Molecular Basis for Substrate Specificity of Protein-tyrosine Phosphatase 1B*

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Protein-tyrosine phosphatases can exhibit stringent substrate specificity in vivo, although the molecular basis for this is not well understood. The three-dimensional structure of the catalytically inactive protein-tyrosine phosphatase 1B (PTP1B/C215S) complexed with an optimal substrate, DADEpYL-NH₃, reveals specific interactions between amino acid residues in the substrate and PTP1B. The goal of this work is to rigorously evaluate the functional significance of Tyr⁴⁶, Arg⁴⁷, Asp⁴⁸, Phe¹₈², and Gln²₆² in substrate binding and catalysis, using site-directed mutagenesis. Combined with structural information, kinetic analysis of the wild type and mutant PTP1B using p-nitrophenyl phosphate and phosphotyrosine-containing peptides has yielded further insight into PTP1B substrates, which recognize general features, as well as specific properties, in peptide substrates. In addition, the kinetic results suggest roles of these residues in E-P hydrolysis, which are not obvious from the structure of PTP1B/peptide complex. Thus, Tyr⁴⁶ and Asp⁴⁸ recognize common features of peptide substrates and are important for peptide substrate binding and/or E-P formation. Arg⁴⁷ acts as a determinant of substrate specificity and is responsible for the modest preference of PTP1B for acidic residues NH₂-terminal to phosphotyrosine. Phe¹₈² and the invariant Gln²₆² are not only important for substrate binding and/or E-P formation but also important for the E-P hydrolysis step.

Protein-tyrosine phosphorylation is a universal mechanism employed for the regulation of cellular processes such as proliferation, differentiation, motility, cell-cell interactions, metabolism, gene transcription, and the immune response (1, 2). The propagation and termination of signaling events controlling these cellular processes are determined by the level of phosphorylated proteins in a cell. The phosphorylation level, in turn, is maintained in an exquisite balance by the reciprocal activities of protein-tyrosine kinases and phosphatases. Thus, in addition to the study of protein-tyrosine kinases, one can appreciate the need to further characterize the dephosphorylation reaction catalyzed by the protein-tyrosine phosphatases (PTPases).¹

Much is known about the catalytic mechanism of the PTPases (3). However, the molecular basis for PTPase substrate specificity is not well understood and remains a major unresolved issue in the field. The PTPase family is presently composed of approximately 100 enzymes, which can be either transmembrane (receptor-like) or intracellular (cytoplasmic). Membership in this family of enzymes requires the presence of the PTPase signature motif, (H/V)CX₅R(S/T), housed within the catalytic domain. Outside this shared catalytic domain are various targeting and localization domains, which may be utilized for controlling and restricting PTPase substrate specificity. There have been relatively few biochemical analyses of the mechanisms that govern PTPase substrate specificity, although recent genetic and biochemical evidence suggests that in vivo PTPases can exhibit extremely stringent substrate specificity (4–9). Importantly, it seems that, at least for PTP-PEST and the Yersinia PTPase, the catalytic domains alone exhibit very high intrinsic substrate specificity (5, 7). Further understanding of the specific functional roles of PTPases in cellular signaling requires definition of physiological substrates for each individual member of the PTPase family and detailed understanding of structural features that control PTPase substrate specificity.

Using synthetic pTyr-containing peptides that correspond to natural phosphorylation sites in proteins, several groups have demonstrated that PTPases display a range of k₅₀/Kₘ values for these relatively short peptide substrates (10–19). We have shown that the undecapeptide DADEpYLIPQQG, modeled on an autophosphorylation site (Tyr⁶⁹²) of the epidermal growth factor receptor (EGFR) is an optimal peptide substrate for both the Yersinia PTPase and the mammalian PTP1 (13, 14). In fact, the k₅₀/Kₘ values for this peptide approach the diffusional limit and are 3–4 orders of magnitude higher than that of phosphotyrosine alone, suggesting that amino acid residues flanking the pTyr moiety contribute to high affinity binding. Furthermore, the Yersinia PTPase and PTP1 display a preference for acidic residues at positions NH₂-terminal to the phosphorylated tyrosine (14) and require a minimum of six amino acid residues (DADEpYL) for efficient binding and catalysis (17). The recently solved crystal structure of the catalytically inactive PTP1B/C215S complexed with this minimal peptide substrate reveals specific interactions between amino acid residues in the substrate and PTP1B (20). These results suggest that a number of residues in PTP1B are important for peptide substrate recognition and that the sequence surrounding the pTyr residue plays a key role in determining its recognition by PTPases.

¹ The abbreviations used are: PTPase, protein-tyrosine phosphatase; pNPP, p-nitrophenyl phosphate.
The structural observations serve as the basis for further functional studies of PTP1B substrate specificity and catalytic mechanism. In order to clarify the role of primary structure in substrate recognition and to test experimentally the importance of specific amino acid residues in PTP1B for substrate recognition, we have mutated a number of residues in PTP1B depicted in the crystal structure to interact with the peptide substrate (Fig. 1). Furthermore, because PTPase catalysis involves a covalent thiol phosphate intermediate (3), it is not immediately obvious whether residues implicated in substrate binding would have an effect on the hydrolysis of the phosphoenzyme intermediate. The role of these active site amino acids in substrate recognition and processing has been studied by kinetic analyses using several pTyr-containing peptides and p-nitrophenyl phosphate as substrates. In combination with the previously made structural observations, our kinetic data and PTPase amino acid sequence alignment have greatly enhanced our understanding of the function of several conserved PTPase residues that recognize common features in pTyr-containing peptides and of some less conserved residues that may serve as specificity determinants for substrate selectivity.

EXPERIMENTAL PROCEDURES

**Material**—p-Nitrophenyl phosphate (pNPP) was purchased from Fluka Co. Phosphopeptides DADEpYL-NH₂, DADApYLIPQQG, AAAApYLIPQQG were synthesized, purified, and characterized as described (13). Phosphopeptide KRSpYEEHIP was synthesized and purified by AnaSpec Inc. All mutant forms of PTP1B were generated using the Muta-Gene kit from Bio-Rad. The oligonucleotide primers used were as follows: Y46F, 5′-AAATAGGGTCAGACGT-3′; Y46A, 5′-ACCGAAATAGGGTCAGACGT-3′; R47E, 5′-AAATAGGGTCAGACGT-3′; R47A, 5′-AAATAGGGTCAGACGT-3′; D48A, 5′-GGTTACAGGCCCCGTAGCC-3′; D48A, 5′-CATGCGCTGACCGGGCAGCC-3′; and Q262A, 5′-GGCGTCATGCAGGCCCC-3′.

**Site-directed Mutagenesis**—All mutant forms of PTP1B were generated using the Mutagen-Gene in vitro mutagenesis kit from Bio-Rad. The oligonucleotide primers used were as follows: Y46F, 5′-AAATAGGGTCAGACGT-3′; Y46A, 5′-ACCGAAATAGGGTCAGACGT-3′; R47E, 5′-AAATAGGGTCAGACGT-3′; R47A, 5′-AAATAGGGTCAGACGT-3′; D48A, 5′-GGTTACAGGCCCCGTAGCC-3′; D48A, 5′-CATGCGCTGACCGGGCAGCC-3′; and Q262A, 5′-GGCGTCATGCAGGCCCC-3′.
TABLE I

| PTP1B   | $K_i$ for arsenate | $K_i$ for vanadate |
|---------|-------------------|--------------------|
| Wild type | 8.10 ± 5.7        | 1.6 ± 0.17         |
| Y46F    | 138 ± 20          | 2.2 ± 0.52         |
| Y46A    | 150 ± 15          | 4.0 ± 1.3          |
| R47E    | 81.0 ± 6.7        | 0.82 ± 0.11        |
| R47A    | 129 ± 17          | 0.48 ± 0.04        |
| D48A    | 82.0 ± 0.65       | 1.8 ± 0.47         |
| F182A   | 18 ± 1            | 3.4 ± 0.43         |
| Q262A   | 73 ± 8            | 6.8 ± 1.0          |

PTP1B Substrate Specificity

Arsenate and vanadate inhibition constants for the wild type and the mutant PTP1B phosphatases at pH 7 and 30 °C

All measurements were made using pNPP as a substrate.

| PTP1B | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|-------|-----------|-------|--------------|
| Wild type | 17.3 ± 0.9 | 2.2 ± 0.4 | 7.9 ± 1.5 |
| Y46F | 10.2 ± 0.4 | 2.6 ± 0.3 | 3.9 ± 0.38 |
| Y46A | 2.4 ± 0.12 | 20.5 ± 2.7 | 0.12 ± 0.017 |
| R47E | 22 ± 0.6 | 2.7 ± 0.18 | 8.1 ± 0.58 |
| R47A | 17.0 ± 0.99 | 1.8 ± 0.27 | 9.4 ± 1.5 |
| D48A | 14.5 ± 0.3 | 1.3 ± 0.07 | 11.2 ± 0.64 |
| F182A | 1.1 ± 0.06 | 1.2 ± 0.07 | 0.92 ± 0.07 |
| Q262A | 0.21 ± 0.01 | 0.058 ± 0.006 | 3.6 ± 0.3 |

TABLE II

Kinetic parameters of the wild type and mutant PTP1B phosphatases using pNPP as a substrate at pH 7 and 30 °C

The E-P formation step accompanied by release of phenol or Tyr-containing peptide. For pNPP, $k_{cat}/K_m$ is primarily limited by the chemical step (i.e. E-P formation) (27). The $k_{cat}/K_m$ for phosphopeptides may be greatly influenced by substrate binding, as they are 3–4 orders of magnitude more efficient substrates than aryl phosphates (17). The $k_{cat}$ term describes the rate-limiting step under saturating concentrations of substrate and is mostly determined by E-P hydrolysis step (17, 28).

PTyr$^{46}$—The side chain of Tyr$^{46}$ is engaged in interactions with the main chain atoms and the aromatic ring of pTyr in peptide substrates (Fig. 1). A hydrogen bond is also observed between the OH group of Tyr$^{46}$ and the side chain of Ser$^{216}$, which may be required for the stabilization of the conformation of Tyr$^{46}$ and its interaction with the substrate (20). To probe the role of the OH group in Tyr$^{46}$, it was mutated to Phe, which eliminates the OH group while retaining the aromatic ring. Using pNPP as a substrate, the $k_{cat}$ for Y46F was slightly lower than that of the wild type, whereas the $K_m$ value was similar (Table II). Using the EGF receptor peptide DADEpYLIPQQG (EGFR988–998) as a substrate, the $k_{cat}$ value for Y46F was only 2-fold lower than that of PTP1B, whereas the $K_m$ was 2-fold higher (Table III). It appears that the OH group of Tyr$^{46}$ may not be essential for activity, and a Phe residue can adequately substitute for Tyr at position 46. Indeed, in the Yersinia PTPase (Yop51), which is the most active PTPase, and in human PTPDI (Fig. 3), a Phe residue, instead of a Tyr, occupies the corresponding position. It is possible that the OH group on Tyr$^{46}$ may be required for PTP1B protein stability, because the recombinant Y46F protein was degraded in E. coli during normal overnight culture after isopropyl-1-thio-β-D-galactopyranoside induction (data not shown). In order to prepare sufficient amount of Y46F protein and minimize protein degradation, a shorter incubation time was employed (see under “Experimental Procedures”).

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The lack of substitutional effect on $k_{cat}$ (which follows E-P hydrolysis) at residue 47 is understandable because the dephosphorylated tyrosyl peptide has already left the active site during E-P hydrolysis and Arg$^{47}$ is positioned away from the site where E-P hydrolysis takes place. It is worth noting that the effect of charge reversal (Arg to Glu mutation) at residue 47 is more severe than simple removal of the positive charge (Arg to Ala substitution). The fact that R47E displayed a greater loss in $k_{cat}/K_m$ than R47A may be explained by charge repulsion between the Glu residue at position 47 and the acidic residues in the substrate. This is consistent with the notion that Arg$^{47}$ specifically interacts with acidic residues in the substrate.

To further probe the role of Arg$^{47}$ in substrate recognition, we also analyzed the kinetics of PTP1B-catalyzed hydrolysis of Ala-substituted EGFR$^{988-998}$ peptides (Table IV). Wild type PTP1B suffered a 4-fold and 2-fold decrease in $k_{cat}/K_m$ when the acidic residue at the −1 or −2 position, respectively, was changed to Ala. A 17-fold decrease in $k_{cat}/K_m$ was observed for the wild type PTP1B when all four NH$_2$-terminal residues were Ala residues. These results are consistent with the crystal structure that depicts Arg$^{47}$ interacting with both the −2 and −1 acidic residues of the substrate. The kinetic data also suggest that the interaction between Arg$^{47}$ and the −1 residue is stronger than that between Arg$^{47}$ and the −2 residue. Interestingly, the effect of the Arg$^{47}$ to Glu mutation on $k_{cat}/K_m$ was mitigated as the overall negative charge NH$_2$-terminal to pTyr in the substrate was reduced or removed with the substitution of Ala at the −1, −2 or all three NH$_2$-terminal acidic residues of the EGFR$^{988-998}$ peptide (Table IV). Moreover, R47A exhibited little preference for the native and Ala-substituted EGFR$^{988-998}$ peptides. These results suggest that Arg$^{47}$ is critical for promoting selection of negatively charged residues at positions amino-terminal to pTyr. Finally, when KRSpYEEHIP (modeled after the phosphorylation site Tyr$^{1316}$ in insulin receptor) was used as a substrate, a reversal of substrate specificity was observed for PTP1B, R47A, and R47E (Table IV). Thus, KRSpYEEHIP was processed by R47E 5- and 2-fold more efficiently than the wild type PTP1B and R47A, respectively. Based on the results described above, one would predict a more pronounced reversal of specificity if a peptide with a basic residue at the −1 position were used as a substrate. Collectively, the observed effects of mutations at Arg$^{47}$ in PTP1B or substitutions of −1 and −2 acidic residues by Ala in peptide substrates on $k_{cat}/K_m$ are consistent with the disruption of the observed hydrogen-bonds between Arg$^{47}$ and the side chains at the −1 and −2 positions seen in the crystal structure of PTP1B and the DDAEpYL-NH$_2$ peptide. These results provide strong evidence that amino acid at position 47 is an important substrate specificity determinant for PTP1B that recognizes residues at the −1 and −2 positions of pTyr-containing peptides. However, it is important to point out that the contribution from Arg$^{47}$ in determining the in vivo substrate specificity is unknown. Unlike PTP-PEST, which exhibits stringent substrate specificity (5), the substrate specificity of PTP1B is more relaxed in vivo when its COOH-terminal localization domain is removed (5, 9).

Asp$^{48}$—The pTyr residue in the peptide substrate adopts a right-handed α-helical conformation in the PTP1B-bound state. It is proposed, based on the crystal structure, that the primary determinant of the peptide conformation at the pTyr site is Asp$^{48}$ because it forms two hydrogen bonds with the main chain nitrogens of pTyr and the +1 residue (Fig. 1). Thus, substitution at Asp$^{48}$ is not expected to have any adverse effects on the hydrolysis of pNPP, which lacks the corresponding main chain nitrogens. Indeed, D48A exhibited $k_{cat}$ and $K_m$ values similar to those of the wild type PTP1B with pNPP as a substrate.

### Table III

| PTP1B | $k_{cat}$ (s$^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ |
|-------|------------------|-------------|---------------|
| Wild type | 44.6 ± 1.8 | 3.9 ± 0.9 | 11.1 ± 0.25 |
| Y46F | 20.4 ± 1.0 | 6.7 ± 0.26 | 3.0 ± 0.015 |
| Y46A | 21.0 ± 2.7 | 1500 ± 230 | 0.0014 ± 0.0003 |
| R47E | 51.1 ± 0.2 | 40 ± 0.5 | 1.3 ± 0.002 |
| R47A | 75.5 ± 0.39 | 10 ± 0.79 | 0.58 ± 0.04 |
| D48A | 30.2 ± 2.0 | 59 ± 10 | 0.051 ± 0.0092 |
| F182A | 2.4 ± 0.06 | 2.8 ± 0.45 | 0.086 ± 0.014 |
| Q262A | 0.29 ± 0.002 | 35.3 ± 0.05 | 0.003 ± 0.011 |
### Table IV

| Kinetic constants of PTP1B and the Arg 47 mutants with phosphopeptides as substrates at 30 °C, pH 7.0 |
|---------------------------------------------|
| Substrate                      | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|----------------------------------|-------|-----------|---------------|
| DDEEYIPQQG                      | 0.28  | 57.5      | 0.001         |
| DADDEYIPQQG                     | 0.11  | 46.5      | 0.0001        |
| DADAYLIPQQQ                     | 0.22  | 4.5       | 0.0001        |
| DADAEYIPQQQ                     | 0.32  | 3.4       | 0.0001        |

PTP1B Substrate Specificity

E-P formation (Scheme 1) by bringing Asp181 into proximity for substrate complex, are required for substrate binding and/or of pTyr, as observed in the crystal structure of PTP1B/which should be catalytically competent.

Kinetic results indicate that Phe182 also plays an important role in the hydrolysis of the phosphoenzyme intermediate. These kinetic results are consistent with the structural data and suggest that Asp48 plays an important role in positioning the peptide substrates in an optimal conformation for peptide binding and/or the initial nucleophilic attack by the active site Cys residue.

$Phe^{182}$—Phe$^{182}$ resides in a flexible surface loop (the WPD loop) that harbors the general acid/base Asp$^{181}$ (20, 29–32). In the unliganded PTP1B and Yersinia PTPase structures, the WPD loop was observed in an open conformation such that Asp$^{181}$ (Asp$^{356}$ in Yersinia PTPase) is greater than 10 Å away from the phosphate binding site (30, 33). However, upon substrate binding, the WPD loop adopts the closed conformation that covers the active site like a flap, which places the general acid/base Asp$^{181}$ carboxylate close to the phenolic oxygen of pTyr (Fig. 1) (20). This movement shifts the side chain of Phe$^{182}$ toward the pTyr binding site, allowing the formation of hydrophobic stacking interactions with the phenyl ring of the pTyr residue of the substrate. In the Yersinia PTPase, the WPD loop adopts a closed conformation in the presence of oxyanions (30, 31). In contrast, WPD loop closure is not observed in PTP1B when tungstate is bound (33). The interaction between pTyr and Phe$^{182}$ is therefore proposed to be crucial for the formation of the WPD loop closed PTP1B conformation (20), which should be catalytically competent.

The $K_m$ values for the F182A-catalyzed reactions were not significantly different from those of the native enzyme (Tables II and III). In contrast, the $k_{cat}/K_m$ for the F182A-catalyzed pNPP and EGFR$^{988-998}$ peptide was 9- and 13-fold lower than those of the wild type enzyme. These results suggest that the hydrophobic interactions between Phe$^{182}$ and the phenyl ring of pTyr, as observed in the crystal structure of the PTP1B/substrate complex, are required for substrate binding and/or E-P formation (Scheme 1) by bringing Asp$^{181}$ into proximity for proton donation to the leaving group phenolic oxygen. Interestingly, the $k_{cat}$ for the F182A catalyzed pNPP and peptide substrate hydrolysis was also decreased by 16- and 19-fold, respectively, as compared with the wild type PTP1B. These are somewhat surprising results because the tyrosyl part of the substrate, which interacts with the side chain of Phe$^{182}$, has already left the active site during the hydrolysis of E-P. Our kinetic results indicate that Phe$^{182}$ also plays an important role in the hydrolysis of E-P. Because Asp$^{181}$ functions as a general base to activate the nucleophilic water for E-P hydrolysis, its precise location in the active site is crucial to the dephosphorylation activity. It is possible that alteration of Phe$^{182}$ structure affects the conformation of the WPD loop and therefore the exact positioning of Asp$^{181}$. Interestingly, although Phe$^{182}$ plays a key role in both the E-P formation and E-P hydrolysis, it is not well conserved within the PTPase family (29). For example, the structurally equivalent residue is Gln in the Yersinia PTPase, Phe in PTP1B, His in LAR, and Met in yeast.
PTP1. The catalytic efficiency exhibited by these phosphatases is *Yersinia* PTPase > PTP1B > LAR > yeast PTP1 (34). Thus, the residue immediately following the catalytic Asp in the WPD loop may be important for the attainment of the catalytic competent active site geometry. Variations in the structure of the WPD loop may modulate the dynamic and catalytic properties of the PTPases (20, 31, 32). Further study on the effect of loop dynamics on catalytic efficiency for PTPases should clarify the exact functional role of the WPD loop residues.

Gln262—The role of Gln262 in PTP1B catalysis was also investigated, because the side chain of Gln262 interacts with the phenyl ring of pTyr and defines a portion of the rim for the pTyr binding pocket shown in the structure of PTP1B/C215S-substrate complex (Fig. 1). Previously, it was shown that the $k_{cat}/K_m$ and $k_{cat}$ for the PTP1B/Q262A catalyzed hydrolysis of phosphorylated lyszyme were reduced by 7- and 83-fold, respectively (9). Similarly, Q262A exhibited 2- and 13-fold decrease in $k_{cat}/K_m$ for pNPP and the EGFR988–998 peptide, respectively (Tables II and III), suggesting that Gln262 may help to align the phenyl ring of pTyr in the E-P formation step. In addition, the $k_{cat}$ for the Q262A catalyzed hydrolysis of pNPP and EGFR988–998 peptide was reduced 80–150-fold, suggesting that Gln262 may also participate in the E-P hydrolysis step. Work on the structural equivalent Gln446 in the *Yersinia* PTPase (35) and Gln262 in PTP1B (36) suggests that the invariant glutamine residue is important for the optimal positioning of the nucleophilic water molecule for efficient E-P hydrolysis. These observations are consistent with the dramatic decrease in $K_m$ for the Q262A mutant (Tables II and III and Ref. 9), which is indicative of increased accumulation of the E-P intermediate in the Q262A-catalyzed reactions.

In summary, results from kinetic studies of the wild type PTP1B and site-directed mutants of Tyr64, Arg57, Asp38, Phe182, and Gln262 are consistent with conclusions reached from the crystal structure of PTP1B/C215S bound with DADEpYL-NH2 (20) regarding the role of these residues in substrate binding and/or the E-P formation step. In addition, the kinetic results reveal functional significance of these residues in E-P hydrolysis, which is not obvious from the structure of PTP1B/substrate complex. Thus, Tyr64 forms part of the pTyr binding pocket, and Asp38 helps to define the main chain peptide conformation at the pTyr site. Both residues recognize the pTyr binding pocket, and Asp48 helps to define the main chain conformation at the pTyr site. Both residues recognize the phospho moity of the substrate and therefore have little influence on the E-P hydrolysis step. Tyr64 (or Phe) and Asp38 (or Asn) are highly conserved throughout the PTPase family (Fig. 1), suggesting that results described in this paper should be applicable to most PTPases. However, there are exceptions, most notably the cytoplasmic EC-PTP and LC-PTP, and the receptor-like PTPases, PTPβ, IA-2, and IA-2β. It is not clear whether these proteins are active tyrosine phosphatases, because there are also sequence deviations in the WPD loop region of PTPβ, IA-2, and IA-2β. Arg57 acts as a determinant for substrate specificity and is responsible for the modest preference of PTP1B for acidic residues NH$_2$-terminal to pTyr. There are more sequence variations at Arg57 (Fig. 2), suggesting that PTPases with different residues at this position may show different sequence selectivity. The interaction between the benzene ring of Phe182 and pTyr in the substrate may be important for positioning the general acid Asp81 in the WPD loop for effective protonation of the tyrosine phenolate leaving group. Surprisingly, our data indicate that Phe182 also plays a role in the E-P hydrolysis, possibly by maintaining the general base Asp81 and the WPD loop in a catalytically competent form. It is possible that residues in the WPD loop may play a role in controlling the activity of PTPases. Finally, the invariant Gln262 is not only involved for peptide substrate binding and/or E-P formation, but also in E-P hydrolysis. Evidence suggests that Gln262 is important for maintaining the strictly hydrolytic activity in PTPases (35, 36).

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