B and all 4 Chimeras studied, starting from either from the closed WPD-loop conformation (top panel) or open WPD-loop conformation (bottom panel). (RMSD is measured to the relevant starting crystal structure). (B) Plots of the WPD-loop C_{α} RMSD for simulations starting from the open WPD-loop conformation, with the reference structure used the closed WPD-loop. (A low RMSD value would therefore indicate a transition to the closed WPD-loop conformation.) The dotted lines across each plot indicate the start/end of an individual replica (20 x 100 ns long replicas were performed per complex).

Furthermore, it appears that there are multiple instances (*i.e.*, it occurred in multiple independent replicas) in which MD simulations of Chimeras 1, 3 and 4 transitioned from the open to closed WPD-loop conformation, even on 100 ns simulation timescales (**Figure 5B**), suggesting this conformation is strongly favored. Whilst this was also observed in either 1 or 2 replicas (of 20 replicas each) for both WT PTP1B and Chimera 7 (**Figure 5B**), histograms of this data clearly demonstrate Chimeras 1, 3 and 4 to exchange from the open to closed WPD-loop conformation significantly more frequently (**Figure S7**).

Figures S8 and S9 show the C_{α} root mean squared fluctuations (RMSF) for each PTP in both the closed and open WPD-loop conformational states. The corresponding difference in RMSF (Δ RMSF) of each residue between WT PTP1B and a given Chimera, with the results for the closed WPD-loop, are presented in **Figure S10A**. To ensure the differences in flexibility that we identified were meaningful, we performed a two sample *t*-test on the Δ RMSF values, projecting only those that were identified as significantly different onto **Figure S10A**. To relate any identified significant Δ RMSF values to changes in the protein interaction network, we also calculated how the hydrogen bonding (H-bonding) network differed for WT PTP1B to each Chimera, with differences for the closed WPD-loop simulations presented in **Figure S10B**.

Focusing first on Chimera 7, our Δ RMSF data demonstrate the flexibility/rigidity of Chimera 7 and WT PTP1B to be highly similar (**Figure S10A**). Likewise, our H-bonding analysis only identifies one major difference, which is the newly formed hydrogen bond (H-bond) between F182Q and D181 in Chimera 7. For Chimeras 1, 3 and 4, however, we identify significant differences in the rigidity of not just the WPD-loop but also other nearby regions including the E-loop and Loop 11 compared to WT PTP1B (**Figure S10A**). Chimera 4's WPD-loop and part of the E-loop became more rigid for example. This observation is consistent with our RMSD data showing a "tighter" histogram (*i.e.*, meaning it samples a narrower range of conformations) for the closed WPD-loop conformation of Chimera 4 compared to the WT and all other Chimeras (**Figure 5A**). The new intra WPD-loop H-bond between G183T and P180 is likely partially responsible for this rigidification alongside the new side chain H-bond between T178N and T177 (**Figure 5B**).

Chimeras 3 and 4 differ in sequence by the single point mutation T177G. This mutation increases the flexibility of both the N- and C-terminal portions of the WPD-loop and the nearby Loop 11 (**Figure S10A**). Both our RMSD and RMSF analysis suggest both the overall WPD-loop

stability (**Figure 5A**) and flexibility (**Figure S10A**) are highly similar for Chimeras 1 and 3, which are separated by only two point mutations, P185V and E186S.

Figure 6 shows analysis of the analogous flexibility and hydrogen bonding networks of the open WPD-loop conformation. Once again, Chimera 7 and WT PTP1B have highly similar RMSFs and H-bonding interaction networks in the open WPD-loop conformation (**Figure 6**). In contrast to Chimera 7, the open WPD-loop conformation of Chimeras 1, 3 and 4 appears notably more unstable with increased RMSF values on large parts of both the WPD- and E-loops (**Figure 6A**). Analysis of the differences in the H-bonding networks of these Chimeras to WT PTP1B (**Figure 6B**) would suggest the mutation G183T (present in Chimeras 1, 3 and 4) is primarily responsible for this effect. That is, the new hydrogen bond between the side chain alcohol of G183T to D265 appears to “pull” the WPD-loop and E-loop apart, substantially weakening many key interactions (relative to WT PTP1B) between these loops (**Figure 6B**), thereby increasing their observed flexibility, and likely giving rise to the overall reduced stability of this conformation as seen in our RMSD analysis (**Figure 5**).

Figure 6. (A) Color mapping of the statistically significant differences (WT – chimera) in the calculated per residue C_{α} RMSF (Δ RMSF) for the simulations of the open WPD-loop. Color mapping was performed from blue (positive Δ RMSF) through to white (0 Δ RMSF value or not significantly different) to red (negative Δ RMSF). In practice, a blue residue would mean increased rigidity for the given chimera variant over WT PTP1B and vice versa. Statistical significance was defined by a two-sample t -test ($p < 0.05$), using the 20 replicas performed per complex, (see **Figure**

S9 for this data in graphical form). **(B)** Differences in the hydrogen bonding network between WT PTP1B and each chimera for simulations of the open WPD-loop conformation. Hydrogen bonds with a higher occupancy in the WT are shown as black cylinders between the donor and acceptor atoms, with red cylinders used to indicate H-bonding interactions which have a higher occupancy in a given chimera. The width of the dash indicates the magnitude of the difference in the occupancy of the hydrogen bond between the two enzymes. The P-loop, Q-loop and E-loop are colored green, magenta and orange respectively, with the WT PTP1B WPD-loop colored cyan and chimera WPD-loop colored yellow.

We note here that in a recent study of WT PTP1B and YopH we demonstrated that motions of the WPD- and E-loops are concerted, with increased flexibility of the E-loop leading to reduced flexibility of the WPD-loop (and *vice versa*).⁴² Moving from Chimera 4 to Chimera 3, we observe further destabilization of both the WPD- and E-loop, and in particular at the N-terminal portion of the WPD-loop. Given the nature and location of the mutation that separates Chimeras 4 and 3 (T177G, located at the N-terminus of the WPD-loop) it is perhaps not surprising to see this effect. Finally, differences between Chimera 3 and Chimera 1 are largely limited to the C-terminal portion of the WPD-loop, where Chimera 1 appears more flexible (**Figure 5A**). Analysis of the H-bonding interaction networks identify the E186S mutation as likely responsible for this by removing and not replacing side chain hydrogen bonds between the glutamic acid and other WPD-loop residues (**Figure 5B**).

Discussion

Previous results have shown a correlation between WPD-loop dynamics and catalysis in YopH and PTP1B.^{20, 25, 42} These enzymes have variable residues within their mobile loops except for the WPD-sequence (**Figure S4**). A suggestive assumption is that the identity of the intervening sequences affects loop dynamics, and thus the catalytic rate, by altering hydrogen bonding

interactions. A less obvious way that these residues may affect enzyme function is by altering their pH-rate dependencies, by affecting binding/release rates, protonic equilibria, and the relative contribution of individual steps to the rate across the pH range.²⁰ A previous study of chimeras of YopH bearing parts of the PTP1B WPD-loop sequence found some instances of unexpected structural perturbation to the WPD-loop structure, including one Chimera that had lost functional WPD-loop closure.²⁶ PTP1B is more tolerant to mutations in the WPD-loop than YopH⁵⁵, creating an expectation that transposing the YopH loop into PTP1B would result in chimeras that retain structural and functional integrity, facilitating assessment of the effects on enzymatic function and loop structure. In this study, the initial Chimera 0 with the YopH WPD-loop residues transposed into PTP1B was systematically restored to the native PTP1B. A combined experimental and computational study provides an assessment of how the catalytic function and conformational dynamics of the chimeras compare to that of the native enzymes. The effect of the transposed loop on the catalytic reaction will be discussed first, followed by the effect on structure and conformational dynamics.

Effects on Catalysis

The active chimeras all exhibit slower steady-state rates than either of the parent enzymes, but all show the bell-shaped pH-rate profile characteristic of the PTP family (**Figure 3**). This indicates the general mechanism shown in **Figure 1** is functional and that the WPD-loop can adopt the closed conformation necessary for general acid/base catalysis. The chimera pH-rate profiles differ slightly in their pH optima and kinetic pK_a values. Several factors in enzymatic catalysis cause kinetic pK_a values extracted from pH-rate data to be distorted from the true thermodynamic pK_a values of ionizable catalytic residues.⁵⁷ Because WPD-loop motions correlate with catalysis in PTP1B and YopH, mutations that affect loop motion could not only affect the rates of the chemical

steps but also change the degree to which particular steps are rate-limiting within the pH range, leading to changes in pH-rate profiles such as those observed. The results here are consistent with the notion that differences in the loop residues and the altered hydrogen bonding patterns affect loop dynamics, and thereby alter the pH profile in ways that include broadening or moving the pH optimum. This provides an explanation for the fact that the broadness/narrowness and pH optima of pH-rate profiles within the PTP family vary despite conservation of their ionizable catalytic residues.

Our simulations (**Figures 5, 6, and S7 – S10**) similarly indicate specific differences in WPD-loop flexibility and related interaction networks, that could account for the observed changes in the experimental pH rate profiles. Generally speaking, our simulations indicate that the more mutations that are accumulated on the WPD-loop the less stable the open WPD-loop conformation becomes. This is especially visible in the case of going from Chimera 4 to 3 (which differ only by the point mutation T177G), where a relatively large reduction in stability of the open WPD-loop was observed. These results track with the crystal structures in which the unliganded form of Chimera 3 was observed in the closed WPD-loop state. This further hints at a link between WPD-loop dynamics and catalytic activity. The structural and molecular dynamics details of residue 177 are described in detail in the following section.

While the pH-rate profiles indicate functional WPD-loops, KIEs provide a closer measure of general acid catalysis in the transition state of the first chemical step. The ^{15}N isotope effect detects charge delocalization into the aryl ring, assessing the synchronization of protonation with P-O bond fission in the transition state. The major contribution to the ^{18}O KIE is the extent of P-O bond fission, and is also affected by the protonation of the oxygen atom. In native PTP1B and YopH reactions, the ^{15}N KIEs are within experimental error of unity, reflecting full neutralization of the

leaving group implying protonation is fully synchronized with P-O bond fission. Some native PTP family members, including DSPs that utilize the same active site, catalytic machinery, and mechanism, exhibit small normal ^{15}N KIEs indicating a partial charge on the leaving group in the transition state, which would arise if the extent of proton transfer lags slightly behind P-O bond fission (**Table 3**). Some PTPs with very similar k_{cat} values show ^{15}N KIEs of unity, while others have small normal ^{15}N KIEs. In other words, small normal values are not indicative of a failure of general acid catalysis, only that protonation lags behind P-O bond fission in the transition state. Loss of general acid catalysis (as seen in D to N mutants, **Table 3**) produces larger ^{15}N KIEs of approximately 1.0030, indicative of a nearly full negative charge on the leaving group in the transition state.⁵⁴

The KIEs for the three active chimeras indicate functional general acid catalysis, consistent with the retention of the basic limbs of their pH-rate profiles. More specifically than the pH profiles, the KIE data tell us that the synchronicity of leaving group protonation with P-O bond fission, although slightly variable among the chimeras, remains within the range of values seen among native PTPs. The extent of P-O bond fission in the transition state is not significantly different in the chimeras and WT enzymes. It can be concluded that the chimeras' lower catalytic rates relative to their parent enzymes do not result from an altered transition state or impaired general acid. Taken together, this is consistent with the structural data that shows all of the chimeras are able to adopt a normal, catalytically active WPD-loop closed conformation, although they have altered open-closed equilibria and thermodynamics.

Insights into the origin of the slower catalytic rates of the chimeras comes from molecular dynamics (MD) simulations. Focusing first on Chimera 7, our ΔRMSF data from our MD simulations demonstrate the flexibility/rigidity of Chimera 7 and WT PTP1B to be highly similar

(**Figure S10A**). Likewise, our H-bonding analysis only identifies one major difference, which is the newly formed hydrogen bond (H-bond) between F182Q and D181 in Chimera 7. This new H-bond does not appear to notably alter the conformational dynamics or protein interaction network in PTP1B, however given its direct interaction with the catalytic acid/base, it may have an important electrostatic effect on both chemical steps of catalysis. Chemical intuition would suggest that mutation to glutamine would increase the acidity of the catalytic aspartic acid by placing more negative charge on the acid's oxygen atoms through a side chain H-bond. This would be expected to have a beneficial effect on the rate of the first step in which the WPD-loop acts as an acid. However, in the second (rate-limiting) step where the WPD-loop acts as a base, the decreased basicity on the aspartic acids' oxygen due to mutation of the adjacent residue to glutamine could ultimately result in an increased activation free energy, which would explain the decreased k_{cat} values observed for Chimera 7 compared to the WT PTP1B.

Our simulation data do not reveal why Chimera 1 is catalytically inactive, as the closed WPD-loop is predicted to have similar stability to that of Chimera 3 (**Figure S10A**). Whether Chimera 1's WPD-loop can adopt another conformation that is more favorable than the closed WPD-loop conformation (that is separated by a reasonably large energetic barrier), or if Chimera 1 has a high activation free energy for the chemical step(s) of catalysis is unknown.

Effects on Structure and Molecular Dynamics

The vanadate complexes provide a structural transition state analog for the phosphoryl transfer reaction. Vanadate adopts the trigonal bipyramidal geometry expected in these structures with distances between vanadium and the nucleophilic cysteine and the apical oxygen atom very close to those in the vanadate complex of native PTP1B.²¹ The high degree of superimposability of the vanadate complexes of Chimeras 3 and 4 with the vanadate complex of native PTP1B is consistent

with conclusions from KIE data that the transition states for the chimera-catalyzed reactions are similar to the native enzyme.

In ligand-bound structures of both WT PTP1B and YopH the general acid sidechain is typically oriented towards the P-loop where it can function catalytically. However, in the Chimera 3 and 4 tungstate-bound structures, the Asp181 side chain is pointing away from the active site, although there is no significant change in the conformation of the WPD-loop backbone. This unproductive orientation provides another possible rationale for their reduced rates. The non-productive position of the Asp181 side chain in the tungstate complexes is aided by coordination to a magnesium ion from the crystallization solution. This led us to consider the possibility that these chimeras might exhibit inhibition by Mg^{2+} , in contrast with the accelerating effect of Mg^{2+} ion reported for WT PTP1B.⁵³ The interaction of Asp181 with Mg^{2+} ion is not seen in WT PTP1B tungstate-bound structure, nor with the corresponding Asp356 in tungstate-bound YopH.¹⁶ To assess whether this unproductive conformation of Asp181 occurs in solution, and whether the presence of magnesium ion contributes to its population, steady-state kinetic data were collected on Chimera 4 in the presence of Mg^{2+} and showed an inhibitory effect of Mg^{2+} (**Figure S5**). The structural data explain why this nonproductive conformation is unique to the chimeras. In WT YopH, the Lys loop providing one of the Mg^{2+} -coordinating residues is absent, and no other residue is in close proximity for hydrogen bonding with this nonproductive conformation of Asp181. In WT PTP1B residue 182 is phenylalanine, which lacks a hydrogen bond acceptor for this conformation of the Asp sidechain. Hence, the network for coordinating a magnesium ion can only occur in the chimeras. Mg^{2+} inhibition is expected in both Chimeras 3 and 4, but only Chimera 4 was tested.

The WPD-loop is typically found in the open conformation in structures of ligand-free PTPs. In the one reported ligand-free structure of PTP1B with its loop closed, non-continuous electron density indicated the presence of a mixture of closed and open conformations.⁵⁶ Interestingly, the WPD-loop is in the closed position of the ligand-free structure of Chimera 3, with no indication in the electron density of an open-conformation species. In contrast, the loop is open in the ligand-free structure of Chimera 4. These chimeras differ only in the identity of residue 177 (G from YopH in Chimera 3; T from PTP1B in Chimera 4). Our MD simulation data suggest the population shift observed for Chimera 3 is primarily the result of destabilization of the open WPD-loop conformation. Further, we have recently studied the impact of the single point mutation T177G on *wt*-PTP1B.²⁰ This mutation was observed to induce a population shift towards the closed state through a combination of interactions that stabilize the closed conformation and destabilize the open conformation of the loop. Taken together, therefore, this mutation appears to exert the same population shift effect as seen in *wt*-PTP1B vs T177G-PTP1B and in Chimera 3 vs. Chimera 4.

This important role of residue 177 is depicted in **Figure S11**. In Chimera 4, the sidechain of Thr177 forms a 2.9 Å hydrogen bond with the backbone carbonyl of Tyr152 in the loop-closed conformation. This interaction is absent in Chimera 3, which has glycine at this position. Backbone shifts were only seen in the WPD-loop region (from Asn178 to Pro188); compared to Chimera 4, Pro180 in Chimera 3 was shifted 3.4 Å towards the P-loop, and the catalytic Asp181 was brought in 5.5 Å closer. The one-residue-difference between these two Chimeras showed almost no discrepancy in ligand-free secondary structures besides the WPD-loop; and besides residue 177, the rest of the hydrogen bonding pattern in the active site has no observable change. The loss of this hydrogen bond could affect loop dynamics. It was recently shown that mutation of residue 177 to alanine shifts the equilibrium of the WPD-loop in favor of the closed conformation.⁵² That is

consistent with the finding that Chimera 3, with glycine rather than the native threonine in this position, adopts a loop-closed conformation even in ligand-free crystal structures.

Molecular dynamics simulations provide insight into how the conformational dynamics of the WPD-loops in the chimeras differ from the WT enzymes. Our analysis of the relative stability of the closed and open conformations of the WPD-loop (**Figure 5**) show all but Chimera 7 to have significantly different WPD-loop dynamics in both conformational states. Further, our simulation data indicates that destabilization of the open WPD-loop conformation (relative to WT PTP1B) of Chimera 3 is responsible for the experimentally observed crystal structure in which the ligand-free form of Chimera 3 was observed in the closed WPD-loop state.

Simulations identified key changes in the local rigidity/flexibility of the WPD-loop and the surrounding scaffold, and showed links to changes in the hydrogen bonding network (**Figures 6** and **S10**). Significant differences in both the flexibility and hydrogen bonding network were observed for Chimeras 1, 3 and 4, and these changes were not just limited to the WPD-loop, but also some surrounding regions, most notably the E-loop and Loop 11 compared to WT PTP1B (**Figures 6** and **S10**). The sensitivity of the E-loop to changes in the WPD-loop is consistent with a recent NMR study⁵⁸ and our recent work⁴² in which we identified these loops' dynamics were correlated with one another. Likewise, Loop 11 is part of a previously characterized allosteric network⁵⁹ which modulates the WPD-loop conformation.

The roles of key mutations such as G183T (present in Chimeras 1, 3 and 4) and T177G (present in Chimeras 1 and 3) were identified and characterized. For G183T, the newly formed intra WPD-loop hydrogen bond between G183T and P180 helps explain the observed increase in stability of the closed WPD-loop state Chimeras 4, 3 and 1 relative to WT PTP1B. Further, in the open state the newly formed hydrogen bond between G183T and D265 helps to explain the destabilization of

both the WPD- and E-loops, as this hydrogen bond pulls both loops apart, ultimately weakening many of their shared interactions. For T177G, the observed destabilization of the open WPD-loop conformation for Chimeras 1 and 3 (relative to WT PTP1B) is consistent with our prior work on the T177G single point mutant,²⁰ and here, the same set of changes were observed to occur with the introduction of the mutant into the chimeras (**Figure 6B**).

Conclusions

In this work we have examined the kinetic, thermodynamic, structural and conformational properties of a series of chimeric proteins constructed by exchanging the WPD-loop of YopH into the corresponding loop of PTP1B. Curiously, the exchange of the WPD-loop from the faster YopH enzyme into the corresponding loop of PTP1B does not result in a faster enzyme; all of the PTP1B chimeras exhibited slower rates than either WT parent (**Table 2**). Backbone structural integrity is maintained in all of the chimeras, in contrast to previous findings²⁶ in reverse YopH-based chimeras, which also were found to sample a catalytically inactive hyper-open conformation of the WPD loop not observed in any of the PTP1B-based chimeras.

The measured kinetics and KIEs find no change to the chemical mechanism or the transition state of the first step in the chimeras, indicating that the reduced catalytic rates compared to the parent enzymes, and differences in rate between the chimeras themselves, do not arise from changes to the chemistry of catalysis. Even moderate differences in WPD-loop sequence identity between the chimeras and native PTP1B result in significant effects on their kinetics and computed molecular dynamics. Residue 177, in particular, plays an important role in controlling loop dynamics and catalytic rate *via* a crucial hydrogen bond²⁰, that causes a population shift from a preferentially open to a preferentially closed conformation of the WPD-loop in even the

unliganded form of the enzyme. The chimera loops also differ in their stability and rigidity. The most stable open WPD-loops are found in WT and Chimera 7. The loops in WT and Chimera 7 are also the most similar in rigidity in both open and closed states. The open-loop conformations in the other chimeras were less stable, resulting in more frequent transitions to the closed conformation. Chimeras 1, 3, and 4 also have differences in rigidity of not only the WPD-loop, but in their E-loop and in Loop 11. The motions in the WPD and E-loops are likely coupled, as previously found in the native enzymes.⁴²

Overall, PTP1B proved to be more tolerant to mutations in the WPD-loop region than YopH, as evidenced by the similar backbone structures of WT PTP1B and the chimeras (**Figure 4**). Molecular dynamics calculations find that while the closed loops in the chimeras have similar stability, the conformational space sampled by the open loops differ between these systems, and exhibit multiple conformationally distinct metastable open states. Because only the closed state is catalytically competent, sampling between these open states, and between the open and closed state, affects catalytic turnover, as was similarly observed, for instance, in prior work on triosephosphate isomerase.^{60, 61} By influencing the population of catalytically competent enzyme-substrate complexes, the pH profile of catalysis can be affected, as well as the limiting rate. Thus, variations in sequence of noncatalytic residues within the WPD-loop provides a means for nature to fine tune these enzymes, and likely contributes to the variation in rate and pH-dependency (broad versus narrow) in the PTP family despite strict conservation of the ionizable residues directly involved in the chemical steps.

Finally, we demonstrate that although the WPD-loop may appear to be a simple decorating loop that positions a key catalytic residue, in fact, changes in one part of the protein (substitutions on this loop) affect dynamics of other parts of the protein (*e.g.* the coupled dynamics of the E-loop

and loop 11). Such coupled dynamics can be exploited in protein engineering, through the introduction of mutations distal to the active site, that can shift the overall conformational ensemble of the protein, including potentially controlling the dynamics of key catalytic loops.^{62, 63} This is also significant in light of increased awareness of the role of the dynamics of decorating loops in the natural evolution of enzyme function, and the potential of altering loop dynamics and loop grafting as a powerful tool in protein design.²⁷⁻³⁰ Here, we demonstrate that non-catalytic loop residues are potential mutational targets for manipulating the conformational equilibria of key catalytic loops in PTPs, in addition to analogous effects from mutation of allosteric residues.⁶⁴

Supporting Information

Additional simulation methodology, simulation analysis, starting structures for all simulations in AmberMD format, parameter files with examples, MD simulation input files, and structural information.

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