The Active Site Specificity of the Yersinia Protein-tyrosine Phosphatase*

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Yersinia protein-tyrosine phosphatase substrates have been synthesized employing an expedient methodology that incorporates phosphorylated non-amino acid residues into an active site-directed peptide. While the peptidic portion of these compounds serves an enzyme targeting role, the nonpeptidic component provides a critical assessment of the range of functionality that can be accommodated within the active site region. We have found that the Yersinia phosphatase hydrolyzes both L- and D-stereoisomers of phosphotyrosine in active site-directed peptides, with the former serving as a 10-fold more efficient substrate than the latter. In addition, this enzyme catalyzes the hydrolysis of a variety of aromatic and aliphatic phosphates. Indeed, a peptide bearing the achiral phosphotyrosine analog, phosphotyramine, is not only the most efficient substrate described in this study, it is also one of the most efficient substrates ever reported for the Yersinia phosphatase. Straight chain peptide-bound aliphatic phosphates of the general structure, (Glu)₄-NH-(CH₂)₇-OPO₃⁻ (n = 2–8), are also hydrolyzed, where the most efficient substrate contains seven methylene groups. Finally, a comparison of the substrate efficacy of the peptide-bound species with that of the corresponding non-peptidic analogs, reveals that the peptide component enhances k_cat/K_m by up to nearly 3 orders of magnitude.

Protein-tyrosine phosphatases (PTPases)1 are emerging as essential regulators of a variety of fundamental cellular processes such as cell growth, mitogenesis, metabolism, gene transcription, cell cycle control, and the immune response (1–3). Together with the protein-tyrosine kinases (PTKs), the PTPases control the state of tyrosine phosphorylation on cellular proteins. PTPases constitute a growing family of enzymes that rival PTKs in terms of structural diversity and complexity. Unlike tyrosine-specific and serine/threonine-specific kinases, which share conserved sequences in their catalytic domains, PTPases show no sequence similarity with serine/threonine kinases, that rival PTKs in terms of structural diversity and complexity. Unlike amino-specific and serine/threonine-specific kinases, which share conserved sequences in their catalytic domains, PTPases show no sequence similarity with serine/threonine kinases, or the broad-specificity phosphatases such as acid or alkaline phosphatases (4, 5). However, all members of the PTPase subfamily, from bacteria to mammals, do share a strong sequence similarity within a 250 residue span of the catalytic domain (6).

Despite a rapidly growing appreciation of the biological importance of PTPases in signal transduction, a detailed understanding of their substrate specificity is lacking. How do PTPases distinguish between the diversity of phosphoproteins that they encounter within the cell? Studies using synthetic phosphopeptides have demonstrated that PTPases are sensitive to the amino acid sequence that encompasses the phosphotyrosine moiety (7–11). In addition, it is clear that the phosphotyrosine residue itself is absolutely essential for PTPase recognition of protein-based substrates as well as peptide-based substrates that are designed to mimic the former. For example, PTPases do not bind tyrosine-bearing peptides that lack the phosphate functionality (9, 12). Furthermore, replacement of the critical phosphotyrosine residue with an O-methylated phosphotyrosine generates a totally inert derivative (9). Finally, PTPases appear to exhibit a strict bias for phosphotyrosine since they do not utilize phosphoserine or phosphothreonine as substrates in protein- and peptide-based environments (13–15). Such discriminatory behavior is not surprising given the difference in orientation and distance of the phosphate moiety, relative to the adjacent peptide backbone, in phosphotyrosine compared to those in phosphoserine or phosphothreonine. This certainly implies that a tyrosine moiety, in conjunction with the negatively charged phosphate group, is crucial for PTPase recognition. Based upon these structural considerations, several nonhydrolyzable analogs of phosphotyrosine have been prepared and inserted into PTPase-targeted peptides. For example, an aromatic moiety and a negatively charged phosphonate are contained within phosphonomethyl phenylalanine, a species that lacks a hydrolyzable aromatic ester. Indeed, phosphonomethyl phenylalanine-containing peptides have been shown to be effective, reversible inhibitors of PTPases (11, 16, 17).

In spite of the fact that the phosphotyrosine moiety appears to be essential for PTPase recognition of peptide- and protein-based systems, a number of small, nonpeptidic residues have been found to serve as reasonably efficient PTPase substrates. In this regard, the most widely utilized substrate is p-nitrophosphorylesters. In addition, we have recently demonstrated that PTPases will also catalyze the dephosphorylation of a variety of alkyl phosphates, including the individual amino acids phosphoserine and phosphothreonine (18). However, these substrates are not contained within the context of a protein-like environment, a structural framework that could assist in targeting specific PTPases. We have recently devised a chemical strategy that fuses peptidic and nonpeptidic components into compounds that act as unusually efficient substrates for protein kinases (19–21). These fusion compounds have provided a critical assessment of the range of functionality that can be accommodated within the active site of protein kinases, infor-
mation that should prove to be of decided assistance in the design of potent inhibitor agents. In addition, these amalga-
mated species have been utilized to distinguish between ki-
nases that are otherwise indistinguishable with conventional peptide substrates (22). We now report that this strategy, for the first time, has been successfully applied to a member of the protein phosphatase family. We have constructed a structur-
ally diverse array of phosphorylated peptide-aminoalcohol fu-
sion compounds to assess the active site substrate specificity of the Yersinia PTPase. This phosphatase is required for the pathogenicity of Yersinia pestis, the bacterium responsible for the bubonic plague, also known as the Black Death (23). In contrast to the currently held beliefs that phosphotyrosine is absolutely essential for PTPase recognition of protein- and peptide-based substrates, we have found that the Yersinia PTPase will catalyze the hydrolysis of a wide variety of both aromatic and aliphatic phosphates in peptides. This not only greatly expands the range of compounds that can be recognized by PTPases, but also provides a mechanism for utilizing the specificity inherent within the peptide component of these compounds to target specific PTPases.

MATERIALS AND METHODS

All chemicals were purchased from Aldrich, except for piperdine and protected amino acid derivatives (Advanced ChemTech) and Fmoc-Glu(O-t-buty1)-2-methoxy-4-alkoxy benzyl alcohol resin (Peninsula Labo-

ratories, Inc.). The structure of all new compounds were confirmed by 'H NMR (300 Mhz), 'C NMR (22.5 Mhz), '3P NMR (161.9 Mhz), fast atom bombardment mass spectral analyses (positive and negative ion). Enzyme assay solutions were prepared with deionized/distilled water.

Phosphorylated Aminoalcohol Synthesis

All aminoalcohol derivatives were synthesized using the methodology outlined in Scheme 1. The amino moiety was converted to the Fmoc-protected derivative via the procedure described by Ottinger et al. (24). The alcohol was subsequently phosphorylated with di-tet-butyl-N,N-diethylphosphoramidite and oxidized to the phosphoroester with m-chloroperbenzoic acid, under the conditions described previously by Perch et al. (25). The Fmoc protecting group was removed by treating the Fmoc-aminoalcohol (0.6 mmol) with 40% piperidine/CH2Cl2 (10 ml) and 40% di-tet-butyl phosphotriester moiety was deprotected with tert-

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ment mass spectral analyses. The di-tet-butyl phosphotriester moiety was deprotected with tert-

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ment mass spectral analyses.

Phosphorylated Aminoalcohol-containing Peptides—Boc-Glu(O-t-buty1)-Glu(O-t-buty1)-COOH (50 mg, 0.05 mmol) was activated with treatment with benztriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate (Bop; 0.11 mmol) in 3 ml of CH2Cl2/dimethyl formamide (1:1) for 3 min. Individual phosphorylated aminoalcohols (0.55 mmol, dissolved in 3 ml of 1:1 CH2Cl2/dimethyl formamide) were introduced and allowed to react with the activated peptide for 2 h at room temperature. The solvent was removed in vacuo and 95% trifluoroacetic acid, 5% H2O (10 ml) was added to the residue and stirred for 1 h. The solvent was subsequently removed, methanol (1 ml) added, and the peptide precipitated with anhydrous ethyl ether (100 ml). The peptides were purified using a preparative high performance liquid chromatography column as described above. All peptides furnished satisfactory fast atom bombard-
ment mass spectral analyses.

Yersinia PTPase

Homogenous recombinant Yersinia PTPase was purified as described previously (26).

PTPase Assays

Assay of Phosphopeptides of Tyrosine Analogs—All enzyme assays were performed at 30°C in 50 mM succinate, 1 mM EDTA, pH 6.0, buffer with a constant ionic strength of 0.15 M (adjusted with NaCl). Initial rates of the enzymatic hydrolysis of phosphorylated mono-

Peptide Synthesis

Synthesis of Phosphorylated Tyrosine Peptides—The tyrosine-containing amino acid (i.e., Fmoc-tyrosine, 0.495 mmol) was coupled to the resin (0.5 g, substitution level = 0.33 mmol/g of resin) and immediately phosphorylated with di-tet-butyl phosphotriester moiety. The crude product was purified on a silica gel column (100–200 mesh) with 1%NH3/H2O in 5:1 CHCl3/MeOH. Solvent evaporation gave the desired product as a slightly yellow solid in 60% yield.

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Yersinia phototyrosine are important recognition elements for the phosphates to the C-terminus of an active site-directed peptide.

In order to take advantage of this specificity, we have attached an array of aliphatic and aromatic compounds to furnish the desired peptide-based phosphorylated amino acid. In this regard, PTPases, such as the phosphatase isolated from Yersinia, are known to utilize a variety of low molecular weight aromatic and aliphatic phosphates as substrates. Unfortunately, these rather simple substrates lack the protein-like environment that may be crucial for targeting individual PTPases in a highly specific fashion. For comparative purposes, the Km and Vmax values associated with the Yersinia PTPase-catalyzed dephosphorylation of Asp-Ala-Asp-Glu-\(\text{Tyr(P)}\)-NH$_2$ and epidermal growth factor receptor (EGF) peptide were determined by analyzing the experimental data through a nonlinear least-squares fit algorithm of the Michaelis-Menten equation.

\[
t = \frac{1}{k_{cat}E_0} + \left(\frac{K_m}{k_{cat}E_0}\right)\ln[p_a/p_p - p_a]
\]  

(Eq. 1)

Kinetic parameters \(k_{cat}\) and \(K_m\) were determined by analyzing the experimental data through a nonlinear least-squares fit algorithm of this equation, where \(k_{cat}\) is the catalytic turn-over number, \(K_m\) is the Michaelis constant, \(E_0\) is the enzyme concentration, and \(p_a\) and \(p_p\) are the product concentrations at time \(t\) and infinity, respectively.

**RESULTS AND DISCUSSION**

An in-depth analysis of the active site substrate specificity of PTPases may furnish critical information that could assist in the design of potent active site-directed inhibitors. For example, such an analysis can provide detailed structural data regarding the range of functionality that can be readily tolerated by the active site of these enzymes. In this regard, PTPases, such as the phosphatase isolated from Yersinia, are known to utilize a variety of low molecular weight aromatic and aliphatic phosphates as substrates. Unfortunately, these rather simple compounds lack the protein-like environment that may be crucial for targeting individual PTPases in a highly specific fashion. Clearly, peptide-based species offer one possible solution to the issue of specificity. In order to take advantage of this specificity, we have attached an array of aliphatic and aromatic phosphates to the C-terminus of an active site-directed peptide. Acidic amino acid residues on the amino-terminal side of phototyrosine are important recognition elements for the Yersinia PTPase (8, 11). Consequently, we prepared the peptide (Glu)$_4$-Tyr(P)-NH$_2$. This species is hydrolyzed by Yersinia PTPase (pH 6 and 30 °C) with \(k_{cat}\) and \(K_m\) values of \((1.2 \pm 0.1) \times 10^3\) s$^{-1}$ and 0.31 ± 0.06 mM, respectively (Table I). For comparative purposes, the \(K_m\) and \(V_{max}\) values associated with the Yersinia PTPase-catalyzed dephosphorylation of Asp-Ala-Asp-Glu-\(\text{Tyr(P)}\)-NH$_2$ and epidermal growth factor receptor (EGF) peptide were provided in Table I (11). Clearly, (Glu)$_4$-Tyr(P)-NH$_2$ is a useful structural starting point for the preparation of peptide-based analogs in which the phototyrosine residue is replaced with various phosphorylated aminoalcohols. Based upon these results, we prepared peptide-based PTPase substrates of the general formula, Glu-Glu-Glu-Glu-NH-R-OPO$_3$\(^{2-}\) (Scheme 1). The amine portion of an aminoalcohol (A) was first protected as the Fmoc-derivative and the alcohol subsequently phosphorylated to furnish B (24, 25). This species was then sequentially oxidized to the phosphotriester and the Fmoc group removed under basic conditions to yield the free amine C. The latter was readily coupled to the Bop-activated form of the protected tetrapeptide, Boc-Glu-(O-t-butyl)-Glu-(O-t-butyl)-Glu-(O-t-butyl)-COOH and all the protecting groups subsequently removed with 95% trifluoroacetic acid, 5% water to furnish the desired peptide-based phosphorylated aminoalcohol D.

We first determined whether the Yersinia PTPase activity is sensitive to stereochemistry at the \(\alpha\)-carbon of the phototyrosine residue. (Glu)$_4$-Tyr(P)-NH$_2$ (4) exhibits a \(k_{cat}\) of \(1.3 \times 10^3\) s$^{-1}$ and a \(K_m\) of 3.0 mM. The resulting \(k_{cat}/K_m\) is 9-fold smaller than that of (Glu)$_4$-Tyr(P)-NH$_2$ (1), which is exclusively due to an order of magnitude increase in \(K_m\) for the \(\beta\)-phototyrosine-bearing peptide. Interestingly, PTPases catalyze the hydrolysis of free \(\beta\)-phototyrosine and \(\gamma\)-phototyrosine with equal efficiency (29). In contrast, it is clear that the Yersinia PTPase prefers the naturally occurring L-stereoisomer when the phosphorylated amino acid is constrained within a peptide-based environment. Will the Yersinia PTPase recognize achiral residues substrates within such an environment? To address this question, we prepared (Glu)$_4$-NH-(CH$_2$)$_2$C$_6$H$_5$-PO$_3$\(^{2-}\) (5) via the synthetic scheme outlined above. The phototyramine residue contained within this peptide lacks the

| Substrate | \(k_{cat}\) (1 x \(10^3\) s$^{-1}$) | \(K_m\) (mM) | \(k_{cat}/K_m\) (\(\mu M^{-1} s^{-1}\)) |
|-----------|--------------------------------|-------------|-----------------------------------|
| EEEE-\(\text{Tyr-NH}_2\) | 1 | 1.2 ± 0.1 | 0.31 ± 0.06 | 4.0 ± 0.8 |
| DADE-\(\text{Tyr-NH}_2\) | 2 | 1.4 ± 0.1 | 0.29 ± 0.01 | 4.6 ± 0.2 |
| DADE-\(\text{LIPQOG}\) | 3 | 1.3 ± 0.1 | 0.06 ± 0.01 | 22 ± 1 |
| (Glu)$_4$-NH$^+$ | 4 | 1.3 ± 0.4 | 3.0 ± 1.2 | 0.43 ± 0.22 |
| (Glu)$_4$-NH$^+$ | 5 | 1.2 ± 0.1 | 0.05 ± 0.02 | 22 ± 7 |
| (Glu)$_4$-\(\text{Tyr-NH}_2\) | 6 | 0.32 ± 0.03 | 3.2 ± 0.5 | 0.96 ± 0.016 |
| (Glu)$_4$-\(\text{NH}_2\) | 7 | 0.50 ± 0.06 | 0.42 ± 0.17 | 1.2 ± 0.5 |
| (Glu)$_4$-\(\text{NH}_2\) | 8 | 0.0078 ± 0.0008 | 1.5 ± 0.4 | 0.0052 ± 0.0015 |
phosphotyrosine, substituents at the sensing of amino acid residues on the carboxyl-terminal side of substrate described in this study, it is also more efficiently (in terms of simple aromatic phosphate, such as phosphotyramine (a 224-phosphate residue interfere with PTPase activity. Second, a peptide part. This is a consequence of a sharp drop in $k_{cat}$ for (Glu)$_4$NH-(CH$_2$)$_2$C$_6$H$_4$OPO$_3^-$ (5). Peptide 5 is not only the best substrate described in this study, it is also more efficiently hydrolyzed than Asp-Ala-Asp-Glu-Tyr(P)-Leu-NH$_2$, and is nearly as efficiently hydrolyzed as the best substrate reported for Yersinia PTPase. Asp-Ala-Asp-Glu-Tyr(P)-Leu-Ile-Pro-kinase (Table I) (11). Is the phosphorylated tyramine moiety exceptionally reactive? We prepared the peptide-free derivative $^1$NH$_2$(CH$_2$)$_2$C$_6$H$_4$OPO$_3^-$ (6) in order to address this question. As expected, 6 behaves as a typical aryl phosphate. The $k_{cat}$ (0.32 × 10$^3$ s$^{-1}$) and $K_m$ (3.2 mM) for this species are nearly identical to those exhibited by p-nitrophenyl phosphate ($k_{cat}$ (0.35 × 10$^3$ s$^{-1}$) and $K_m$ (2.6 mM)) under similar conditions (6). These results reveal two significant facets of the substrate specificity of the Yersinia PTPase. First, in the absence of amino acid residues on the carboxyl-terminal side of phosphotyrosine, substituents at the $\alpha$-position of the phosphoryl moiety interfere with PTPase activity. Second, a peptide moiety can dramatically enhance the substrate efficacy of a simple aromatic phosphate, such as phosphotyramine (a 224-fold enhancement in $k_{cat}/K_m$ of (Glu)$_4$NH-(CH$_2$)$_2$C$_6$H$_4$OPO$_3^-$ (5) versus $^1$NH$_2$(CH$_2$)$_2$C$_6$H$_4$OPO$_3^-$ (6)).

In addition to the influence exerted by stereochemistry, we have found that the distance between the peptide backbone and the aromatic phosphate moiety also controls the efficacy of PTPase-catalyzed hydrolysis. When the distance between the peptide unit and the aryl phosphate group is shortened by a single methylene unit (i.e., compound (Glu)$_4$NH-CH$_2$C$_6$H$_4$OPO$_3^-$ (7)) an 18-fold reduction in $k_{cat}/K_m$ occurs relative to that observed with (Glu)$_4$NH-(CH$_2$)$_2$C$_6$H$_4$OPO$_3^-$ (5). The drop in substrate efficacy is primarily due to an 8-fold increase in $K_m$ (Table I). However, a minor 2.4-fold decrease in $k_{cat}$ is observed as well. In addition, we prepared an analog of (Glu)$_4$NH-CH$_2$C$_6$H$_4$OPO$_3^-$, one which contains a methylene group between the aromatic ring and the phosphate moiety (i.e., (Glu)$_4$NH-CH$_2$C$_6$H$_4$OPO$_3^-$ (8)). In this case, the distance between the phosphate and the peptide backbone is similar to that present in (Glu)$_4$NH-(CH$_2$)$_2$C$_6$H$_4$OPO$_3^-$ (5). However, the phosphate moiety is no longer directly attached to the aromatic ring. Compound 8 does serve as a substrate for the Yersinia PTPase, an observation consistent with our earlier results that demonstrated that alkyl phosphates are hydrolyzed by tyrosine-specific phosphatases (18). However, compared to its structural isostere 5, compound 8 exhibits a 150-fold smaller $k_{cat}$ and a 30-fold larger $K_m$. Clearly, the Yersinia PTPase is more efficient in processing the aryl phosphate in (Glu)$_4$NH-(CH$_2$)$_2$C$_6$H$_4$OPO$_3^-$ (5) than its benzyl counterpart in (Glu)$_4$NH-CH$_2$C$_6$H$_4$OPO$_3^-$ (8). However, it is not clear if this is a consequence of some intrinsically lower chemical reactivity associated with the benzyl phosphate moiety or if this is due to an altered active site-bound orientation of the phosphate group induced by the nature of the aromatic ring substituents. The former possibility is the most likely since alkyl phosphates are hydrolyzed at a significantly reduced rate compared to their aromatic counterparts (Ref. 18 and see below). Finally, we note that (Glu)$_4$NH-CH$_2$C$_6$H$_4$OPO$_3^-$ (8) is a significantly (46-fold) more efficient substrate than pyridoxal 5'-phosphate (18), a non-peptidic species that contains a benzyl phosphate moiety. This comparison once again illustrates the importance of the peptide component and demonstrates that interactions removed from the site of bond cleavage and formation can be beneficial for catalysis. Barford et al. (30) recently proposed that PTPase specificity for phosphotyrosine-containing peptides probably results from the depth of the active site cleft since the smaller phosphoserine and phosphothreonine side chains should be unable to reach the phosphate binding site. Since simple alkyl phosphates can be processed by PTPases (although not as efficiently as aryl phosphates) (18), and since the presence of a peptide enhances the substrate reactivity (Ref. 11 and see above), we expected that a peptide-linked alkyl phosphate should make a reasonable PTPase substrate. This should especially be the case if the distance between the peptide backbone and the phosphate moiety corresponds to that present in a peptide-linked phosphorylated tyrosine. Such an "optimal" distance should approximately be six CH$_2$ units. We prepared a series of peptide-based phosphorylated aliphatic alcohols (Table II) in order to explore the validity of this supposition. The $k_{cat}/K_m$ for the Yersinia PTPase-catalyzed hydrolysis of (Glu)$_4$NH-(CH$_2$)$_2$OPO$_3^-$ (9) is 0.20 m$^{-1}$ s$^{-1}$ (Table II). Although this value is significantly lower than all of the aromatic phosphates illustrated in Table I, peptide 9 is a considerably better substrate than its non-peptidic counterpart, $^1$NH$_2$(CH$_2$)$_2$OPO$_3^-$ (18). Indeed, the hydrolytic turnover of the latter is so slow that we could not accurately measure the kinetic constants. However, since we were able to obtain a $k_{cat}$ (1.8 s$^{-1}$) and $K_m$ (48 mM) for DL-$\omega$-glycerophosphate (18), we have attributed the lack of activity of $^1$NH$_2$(CH$_2$)$_2$OPO$_3^-$ to the positively charged $\beta$-substituent. The specific activity of the Yersinia PTPase at 20 mM substrate concentration toward $^1$NH$_2$(CH$_2$)$_2$OPO$_3^-$ is 149-fold lower than DL-$\omega$-glycerophosphate. Since the $K_m$ values for these simple aliphatic alkyl phosphates are significantly higher than 20 mM, the specific activity measurement for these substrates at this concentration provides an assessment of the kinetic parameter $k_{cat}/K_m$. To our approximation, the $k_{cat}/K_m$ for $^1$NH$_2$(CH$_2$)$_2$OPO$_3^-$ is $2.6 \times 10^{-4}$ mM$^{-1}$ s$^{-1}$. Consequently, (Glu)$_4$NH-(CH$_2$)$_2$OPO$_3^-$ (9), which lacks the positively charged $\beta$-substituent and contains an appended active site-directed peptide, is approximately 770-fold more efficiently hydrolyzed than O-phosphorylethanolamine.

### Table II

| Substrate | $k_{cat}$ (s$^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$) |
|-----------|---------------------|------------|----------------------------------|
| (Glu)$_4$NH-(CH$_2$)$_2$OPO$_3^-$ | 9 | 0.32 ± 0.03 | 1.6 ± 0.2 | 0.20 ± 0.03 |
| (Glu)$_4$NH-(CH$_2$)$_2$OPO$_3^-$ | 10 | 3.2 ± 0.3 | 4.1 ± 0.4 | 0.78 ± 0.11 |
| (Glu)$_4$NH-(CH$_2$)$_2$OPO$_3^-$ | 11 | 4.2 ± 0.5 | 4.4 ± 0.7 | 0.95 ± 0.20 |
| (Glu)$_4$NH-(CH$_2$)$_2$OPO$_3^-$ | 12 | 3.6 ± 0.2 | 1.9 ± 0.2 | 1.9 ± 0.2 |
| (Glu)$_4$NH-(CH$_2$)$_2$OPO$_3^-$ | 13 | 11 ± 2 | 2.1 ± 0.6 | 5.2 ± 1.8 |
| (Glu)$_4$NH-(CH$_2$)$_2$OPO$_3^-$ | 14 | 25 ± 1 | 1.5 ± 0.1 | 14 ± 1 |
| (Glu)$_4$NH-(CH$_2$)$_2$OPO$_3^-$ | 15 | 2.9 ± 0.4 | 3.5 ± 0.8 | 0.83 ± 0.22 |
| (Glu)$_4$NH-(CH$_2$)$_2$OPO$_3^-$ | 16 | 4.3 ± 0.4 | 3.2 ± 0.6 | 1.3 ± 0.3 |
We next addressed the issue of optimal distance between the phosphate moiety and the peptide backbone (Table I). As the number of methylene groups n in (Glu)ₙ-NH-(CH₂)ₙ-OPO₃⁻ increases from 2 up through 5 (compounds 9–12), an overall 10-fold increase in Kᵦ/Kᵦᵠ is apparent. Most of the improvement in the kᵦᵠ/Kᵦᵠ parameter is a consequence of enhanced kᵦᵠ values (Table I). However, this improvement accelerates rapidly at n = 6 and peaks at n = 7. The 26-fold enhancement in kᵦᵠ/Kᵦᵠ for (Glu)₆-NH-(CH₂)₆-OPO₃⁻ (13) and 70-fold enhancement in substrate efficacy for (Glu)₆-NH-(CH₂)₆-OPO₃⁻ (14) (both relative to (Glu)₅-NH-(CH₂)₅-OPO₃⁻ (9)) are due to substantial improvements in the kᵦᵠ parameter. However, the catalytic efficiency of compound 15 (n = 8) is more than an order of magnitude less than its n = 7 counterpart (Table I). These results are consistent with the hypothesis that the optimal distance between the phosphate moiety and the peptide backbone corresponds to the length of a tyrosine side chain. One possible explanation for the ability of peptides containing a shorter-than-ideal chain length to serve as substrates may be due to the insertion of a portion of the peptide backbone into the enzyme active site. Similarly, (Glu)₅-NH-(CH₂)₅-OPO₃⁻ (15), which possesses a longer-than-ideal chain length, may be able to position itself somewhat further from the active site, which would allow the phosphate moiety, on the relatively long side chain, to interact with the catalytic apparatus in a favorable fashion. In short, the peptide may be able to “slide” along the enzyme surface until the phosphate is properly positioned in the active site. We have applied a similar type of reasoning to explain the relatively broad active site substrate specificity of pp60⁰-src (21). Finally, we prepared the phosphate monooester of the cyclohexyl derivative 16 (Table I). The efficacy of this peptide as a substrate is similar to species 11 (n = 4) and 12 (n = 5), compounds in which the phosphate is fixed at a similar distance from the peptide backbone. However, this result is surprising in light of the fact that phosphorylated secondary alcohols are generally much poorer substrates than their primary alcohol-containing counterparts (18). At this point, we do not know whether the favorable kinetics associated with 16 is due to the presence of the peptide unit or a consequence of unusual reactivity associated with the cyclohexyl group.

PTPases are much more effective catalysts for the hydrolysis of aryl phosphates than alkyl phosphates. For example, the Yersinia PTPase dephosphorylates aryl phosphates, such as p-nitrophenyl phosphate, 2–3 orders of magnitude more rapidly than alkyl phosphates of primary alcohols (18). The Yersinia PTPase also catalyzes the exchange reaction between ¹⁸O-labeled phosphate and solvent water (31). Similarly, the kᵦᵠ value for exchange at pH 6.0 is 350-fold slower than the kᵦᵠ value for the Yersinia PTPase-catalyzed hydrolysis of p-nitrophenyl phosphate. The PTPase-catalyzed reaction involves a phosphoenzyme intermediate (32–34). The formation of the phosphoenzyme intermediate involves attack by the active site cysteine on the phosphorus atom and subsequent release of the leaving group (e.g. phenoxide, alkoxide, or hydroxide). Since the pKᵠ values of the conjugate acid of the leaving group (alkoxide (RO⁻) for alkyl phosphate and hydroxide (HO⁻) for inorganic phosphate) are both approximately 15–16, the repulsion of alkoxides or a hydroxide would require much greater assistance from the enzyme than phenoxides (which have pKᵠ values typically below 10). The phosphorylated aliphatic alcohols in this study should all have leaving group pKᵠ values of 15–16. In short, their intrinsic chemical reactivity should be similar to simple alkyl phosphates. Consequently, it is remarkable that (Glu)₅-NH-(CH₂)₅-OPO₃⁻ exhibits a turnover number of 25 s⁻¹, which is only 14-fold slower than p-nitrophenyl phosphate.

This is another clear example that binding interactions substantially removed from the site of bond cleavage and formation are utilized to facilitate catalysis.

We also performed a preliminary analysis of the binding affinity of two phosphorylated aliphatic alcohol peptides to the Yersinia PTPase. We measured the ability of peptides 13 (n = 6) and 15 (n = 8) to inhibit the enzyme-catalyzed hydrolysis of p-nitrophenyl phosphate. Both (Glu)₅-NH-(CH₂)₅-OPO₃⁻ and (Glu)₆-NH-(CH₂)₆-OPO₃⁻ serve as competitive inhibitors versus the phosphomonoester substrate, with Kᵠ values of 360 ± 88 and 960 ± 260 μM, respectively. As a comparison, DL-α-glycerophosphate competitively inhibits the Yersinia PTPase-catalyzed hydrolysis of p-nitrophenyl phosphate at pH 7.0 with a Kᵠ of 20.4 ± 5.6 μM (18). Therefore, we conclude that the presence of the peptide moiety does contribute to additional substrate binding. This additional binding energy may be utilized to position the catalytic groups in the enzyme active site to efficiently carry out the chemical steps.

In summary, we have prepared an array of structurally diverse phosphorylated aminoalcohols and have attached these to the carboxyl terminus of Glu-Glu-Glu-Glu. These amalgamated peptide-aminoalcohol phosphates have been utilized to probe the active site specificity of the Yersinia PTPase. We have found that efficient binding and catalysis by the Yersinia PTPase is dependent upon the distance between the phosphate moiety and the peptide main chain backbone. Although substrate efficacy is distance-dependent, this enzyme does hydrolyze a wide variety of aliphatic and aromatic phosphates. In addition, when compared with the catalytic efficacy of non-peptidic analogs, it is clear that the peptide component of these amalgamated substrates significantly contributes to efficient enzyme-catalyzed hydrolysis. For example, ethanolamine phosphate is hydrolyzed nearly 3 orders of magnitude more efficiently when attached to the active site directed peptide Glu-Glu-Glu. The strategy that we have developed provides the opportunity to deliver relatively simple functionality to specific PTPases through the use of appropriate peptide templates. Furthermore, a comparative analysis of the active site specificities of protein phosphatases may reveal key differences in the ability of individual enzymes to tolerate specific structural motifs. Clearly, any observed differences should prove useful in the design of PTPase-specific inhibitors. The latter would be of decided benefit in helping to define the role of PTPases in cellular signaling.

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