The structure of apo protein-tyrosine phosphatase 1B C215S mutant: More than just an S → O change

GIOVANNA SCAPIN,¹ SANGITA PATEL,¹ VIRA PATEL,² BRIAN KENNEDY,² AND ERNEST ASANTE-APPIAH²

¹Department of Endocrinology and Chemical Biology, Merck Research Laboratories, Rahway, NJ 07065, USA

²Department of Biochemistry and Molecular Biology, Merck Frosst, Pointe-Claire-Dorval, Quebec H9R 4P8

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Abstract

Protein-tyrosine phosphatases catalyze the hydrolysis of phosphate monoesters via a two-step mechanism involving a covalent phospho-enzyme intermediate. Biochemical and site-directed mutagenesis experiments show that the invariant Cys residue present in the PTPase signature motif (H/V)CX₅R(S/T) (i.e., C215 in PTP1B) is absolutely required for activity. Mutation of the invariant Cys to Ser results in a catalytically inactive enzyme, which still is capable of binding substrates and inhibitors. Although it often is assumed that substrate-trapping mutants such as the C215S retain, in solution, the structural and binding properties of wild-type PTPases, significant differences have been found in the few studies that have addressed this issue, suggesting that the mutation may lead to structural/conformational alterations in or near the PTP1B binding site. Several crystal structures of apo-WT PTP1B, and of WT- and C215S-mutant PTP1B in complex with different ligands are available, but no structure of the apo-PTP1B C215S has ever been reported. In all previously reported structures, residues of the PTPase signature motif have an identical conformation, while residues of the WPD loop (a surface loop which includes the catalytic Asp) assume a different conformation in the presence or absence of ligand. These observations led to the hypothesis that the different spectroscopic and thermodynamic properties of the mutant protein may be the result of a different conformation for the WPD loop. We report here the structure of the apo-PTP1B C215S mutant, which reveals that, while the WPD loop is in the open conformation observed in the apo WT enzyme crystal structure, the residues of the PTPases signature motif are in a dramatically different conformation. These results provide a structural basis for the differences in spectroscopic properties and thermodynamic parameters in inhibitor binding observed for the wild-type and mutant enzymes.

Keywords: Crystal structure; substrate-trapping mutant; conformational change; loop flexibility

Reversible tyrosine phosphorylation, controlled by protein tyrosine kinases and protein tyrosine phosphatases (PTPases), plays a critical role in the transduction of signals that control many diverse processes, including passage through the cell cycle, proliferation and differentiation, metabolism, cytoskeletal organization, neuronal development,

and immune response (for reviews see Fisher 1991; Hunter 1995; Barford 1995; Neel and Tonks 1997). PTPases constitute a large, structurally diverse family of receptor-like and cytoplasmic enzymes expressed in all eukaryotes. There are ~100 PTPase genes encoded within the human genome, including transmembrane, receptor-like, and intracellular enzymes. Each PTPase is composed of at least one conserved domain characterized by a unique 11-residue sequence motif (PTPase signature motif, or P-loop, (I/V)HCX-AGXXR(S/T)G) containing the cysteine and arginine residues known to be essential for catalytic activity. The PTPase catalyzed reaction proceeds through a double-displacement mechanism in which the phosphoryl group of the

Reprint requests to: Giovanna Scapin, Department of Endocrinology and Chemical Biology, Merck Research Laboratories, PO Box 2000, Rahway, New Jersey 07065, USA; e-mail: Giovanna_scapin@merck.com; fax: (732) 594-5042.

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phosphorylated substrate first is transferred to the active site Cys residue (Cys215 in PTP1B) within the PTPase signature motif, leading to the formation of a cysteinyl-phosphate intermediate. The intermediate is subsequently hydrolyzed by water (Guan and Dixon 1991; Cho et al. 1992). The invariant Arg residue (Arg221 in PTP1B) functions in substrate binding and in transition-state stabilization (Zhang et al. 1994a; Hoff et al. 1999). The initial phosphoryl transfer step is assisted by the conserved Asp (Asp-181 in PTP1B) in the WPD loop, which acts as a general acid catalyst protonating the leaving group (Zhang et al. 1994b; Hengge et al. 1995; Lohse et al. 1997). The WPD loop is a flexible surface loop, spanning residues 175–184 in PTP1B, which derives its name from the conserved tripeptide Trp-Pro-Asp (179–181 in PTP1B). This loop has been shown to adopt different conformations in the unliganded and liganded forms of the enzyme (Barford et al. 1994; Jia et al. 1995). In the unliganded structure, the WPD loop is in an open conformation, such that the catalytic Asp is ~10 Å away from the phosphate-binding loop (P-loop); upon substrate binding, the WPD loop assumes a closed conformation, covers the active site like a “flap”, and positions the catalytic Asp close to the leaving group oxygen of the substrate (Fig. 1A).

“Substrate-trapping” mutants of two types have been used to characterize PTPases: in the first, the active site Cys is replaced by a Ser, whereas in the second, the general acid Asp residue is substituted by an Ala. Although the Cys to Ser mutant has no measurable phosphatase activity (Zhang and Wu 1997), and the catalytic activity of the Asp to Ala mutant is reduced 10⁵-fold toward a protein substrate (Flint et al. 1997), both mutants retain the ability to bind substrates. Although it often is assumed that substrate-trapping mutants retain the structural and substrate binding properties of wild-type PTPases, significant differences have been found in the few solution-phase studies that have addressed this issue directly. For example, Zhang and Wu (1997) showed that stability and spectroscopic properties of the wild type and the Cys to Ser mutant of the *Yersinia* PTPase are different; Juszczak et al. (1997) used time-resolved fluorescence anisotropy and steady-state ultraviolet resonance Raman (UVR) spectroscopies to show that the WPD loop flexibility is drastically different in the WT *Yersinia* PTPase and in the C403S (active site Cys residue) mutant. Wang et al. (1998), using hydrogen/deuterium (H/D) exchange and electrospray ionization mass spectrometry showed that the single active site mutation in *Yersinia* PTPase reduces solvent access to the WPD loop but increases access to several other segments, including the segment containing the catalytic Cys. More recently, Zhang et al. (2000) showed that while binding of high affinity ligands to WT-PTP1B is favored by both enthalpic and entropic contributions, the binding to the C215S mutant is enthalpy-driven, with negative entropic contribution. This evidence points to struc-

tural/conformational alterations in the active site mutants, which, in the case of the *Yersinia* PTPase, have been attributed mostly to differences in flexibility and possibly positioning of the WPD loop in the wild-type and active-site mutant in the absence of ligand. Crystal structures of WT PTP1B and WT *Yersinia* PTPase, both in the apo form and in complex with several inhibitors, have been reported (Barford et al. 1994; Stuckey et al. 1994; Fauman et al. 1995; Groves et al. 1998; Pannifer et al. 1998; Iversen et al. 2000; Andersen et al. 2000). Crystal structures of PTP1B C215S mutant and of the corresponding *Yersinia* mutant in complex with high affinity substrates also are available (Schubert et al. 1995; Jia et al. 1995; Puius et al. 1997; Sarmiento et al. 2000; Salmeen et al. 2000). These structures show that the human and the *Yersinia* enzymes are very similar structurally (Fig. 1B); there also are no noticeable differences, in the liganded state, between WT and mutant enzymes. However, to our knowledge, no published study has directly correlated the liganded and unliganded structure of the mutant PTP1B. Jia et al. (1995) contains a reference to unpublished structural studies on mutant PTP1B that indicates that the structure is unchanged from the wild-type protein, leaving no structural explanation for the observed functional differences between the two forms of the enzyme. We report here the 2.1 Å structure of the unliganded PTP1B C215S mutant. The structure reveals that, in absence of ligand, the conformation of the P-loop in the mutant is significantly different from that observed in the WT enzyme (Fig. 1C and Fig. 2). The substantial conformational change observed provides the molecular basis for the observed differences in thermodynamic parameters for ligand binding between the WT and C215S mutant (Zhang et al. 2000). In addition, given the high structural homology between the human and the *Yersinia* enzyme, this structure may explain the H/D exchange results (Wang et al. 1998) and the spectroscopic data (Juszczak et al. 1997) reported for the *Yersinia* PTPase.

Results and Discussion

The crystal structure of apo-PTP1B C215S was solved and refined to 2.1 Å using standard crystallographic procedures. The current model (R-factor is 19.5%, R_{free} is 26.6%) contains protein residues 2–285, 250 water atoms, 1 Mg²⁺ ion and 5 Cl⁻ ions. The N-terminal FLAG residues and the C-terminal 13 residues are not visible in the electron density map and are assumed to be disordered.

PTP1B C215S mutant and WT PTP1B are structurally different in the absence of ligand

The structures of apo PTP1B C215S mutant (this work) and apo PTP1B wild type (2HNP) are very similar, except for the conformation of the P-loop: the rmsd on Cα for residues 5–214 and 222–282 (i.e., with exclusion of the phosphate-

Fig. 1. (A) Stereoview of the overlaid C α trace for the liganded wild-type (green, PDB code 1BZI) and unliganded wild-type (magenta, PDB code 2HNP) form of human PTP1B. Upon ligand binding, the WPD loop (orange in figure) assumes a closed conformation and covers the active site like a “flap”. (B) Stereoview of the overlaid C α trace for the unliganded human wild-type PTP1B (magenta, PDB code 2HNP) and the unliganded *Yersinia* wild-type PTPase (cyan, PDB code 1YPT), centered on the binding site. The primary sequence alignment for the residues that comprise the binding site also is displayed. Residues are colored to correspond to the structural elements identified in the ribbon diagrams. (C) Ribbon diagram of the C α trace of apo PTP1B C215S mutant (yellow, this work) overlaid onto the structure of the liganded PTP1B C215S mutant (red; PDB file 1PTY)

binding loop) is 0.78 Å. The other significant differences observed between the WT and mutant structures are located in the region spanning residues 110–120 and 235–241. These residues belong to flexible loops, and the differences

observed between the two structures in this region are likely the result of different crystal packing. Figure 3 shows stereoviews of the PTPase signature motif region in the unliganded human WT enzyme (A; 2HNP), *Yersinia* WT en-

Fig. 2. Stereoview of the 2Fo-Fc electron density map calculated with data between 20.0 and 2.3 Å for the region corresponding to the phosphate-binding loop (P-loop) (residues 213–222) in the PTP1B C215S mutant structure, with superimposed the P-loop as found in the apo WT enzyme. The map is contoured at 1.5 σ .

zyme (B, 1YPT) and human C215S mutant enzyme (C, this work). The active site cysteine has been shown, in the *Yersinia* enzyme, to be negatively charged at physiological pH (Zhang and Dixon 1993). Given the high primary and tertiary sequence similarity between the *Yersinia* and human enzyme in the active site area (Fig. 1B), it is likely that the catalytic Cys residue is present as a thiolate in the human enzyme as well. Indeed, iodoacetate titration and computational studies (Dillet et al. 2000) show that the pKa for C215 in PTP1B is about 5.5. In both WT enzymes, residues of the PTPase signature motif (213–221, numbered according to PTP1B) form a distinctive phosphate-binding loop (or P-loop), similar to the phosphate-binding loop found in phosphoribosyltransferases (Vos et al. 1997, and references therein) and the anion-binding loop in rhodanese (Ploegman et al. 1978). The side-chain of Cys 215 is facing the inside of the loop. The main-chain nitrogens of residues 216–221, which also point to the inside of the loop, together with nearby invariant arginine residues, may stabilize the thiolate-negative charge: C215 S γ is located between 3.5 and 4.5 Å from every amide nitrogen of the P-loop, and should make reasonable S-NH hydrogen bonds (Gregoret et al. 1991). Alternatively, the P-loop amides may be considered individual microdipoles with their δ^+ ends oriented towards the thiolate (Stuckey et al. 1994; Dillet et al. 2000). Barford et al. (1994) suggest that the orientation of the P-loop is maintained by polar groups on neighboring conserved residues, which form hydrogen bonds to the main-chain carbonyl groups of the loop: such hydrogen bonds are formed between the carbonyl oxygens of C215, S216, G217, and

I219 and the side-chain ND1 of H214, the main-chain nitrogen of G86, the side-chain NH2 of R257, and the main-chain nitrogen of I261, respectively (Fig. 3A).

In the mutant enzyme (Fig. 3C) residues 215–220 assume a completely different conformation and extend into the substrate-binding area. The loop is solvent-exposed, and several interactions are found between ordered waters and loop residues. Residues of the loop change their main-chain conformation from mostly α -helical to β -strand, as shown by a pair-wise comparison of the values for their ϕ , ψ angles (Table 2). Interactions with surrounding residues also are different and appear to stabilize the extended conformation of the loop. The average temperature factor ($\langle B \rangle$) for residues 214–222 is 27.5 Å², slightly lower than the overall average B for the protein (36.4 Å²): these numbers

Table 1. Statistics for the data used to solve the structure of the apo PTP1B C215S mutant

Space Group	P3 ₁ 21
Unit Cell Parameters	a = b = 87.4 Å, c = 95.9 Å $\alpha = \beta = 90.0^\circ$, $\gamma = 120.0^\circ$
Molecule/au	1
Resolution range (Å)	20–2.1
No. of observations	259144 (43394)
No. of reflections	25181 (4136)
Completeness (%)	99.9 (100)
$\langle I/\sigma I \rangle$	11.7 (1.9)
Rmerge (I)	6.1 (39.8)

Numbers refer to all data with $I > -3 \sigma I$. Numbers in parentheses represent measurements in the last shell of resolution (2.2–2.1 Å)

Fig. 3 Stereoview of the PTPase signature motif region in the unliganded (A) PTP1B wild-type, (B) *Yersinia* PTPase wild-type and (C) PTP1B C215S mutant. The orientation of the loop is the same as in Figure 1C. The different conformation of the loop can be seen easily by comparing the position of A217 (A405 in the *Yersinia* enzyme) in the WT (A and B) and mutant (C) enzyme.

are consistent with those for the WT enzyme (16.3 and 21.7 Å³, respectively), suggesting that the extended loop conformation is fairly stable. The main-chain carbonyl oxygen of

S215 is still hydrogen bonded to the side chain of H214, but its side chain now is facing the solvent. The main-chain nitrogen and oxygen of A217 are within hydrogen-bonding

distance from the main-chain oxygen and the side-chain nitrogen of K120, respectively. The main-chain nitrogen of G218 is hydrogen bonded to the side-chain hydroxyl of Y46. The main-chain nitrogen and oxygen of G220 are interacting with the side-chain oxygen of Q262 and the side-chain nitrogen of Q266, respectively. R221 and S222 have the same conformation as in the wild-type enzyme.

The crystal packing in 2HNQ and in the structure we report here are different, and this difference probably is caused by the extended conformation of the loop itself, which brings hydrophobic residues (A217, G218, I219) to the surface. The mutant T-loop conformation would be compatible with the 2HNQ crystal packing, but if this were the case, it would expose hydrophobic residues to the solvent. In contrast, in the new crystal form, residues 13–27 of a symmetry-related molecule are adjacent to the loop region, shielding the side chain of I219 from the solvent. This arrangement is stabilized by hydrogen bonds involving the side chains of R47 and D48 and the main-chain oxygen of A27 and side-chain nitrogens of H25 in the symmetry-related molecule.

Bound cations and anions

The refined crystal structure of the mutant PTP1B contains one magnesium and five chlorine ions bound to the protein. The presence of a magnesium ion in PTP1B structures had been reported in two other instances (PDB code 1PTY and 1AAX), and magnesium salts have been proven to be a necessary component for the crystallization of PTP1B. In our structure, similarly to the other cases, the Mg^{+2} is hexahydrated: the complex is bound between two symmetry-related molecules; although the crystal packing in our space group is different from that reported for 1PTY and 1AAX, the magnesium-binding site is formed by the same set of residues (E129 and E130, and H54 from the symmetry-related molecule), suggesting that one possible role for magnesium is to induce the ordered protein-protein interactions necessary for crystallization.

Toward the end of refinement, the most significant feature seen in Fo-Fc residual maps was additional positive density ($>5\sigma$) at several modeled water molecules, generally loosely hydrogen bonded ($d = 3.1\text{--}3.3 \text{ \AA}$) to main-chain nitrogens or guanidinium groups of arginine residues; these water molecules also were characterized by very low temperature factors. Bound anions in these locations could interact with the partial-positive charge associated with the peptide and arginine side-chain nitrogens. The only anion present in the mother liquor of crystallization was chloride, which also nicely fit the observed compact spherical density. When chloride ions were included in the model at full occupancy and a round of CNX refinement was carried out, residual maps showed no significant positive or negative density at these putative chloride sites. The temperature

factors for the refined chloride sites were comparable to the temperature factors for surrounding protein atoms. Locations and hydrogen-bonding patterns for the five chlorine ions found in the PTP1B structure are very similar to those observed in other protein-crystal structures (Lim et al. 1998; Fiedler et al. 2000). Two of the chlorine atoms have been located in the solvent-filled pocket created by the newly observed conformation of the P-loop (Fig. 4). They occupy the same positions occupied in the WT structure by the carbonyl oxygens of S216 and I219, and maintain the same, although more extended, hydrogen-bonding structure: as the carbonyl oxygen of S216, CL403 interacts with the main-chain nitrogen of S86 ($3.2 \text{ \AA}$), but in addition is hydrogen bonded to the side-chain NE of R45 ($3.1 \text{ \AA}$) and one ordered solvent molecule ($3.2 \text{ \AA}$); CL404 interacts with the main-chain nitrogens of G223 ($3.2 \text{ \AA}$) and I261 ($3.3 \text{ \AA}$), and forms one more hydrogen bond to a water molecule ($3.1 \text{ \AA}$). The remaining three of the chlorine ions are located on the surface of the protein, in shallow, positively charged, or neutral pockets. CL406 is located in the phosphatase's secondary aryl phosphate-binding site (Puius et al. 1997), and is within $3.2 \text{ \AA}$ of the side-chains of R24 and R254. CL405 is the only ion that does not interact with basic residues: it forms three hydrogen bonds, one with the hydroxyl of T263 ($3.1 \text{ \AA}$), and two with solvent molecules (3.0 and $3.1 \text{ \AA}$). CL402 interacts with the side-chain nitrogens of R45 ($3.2 \text{ \AA}$), the main-chain nitrogen of A122 ($3.5 \text{ \AA}$) and one water molecule ($3.2 \text{ \AA}$). An analysis of the PTP1B structures available in the Protein Data Bank shows that, out of 16 structures for which water molecules have been reported, 10 of them have a water molecule in the position occupied by CL402: in most of the cases, the temperature factor of the water is 5 to 20 $\text{\AA}^2$ lower than the average temperature factor for all atoms (in two cases it is almost the same), and in all cases the average hydrogen-bonding distance with R45 is $3.0\text{--}3.3 \text{ \AA}$. This suggests that even in the other reported structures, these features may be chlorine atoms that may play a structural role as counter-ions, thus neutralizing charged pockets and possibly minimizing random protein aggregation.

The structural findings explain the H/D exchange data

Wang et al. (1998), using H/D exchange and electrospray ionization mass spectrometry, showed that the single active site mutation in *Yersinia* PTPase increases solvent access to several segments, including the segment containing the catalytic Cys, and regions surrounding the active site: the pTyr recognition loop (Asn 44–Pro 51, numbers according to the PTP1B sequence) and the region spanning residues Leu 250–Leu 267. The solvent access to the WPD loop is, on the other hand, reduced. Crystal structures of PTP1B and of the corresponding *Yersinia* phosphatase show that the two enzymes are very similar structurally (Fig. 1B). The structure of PTP1B C215S mutant thus may provide a struc-

Fig. 4 Stereoview of the overlaid PTPase signature motif region in the PTP1B C215 mutant (yellow) and WT (magenta). The two chloride ions that have been identified in this area (orange) also are displayed. They occupy the same positions occupied in the WT structure by the carbonyl oxygens of S216 and I219, and maintain the same, although more extended, hydrogen-bonding structure. See text for a complete discussion.

tural basis for the observed H/D exchange results. As discussed before, residues of the P-loop are more solvent exposed in the extended conformation than in the WT conformation. Surface accessibility calculations (program Surface, CCP4, 1994) show that the overall solvent-accessible area for residues Val 213–Arg 221 more than doubles in the mutant enzyme ($187.9 \text{ \AA}^2$ versus $88.5 \text{ \AA}^2$ for the WT enzyme). Moreover, most of the backbone amides (for which the H/D exchange rate is measured), which are facing the inside of the loop and the thiolate in the WT enzyme, are interacting with ordered solvent molecules in the mutant enzyme structure. For residues of the pTyr recognition loop and of the peptide-spanning L250–L267, there is also a slight increase in solvent-accessible area ($502.6 \text{ \AA}^2$ and $526.5 \text{ \AA}^2$ versus $437.0 \text{ \AA}^2$ and $502.3 \text{ \AA}^2$, respectively, for mutant and WT enzyme). Increased solvent access may be caused not only by increased surface accessibility, but also by increased flexibility of the residues involved. In both human and *Yersinia* phosphatases there are hydrogen-bonding arrays between carbonyl oxygens of the P-loop and the surrounding residues, which may help in stabilizing both the P-loop and the neighboring areas, i.e., the main-chain carbonyl oxygen of A217 and G218 are interacting with the side-chain guanidinium group of R253, and L219 carbonyl oxygen is hydrogen bonding with the main-chain nitrogen of I261 (with PTP1B residue numbering). Because of the different P-loop conformation, these interactions no longer are present in the structure of PTP1B C215S mutant.

The H/D exchange data show that the solvent access is decreased for the WPD loop (residues His 175–Val 184), in the mutant enzyme, suggestive of either a decreased solvent-accessible surface or a reduced mobility. The surface-accessibility calculation gives very similar values for both forms of the enzyme, although the value for the mutant is slightly reduced ($469.8 \text{ \AA}^2$ for the WT and $434.6 \text{ \AA}^2$ for the mutant enzyme), suggesting that loop flexibility must be the most important factor in affecting the H/D exchange. The WPD loop has been shown to adopt different conformations in the unliganded and liganded form of the enzyme, for both

human and *Yersinia* phosphatases. In the unliganded structure, the WPD loop is in an open conformation, such that the catalytic Asp is $\sim 10 \text{ \AA}$ away from the P-loop; upon substrate binding, the WPD loop assumes a closed conformation, covers the active site like a “flap”, and positions the catalytic Asp close to the leaving-group oxygen of the substrate (Fig 1A). Juszczak et al. (1997), using time resolved fluorescence anisotropy and steady-state UVRR, suggested that under physiological conditions in the nonliganded WT *Yersinia* PTPase, the WPD loop alternates between the open and the closed conformation, while in the active-site mutant it assumes only one conformation, with a much-reduced loop motion. Overlap of the structure of the liganded PTP1B C215S mutant (PDB file 1PTY) and the unliganded PTP1B C215S mutant (this work; Fig. 1C) shows that in the apo mutant enzyme, the closed conformation for the WPD loop is not likely, because of steric interference with the extended P-loop. In the mutant enzyme, the WPD loop thus is locked in a single conformation, with much reduced flexibility, in accord with both H/D exchange and spectroscopic data.

The structure of the mutant enzyme explains the isothermal titration calorimetry data

Binding of ligands to WT and C215S mutant of *Yersinia* PTPase and human PTP1B have been analyzed by isothermal titration calorimetry (Wang et al. 1998; Zhang et al. 2000). In all cases, ligands bind with similar affinities to WT and mutant enzymes. However, though the total free energies of binding substrates to WT and mutant enzymes are very similar, binding to the mutant enzyme is predominantly driven by enthalpy, and the binding entropy is significantly lower than for the WT enzyme (Table 3). As previously observed (Zhang et al. 2000), the enthalpic contribution to the binding of ligand to PTP1B/C215S mutant is 6.5 kcal/mol more favorable than the wild-type enzyme, and the entropic contribution is disfavored by 6.3 kcal/mol . Similarly, for the *Yersinia* PTPase, the enthalpic contribution for binding of vanadate to the C403S mutant is 4.9

Table 2. Values of the ϕ, ψ angles for residues 214 through 222 in the WT and mutant PTP1B

Residue number	PTP1B WT	PTP1B C215S mutant
His 214	-163, 153 (β)	-108, 156 (β)
Cys 215	-129, -118 (β)	-89, -172 (β)
Ser 216	-92, -55 (α)	-152, -154 (β)
Ala 217	-97, -13 (α)	-46, 148 (β)
Gly 218	68, 46 (Gly)	80, -115 (Gly)
Ile 219	-143, -39 (α)	-104, 105 (β)
Gly 220	-70, -74 (α)	-80, 161 (β)
Arg 221	-70, -32 (α)	-134, 17 (α)
Ser 222	-59, -45 (α)	-61, -31 (α)

kcal/mol more favorable, and the entropic contribution is disfavored by 5.2 kcal/mol. Enthalpy/entropy compensation, in which perturbations that increase the enthalpy also can increase the entropy with little or no effect on the free energy, is a common phenomenon in biopolymer processes occurring in aqueous solvent (Lumry and Rajender 1970). Similar enthalpy-entropy compensation has been observed both as a function of ligand structure and of protein structure (Kelley and O'Connell 1993; Brummell et al. 1993; Ito et al. 1993). The enhanced enthalpic contribution for the association of ligands to the catalytic cysteine mutants results from the removal of the electrostatic repulsion between the thiolate ion and the negatively charged substrate. In accord with the enthalpy/entropy compensation rule, the decrease in entropic contribution to ligand binding by the Cys to Ser mutant is caused by the same perturbation that decreases the enthalpy, i.e., the replacement of the negatively charged thiolate with the neutral hydroxyl. The Cys to Ser mutation causes the PTPase signature motif to assume the extended conformation seen in the crystal structure: hence, binding of the substrate must be accompanied by rearrangement of the residues of the PTPase signature motif, from the extended conformation to the loop conformation observed in the bound enzyme, with consequent decrease in entropy.

Conclusions

In this paper, we report the three-dimensional structure of the unliganded PTP1B C215S mutant. The structure reveals

that, in the absence of ligand, the mutant is conformationally distinct from the WT enzyme. In the mutant enzyme, residues 215–220 (the PTPase signature motif) assume a completely different conformation from that observed in the WT enzyme and extend into the substrate binding area. Although it often is assumed that conservative amino-acid substitutions retain the structural properties of their wild-type counterparts, the structure of PTP1B C215S mutant shows that dramatic conformational changes may occur. These changes may influence interpretations based on such mutants. Why does this conformational change occur in PTP1B? The stabilization of the thiolate in the free enzyme is accomplished by substantially lowering the pK_a of the cysteine. The largest contribution to this pK_a variation is made by backbone dipoles of the P-loop, the side chain of the conserved S222, and the backbone dipoles of the nearby α -helix (Dillet et al. 2000). The extremely low calculated pK_a value leads to a prediction that protonation of this cysteine residue cannot occur without a significant structural change in the active site geometry (Dillet et al. 2000). Substitution of the negatively charged thiolate with a neutral (although polar) alcohol may be seen as the equivalent of the cysteine titration: it destabilizes the PTPase signature motif loop and the surrounding areas, favoring the extended conformation. These structural findings, together with the fact that in structures of the liganded mutant enzyme the PTPase signature motif assumes the WT-loop conformation, also suggest that the conformation of the P-loop is inducible and dependent on the presence of a negative charge (either the thiolate in the wild-type enzyme or the phosphate of the substrate in the mutant enzyme). The substantial conformational change we have observed is in agreement with, and provides a molecular basis for, the observed differences in thermodynamic parameters for ligand binding between the WT and C215S mutant (Zhang et al. 2000), the H/D exchange results (Wang et al. 1998) and the spectroscopic data (Juszczak et al. 1997).

Materials and methods

Crystallization and data collection

The C215S substitution in PTP1B was introduced by polymerase chain reaction (PCR)-based oligonucleotide-directed mutagenesis.

Table 3. Thermodynamic parameters for the binding of ligands to the wild-type phosphatases and their catalytic cysteine mutants

Enzyme	Ligand	Kd (μ M)	ΔH (Kcal mol ⁻¹)	T ΔS (Kcal mol ⁻¹)	ΔG
PTP1B WT	Ac-DADE-F ₂ PMP-L-NH ₂	0.24 $\pm$ 0.05	-3.9 $\pm$ 0.2	5.1 $\pm$ 0.2	-9.0 $\pm$ 0.1
PTP1B C215S	Ac-DADE-F ₂ PMP-L-NH ₂	0.19 $\pm$ 0.03	-10.4 $\pm$ 0.2	-1.2 $\pm$ 0.2	-9.2 $\pm$ 0.1
<i>Yersinia</i> PTPase WT	Vanadate	1.3 $\pm$ 0.1	-10.0 $\pm$ 0.2	-2.0	-8.0
<i>Yersinia</i> PTPase C403S	Vanadate	2.3 $\pm$ 0.1	-14.9 $\pm$ 0.1	-7.2	-7.7

PTP1B data are from Zhang et al (2000).

Yersinia PTPase data are from Wang et al. (1998).

F₂PMP: difluoro-phosphonomethyl phenylalanine (non-hydrolyzable phospho-tyrosine analog).

The correct construction of the mutant derivative was verified by DNA sequencing. DNA isolated from positive clones was introduced into *Escherichia coli* BL21 cells for protein expression and purification. The protein construct included PTP1B residues 1–298 (catalytic domain) and an N-terminal FLAG tag (Kodak). A two-step purification protocol (affinity and anionic-exchange chromatography) was employed to obtain a homogenous preparation (details of the purification protocol will be published elsewhere).

Apo PTP1B C215S mutant crystals were obtained by vapor diffusion in sitting drops at 4°C by mixing 2 μ L of protein (10 mg/mL in 20 mM Hepes, pH = 7.0, 50 NaCl, 1 mM EDTA, 5 mM DMH) and 2 μ L of precipitant solution (13%–16% PEG3350, 100 mM Hepes pH = 7.0, 200 mM MgCl_2). X-ray diffraction data were collected on a Mar CCD from a single crystal (of $\sim 0.2 \times 0.2 \times 0.08$ mm in size) using synchrotron radiation. Data were collected at beamline 17-ID in the facilities of the Industrial Macromolecular Crystallography Association Collaborative Access Team (IMCA-CAT) at the Advanced Photon Source. A preliminary indexing of the data with HKL2000 (Otwinowski and Minor 1997) showed that the crystal was trigonal or hexagonal, with unit cell parameters $a = b = 87.4$ Å, $c = 95.9$ Å, $\alpha = \beta = 90.0^\circ$, $\gamma = 120.0^\circ$. All further data processing, scaling, and merging were done with the software X-GEN (Howard 2001). Data initially were integrated in a low-symmetry group (i.e., assuming P3 symmetry): analysis of the integrated data set showed that the pattern of reflections was consistent with trigonal crystals, space group P3₁21 or P3₂21. The calculated unit-cell volume was 634 414 Å³. The monomeric molecular weight of the mutant used in the crystallographic studies is 36172 Da, and assuming one molecule per asymmetric unit, we obtained a V_m ratio (Matthews 1968) of 2.9 Å³/Da, corresponding to a solvent content of $\sim 65\%$, within the range expected for globular protein. Table 1 summarizes the statistics for the data collected.

Structure solution and refinement

The three-dimensional structure of apo PTP1B C215S mutant was solved by molecular-replacement (MR) techniques, using as a search model the 1.8 Å structure of the mutant enzyme in complex with phosphotyrosine (PDB code 1PTY). Bound ligand, solvent molecules, and protein residues 286–298 (C-terminus), and 175–184 (WPD loop) were deleted from the coordinate file. Rotation and translation functions were calculated using AMORE (Navaza 1994). Results from the MR analysis were also used to define the correct space group. The model corresponding to the correct solution was subjected to 30 cycles of rigid body refinement as implemented in CNX (Brünger et al. 1998; Molecular Structure, Inc.) using data between 20.0 and 2.3 Å. Five percent of the data were set aside for Rfree calculation (Brünger 1992; Kleyvegt and Brünger 1996) and the rigid-body fitted model was subjected to torsion-angle dynamics (Rice and Brünger 1994), followed by positional and temperature-factor refinement. The resulting model had a crystallographic R-factor of 29.2% (Rfree was 34.0%) for all data between 20.0 and 2.3 Å. Initial electron density maps calculated from this model clearly showed that the WPD loop is in an open conformation, and that the peptide containing the catalytic site mutation assumes a completely new and different conformation (Fig. 2). This model subsequently was confirmed by SA-omit maps. Both loops were built into the available density using the graphic software O (Jones and Kjeldgaard 1997). Refinement of the model was carried out by alternating cycles of manual rebuilding of the model in O and computer-based refinement using CNX. Typically, two cycles of torsion-angle dynamics, positional and temperature-factor refinement were run in each cycle. Bulk sol-

vent correction was applied throughout the entire refinement, and the refinement was performed using the cross-validated maximum likelihood approach (Pannu and Read 1996; Adams et al. 1997). The current model has a crystallographic R-factor of 19.5% (Rfree is 26.6%) for 23,604 reflections between 20.0 and 2.1 Å (5% flagged for Rfree calculation) and maintains good geometry (rmsd for bond lengths and bond angles are 0.012 Å and 1.65 degrees, respectively). The backbone conformation of 89.9% of the residues is within the most favored regions of the Ramachandran plot, with none in disallowed regions, as defined using PROCHECK (Laskowski et al. 1993). Coordinates and structure factors for this structure have been deposited in the Protein Data Bank (accession code 1I57).

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