Protein-tyrosine phosphatases (PTPs) are signal transduction enzymes that catalyze the dephosphorylation of phosphotyrosine residues via the formation of a transient cysteinyl-phosphate intermediate. The mechanism of hydrolysis of this intermediate has been examined by generating a Gln-262 → Ala mutant of PTP1B, which allows the accumulation and trapping of the intermediate within a PTP1B crystal. The structure of the intermediate at 2.5-Å resolution reveals that a conformationally flexible loop (the WPD loop) is closed over the entrance to the catalytic site, sequestering the phosphocysteine intermediate and catalytic site water molecules and preventing non-specific phosphoryltransfer reactions to extraneous phosphoryl acceptors. One of the catalytic site water molecules, the likely nucleophile, forms a hydrogen bond to the putative catalytic base, Asp-181. In the wild-type enzyme, the nucleophilic water molecule would be coordinated by the side chain of Gln-262. In combination with our previous structural data, we can now visualize each of the reaction steps of the PTP catalytic pathway. The hydrolysis of the cysteinyl-phosphate intermediate of PTPs is reminiscent of GTP hydrolysis by the GTPases, in that both families of enzymes utilize an invariant Gln residue to coordinate the attacking nucleophilic water molecule.

The formation of phosphoryl-enzyme intermediates is an essential component of numerous enzymatic mechanisms that involve phosphoryl-transfer reactions, for example the dephosphorylation of Tyr(P) residues catalyzed by protein-tyrosine phosphatases (1). PTPs together with the protein-tyrosine kinases, which catalyze the opposing tyrosine phosphorylation reaction, control the overall levels of cellular tyrosine phosphorylation, and the molecular basis of the regulation and substrate specificity of these enzymes is subject to much investigation (2, 3). Reversible tyrosine phosphorylation is essential to the signal transduction pathways triggered by hormones, mitogens, and oncogenes that regulate such processes as cell growth, differentiation, and proliferation. The PTPs are a diverse family of enzymes that comprise transmembrane receptor-like PTPs (RPTPs) and soluble cytosolic proteins. The catalytic domains of the PTPs are highly conserved, consisting of ~250 amino acids and are characterized by an N1-residue PTP signature motif, (IV/GXGXXRxS/T/G, containing the Cys and Arg residues that are essential for catalysis (4, 5). Diversity within the family is generated by the nature of the non-catalytic segments attached to the N and C termini of the PTP domains, which provide regulatory and subcellular targeting functions. Additional diversity is generated within the RPTPs, which frequently possess tandem PTP domains, although for some RPTPs such as CD45, the C-terminal PTP domain lacks catalytic activity (3). The dual specificity phosphatases (DSPs) are related to the PTPs by their possession of the PTP signature motif and related tertiary structure and catalytic mechanism (1, 6).

Insights into the catalytic mechanism of PTPs and DSPs have been obtained from structural and kinetic studies of these enzymes (1, 4, 5). A PTP1B-phosphotyrosine peptide complex revealed that the Tyr(P) residue of the peptide is buried within a deep catalytic site cleft present on the protein’s molecular surface. The base of the catalytic site is formed by residues of the PTP signature motif with the phosphate group of Tyr(P) being coordinated by main-chain amide groups and the Arg side chain of this motif, such that the phosphorus atom is situated adjacent to the Sγ atom of the catalytic Cys residue (7). Four other loops bearing invariant residues form the sides of the catalytic cleft and contribute to catalysis and substrate recognition. Engagement of phosphopeptides by PTP1B promotes a major conformational change of one of these loops (the WPD loop) consisting of residues 179–187 that shift by as much as 8 Å to close over the phenyl ring of Tyr(P) and allow the side chain of Asp-181 to act as a general acid in the catalytic reaction. The Arg-221 side chain reorients to optimize salt bridge interactions with the phosphate bound to the catalytic site. This shift is coupled to motion of the WPD loop via a hydrogen bond between NH2 of Arg-221 and the carboxyl oxygen of Pro-180 and hydrophobic interactions between the aliphatic moiety of Arg-221 and the side chain of Trp-179. These interactions and the hydrophobic packing between Phe-182 and the phenyl ring of Tyr(P) stabilize the closed, catalytically competent conformation of the loop. The phosphotyrosine dephosphorylation reaction commences with nucleophilic attack by the Sγ atom of the catalytic cysteine on the Tyr(P) phosphorus atom. Cleavage of the scissile P–O bond is facilitated by protonation of the phenolic oxygen by Asp-181 with the consequent formation of a phosphocysteine intermediate. The tyrosine residue diffuses out of the catalytic site and subsequently the transient phosphoryl-enzyme intermediate is hydrolyzed by an activated water molecule. The structure of a PTP1B-WO4 complex suggests that after hydrolysis of the phosphoryl-enzyme interme-
diolate, the WPD loop opens, allowing product release (8).

Numerous kinetic data support the reaction mechanism outlined above (1). Cysteinyl-phosphate intermediates have been trapped by rapid denaturation of PTPs and a DSP (vaccinia H1-related) during catalytic turnover (9–11). Moreover, substitution of the catalytic Cys residue for a serine abolishes catalytic activity and the formation of an enzyme intermediate (9). The nucleophilicity of the Cys residue results from its close proximity to main-chain amide and a hydrogen bond with the side chain of Ser-222 of the PTP signature motif, resulting in an unusually low pK<sub>a</sub> of 4.6 (12). The catalytic Asp residue (Asp-181 of PTP1B) contributes to the basic limb of the pH activity profile, and its substitution to Ala causes a 10<sup>5</sup>-fold reduction in k<sub>cat</sub>, suggestive of a role as an acid catalyst (13, 14). These results imply that Asp-181 is necessary for the first step of the reaction, namely cleavage of the Tyr(P) P-O bond and intermediate formation, a notion consistent with the finding that Asp-181 mutants of PTP1B allow phosphorylated substrates to form stable complexes with the enzyme in vivo (14).

An understanding of the chemical steps of the phosphocysteine dephosphorylation reaction has been more elusive, mainly because of the transient character of this intermediate as PTPs are efficient catalysts. For example, PTP1B hydrolyzes 40 molecules of substrate(s) at 25 °C (13, 14). Of particular interest are the structure of the cysteinyl-phosphate PTP intermediate, the mechanism of activation of the nucleophilic water molecule responsible for the hydrolysis of the intermediate, and mechanisms by which nonspecific phosphoryl-transfer reactions are prevented. The phosphoryl transfer reaction to a water molecule catalyzed by PTPs is highly specific, as PTPs are unable to phosphorylate a range of primary alcohols and other phosphate acceptors (15). Here, we describe an approach to obtain structural information on the cysteinyl-phosphate intermediate of PTP1B. The structure of a PTP1B-orthovanadate complex, which revealed that vanadate mimics the pentavalent phosphorus transition state intermediate, indicated that Gln-262 may play an important role in stabilizing and/or activating a nucleophilic water molecule for attack on the cysteinyl-phosphate intermediate. By replacing Gln-262 with Ala, we have prolonged the life-time of the cysteinyl-phosphate intermediate, allowing us to trap and visualize it using x-ray cryocrystallography. The structure (i) reveals that the WPD loop adopts a closed conformation that sequesters both the cysteinyl-phosphate residue and the nucleophilic water molecule, (ii) suggests roles for Asp-181 and Gln-262 in ligand binding. The model was subjected to 100 cycles of positional refinement, after which the coordinates for the WPD loop and Arg-221 side chain were rebuilt into the difference electron density. This model reveals that vanadate mimics the cysteinyl-phosphate intermediate, allowing us to trap and visualize it with Ala, we have prolonged the life-time of the cysteinyl-phosphate intermediate, (iii) catalyzing the enzyme dephosphorylation reaction, and (iii) explains why phosphoryl transfer from the intermediate occurs to water molecules and not to other phosphoryl acceptors. The role of a Gln residue in catalyzing the hydrolysis of the cysteinyl-phosphate intermediate of PTPs is reminiscent of that performed by the Gln residue in GTP hydrolysis at the catalytic site of GTPases (16–19). The approach that we describe here to trap the phosphoryl-enzyme intermediate of PTP1B may have general applicability to other transient covalent-enzyme intermediates and enzyme-substrate complexes that will allow their isolation and structural characterization.

**EXPERIMENTAL PROCEDURES**

**Preparation of Crystals**—Wild-type protein and the Q262A mutant PTP1B protein and crystals were prepared as described (20). The PTP1B-orthovanadate complex was prepared by incubating wild-type PTP1B crystals in 100 mM HEPES (pH 7.5), 200 mM magnesium acetate, 18% (w/v) polyethylene glycol 8000, and 1 mM sodium orthovanadate for 10 min prior to transfer to the same buffer with 25% (v/v) glycerol and freezing in a nitrogen gas stream at 100 K (22). To prepare crystals of the PTP1B-cysteinyl-phosphate complex, Q262A mutant crystals that had been grown at pH 7.5 were incubated for 5 min in a buffer of 100 mM BisTris (pH 7.0), 200 mM magnesium acetate, 18% (w/v) polyethylene glycol 8000 and subsequently for another 5 min in the same buffer at pH 6.5. To prepare for cryofreezing and for trapping the intermediate, crystals were incubated in this buffer with 5% and then 10% (v/v) methyl-pentanediol each for 5 min before being transferred to 3 ml of 100 mM BisTris (pH 6.5), 200 mM magnesium acetate, 18% (w/v) polyethylene glycol 8000, 25 mM para-nitrophenol phosphate, 15% (v/v) methyl-pentanediol for 12 min. At this time, crystals were mounted directly into nylon loops and frozen.

**Data Collection and Refinement**—Data for the intermediate crystal were collected using a MAR 30-cm detector mounted on a Rigaku x-ray generator, and data for the orthovanadate crystal were also collected using a MAR 30-cm detector but on PX 9.6 at the Synchrotron Radiation Source, Daresbury Laboratory. Both data sets were processed using DENZO and SCALEPACK (21) (see Table I for details). All refinement of the coordinates was performed using X-PLOR (22) and the protein coordinates and electron density maps analyzed with O (23) and TURBO-FRODO (24).

**Orthovanadate Complex**—Initial phases were derived from the 2.2-Å structure of PTP1B in an unliganded form to minimize model bias. In this structure, no ligands were bound at the catalytic site and the WPD loop adopts the open conformation. 2F<sub>o</sub>−F<sub>c</sub> and F<sub>c</sub>−F<sub>o</sub> OMIT maps (without orthovanadate in the model) were calculated after 75 cycles of rigid body refinement, and these maps demonstrated density for the vanadate ion at the catalytic site, a shift in the position of the WPD loop by 8 Å from the open to the closed conformation, and a rotation of the Arg-221 side chain from an all-trans to a cis conformation, indicative of ligand binding. The model was subjected to 100 cycles of positional refinement, after which the coordinates for the WPD loop and Arg-221 side chain were rebuilt into the difference electron density. This model was then subjected to simulated annealing with an initial temperature of 2500 K. A model for the orthovanadate ion was then constructed using perfect trigonal bipyramidal geometry and V-O bond lengths of 1.61 Å. The ion was built into the difference density and allowed to refine freely as a rigid body with interactions to the protein turned off. The ion refined to a position 2.4 Å away from the Cys-215 S atom, consistent with the formation of a covalent bond between the vanadium and Cys-215 S atom, and a bond of this length was included in subsequent refinement. The model was then subjected to further rounds of positional refinement and 15 cycles of individual isotropic B-factor refinement. The bond length between the vanadium and Cys-215 S atom refined to a value of 2.4 Å both in the presence (at 200 kcal mol<sup>−1</sup>) and absence of bond length constraints. This value is similar to water molecules and not to other phosphoryl acceptors. The role of a Gln residue in catalyzing the hydrolysis of the cysteinyl-phosphate intermediate of PTPs is reminiscent of that performed by the Gln residue in GTP hydrolysis at the catalytic site of GTPases (16–19). The approach that we describe here to trap the phosphoryl-enzyme intermediate of PTP1B may have general applicability to other transient covalent-enzyme intermediates and enzyme-substrate complexes that will allow their isolation and structural characterization.

**TABLE I. Crystallographic data and refinement statistics**

| Crystallographic data | PTP1B-VO₄ | PTP1B Q262A cysteinyl-phosphate complex |
|-----------------------|-----------|----------------------------------------|
| **Space group**       | P₃₁₂₁    | P₃₁₂₁                                  |
| **Cell parameters**   |           |                                        |
| a (Å)                 | 88.28     | 88.54                                  |
| b (Å)                 | 88.28     | 88.54                                  |
| c (Å)                 | 104.91    | 104.69                                 |
| Resolution (Å)        | 50 to 2.3 | 50 to 2.5                              |
| Observations (N)      | 53355     | 67136                                  |
| Unique reflections (% complete) | 22284 (98.3) | 16797 (99.4) |
| R<sub>free</sub> (%)  | 0.079 (0.234)<sup>a</sup> | 0.086 (0.313)<sup>b</sup> |
| R<sub>i</sub>s<sub>igf</sub> | 7.8 (2.7)<sup>c</sup> | 7.3 (2.9)<sup>c</sup> |
| Reflections with I/σ(I) > 2.0 (%) | 75.0 (49.2)<sup>b</sup> | 79.1 (51.8)<sup>b</sup> |
| **Refinement statistics** |           |                                        |
| Resolution (Å)        | 6.0 to 2.3 | 6.0 to 2.5                             |
| Number of reflections (% complete with 2σ cutoff) | 20563 (90.2) | 15546 (92.6) |
| Protein atoms (N)     | 2980      | 2837                                   |
| Water molecules (N)   | 131       | 74                                     |
| R<sub>cryst</sub> (work-free)% | 20.9/27.6 | 20.5/28.1                              |
| r.m.s.d. bond lengths (Å) | 0.018 | 0.011                                  |
| r.m.s.d. bond angles (°) | 1.90 | 1.64                                   |
| r.m.s.d. dihedrals (°) | 24.4 | 24.4                                   |
| r.m.s.d. water oxygen (°) | 1.69 | 1.41                                   |

<sup>a</sup> R<sub>free</sub> = Σ(|F<sub>o</sub>|−|F<sub>c</sub>|)/Σ|F<sub>o</sub>|
<sup>b</sup> Values for the outer resolution shell (2.38 to 2.30 Å and 2.59 to 2.50 Å for the PTP1B-VO₄ and PTP1B Q262A cysteinyl-phosphate coordinates, respectively).
<sup>c</sup> R<sub>cryst</sub> = Σ(|F<sub>o</sub>|−|F<sub>c</sub>|)/Σ|F<sub>c</sub>|

ilar to that reported by Denu et al. (25) in their crystal structure of the Yersinia PTP-vanadate complex. Water molecules were added manually with criteria for addition being $F_o - F_c$ density at 3σ, $2F_o - F_c$ density at 1σ, a favorable hydrogen bonding environment, and a decrease in both the free and working $R$-factors after each cycle. The model was then subjected to 50 cycles of positional refinement and 15

**Fig. 1.** A, simulated annealing OMIT $2F_o - F_c$ (green) and $F_o - F_c$ (red) electron density maps of the PTP1B-vanadate complex superimposed onto the refined coordinates. Phases and $F_c$ coefficients were calculated using protein coordinates (without those for vanadate) from a simulated annealing refinement run. Contour levels are at 1σ and 3σ for the $2F_o - F_c$ and $F_o - F_c$ electron density maps, respectively. This figure was generated using TURBO-FRODO (24). B, refined structure of the PTP1B-vanadate complex. This figure and all others except as indicated were generated using X-OBJECTS (M. E. Noble, unpublished).

**Fig. 2.** Superimposition of the PTP1B-vanadate complex and PTP1B-Tyr(P) complex in the vicinity of the catalytic site to show conformational change of Gln-262. In the PTP1B-Tyr(P) complex (cyan), Gln-262 is rotated out of the catalytic site relative to the PTP1B-vanadate complex.
cycles of B-factor refinement. Simulated annealing OMIT maps were calculated after performing a simulated annealing refinement run (at 2000 K) using the refined protein and water coordinates and in the absence of the vanadate coordinates. The final model satisfied or exceeded all the stereochemical quality indicators of PROCHECK (26).

Cysteinyl-phosphate Intermediate—Initial phases were also calculated from the 2.2-Å structure of unliganded PTP1B. The model was subjected to 75 cycles of rigid body and 100 cycles of positional refinement, and 2Fo - Fc and Fo - Fc OMIT maps (without intermediate) were calculated. The Fo - Fc difference map showed a 3σ peak adjacent to the Cys-215 S atom and contiguous 2Fo - Fc density extending from the sulfur atom and enveloping the difference density (Fig. 3A). The maps also showed movement of the WPD loop by 8 Å to the closed conformation and a rotation of the Arg-221 side chain. Negative difference density was also seen on the side chain of Gln-262 confirming the mutation. The model was then subjected to simulated annealing from 2500 K. A model for the phosphate intermediate was constructed with perfect tetrahedral geometry around the phosphorus atom, P–O bond lengths of 1.55 Å, and a P–S bond length of 1.85 Å. This model was built into the density, the WPD loop was rebuilt into difference density, adopting the closed conformation, and Gln-262 was mutated to Ala. The model was then subjected to another 100 cycles of positional refinement and 15 cycles of individual isotropic B-factor refinement. Water molecules were then added manually, subject to the same criteria applied to the PTP1B-vanadate complex, and the model was subjected to positional and B-factor refinement. A 2Fo - Fc electron density map using phases from refined coordinates is shown in Fig. 3B. The final model satisfied or exceeded all the stereochemical quality indicators of PROCHECK (26).

RESULTS

Structure of a Transition State Analogue—We investigated initially the structure of the PTP1B-orthovanadate complex, which provides information concerning the transition state structure of the enzyme catalyzed Tyr(P) dephosphorylation reaction. A similar structure of the distantly related Yersinia PTP has been reported (25). Fig. 1A shows 2Fo - Fc, Fo - Fc electron density maps calculated using phases from a simulated annealing refinement run of PTP1B coordinates excluding those for vanadate. A covalent bond is formed between the S atom of the nucleophilic Cys-215 residue and the vanadium atom, so that the vanadate ion forms a pentavalent trigonal bipyramidal structure that is an analogue of the pentavalent transition state intermediate. At 2.3-Å resolution, we do not observe distortion from perfect bipyramidal geometry. The three equatorial oxygen atoms of vanadate form some eight hydrogen bonds to main-chain NH groups of the PTP signature motif and guanidinium side chain of Arg-221, whereas the apical oxygen atom forms hydrogen bonds with the side chains of Gln-262 and Asp-181 (Fig. 1B). The latter interaction suggests that the apical oxygen atom is protonated, forming a hydrogen bond with the carboxylate group of Asp-181 at pH 7.5. The apical oxygen atom most closely resembles the position of an attacking nucleophilic water molecule during the hydrolysis step of the cysteinyl-phosphate intermediate. Thus, the hydrogen bonds to Asp-181 and Gln-262 suggest that these residues may play a role in positioning and/or activating a water molecule during this step. The interaction between Asp-181 and the apical vanadate oxygen atom is possible as a result of the closed conformation of the WPD loop, similar to that observed in the PTP1B-phosphopeptide complex (7). Closure of the loop is probably stabilized by a combination of interactions between Arg-221 and loop residues, Trp-179 and Pro-180 and the hydrogen bond between Asp-181 and the vanadate apical oxygen atom (Trp-179). A buried water molecule (WAT1), analogous to that in the PTP1B-Tyr(P) complex, links the main-chain amide groups of Phe-182 and Arg-221 with a vanadate equatorial oxygen via a network of hydrogen bonds (Fig. 1B).

Structure-based Design to Slow Rate of Phosphocysteine Hydrolysis—To understand the mechanism of cysteinyl-phosphate hydrolysis more fully, we have sought to determine the structure of this intermediate using x-ray crystallography. To achieve this, the rate of hydrolysis of the phosphoryl-enzyme intermediate should be substantially slower than the rate of intermediate formation to allow the intermediate to accumulate. Moreover, its life-time must be of sufficient length to be amenable to structural analysis, being in effect the rate-limiting step of the enzyme catalyzed reaction within the crystal. The nature of the rate-limiting step catalyzed by PTPs and DSPs is not entirely resolved. There are kinetic data to suggest that the rate-limiting step of PTP catalysis is the rate of phosphoryl-enzyme formation (25). This would imply that the cysteinyl-phosphate intermediate would not accumulate appreciably within the wild-type enzyme. However, other investigators, have reported that the presence of a pre-steady state burst phase observed during hydrolysis of para-nitrophenol phosphate (p-NPP) indicates that hydrolysis of the phosphoryl-enzyme intermediate is rate-limiting (27). The latter result implies that during steady state catalysis, a phosphoryl-enzyme intermediate would accumulate, and this is consistent with the trapping, using rapid denaturation techniques, of phosphoryl-enzyme intermediates of PTPs during the catalytic reaction (9–11, 25). However, since the incorporation of phosphate in the phosphoryl-enzyme intermediate was substoichiometric, the rates of phosphoryl enzyme formation and hydrolysis are probably quite similar.

As discussed earlier, the structure of the PTP1B-vanadate complex suggests crucial roles for Asp-181 and Gln-262, two invariant residues, in positioning and/or activating the nucleophilic water molecule during hydrolysis of the cysteinyl-phosphate intermediate. Recent kinetic data have implicated Asp-92 of vaccinia H1-related (equivalent to Asp-181 of PTP1B) as a catalytic base during phosphoryl-enzyme hydrolysis in addition to its role as a catalytic acid to promote Tyr(P) cleavage (25). We have reported previously that mutation of Gln-262 of PTP1B to Ala reduces kcat by 100-fold and Km 10-fold (13). In the PTP1B-phosphotyrosine peptide complex, the position of Gln-262 differs from its position in the PTP1B-vanadate complex, as the side chain is swung out of the catalytic site to avoid a steric clash with the phenyl side chain of the Tyr(P) substrate (7) (Fig. 2). This implies that Gln-262 plays little or no catalytic function during cleavage of the scissile P–O bond, whereas a role in positioning and/or activating a water molecule during cysteinyl-phosphate hydrolysis is likely. From this analysis, we reasoned that the 100-fold reduction in kcat of the Q262A mutant may result from reducing the rate of hydrolysis of the cysteinyl-phosphate intermediate without affecting the rate of enzyme phosphorylation. This would render the rate-limiting step of the reaction as phosphoryl-enzyme hydrolysis and increase the life-time of the intermediate 100-fold. The wild-type enzyme kcat is 40 s-1, whereas that of the Q262A mutant is -0.5 s-1 at 25 °C (13). At 4 °C this corresponds to one enzymatic reaction in ~10 s.

Our approach to trapping the phosphocysteine intermediate was as follows. By assuming that the rate of substrate diffusion into the crystal exceeds the rate of cysteinyl-phosphate hydrolysis, and with an excess of substrate added to the crystal incubation buffer, a steady state accumulation of intermediate should be obtained, which may be captured by flash-freezing a crystal in a stream of nitrogen gas at 100 K. The cysteinyl-phosphate intermediate is preserved in the frozen crystals, which are suitable for x-ray data analysis. PTP1B crystals are grown at pH 7.5 and 4 °C (20). However, at this pH, the rate of PTP-catalyzed hydrolysis of p-NPP is 10% of its maximum rate observed between pH 5.5 and 6.5 (27). The reduction in rate is attributable in large part to the deprotonation of Asp-181, the presumed acid catalyst in the p-NPP hydrolysis reaction, which
would imply that the rate-limiting step becomes that of enzyme phosphorylation and hence a phosphoryl-enzyme intermediate would not accumulate under these conditions (14, 27). Thus, to optimize the conditions for enzyme phosphorylation, we equilibrated crystals at pH 6.5 prior to the start of the kinetic experiment. To start the experiment, we incubated small crystals in a large molar excess (\(\times 1000\)-fold) of \(\text{p}-\text{NPP}\) for 12 min at a high concentration (25 mM) to saturate the enzyme catalytic site to achieve \(V_{\text{max}}\) for enzyme phosphorylation. The \(K_m\) for \(\text{p}-\text{NPP}\) is \(1\) mM (27). Small crystals were used to optimize uniform distribution of substrate within the crystal. Advantages of PTP1B crystals for this study are (i) the high solvent content of 60%, which facilitates diffusion, and (ii) the ability of small substrates such as Tyr(P) and \(\text{p}-\text{NPP}\) to bind to the catalytic site and promote closure of the WPD loop (7). It was assumed that, after 12 min, the kinetic reaction had reached steady state and hence accumulation of the phosphoryl-enzyme intermediate had been achieved. At this time, crystals were flash-frozen at 100 K. As a control, we performed an identical experiment using wild-type PTP1B crystals.

**Structure of the Phosphocysteinyl Intermediate**—In the control experiment, using the wild-type PTP1B crystals incubated with \(\text{p}-\text{NPP}\), no evidence for electron density corresponding to a cysteinyl-phosphate intermediate or either substrate or product bound to the catalytic site was observed. The WPD loop was in the open conformation, reminiscent of the ligand free PTP1B state (8). The absence of substrate is attributable to the rapid turnover of the wild-type enzyme, and that of product, to the low phosphate product concentration achieved at the time of crystal freezing.

However, for the PTP1B Q262A mutant, \(2F_o - F_c\) and \(F_o - F_c\) electron density OMIT maps indicated that the catalytic site Cys-215 residue had been modified to a phosphocysteine residue representing the phosphoryl-enzyme intermediate (Fig. 3).
Clearly resolved density was observed for three terminal oxygen atoms and for density bridging the phosphorus atom of the phosphate with the $\text{S}_\gamma$ atom of Cys-215. The refined atomic temperature factors of the phosphate oxygen atoms are between 30 and 33 Å$^2$, higher than those for the oxygens of the covalently bound vanadate ion, which are similar to the atoms of Cys-215 (16 Å$^2$). The most likely explanations are either an incomplete occupancy of the cysteinyl-phosphate residue within the protein crystal or an increased mobility of the phosphoryl group relative to the vanadate, which as a transition state analogue is likely to be bound tightly. Electron density corresponding to three water molecules interacting with the thiophosphate group is observed in the $2F_o - F_c$ maps calculated using structure factors from the refined protein coordinates (Fig. 3B). One of these (W1) is equivalent to WAT1 of the PTP1B-vanadate complex and forms hydrogen bonds to the main-chain NH groups of Phe-182 and Arg-221, the side chains of Arg-221 and Gln-266, and a terminal oxygen of the cysteinyl-phosphate group (Fig. 4). A second water molecule (W2), situated above the cysteinyl-phosphate group, participates in hydrogen bonds with the side chain of Asp-181, W1, and a terminal oxygen atom of the cysteinyl-phosphate. The position occupied by the Gln-262 side chain in the PTP1B wild-type enzyme is the site of a third water molecule (W3), which forms a hydrogen bond to the third cysteinyl-phosphate oxygen atom.

The overall protein conformation of the PTP1B-intermediate structure is virtually identical with that of the wild-type PTP1B vanadate complex. Equivalent atoms superimpose with a root-mean-square deviation of 0.8 Å. These overall similarities extend to the catalytic site residues of the PTP signature motif (including the side chains of Cys-215 and Arg-221). Within the resolution limits of the data, the terminal oxygen atoms of the cysteinyl-phosphate intermediate superimpose almost exactly onto the equivalent vanadate and phosphate oxygen atoms of the PTP1B-vanadate and PTP1B-Tyr(P) complexes, respectively. Thus, although a stereochemical inversion at the phosphorus atom occurs during the phosphoryl-transfer reaction, the formation of a covalent bond to the $\text{S}_\gamma$ atom of Cys-215 shifts the center of mass of the phosphoryl group toward the base of the catalytic site.

X-ray Structure of the PTP1B Phosphocysteinyl Intermediate

![Fig. 4. Refined structure of the PTP1B-cysteiny1-phosphate intermediate in the vicinity of the catalytic site.](image)

![Fig. 5. Stereo view of a superimposition of PTP1B-vanadate complex (cyan) and PTP1B-cysteiny1-phosphate intermediate to show displacement of the putative nucleophilic water molecule from in-line geometry of the $\text{S}_\gamma$-P bond represented by the apical oxygen of vanadate.](image)
water molecules (Figs. 3 (A and B) and 4). The phenyl ring of Phe-182, immediately C-terminal to the WPD motif, is positioned exactly 7 Å above the S\textsubscript{γ}-P bond of the phosphocysteine intermediate. A small difference between the wild-type PTP1B-vanadate complex and the intermediate complex is that the carboxylate group of Asp-181 of the intermediate complex is
a rationale for the reduced rate of hydrolysis of the cysteinyl-phosphate intermediate of the PTP1B Q262A mutant and suggests a plausible function for Gln-262 in positioning the nucleophilic water molecule for attack on the cysteinyl-phosphate intermediate. Model building shows that if W2 were to form hydrogen bonds with Gln-262, as observed for the apical oxygen atom of the PTP1B-vanadate complex, then the water molecule would be ideally positioned for nucleophilic in-line attack on the cysteinyl-phosphate group, being colinear with the P-Sy bond and 3.5 Å from the phosphorus atom of the cysteinyl-phosphate. Thus, loss of Gln-262 in the PTP1B Q262A mutant destabilizes the attacking water molecule site and causes a shift to a position where in-line attack on the cysteinyl-phosphate group is less favorable. An in-line position for W2 would increase its distance to the carboxylate of Asp-181 (4.5 Å) if Asp-181 adopted the same conformation in the native PTP1B-phosphocysteine complex as it does in the mutant PTP1B Q262A phosphocysteine complex. Such as distance would not be favorable for the Asp-181 to act as a general base. However, as noted above, the position of the Asp-181 carboxylate group in the mutant PTP1B Q262A phosphocysteine complex is shifted 1 Å out of the catalytic site compared with its position in the wild-type PTP1B vanadate complex (Fig. 5). A plausible model is that, in the wild-type PTP1B phosphocysteine complex, the position of the Asp-181 carboxylate group is similar to that in the PTP1B-vanadate complex. Such a position would allow hydrogen bonds between W2 occupying an in-line attacking site above the phospho-cysteine and simultaneous hydrogen bonds to the side chains of Asp-181 and Gln-262. It is possible that in the PTP1B Q262A phosphocysteine complex, loss of the Gln-262 residue removes the anchor that stabilizes the position of the attacking water molecule (W2) above the phosphocysteine and in-line with the P–Sy bond. This shift of W2 to a position where it forms a hydrogen bond with one of the phosphocysteine oxygen atoms causes a coupled shift of the position of the Asp-181 side chain.

**Conclusions and Perspectives**—The results that we describe here, together with that of earlier work (1, 4, 5), allow each step of the catalytic reaction of PTPs to be delineated in some detail, outlined in Fig. 6 (A–E). The focus of this investigation has been to understand the mechanism of hydrolysis of the phosphocysteine intermediate. We show that in the intermediate, the WPD loop adopts the closed, catalytically competent conformation. This conformation serves two functions. The first is to bring the side chain of the general base Asp-181 into the catalytic site, and the second is to cap the catalytic site entrance, a role played by the side chain of Phe-182, sequestering the phosphocysteine intermediate and the buried water molecules and preventing phosphoryl-transfer to extraneous phosphoryl acceptors. In contrast, the coordination of a water molecule via a bidentate hydrogen bond to Gln-262 favors specific phosphoryl transfer to a water molecule. The phosphocysteine hydrolysis reaction can be described as follows. A water molecule, coordinated by bifurcated hydrogen bonds to the side chain of Gln-262 and a single hydrogen bond with Asp-181 is situated directly above and in-line with the P–Sy bond of the phosphocysteine. The relative $pK_a$ values of Asp and Gln would suggest that it is most likely that the role of Gln-262 is to position the water molecule correctly, and that of Asp-181 to function as a general base. Abstraction of a proton from the water by Asp-181 increases the nucleophilicity of the water molecule, facilitating attack onto the phosphorus atom of the intermediate. Dixon and colleagues (25) have proposed that the negative charge that develops on the Sy atom of Cys-215 during the phosphoryl-displacement is compensated by a hydrogen bond to the hydroxyl group of the Ser/Thr residue within the

**FIG. 7. Schematic of the reaction mechanism catalyzed by PTP1B.** A, formation of the cysteinyl-phosphate intermediate. B, hydrolysis of the cysteinyl-phosphate intermediate.
PTP motif (Fig. 4). This is consistent with the invariance of a either a Ser or Thr at this position within the PTP family and the finding that substitution to Ala reduces the rate of phosphocysteine hydrolysis 100-fold without affecting the rate of Tyr(P) P–O bond cleavage (25, 29). In our structure, we observe a hydrogen bond between the Sγ atom of the cysteinylin-phosphate residue and OH of Ser-222, supporting this mechanism (Fig. 4). Where displacement reactions are dissociative rather than associative in character, considerable rate enhancement is achieved when the negative charge that develops on the leaving group is compensated. Less important for catalysis is the need to activate the attacking nucleophilic species (19). Thus, the observation that substitution of an Ala for the Ser/Thr residue within the PTP motif affects only the rate of cysteinylin-phosphate hydrolysis and not its rate of formation (25, 29) may be explained by a model where the two reaction steps catalyzed by PTPs are both dissociative in character. A schematic of the reaction mechanism is shown in Fig. 7. Our model for the role of Gln-262 is supported by the findings that substitution of the equivalent residue in the Versinia PTP for Ala reduces the steady state but not the burst phase rate, and moreover, allows phosphoryl-transfer to alcohols (15).

The reactions catalyzed by PTPs share many features in common with that of GTP hydrolysis by the GTPases. For example, the role of Gln-262 in positioning a water molecule for nucleophilic attack onto the phosphocyesteine intermediate of PTP1B is reminiscent of the Gln residue at the catalytic site of most GTPases, including the Ras family and Go subunits of the heterotrimeric G-proteins. Mutation of the catalytic site Gln-61 residue in Ras causes cellular transformation (30) and a 10-fold reduction in the rate of GTP hydrolysis (31, 32). Similarly, mutations of the equivalent Gln residue within the catalytic sites of Goα and Goi reduce the intrinsic rate of GTP hydrolysis and are associated with thyroid and pituitary tumors (33, 34). Crystal structures of complexes of Goα and Goi with GDP, Mg2+, and AlF4− (16, 17) and a recent structure of a Ras-RasGAP, GDP, Mg2+, AlF4− complex (18) have revealed the active site conformations of these enzymes and the mechanisms of GTP hydrolysis. Gln-61 (and its homolog in the heterotrimeric G-proteins) plays a dual role in catalyzing GTP hydrolysis. First, it coordinates the attacking water molecule and positions it optimally for in-line approach onto the GTP γ-phosphate. Second, it forms a hydrogen bond with the γ-phosphate oxygen atom as it passes through the transition state, stabilizing the pentavalent phosphorus transition state. In PTP1B, Gln-262 also coordinates the attacking water molecule to position it for in-line attack, although, unlike GTPase, Gln-262 does not hydrogen-bond to the oxygens of the cysteinylin-phosphate. In addition to a common Gln residue, both PTPs and GTPases utilize an essential Arg residue that coordinates and stabilizes the pentavalent phosphorus intermediate (16–18, 35).

In conclusion, we have succeeded in trapping the PTP1B phosphoryl-enzyme intermediate by introducing a catalytic site mutation to reduce the rate of cysteinylin-phosphate hydrolysis, rendering this step as the rate-limiting step of the PTP catalyzed reaction. In combination with our earlier work (7, 8), this allows us to delineate each step of the reaction pathway.

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