Residue 259 Is a Key Determinant of Substrate Specificity of Protein-tyrosine Phosphatases 1B and α*

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The aim of this study was to define the structural elements that determine the differences in substrate recognition capacity of two protein-tyrosine phosphatases (PTPs), PTP1B and PTPα, both suggested to be negative regulators of insulin signaling. Since the Ac-DADE(pY)L-NH₂ peptide is well recognized by PTP1B, but less efficiently by PTPα, it was chosen as a tool for these analyses. Co-regiovariation analyses and primary sequence alignments indicate that residues 47, 48, 258, and 259 (PTP1B numbering) define a selectivity-determining region. By analyzing a set of DADE(pY)L analogs with a series of PTP mutants in which these four residues were exchanged between PTP1B and PTPα, either in combination or alone, we here demonstrate that the key selectivity-determining residue is 259. In PTPα, this residue is a glutamine causing steric hindrance and in PTP1B a glycine allowing broad substrate recognition. Significantly, replacing Gln²⁵⁹ with a glycine almost turns PTPα into a PTP1B-like enzyme. By using a novel set of PTP inhibitors and x-ray crystallography, we further provide evidence that Gln²⁵⁹ in PTPα plays a dual role leading to restricted substrate recognition (directly via steric hindrance) and reduced catalytic activity (indirectly via Gln²⁵⁹). Both effects may indicate that PTPα regulates highly selective signal transduction processes.

Protein-tyrosine phosphatases (PTPs)¹ and protein-tyrosine kinases control the phosphorytrosine (Tyr(P)) levels of cellular proteins and are thus key regulators of signal transduction (reviewed in Refs. 1 and 2). PTPs are a diverse family of intracellular and receptor-type enzymes that are characterized by one or two structurally conserved catalytic domain(s) of about 250 amino acid residues. The catalytic domain in intracellular PTPs is often associated with proximal or distal sequence homology containing regulatory elements such as SH2 and FERM domains (3) directing protein-protein interactions, subcellular localization, or enzyme stability. Most receptor-type PTPs contain two catalytic domains in their intracellular region and, in addition, have a single transmembrane region and an extracellular domain. The diversity of the extracellular domains suggests that these PTPs are regulated by specific extracellular ligands.

The intricate regulation of signal transduction processes into temporarily and spatially ordered pathways requires recruitment of regulatory molecules, including PTPs, into macromolecular assemblies (reviewed in Ref. 4). Thus, subcellular localization is considered to play a significant role in defining the substrates that are dephosphorylated by specific PTPs (5). However, there is increasing evidence that substrate specificity is also conveyed directly by the catalytic domains of PTPs. Thus, several studies with synthetic tyrosine-phosphorylated substrates (6–9) or peptides containing nonhydrolyzable phosphotyrosine analogs (10) have shown significant preferences for specific residues adjacent to the phosphorylated tyrosine or Tyr(P) mimetic. One of the most convincing demonstrations of the functional importance of direct substrate recognition by PTPs came from recent studies of the highly homologous PTPs, SHP-1 and SHP-2. By using chimeric constructs of these two PTPs, Böhmer and co-workers (11) demonstrated that the differential interaction of SHP-1 and SHP-2 with the epidermal growth factor receptor is due to the specificity of the catalytic domains rather than the SH2 domains. In a detailed study of the influence of SHP-1 and SHP-2 on Xenopus oocyte maturation, O'Reilly and Neel (12) showed that the most important substrate recognition elements reside in the catalytic domain. Furthermore, in a functional cell-based rescue assay, we found PTPα and PTPβ to be the most efficient negative regulators of insulin signaling, whereas other receptor-type PTPs had little or no influence (13, 14). Finally, studies with so-called substrate-trapping mutants show selective substrate recognition that seems to be mediated by the catalytic domains (15, 16).

PTPs are generally considered potentially important therapeutic targets due to their pivotal roles as control elements in signal transduction pathways (17). As an example, several candidate PTPs have been proposed as negative regulators of the insulin receptor signaling pathway, including PTP1B and PTPα (reviewed in Ref. 18). Thus, selective inhibitors of these enzymes could potentially be useful in the treatment of diabetes. Recently, analysis of PTP1B knock-out mice (19) and treatment of diabetic ob/ob mice with selective PTP1B inhibitors (20) provided support for the view that PTP1B is a major regulator of insulin signaling. By using a structure-based design, we have recently been able to make selective, low molecular weight non-phosphorus PTP1B inhibitors. A basic nitrogen in the inhibitor forms a salt bridge with the selectivity-determining residue Asp⁴⁸ in PTP1B. In other PTPs with an asparagine in the equivalent position, this basic nitrogen

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² The abbreviations used are: PTP, protein-tyrosine phosphatase; p-NPP, para-nitrophenyl phosphate; pY, phosphotyrosine; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
causes repulsion. The net effect is a remarkable selectivity for PTP1B (21). Furthermore, replacing the basic nitrogen in the inhibitor structure with an oxygen atom increased the potency of the inhibitor for all PTPs with an asparagine in position 48.

In the present study, we decided to get further insight into the elements that determine the substrate specificity of different PTPs and, hence, by inference also information that can be used in structure-based design of selective inhibitors. In particular, we wanted to study the difference in substrate recognition of PTP1B and PTPα. Tyrosine-phosphorylated synthetic peptides have been used extensively to analyze the substrate specificity of PTPs. The peptide Ac-DADE(pY)L-NH₂, which is derived from the epidermal growth factor receptor, has been particularly useful. This peptide is well recognized by PTP1B but less efficiently by PTPα. Based on Cα regiovariation analyses and primary sequence alignments, we have previously identified regions in close proximity to the catalytic cleft that are likely to confer specificity onto PTPs. The combination of residues 47, 48, 258, and 259 (PTP1B numbering) might be a specificity-determining region. PTP1B and PTPα mutants, in which these four residues are exchanged either in combination or alone, were used to analyze the substrate specificity against Ac-DADE(pY)L-NH₂ peptide and a set of analogs. Residue 259, which is a glutamine in PTPα and a glycine in PTP1B, was found to be a major determinant of substrate recognition capacity and hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phosphopeptides were purchased from Neosystem (Strasbourg, France). The purity of the peptides was greater than 97%. Water was purified in a Millipore purification system (18 megohm-cm; Millipore Inc.). Glutathione-Sepharose, Sephadex G-25, Q-Sepharose Fast Flow, Mono Q, and Superdex 200 were from Amersham Pharmacia Biotech.

**Phosphate Reagent**—One volume of 10% (w/v) ammonium molybdate was mixed with 3 volumes of 0.2% (w/v) peptone in 4 × HCl. After stirring for 30 min at room temperature, the solution was filtered through a 0.22-μm Millipore filter (Milllex-GV). The solution was stored at 4 °C in a foil-wrapped container. Before usage, the solution was stirred for 30 min and filtered through a 0.22-μm Millipore filter.

**Buffers**—All enzyme assays with synthetic peptide substrates were performed at a constant ionic strength of 100 mM using a three-component buffer system consisting of 50 mM Tris, 50 mM Bis-Tris, and 100 mM sodium acetate or magnesium acetate, 50 mM sodium chloride, 0.1% (w/v) human serum albumin, 5 mM 2-mercaptoethanol, and 1 mM EDTA, pH 5.5. The reaction was initiated by addition of the enzyme and carried out in microtiter plates at 25 °C for 20 min. The reactions were stopped by addition of 1 N NaOH.

**Cloning, Expression, and Purification of Recombinant Proteins**—Cloning, expression, and purification of the catalytic domains of PTP1B (26) and PTPα domains 1 (27) were done as described previously (28). The following PTP mutants were made by overlap extension polymerase chain reaction using appropriate restriction sites for cloning purposes (29): PTP1B to PTPα, (i) R47V, D48N, C258M, and G259Q; (ii) R47V and D48N; (iii) C258M and G259Q; and (iv) G259Q. PTPα to PTP1B (PTP1B numbering): (v) V47R, N48D, M258C, and G259Q; (vi) M258C and G259Q; and (vii) Q259G. All constructs were inserted into pET11a expression vector. All coding sequences were inserted in the pET11a expression vector. All coding sequences were inserted into at least four independent experiments using the nonbiased method.

**Determination of Inhibitor Constants, Kₐ**—The enzyme reactions were carried out using standard conditions essentially as described by Burke et al. (31). The assay conditions were as follows. Diluted inhibitors (6 different concentrations, 2-fold dilution) were added to the reaction mixtures containing different concentrations of the substrate, p-nitrophenyl phosphate (p-NPP) (usual range, 0.16–10 mM final assay concentration). The buffer used (total volume, 100 μl) consisted of 100 mM sodium acetate, 50 mM sodium chloride, 0.1% (w/v) human serum albumin, 5 mM 2-mercaptoethanol, and 1 mM EDTA, pH 5.5. The reaction was initiated by addition of the enzyme and carried out in microtiter plates at 25 °C for 20 min. The reactions were stopped by addition of 1 N NaOH. The enzyme activity was determined by measuring the absorbance at 405 nm with appropriate corrections for absorbance of the compounds and p-NPP. The data were analyzed using nonlinear regression hyperbolic fit to classical Michaelis-Menten enzyme kinetic models. Inhibition is expressed as Kᵢ, values in μM.

**Co-crystallization of PTP1B with Compound 1**—A 10 mg/ml preparation of PTP1B (PTP1B, R47V, D48N, C258M, G259Q) in 10 mM Tris, pH 7.5, 25 mM NaCl, 0.2 mM EDTA, and 3 mM dithiothreitol, was used for crystallization. Crystals were grown by the sitting drop vapor diffusion method. A 1:10 (PTP1B, R47V, D48N, C258M, G259Q) compound 1 molar ratio mixture was prepared at least 1 h prior to crystallization. Two μl of PTP1B (PTP1B, R47V, D48N, C258M, G259Q)/compound 1 solution were mixed with 2 μl of reservoir solution consisting of 0.1 M Hepes buffer, pH 7.5, 0.3–0.4 M sodium acetate or magnesium acetate, 12–16% polyethylene glycol 8000. The reservoir volume was 1 ml. Crystals grew to the size of 0.5 × 0.7 × 0.07 mm within 3 days.

**X-ray Data Collection**—Data collection was performed at 100 K. The following X-ray conditions were used: to the sitting drop 3 μl of 50% glycerol (containing 3 μM of (6) Kᵢ of inhibitor) were added. The crystal was removed from the drop after 20 min and transferred to 50% glycerol (containing 0.5 mM of inhibitor) for 5 to 10 s and flash-frozen. Data were collected using a mar345 image plate detector at the 711 beam line at the MAX-lab synchrotron facilities at Lund University (Sweden). A 1° oscillation was used for 60 images. A full data set to 2.1 Å resolution was obtained. The space group was determined to be P3121. Data processing was performed using Denzo, Scalepack, and the CCP4 program suite (32, 33). For further details see Table I.

**Structure Refinements**—As P3121 contains a polar axis and, thus, possesses more than one indexing possibility, a molecular replacement solution using Amore (33, 34) was found prior to the refinements. A high resolution PTP1B structure was used as a starting model (28), where ligand and water molecules were omitted from the structure. Furthermore, alanine substitutions in the mutated positions were performed. All refinements were performed with Xplor. version 3.85 (Molecular Simulations Inc.). Interchanging cycles of model building using X-build (Molecular Simulations Inc.) and refinement were performed. The alamines for the mutated residues were substituted with the correct side chains and build into the 2Fᵢ – Fᵢ maps. The 2Fᵢ – Fᵢ maps were inspected by the use of X-ligand (Molecular Simulations Inc.) at a 1.3σ level for densities that could correspond to the structure of the inhibitor. A well suited inhibitor electron density was identified in the active site pocket, see Fig. 6 below. No other densities were identified to fit the inhibitor. Water molecules were inserted using the X-solvate program (Molecular Simulations Inc.). For further details see Table I.

**Compound Synthesis**—2-Oxacyclo-aminophen 4,7-dihydroythieno[2,3-c]thiophene-3-carboxylic acid tet-butyl ester was synthesized in the following way. Tetrahydro-thiopyran-4-one was treated under conditions described by Gewald et al. (35) for synthesis of 2-aminothiophenes which afforded 2-amino-4,7-dihydro-5H-thieno[2,3-c]thiophene-3-carboxylic acid tert-butyl ester. This ester was condensed with imidazol-1-yl-oxo-acetic acid tert-butyl ester in dry tetrahydrofuran affinity 2-(tert-butoxycarbonyl)-amino, 4,7-dihydro-5H-thieno[2,3-c]thiophen-
random 5% of the observations omitted from the refinement process.

synthetic tyrosine-phosphorylated substrates, PTP inhibitors. Whereas PTP1B seems to recognize a broad variety of studies, we found that PTP1B dephosphorylates this peptide. In the present context it is of significance that this peptide is used extensively to study substrate recognition by PTPs (36).

The Ac-DADE(pY)L-NH2 peptide can be used to discriminate between PTP1B and PTPα—The synthetic peptide Ac-DADE(pY)L-NH2 and analogs thereof have previously been used extensively to study substrate recognition by PTPs (36). In the present context it is of significance that this peptide is recognized well by PTP1B but not by PTPα (6, 7, 37) and that the binding mode of this peptide in PTP1B has been elucidated by x-ray crystallography (38). Therefore, the Ac-DADE(pY)L-NH2 peptide offers a unique possibility for identifying key structural elements that determine the differences in substrate recognition by PTP1B and PTPα. In accordance with previous studies, we found that PTP1B dephosphorylates this peptide about 30 times more efficiently than PTPα (Table II).

Residues 258 and 259—The published x-ray structure of PTP1B co-crystallized with the DADE(pY)L-NH2 peptide shows that the leucine residue (pY+1) is located on a hydrophobic region of the protein surface forming van der Waals contacts with the side chains of Val49, Ile219, and Gln262. In addition, a water-mediated hydrogen bond was observed between the pY+1 amide group and the side chain of Gln262. Since these residues are positioned adjacent to Gly259 and Met258, we reasoned that in particular introduction of the corresponding residues from PTPα into PTP1B could influence/impair the interaction of the pY+1 residue with PTP1B. Indeed, Cys258 and Gln259 seemed to be responsible for the limited catalytic activity against the Ac-DADE(pY)L-NH2 peptide of the PTP1B(M258C,G259Q) mutant and possibly PTPα (Table III). It should be noted that previous studies with murine PTPα have shown that the kcat/Km value for DADE(pY)-NH2 is about 3-fold higher than for DADE(pY)L-NH2, thus indicating a negative influence of the pY+1 residue (36). Therefore, to study in closer detail the influence of the pY+1 position on substrate recognition by PTP1B, we compared a series of Ac-DADE(pY)L-NH2 analogs as shown in Fig. 5. Although some differences are observed, the wild type enzyme in general seemed to be influenced very little by the actual pY+1 residue, probably indicating that the contribution of this residue to the overall binding affinity is mainly due to the above water-mediated hydrogen bond to Gln262. By introduction of residues 258–259 from PTPα into PTP1B, a significant increase in Km was observed, whereas the introduction of residues 258–259 reduced the overall catalytic efficiency about 4-fold. In comparison to the wild type enzyme, the lower catalytic efficiency for PTP1B(R47V,D48N) is due to the 1.4-fold reduction in the turnover number (Table III), whereas Km is apparently not affected by these mutations. In contrast, introduction of a cysteine (residue 258) and a glutamine (residue 259) resulted in an almost 2-fold increase in Km value. Thus, when comparing the PTP1B(R47V,D48N) and PTP1B(M258C,G259Q) mutants, it seems that the region comprising residues 258–259 is the most important determinant for the catalytic efficiencies of these enzymes toward the DADE(pY)L peptide. Hence, we decided to focus our attention on residues 258 and 259.

| Substrate Specificity of PTP1B and PTPα |
|----------------------------------------|
| **TABLE I**                             |
| Statistics of x-ray data and structure refinements |
| Space group | P3121 |
| a = b | 88.2 | c = 103.7 Å |
| Completeness (20–2.13 Å) | 100% |
| Completeness (2.17–2.13 Å) | 100% |
| Multiplicity (20–2.13 Å) | 5.3 |
| Rmerge (20–2.13 Å) | 7.3% |
| Rmerge (1.7–2.13 Å) | 8.3% |
| <I/Io|> (20–2.13 Å) | 14.7 |
| <I/Io|> (1.7–2.13 Å) | 3.1 |
| Uniques reflections | 25,558 |
| Atoms in structure | 2,644 |
| R-factora | 19.1% |
| Rfreea | 25.0% |
| r.m.s. deviations from idealized geometry |
| Bond lengths (Å) | 0.019 |
| Bond angles (°) | 2.97 |
| Dihedral angles (°) | 3.72 |

a R-factors were calculated using all data from 6 to 2.13 Å. Crystallographic R-factor = [Σ(|Fo| - |Fc|) / Σ|Fo|]1/2. Rfree = [Σ(|Fo| - |Fc|) / Σ|Fo|]1/2 where T is a test set containing a random 5% of the observations omitted from the refinement process.

RESULTS

The goal of this study was to identify structural elements that are critically involved in determining the substrate specificity of PTPs. We have chosen to describe at the molecular level the difference in substrate recognition by two widely expressed PTPs, PTP1B and PTPα. Both enzymes have been implicated as negative regulators of the insulin receptor tyrosine kinase, and hence they are potential therapeutic targets in type 2 diabetes. An understanding of the difference in substrate recognition should assist in designing selective PTP inhibitors. Whereas PTP1B seems to recognize a broad variety of synthetic tyrosine-phosphorylated substrates, PTPα shows limited recognition capability.

The Ac-DADE(pY)L-NH2 peptide can be used to discriminate between PTP1B and PTPα. The synthetic peptide Ac-DADE(pY)L-NH2 and analogs thereof have previously been used extensively to study substrate recognition by PTPs (36). In the present context it is of significance that this peptide is recognized well by PTP1B but not by PTPα (6, 7, 37) and that the binding mode of this peptide in PTP1B has been elucidated by x-ray crystallography (38). Therefore, the Ac-DADE(pY)L-NH2 peptide offers a unique possibility for identifying key structural elements that determine the differences in substrate recognition by PTP1B and PTPα. In accordance with previous studies, we found that PTP1B dephosphorylates this peptide about 30 times more efficiently than PTPα (Table II).

Residues 47, 48, 258, and 259 define an important substrate recognition site—By using primary sequence alignments and Cα regiovariation-score analyses, we have previously identified residues 47, 48, 258, and 259 (PTP1B numbering) as a potential selectivity determining area of PTPs. To get initial insight into the significance of this region for substrate recognition, we first made a PTP1B mutant in which these four amino acid residues were substituted for the corresponding residues in PTPα (i.e. Arg-Asp-Met-Gly to Val-Asn-Cys-Gln). Table II shows that introduction of the putative selectivity-determining residues from PTPα into PTP1B caused a remarkable decrease in activity against the Ac-DADE(pY)L-NH2 peptide. Importantly, x-ray crystallography of this mutant shows that the overall folding and structure is similar to that of the wild type enzyme (see below). Thus, the observed differences in Km and kcat values are due to the introduction of the four “PTPα residues” into PTP1B thereby providing significant support for the view that these four residues are critically involved in the substrate recognition capacity and hydrolysis.

Residues 258 and 259 are the major determinants for selectivity—To dissect further the role of the individual amino acid residues in the putative selectivity-determining region, we next introduced either residues 47–48 or 258–259 from PTPα into PTP1B. Table III shows that the replacement of residues 47–48 resulted in a 1.5-fold reduction in catalytic efficiency (kcat/Km), whereas the introduction of residues 258–259 reduced the overall catalytic efficiency about 4-fold. In comparison to the wild type enzyme, the lower catalytic efficiency for PTP1B(R47V,D48N) is due to the 1.4-fold reduction in the turnover number (Table III), whereas Km is apparently not affected by these mutations. In contrast, introduction of a cysteine (residue 258) and a glutamine (residue 259) resulted in an almost 2-fold increase in Km value. Thus, when comparing the PTP1B(R47V,D48N) and PTP1B(M258C,G259Q) mutants, it seems that the region comprising residues 258–259 is the most important determinant for the catalytic efficiencies of these enzymes toward the DADE(pY)L peptide. Hence, we decided to focus our attention on residues 258 and 259.

Residues 258–259—The published x-ray structure of PTP1B co-crystallized with the DADE(pY)L-NH2 peptide shows that the leucine residue (pY+1) is located on a hydrophobic region of the protein surface forming van der Waals contacts with the side chains of Val49, Ile219, and Gln262. In addition, a water-mediated hydrogen bond was observed between the pY+1 amide group and the side chain of Gln262. Since these residues are positioned adjacent to Gly259 and Met258, we reasoned that in particular introduction of the corresponding residues from PTPα into PTP1B could influence/impair the interaction of the pY+1 residue with PTP1B. Indeed, Cys258 and Gln259 seemed to be responsible for the limited catalytic activity against the Ac-DADE(pY)L-NH2 peptide of the PTP1B(M258C,G259Q) mutant and possibly PTPα (Table III). It should be noted that previous studies with murine PTPα have shown that the kcat/Km value for DADE(pY)-NH2 is about 3-fold higher than for DADE(pY)L-NH2, thus indicating a negative influence of the pY+1 residue (36). Therefore, to study in closer detail the influence of the pY+1 position on substrate recognition by PTP1B, we compared a series of Ac-DADE(pY)L-NH2 analogs as shown in Fig. 5. Although some differences are observed, the wild type enzyme in general seemed to be influenced very little by the actual pY+1 residue, probably indicating that the contribution of this residue to the overall binding affinity is mainly due to the above water-mediated hydrogen bond to Gln262. By introduction of residues 258–259 from PTPα into PTP1B, a significant increase in Km was observed, whereas the introduction of residues 258–259 reduced the overall catalytic efficiency about 4-fold. In comparison to the wild type enzyme, the peptide side chain of the pY+1 residue had significant influence on substrate recognition by PTPα (Fig. 3). Thus, it was not possible to measure Km with an isoleucine or a threonine residue in the pY+1 position. It is of significance that the highest catalytic efficiency for PTPα was observed for the peptide without a residue in the pY+1 position, pointing to steric hindrance as a cause of the reduced substrate recognition in PTPα. When Met258 and Gly259 were replacing Cys258 and Gln259, these differences are less pronounced, i.e. with little influence of the nature of pY+1 side.
chain as in wild type PTP1B.

Gln259 Causes Steric Hindrance in PTPα—The above results indicated to us that the architecture of the binding region 258–259 causes steric hindrance and hence contributes to the general mechanism by which PTPα discriminates between different substrates. We speculated that Gln 259, due to its structural position and bulkiness, would be the major determinant for substrate recognition. For the substitution of Met258 to Cys258, we would expect a less significant effect, since this side chain in the x-ray structure of PTP1B complexed with DADE(pY)L-NH₂ does not interact with the pY₁ side chain (38). Thus, in order to define unambiguously the role of residue 259, we made single mutants of PTP1B and PTPα in which residue 259 was exchanged. As seen in Fig. 4, introduction of a glutamine in position 259 in PTP1B gave rise to increases in $K_m$ values and a concomitant decrease in $k_{cat}/K_m$ values similar to that of the double mutant shown in Fig. 2. Consistently, by replacing Gln259 in PTPα with a glycine resulted in an enzyme with almost similar $K_m$ values as the double mutant PTPα(C258M,Q259G) for this series of synthetic peptide substrates (Figs. 3 and 4). Noticeably, the catalytic efficiency is almost comparable to that of PTP1B, irrespective of the residue in the pY₊₁ position in the substrate (Fig. 4 and Table IV). We conclude that the most significant difference between PTP1B and PTPα in regard to substrate recognition and hydrolysis resides in residue 259.

Gln259 Has Both Direct and Indirect Negative Influence on Substrate/Inhibitor Binding in PTPs—The above studies show that introduction of Gln²⁵⁹ into PTP1B not only had negative

![Chemical structures of compounds 1–4.](image)

**Fig. 1.** Chemical structures of compounds 1–4.

**TABLE II**

Kinetic constants for the hydrolysis of Ac-DADE(pY)L-NH₂ with PTP1B, PTPα, and PTP1B(R47V,D48N,M258C,G259Q) at pH 5.5, 30 °C

| Enzyme               | $k_{cat}$ | $K_m$ | $k_{cat}/K_m \times 10^{-3}$ |
|----------------------|-----------|-------|-------------------------------|
| PTP1B                | 11.6 ± 2.8| 0.016 | 177                           |
| PTP1B(R47V,D48N)     | 9.9 ± 1.9 | 0.019 | 219.6                         |
| PTP1B(R47V,D48N,M258C,G259Q) | 11.6 ± 2.8| 0.016 | 177                           |

**TABLE III**

Kinetic constants for the hydrolysis of Ac-DADE(pY)L-NH₂ with PTP1B, PTP1B(R47V,D48N), and PTP1B(M258C,G259Q) at pH 5.5, 30 °C

| Enzyme               | $k_{cat}$ | $K_m$ | $k_{cat}/K_m \times 10^{-3}$ |
|----------------------|-----------|-------|-------------------------------|
| PTP1B                | 11.6 ± 2.8| 0.016 | 177                           |
| PTP1B(R47V,D48N)     | 23.9 ± 4.3| 0.046 | 24.6                          |
| PTP1B(M258C,G259Q)   | 15.2 ± 1.3| 0.024 | 42.4                          |

![Kinetic constants for the hydrolysis of Ac-DADE(pY)L-NH₂ with PTP1B, PTP1B(R47V,D48N), and PTP1B(M258C,G259Q) at pH 5.5, 30 °C.](image)
influence on the binding of peptides with a residue in the pY+1 position but also on the recognition of Ac-DADE(pY)-NH2 (Figs. 2–4). This indicated that Gln259 indirectly would interfere with the binding of other parts of the peptide than the pY+1 residue, perhaps even with the tyrosine phosphate group itself. We hypothesized that the bulky side chain of Gln259 could affect the positioning and the rotational flexibility of the side chains of structurally neighboring residues. By comparing the published apo structures of PTP1B (39) and PTPα (40), it became apparent that Gln262 in PTPα was pointing into the active site cleft and thus seemed to impair the access of Tyr(P) substrates to the PTP signature motif (Fig. 5A). In contrast, Gln262 in PTP1B points away from the active site thereby promoting substrate binding (Fig. 5B). In other words, it seems conceivable that the side chain of Gln259 forces the Gln262 side chain into a conformation that impairs substrate binding and/or hydrolysis.

**Protein X-ray Crystallography**—To gain further insight at the structural level of the importance of Gln259/Gln262 on inhibitor/substrate binding, we next attempted to co-crystallize PTPα with low molecular weight inhibitors. However, although we were able to obtain crystals that diffracted well, in all cases there was no inhibitor bound to the active site. Instead, as reported by Bilwes and co-workers (40), PTPα crystallized as a dimer with the so-called wedge inserted into the active site and probably therefore preventing binding of the inhibitors. Assuming that the PTP1B147Y,D48N,M258C,G259Q mutant could be used as a model for PTPα in the surrounding of Gln259, we then co-crystallized this mutant with compound 1, which inhibits PTPα with a $K_i$ value of $\sim80 \mu M$ (see below). The mutated residues and the inhibitor are clearly defined in the electron density maps shown in Fig. 6. As shown in Fig. 6B, there are no direct interactions between the inhibitor and Gln259/Gln262. Most importantly, no changes were observed in the secondary or tertiary structure of the mutated enzyme validating the general concept of using mutants for enzyme kinetics analysis. After binding of compound 1, Gln262 is found in a conformation pointing away from the active site pocket similar to the side chain conformation seen for apoPTP1B. This conformation is stabilized by a hydrogen bond to the backbone of Gln259 and van der Waals interactions between these two residues (Fig. 6). We assume that similar structural constraints are at play in PTPα. Therefore, it is likely that this arrangement in turn will increase the energy penalties for binding of substrates, which have bulky pY+1 residues. In addition, when the Gln259 and Gln262 conformations first are formed, this may impair the capability of the latter to position a nucleophilic water molecule correctly (see below). The side chain of Gln259 is fairly flexible in the PTP1B mutant structure (average $B$-factor 41 Å$^2$), whereas Gln262 is stable (average $B$-factor 20 Å$^2$). The average $B$-factor for the whole PTP1B mutant structure is 20 Å$^2$.

**Gln262 in PTPα**—As indicated above, energy penalties could be expected for ligand binding in PTPα due to the positioning of Gln259/Gln262. In accordance with this notion, replacement of
Gln<sup>259</sup> in PTPα with a glycine residue significantly decreases the $K_m$ values for all peptides analyzed (Fig. 4). However, since Gln<sup>259</sup> seems to have both negative direct (steric hindrance) and indirect (via Gln<sup>262</sup>) effects on the binding and/or hydrolysis of peptide substrates, it is difficult to assess the contribution from Gln<sup>262</sup>. The above protein x-ray crystallographic analysis of compound 1 complexed with the PTP1B<sub>R47V,D48N,M258C,G259Q</sub> mutant (Fig. 6) shows that the minimal unit of this inhibitor (2-(oxalyl-amino)-thiophene-3-carboxylic acid) is too small to be directly influenced by Gln<sup>259</sup>. We therefore reasoned that low molecular weight inhibitors representing the minimal unit could be used to semi-quantify the influence of Gln<sup>262</sup> on the accessibility of the active site pocket. Consequently, compounds 2–4 were tested against PTP1B, PTPα, and the single mutants PTP1B<sub>Q259G</sub> and PTPα<sub>Q259G</sub>. It appears from Table V that introduction of Gln<sup>259</sup> into PTP1B caused a significant reduction in the affinity for all three minimal unit inhibitors, most conceivably due to a direct interference of Gln<sup>262</sup> with ligand binding. When Gln<sup>259</sup> in PTPα was replaced by a glycine, a substantial increase in affinity was observed for all inhibitors. A schematic representation of the proposed positioning of residues 259 and 262 in the apo structures of PTP1B and PTPα and during substrate binding and hydrolysis is shown in Fig. 7.

**DISCUSSION**

Intensive studies of the insulin signaling pathway have pointed to several PTPs as key regulators, including PTP1B, PTPα, and PTP-LAR (18). It is generally believed that selective inhibitors of PTPs that negatively regulate insulin signaling could be useful in the treatment of diabetes. Using structure-based design we have recently developed a novel, low molecular weight PTP inhibitor. The above study is designed to provide information on the role of Gln<sup>259</sup> and Gln<sup>262</sup> in the binding and hydrolysis of peptide substrates. The results suggest that the direct and indirect effects of these residues on the active site pocket must be considered when designing inhibitors of PTPs.
weight, non-phosphorus, and highly selective inhibitor of PTP1B (21). This was achieved by introducing a basic nitrogen into a general PTP inhibitor leading to salt bridge formation with Asp48 in PTP1B. In other PTPs, containing an asparagine in the equivalent position, the basic nitrogen causes repulsion. The net result is a marked selectivity for PTP1B. The present study was undertaken to expand further our knowledge regarding the structural requirements for substrate and inhibitor recognition by PTPs. In particular, our focus has been to delineate the structural basis for differences in substrate recognition by PTP1B and PTPα.

The synthetic peptide DADE(pY)L (derived from the epidermal growth factor receptor) and analogs thereof have previously been used extensively to study substrate recognition of PTPs (6, 7, 41, 42). Furthermore, structural information is provided by x-ray crystallography of the PTP1B-DADE(pY)L-NH2 complex that defines the binding mode (38). Importantly, the DADE(pY)L peptide is recognized well by PTP1B but less efficiently by PTPα (36, 37). Thus, this peptide seems an ideal tool to probe and identify the structural requirements involved in defining substrate specificity. By using a series of PTP mutants in which residues 47, 48, 258, and 259 were exchanged between PTP1B and PTPα in combination with a series of Ac-DADE(pY)L-NH2 analogs, we here demonstrate that the key selectivity-determining residue is residue 259. In PTPα this residue is a glutamine, and in PTP1B it is a glycine. By replacing Gln259 with a glycine in PTPα, this enzyme exhibits the same broad substrate recognition capacity and catalytic activity as PTP1B. Conversely, introduction of glutamine in position 259 almost turns PTP1B into a PTPα-like enzyme. Thus, glutamine in position 259 causes steric hindrance and limits the substrate recognition capability, whereas a glycine allows broad substrate recognition. In comparison, in the evolution of phosphatases steric hindrance is utilized to provide selectivity for phosphotyrosine. As noted by Barford and co-workers (39), the catalytic cleft is about 9 Å deep, which only allows phosphotyrosine, but not phosphoserine, to reach the active cysteine and bind to the PTP signature motif.

As indicated above, the Ac-DADE(pY)L-NH2 peptide and analogs thereof have previously been widely used to study the substrate specificity of PTPs (6, 7, 10, 42). However, these studies in most cases focused on the residues positioned N-terminally to the Tyr(P) residue. Consequently, little is known about the importance of residues located C-terminally to Tyr(P). In a detailed Ala scan of the longer Ac-DADE(pY)LIPQQG-NH2 peptide, only a minor influence of

| Peptide          | PTP1B | PTPα | PTP1B(M258C,G259Q) | PTPα(C258M,Q259G) | PTP1B(G259Q) | PTPα(Q259G) |
|------------------|-------|------|-------------------|------------------|-------------|-------------|
| Ac-DADE(pY)-NH2  | 38.5 ± 2.4 | 25.9 ± 1.0 | 17.2 ± 2.3 | 16.1 ± 0.8 | 16.3 ± 2.4 | 57.2 ± 4.2 |
| Ac-DADE(pY)L-NH2 | 48.1 ± 1.0 | — | 20.1 ± 6.5 | 22.2 ± 5.4 | 12.0 ± 0.9 | 63.1 ± 1.2 |
| Ac-DADE(pY)L-NH2 | 33.6 ± 0.6 | 9.9 ± 1.9 | 15.2 ± 1.3 | 13.3 ± 3.3 | 15.0 ± 2.5 | 37.1 ± 4.9 |
| Ac-DADE(pY)-NH2  | 33.5 ± 0.1 | — | 14.9 ± 2.3 | 14.0 ± 0.5 | 14.5 ± 1.5 | 40.0 ± 2.7 |
| Ac-DADE(pY)L-NH2 | 34.3 ± 0.1 | 11.0 ± 3.2 | 14.6 ± 1.8 | 11.7 ± 2.0 | 14.0 ± 1.9 | 38.1 ± 3.3 |
| Ac-DADE(pY)-NH2  | 33.4 ± 0.0 | 7.8 ± 0.1 | 11.8 ± 4.0 | 10.2 ± 2.2 | 13.2 ± 1.0 | 27.4 ± 5.5 |
| Ac-DADE(pY)L-NH2 | 32.3 ± 3.0 | 9.8 ± 2.1 | 11.2 ± 1.3 | 10.5 ± 2.2 | 13.2 ± 1.0 | 27.4 ± 5.5 |

*K_m* : substrate concentration.
changes also take place in PTPα upon substrate or inhibitor binding, we speculate that the interaction between the glutamines further decreases their rotational freedom leading to an even more efficient blockade of this area of the enzyme (Fig. 7).

During hydrolysis of the cysteinyl-phosphate intermediate, Gln262 in PTP1B swings into the catalytic site to position or activate a nucleophilic water molecule (43). Mutation of Gln262 in PTP1B to an alanine reduces $k_{\text{cat}}$ by 100-fold and $K_m$ 10-fold (44). This indicates that the positioning of Gln262 plays a major role for efficient hydrolysis. Since it is conceivable that Gln262 in PTPα forms a hydrogen bond and van der Waals contacts to Gln259 this would implicate that Gln262 moves less freely in PTPα than in PTP1B. We here demonstrate that replacement of Gly259 with a glutamine in PTP1B causes a significant decrease in the $k_{\text{cat}}$ value, irrespective of the residue in the pY+1 position in the substrate (Fig. 4). Similarly the catalytic efficiency against p-NPP is decreased in this mutant (Table V). Although not formally proven, we hypothesize that the low catalytic efficiency observed for wild type PTPα could in part be due to ligand-induced stabilization of Gln259 and Gln262. In addition, Gln259 may also impair correct positioning of Gln262 relative to the above nucleophilic water molecule (Fig. 7).

The steric hindrance caused by residues in position 259 may potentially be useful in inhibitor design. Thus, inhibitors containing substituents addressing this area of the enzyme may be accommodated in PTP1B with a glycine in this position, but not by PTPs with bulky residues. In this context, it is of interest that Zhang and co-workers (45) utilizing a second aryl phosphate-binding site in PTP1B have recently made a series of phosphonate-based non-peptide inhibitors that showed a remarkable selectivity for PTP1B (46). The most important residues in this second binding site are Arg24 and Arg254 which were found to coordinate phosphate (45). Furthermore, additional points of interactions were found to include Met258 and Val69. In other words, to bind simultaneously to the PTP loop and the second aryl phosphate-binding site, the inhibitors needed to “pass over” Gly259 and Met258 (46). Although no structural information was provided, we hypothesize that the high selectivity for PTP1B obtained with the above phosphonate-based inhibitors may, at least in part, be due to steric hindrance imposed by more bulky residues in position 259 in other PTPs.

In summary, we have shown that the residue in position 259 (PTP1B numbering) is a key determinant in substrate recognition capacity and hydrolysis by PTPs. PTPs with a bulky residue such as a glutamine show restricted substrate recognition, whereas PTPs with a glycine in this position have broad specificity. In PTPα, Gln259 further influences the positioning of Gln262. As a consequence, the ligand-induced stabilization of both residues leads to an increased efficiency of steric hindrance and a decreased catalytic efficiency. Thus, we propose that Gln259 in PTPα plays a dual role leading to restricted substrate recognition and reduced catalytic rate. Both effects could indicate that PTPα is involved in the control of highly selective signal transduction processes.

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**Table V**

| Enzyme               | $K_m$ compound 1 $\mu M$ | $K_m$ compound 2 $\mu M$ | $K_m$ compound 3 $\mu M$ | $K_m$ p-NPP $\mu M$ | $K_m$ p-NPP $\mu M$ |
|----------------------|--------------------------|--------------------------|--------------------------|---------------------|---------------------|
| PTP1B                | 2                        | 108                      | 37                       | 30                  | 0.77                |
| PTP1B (G259Q)        | 33                       | 448                      | 376                      | 146                 | 3.02                |
| PTPα                 | 81                       | 741                      | 892                      | 1117                | 2.58                |
| PTPα (G259Q)         | 19                       | 606                      | 408                      | 119                 | 0.70                |

* Mixed inhibition.
Substrate Specificity of PTP1B and PTPα

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Residue 259 Is a Key Determinant of Substrate Specificity of Protein-tyrosine Phosphatases 1B and α

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