Residue 182 influences the second step of protein-tyrosine phosphatase-mediated catalysis

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Previous enzyme kinetic and structural studies have revealed a critical role for Asp¹⁸¹ (PTP1B numbering) in PTP (protein-tyrosine phosphatase)-mediated catalysis. In the E-P (phosphoenzyme) formation step, Asp¹⁸¹ functions as a general acid, while in the E-P hydrolysis step it acts as a general base. Most of our understanding of the role of Asp¹⁸¹ is derived from studies with the *Yersinia* PTP and the mammalian PTP1B, and to some extent also TC (T-cell)-PTP and the related PTP α and PTP ϵ . The neighbouring residue 182 is a phenylalanine in these four mammalian enzymes and a glutamine in *Yersinia* PTP. Surprisingly, little attention has been paid to the fact that this residue is a histidine in most other mammalian PTPs. Using a reciprocal single-point mutational

approach with introduction of His¹⁸² in PTP1B and Phe¹⁸² in PTPH1, we demonstrate here that His¹⁸²-PTPs, in comparison with Phe¹⁸²-PTPs, have significantly decreased k_{cat} values, and to a lesser degree, decreased $k_{\text{cat}}/K_{\text{m}}$ values. Combined enzyme kinetic, X-ray crystallographic and molecular dynamics studies indicate that the effect of His¹⁸² is due to interactions with Asp¹⁸¹ and with Gln²⁶². We conclude that residue 182 can modulate the functionality of both Asp¹⁸¹ and Gln²⁶² and therefore affect the E-P hydrolysis step of PTP-mediated catalysis.

Key words: enzyme kinetics, molecular dynamics, protein-tyrosine phosphatase, PTP1B, PTPH1, X-ray crystallography.

INTRODUCTION

PTPs (protein-tyrosine phosphatases) are key regulators of most intracellular signal transduction processes [1–3]. The recent sequencing of the human genome has revealed the total number of PTPs in mammals to be close to 40 (J. N. Andersen, P. G. Jansen, S. M. Echwald, O. H. Mortensen, T. Fukada, R. Del Vecchio, N. K. Tonks and N. P. H. Møller, unpublished work). However, the actual number of different enzymes in the human proteome is expected to be much higher due to regulatory processes such as (i) alternative promoter usage [4], (ii) alternative splicing [5–7], (iii) proteolytic processing [8], and (iv) covalent modification [9]. Classical PTPs are traditionally divided into two classes based on the presence or absence of a transmembrane region: intracellular PTPs and receptor-like PTPs. Overall, this structural classification is in agreement with a phylogenetic classification based on PTP domain sequence identity [10].

The catalytic mechanism of classical PTPs has been elucidated by a number of elegant enzyme kinetic and X-ray crystallographic studies (Scheme 1; reviewed in [11–13]). The so-called P-loop or PTP loop, consisting of Cys(Xaa)₅-Arg, the hallmark sequence of classical and dual-specificity PTPs, is positioned at the bottom of the active-site pocket. The P-loop forms a half-circle, with the main-chain nitrogens pointing towards the cysteine (Cys²¹⁵; PTP1B numbering is used throughout) in the centre. This structural arrangement allows the formation of a number of hydrogen bonds between the phosphate moiety of pTyr (phosphotyrosine) and the P-loop amide hydrogens. In addition, pTyr forms important salt bridges with the side chain of the arginine in the P-loop (Arg²²¹). The binding of pTyr is further stabilized by an electrostatic dipole of a central α -helix and by interaction of the phenyl

ring of pTyr with Phe¹⁸², Tyr⁴⁶ and the hydrophobic residues Val⁴⁹, Ala²¹⁷ and Ile²¹⁹ [14]. The active-site cysteine is negatively charged and acts as a nucleophile in the first step of catalysis, leading to the formation of a thiophosphoryl enzyme intermediate (Scheme 1) [15]. Binding of pTyr to the active-site pocket of PTPs is followed by a remarkable conformational change of the so-called WPD loop, which brings Asp¹⁸¹ close to the scissile oxygen of pTyr and thus in a favourable position to function as a general acid. In this first step of catalysis, Asp¹⁸¹ protonates the leaving group [16–18]. In the second step, the invariant Gln²⁶² is believed to play an essential role in positioning a catalytic water molecule correctly relative to the phosphate-cysteinyl intermediate [19,20]. In this part of the process, Asp¹⁸¹ functions as a general base by abstracting a proton from this water molecule [19,21], leading subsequently to hydrolysis and release of inorganic phosphate (Scheme 1).

As described, the movement of the WPD loop plays a central role in the PTP-mediated hydrolysis of pTyr-containing substrates. In particular, two residues (i.e. the tryptophan and aspartic acid) in the WPD loop have received much attention and have been studied intensively. Thus mutation of the aspartic acid (Asp¹⁸¹ in PTP1B) to an alanine dramatically reduces the k_{cat} , and this mutation forms the basis for so-called trapping mutants in PTP1B [22]. Further, the conserved tryptophan in *Yersinia* PTP (corresponding to Trp¹⁷⁹ in PTP1B) is absolutely required for the correct positioning of Asp¹⁸¹ to act as a general acid/base in catalysis by PTPs [23,24].

Less attention has been paid to residue 182, which in PTP1B, TC (T-cell)-PTP, PTP α and PTP ϵ is a phenylalanine, but in most other classical PTPs is a histidine (<http://science.novonordisk.com/ptp/>). This is surprising considering the fact that closely

Abbreviations used: PTP, protein-tyrosine phosphatase; pTyr, phosphotyrosine; E-P, phosphoenzyme; pNPP, *p*-nitrophenyl phosphate; GST, glutathione S-transferase; TC, T-cell.

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Scheme 1 Schematic representation of the PTP1B-catalysed hydrolysis of aryl phosphates

positioned histidine and aspartic acid residues have been found to be important for catalysis in other enzyme systems [25]. Indeed, Nature often utilizes the imidazole ring in catalysis, as exemplified by the catalytic triad in serine proteases [25].

We reasoned that a histidine in position 182 could influence the efficiency of catalysis in two different ways. First, in contrast with the uncharged phenylalanine, the histidine side chain with a theoretical pK_a of 6.2 might influence the general acid/base function of the neighbouring Asp¹⁸¹, and perhaps also the function of Gln²⁶². Secondly, the contribution of a histidine in position 182 to substrate binding (via interaction with the phenyl ring of pTyr) may be less efficient than that of a phenylalanine, due partly to its shorter side chain and partly to the fact that the imidazole ring of histidine is less aromatic than the phenyl ring of phenylalanine.

We have previously successfully utilized a reciprocal single-point mutational approach to analyse the effects of specific residues on substrate and inhibitor binding [26–28]. Therefore we reasoned that a similar approach could be used to investigate the function of residue 182 in PTPs. In the present study we have performed a detailed functional and structural comparison of wild-type and mutant forms of PTP1B and PTPH1 in which the residues at position 182 were exchanged.

EXPERIMENTAL

Cloning, expression and purification

PTP1B-(1–321) and PTPH1-(634–913) were cloned, expressed and purified essentially as described previously [26,27]. The following PTP mutants were made [as GST (glutathione S-transferase) fusion proteins] by overlap extension PCR using appropriate restriction sites for cloning purposes [29]: PTP1B to PTPH1, PTP1B_{F182H}; PTPH1 to PTP1B, PTPH1_{H182F} (PTP1B numbering). All GST fusion proteins were purified using glutathione–Sepharose 4B columns according to the manufacturer's instructions (Amersham Biosciences), followed by a Superdex 200 column (10 mM Tris, pH 7.5, 25 mM NaCl, 3 mM dithiothreitol, 0.2 mM EDTA). A final polishing step was included for PTPH1 and PTPH1_{H182F}, consisting of an anion-exchange column (Mono-Q; Amersham Biosciences). Protein concentrations were determined by amino acid analysis.

For X-ray crystallography, the cDNAs encoding PTP1B and PTP1B_{F182H}-(1–321) were inserted into a pET11a expression vector and transformed into the *Escherichia coli* BL21 (DE3) strain. Expression and purification were done essentially as described previously for wild-type PTP1B-(1–321) [27,30].

Compounds

Compound **1** {2-(Oxalyl-amino)-4,5,6,7-tetrahydro-thieno[2,3-c]pyridine-3-carboxylic acid}, compound **2** {2-(oxalyl-amino)-

Figure 1 Chemical structures

The chemical structures of 2-(oxalyl-amino)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylic acid (compound **1**), 2-(oxalyl-amino)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid (compound **2**), and 3-(oxalyl-amino)naphthalene-2-carboxylic acid (compound **3**) are shown.

4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid} and compound **3** [3-(oxalyl-amino)naphthalene-2-carboxylic acid] (Figure 1) were synthesized as described previously [27,31]. *p*NPP (*p*-nitrophenyl phosphate) was purchased from Fluka or Sigma. Phenyl phosphate was obtained from Sigma. Other aryl phosphates, 4-trifluoromethylphenyl phosphate, 4-chlorophenyl phosphate and 4-cyanophenyl phosphate were synthesized as described [32]. Vanadate was treated with NaOH and boiled repeatedly as described [33].

Determination of kinetic constants

The phosphatase activities of PTP1B, PTP1B_{F182H}, PTPH1 and PTPH1_{H182F} were determined using *p*NPP and the phosphorylated epidermal growth factor receptor-derived peptide acetyl-DADEpYL-NH₂ as substrates. The enzyme assays were carried out at 30 °C using a three-component buffer, consisting of 50 mM Tris, 50 mM Bis-Tris and 100 mM acetic acid. This buffer has a constant ionic strength over a pH range of 4.5–9.0 [17,34]. For all assays, the buffer included 5 mM dithiothreitol. Initial-rate measurements for the enzyme-catalysed hydrolysis of *p*NPP were conducted as described previously [32]. Steady-state kinetic parameters were determined from a direct fit of the data to the Michaelis–Menten equation using the non-linear least-squares regression program (Kaleidagraph, Synergy Software).

The activity towards the peptide substrate acetyl-DADEpYL-NH₂ was monitored continuously at 282 nm using a PerkinElmer Lambda 14 spectrophotometer [35]. The instrument was equipped with a water-jacketed cell holder, permitting maintenance of the reaction mixture at the desired temperature (30 °C). When $K_m \ll [S]$, both k_{cat} and K_m values can be determined using the integrated Michaelis–Menten equation [1,35]:

$$t = p/k_{cat}E_0 + (K_m/k_{cat}E_0) \cdot \ln[p_\infty/(p_\infty - p)] \quad (1)$$

where E_0 is enzyme concentration and p and p_∞ are the product concentrations at times t and infinity respectively. The reaction

mixture (0.6 ml) contained 100–600 μ M peptide substrate in the pH 7.0 buffer as described above. The reaction was initiated by addition of the PTP at a final concentration in the range 5–25 nM. The data were analysed using a non-linear least-squares regression program (KaleidaGraph, Synergy Software).

Leaving-group dependence

Leaving-group dependence studies were performed at pH 7.0, 30 °C, using an inorganic phosphate assay. The reaction was initiated the same way as in the PTP-catalysed *p*NPP reaction. The assay mixture was quenched by 0.1 ml of 10% (v/v) trichloroacetic acid. The amount of inorganic phosphate released was quantified based on the method of Black and Jones [36]. Briefly, 0.25 ml of a mixture containing 0.15 ml of 14% (v/v) ascorbic acid in 50% (v/v) trichloroacetic acid and 0.1 ml of 2% (v/v) ammonium molybdate $\cdot$ 4H₂O was added to the quenched solution and the assay mixture was vortexed. After 2.5 min, 0.5 ml of 2% trisodium citrate $\cdot$ 2H₂O plus 2% (w/v) sodium arsenite in 2% (v/v) acetic acid was added. Absorbance at 700 nm was measured 5 min later. The $\log(k_{\text{cat}})$ and $\log(k_{\text{cat}}/K_{\text{m}})$ values were plotted against the leaving-group $\text{p}K_{\text{a}}$ of the aryl phosphate substrates. The β_{lg} value was obtained as the slope of the linear least-squares fit to the data.

Determination of inhibition constants

The determination of K_{i} values was carried out in microtitre plates in the same buffer system as for the *p*NPP activity assay, essentially as described previously [27]. Four different inhibitor concentrations (3-fold dilutions) were added to reaction mixtures containing *p*NPP at various concentrations (0.26–6.4 mM final assay concentration). The final reaction volume was 100 μ l. The reaction was started by addition of enzyme and carried out at 25 °C for 15 min. The reactions were stopped by addition of 0.5 M NaOH in 50% (v/v) ethanol. The enzyme activity was determined by measuring the absorbance at 405 nm, with correction for compound and *p*NPP absorbance. The data were analysed using non-linear regression hyperbolic fit to classical Michaelis–Menten enzyme kinetic models. The presented K_{i} values ($\pm$ S.D.) were calculated from at least three independent experiments.

Crystallization

PTP1B and PTP1B_{F182H} protein solutions (20 mg/ml) in 10 mM Tris, pH 7.5, 25 mM NaCl, 0.2 mM EDTA and 3 mM dithiothreitol were used for the crystallizations. Crystals were grown by the hanging-drop vapour diffusion method at room temperature. A 1:10 (protein/inhibitor) molar ratio mixture was prepared at least 1 h prior to crystallization. The drops contained 1 μ l of protein/inhibitor solution and 1 μ l of reservoir solution consisting of 0.1 M Hepes buffer, pH 7.5, 0.2 M magnesium acetate, 14–17% (w/v) polyethylene glycol 8000 and 0.1% (v/v) 2-mercaptoethanol. The reservoir volume was 1 ml.

Data collection

Diffraction data were collected at 100 K. For cryo cooling, 1.5 μ l of 50% (v/v) glycerol containing 1 mM inhibitor was added to the hanging drop. The crystals were transferred rapidly through a solution of 50% (v/v) glycerol containing 1 mM inhibitor and flash frozen. Data were collected using a Mar345 image plate detector on a rotating anode (RU300; CuK α 50 kV/100 mA) equipped with an Osmic multiplayer mirror system. Data were

processed using Denzo, Scalepack [37], and the CCP4 program suite [38].

Structure determination

The crystal structures of PTP1B_{F182H} in complex with compound 1 (Figure 1) or vanadate, and of wild-type PTP1B in complex with vanadate, were solved by molecular replacement using AMoRe [39]. For all structures, the co-ordinates of a high-resolution structure of PTP1B (PDB code 1C88) were used as search model (water molecules and the inhibitor were omitted from the model). The structures were refined using CNX (Accelrys) and model building was done in the program Quanta (Accelrys). The crystallographic data and statistics of structure refinement are given in Table 1. The C-terminal 22 residues could not be seen in the electron density maps of any of the structures.

Molecular dynamics simulations

Simulations were carried out on PTP1B_{F182H} as the cysteinyl-phosphate intermediate (i.e. phosphate covalently bound to Cys²¹⁵). The model was constructed from the structure of the PTP1B_{Q262A}–phosphocysteine intermediate (1A5Y). All simulations were performed using the CHARMM22 molecular modelling package with the polar hydrogen atom force field [40]. The enzyme was solvated by a shell of explicit water molecules described by the TIP3 model. Atoms within a 20 Å (1 Å $\equiv$ 0.1 nm) shell measured from the C α atom of Cys²¹⁵ were allowed to move, whereas atoms in a shell between 20 and 24 Å were constrained to their initial positions by a harmonic constraint using a force constant of 550 kcal/mol (2300 kJ/mol). A time step of 1 fs was used throughout. The non-bonded forces were shifted and truncated at 10 Å. To release any constraint in the system, atomic positions were optimized using 250 steps of the steepest descent method, followed by 500 steps of the conjugate gradient method. Initially, the systems were heated to the final temperature of 300 K over a period of 3000 steps. The simulations were performed with His¹⁸² in the uncharged form (hydrogen atom on N δ or N ϵ).

Cambridge Structural Database search

A search using the Cambridge Structural Database (Cambridge Crystallographic Data Centre, Cambridge, U.K.) was performed to analyse the nature of interactions between an imidazole ring of His and a carboxylic acid moiety of Asp. All six theoretical combinations were investigated: a carboxylic acid or carboxylate group together with a charged or an uncharged imidazole with the hydrogen atom located on N δ or N ϵ . A C-substituent was specified in the 4-position of the imidazole and on the carboxylic acid/carboxylate groups, and explicit hydrogen atoms were required in the other positions of the imidazole. The individual C–O bond lengths were measured to estimate if they belonged to a carboxylic acid or carboxylate group.

RESULTS

The movement of the WPD loop is a key event in the PTP-mediated hydrolysis of *p*Tyr, bringing Asp¹⁸¹ in PTP1B into a favourable position relative to the scissile oxygen to act as a general acid in the *E-P* formation step (k_2) and as a general base in the *E-P* hydrolysis step (k_3) (Schemes 1 and 2). The side chain of the neighbouring Phe¹⁸² facilitates WPD loop closure by interacting with the phenyl ring of *p*Tyr, and therefore seems to be a key residue in PTP1B [14]. Indeed, removal of the aromatic side

Table 1 Crystallographic data and statistics of structure refinement

Values in parentheses correspond to the outermost resolution shell. *R*-factors were calculated using all data. The crystallographic *R*-factor was calculated using the eqn:

$$\text{Crystallographic } R\text{-factor} = \frac{\sum_{(hkl)} \|F_o\| - |F_c|}{\sum_{(hkl)} \|F_o\|}$$

R-free was calculated as follows:

$$R\text{-free} = \frac{\sum_{(hkl) \notin T} \|F_o\| - |F_c|}{\sum_{(hkl) \in T} \|F_o\|}$$

where *T* is a test set containing a random 5% of the observations omitted from the refinement process. The Ramachandran plots were calculated according to Kleywegt and Jones [56]. R.m.s.d., root mean square deviation.

Parameter	Protein. . . Ligand. . .	PTP1B Orthovanadate	PTP1B _{F182H} Orthovanadate	PTP1B _{F182H} Compound 1
Space group		P3 ₁ 21	P3 ₁ 21	P3 ₁ 21
Unit cell parameters (Å)		<i>a</i> = <i>b</i> = 88.10, <i>c</i> = 103.81	<i>a</i> = <i>b</i> = 88.26, <i>c</i> = 103.82	<i>a</i> = <i>b</i> = 88.61, <i>c</i> = 104.21
Resolution range (Å)		50.0–2.08	50.0–2.22	50.0–2.07
Unique reflections		28 516	23 406	29 034
Average redundancy		7.2	7.1	5.9
Completeness (%)		99.8 (99.5)	99.0 (92.6)	98.8 (82.7)
<i>R</i> _{merge} (%)		9.6 (40.7)	9.2 (38.5)	4.9 (33.1)
<i>I</i> /σ(<i>I</i>)		29.0 (5.3)	22.8 (4.7)	16.9 (2.5)
Atoms in structure		2729	2695	2766
<i>R</i> -factor (%)		19.2	19.4	20.1
<i>R</i> -free (%)		21.1	23.3	23.3
R.m.s.d. bond length (Å)/angle (°)		0.005/1.15	0.005/1.19	0.006/1.18
Residues in allowed regions of Ramachandran plot (%)		98.6	98.6	98.2

Scheme 2 Kinetic mechanism of the PTP-catalysed reaction

chain at position 182 by mutation to an alanine results in dramatic decreases in both binding affinity for a non-hydrolysable pTyr-mimetic inhibitor [41] and rates for E–P formation and hydrolysis [42]. Surprisingly, little attention has been paid to the fact that residue 182 is a histidine in 25 of the known 37 full-length human PTPs, whereas a phenylalanine is found in only four members of the PTP family (PTP1B, TC-PTP, PTPα and PTPε) (Figure 2).

Influence of residue 182 in PTP1B on hydrolysis of pNPP

We first decided to study the effects of a histidine residue in position 182 on the kinetic parameters k_{cat}/K_m and k_{cat} as a function of pH by introducing the appropriate substitution in PTP1B. k_{cat}/K_m monitors the reaction beginning with binding of the substrate and up to and including the first irreversible step in the kinetic mechanism (Scheme 1), which is E–P formation accompanied by release of the leaving group. The kinetic parameter k_{cat} describes the rate-limiting step under a saturating concentration of the substrate, which corresponds to E–P hydrolysis (k_3) under acidic to neutral conditions [16,43,44].

A comparison of the k_{cat}/K_m values of PTP1B_{F182H} and wild-type PTP1B covering the pH range from 4.3 to 8 revealed that His¹⁸² caused a modest reduction in k_{cat}/K_m with pNPP as a substrate (Figure 3A). Of note, the overall pH versus k_{cat}/K_m profiles of wild-type and mutant PTP1B were similar, with a maximum at pH ~5.5 and with a limiting slope of +2 on the acidic side and

–1 on the basic side. For the analysis of k_{cat}/K_m , eqn (2) was used:

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

where $(k_{\text{cat}}/K_m)^{\text{lim}}$ is the pH-independent maximal second-order rate constant, *H* is the concentration of H⁺ ions, *K*_{S2} is the second ionization constant of the substrate (p*K*_{S2} = 5.1), and *K*_{E1} and *K*_{E2} are the ionization constants of the active-site Cys²¹⁵ and the general acid Asp¹⁸¹ in the free enzyme [17,43,45,46]. From Table 2, it appears that introduction of a histidine in position 182 of PTP1B led to a slight increase in p*K*_{E1}, a slight decrease in p*K*_{E2}, and a 1.7-fold decrease in $(k_{\text{cat}}/K_m)^{\text{lim}}$.

A more dramatic effect was demonstrated on k_{cat} , with a 7-fold reduction for PTP1B_{F182H} at pH 5.5 (Figure 3B). Similar to previous studies with the rat orthologue of human PTP1B, PTP1 [17,43], the pH– k_{cat} profile for wild-type PTP1B was bell-shaped, with a pH maximum between 5 and 6 and a slope of +1 on the acidic limb and –1 on the basic limb. The pH– k_{cat} profile also seemed to be bell-shaped for the PTP1B_{F182H} mutant. However, in this case there was a very broad pH maximum between 5 and 7.5, with only little pH dependence on the acidic side. For the analysis of k_{cat} , eqn (3) was used:

$$k_{\text{cat}} = \frac{k_{\text{cat}}^{\text{lim}}}{1 + \frac{H}{K_1^{\text{app}}} + \frac{K_2^{\text{app}}}{H}} \quad (3)$$

where $k_{\text{cat}}^{\text{lim}}$ is the pH-independent maximum turnover number, *H* is the concentration of H⁺ ions, and *K*₁^{app} and *K*₂^{app} are the apparent ionization constants of the enzyme–substrate complex in the rate-limiting step [43,45]. In agreement with previous findings

Figure 2 Amino acid sequence alignment of the WPD loop of 37 human PTPs

The WPD loop corresponds to residues 178–187 in PTP1B.

for wild-type PTP1B, eqn (3) is an excellent model for the pH- k_{cat} profile of the wild-type enzyme (Figure 3B and Table 2) [17,43]. Importantly, this model also fits well with the profile for the PTP1B_{F182H}. Indeed, the flat and almost pH-independent pH- k_{cat} profile is adequately described by the two-ionization equation, assuming that the basic ionization constant increases from 6.64 to 7.88 when Phe¹⁸² is replaced with a histidine in PTP1B (Figure 3B, Table 2). Above pH 7.5, the k_{cat} values for wild-type PTP1B and PTP1B_{F182H} were very similar, which is in accordance with the two-ionization equations. A 6.7-fold decrease was observed for $k_{\text{cat}}^{\text{lim}}$ with this mutant.

Introduction of a phenylalanine residue in position 182 of PTPH1

To investigate if the above effects of replacing Phe¹⁸² with a histidine are general in nature, we decided to make a reciprocal mutation in a representative member of the PTPs with a histidine in position 182, i.e. PTPH1 [47], which was selected for several reasons. First, like PTP1B, this is an intracellular enzyme. Secondly, PTPH1 has an aspartic acid residue in position 48, which allows direct comparison of binding affinities towards an inhibitor that specifically addresses this residue [27]. Thirdly, we have previously utilized PTP1B and PTPH1 in a reciprocal single-point mutation study to characterize subtle structural differences in the proximity of the active site [26].

The pH- $(k_{\text{cat}}/K_{\text{m}})$ profiles for the wild-type PTPH1 and the PTPH1_{H182F} mutant are almost superimposable at neutral and basic pH (Figure 3C). Slightly increased $k_{\text{cat}}/K_{\text{m}}$ values were observed for PTPH1_{H182F} at lower pH values. Both profiles were found to have their maximum near pH 6, and a limiting slope of +2 on the acidic side and -1 on the basic side. As for the PTP1B enzymes, there were only minor changes in the pK_{E1} , pK_{E2} and $(k_{\text{cat}}/K_{\text{m}})^{\text{lim}}$ values when replacing a histidine with a phenylalanine in PTPH1 (Table 2).

The pH- k_{cat} profiles of wild-type PTPH1 and PTPH1_{H182F} are shown in Figure 3(D). Both are bell-shaped, and have limiting slopes of 1 and -1 on the acidic and basic limbs respectively. In contrast with wild-type PTP1B, which has a pH optimum at ~5.5, wild-type PTPH1 has a relatively broad pH- k_{cat} optimum in the range 6.5–7.5. When His¹⁸² in PTPH1 was replaced by a phenylalanine, a significant increase in k_{cat} was observed throughout the pH range investigated. Further, a more narrow pH optimum at 6.3 appeared. Consistent with the results obtained with wild-type and mutant PTP1B, the basic ionization constant pK_2^{app} decreased significantly when a phenylalanine was introduced in position 182 in PTPH1, resulting in a more narrow pH- k_{cat} profile. In addition, a 5.6-fold increase in $k_{\text{cat}}^{\text{lim}}$ was observed for the PTPH1_{H182F} mutant.

Influence of residue 182 on k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ for the PTP-catalysed hydrolysis of a pTyr-containing peptide substrate

To substantiate further the above findings in a more biologically relevant context we next analysed the kinetic properties at neutral pH of the wild-type and mutant PTPs using acetyl-DADEpYL-NH₂, a tyrosine-phosphorylated synthetic substrate derived from the epidermal growth factor receptor (Table 3). Similar to the results obtained with *p*NPP as a substrate, introduction of a histidine in position 182 of PTP1B decreased both k_{cat} and K_{m} for the peptide substrate, while replacement of His¹⁸² in PTPH1 with a phenylalanine resulted in an increase in both k_{cat} and K_{m} values for the peptide substrate. Consequently, much smaller effects were observed on the $k_{\text{cat}}/K_{\text{m}}$ values.

Leaving-group dependence

To assess directly the effect of substitution at position 182 on general acid catalysis, we determined the leaving-group dependencies of wild-type PTP1B and PTPH1, as well as of the PTP1B_{F182H} and PTPH1_{H182F} mutants, at pH 7.0 using a series of aryl phosphates [*p*NPP, 4-cyanophenyl phosphate, 4-(trifluoromethyl)phenyl phosphate, 4-chlorophenyl phosphate and phenyl phosphate] with leaving-group pK_{a} values ranging from 7.14 to 9.99. Figure 4 shows the Brønsted plots that relate the logarithms of k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ to the pK_{a} values of the leaving group. Linear least-squares fitting of the Brønsted plots yielded the slopes, which correspond to the β_{lg} values (Table 4). The β_{lg} values for $k_{\text{cat}}/K_{\text{m}}$ were essentially identical for the wild-type and mutant PTPs, and

Figure 3 pH versus k_{cat}/K_m and k_{cat} profiles of wild-type and mutant PTP1B and PTPH1 for the hydrolysis of pNPP at 30 °C

(A) pH versus k_{cat}/K_m and (B) pH versus k_{cat} profiles for wild-type PTP1B ($\blacktriangle$) and the mutant PTP1B_{F182H} ($\blacksquare$). (C) pH versus k_{cat}/K_m and (D) pH versus k_{cat} profiles for wild-type PTPH1 ($\triangle$) and the mutant PTPH1_{H182F} ($\square$). The fitted curves, shown as broken lines, were calculated from eqn (2) for the k_{cat}/K_m profiles and from eqn (3) for the k_{cat} profiles.

Table 2 Kinetic parameters for the PTP-catalysed hydrolysis of pNPP at 30 °C ($pK_{\text{S1}} = 5.1$)

Enzyme	k_{cat}/K_m			k_{cat}		
	pK_{E1}	pK_{E2}	$10^{-3} \times (k_{\text{cat}}/K_m)^{\text{lim}} (\text{M}^{-1} \cdot \text{s}^{-1})$	$pK_{1\text{app}}$	$pK_{2\text{app}}$	$k_{\text{cat}}^{\text{lim}} (\text{s}^{-1})$
PTP1B	4.24 ± 0.24	6.03 ± 0.07	119 ± 9.7	4.51 ± 0.05	6.64 ± 0.04	44 ± 1.4
PTP1B _{F182H}	4.94 ± 0.12	5.75 ± 0.07	70 ± 7.3	4.10 ± 0.28	7.88 ± 0.22	6.5 ± 0.5
PTPH1	6.18 ± 0.11	6.41 ± 0.10	118 ± 20	6.05 ± 0.07	8.04 ± 0.07	9.0 ± 0.5
PTPH1 _{H182F}	5.87 ± 0.19	6.24 ± 0.17	149 ± 40	5.57 ± 0.09	7.31 ± 0.09	50 ± 3.6

Table 3 Kinetic constants for the hydrolysis of acetyl-DADEpYL at pH 7.0 and 30 °C

Enzyme	$k_{\text{cat}} (\text{s}^{-1})$	$K_m (\mu\text{M})$	$10^{-6} \times k_{\text{cat}}/K_m (\text{s}^{-1} \cdot \text{M}^{-1})$
PTP1B	41.0 ± 0.02	4.04 ± 0.01	10
PTP1B _{F182H}	9.9 ± 0.003	1.84 ± 0.006	5.3
PTPH1	9.7 ± 0.005	1.10 ± 0.009	8.9
PTPH1 _{H182F}	97.5 ± 0.06	24.0 ± 0.06	4.1

the β_{lg} values for the k_{cat} parameter were effectively zero for all of the enzymes.

Protein X-ray crystallography

As shown above, replacement of Phe¹⁸² in PTP1B with a histidine resulted in a significant decrease in k_{cat} and a less pronounced decrease in k_{cat}/K_m with both pNPP and acetyl-DADEpYL-NH₂ as substrates. We hypothesize that this effect can be explained, at least in part, by a direct interaction between His¹⁸² and its adjacent

residues (i.e. Asp¹⁸¹ and Gln²⁶²). However, enzyme kinetic analyses alone cannot unequivocally determine the positions and putative interactions between the side chains of these residues. Although recent studies provide evidence against the idea that the *Yersinia* PTP–vanadate complex is a true transition-state analogue beyond the geometric similarity [48], these complexes have nonetheless been very useful for describing aspects of E–P hydrolysis [19,46]. Therefore we reasoned that PTP1B_{F182H} complexed with vanadate could provide further insight at the structural level of the importance of residue 182 for catalysis. To allow direct comparison, structures were obtained for both wild-type PTP1B and PTP1B_{F182H} in complex with vanadate.

In Figure 5(A) a well defined $2F_o - F_c$ electron density map of vanadate at the bottom of the active-site pocket of PTP1B_{F182H} is shown. An identical electron density was observed for wild-type PTP1B complexed with vanadate (results not shown). As expected, and consistent with the previous demonstration of a covalent bond between the vanadium atom and the S γ atom of Cys²¹⁵ [19,46], continuous electron density was found between vanadate and the cysteine residue, with a bond length of 2.4 Å. Vanadate forms a trigonal bipyramidal structure and

Figure 4 Leaving-group dependence of PTP-catalysed phosphate monoester hydrolysis

(A, B) Leaving-group dependence of (A) k_{cat}/K_m and (B) k_{cat} for wild-type PTP1B (▲) and the mutant PTP1B_{F182H} (■). (C, D) Leaving-group dependence of (C) k_{cat}/K_m and (D) k_{cat} for wild-type PTPH1 (△) and the mutant PTPH1_{H182F} (□).

Table 4 β_{lg} values for the PTP-catalysed hydrolysis of aryl phosphates at pH 7.0 and 30 °C

Enzyme	β_{lg}	
	k_{cat}/K_m	k_{cat}
PTP1B	-0.20 ± 0.09	-0.035 ± 0.080
PTP1B _{F182H}	-0.15 ± 0.07	-0.076 ± 0.050
PTPH1	-0.25 ± 0.13	-0.073 ± 0.048
PTPH1 _{H182F}	-0.27 ± 0.05	-0.066 ± 0.047

thus at the structural level mimics the pentavalent transition-state intermediate. The overall structure of the PTP1B_{F182H}–vanadate complex is almost identical to that of the PTP1B_{Q262A}–phosphocysteine intermediate (PBD 1A5Y; [19]) and to that of PTP1B_{C215S} complexed with the DADEpYL-NH₂ peptide (IPTU; [14]), with root-mean-square deviations of 0.39 Å and 0.40 Å respectively on C α atoms. Importantly, the apical oxygen mimics the scissile oxygen in the structure of the PTP1B–DADEpYL-NH₂ complex (IPTU) by being similarly positioned (results not shown).

His¹⁸² interacts with Asp¹⁸¹ and Gln²⁶²

Although many of the previously defined features of the structure of the PTP1B_{Q262A}–phosphocysteine intermediate and those reported for the PTP–vanadate complexes are also found in our complex structure, there are significant structural differences that correspond well with the enzyme kinetic observations described above. Thus, while the WPD loop has adopted the closed conformation, the $2F_o - F_c$ omit map of His¹⁸² indicates that the imidazole ring rotates significantly (Figure 5B). However, closer inspection reveals that, in one position with an apparent occupancy of 0.5, the side chain of His¹⁸² may form a hydrogen bond with Gln²⁶², while in another position with an apparent occupancy of

0.5, a hydrogen bond may be formed to Asp¹⁸¹ (Figure 5C). In the PTP1B–vanadate complex, Phe¹⁸² is well defined in the $2F_o - F_c$ electron density map (results not shown).

Asp¹⁸¹ in the PTP1B_{F182H}–vanadate complex is positioned to function as a general base

In the PTP–vanadate complex structures reported for wild-type PTP1B and *Yersinia* PTP, as well as the wild-type PTP1B–vanadate complex in the present study, the Asp¹⁸¹ side chain is within hydrogen-bonding distance of the apical oxygen of vanadate, which corresponds to the oxygen atom of the attacking water molecule ([19,46]; results not shown). These complexes thus provide the structural basis for our understanding of the general-base function of Asp¹⁸¹ (PTP1B) and Asp³⁵⁶ (*Yersinia* PTP). Similarly, Asp¹⁸¹ in the PTP1B_{F182H}–vanadate complex is positioned 2.8 Å from the apical oxygen of vanadate, i.e. consistent with the notion that this residue may function as a general base also in PTPs with a histidine in position 182 (Figure 5C). However, in contrast with wild-type PTP1B with an uncharged phenylalanine in position 182, hydrogen-bond formation between His¹⁸² and Asp¹⁸¹ may influence the general-base functionality.

Gln²⁶²

In both publications reporting PTP–vanadate complex structures [19,46], Gln²⁶² (PTP1B) and the equivalent residue in the *Yersinia* PTP (Gln⁴⁴⁶) are depicted as pointing towards the active site, and in the PTP1B structure this residue forms hydrogen bonds with the apical oxygen of vanadate. In agreement with its highly conserved nature [10], Gln²⁶² in conjunction with Asp¹⁸¹ is hypothesized to co-ordinate and activate the nucleophilic water molecule during E–P hydrolysis. A similar function is indicated in the present PTP1B_{F182H}–vanadate complex structure by the hydrogen bond between the Gln²⁶² side chain and the apical oxygen of vanadate (Figure 5C). However, analogous to the above interaction between

Figure 5 Crystal structure of PTP1B_{F182H} in complex with vanadate

(A) $2F_o - F_c$ map for the structure of the PTP1B_{F182H}-vanadate complex. The electron density maps are contoured in yellow at the 1σ level and in red at the 3σ level. Several cycles of refinement were performed before map calculation. Atoms are coloured according to type (carbon in green, oxygen in red, sulphur in yellow, nitrogen in blue and vanadate in magenta). (B) $2F_o - F_c$ omit map for His¹⁸² in the structure of the PTP1B_{F182H}-vanadate complex. The electron density maps are contoured in yellow at the 1σ level and in red at the 3σ level. The two conformations of the side chain are shown. (C) Two conformations of His¹⁸² showing hydrogen-bond formation to Asp¹⁸¹ (yellow lines) and Gln²⁶² (white lines). Distances are in Å.

His¹⁸² and Asp¹⁸¹, the function of Gln²⁶² may also be influenced by the neighbouring His¹⁸². The observed interaction between Gln²⁶² and His¹⁸² may impair the ability of Gln²⁶² to co-ordinate

the catalytic water molecule correctly, leading to less efficient catalysis.

PTP1B_{F182H} in complex with an active-site-directed inhibitor

To study if the positioning of His¹⁸², and indirectly also residue 181, could be influenced by binding of ligands with aromatic rings, we next decided to undertake X-ray crystallographic studies of PTP1B_{F182H} complexed with compound **1**, which is a selective PTP1B inhibitor. Compound **1** was chosen for these studies rather than a peptide substrate, as we have demonstrated previously that compound **1** mimics the binding of the natural substrate, pTyr, and induces closure of the WPD loop [27].

Superimposition of the PTP1B_{F182H}-(compound **1**) complex and the previously reported wild-type PTP1B-(compound **1**) structure (PDB 1C88) showed that compound **1** is positioned identically in the two structures (root mean square deviation of 0.23 Å between Cα atoms) (Figure 6A). Hence this allows a direct comparison of the structural importance of histidine compared with phenylalanine in position 182. Of note, a phenylalanine in position 182 is in a more favourable position than a histidine for an aromatic-aromatic interaction with the thiophene ring of the inhibitor (Figure 6A). In agreement with this, we observed an ~3-fold reduction in potency for compound **1** when a histidine was introduced in position 182 in PTP1B, and a more than 2-fold increase in potency against PTPH1_{H182F} compared with PTPH1 (Table 5). Similarly, the aromatic-aromatic interaction between His¹⁸² and the phenyl ring of the natural substrate is expected to be weaker than that caused by Phe¹⁸² [49]. Therefore the WPD loop in the closed conformation may be less stable in His¹⁸²-PTPs. Thus, in addition to the previously described direct influence on Asp¹⁸¹ via hydrogen bonding or salt-bridge formation, His¹⁸² may in this way indirectly influence the general acid/base functionality of PTPs.

A more complicated picture emerges when testing compounds **2** and **3**, which are not able to interact with residue 48 (Table 5). Thus, while the interaction with the PTP1B enzymes show higher affinity with a phenylalanine than with a histidine, this is not always the case with the PTPH1 enzymes.

A water molecule next to the imidazole ring of His¹⁸² is apparent in the electron density maps (Figure 6B; electron density not shown). This water molecule, which has not been observed previously in any published PTP1B structure, is within hydrogen-bonding distance of the conserved water molecule observed in all PTP structures with closed WPD loops. The position of the novel water molecule, with possible interactions with both His¹⁸² and Asp¹⁸¹, may indicate participation in hydrolysis. This is illustrated further by superimposing the present complex structure with that of PTP1B_{C215S} in complex with pTyr (PDB 1PTY), which shows that the water molecule is within hydrogen-bonding distance of the scissile oxygen of pTyr (Figure 6C). It should be noted that the phenyl ring of Phe¹⁸² in wild-type PTP1B will displace this water molecule. Thus the presence of the water molecule may simply be the result of the empty space introduced by the smaller volume of the histidine and movement of the histidine side chain compared with the phenylalanine, without any influence on catalysis.

Molecular dynamics studies

We have performed molecular dynamics simulations to identify key sites of interaction of His¹⁸² with nearby residues. In light of the above results, we focused on Asp¹⁸¹ and Gln²⁶² in the E-P hydrolysis step. A PTP1B_{F182H}-cysteinyI-phosphate complex was

Figure 6 PTP1B_{F182H} in complex with a selective PTP1B inhibitor

(A) Superimposition of wild-type PTP1B (PDB 1C88) and PTP1B_{F182H} in complex with compound **1**. Van der Waals distances (Å) between the ring systems are indicated. In addition, the distance between the phenyl ring of Phe¹⁸² and the imidazole ring of His¹⁸² is shown. (B) A novel water molecule in hydrogen-bonding distance to His¹⁸² and Asp¹⁸¹. Also shown is a conserved water molecule observed in all PTP1B structures. (C) Superimposition of PTP1B_{C215S} in complex with pTyr (1PTY) and PTP1B_{F182H} in complex with compound **1**. The ligand is from 1PTY, and Asp¹⁸¹ and His¹⁸² are from the PTP1B_{F182H}-(compound **1**) complex. The novel water molecule is within hydrogen-bonding distance of the scissile oxygen of pTyr. Colour coding is as in Figure 5.

Table 5 Inhibitory effect of compounds **1–3** at pH 6.5 and pH 7.0, measured at 25 °C using *p*NPP as substrate

Enzyme	pH 6.5			pH 7.0		
	Compound 1	Compound 2	Compound 3	Compound 1	Compound 2	Compound 3
PTP1B	1.0 ± 0.2	6.0 ± 0.9	17.8 ± 1.3	2.6 ± 0.3	12.9 ± 1.8	17.8 ± 2.4
PTP1B _{F182H}	2.8 ± 0.3	9.8 ± 0.5	18.1 ± 0.9	5.8 ± 0.6	25.8 ± 2.3	28.9 ± 3.2
PTPH1	9.7 ± 0.7	7.3 ± 1.4	3.1 ± 0.5	22.9 ± 1.8	30.0 ± 4.0	12.9 ± 1.6
PTPH1 _{H182F}	4.0 ± 0.5	17.7 ± 5.6	9.4 ± 0.1	10.9 ± 1.2	30.9 ± 0.9	11.8 ± 2.6

used for the simulation, which was initiated from a conformation where Gln²⁶² is pointing towards the cysteinyl–phosphate intermediate. The initial position of His¹⁸² was chosen according to the position of Phe¹⁸² in PTP1B.

The free histidine residue has a pK_a of 6.2, whereas in proteins the pK_a is shifted and often found to be in the range 6.5–7.5 [50]. However, molecular dynamics simulations based on classical mechanics cannot describe proton (charge) transfer. Therefore, to reflect the different protonation states, His¹⁸² can be either uncharged (i.e. hydrogen on the N δ or N ϵ atom) or charged (i.e. hydrogens on the N δ and N ϵ atoms). Similarly, Asp¹⁸¹ – depending on its function as a general acid or a general base – can be uncharged or negatively charged. From a theoretical point of view, a total of six different combinations of the protonation states of His¹⁸² and Asp¹⁸¹ are therefore possible. However, a search of the Cambridge Structural Database with combinations of charged and uncharged imidazole and carboxylic acid groups revealed that, in 27 out of a total of 35 structures, both the imidazole and the carboxylate were found to be charged. In no case was a combination of uncharged species observed, and in only four cases were a charged imidazole and neutral carboxylic acid or an uncharged imidazole and a carboxylate group observed, meaning that the most likely interaction between His¹⁸² and Asp¹⁸¹ is with both residues in the charged form. Therefore, following the *E–P* formation step where

a proton has been donated, only a negatively charged Asp¹⁸¹ and an uncharged His¹⁸² (i.e. with a hydrogen atom on N δ or N ϵ) are considered in the simulations of the *E–P* hydrolysis step. Although the proton on the nitrogens of the uncharged imidazole ring is in practice shared between the N δ and N ϵ atoms, these cases have to be treated separately in the simulations (see above).

When the hydrogen of an uncharged His¹⁸² is positioned on N δ , we find alternating interactions with Asp¹⁸¹(O δ) and Gln²⁶²(O ϵ) (Figure 7, left panels). As the histidine side chain moves away from Asp¹⁸¹, the distance between His¹⁸²(H δ) and Gln²⁶²(O ϵ) decreases and a hydrogen bond is formed between these residues. When the histidine side chain swings back towards Asp¹⁸¹, the hydrogen patterns are reversed.

In the case where the hydrogen is positioned on N ϵ , a completely different pattern is observed (Figure 7, right panels). Thus there is no interaction between His¹⁸² and Asp¹⁸¹, and the interaction between His¹⁸² and Gln²⁶² is decreased and sporadic. Importantly, the X-ray structure of the PTP1B_{F182H}–vanadate complex suggests a similar preference for Asp¹⁸¹ due to a more favourable positioning of N δ for Asp¹⁸¹ and N ϵ for Gln²⁶².

Thus, in contrast with Phe¹⁸²-PTPs, where residue 182 is not expected to influence the positioning of Asp¹⁸¹ and Gln²⁶², both X-ray crystallography and molecular dynamics simulations show an interaction between these residues in His¹⁸²-PTPs. In accordance

Figure 7 Molecular dynamics

Distances were calculated between selected residues as a function of simulation time. Left panels: His¹⁸²(Hδ)–Asp¹⁸¹(Oδ) (upper) and His¹⁸²(Hδ)–Gln²⁶²(Oε) (lower); His¹⁸² is uncharged with the hydrogen atom situated on Nδ. Right panels: His¹⁸²(Hε)–Asp¹⁸¹(Oδ) (upper) and His¹⁸²(Hε)–Gln²⁶²(Oε) (lower); His¹⁸² is uncharged with the hydrogen atom on Nε.

with this notion, the effect of a histidine in position 182 has significant effect on k_{cat} (Figure 3).

DISCUSSION

By using a reciprocal single-point mutant substitution approach, we here demonstrate that the enzyme kinetic parameters k_{cat}/K_m and k_{cat} of classical PTPs can be influenced by the identity of residue 182. Thus introducing a histidine in this position in PTP1B lowers k_{cat} , and to a lesser extent k_{cat}/K_m , in comparison with the natural phenylalanine residue. Conversely, replacing the histidine in this position in PTPH1 with a phenylalanine increases k_{cat} , and to a lesser degree k_{cat}/K_m , values. Intriguingly, these reciprocal substitutions turn the PTP1B_{F182H} mutant into a PTPH1-like enzyme and the PTPH1_{H182F} mutant into a PTP1B-like enzyme. Noticeably, Yang et al. [51] have demonstrated that replacing His⁴²² in SHP-1 (equivalent to residue 182 in PTP1B) with a phenylalanine or a glutamine increased the k_{cat} value at pH 5. Although not as dramatic as the changes observed in the present study, this supports the notion that residue 182 of PTPs is important for catalysis.

The k_{cat}/K_m value is the apparent second-order rate constant that reflects all steps up to the formation of the E-P intermediate. Kinetic isotope effect studies indicated that the chemical step of phosphoryl transfer from the substrate to the enzymic nucleophile is rate-limiting for the k_{cat}/K_m term [18]. To assess directly the effects of substitution at position 182 on general-acid catalysis, we determined the pH and leaving-group dependencies of the PTP-catalysed reaction (Figures 3 and 4). Of note, we observed a basic limb at pH values above 6 for the PTP1B_{F182H} mutant and wild-type PTPH1, and the $\text{p}K_{\text{E2}}$ values for the general acid were similar for both the Phe¹⁸²- and the His¹⁸²-containing PTPs (Table 2), thus demonstrating that His¹⁸²-PTPs are capable of

general-acid catalysis. In addition, the maximal pH-independent (k_{cat}/K_m)^{lim} values for the His¹⁸²-PTPs were only slightly lower than those of the Phe¹⁸²-PTPs (Table 2). Also, small changes in k_{cat}/K_m were noted when a pTyr-containing peptide was used as a substrate at neutral pH (Table 3). Finally, essentially identical β_{lg} values for k_{cat}/K_m (in the range -0.15 to -0.27 ; Table 4) were observed for the wild-type and mutant PTPs. These values are similar to those determined for PTPs with functional general-acid catalysis [20,21,32,43] and the uncatalysed monoanion reaction ($\beta_{\text{lg}} = -0.27$; [52]), in which the leaving group is protonated in the transition state. In contrast, abrogation of general-acid catalysis through substitution of either the aspartic acid or the tryptophan in the WPD loop results in β_{lg} values similar to that for the solution dianion reaction ($\beta_{\text{lg}} = -1.23$; [52]), which is indicative of the development of a full negative charge in the transition state. Collectively, the results suggest that general-acid catalysis is fully operative in both the His¹⁸²- and Phe¹⁸²-PTPs.

The k_{cat} parameter reflects the rate-limiting step of enzyme catalysis and, in the case of PTPs, observation of burst kinetics and lack of leaving-group dependence on k_{cat} at pH < 7 have shown this to be associated mainly with hydrolysis of the E-P intermediate under acidic to neutral conditions [43–45]. Consistent with E-P hydrolysis being the rate-limiting step, the β_{lg} values for the k_{cat} parameter were effectively zero for both the wild-type and the mutant PTPs (Table 4). Under more basic conditions, the catalytic aspartate residue (Asp¹⁸¹ in PTP1B) will be deprotonated, so that the E-P formation step will eventually become the rate-limiting step. While only modest effects were observed on k_{cat}/K_m , significant changes were seen in k_{cat} when replacing residue 182. Thus the $k_{\text{cat}}^{\text{lim}}$ values of the His¹⁸²-PTPs are 6–7-fold lower than those of the Phe¹⁸²-PTPs (Table 2). Since the largest changes in k_{cat} were seen at acidic to neutral pH values when Phe¹⁸² was replaced by a histidine residue, the large decreases in k_{cat} and $k_{\text{cat}}^{\text{lim}}$ for the His¹⁸²-PTPs appear to be due primarily to impairment of the E-P

hydrolysis step. Consistent with this conclusion, the K_m values for the His¹⁸²-PTPs are lower than those for the Phe¹⁸²-PTPs, indicative of increased accumulation of the E-*P* intermediate in the His¹⁸²-PTP-catalysed reactions. The increases in both k_{cat} and K_m values when replacing a phenylalanine with a histidine result in smaller effects on k_{cat}/K_m , as described above.

Extensive structural and mechanistic studies have shown that Asp¹⁸¹ functions as a general acid during E-*P* formation and as a general base in E-*P* hydrolysis [11,13,17]. Thus the pK_1^{app} from the acidic limb of the k_{cat} profile corresponds to the general base Asp¹⁸¹. Since Asp¹⁸¹ is involved in both E-*P* formation and breakdown, with opposite requirements for protonation, a pH-dependent change in the rate-limiting step occurs. At low pH values, Asp¹⁸¹ is protonated and E-*P* formation is optimal. In contrast, under these conditions E-*P* hydrolysis is impaired. At high pH values, Asp¹⁸¹ is deprotonated and the rate of E-*P* formation (which requires Asp¹⁸¹ to be protonated as a general acid) will decrease and the rate of E-*P* hydrolysis reaches the maximal plateau. Thus pK_2^{app} is a kinetic ' pK_a ' which reflects a change in the rate-limiting step from E-*P* hydrolysis to E-*P* formation as the pH is raised. It is important to point out that the pK_2^{app} is not a real ionization constant and cannot be assigned to a particular group. It is also important to emphasize that a change in rate-limiting step with pH is a common phenomenon in both enzyme- and non-enzyme-catalysed reactions. A well known example is the reaction catalysed by chymotrypsin discussed by Fersht [53]. Consistent with the importance of the general-acid/base function for k_{cat} , removal of the invariant Asp residue eliminates both the acidic and basic limbs of the pH- k_{cat} profile (Z.-Y. Zhang, unpublished work). Substitution at residue 182 does not result in significant changes in the pK_1^{app} value (Table 2). Since the rate of E-*P* hydrolysis is decreased when a histidine residue occupies position 182, the His¹⁸²-PTPs display pK_2^{app} values that are substantially higher than those of the Phe¹⁸²-PTPs, giving rise to a wider plateau in the pH versus k_{cat} profiles.

The effects of reciprocal substitutions of residue 182 in PTP1B and PTPH1 are in agreement with findings from previous mutational studies of Phe¹⁸² and Gln²⁶² [20,22,41,42,54]. The k_{cat}/K_m for PTP1B_{F182A} is ~10-fold lower than that for the wild-type PTP1B [42], which is consistent with demolition of the hydrophobic stacking between Phe¹⁸² and the phenyl ring of pTyr [14]. However, the large effect on k_{cat} (~20-fold decrease for PTP1B_{F182A}) was somewhat unexpected, given that the phenyl ring of the substrate, which interacts with the side chain of Phe¹⁸², has already left the active site during E-*P* hydrolysis. This suggests that Phe¹⁸² also plays a role in the precise positioning of Asp¹⁸¹ during E-*P* hydrolysis. The results described in the present study suggest that a histidine residue can substitute for a phenylalanine at position 182 in the E-*P* formation step, although a histidine seems less efficient at lower pH values, as reflected by the k_{cat}/K_m versus pH curves. In contrast with the effect on E-*P* formation, a histidine residue in position 182 is significantly less efficient in promoting E-*P* hydrolysis. In fact, the phenotypes exhibited by the His¹⁸²-PTPs are similar to those of mutant PTPs in which the side chain of Gln²⁶² has been removed. Large decreases in k_{cat} and K_m were observed for PTP1B_{Q262A}, consistent with the notion that Gln²⁶² is an important participant in E-*P* hydrolysis [20,22,42,54].

The structure of the PTP1B_{F182H}-vanadate complex, which reflects the E-*P* hydrolysis step, offers a molecular basis for the significantly lower k_{cat} values of the His¹⁸²-PTPs. Similar to other PTP-vanadate structures, the side chain of Asp¹⁸¹ in the PTP1B_{F182H}-vanadate complex is positioned 2.8 Å from the apical oxygen of vanadate, indicating that Asp¹⁸¹ may still function as a general base in PTPs with a histidine in position 182. As observed in other PTP-vanadate structures, the side chain of Gln²⁶² forms

a hydrogen bond with the apical oxygen of vanadate, suggesting that Gln²⁶² is capable of positioning and activating the nucleophilic water molecule during E-*P* hydrolysis. However, the structure of the PTP1B_{F182H}-vanadate complex also reveals that His¹⁸² can exist in at least two distinct conformations. In one orientation, the side chain of His¹⁸² forms a hydrogen bond with Gln²⁶², while in another the side chain of His¹⁸² is engaged in a hydrogen-bond interaction with Asp¹⁸¹. Consequently, the hydrogen-bond formation between His¹⁸² and Asp¹⁸¹ may influence the general-base functionality. In addition, the hydrogen-bond interaction between His¹⁸² and Gln²⁶² may affect the Gln²⁶²-mediated activation and positioning of the catalytic water molecule in E-*P* hydrolysis. Molecular dynamics simulation experiments provide further evidence for hydrogen bonding between His¹⁸²/Asp¹⁸¹ and His¹⁸²/Gln²⁶² and thereby potential interference of the functions of Asp¹⁸¹ and Gln²⁶² by His¹⁸².

Compound **1** was used for direct evaluation of the influence of residue 182 on ligand binding affinity, since it is expected to be positioned similarly in the PTP1B and PTPH1 enzymes due to a strong ionic interaction with Asp⁴⁸ [27]. The affinity of compound **1** was found to be 2–3-fold lower for His¹⁸²-PTPs than for Phe¹⁸²-PTPs (Table 5), reflecting weaker aromatic-aromatic interactions with a histidine than with a phenylalanine in position 182. Why do we find that the K_i values for compound **1** are higher in His¹⁸²-PTPs than in Phe¹⁸²-PTPs, when the K_m values are lower? First, it should be noted that recent studies indicate that the influence of residue 182 on binding of inhibitors may be dependent on the nature of inhibitor [41]. In agreement with this notion, a more complicated picture emerges when analysing compounds that do not form the salt bridge to Asp⁴⁸ (Table 5). Thus, while analysis of compounds **2** and **3** with PTP1B and PTP1B_{F182H} clearly showed that a phenylalanine in position 182 is more efficient than a histidine, this was not always the case when analysing PTPH1 and PTPH1_{H182F}. We speculate that the discrepancies between the PTP1B and PTPH1 enzymes in this respect are caused by the bulky Met²⁵⁹ side chain (in PTPH1) leading to a different binding mode of the compounds and thereby a steric clash with Phe¹⁸² in the PTPH1_{H182F} mutant. This effect of Met²⁵⁹ is likely to be overcome by compound **1** due to salt-bridge formation, and in the PTP1B enzymes residue 259, a glycine, does not interfere with binding of any of the compounds. Indeed, we have shown previously that bulky residues in position 259 impair the binding of similar low-molecular-mass PTP inhibitors [26]. Secondly, it is important to note that the position of the aromatic ring of compound **1** does not completely overlap with that of the phenyl ring of pTyr [27]. Thirdly, K_m values are not merely a reflection of affinity, making a direct comparison of K_i and K_m values difficult. Nevertheless, it is likely that the lower K_m values for His¹⁸²-PTPs in fact reflect a stronger binding of pTyr-containing substrates. Thus we hypothesize that the side chain of His¹⁸² binds to the scissile oxygen of pTyr via a novel water molecule. This binding is illustrated by superimposing the PTP1B_{F182H}-(compound **1**) complex and PTP1B_{C215S} in complex with pTyr (Figure 6C), which shows a putative hydrogen bond between His¹⁸² of PTP1B_{F182H} and pTyr via the novel water molecule. Such water-mediated hydrogen-bond formation will contribute significantly to the overall affinity for the substrate, and thus compensate for the minor loss in affinity due to replacement of Phe¹⁸² with the shorter and less aromatic side chain of histidine. In contrast, Phe¹⁸²-PTPs are unable to form hydrogen bonds to the substrate via this mechanism.

Histidine is generally considered to be a polar amino acid. Since its pK_a is close to physiological pH, histidine is an effective intracellular buffer of hydrogen ions. This property is utilized in Nature, where histidines are often found at the active sites of

enzymes as part of a charge relay system, for instance within catalytic triads (e.g. in serine proteases). In accordance with this role in other enzyme systems, we demonstrate here an important role of His¹⁸² in PTP-mediated catalysis. The lowered k_{cat} values of His¹⁸²-PTPs in comparison with Phe¹⁸²-PTPs may reflect either a loss-of-function of the former or a gain-of-function of the latter. The proposed function of PTP1B as a 'general' PTP that keeps newly synthesized tyrosine kinases silent during their transport to the cell membrane [55] is consistent with a gain-of-function hypothesis.

A more direct practical outcome of the present study is the demonstration of a previously unnoticed water molecule, which in conjunction with His¹⁸² potentially may be used for the design of a novel type of selective PTP inhibitors addressing the WPD loop. In this context, it is of particular interest that we and others have demonstrated previously that structural water molecules can be utilized efficiently in structure-based drug design, and contribute significantly to both affinity and selectivity [26].

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