Functional Characterization and Crystal Structure of the C215D Mutant of Protein Tyrosine Phosphatase-1B

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Running title: Structure and function of C215D mutant of PTP-1B

Key words: PTP-1B, crystallization, active-site, mutant, protein tyrosine phosphatase

*Recipient of a Natural Sciences and Engineering Research Council of Canada (NSERC) Post-doctoral Industrial Research Fellowship.

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SUMMARY

We have characterized the C215D active-site mutant of protein tyrosine phosphatase 1B (PTP-1B) and solved the crystal structure of the catalytic domain of the apo-enzyme to a resolution of 1.6 Å. The mutant enzyme displays maximal catalytic activity at a pH of ~ 4.5, which is significantly lower than the pH optimum of 6 for wild-type PTP-1B. Although both forms of the enzyme exhibited identical \( K_m \) values for hydrolysis of \( p \)-nitrophenylphosphate (\( p \)NPP) at pHs of 4.5 and 6, \( k_{cat} \) of C215D was \( \sim 70 \)-fold and \( \sim 7000 \)-fold lower respectively than that of wild-type PTP-1B. Arrhenius plots revealed that the mutant and wild-type enzymes displayed activation energies of \( 61 \pm 1 \) kJ/mol and \( 18 \pm 2 \) kJ/mol respectively at their pH optima. Unlike wild-type-PTP-1B, C215D-mediated \( p \)NPP hydrolysis was inactivated by 1,2-epoxy-3-(\( p \)-nitrophenoxy)propane, suggesting a direct involvement of Asp\(^{215} \) in catalysis. Increasing solvent microviscosity with sucrose (up to 40 % (w/v)) caused a significant decrease in \( k_{cat} / K_m \) of the wild-type enzyme, but did not alter catalytic efficiency of the mutant protein. Structurally, the apo-enzyme was identical to wild-type PTP-1B, aside from the flexible WPD loop region, which was in both “open” and “closed” conformations. The C215D mutant form of PTP-1B should be an effective substrate-trapping mutant at physiological pH that can be used to identify cellular substrates of PTP-1B. In addition, due to its insensitivity to oxidation, this mutant may be used for screening of fermentation broth and other natural products to identify inhibitors of PTP-1B.
INTRODUCTION

The Protein tyrosine phosphatases (PTPases) comprise a family of ~100 enzymes that play an important role in controlling several biological processes including cell cycles and signal transduction pathways (1, 2). One member of this family of phosphatases, which is receiving increased attention owing to its potential role in controlling the insulin signaling pathway, is protein tyrosine phosphatase-1B (PTP-1B). Studies have suggested that this ubiquitously expressed enzyme may play a role in regulating the function of the insulin receptor. Thus, PTP-1B is an attractive target for drug design in the treatment of Type 2 diabetes (for reviews see Refs. 3, 4).

Like other PTPases, PTP-1B contains a conserved 11-residue sequence motif (i.e. (I/V)HCXAGXXR(S/T)G) which harbors Cys\textsuperscript{215} that acts as the nucleophile and is essential for catalysis. The signature motif also forms the “P-loop” that is involved in substrate binding and catalysis. It is thought that PTP-1B-mediated catalysis occurs via a double displacement mechanism, in which the phosphoryl group of the substrate is first transferred to the active-site Cys residue (Cys\textsuperscript{215}) (5, 6). The initial phosphoryl transfer is assisted by an invariant Asp residue (Asp\textsuperscript{181}) residing in a flexible loop region (WPD loop) which spans the conserved tripeptide Trp-Pro-Asp. It is generally believed that Asp\textsuperscript{181} first acts as a general acid and protonates the leaving group in the phosphorylation step. Subsequently, Asp\textsuperscript{181} functions as a general base, abstracting a proton from an attacking water molecule in the dephosphorylation step to enhance the rate of hydrolysis of the enzyme-thiophosphate intermediate (7–10).

Previous structural studies on PTP-1B have revealed interesting details regarding the conformations and structural organizations of the WPD loop and P-loop regions. Specifically, the WPD loop has been shown to adopt different conformations in the unliganded and liganded
forms of the enzyme. In the unliganded structure, the WPD loop is in an open conformation, in which Asp\textsuperscript{181} is \(\sim\) 10 Å away from the P-loop. Upon substrate binding, the WPD adopts a closed conformation and covers the active-site like a “flap” thereby positioning Asp\textsuperscript{181} closer to the leaving group (11, 12).

In wildtype PTP-1B, Cys\textsuperscript{215} is present as a thiolate (13), and it is known that this active-site residue is absolutely necessary for PTP-1B-mediated catalysis. Mutation of this residue to a neutral Ser generates a “substrate-trapping” mutant, which is able to bind substrates with affinities similar to the wild-type enzyme but does not display any measurable phosphatase activity (14, 15). The crystal structure of the unliganded PTP-1B C215S mutant shows the P-loop in a conformationally distinct orientation from that found in the wild-type protein. However, in the liganded form, the P-loop adopts the same conformation as the wild-type protein. In the C215S mutant, substitution of the negatively charged thiolate with a neutral (although polar) alcohol destabilizes the PTPase signature motif loop (P-loop) and the surrounding areas, favoring the extended conformation (16). The structural studies therefore suggest that the conformation of the P-loop region of the enzyme is inducible and may be dependent on the presence of the negative charge of the active-site nucleophile. The goal of this study was to explore the importance of the presence of a negatively charged residue other than Cys at position 215 on the conformation of the P-loop and on the catalytic activity of PTP-1B. We therefore substituted Cys\textsuperscript{215} with Asp, as this residue is similar in charge and size density to the active-site thiolate. Here, we report the functional characterization and crystal structure of the C215D mutant enzyme, and compare these properties to those of wild-type PTP-1B.
EXPERIMENTAL PROCEDURES

Materials.

Bovine serum albumin and the SuperSignal™ west pico immunoblotting kit were obtained from Pierce. SDS-PAGE gels were purchased from Invitrogen, and Escherichia coli (E. coli) BL21 cells were obtained from Stratagene. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) and protease inhibitors were from Roche Biochemicals. The Cibacron blue and Sepharose Q columns were bought from Amersham Biosciences. All other chemicals were obtained from Sigma.

General methods.

Protein concentrations were determined by a microplate adaptation of the Bradford assay, using BSA as a standard. For SDS-PAGE and immunoblotting of purified mutant and wild-type PTP-1B, proteins were boiled in SDS-PAGE loading buffer for 5 – 10 minutes and then separated on 10 – 20% (w/v) polyacrylamide gradient gels containing SDS. The proteins were then transferred onto nitrocellulose at a constant voltage of 100 volts for 90 minutes. The blot was subsequently probed with an anti-FLAG monoclonal antibody and detected by chemiluminescence. The SuperSignal™ west pico kit was used for immunoblotting of proteins by following the manufacturer’s suggested protocol.

Mutagenesis and protein expression.

A plasmid containing the isolated catalytic domain of human PTP-1B (residues 1 – 298) in a pFLAG vector was used as a template for site-directed mutagenesis. The Cys to Asp mutation was introduced using a PCR-based approach by following established procedures (17).
resulting construct was verified by DNA sequencing on an ABI 373 DNA sequencer (Applied Biosystems), and the sequence data was analyzed by the software application package Sequencher 4.0.5 (Gene code Corp.).

The resulting plasmid was transformed into *Escherichia coli* (*E. coli*) BL21 cells for protein expression. The bacterial cultures were grown in Luria Bertani broth at 37 °C with shaking at 250 rpm to an optical density (O.D.) of 0.7. The cultures were then induced by the addition of 1 mM IPTG and were grown for an additional 2 hours. Cells were harvested by centrifugation and cell lysates were analyzed by electrophoresis and immunoblotting using the protocol outlined above.

**Purification of mutant and wild-type PTP-1B.**

Purification of mutant and wild-type PTP-1B was performed as outlined previously (18). Briefly, *E. coli* BL21 cells transformed with the expression vector were suspended in lysis buffer consisting of 20 mM Tris/HCl, 0.1 mM EDTA, 5 mM DTT with 2 tablets of Complete™ protease inhibitors per 50 ml of solution at a final pH of 7.5. Cell lysis was achieved by passing cells twice through an ice-chilled French pressure cell (SLM Aminco Spectronic Instruments) at a pressure of 16,000 psi. The supernatant was retained following centrifugation of the cell lysate at 31,000 × g for 30 minutes and then applied to a Cibacron-blue affinity column pre-equilibrated with lysis buffer without protease inhibitors (Binding buffer 1). Following the application of the supernatant, the affinity column was washed with 20 column volumes of binding buffer or until the absorbance at 280 nm returned to baseline. The protein was then eluted with a linear gradient of NaCl (0 to 500 mM) in binding buffer over 10 column volumes. PTP-1B-rich fractions were determined by analyzing the fractions by SDS-PAGE and also measuring PTPase activity. The
appropriate fractions were pooled and the ionic strength was adjusted to 100 mM NaCl. The partially purified preparation was subsequently applied to a Sepharose Q anion-exchange column pre-equilibrated in 20 mM Tris / HCl, 0.1 mM EDTA, 5 mM DTT, 100 mM NaCl at a pH of 7.5 (Binding buffer 2). The column was then washed with Binding buffer 2 to return the absorbance at 280 nm to baseline levels. Next, the protein was eluted with a linear gradient of increasing NaCl concentrations (100 to 500 mM) over 8 column volumes. The eluted fractions were analyzed by SDS-PAGE and by measuring enzyme activity; the appropriate fractions were then pooled. The purified protein preparation was dialyzed into 20 mM Tris / HCl, 0.1 mM EDTA, 5 mM DTT, 150 mM NaCl, 20 % (v/v) glycerol at a pH of 7.5 prior to storage at – 80°C. All steps in the purification scheme were carried out at 4 °C or on ice with the aid of an FPLC (Pharmacia); a flow rate of 1 ml/min was used in all chromatographic steps.

Activity assays.

The assays were carried out in a 96-well format at 22 °C in a buffer that consisted of 100 mM Tris/HCl, 50 mM MES, 50 mM acetic acid, N,N’-dimethyl-bis(mercaptoacetyl)hydrazine (DMH), 2 mM EDTA, 5% (v/v) DMSO, 2% (v/v) glycerol, 0.01% (v/v) Triton X-100 at the appropriate pH. The use of this triple-component buffering system minimizes changes in ionic strength across a pH range of 3 – 9 (19). Enzyme activity was quantitated by monitoring C215D- or wild-type PTP-1B-catalyzed hydrolysis of p-nitrophenylphosphate (pNPP) to p-nitrophenol. Briefly, pNPP hydrolysis was measured by incubating the C215D mutant or PTP-1B with 0 to 10 mM pNPP for 30 or 4 minutes respectively and then stopping the reaction by adding NaOH to a final concentration of 1M. The absorbance at 405 nm was measured on a Cytofluor II™ plate reader and the catalytic activity was calculated using the molar extinction
coefficient of the $p$-nitrophenolate anion ($18,800 \text{ M}^{-1}\text{cm}^{-1}$). The observed rates of reactions were fitted to the Michaelis-Menten equation using the non-linear curve fitting software program, Grafit 4.0.10 (Erithacus, software Inc.) to determine kinetic constants. To extract $p\text{K}^{\text{app}}$ values from the pH-response studies, the data was also fitted using non-linear regression analysis (SigmaPlot, Jandel Scientific).

For collection of Arrhenius plot data, C215D and PTP-1B activities were measured at their respective pH optima at temperatures in the range $10 - 37 \, ^\circ\text{C}$ (± 0.5 °C) using 10 mM $p\text{NPP}$ as a substrate and an assay time of 30 or 4 minutes. The data was transformed into Arrhenius plots, and the slopes of the lines were obtained using linear regression with the Marquardt-Levenberg algorithm (SigmaPlot).

For inactivation assays using 1,2-epoxy-3-($p$-nitrophenoxy)propane (EPNP), the epoxide was dissolved in DMSO and added to the reaction mixtures to a final concentration of 2.4 mM. Following incubation of the enzymes for 1 hour at 4 °C in the presence of EPNP, the catalytic activities were determined at their respective pH optima as described above.

**Viscosity studies.**

The effect of viscosity on C215D or PTP-1B activity was determined by measuring $p\text{NPP}$ hydrolysis using the protocol outlined above in reaction mixtures containing 0 – 40% (w/v) sucrose. Stock solutions of sucrose were prepared at twice the desired final concentration in assay buffer (pH = 4.5 or 6) and were added to the reaction mixtures to obtain a 2-fold dilution. The plates were continuously shaken during the incubation time to maintain homogeneity of the reaction mixtures. Relative solvent viscosities ($\eta_{\text{rel}} = \eta/\eta^0$), where the superscript $^0$ denotes the reaction in buffer lacking sucrose, were calculated using the solution densities at 22 ° (20, 21).
The calculated relative viscosities used in these experiments were 1.00, 1.37, 2.02, 3.32, and 6.39 for 0%, 10%, 20%, 30% and 40% (w/v) sucrose solutions, respectively.

**Crystallization and data collection.**

Apo PTP-1B C215D mutant crystals were obtained by vapor diffusion in sitting drops at 4 °C by mixing 2 µl of protein (10 mg/ml in 20 mM Hepes, 50 NaCl, 1mM EDTA, 5 mM DMH, pH = 7.0) and 2 µl of precipitant solution (13-16% PEG3350, 100 mM Hepes, 200 mM MgCl₂, pH = 7.0). X-ray diffraction data were collected on an ADSC Q210 detector from a single crystal (of approximately 0.3 × 0.2 × 0.1 mm in size) using synchrotron radiation. Data to 1.6 Å 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. Data processing, scaling and merging were done with the software DPS/MOSFLM (22, 23). The crystal was trigonal, space group P3₁2₁, with unit cell parameters a = b = 88.45 Å, c = 104.36 Å, α = β = 90.0°, γ = 120°. Table 3 summarizes the statistics for the data collected.

**Structure solution and refinement.**

The crystal was isomorphous with previously reported PTP-1B crystals (i.e. 1PTY, (24)) and the three-dimensional structure of the C215D mutant was solved by Difference Fourier, using as initial model the 1.8 Å structure of the mutant enzyme in complex with phosphotyrosine (PDB code 1PTY). Bound ligand, solvent molecules, and protein residues 175-184 (WPD loop) and 213-223 (P-loop) were deleted from the coordinate file. The initial electron density maps to 2.4 Å calculated from this model showed that the WPD loop was in a closed conformation, and that the peptide containing the catalytic site mutation assumed the same conformation observed in the
WT enzyme (Figure 4). Both loops were built into the available density using the graphic software O (25). Refinement of the model was carried out by alternating cycles of manual rebuilding of the model in O and computer-based refinement using CNX, slowly including all available data to 1.6 Å resolution. Typically, two cycles of torsion angle dynamics, positional and temperature factor refinement were run in each cycle. Bulk solvent correction was applied throughout the entire refinement, and the refinement was performed using the cross-validated maximum likelihood approach (26, 27). When high resolution data were included, it became evident in the electron density maps that the WPD loop (residues Thr^{177}–Ser^{188}) was present in both "open" and closed" conformations. Several other residues were also modeled as having dual conformation for their side chains; the occupancy for atoms in dual conformations was initially set to 50% for each conformer, and then manually adjusted to reflect the temperature factors. At the end of the refinement, 30 additional cycles of occupancy refinement were carried out for the atoms modeled in the alternate configurations. Table 3 summarizes the statistics for the refined model. Coordinates and structure factors for this structure have been deposited in the Protein Data Bank (accession code XXXX).
RESULTS

To evaluate the importance of a negatively-charged residue at position 215 of PTP-1B, we substituted Cys\textsuperscript{215} with Asp. The isosteric nature of this amino acid replacement minimizes the introduction of structural perturbations in the conformation of the P-loop. Following expression and purification of the C215D mutant, the functional and structural properties of the enzyme were compared to those of wild-type PTP-1B.

**Effect of pH on C215D activity.**

We investigated the pH dependence of C215D- and wild-type-PTP-1B-catalyzed hydrolysis of \( pNPP \) at various pHs ranging from 3.5 to 9 (Figure 1). Although the pH profiles were generally bell-shaped for both enzymes, suggesting the existence of two ionizable amino acid side chains that are involved in catalysis, significant differences in the profiles were noted. As expected, wild-type PTP-1B displayed maximal catalytic activity at pH of 6.0; however, when the active-site Cys was replaced by Asp, the pH optimum of the enzyme was shifted to 4.5 – 4.7. Interestingly, the pH profiles showed very little overlap, and significant differences in the relative levels of catalytic activity were observed at the pH optima of the two enzymes. For example, at a pH of 4.5, where the C215D mutant displayed maximal activity, the catalytic activity of wild-type PTP-1B was < 5% of its maximum. Similarly, whereas wild-type PTP-1B exhibited maximal catalytic activity at pH of 6, only 15% of the maximal catalytic activity of the C215D mutant was present. From the pH profiles, the first and second apparent ionization constants of the enzyme-substrate complexes (i.e. \( pK_{1,app} \) and \( pK_{2,app} \)) for wild-type PTP-1B were estimated to be ~ 5.5 and 6.8 respectively. Due to the steep slope of the acid limb of the pH profile for the C215D mutant, the \( pK_{1,app} \) could not be determined accurately, and it could only be
estimated that the value was between 4.3 and 4.5. According to the pH profile, the pK$_2^{app}$ of the mutant enzyme was also significantly lower than for wild-type PTP-1B, displaying a pK$_2^{app}$ of ~5.5. These results clearly show that the two forms of the enzyme displayed distinct pH dependencies with respect to substrate hydrolysis.

**Comparison of K$_m$, k$_{cat}$, and E$_{act}$ of mutant and wild-type PTP-1B.**

Next, we compared the kinetic parameters of the C215D mutant to wild-type PTP-1B. Table 1 shows a comparison of the kinetic parameters of C215D- and wild-type PTP-1B-mediated pNPP hydrolysis at the determined pH optima. Although both enzymes displayed similar K$_m$ values of 1.1 – 1.4 mM, the k$_{cat}$ of the C215D mutant was ~70-fold and ~7000-fold less than wild-type PTP-1B at pHs of 4.5 and 6 respectively.

To determine whether the observed differences in k$_{cat}$ between the mutant and wild-type enzymes could be at least partially explained by potential differences in the thermodynamics of catalysis, we compared the activation energies of C215D- and wild-type PTP-1B-catalyzed pNPP hydrolysis by measuring the catalytic activities as a function of temperature. The Arrhenius plots and the corresponding calculated E$_{act}$ for the C215D mutant and wild-type PTP-1B are shown in Figure 2 and Table 2 respectively. The Arrhenius plot for wild-type PTP-1B-mediated catalysis was continuous over the temperature range of 10 – 35 °C, with E$_{act}$ = 18 ± 2 kJ/mol. At temperatures above 35 °C, significant denaturation of the enzyme was observed (data not shown). The C215D mutant was more thermally labile, and a continuous Arrhenius plot was obtained only up to 32 °C. Above this temperature, the Arrhenius plot also began to level off due to thermal denaturation of the enzyme. Interestingly, the E$_{act}$ for the C215D mutant (i.e. 61 ± 1 kJ/mol) was over 3-fold higher than the E$_{act}$ for wild-type PTP-1B.
**Inactivation of C215D-mediated catalysis by EPNP.**

EPNP is a potent inactivator of enzymes that possess an active-site carboxyl residue (\((28 \rightarrow 30)\), for example). Irreversible inactivation is presumably due to alkylation of the active-site carboxyl residue by the epoxide moiety of EPNP. Since the C215D mutant contains a carboxyl residue in its active-site, we wished to determine the effect of EPNP on the catalytic activity of this mutant derivative of PTP-1B. We therefore monitored the amount of C215D- and wild-type PTP-1B catalytic activity that remained following a 1-hour incubation of the enzymes at 4 °C in the presence of 2.4 mM EPNP. The activity of the mutant protein was reduced by >80% following incubation of the enzyme in the presence of the inactivator (Figure 3). However, the activity of wild-type PTP-1B was not significantly affected under similar conditions. As the only amino acid difference between the mutant and wild-type enzymes was the substitution of the catalytic Cys\(^{215}\) by Asp in the derivative, we conclude that EPNP inactivated C215D through alkylation of the carboxyl group of Asp\(^{215}\).

**Crystal structure of the apo-C215D mutant.**

To evaluate the effect of the Cys to Asp substitution on the tertiary structure of PTP-1B, we solved the X-ray crystal structure of the mutant enzyme and refined it against 1.6 Å data. The final model had a crystallographic R-factor of 18.6% (Rfree is 20.4%) for 59409 reflections between 15.0 and 1.6 Å (5% flagged for Rfree calculation) and maintained good geometry (rmsd for bond lengths and bond angles were 0.01 Å and 1.5 degrees, respectively). The backbone conformation of 91.6% of the residues was within the most favored regions of the Ramachandran plot, with none in disallowed regions, as defined using PROCHECK (31). The side chains of Met\(^3\), Asp\(^{48}\), Pro\(^{87}\), Arg\(^{105}\), Ser\(^{118}\), Leu\(^{119}\), Gln\(^{157}\), Glu\(^{159}\), Ser\(^{190}\), Ser\(^{216}\), Arg\(^{221}\), Cys\(^{226}\), Ile\(^{246}\),...
Met$^{253}$, and Ser$^{285}$ were modeled as having dual conformations. Two peptides (His$^{60}$ – Asp$^{63}$, and Thr$^{177}$-Ser$^{188}$, the WPD loop) and 5 solvent molecules were also modeled as having alternate conformations. The P-loop is in the WT conformation (Figure 4), and extensive hydrogen bond interactions are present between the Asp$^{215}$ carboxylate and main-chain and side chain nitrogens of Ser$^{216}$, Ala$^{217}$, Gly$^{218}$, Ser$^{222}$, and the side-chain oxygen of Ser$^{222}$ as depicted schematically in Figure 4a. Asp$^{215}$ is also hydrogen bonded to one of several ordered water molecules located in the binding site. This water is located at the position normally occupied by one of the phosphate oxygens of pTyr in the 1PTY structure (Figure 4b), and makes similar hydrogen bond interactions with the main-chain nitrogens of Ile$^{219}$ and Gly$^{220}$. A second water molecule occupies the position of another phosphate oxygen, but appears to be quite mobile and it has been modeled as having two distinct positions. This water molecule interacts with the main chain and the ε nitrogen of Arg$^{222}$, the side chain oxygen of Asp$^{215}$ and three water molecules.

The other four solvent molecules, of which at least one appears to be quite delocalized, have been located in the binding site; together, these molecules form an extensive hydrogen bond network connecting Asp$^{215}$ to Asp$^{181}$ in the WPD loop (Figures 4a and 4b). The WPD loop assumes both conformations: the catalytically active, closed conformation (preferred, with an average occupancy of 73.1% and an average temperature factor of 17.6 Å$^2$), and the inactive, open conformation typical of the unliganded enzyme. This alternate position refined to a much lower occupancy (26.9% in average, with an average temperature factor of 22.8 Å$^2$) but electron density was clearly available in the difference Fourier maps that could be justified only by assuming a partially open loop. Following the two different positions of the WPD loop, the side chain of Arg$^{221}$ also assumes two distinct conformations (with similar occupancies), that indeed
correspond to the conformations previously observed in the apo- and liganded PTP-1B (PDB codes 2HNP and 1PTY, respectively).

**Effect of viscosity on C215D and wild-type-PTP-1B-mediated substrate hydrolysis.**

As the major structural difference between the wild-type enzyme and the C215D derivative was observed in the conformation of the WPD loop, we wished to investigate this further. Therefore, we evaluated the contribution of loop motion in C215D- and wild-type PTP-1B-mediated catalysis by monitoring the effect of solvent microviscosity. We measured the $K_m$ and $k_{cat}$ of enzyme-catalyzed hydrolysis of $p$NPP in the presence of increasing concentrations of sucrose. As shown in Figure 5a, the $k_{cat}$ of the wild-type enzyme was slightly higher in the presence of 40% (w/v) sucrose. However, there was a 30% decrease in $k_{cat}/K_m$ of wild-type PTP-1B (Figure 5b), suggesting that the catalytic efficiency of wild-type PTP-1B was significantly hindered in the presence of sucrose. In contrast, there was no change in either the $K_m$ or $k_{cat}$ of C215D-mediated catalysis under similar conditions, resulting in a lack of deviation of $(k_{cat}/K_m)^0 / (k_{cat}/K_m)^n$ as a function of relative solvent microviscosity (Figures 5c and 5d).
DISCUSSION

Previously, we reported that the substitution of Cys$^{215}$ with Ser resulted in a conformationally distinct P-loop in the unliganded mutant enzyme, and we suggested that a negative charge in the active-site is required to maintain the P-loop conformation observed in the WT enzyme (16). In this study, we have generated and characterized the C215D mutant of PTP-1B to obtain further insights into the structural basis of PTP-1B catalysis. We substituted Cys$^{215}$ with Asp to preserve both the charge and size of this active-site residue.

Substitution of the active-site Cys with Asp resulted in a dramatic change in the pH profile for PTP-1B-catalyzed hydrolysis of pNPP. As expected, wild-type PTP-1B displayed a pH optimum of ~ 6. This value agrees favorably with the optimum values of 5.5 – 6.5 determined previously for the enzyme (32, 33). In contrast, the pH optimum for the C215D mutant was shifted to a significantly lower value of 4.5 – 4.7, and likely reflects the fact that the active-site Cys, which displays a pK$_a$ of ~ 5.5 in wild-type PTP-1B (13), is replaced with the strongly acidic Asp residue, with a pK$_a$ ~ 4 in model systems. The lower pH optimum of the C215D derivative is similar to the pH optima of some enzymes that contain active-site Asp residues such as pepsin (28) and the simian immunodeficiency virus (SIV) protease (34).

Interestingly, both forms of PTP-1B displayed identical $K_m$ values at the pH optimum of the C215D mutant. The $K_m$ values of 5.4 and 5.8 mM for pNPP for both the mutant and wild-type PTP-1B respectively were 4-fold higher than the $K_m$ determined at pH of 6. Since both PTPases displayed the same shift in $K_m$ at a lower pH, this suggests that the increase in $K_m$ was not a result of substitution of the active-site Cys with Asp. Rather, the increase in $K_m$ may be due to the influence of pH on the ionization state of the substrate, or may be a result of alterations in the ionization states of other important residues in the vicinity of the active-site regions of the
proteins. At a pH of 4.5, C215D-mediated pNPP hydrolysis still remained >70-fold lower than that of the wild-type enzyme at the same pH. Thus, substitution of the strongly nucleophilic active-site Cys with the negatively charged Asp caused a significant reduction in the rate of substrate turnover, that could not be reversed to that of the wild-type enzyme at the pH optimum of the mutant protein.

The Arrhenius plots for the C215D mutant and wild-type PTP-1B revealed that the activation energy of C215D-mediated pNPP hydrolysis was over 3-fold higher than that of the reaction catalyzed by wild-type PTP-1B. The plots were linear over a temperature range of 10 – 32 °C for the C215D mutant and 10 – 35 °C for the wild-type enzyme, and did not reveal any discontinuities over the temperature ranges. Thus, the large drop in $k_{\text{cat}}$ as observed for the mutant protein with respect to pNPP hydrolysis may be partially attributable to the 3-fold higher activation energy of C215D-mediated catalysis. The crystal structure of the apo-C215D mutant shows several water molecules sequestered at the active-site. These water molecules would have to be excluded from the active-site to enable substrate binding and catalysis. Thus, we hypothesize that the energy penalty that would result from the desolvation of the active-site would translate to the higher activation energy observed for the mutant protein.

EPNP is a potent inactivator of enzymes that possess an active-site carboxyl residue, and has been used to study the kinetic mechanisms of various aspartyl proteases such as pepsin (28) and the simian and human immunodeficiency virus proteases ((30, 35), for example). Inactivation by this uncharged molecule is presumably due to alkylation of the active-site carboxyl residue by the epoxide moiety of EPNP. Inactivation of the C215D derivative by EPNP, and a lack of an effect on wild-type PTP-1B suggests a direct involvement of Asp$^{215}$ in the catalytic mechanism of the mutant protein. Previously, Zhang et al. (36) showed that EPNP
also acts as an irreversible inactivator of the Low Molecular Weight (LMW) PTPase from bovine heart. In the case of this PTPase, however, two cysteine residues were proposed to be the target of the epoxide. As EPNP did not significantly inhibit or inactivate wild-type PTP-1B, it seems reasonable to suggest that the irreversible inactivation of the C215D mutant was a result of a chemical modification of the active-site Asp by the epoxide as observed for aspartyl proteases. These results corroborate the pH studies and provide strong evidence that Asp\textsuperscript{215} is crucial to the reaction mechanism of the mutant protein.

Structurally, wild-type PTP-1B and the C215D derivative were identical with the exception of the so-called “WPD loop”, which appears in both “open” and “closed” conformations in the mutant protein. The closed conformation is clearly favored over the open conformation (the relative occupancies were 73.1\% and 26.9\%, respectively), and is probably induced by the extensive hydrogen-bonding network, involving both solvent and protein atoms, identified in the binding site (Figure 4). These differences in the flexible WPD loop prompted us to investigate whether solvent microviscosity could affect the catalytic properties of these two enzymes. If rapid movement of the WPD loop is crucial to the catalytic mechanism of PTP-1B, then it is possible that increasing solvent microviscosity could have a detrimental effect on PTP-1B-mediated catalysis, by imposing a physical energy barrier to the movement of this region. The catalytic domain (residues 1–298) of wild-type PTP-1B was influenced by solvent microviscosity, resulting in a 30 % decrease in $k_{\text{cat}} / K_m$ in the presence of 40\% (w/v) sucrose primarily stemming from a higher $K_m$ value. In addition, we have performed similar viscosity studies on the highly homologous T-cell protein tyrosine phosphatase (TCPTP), and have found that the catalytic efficiency of the corresponding region of this enzyme (i.e. amino acids 1 – 296) responds to changes in solvent microviscosity in a manner similar to PTP-1B (data not shown).
Specifically, $k_{\text{cat}} / K_m$ of TCPTP-catalyzed $p$NPP hydrolysis is ~ 1.5-fold lower in the presence of 40 % (w/v) sucrose. The decrease in catalytic efficiency in the presence of sucrose is consistent with the hypothesis that movement of the WPD loop is crucial to the catalytic mechanism of PTP-1B. However, increasing the relative viscosity to the same extent did not influence either $K_m$ or $k_{\text{cat}}$ of the C215D mutant and hence, resulting in no change of the second-order rate constant of C215D-mediated $p$NPP hydrolysis. A comparison of the crystal structures of apo C215D and wild-type PTP-1B gives an insight into the effect of increasing solvent viscosity on catalytic efficiency. The closed conformation necessary for catalysis is observed in the apo C215D structure but not in the apo wild-type structure. This suggests that there is a higher propensity for the loop to assume the catalytically competent conformation in the mutant derivative even in absence of substrate than in the wild-type enzyme. It seems possible that this structural change in the WPD loop’s conformation in the C215D mutant, which results in a pre-formed active-site, influences the lack of sensitivity to the increasing solvent microviscosity. This induced-fit mechanism may explain the higher $K_m$ value for the wild-type enzyme in the presence of the viscogen.

An important strategy in the identification of potential substrates of PTPases is the use of substrate-trapping mutants that are structurally similar to the wild-type enzyme, but display either a lower dissociation constant between the enzyme and the substrate or a slower substrate turnover. To date, four examples of substrate-trapping mutants have been used to characterize PTP-1B: in the first case, the active-site Cys has been replaced by a Ser (37–39). This mutant still retains the ability to bind substrates but displays no measurable catalytic activity. However, differences in the thermodynamic parameters for ligand binding between the human form of the C215S mutant and wild-type PTP-1B have been noted (40), which could be partially explained
by the observed altered conformation of the P-loop in the mutant enzyme (16). In the second type of substrate-trapping mutant, the general acid Asp residue (i.e. Asp\textsuperscript{181}) is replaced by Ala (41, 42). Like the C215S mutant, this enzyme also binds substrate, but its catalytic activity is drastically reduced (42, 43). A third type of substrate-trapping mutant is the Q262A derivative, which has been used to obtain a crystal structure of the phosphoryl-enzyme intermediate (44). Recently, a double mutant (D181A/Q262A) of PTP-1B has been generated. This substrate-trapping mutant exhibits higher affinity than both the independent D181A and C215S mutants for the epidermal growth factor receptor (EGFR), and displays a 3000- and 11000-fold lower $k_{\text{cat}}$ for $p$NPP and EGFR respectively (45). It is currently unknown, however, how this mutant compares structurally to wild-type PTP-1B.

In wild-type PTP-1B, Cys\textsuperscript{215} functions as a strong nucleophile, forming a cysteinyl-phosphate intermediate in the reaction mechanism. We hypothesized that substitution of this residue with the negatively charged Asp would decrease the catalytic activity of the enzyme. We observed that the turnover number of the C215D mutant was indeed significantly lower (>7000-fold) than that of the wild-type protein at pH of 6, suggesting that this PTP-1B derivative may also serve as a highly efficient substrate-trapping derivative of the enzyme.

In this study, we have characterized some of the functional properties of the C215D derivative of PTP-1B. Although the results of this study suggest that Asp\textsuperscript{215} is involved in catalysis, it is currently unknown whether the catalytic mechanism of this derivative is identical to that of the wild-type enzyme or is somewhat altered. Experiments are underway in our laboratory to address this question. Nonetheless, the C215D enzyme is a novel substrate-trapping mutant whose structure is nearly identical to, but displays significantly lower catalytic activity than wild-type PTP-1B. Thus, C215D could be used to isolate and identify physiological
substrates of PTP-1B. Introduction of a carboxylate residue in place of a thiolate has rendered the enzyme resistant to oxidation, and therefore, this mutant may also be used for screening of fermentation broth and natural products to identify inhibitors of PTP-1B. Preliminary studies in our laboratory indicate that the profiles of inhibitors screened with the mutant enzyme are equivalent to those obtained with the wild-type enzyme.

Acknowledgments: We would like to thank the staff at the facilities at the Industrial Macromolecular Crystallography Association Collaborative Access Team (IMCA-CAT) for help during data collection. The facilities at IMCA-CAT are supported by the companies of the Industrial Macromolecular Crystallography Association through a contract with Illinois Institute of Technology (IIT), executed through IIT’s Center for Synchrotron Radiation Research and Instrumentation. Use of the Advanced Photon Source was supported by the U. S. Department of Energy, Basic Energy Sciences, Office of Science, under Contract No. W-31-109-Eng-38.
REFERENCES


The abbreviations used are:

DMH, N,N’-dimethyl-bis(mercaptoacetyl)hydrazine; DTT, dithiothreitol; E. coli, Escherichia coli; EGFR, epidermal growth factor receptor; EPNP, 1,2-epoxy-3-(p-nitrophenoxy)propane; IPTG, Isopropyl-1-thio-β-D-galactopyranoside; LMW, low molecular weight; pNPP, p-nitrophenylphosphate; PTPase, protein tyrosine phosphatase; PTP-1B, protein tyrosine phosphatase-1B; TCPTP, T-cell protein tyrosine phosphatase
FIGURE LEGENDS

Figure 1: Effect of pH on catalytic activity of C215D and wild-type PTP-1B. Catalytic activities of wild-type PTP-1B (○) and C215D (●) were determined using 10 mM pNPP as a substrate in assay buffers adjusted to the appropriate pH values. The data is presented as % activity observed at the optimal pH for each protein. Data points represent the means for triplicate determinations; where error bars are not visible, they fall within the symbols.

Figure 2. Arrhenius plots of the initial rate of (A) C215D- and (B) wild-type PTP-1B-mediated pNPP hydrolysis at various temperatures. Rates were determined by measuring the absorbance of the reaction mixtures at 405 nm. Data points represent the means for triplicate determinations; where not visible, error bars are masked by the symbols. The solid lines indicate the calculated best-fit lines using linear regression analysis.

Figure 3. Effect of EPNP on C215D- and wild-type PTP-1B-catalyzed pNPP hydrolysis. Catalytic activity of the C215D mutant and wild-type PTP-1B was measured following a 1-hour incubation at 4 °C in the absence (shaded) or presence (unshaded) of 2.4 mM EPNP using 10 mM pNPP as a substrate. The data represents the means for triplicate determinations, and activity is expressed as % maximal activity of C215D and wild-type PTP-1B at pH = 4.5 and pH = 6 respectively.

Figure 4. The crystal structure of apo C215D PTP-1B mutant. Panel A is a schematic illustration of the bonding network between various residues and the water molecules in the active site. Panel B shows a stereo view of the PTP-1B catalytic center. Asp^{215} is identified with
a van der Waals surface. The solvent molecules are represented as red spheres, and the phospho-tyrosine (from 1PTY) is shown in green. For clarity, only hydrogen bonds involving solvent and protein atoms are shown. The alternate conformations observed for the WPD loop and the side chains of $\text{Arg}^{221}$ and $\text{Ser}^{216}$ are in cyan. This figure was made with RIBBONS (46).

Figure 5. Dependence of relative kinetic parameters for C215D- and wild-type PTP-1B-catalyzed pNPP hydrolysis on solvent viscosity. The left panels show the dependence of $k_{\text{cat}}$ on solvent viscosity for (A) wild-type PTP-1B and (C) C215D. The right panels show the effect of solvent viscosity on $k_{\text{cat}} / K_m$ for (B) wild-type PTP-1B and (D) C215D. The parameters were determined by measuring the catalytic activities of the enzymes in the presence of increasing concentrations of sucrose. The data points represent the means of duplicate experiments. The solid lines indicate the calculated best-fit lines using linear regression analysis.
Table 1  Kinetic parameters of C215D- and wild-type PTP-1B-mediated pNPP hydrolysis at pH 4.5 and 6\textsuperscript{y}.

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (mM)</th>
<th></th>
<th>$k_{cat}$ (s\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH = 4.5</td>
<td>pH = 6</td>
<td>pH = 4.5</td>
</tr>
<tr>
<td>C215D</td>
<td>5.4 ± 0.4</td>
<td>1.1 ± 0.1</td>
<td>0.025 ± 0.001</td>
</tr>
<tr>
<td>Wt PTP-1B</td>
<td>5.8 ± 1.7</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.3</td>
</tr>
</tbody>
</table>

\textsuperscript{y}The data represents the means of three independent determinations (± S.E.M)
Table 2  Activation energies for \( p\)NPP hydrolysis by C215D and wild-type PTP-1B\textsuperscript{¶}.

<table>
<thead>
<tr>
<th></th>
<th>( E_{\text{act}} ) (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C215D</td>
<td>61 ± 1</td>
</tr>
<tr>
<td>Wt-PTP-1B</td>
<td>18 ± 2</td>
</tr>
</tbody>
</table>

\textsuperscript{¶}Values of \( E_{\text{act}} \) were calculated from the slopes of the lines in Figure 2.
Table 3. Statistics for the data set used to solve the structure of apo C215D mutant of PTP-1B and final statistics for the refined model. Numbers in parentheses refer to the last resolution shell.

<table>
<thead>
<tr>
<th>Resolution Range (Å)</th>
<th>28.0 - 1.6 (1.7 - 1.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td># reflections</td>
<td>62681 (9061)</td>
</tr>
<tr>
<td>% possible</td>
<td>99.9 (100)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>10 (9.5)</td>
</tr>
<tr>
<td>(I/\sigma I)</td>
<td>11.2 (2.9)</td>
</tr>
<tr>
<td>(R_{sym})</td>
<td>4.3 (26.9)</td>
</tr>
</tbody>
</table>

Refinement statistics

<table>
<thead>
<tr>
<th>Resolution range (Å)</th>
<th>15.0 - 1.6 (1.66 - 1.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td># of reflections (work)(^a)</td>
<td>59409 (5849)(^a)</td>
</tr>
<tr>
<td>% of possible(^a)</td>
<td>94.9 (94.8)(^a)</td>
</tr>
<tr>
<td>(R_{free} / R)</td>
<td>20.4 / 18.6 (25.2 / 23.4)</td>
</tr>
<tr>
<td>Rmsd bond length/angle</td>
<td>0.010 / 1.50</td>
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<tr>
<td># of protein atoms(^b)</td>
<td>2719(^b)</td>
</tr>
<tr>
<td># of solvent atoms</td>
<td>325</td>
</tr>
<tr>
<td># of heteroatoms(^c)</td>
<td>8(^c)</td>
</tr>
</tbody>
</table>

\(^a\) 5% of reflections were set aside for free-R calculation

\(^b\) 248 atoms belong to alternate conformations

\(^c\) Two magnesium and six chlorine ions have been identified in the structure
Figure 1

% maximal activity vs pH
Figure 2

A

B

$\log k_{\text{cat}} \text{ (s}^{-1}\text{)}$

$1/T \text{ (K x 1000)}$

3.25 3.30 3.35 3.40 3.45 3.50 3.55
Figure 3

% activity (pNPP hydrolysis)

- EPNP
+ 2.4 mM EPNP

C215D  WT-PTP1B
Figure 5

A

B

C

D

\[ \frac{k_{\text{cat}}}{k_{\text{cat}}} \cdot \frac{\eta}{\eta_0} \]

\[ \frac{(k_{\text{cat}} / K_m)^n}{(k_{\text{cat}} / K_m)} \cdot \frac{\eta}{\eta_0} \]

(trimmed edge)

(diffusion limit)

(trimmed edge)
Functional characterization and crystal structure of the C215D mutant of protein tyrosine phosphatase-1B
Yolanda Romsicki, Giovanna Scapin, Veronique Beaulieu-Audy, Sangita B. Patel, Joseph W. Becker, Brian P. Kennedy and Ernest Asante-Appiah

J. Biol. Chem. published online May 13, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303817200

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