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 apoenzyme to a resolution of 1.6 Å. The mutant enzyme displayed maximal catalytic activity at pH ~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 $p$-nitrophenyl phosphate at pH 4.5 and 6, the $k_{cat}$ values of C215D were ~70- and ~7000-fold lower than those of wild-type PTP-1B, respectively. Arrhenius plots revealed that the mutant and wild-type enzymes displayed activation energies of 61 ± 1 and 18 ± 2 kJ/mol, respectively, at their pH optima. Unlike wild-type PTP-1B, C215D-mediated $p$-nitrophenyl phosphate hydrolysis was inactivated by 1,2-epoxy-3-$p$-nitrophenoxypyropane, 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 the catalytic efficiency of the mutant protein. Structurally, the apoenzyme was identical to wild-type PTP-1B, aside from the flexible WPD loop regions. Specifically, the WPD loop has been shown to adopt different conformations in the unliganded and liganded forms of protein-tyrosine phosphatase-1B (PTP-1B). 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$^{181}$ functions as a general base, abstracting a proton from an attacking water molecule in the dephosphorylation step. Subsequently, Asp$^{181}$ functions as a general base, abstracting a proton from an attacking water molecule in the dephosphorylation step. Enhancing 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$^{181}$ is ~10 Å away from the P-loop. Upon substrate binding, the WPD loop adopts a closed conformation and covers the active site like a "flap," thereby positioning Asp$^{181}$ closer to the leaving group (11, 12).

In wild-type PTP-1B, Cys$^{215}$ 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 those of the wild-type enzyme, but does not display any measurable phosphatase activity (14, 15). The crystal structure of the unliganded C215S PTP-1B mutant shows the P-loop in a conforma-

\[ x = \frac{\text{The abbreviations used are: PTPases, protein-tyrosine phosphatases; PTP-1B, protein-tyrosine phosphatase-1B; MES, 4-morpholineethanesulfonic acid; pNPP, p-nitrophosphophate; EPNP, 1,2-epoxy-3-$p$-nitrophenoxy)propane.}\]
tionally distinct orientation compared with 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 PT-Pase signature motif loop (P-loop) and the surrounding areas, favoring the extended conformation (16). The structural studies suggested that the conformation of the C215S mutant and wild-type PTP-1B is similar and may be dependent on the presence of the negative charge of the active-site nucelophile. The goal of this study was to explore the importance of the presence of a negatively charged residue other than Cys at position 215 in the conformation of the P-loop and in the catalytic activity of PTP-1B. We therefore substituted Cys 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 with those of wild-type PTP-1B.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin and the SuperSignal West Pico immunoblotting kit were obtained from Pierce. SDS-polyacrylamide gels were obtained from Invitrogen, and Cibacron blue colI BL21 cells were obtained from Stratagene. Isopropyl-1-thio-β-D-galactopyranoside and protease inhibitors were from Roche Applied Science. 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 bovine serum albumin as a standard (47). For SDS-PAGE and immunoblotting of purified mutant and wild-type PTP-1B, proteins were boiled in SDS-PAGE loading buffer for 5–10 min and then separated on 10–20% (w/v) polyacrylamide gradient gels containing SDS. The proteins were transferred onto nitrocellulose at a constant voltage of 100 V for 90 min. The acrylamide gradient gels containing SDS-polyacrylamide gels were purchased from Invitrogen, and the SuperSignal West Pico kit were obtained from Pierce. SDS-polyacrylamide gel electrophoresis (SDS-PAGE), gel staining, 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 grown for an additional 2 h. Cells were harvested by centrifugation of the cell lysate at 31,000 g. The resulting pelleted protein sample was then subjected to the Michaelis-Menten equation using the nonlinear curve fitting software program Grafit Version 4.0.10 (Erithacus Software Inc.) to determine kinetic constants. To extract pK values from the pH-response studies, the data were also fitted using nonlinear regression analysis (SigmaPlot, Jandel Scientific).

**Activity Assays**—Assays were carried out in a 96-well format at 22 °C in buffer consisting of 100 mM Tris-HCl, 50 mM MES, 50 mM acetic acid, 5 mM dithiothreitol, 2% (v/v) MeSO₂, 2% (v/v) glycerol, and 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-nitrophenyl phosphate (pNPP) to p-nitrophenol. Briefly, pNPP hydrolysis was measured by incubating C215D or wild-type PTP-1B with 0–10 mM pNPP for 30 or 4 min, respectively, and then stopping the reaction by adding NaOH to a final concentration of 1 M. 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-nitrophenol anion (15,800 M⁻¹ cm⁻¹). The observed rates of reactions were fitted to the Michaelis-Menten equation using the nonlinear curve fitting software program Grafit Version 4.0.10 (Erithacus Software Inc.) to determine kinetic constants. To extract pK values from the pH-response studies, the data were fitted using nonlinear regression analysis (SigmaPlot, Jandel Scientific).

**Viscosity Studies**—The effect of viscosity on C215D or wild-type PTP-1B activity was determined by measuring pNPP 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 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. Reaction rates were determined by measuring the absorbance at 405 nm using a 1-nitrophenyl phosphate (pNPP, the epoxide was dissolved in MeSO, and added to the reaction mixtures to a final concentration of 2.4 mM. Following incubation of the enzymes for 1 h at 4 °C in the presence of pNPP, the catalytic activities were determined at their respective pH optima as described above.

**Crystallization and Data Collection**—Apocr215D 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 mM NaCl, 1 mM EDTA, and 5 mM N,N,N’,N’-dimethylbiis(mercaptopacetyl)phosphonate (pEPN), the epoxide was dissolved in MeSO, and added to the reaction mixtures to a final concentration of 2.4 mM. Following incubation of the enzymes for 1 h at 4 °C in the presence of pNPP, the catalytic activities were determined at their respective pH optima as described above.

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conformation and that the peptide containing the catalytic site mutation assumed the same conformation observed in the wild-type enzyme (see Fig. 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 1.6Å resolution. Typically, two cycles of torsion angle dynamics and 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 maximal likelihood approach (26, 27). When high resolution data were included, it became evident in the electron density maps that the WP loop (Thr177–Ser189) 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. See “Results” for the statistics for the refined model.

RESULTS

To evaluate the importance of a negatively charged residue at position 215 of PTP-1B, we substituted Cys215 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 with 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 pH 3.5–9 (Fig. 1). Although the pH profiles were generally bell-shaped for both enzymes, suggesting the existence of two ionizable amino acid side chains involved in catalysis, significant differences in the profiles were noted. As expected, wild-type PTP-1B displayed maximal catalytic activity at pH 6.0; however, when the active-site Cys was replaced with 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 pH 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 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 (−5.5) was also significantly lower than that of wild-type PTP-1B. These results clearly show that the two forms of the enzyme displayed distinct pH dependences with respect to substrate hydrolysis.

Comparison of K_{cat}, k_{cat}, and E_{act} of Mutant and Wild-type PTP-1B—Next, we compared the kinetic parameters of the C215D mutant with those of wild-type PTP-1B. Table I 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_{cat} values of 1.1–1.4 μM, the k_{cat} values of the C215D mutant were ~70- and ~7000-fold lower than those of wild-type PTP-1B at pH 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 (E_{act}) 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} values for C215D and wild-type PTP-1B are shown in Fig. 2 and Table II, 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 >3-fold higher than that 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 (for example, see Refs. 28–30). Irreversible inactivation is presumably due to alkylation of the active-site carboxyl residue by the epoxide moiety of EPNP. Because 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 activities that remained following a 1-h 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 (Fig. 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\(^{215}\) with 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% (\(R_{	ext{free}}\) is 20.4%) for 59,409 reflections between 15.0 and 1.6 Å (5% flagged for \(R_{	ext{free}}\) calculation) and maintained good geometry (root mean square deviation) of 0.01°/1.5 Å. The backbone conformation of 91.6% (94.8%) 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\(^{18}\), Pro\(^{37}\), Arg\(^{105}\), Ser\(^{118}\), Leu\(^{119}\), Glu\(^{159}\), Ser\(^{200}\), Ser\(^{216}\), Arg\(^{221}\), Cys\(^{226}\), Ile\(^{246}\), Met\(^{253}\), and Ser\(^{265}\) were modeled as having alternate conformations. The P-loop is depicted schematically in Fig. 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 bonded to one of several ordered water molecules located in the binding site. This water is located at the position normally bonded to one of several ordered water molecules located in the binding site.

**Statistics for the data set used to solve the structure of the apo-C215D mutant of PTP-1B and final statistics for the refined model**

| No. of reflections | 62,681 (9061) |
|--------------------|--------------|
| Redundancy         | 10 (9.5)     |
| B factor           | 11.2 (2.9)   |
| \(R_{	ext{sym}}\)  | 4.3 (26.9)   |

Numbers in parentheses refer to the last resolution shell. r.m.s.d., root mean square deviation.

**TABLE III**

| Resolution range (Å) | 28.0–1.6 (1.7–1.6) |
|-----------------------|---------------------|
| No. of reflections    | 59,409 (5849)       |
| % possible           | 99.9 (100)          |
| Refinement statistics | 15.0–1.6 (1.66–1.6) |
| No. of reflections (work) | 59,409 (5849) |
| % possible           | 94.9 (94.8)         |
| No. of bond length (Å)/angle | 0.010/1.50° |
| No. of protein atoms | 2719                |
| No. of solvent atoms | 825                 |
| No. of heteroatoms   | 8                   |

\(^a\) 5% of reflections were set aside for \(R_{	ext{free}}\) calculation.

\(^\circ\) 248 atoms belong to alternate conformations.

\(^\circ\) Two magnesium and six chlorine ions have been identified in the structure.

**FIG. 3.** Effect of EPNP on C215D and wild-type PTP-1B-catalyzed pNPP hydrolysis. The catalytic activities of C215D and wild-type (WT) PTP-1B were measured following a 1-h incubation at 4°C in the absence (black bars) or presence (gray bars) of 2.4 mM EPNP using 10 mM pNPP as a substrate. The data represent the means of triplicate determinations, and activity is expressed as percent maximal activity of C215D and wild-type PTP-1B at pH 4.5 and 6, respectively.

**TABLE II**

| PTP-1B     | \(E_{\text{act}}\) (kJ/mol) |
|------------|-----------------------------|
| C215D      | 61 ± 1                      |
| Wild-type  | 18 ± 2                      |

activation values were calculated from the slopes of the lines in Fig. 2.
average temperature factor of 22.8 Å²), 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 Arg221 also assumes two distinct conformations (with similar occupancies) that indeed correspond to the conformations previously observed in the apo- and liganded PTP-1B (Protein Data Bank 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 to 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 pNPP in the presence of increasing concentrations of sucrose. As shown in Fig. 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 (Fig. 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 $(k_{cat}/K_m)^o/(k_{cat}/K_m)^s$ as a function of relative solvent microviscosity (Fig. 5, C and D).

DISCUSSION

Previously, we reported that substitution of Cys215 with Ser results 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 wild-type enzyme (16). In this study, we generated and characterized the C215D mutant of PTP-1B to obtain further insights into the structural basis of PTP-1B catalysis. We substituted Cys215 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
Structure and Function of Mutant C215D of PTP-1B

Fig. 5. Dependence of relative kinetic parameters for C215D and wild-type PTP-1B-catalyzed pNPP hydrolysis on solvent viscosity. A and C show the dependence of $k_{cat}$ on solvent viscosity for wild-type and C215D PTP-1B, respectively. B and D show the effect of solvent viscosity on $K_m$ for wild-type and C215D PTP-1B, respectively. The parameters were determined by measuring the catalytic activities of the enzymes in the presence of increasing concentrations of sucrose. Data points represent the means of duplicate experiments. The solid lines indicate the calculated best fit lines using linear regression analysis.

reflects the fact that the active-site Cys, which displays a $pK_a$ of $-5.5$ in wild-type PTP-1B (13), was replaced with the strongly acidic Asp residue, with a $pK_a$ of $-4$ 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 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 m$M$ for pNPP for both mutant and wild-type PTP-1B, respectively, were 4-fold higher than the $K_m$ values determined at pH 6. That both PTPases displayed the same shift in $K_m$ at a lower pH 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 pH 4.5, C215D-mediated pNPP hydrolysis still remained >70-fold lower than wild-type enzyme-mediated pNPP hydrolysis at the same pH. Thus, substitution of the strongly nucleophilic active-site Cys with 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 C215D and wild-type PTP-1B revealed that the activation energy of C215D-mediated pNPP hydrolysis was >3-fold higher than that of the reaction catalyzed by wild-type PTP-1B. The plots were linear over temperature ranges 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 $k_{cat}$ 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 (for example, see Refs. 30 and 35). 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 the lack of an effect on wild-type PTP-1B suggest a direct involvement of Asp$^{325}$ 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 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$^{325}$ is crucial to the reaction mechanism of the mutant protein.

Structurally, wild-type PTP-1B and the C215D derivative are 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 (relative occupancies of 73.1 and 26.9%, respectively) and is probably induced by the extensive hydrogen bond network, involving both solvent and protein atoms, identified in the binding site (Fig. 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 $k_{cat}/K_m$ 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 PTPase and have found that the catalytic efficiency of the corresponding region of this enzyme (i.e. amino acids 1–296) responded to changes in solvent microviscosity in a manner similar to PTP-1B (data not shown).

Specifically, $k_{cat}/K_m$ of T-cell PTPase-catalyzed pNPP hydrolysis was $-1.5$-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 the $K_m$ or $k_{cat}$ of the C215D mutant and hence resulted in no change in the second-order rate constant of C215D-mediated pNPP 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 wild-type apo-PTP-1B 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 conformation in the C215D mutant, which results in a preformed 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 viscosogen.

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 is replaced with 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) and 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 (i.e. $\text{Asp}^\text{181}$) is replaced with 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 Q626A derivative, which has been used to obtain a crystal structure of the phosphoryl-enzyme intermediate (44).

Recently, a double mutant (D181A/Q626A) of PTP-1B has been generated. This substrate-trapping mutant exhibits higher affinity than both the independent D181A and C215S mutants for the epidural growth factor receptor and displays 3000- and 11,000-fold lower $K_m$ values for nPPN and epidermal growth factor receptor, respectively (45). It is currently unknown, however, how this mutant compares structurally with wild-type PTP-1B.

In wild-type PTP-1B, Cys$^{215}$ functions as a strong nucleophile, forming a cysteinyl-phosphate intermediate in the reaction mechanism. We hypothesized that substitution of this residue with 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 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$^{215}$ 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 carbonyl 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.

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