Thermodynamic Study of Ligand Binding to Protein-Tyrosine Phosphatase 1B and Its Substrate-Trapping Mutants

Running Title: Thermodynamics of Ligand Binding to PTP1B

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Abbreviations

PTPase, protein-tyrosine phosphatase; PTP1B, protein tyrosine phosphatase 1B; pY or pTyr, phosphotyrosine; Pmp, phosphonomethyl phenylalanine; F₂Pmp, phosphonodifluoromethyl phenylalanine; pNPP, p-nitrophenyl phosphate; EDTA, ethylenediaminetetraacetic acid.

Footnote:

¹PTP1B hydrolyzes Asp-Ala-Asp-Glu-pTyr-Leu-NH₂ and the N-terminal acetylated form Ac-Asp-Ala-Asp-Glu-pTyr-Leu-NH₂ with the same kinetic constants (45).
Abstract

The binding of several phosphonodifluoromethyl phenylalanine (F2Pmp) containing peptides to protein tyrosine phosphatase 1B (PTP1B) and its substrate-trapping mutants (C215S and D181A) have been studied using isothermal titration calorimetry. The binding of a high affinity ligand, Ac-Asp-Ala-Asp-Glu-F2Pmp-Leu-NH2, to PTP1B (Kd = 0.24 µM) is favored by both enthalpic and entropic contributions. Disruption of ionic interactions between the side chain of Arg47 and the N-terminal acidic residues, reduces the binding affinity primarily through the reduction of the TΔS term. The role of Arg47 may be to maximize surface contact between PTP1B and the peptide, which contributes to high affinity binding. The active site Cys215 to Ser mutant PTP1B binds ligands with the same affinity as the wild-type enzyme. However, unlike wild type PTP1B, peptide binding to C215S is predominately driven by enthalpy change, which likely results from the elimination of the electrostatic repulsion between the thiolate anion and the phosphonate group. The increased enthalpic contribution is offset by reduction in the binding entropy, which may be the result of increased entropy of the unbound protein caused by this mutation. The general acid deficient mutant D181A binds the peptide 5-fold tighter than the C215S mutant, consistent with the observation that the Asp to Ala mutant is a better “substrate-trapping” reagent than C215S. The increased binding affinity for D181A as compared to the wild-type PTP1B results primarily from an increase in the ΔH of binding in the mutant, which may be related to decreased electrostatic repulsion between the phosphate moiety and PTP1B. These results have important implications for the design of high affinity PTP1B inhibitors.
Protein tyrosine phosphatases (PTPases) catalyze the removal of the phosphoryl moiety from phosphotyrosine (pTyr) and play important roles in signal transduction pathways that regulate cell proliferation, differentiation, migration, metabolism and cell death (1-3). The PTPase superfamily is presently comprised of approximately 100 enzymes which includes tyrosine specific, dual specificity, and low molecular weight phosphatases (4). PTP1B is one member of this enzyme family, which in common with all PTPases has the active site signature motif C(X₅)R(S/T) (5). Extensive biochemical and structural studies have led to an understanding of the mechanism by which PTPases catalyze phosphate monoester hydrolysis (4, 6). PTPases share a common catalytic mechanism, utilizing a Cys nucleophile (Cys215 in PTP1B) in the formation of a thiophosphoryl covalent enzyme intermediate (Fig. 1), which undergoes hydrolysis in a second step (7, 8). The invariant Arg residue (Arg221 in PTP1B) functions in substrate binding and in transition state stabilization (5, 9). The initial phosphoryl transfer step is assisted by the conserved Asp (Asp181 in PTP1B) which protonates the leaving group (10-12) thereby acting as a general acid catalyst (Fig. 1).

Research into PTPases has relied heavily upon the use of “substrate-trapping” mutants (13-21). Two types of substrate-trapping mutants have been used to isolate PTPase substrates. In the first, the active site Cys residue is replaced by a Ser while in the second, the general acid Asp residue is substituted by an Ala. Although the Cys to Ser mutant has no measurable phosphatase activity (22) and the catalytic activity of the Asp to Ala mutant is reduced 10⁵-fold towards a protein substrate (21), both mutants retain the ability to bind substrates, enabling their use as “affinity reagents” for the isolation of in vivo PTPase substrates. Interestingly, in several instances, the Asp to Ala mutant has been found to be a better substrate trapping reagent than the Cys to Ser mutant (17, 21). Substrate-trapping mutants have also been employed extensively in structural studies of PTPases. In fact, most of the structural information regarding PTPase-substrate interactions has been obtained not from wild type PTPases, but from substrate complexes with the active site Cys to Ser substrate-trapping mutants (23-26). Though it is often assumed that substrate-trapping mutants
retain the structural and binding properties of the wild type PTPases, significant differences have been found in the few studies that have addressed this issue (22, 27, 28). Given the importance of PTPases in signal transduction, and the importance of substrate-trapping mutants in the studies of PTPases, a clear understanding of the molecular basis of the ability of these mutants to bind substrates is highly desirable.

PTP1B has been implicated as a negative regulator of insulin signaling (29-32). In addition, mice lacking functional PTP1B exhibit increased sensitivity toward insulin and are resistant to obesity (33, 34). These results, taken together, suggest that specific inhibition of PTP1B may be therapeutically beneficial for the treatment of Type II diabetes and obesity. Thus, there is considerable interest in understanding the molecular basis of PTP1B-ligand interactions. An improved understanding of the interactions between PTP1B and its ligands (substrates and/or inhibitors) should facilitate the development of potent and selective PTP1B inhibitors.

We describe here a detailed thermodynamic study of ligand binding to PTP1B and its substrate-trapping mutants using isothermal titration calorimetry. The major advantage of isothermal titration calorimetry is that the association constant as well as the energetics of binding can be measured simultaneously. Differences in ligand binding affinity can be understood in terms of the relative contributions of the enthalpy change (\(\Delta H\)) and the entropy change (\(\Delta S\)) for binding of each ligand, thereby providing a thermodynamic basis for the ability of PTP1B and several mutants to bind ligands. Significant differences between the thermodynamic consequences of the interactions between various forms of PTP1B and a specific phosphonopeptide are reported herein. In addition, the energetics of binding of several ligands to a number of different PTP1B mutants is described, which permits an improved understanding for the observed differences and similarities in ligand binding affinity in terms of the relative contributions of the \(\Delta H\) and the \(\Delta S\) of binding for each ligand.
MATERIALS AND METHODS

**Materials.** p-nitrophenyl phosphate (pNPP) was purchased from Fluka Co. Other chemicals were from Fisher Co. Solutions were prepared using deionized and distilled water. The nonhydrolyzable pTyr mimetic phosphonodifluoromethyl phenylalanine (F2Pmp)-containing peptides, Ac-Glu-F2Pmp-Leu-NH2, Ac-Asp-Ala-Asp-Glu-F2Pmp-Leu-NH2, and Ac-Ser-Ser-Val-Leu-F2Pmp-Thr-NH2 were synthesized using a modification of previously reported solid-phase technique (35), in which F2Pmp reagent (36) lacking phosphonate protection (37) was used. Final HPLC-purified products provided NMR and mass spectral data consistent with their assigned structures.

**Protein Expression and Purification.** The catalytic domain of PTP1B (residues 1 to 321) was used in this study. Site-directed mutagenesis for C215S, D181A, D181N, and R47E was described previously (11, 24, 38, 39). The recombinant wild type and mutant PTP1Bs were expressed in *E. coli* and purified to homogeneity as described (24, 40). Protein concentration was determined from absorbance measurement at 280 nm using an absorbance coefficient 1.24 for 1mg/ml PTP1B.

**Inhibition Study.** The PTPase activity was assayed at 25 °C in a reaction mixture (0.2 ml) containing an appropriate concentration of pNPP as a substrate. The reaction was initiated by addition of the enzyme and quenched after 2 - 3 min by addition of 1 ml of 1N NaOH. The nonenzymatic hydrolysis of the substrate was corrected by measuring the control without the addition of the enzyme. The amount of p-nitrophenol produced was determined from the absorbance at 405 nm using a molar extinction coefficient of 18,000 M⁻¹cm⁻¹. The inhibition constants for the F2Pmp-containing peptides were determined for PTP1B in the following manner. At various fixed concentration of inhibitors, the initial rate at various pNPP concentrations was measured as described (40). All inhibition experiments were performed in 50 mM 3,3-dimethylglutarate pH 7.0 buffer. The ionic strength of each solution was adjusted to 0.15 M by addition of NaCl. Inhibition was competitive with respect to the substrate, and data was fit to
equation [1] using KINETASYST (IntelliKinetics, State College, PA) to obtain the inhibition constant ($K_i$):

$$v = \frac{V_{\text{max}} \cdot S}{K_m (1 + \frac{I}{K_i}) + S}$$

where $V_{\text{max}}$ is the maximal velocity, $K_m$, the Michaelis constant, $S$, the substrate concentration, $I$, the inhibitor concentration, and $K_i$, the inhibition constant.

*Isothermal Titration Calorimetry.* All isothermal titration calorimetry experiments were performed using a MCS Isothermal Titration Calorimetry System from Microcal Inc. Northampton, MA. Experiments at pH 7.0 were conducted at 25 °C, in 50 mM 3,3-dimethylglutarate buffer, containing 1 mM DTT. The ionic strength of the buffer was adjusted to 0.15 M by addition of NaCl. Protein concentration in the calorimeter cell was 27 - 85 µM, while the ligand in the syringe was 0.37 - 1 mM. The PTP1B samples used in the isothermal titration calorimetry experiments were dialyzed completely against buffer. High concentration stock solutions were prepared for ligands with distilled water and adjusted to pH 7.0. Stock was diluted at least 26-fold with 50 mM 3,3-dimethylglutarate buffer before titration. Protein dilution during titration was determined by titration of buffer into the protein solution. The heat of protein dilution was found to be negligible. The heat of ligand dilution was corrected by subtracting the average heat of injection after saturation. The binding data were analyzed using Origin software (41). Binding constants $K$ and enthalpy changes $\Delta H$ were used to calculate free energy change $\Delta G$ and entropy change $\Delta S$ according to equation [2]:

$$-RT\ln K = \Delta G = \Delta H - T\Delta S$$

where $R$ is the gas constant and $T$ is the absolute temperature.

*Calculation of Accessible Surface Areas.* Accessible surface area (ASA) calculations were carried out using Delphi – Insight II (Biosym Technologies, Inc.). A 1.4 Å probe radius was employed. The structures used in this study have the following accession numbers in the Brookhaven Protein Data Bank: PTP1B (2HNP); PTP1B/C215S complexed with Asp-Ala-Asp-Gly-pTyr-Leu-NH2 (1PTU); PTP1B/C215S complexed with Ac-Asp-Glu-pTyr-Leu (1PTT). To
calculate the change in accessible surface area (ΔASA) upon peptide binding, the surface areas of ligand-free PTP1B (ASA_{PTP1B}), free peptide (ASA_{peptide}), and PTP1B-peptide complex (ASA_{PTP1B-peptide}) were determined for both the hexapeptide and the tetrapeptide. The change in accessible surface area was then calculated from ΔASA = ASA_{PTP1B} + ASA_{peptide} - ASA_{PTP1B-peptide} (42). ASA_{peptide} was calculated as the sum of the accessible surface areas of the individual residues in the extended Ala-X-Ala geometry. ASA_{PTP1B} and ASA_{PTP1B-peptide} were calculated from the corresponding crystal structures using coordinates for residues 5-282 in PTP1B to keep the ligand-free and ligand-bound form constant.

**RESULTS AND DISCUSSION**

Isothermal titration calorimetry allows a simultaneous determination of the binding constant (K), stoichiometry, as well as the enthalpy change (ΔH) associated with the binding of a ligand to a macromolecule (41). From these parameters, the Gibbs free energy of binding (ΔG) and the entropy change (ΔS) of binding can also be derived from the expression ΔG = -RT\ln K = ΔH - TΔS. Evaluation of these thermodynamic parameters yields important insight into the nature of binding reaction that are not evident from the measurement of binding constants alone (43, 44). For example, from changes in the magnitude and the sign of the ΔH and ΔS and in conjunction with structural data provided by NMR and X-ray crystallography, one can obtain information about structural alterations that accompany ligand binding in terms of 1) changes in solvation state of the ligand or protein, 2) interactions between ligand and macromolecule, such as hydrogen bonding, dipole-dipole, electrostatic, van der Waals forces, and hydrophobic interaction, and 3) changes in conformation/dynamics induced by ligand binding or by mutation. In the following, we describe a thermodynamic analysis of binding reactions between PTP1B and several site directed mutants with phosphonodifluoromethyl phenylalanine (F₂Pmp)-containing peptides using isothermal titration calorimetry.
The phosphopeptide Ac-Asp-Ala-Asp-Glu-pTyr-Leu-NH₂ (modeled after the epidermal growth factor receptor autophosphorylation site Tyr992, residues 988 to 993) is an excellent substrate for PTP1B with a \( k_{\text{cat}}/K_m \) value (1.88 x 10⁷ M⁻¹ s⁻¹) 2100-fold higher than that of pTyr (45). This indicates that amino acid residues flanking pTyr contribute to efficient PTP1B binding and catalysis. The crystal structure of the catalytically inactive PTP1B/C215S complexed with Asp-Ala-Asp-Glu-pTyr-Leu-NH₂ provides a structural snapshot of the interaction of PTP1B/C215S with this peptide (23). However, to fully appreciate the interactions between a protein binding site and its ligands, a detailed thermodynamic description is a highly desirable complement to the structural data.

**F₂Pmp-Containing Peptides Are Excellent Non-Hydrolyzable Substrate Analogs for PTPases.** Because of inherent hydrolytic activity, it has not been possible to study binding interactions between a PTPase and its substrate directly. In fact, most structural studies have employed catalytically inactive, active site Cys to Ser mutant PTPases to visualize enzyme-substrate complexes (23-26). In order to compare binding interactions between wild-type PTP1B and its various mutants (either active or inactive), we must use nonhydrolyzable phosphopeptide analogs. The most commonly used phosphorus-based pTyr analogs are phosphonomethyl phenylalanine (Pmp) (45) and phosphonodifluoromethyl phenylalanine (F₂Pmp) (46) (Fig. 2). Peptides bearing F₂Pmp are over 1000 times more potent PTPase inhibitors than the analogous peptides containing Pmp (46, 47). This has been attributed to a direct interaction between the fluorine atoms and PTPase active site residues (47). Specific interactions between one of the fluorine atoms in difluoronaphthylmethyl phosphonic acid and PTP1B have been observed (48). Biochemical and structural studies indicate that the phenolic oxygen in the substrate receives a proton from the general acid Asp181 (10, 11, 23) (Fig. 1). The enhanced affinity of F₂Pmp over Pmp likely arises from the ability of the fluorine atoms in F₂Pmp to interact with the PTPase active site residues in a fashion analogous to that involving the phenolic oxygen and side chains in the active site of PTPases. Thus, F₂Pmp-containing peptides are excellent non-hydrolyzable substrate analogs for PTPases.
Binding of Ac-Asp-Ala-Asp-Glu-F2Pmp-Leu-NH2 to PTP1B. The F2Pmp-containing peptide Ac-Asp-Ala-Asp-Glu-F2Pmp-Leu-NH2 is a nonhydrolyzable, competitive inhibitor of PTP1B with a $K_i$ value of 0.25 µM at pH 7.0 and 25 °C (Table1). This value is similar to a previous measurement of 0.18 µM at pH 7.0 and 30 °C (47). Unless stated otherwise, all experiments were performed at pH 7.0 and 25 °C in this study. The dissociation constant ($K_d$) and thermodynamic parameters for the binding of Ac-Asp-Ala-Asp-Glu-F2Pmp-Leu-NH2 to PTP1B were determined by isothermal titration calorimetry and summarized in Table 1. A typical titration curve and binding isotherm is shown in Figure 3. Binding of Ac-Asp-Ala-Asp-Glu-F2Pmp-Leu-NH2 was exothermic at 25 °C ($\Delta H = -3.9$ kcal/mol). From curve fitting of such binding isotherms, the stoichiometry for the binding of the peptide to PTP1B was determined to be 1 : 1. The dissociation constant $K_d$ was 0.24 µM, which is very close to the $K_i$ values determined from inhibition studies.

The $\Delta G$ for binding of Ac-Asp-Ala-Asp-Glu-F2Pmp-Leu-NH2 to PTP1B is -9.0 kcal/mol at pH 7.0 and 25 °C. The association process between PTP1B and the F2Pmp-containing peptide is both enthalpically ($\Delta H = -3.9$ kcal/mol) and entropically ($T\Delta S = 5.1$ kcal/mol) favored. The relative magnitude of $\Delta H$ is usually associated with ligand-protein interactions such as hydrogen bonding, ionic and positive van der Waals interactions, while the magnitude of the $T\Delta S$ term is associated with solvent reorganization and other entropic contributions to binding (49). In the crystal structure of PTP1B/C215S with Asp-Ala-Asp-Glu-pTyr-Leu-NH2, the oxygen atoms of the phosphoryl moiety form an extensive array of hydrogen bonds with the main-chain nitrogens of the PTP loop (residues 215-221) and the guanidinium side-chain of Arg221 (Fig. 4 and 23). In addition to the electrostatic interactions between the phosphoryl moiety and the PTP loop in the active site, binding of phosphopeptides also include favorable $\Delta H$ terms due to hydrogen bonding, ionic and van der Waals interactions between amino acid residues of the peptide and PTP1B. The pTyr phenyl ring is effectively buried within an active site cavity, formed by the nonpolar side-chains of Ala217 and Ile219 of the PTP loop, Phe182 of the WPD loop (residues 179-189), and Tyr46, Val49 and Gln262 (Fig. 4). Specific polar interactions between the enzyme and the peptide backbone stabilize the binding interface. Most notably, Asp48 forms two hydrogen bonds to the main chain nitrogens of
the pTyr and the +1 residues (Fig. 4). The main chain nitrogen of Arg47 forms a hydrogen bond with the main chain carbonyl of the Glu(-2) in the peptide. In addition, the guanidinium group of Arg47 forms salt bridges with the carboxylate groups at the -2 and -1 positions of the peptide substrate and a long hydrogen bond with the main-chain carbonyl at the -4 position (Fig. 4).

Hydrophobic interactions as well as other specific interactions between residues of the hexapeptide and PTP1B and the binding stabilized WPD loop closure are responsible for the burial of an extensive amount of total surface area (869 Å², calculated using Delphi–Insight II, see Materials and Methods). This value is similar to the calculated total buried surface area (902 Å²) upon docking of Asp-Ala-Asp-Glu-F₂Pmp-Leu, whose structure was determined by NMR transferred nuclear Overhauser effects, to the active site of PTP1B (50). Thus, the large favorable \( \Delta T \) term (5.1 kcal/mol) may include the release from the interacting surface of water molecules to the bulk solvent, which provides the major driving force for the association of PTP1B with the peptide.

**Binding of Ac-Glu-F₂Pmp-Leu-NH₂ to PTP1B.** The affinity of the tripeptide Ac-Glu-F₂Pmp-Leu-NH₂ for PTP1B is only 6-fold less than Ac-Asp-Ala-Asp-Glu-F₂Pmp-Leu-NH₂ (Table I). A comparison of the crystal structures of PTP1B/C215S with Ac-Asp-Glu-pTyr-Leu-NH₂ and with Asp-Ala-Asp-Glu-pTyr-Leu-NH₂ (23) reveals that the two hydrogen bonds between the Asp48 carboxylate side chain and the main chain nitrogens of the pTyr and the +1 residue, as well as interactions with the pTyr moiety are conserved in the two structures. One noticeable difference is the amount of buried surface area upon complex formation with PTP1B for the hexapeptide (869 Å²) and the tetrapeptide (669 Å²). Presumably, the tripeptide Ac-Glu-F₂Pmp-Leu-NH₂ interacts with PTP1B in a similar mode as the tetrapeptide does and buries less surface area than 669 Å². Thus, the greater than 200 Å² decrease in the buried surface area for the tripeptide may be responsible for the reduced \( \Delta T \) term for the binding of Ac-Glu-F₂Pmp-Leu-NH₂ to PTP1B (Table I). Another noticeable difference between the two complexes is that the distance between the main-chain nitrogen of Arg47 and the main-chain carbonyl of the -2 residue is shortened from 3.0 Å in the
hexapeptide to 2.6 Å in the tetrapeptide. The large enthalpic gain for the binding of Ac-Glu-F2Pmp-Leu-NH2 to PTP1B may be the result of a shorter and therefore stronger hydrogen bond (51) formed between the main-chain nitrogen of Arg47 and the acetyl carbonyl group in the tripeptide. However, given the resolution of the structures (2.6 Å for the hexapeptide complex and 2.9 Å for the tetrapeptide complex), the observed difference in distance between the main-chain nitrogen of Arg47 and the main-chain carbonyl of the -2 residue may be at the threshold of significance. Alternatively, it is also possible that the increased enthalpic contribution to binding for the tripeptide may be the result of elimination of unfavorable enthalpic interactions in the PTP1B/hexapeptide complex. However, this more favorable ∆H is more than offset by the smaller T∆S, which may result from a smaller surface area buried during complexation between the tripeptide and PTP1B.

**Binding of Ac-Ser-Ser-Val-Leu-F2Pmp-Thr-NH2 to PTP1B.** We also measured the binding of Ac-Ser-Ser-Val-Leu-F2Pmp-Thr-NH2 to PTP1B. This sequence originated from the platelet derived growth factor receptor residues 1005 to 1010. The rationale for choosing this peptide was to study the binding of a “nonspecific” peptide to PTP1B, that lacks acidic residues at the N-terminus. However, it became clear during the course of this work that the -1 binding site in PTP1B can also accommodate a Leu residue (52). Furthermore, crystallographic structural analysis of peptide substrate-PTP1B/C215S complexes shows that the two hydrogen bonds between the Asp48 carboxylate side chain and the main chain nitrogens of the pTyr and the +1 residue, and a third between the main-chain nitrogen of Arg47 and the main-chain carbonyl of the -2 residue occur in all PTP1B/peptide interactions (23, 26). In addition, the pTyr moiety is engaged in similar interactions with PTP1B in all complexes. Thus, the binding affinity of Ac-Ser-Ser-Val-Leu-F2Pmp-Thr-NH2 for PTP1B is only 7.5-fold lower than that of Ac-Asp-Ala-Asp-Glu-F2Pmp-Leu-NH2 (Table 1). This lower affinity apparently results from a reduction of the T∆S term (Table 1). Because Ac-Ser-Ser-Val-Leu-F2Pmp-Thr-NH2 lacks acidic residues at its N-terminus, it is unable to engage in ionic interactions with the guanidinium group of Arg47. Consequently, the distal N-terminal residues may not be able to make as extensive surface contact with PTP1B as the acidic peptide does. The
reduced $\Delta S$ term may be the result of decreased binding interface (less solvent displacement) between Ac-Ser-Ser-Val-Leu-F$_2$Pmp-Thr-NH$_2$ and PTP1B.

**Temperature Dependence of Ac-Asp-Ala-Asp-Glu-F$_2$Pmp-Leu-NH$_2$ Binding to PTP1B.** The temperature dependence of the binding of Ac-Asp-Ala-Asp-Glu-F$_2$Pmp-Leu-NH$_2$ to PTP1B was studied over the range of 16 to 30 °C, with the thermodynamic parameters $\Delta H$, $\Delta S$, and $\Delta G$ as a function of temperature being shown in Table 2. It is clear that the association becomes increasingly enthalpically driven as the temperature increases. This pattern of temperature dependence is characteristic of hydrophobic association (53). The temperature dependence data can be used to determine the heat capacity change ($\Delta C_p = \frac{\delta \Delta H}{\delta T}$) for the binding reaction. A plot of $\Delta H$ versus temperature was linear in this temperature range (Fig. 5A). The slope of the line yields $\Delta C_p$ of -220 ± 20 cal mol$^{-1}$ deg$^{-1}$ for the binding reaction between Ac-Asp-Ala-Asp-Glu-F$_2$Pmp-Leu-NH$_2$ and PTP1B. The $\Delta C_p$ for the binding reaction is modest, and is typical of a rigid-body interaction that does not involve significant gross conformational change in the protein (54). This correlates with crystallographic studies of the free and bound states of PTP1B, which show conformational changes limited only to the WPD loop between the two states (23).

It is interesting to note that though the $\Delta H$ decreases as the temperature increases, the $\Delta G$ is relatively invariant with temperature (Table 2). Hence, $\Delta H$ and $\Delta S$ compensate to provide a relatively constant $\Delta G$. Figure 5B shows an enthalpy/entropy compensation plot for the interaction between the peptide and PTP1B. Similar compensation phenomena have been observed for many macromolecule interactions and are attributed to the unique properties of weak intermolecular interactions in aqueous solution (55, 56).

**Binding of F$_2$Pmp-Containing Peptides to PTP1B/R47E.** The structure of PTP1B/C215S in complex with Asp-Ala-Asp-Glu-pTyr-Leu-NH$_2$ revealed that the guanidinium group of Arg47 forms both salt bridges with the carboxylate groups at the -2 and -1 positions of the peptide substrate and a long hydrogen bond with the main-chain carbonyl at the -4 position (Fig. 4). To test the importance of Arg47 in PTP1B substrate recognition, Arg47 was mutated to a Glu. It has been
previously shown that the abrogation of the electrostatic interactions between Arg47 and the acidic residues in the substrate led to 8-fold decrease in $k_{cat}/K_m$ for the substrate Asp-Ala-Asp-Glu-pTyr-Leu-Ile-Pro-Gln-Gln-Gly. In addition, PTP1B/R47E displays a greater decrease in $k_{cat}/K_m$ than PTP1B/R47A for the same peptide which is consistent with charge repulsion between the Glu residue at position 47 and the acidic residues in the substrate (39). Interestingly, the affinity for the binding of Ac-Asp-Ala-Asp-Glu-F$_2$Pmp-Leu-NH$_2$ to PTP1B/R47E is 110-fold lower than the wild type PTP1B (Table 3). The major effect of the Arg47 to Glu mutation on binding is a large decrease in the TΔS term (Table 1 & 3). This may result from a reduction in the amount of buried surface area due to electrostatic repulsion between the acidic residues in the peptide and Glu47 in the mutant. To further probe the role of Arg47 in peptide binding, we also determined the binding affinity of PTP1B/R47E for Ac-Glu-F$_2$Pmp-Leu-NH$_2$ (Table 3), which is 4-12 fold lower than that of the tripeptide for the wild-type PTP1B. This clearly shows that the importance of the interaction between Arg47 and the –1 acidic residue in the peptide. As a control, we also measured the binding of Ac-Ser-Ser-Val-Leu-F$_2$Pmp-Thr-NH$_2$ to PTP1B/R47E (Table 3). Because Ac-Ser-Ser-Val-Leu-F$_2$Pmp-Thr-NH$_2$ lacks the acidic residues that can engage in electrostatic interactions with Arg47, it should bind wild-type PTP1B and the R47E mutant with equal affinity. This is indeed the case (Table 1 and Table 3). Thus, electrostatic interactions between Arg47 and the N-terminal acidic residues are important for high affinity binding between PTP1B and peptides. Because the kinetic parameter $k_{cat}/K_m$ is an apparent second order constant that contains elements of both substrate binding and catalysis, it does not provide an accurate description of the intrinsic affinity between enzyme and substrate.

**Binding of Ac-Asp-Ala-Asp-Glu-F$_2$Pmp-Leu-NH$_2$ to PTP1B/C215S.** As discussed previously, the active site Cys residue in PTPases acts as a nucleophile to attack the substrate phosphorous atom. Although replacing this Cys residue with a Ser abolishes the PTPase activity, the mutant protein can still bind substrates, as it has been used in substrate trapping experiments and X-ray crystal structural studies. However, it is not known how tightly the Cys to Ser mutant binds substrates as compared to the wild-type PTPase. The affinity of PTP1B/C215S for Ac-Asp-Ala-
Asp-Glu-F₂Pmp-Leu-NH₂ and the thermodynamic parameters associated with binding were measured using isothermal titration calorimetry (Table 4). The dissociation constant of PTP1B/C215S for the peptide is 0.19 µM, which is similar to that of the wild-type PTP1B (0.24 µM). Thus, substitution of Cys215 in PTP1B by a Ser does not change PTP1B’s affinity for peptide substrates, even though the phosphatase activity was completely abolished. The Cys to Ser mutation also does not affect PTPase’s ability to bind suramin, an active site-directed, competitive PTPase inhibitor (38). However, although the free energies of binding to the peptide of the wild-type PTP1B and the C215S mutant were found to be similar, significant differences in binding ∆H and ∆TΔS were observed (Table 4). Unlike the wild-type PTP1B, for which peptide binding is driven by both favorable enthalpic and entropic terms, the ∆H and TΔS for peptide binding to PTP1B/C215S are -10.4 and -1.2 kcal/mol, respectively, indicating that peptide binding is primarily driven by enthalpy with an unfavorable entropic contribution. Although the enthalpic contribution to the binding of peptide to PTP1B/C215S is 6.5 kcal/mole more favorable than the wild-type enzyme, the entropic contribution is disfavored by 6.3 kcal/mol. Thus the changes in enthalpy are not independent of changes in entropy. This is a hallmark of enthalpy/entropy compensation which means that perturbations that increase the enthalpy can also increase the entropy, with little or no effect on the free energy (55).

Enthalpy/entropy compensation has also been observed for the binding of vanadate by the Yersinia PTPase C403S mutant (28). The dissociation constant of C403S for vanadate is 2.3 µM, which is similar to that of the wild-type PTPase (1.3 µM). However, the ∆H and TΔS for the binding of vanadate to the Yersinia PTPase are -10.0 kcal/mol and -2.0 kcal/mol, respectively, while those for the C403S mutant are -14.9 kcal/mol and -7.2 kcal/mol, respectively. Thus the enthalpic contribution to the binding of vanadate for C403S is 4.9 kcal/mole more favorable than the wild-type enzyme, whereas the entropic contribution is disfavored by 5.2 kcal/mol.

To understand the observed enthalpy/entropy compensation in ligand binding of the Cys to Ser mutant, it is important to point out that the side chain of the active site Cys exists as a thiolate
anion at physiological pH (12, 57). Thus, the Cys403 to Ser mutation is not a simple substitution of OH for SH, but rather a replacement of the negatively charged thiolate anion with a neutral hydroxyl group. The enhanced enthalpic contribution for the association of PTP1B/C215S with the peptide may be explained by the removal of the repulsive interaction between the thiolate anion and the negatively charged phosphonic acid moiety. The decrease in the entropic contribution to ligand binding by the Cys to Ser mutant may be the result of enthalpy/entropy compensation, i.e., perturbations that decrease the enthalpy (e.g., due to tightening-up of the system) can also decrease the entropy. Alternatively, the reduction in the value of $T\Delta S$ may also be the result of increased entropy of the unbound state (free protein) due to this mutation (see below).

The thiolate anion in the Yersinia PTPase is stabilized by hydrogen bonds between the backbone amides of the PTP loop, helix $\alpha5$ (58-61), and the side chains of His402 and Thr410 (57, 62). In other words, the thiolate anion in the wild type protein may be responsible for holding the PTP loop and the surrounding loops together (low entropy state). In the Cys to Ser mutant, structural and/or dynamic features that are important for the stabilization of the thiolate in the wild-type PTPase may be disrupted. The loss of the negative charge in the active site may perturb the electrostatic balance leading to more relaxed dynamics in the active site region (high entropy state). Thus compared to the wild-type protein, the Cys to Ser mutant may be more flexible and therefore possesses higher entropy. Consistent with this, the Cys to Ser mutant of the Yersinia PTPase is considerable less stable (22) and experiences increased H/D exchange in the active site region relative to the wild-type protein (28). The overall negative $T\Delta S$ for the binding reaction between PTP1B/C215S and the peptide may thus be the result of a large reduction in protein entropy that more than offsets any positive entropic contribution derived from the liberation of water molecules into bulk solvent due to complex formation (Table 4).

**Binding of Ac-Asp-Ala-Asp-Glu-F$_2$Pmp-Leu-NH$_2$ to PTP1B/D181A and PTP1B/D181N.**

The Asp181 to Ala mutant PTP1B has been found to trap substrates more efficiently than the Cys215 to Ser mutant (21). To provide a quantitative comparison with the Cys to Ser mutant, we
have measured the binding of Ac-Asp-Ala-Asp-Glu-F_{2}Pmp-Leu-NH_{2} by PTP1B/D181A (Table 4). PTP1B/D181A binds the peptide 5-fold tighter than PTP1B/C215S. Similarly, both PTP1B/D181A and \textit{Yersinia} PTP/D356A displayed higher affinity (5-fold) than the wild-type enzymes toward suramin (38). These are consistent with the observation that the Asp to Ala mutant PTPase is a better “substrate-trapping” reagent than the active site Cys to Ser mutant for the identification of physiological PTPase substrates \textit{in vivo}.

The affinity of PTP1B/D181A for the peptide is also 6-fold higher than that of the wild-type PTP1B (Table 4). An examination of the thermodynamic parameters in Table 4 shows that the increased binding affinity for the general acid deficient mutant as compared to the wild type enzyme results primarily from the increase in the \( \Delta H \) of binding in the mutant. In the crystal structures of PTP1B/C215S substrate complexes (23), the carboxyl group of Asp181 makes a hydrogen bond with the phenolic oxygen of pTyr and another one with a phosphoryl oxygen through a water molecule (Fig. 4). Presumably, the F_{2}Pmp moiety interacts with PTP1B in a similar fashion as pTyr does. The mutation of Asp181 to an uncharged Ala will result in a decrease in negative charge at this position. As suggested previously, this decrease in negative charge would reduce the electrostatic repulsion between the phosphate moiety and PTP1B and increase binding affinity (21). Our thermodynamic analyses are fully consistent with this suggestion. Furthermore, because Asn lacks the negative charge but retains the hydrogen bonding potential, one would predict that the affinity of PTP1B/D181N for the peptide should be in between those of the wild type and the Asp to Ala mutant. Indeed, the affinity of PTP1B/D181N for the peptide was determined to be 2-fold higher than wild type and 3-fold lower than PTP1B/D181A (Table 4).

\textit{Implications for PTPase Inhibitor Design.} It has been shown that potent PTPase inhibitors can be obtained when the F_{2}Pmp moiety is incorporated into an appropriate template. However, there is a concern that the dianionic nature of the phosphonate group may compromise its ability to cross cell membranes. On the other hand there is also worry that elimination of the negative charges from the phosphonate may also decrease its affinity for the enzyme. The results described above
show that the active site Cys to Ser mutant PTPases display enhanced enthalpic contribution for the binding of negatively charged ligands (e.g., F$_2$Pmp-containing peptides, suramin, and vanadate), possibly because of the removal of the repulsive interaction between the thiolate anion and the negatively charged phosphate mimics. By the same token, a properly functionalized phosphate surrogate with less or no negative charge when attached to an appropriate aromatic framework would be expected to effectively occupy the native PTPase’s active site. Indeed, high affinity nonphosphate-containing small molecule PTP1B inhibitors have been reported recently (63).

Summary. In the present paper, we report the results of a detailed study of binding thermodynamics of a number of F$_2$Pmp-containing peptides to PTP1B and several mutant forms using isothermal titration microcalorimetry. In addition to the determination of energetics of ligand binding, this study leads to a more enhanced understanding of the molecular basis of PTP1B substrate recognition. The binding of the high affinity ligand Ac-Asp-Ala-Asp-Glu-F$_2$Pmp-Leu-NH$_2$ to PTP1B is favored by both enthalpy and entropic contributions. Significant differences between the thermodynamic consequences of the interactions between the peptide and various forms of PTP1B have been determined. Elimination of the ionic interactions between the side chain of Arg47 and the N-terminal acidic residues, reduces the binding affinity primarily through the reduction of the T$\Delta$S term. The role of Arg47 is to maximize surface burial upon complex formation, which contributes to high affinity binding. Substitution of the general acid Asp181 by a neutral residue improves the binding affinity of the mutant for the ligands, possibly due to the reduction of charge - charge repulsion between the carboxylate and the phosphate group. The fact that D181A binds Ac-Asp-Ala-Asp-Glu-F$_2$Pmp-Leu-NH$_2$ 5 times more tightly than the C215S mutant, supports the conclusion that it is a superior substrate-trapping reagent. PTP1B/C215S binds ligands with the same affinity as the wild-type enzyme. However, unlike the wild type PTP1B, peptide binding to C215S is predominately driven by enthalpy change, which likely results from the elimination of the electrostatic repulsion between the thiolate anion and the phosphonate group. The increased enthalpic contribution is offset by reduction in the binding entropy change, which may be the result of increased entropy of the unbound protein due to this mutation. This study also
demonstrates how structural and dynamic data can be complemented by thermodynamic measurements. The thermodynamic data support conclusions reached from spectroscopic experiments (22, 27) and H/D exchange experiments (28), that the conformation and/or dynamic properties of the Cys to Ser mutant is different from those of the native PTPase.

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REFERENCES

1. Walton, K. M., and Dixon, J. E. (1993) *Annu. Rev. Biochem.* **62**, 101-120

2. Hunter, T. (1995) *Cell* **80**, 225-236

3. Neel, B. G. and Tonks, N. K. (1997) *Curr. Opin. Cell Biol.* **9**, 193-204

4. Zhang, Z.-Y. (1998) *CRC Crit. Rev. Biochem. Mol. Biol.* **33**, 1-52

5. Zhang, Z.-Y., Wang, Y., Wu, L., Fauman, E., Stuckey, J. A., Schubert, H. L., Saper, M. A., and Dixon, J. E. (1994) *Biochemistry* **33**, 15266-15270

6. Barford, D., Das, A. K., and Egloff, M. P. (1998) *Annu. Rev. Biophys. Biomol. Struct.* **27**, 133-64

7. Guan, K. L., and Dixon, J. E. (1991) *J. Biol. Chem.* **266**, 17026-17030

8. Cho, H., Krishnaraj, R., Bannwarth, W., Walsh, C. T. and Anderson, K. S. (1992) *J. Am. Chem. Soc.* **114**, 7296-7298

9. Hoff, R. H., Wu, L., Zhou, B. Zhang, Z.-Y. and Hengge, A. C. (1999) *J. Am. Chem. Soc.* **121**, 9514-9521

10. Zhang, Z.-Y., Wang, Y., and Dixon, J. E. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1624-1627

11. Hengge, A. C., Sowa, G, Wu, L. and Zhang, Z.-Y. (1995) *Biochemistry* **34**, 13982-13987

12. Lohse, D. L., Denu, J. M., Santoro, N. and Dixon, J. E. (1997) *Biochemistry* **36**, 4568-4575

13. Bliska, J. B., Clemens, J. C., Dixon, J. E., and Falkow, S. (1992) *J. Exp. Med.* **176**, 1625-1630

14. Sun, H., Charles, C. H., Lau, L. F., and Tonks, N. K. (1993) *Cell* **75**, 487-493

15. Milarski, K. L., Zhu, G., Pearl, C. G., McNamara, D. J., Dobrusin, E. M., Maclean, D., Thieme-Sefler, A., Zhang, Z.-Y., Sawyer, T., Decker, S. J., Dixon, J. E., and Saltiel, A. R. (1993) *J. Biol. Chem.* **268**, 23634-23639

16. Herbst, R., Cartoll, P. M., Allard, J. D., Schilling, J., Raabe, T., and Simon, M. A. (1996) *Cell* **85**, 899-909

17. Garton, A. J., Flint, A. J., and Tonks, N. K. (1996) *Mol. Cell. Biol.* **16**, 6408-6418

18. Liu, F., Hill, D. E. and Chernoff, J. (1996) *J. Biol. Chem.* **271**, 31290-31295

19. Black, D. S. and Bliska, J. B. (1997) *EMBO J.* **16**, 2730-2744

20. Persson, C., Carballeira, N., Wolf-Watz, H. and Fallman, M (1997) *EMBO J.* **16**, 2307-2318
21. Flint, A. J., Taganis, T., Barford, D., and Tonks, N. K. (1997) *Proc. Natl. Acad. Sci. USA* 94, 1680-1685

22. Zhang, Z.-Y. and Wu, L. (1997) *Biochemistry* 36, 1362-1369

23. Jia, Z., Barford, D., Flint, A. J. and Tonks, N. K. (1995) *Science* 268, 1754-1758

24. Puius, Y. A., Zhao, Y., Sullivan, M., Lawrence, D. S., Almo, S. C. and Zhang, Z.-Y. (1997) *Proc. Natl. Acad. Sci. USA* 94, 13420-13425

25. Yang, J., Cheng, Z., Niu, T., Liang, X., Zhao, Z. J. and Zhou, G. W. (2000) *J. Biol. Chem.* 275, 4066-4071

26. Sarmiento, M., Puius, Y. A., Vetter, S. W., Keng, Y.-F., Wu, L., Zhao, Y., Lawrence, D. S., Almo, S. C. and Zhang, Z.-Y. (2000) *Biochemistry* 39, 8171-8179

27. Juszczak, L. J., Zhang, Z.-Y., Wu, L., Gottfried, D. S., and Eads, D. D. (1997) *Biochemistry* 36, 2227-2236

28. Wang, F., Li, W., Emmett, M. R., Hendrickson, C. L., Marshall, A. G., Zhang, Y.-L., Wu, L., and Zhang, Z.-Y. (1998) *Biochemistry* 37, 15289-15299

29. Ahmad, F., Li, P. M., Meyerovitch, J. and Goldstein, B. J. (1995) *J. Biol. Chem.* 270, 20503-20508

30. Kenner, K. A., Anyanwu, E., Olefsky, J. M., and Kusari, J. (1996) *J. Biol. Chem.* 271, 19810-19816.

31. Seely, B. L., Staubs, P. A., Reichart, D. R., Perhanu, P., Milarski, K. L., Satiel, A. R., Kusari, J. and Olefsky, J. M. (1996) *Diabetes* 45, 1379-1385

32. Bandyopadhyay, D., Kusari, A., Kenner, K. A., Liu, F.; Chernoff, J., Gustafson, T. A., and Kusari, J. (1997) *J. Biol. Chem.* 272, 1639-1645

33. Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., Loy, A. L., Normandin, D., Cheng, A., Himms-Hagen, J., Chan, C.-C., Ramachandran, C., Gresser, M. J., Tremblay, M. L., and Kennedy, B. P. (1999) *Science* 283, 1544-1548
34. Klaman, L. D., Boss, O., Peroni, O. D., Kim, J. K., Martino, J. L., Zablotny, J. M., Moghal, N., Lubkin, M., Kim, Y. B., Sharpe, A. H., Stricker-Krongrad, A., Shulman, G. I., Neel, B. G., and Kahn, B. B. (2000) *Mol. Cell. Biol.* **20**, 5479-5489

35. Burke, T. R. Jr., Kole, H. K., Roller, P. P. (1994) *Biochem. Biophys. Res. Commun.* **204**, 129-134

36. Smyth, M. S. and Burke, T. R., Jr. (1994) *Tetrahedron Lett.* **35**, 551-554

37. Gordeev, M. F., Patel, D. V., Barker, P. L. and Gordon, E. M. (1994) *Tetrahedron Lett.* **35**, 7585-7588

38. Zhang, Y.-L., Keng, Y.-F., Zhao, Y., Wu, L. and Zhang, Z.-Y. (1998) *J. Biol. Chem.* **273**, 12281-12287

39. Sarmiento, M., Zhao, Y., Gordon, S. J. and Zhang, Z.-Y. (1998) *J. Biol. Chem.* **273**, 26368-26374

40. Zhang, Y.-L. and Zhang, Z.-Y. (1998) *Anal. Biochem.* **261**, 139-148

41. Wiseman, T., Williston, S., Brandts, J. and Lin, L.-N. (1989) *Anal. Biochem.* **179**, 131-137

42. Bradshaw, J. M., Grucza, R. A., Ladbury, J. E., and Waksman, G. (1998) *Biochemistry* **37**, 9083-9090

43. Connelly, P. R., Varadarajan, R., Sturtevant, J. M. and Richards, F. M. (1990) *Biochemistry* **29**, 6108-6114

44. Ladbury, J. E. and Chowdhry, B. Z. (1996) *Chem. Biol.* **3**, 791-801

45. Zhang, Z.-Y., Maclean, D., McNamara, D. J., Sawyer, T. K., and Dixon, J. E. (1994) *Biochemistry* **33**, 2285-2290

46. Burke, T. R., Jr. Smyth, M., Nomizu, M., Otaka, A., Roller, P. P. (1993) *J. Org. Chem.* **58**, 1336-1340

47. Chen, L., Wu, L., Otaka, A., Smyth, M. S., Roller, P. P., Burke, T. R., den Hertog, J. & Zhang, Z.-Y. (1995) *Biochem. Biophys. Res. Commun.* **216**, 976-984

48. Burke, T. R. Jr., Ye, B., Yan, X., Wang, S., Jia, Z., Chen, L., Zhang, Z.-Y., and Barford, D. (1996) *Biochemistry* **35**, 15989-15996

49. Hinz, H. J. (1983) *Annu. Rev. Biophys. Bioeng.* **12**, 285-317
50. Glover, N. R. and Tracey, A. S. (1999) *Biochemistry* **38**, 5256-5271

51. Hibbert, F. and Emsley, J. (1990) *Adv. Phys. Org. Chem.* **26**, 255-379

52. Vetter, S. W., Keng, Y.-F., Lawrence, D. S. and Zhang, Z.-Y. (2000) *J. Biol. Chem.* **275**, 2265-2268

53. Sigurskjold, B. W., Berland, C. R. and Svensson, B. (1994) *Biochemistry* **33**, 10191-10199

54. Spolar, R. S. and Record, M. T. (1994) *Science* **263**, 777-783

55. Lumry, E. and Rajender, S. (1970) *Biopolymers* **9**, 1125-1227

56. Dunitz, J. D. (1995) *Curr. Biol.* **2**, 709-712

57. Zhang, Z.-Y. and Dixon, J. E. (1993) *Biochemistry* **32**, 9340-9345

58. Stuckey, J. A., Schubert, H. L., Fauman, E. B., Zhang, Z.-Y., Dixon, J. E., and Saper, M. A. (1994) *Nature* **370**, 571-575

59. Barford, D., Flint, A. J. and Tonks, N. K. (1994) *Science* **263**, 1397-1404

60. Peters, G. H., Frimurer, T. M., and Olson, O. H. (1998) *Biochemistry* **37**, 5383-5393

61. Alhambra, C., Wu, L., Zhang, Z.-Y. and Gao, J. (1998) *J. Am. Chem. Soc.* **120**, 3858-3866

62. Zhang, Z.-Y., Palfey, B. A., Wu, L. and Zhao, Y. (1995) *Biochemistry* **34**, 16389-16396

63. Wrobel, J., Sredy, J., Moxham, C., Dietrich, A., Li, Z., Sawicki, D. R., Seestaller, L., Wu, L., Katz, A., Sullivan, D. Tio, C. and Zhang, Z.-Y. (1999) *J. Med. Chem.* **42**, 3199-3202
Table 1

**Thermodynamic Parameters for the Binding of PTP1B with F₂Pmp-Containing Peptides**

| Ligand                                         | $K_d$ (µM) | $\Delta H$ (kcal mol$^{-1}$) | $T\Delta S$ (kcal mol$^{-1}$) | $\Delta G$ (kcal mol$^{-1}$) |
|-----------------------------------------------|------------|------------------------------|-------------------------------|-----------------------------|
| Ac-Glu-F₂Pmp-Leu-NH$_2$                      | 1.4±0.5    | -5.9±0.4                     | 2.0±0.4                       | -7.9±0.2                    |
|                                               | $^a$(0.8±0.1) |                             |                               |                             |
| Ac-Asp-Ala-Asp-Glu-F₂Pmp-Leu-NH$_2$           | 0.24±0.05  | -3.9±0.2                     | 5.1±0.2                       | -9.0±0.1                    |
|                                               | $^a$(0.25±0.02) |                          |                               |                             |
| Ac-Ser-Ser-Val-Leu-F₂Pmp-Thr-NH$_2$           | 1.8±0.2    | -4.3±0.1                     | 3.5±0.1                       | -7.8±0.07                   |
|                                               | $^a$(2.0±0.2) |                              |                               |                             |

All experiments were performed at 25 °C and pH 7.0. $^a$K$_i$ values (inhibition constants) were measured by steady state kinetic experiments under the same conditions. Errors for individual titration experiments were obtained from the direct nonlinear regression fit of the data to the binding isotherms using the Origin program (41). Similar results were obtained from multiple measurements. The reported values are the average of all experiments and the errors were standard deviations.
Table 2

Temperature Dependence for the Binding of PTP1B with Ac-Asp-Ala-Asp-Glu-F$_2$Pmp-Leu-NH$_2$

| Temperature (°C) | $K_d$ (µM) | $\Delta H$ (kcal mol$^{-1}$) | $T\Delta S$ (kcal mol$^{-1}$) | $\Delta G$ (kcal mol$^{-1}$) |
|-----------------|------------|-----------------------------|-----------------------------|-----------------------------|
| 16              | 0.44±0.17  | -1.8±0.06                   | 6.6±0.2                     | -8.4±0.2                    |
| 19              | 0.29±0.08  | -2.5±0.05                   | 6.2±0.2                     | -8.7±0.2                    |
| 22              | 0.34±0.05  | -3.5±0.04                   | 5.2±0.1                     | -8.7±0.1                    |
| 25              | 0.24±0.05  | -3.9±0.1                    | 5.1±0.1                     | -9.0±0.1                    |
| 26              | 0.45±0.09  | -4.1±0.1                    | 4.6±0.1                     | -8.7±0.1                    |
| 30              | 0.25±0.10  | -4.9±0.2                    | 4.3±0.3                     | -9.2±0.2                    |

All experiments were performed at pH 7.0.
Table 3

Thermodynamic Parameters for the Binding of PTP1B/R47E with F$_2$Pmp-Containing Peptides

| Peptide                  | $K_d$ (µM) | $\Delta H$ (kcal mol$^{-1}$) | $T\Delta S$ (kcal mol$^{-1}$) | $\Delta G$ (kcal mol$^{-1}$) |
|--------------------------|------------|-------------------------------|-------------------------------|-------------------------------|
| Ac-Glu-F$_2$Pmp-Leu-NH$_2$ | 6.1±1.2 $^a$ (10±2) | -5.3±0.4                      | 1.8±0.4                       | -7.1±0.1                      |
| Ac-Asp-Ala-Asp-Glu-F$_2$Pmp-Leu-NH$_2$ | 26±4       | -4.7±0.5                      | 1.5±0.5                       | -6.2±0.1                      |
| Ac-Ser-Ser-Val-Leu-F$_2$Pmp-Thr-NH$_2$ | 1.8±0.2    | -3.0±0.1                      | 4.8±0.1                       | -7.8±0.07                     |

All experiments were performed at 25 °C and pH 7.0. $^a$The $K_i$ value (inhibition constant) was measured by steady state kinetic experiments under the same conditions.
Table 4

Thermodynamic Parameters for the Binding of PTP1B and Its Substrate-Trapping Mutants with Ac-Asp-Ala-Asp-Glu-F_{2}Pmp-Leu-NH_{2}

| PTP1B     | K_{d} (µM) | ΔH (kcal mol^{-1}) | TΔS (kcal mol^{-1}) | ΔG (kcal mol^{-1}) |
|-----------|------------|--------------------|---------------------|--------------------|
| Wild Type | 0.24±0.05  | -3.9±0.2           | 5.1±0.2             | -9.0±0.1           |
| D181N     | 0.12±0.03  | -4.7±0.1           | 4.7±0.1             | -9.4±0.1           |
| D181A     | 0.04±0.01  | -6.5±0.1           | 3.7±0.1             | -10.2±0.1          |
| C215S     | 0.19±0.03  | -10.4±0.2          | -1.2±0.2            | -9.2±0.1           |

All experiments were performed at 25 °C and pH 7.0.
Figure Legends

Figure 1. Transition state for the initial phosphoryl transfer from the substrate to the PTPase. The PTP loop consists of residues in the PTPase signature motif. The general acid Asp resides on a flexible loop that undergoes a major conformational change upon binding of substrate (23, 58). This loop is termed the WPD loop for the conserved Trp-Pro-Asp sequence.

Figure 2. Structures of phosphotyrosine (pTyr), phosphonomethyl phenylalanine (Pmp), and phosphonodifluoromethyl phenylalanine (F₂Pmp).

Figure 3. Calorimetric isothermal titration for the reaction of peptide Ac-Asp-Ala-Asp-Glu-F₂Pmp-Leu-NH₂ with PTP1B/D181N. Top: raw data for thirty 8-µl injections of the peptide (1.0 mM stock) into the isothermal cell containing 80 µM PTP1B/D181N at 2.5 min intervals and 25 °C. Both the protein and the peptide were in 50 mM 3,3-dimethylglutarate buffer, pH 7.0, containing 1 mM DTT. Bottom: integrated curve showing experimental points that were obtained by integration of the above peaks plotted against the molar ratio of the peptide to PTP1B/D181N in the reaction cell. The solid line corresponds to the best fit to the data by a nonlinear least-squares regression algorithm ORIGIN (41).

Figure 4: Schematic representations of the interactions between PTP1B/C215S and the hexapeptide DADEpYL-NH₂ (23). Peptide substrate residues are shown in bold. Dashed lines indicate hydrogen bonding and/or electrostatic interactions.

Figure 5: A. Effect of temperature on ΔH for the binding of Ac-Asp-Ala-Asp-Glu-F₂Pmp-Leu-NH₂ to wild type PTP1B. The solid line is a linear regression fit to the data. B. Plot of ΔH versus TΔS for the binding of Ac-Asp-Ala-Asp-Glu-F₂Pmp-Leu-NH₂ to the wild type PTP1B. The solid line is a linear regression fit to the data.
Thermodynamic study of ligand binding to protein tyrosine phosphatase 1B and its substrate-trapping mutants
Yan-Ling Zhang, Zhu-Jun Yao, Mauro Sarmiento, Li Wu, Terrence R. Burke, Jr. and Zhong-Yin Zhang

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