Synthesis of Phosphotyrosine-Containing Peptides and Their Use as Substrates for Protein Tyrosine Phosphatases†

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ABSTRACT: Prior methods for the chemical synthesis of phosphotyrosine-containing peptides involved the incorporation of fully protected phosphoamino acids into the peptide chain or phosphorylation of free phenol side chains after peptide assembly is complete. The present work describes a novel and general methodology for the solid-phase synthesis of phosphopeptides, featuring direct incorporation of N^{α}-(9-fluorenylmethyloxycarbonyl)-*O*-phospho-L-tyrosine (unprotected side chain). This technique obviated the formation of peptide byproducts containing tyrosine H-phosphonate, a previously unrecognized side reaction from literature phosphitylation/oxidation approaches. Phosphopeptides corresponding to the tyrosine phosphorylation site of adipocyte lipid binding protein were synthesized by the newer, preferred method. These peptides were purified and characterized by high-performance liquid chromatography (HPLC), capillary zone electrophoresis (CZE), amino acid analysis (AAA), fast atom bombardment mass spectrometry (FABMS), and ^{31}P nuclear magnetic resonance (^{31}P NMR). The synthetic peptides were tested as substrates for two distinct protein tyrosine phosphatases, rat brain protein tyrosine phosphatase (PTPase) and human acid phosphatase. Substrate specificity was measured at pH 6.0 and 37 °C, using a colorimetric assay for released inorganic phosphate. Kinetic analysis revealed that both the rat brain PTPase and the human adipocyte acid phosphatase catalyzed peptide dephosphorylation but with different rates and affinities. The rat brain PTPase displayed classical Michaelis–Menten kinetics, with K_m's of 68 ± 9 μM and 42 ± 11 μM and k_{cat}/K_m values of 4.9×10^5 s^{-1} M^{-1} and 6.9×10^5 s^{-1} M^{-1} determined for phosphorylated peptides of lengths 4 and 10 residues, respectively. In contrast, the human acid phosphatase demonstrated linear kinetics, with no saturation observed up to 2 mM phosphopeptide. These results demonstrate the utility of the methodology and describe how synthetic tyrosine phosphopeptides can be used to assess the catalytic efficacies of enzymes that hydrolyze protein phosphotyrosine.

Tyrosine phosphorylation is now recognized as an integral component of many intracellular signaling pathways. The receptors for insulin, and for a number of growth factors including epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), function as hormone-stimulated tyrosine kinases [for reviews, see Hunter (1989), Lau et al. (1989), and Ullrich and Schlessinger (1990)]. The complementary actions of protein kinases and phosphatases, which respectively carry out phosphorylation and dephosphorylation of tyrosine residues, control the steady-state phosphorylation level of a number of key cellular proteins implicated in hormone action. Efforts to identify and characterize sequence-specific protein phosphatases have been hampered in part by the difficulty in obtaining sufficient quantities of pure phosphoproteins, due to the small quantities produced in cells, and to the chemical instabilities of *O*-phosphates to extremes of pH. The use of synthetic phosphopeptides corresponding to the substrate phosphorylation site can be an effective means to assess phosphatase activity [Cho et al. (1991) and Ramachandran et al. (1992) and references cited therein]. Therefore, the availability of phosphotyrosine-containing peptides prepared via efficient routes of chemical synthesis becomes a major goal for this approach to phosphatase characterization.

Prior methods for the chemical synthesis of phosphopeptides involve the incorporation of fully protected phosphoamino acids into the peptide chain (Valerio et al., 1989; Kitas et al., 1989, 1991) or phosphorylation of free phenol groups after chain assembly is complete (Bannwarth & Trzeciak, 1987; de Bont et al., 1990; Andrews et al., 1991; Kitas et al., 1991; Perich et al., 1991; Staerkaer et al., 1991). Temporary N^{α}-amino protection can be provided by either *tert*-butyloxycarbonyl (Boc)[1] or 9-fluorenylmethyloxycarbonyl (Fmoc) [for a recent review, see Fields et al. (1992)]. For the synthesis of phosphotyrosine-containing peptides, application of Fmoc methodology is more favorable because of the milder conditions for temporary deprotection and final cleavage/deprotection from the resin. In particular, the aryl oxygen to phosphorus bond is not entirely stable to the strong acids, e.g., anhydrous hydrogen fluoride, used for the final step in Boc methodology (Kitas et al. 1988, 1990). However, problems have also been noted when the Fmoc approach is used, since piperidine (the base for Fmoc removal) promotes partial cleavage of benzyl or methyl phosphate protecting groups (Kitas et al., 1989; Andrews et al., 1991). An optimal protecting group would appear to be the base-stable bis(*tert*-butyl)phosphate. However, N^{α}-Fmoc-*O*-[di-*tert*-butylphosphono]-L-tyrosine [Fmoc-Tyr(PO_3tBu_2)-OH] is difficult to synthesize due to the extreme

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acid lability of the *tert*-butyl group both in the workup and upon storage (Perich & Reynolds, 1991). The alternative post-phosphorylation approaches are appealing, but the required oxidation step may not be compatible with methionine residues, which are frequently found near tyrosine phosphorylation sites (Sun et al., 1991). The present work delineates a hitherto unreported side reaction with post-phosphorylation and continues with the description of a novel and efficient route for synthesis of phosphotyrosine-containing peptides. The new approach applies N^{α}-Fmoc-*O*-phospho-L-tyrosine [Fmoc-Tyr(PO_3H_2)-OH; unprotected phosphate group] for peptide chain assembly on the resin and minimizes various potential problems. This method was used to synthesize phosphopeptides (4 and 10 amino acid residues) corresponding to the tyrosine phosphorylation site of murine adipocyte lipid binding protein (Buelt et al., 1991). The synthetic peptides were used for the kinetic characterization of two enzymes, the rat brain protein tyrosine phosphatase (Guan et al., 1990) and the human adipocyte acid phosphatase, which has been suggested to function as a protein tyrosine phosphatase (Boivin et al., 1987; Zhang & Van Etten, 1990; Shekels et al., 1992).

EXPERIMENTAL PROCEDURES

General. Some of the materials and general peptide synthetic and analytical procedures have been described in recent publications from one of our laboratories [Albericio et al. (1990) and references cited therein; Barany et al., 1992; Ferrer et al., 1992; Solé & Barany, 1992]. Fmoc-amino acid derivatives were from Bachem Bioscience (Philadelphia, PA) or Advanced Chemtech (Louisville, KY). Stepwise synthesis was carried out on a novel poly(ethylene glycol)–polystyrene graft support, MPEG-Orn(Fmoc-PAL)-PS, described elsewhere (Barany et al., 1992). *N*,*N*-Dimethylformamide (DMF) was sequencing grade from Fisher (Pittsburgh, PA) and was flushed with N_2 for at least a day and maintained over Amberlyst-15 (H-form) ion-exchange resin prior to its use for peptide synthesis [confirmed negative to FDNB test, carried out according to Stewart and Young (1984)]. Piperidine, trifluoroacetic acid (TFA), and 1-hydroxybenzotriazole (HOBt) were also sequencing grade from Fisher, and the BOP reagent was from AminoTech (Nepean, Ontario, Canada). [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). L-Tyrosine, isopropyl thiogalactoside (IPTG), glutathione–agarose, 1*H*-tetrazole, di-*tert*-butyl *N*,*N*-diethylphosphoramidite, and all other chemicals or reagents were purchased from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI).

Amino acid analyses were performed on a Beckman 6300 analyzer with a sulfated polystyrene cation-exchange column (0.4 cm × 21 cm). Peptide–resins were hydrolyzed in 12 N HCl–propionic acid (1:1 v/v) + 2 drops liquefied phenol for 1 h at 150 °C, and free peptides were hydrolyzed with 6 N aqueous HCl at 110 °C for 24 h + 2 drops liquefied phenol to prevent degradation of Tyr. Analytical HPLC was performed using a Vydac analytical C-18 reversed-phase column (218TP54, 5 μm, 300 Å; 0.46 cm × 25 cm) on a Beckman system configured with two Model 112 pumps and a Model 165 variable-wavelength detector controlled from an IBM computer with Beckman System Gold software. Peptide samples were chromatographed at 1.0 mL/min with 0.1% aqueous TFA–CH_3CN [49:1 to 1:1 over 30 min for Figures 1, 2, and 4B; in Figure 4A, the gradient was from 49:1 to 19:1 over 5 min and then to 17:3 over the next 20 min] and detected at 220 nm. Semipreparative HPLC was performed using a Vydac semipreparative C-18 reversed-phase column (218TP1010; 10 μm, 300 Å; 1.0 cm × 25 cm) on a Waters Deltaprep system using manual injection (2 mL, ~10 mg of peptide/run) and elution at 5 mL/min using 0.1% aqueous TFA–CH_3CN (49:1 to 1:1 over 30 min) and detection at 220 nm. Capillary zone electrophoresis (CZE) was performed on a Beckman PACE System 2100 in capillary cartridges (fused silica 75 μm i.d. × 50 cm length; 100 × 200 aperture), as follows: prewash for 10 min with running buffer, sodium borate (0.1 M, pH 8.35); equilibration of capillary for 2 min with running buffer; 10-s pressure injection; and separation for 15 min at 25 kV and 25 mA; detection was at 214 nm. Positive ion fast atom bombardment mass spectrometry (FABMS) was carried out on a VG 7070E-HF instrument, scans from 500 to 3000/300 s/decade, operated at a resolution of 3000. Matrices of glycerol–water or thioglycerol–water were used with the various crude and purified synthetic peptide products which were characterized in this work.

N^{α}-(9-Fluorenylmethyloxycarbonyl)-O-phospho-L-tyrosine [Fmoc-Tyr(PO_3H_2)-OH]. First, phospho-L-tyrosine was prepared in 90% yield on an 18-mmol scale by reaction of L-tyrosine, phosphoric acid, and phosphorus pentoxide essentially as described by Alewood et al. (1983). The product was precipitated by addition of *n*-butanol, maintained overnight at 0 °C, filtered, washed, and stored at −20 °C. To introduce the Fmoc group, phospho-L-tyrosine (0.5 g, 1.9 mmol), Fmoc-OSu (0.78 g, 2.3 mmol), and Et_3N (0.27 mL, 1.9 mmol) were suspended in a mixture of H_2O (5 mL) and CH_3CN (5 mL). This heterogeneous mixture was stirred at 25 °C over 30 min, while the pH was adjusted to 8.5–9.0 with additional Et_3N until the pH was constant. The resultant homogeneous solution was concentrated in vacuo, and both EtOAc (50 mL) and H_2O (50 mL) were added. The mixture was acidified to pH ~2 with 12 N HCl, and the organic phase was taken. The aqueous phase was extracted further with EtOAc (2 × 50 mL), and the combined organic phases were filtered through a fine-porosity glass-fritted funnel, washed further with 1 N HCl (2 × 40 mL), H_2O (2 × 40 mL), and saturated aqueous NaCl (2 × 40 mL), and dried ($MgSO_4$). After concentration in vacuo, the title product was obtained as a white crystalline solid (0.76 g, 81%), m.p. 132–135 °C, that was stored at −20 °C (stable for at least 1 month). Exposure to air at ambient temperature turned the product yellow-orange, but the color change did not reflect a change in purity. HPLC showed a major peak (~95%) at 10.0 min

[1] Amino acids and peptides are abbreviated and designated following rules of the IUPAC–IUB Commission of Biochemical Nomenclature (1972) *J. Biol. Chem. 247*, 977–983. Additionally, the following abbreviations are used: ALBP, adipocyte lipid binding protein; Boc, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; CZE, capillary zone electrophoresis; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; EtOAc, ethyl acetate; Fmoc, 9-fluorenylmethyloxycarbonyl; GST, glutathione S-transferase; GST-PTPU323, glutathione S-transferase–rat brain protein tyrosine phosphatase fusion protein; GST-HAAPβ, glutathione S-transferase–human adipocyte acid phosphatase fusion protein; HOBt, 1-hydroxybenzotriazole; IRAA, "internal reference" amino acid; IPTG, isopropyl thiogalactoside; NMM, *N*-methylmorpholine; NMR, nuclear magnetic resonance; Orn, ornithine; OSu, *N*-succinimidoyl ester; PAGE, polyacrylamide gel electrophoresis; PAL, 5-[4-[[(9-fluorenylmethyloxycarbonyl)amino]-methyl]-3,5-dimethoxyphenoxy]valeric acid; MPEG, monofunctional poly(ethylene glycol); pNPP, *p*-nitrophenyl phosphate; PS, polystyrene; PTPase, protein tyrosine phosphatase; SDS, sodium dodecyl sulfate; RP-HPLC, reverse-phase high-performance liquid chromatography; *t*Bu, *tert*-butyl; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; Tmob, 2,4,6-trimethoxybenzyl. Amino acid symbols denote the L-configuration unless indicated otherwise. All solvent ratios and percentages are volume/volume unless stated otherwise.

[0.01 N aqueous HCl–CH_3CN (4:1 to 1:4 over 15 min), 1.2 mL/min, detection at 280 nm]. ^{31}P NMR ($CDCl_3$) referenced to external H_3PO_4, δ – 4.18 (s); ^1H NMR (CD_3CN) δ 7.75 (d, J = 7.3 Hz, 2 H, Fmoc), 7.54 (d, J = 7.3 Hz, 2 H, Fmoc), 7.36 (t, J = 7.2 Hz, 2 H, Fmoc), 7.23 (d, J = 7.2, 2 H, Fmoc), 7.14 (d, J = 8.6 Hz, 2 H, Tyr aromatic), 7.06 (d, J = 8.6 Hz, 2 H, Tyr aromatic), 4.23 (d, J = 6.9 Hz, Fmoc CH_2), 4.14 (t, J = 6.9 Hz, Fmoc CH), 3.4 (q, J unresolved, α-CH), 3.08 (dd, J = 5.0 Hz, 1 H, β-H), 2.86 (dd, J unresolved, 1 H, β-H); FABMS (thioglycerol matrix) calculated monoisotopic mass of $C_{24}H_{22}O_8NP$ 483.11, positive spectrum m/z 504.2 [$(M + Na)^+$], 484.2 [MH^+], negative spectrum m/z 482.2 [$(M - H)^-$]. Anal. Calcd for $C_{24}H_{20}O_8NP$, mol wt 483.42: C, 59.63; H, 4.59; N, 2.90; P, 6.41. Found: C, 59.88; H, 4.71; N, 2.69; P, 6.12.

Synthesis, Purification, and Characterization of Phosphotyrosine-Containing Peptides. Solid-phase synthesis was carried out starting with an MPEG-Orn(Fmoc-PAL)-PS resin (0.5 g, 0.22 mmol of amino sites/g, 0.11 mmol) and using appropriate N^{α}-Fmoc-amino acids (0.44 mmol each, 4.0 equiv). Side-chain protecting groups were *tert*-butyl (*t*Bu) ethers and esters for Asp, Ser, and Glu, Boc for Lys, and 2,4,6-trimethoxybenzyl (Tmob) for Asn. Fmoc removal was with piperidine–DMF (1:4, 2 + 8 min), followed by washing with DMF (5 × 2 min) and CH_2Cl_2 (5 × 2 min) (volume of 5 mL for deprotection and all washing steps). Couplings were achieved by combining solid N^{α}-Fmoc-amino acids (0.44 mmol), 1-hydroxybenzotriazole (HOBt) (67 mg, 0.44 mmol), benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) (194 mg, 0.44 mmol), and *N*-methylmorpholine (NMM) (96 μL, 0.88 mmol) in DMF (0.88 mL) for 10 min under N_2 at 25 °C and then adding this preactivated solution to the peptide–resin. Coupling of N^{α}-Fmoc-phospho-L-tyrosine and all subsequent couplings were carried out by the same protocol, but substituting *N,N*-diisopropylethylamine (DIEA) (0.15 mL, 0.88 mmol) for NMM. Single couplings were carried out for 1 h, although ninhydrin tests (Kaiser et al., 1970) on resin aliquots were often negative at 30 min. Occasionally, the ninhydrin test was slightly positive after 1 h coupling of Fmoc-Tyr(PO_3H_2)-OH or the immediate next Fmoc-amino acid in the sequence, so these residues were recoupled with fresh reagents for an additional 1 h. Upon completion of chain assembly, the N-terminal Fmoc group was removed and the otherwise protected peptide–resin was dried for 24 h in vacuo (1 mm). Reagent K, TFA–phenol–H_2O–thioanisole–1,2-ethanedithiol (33:2:2:2:1) (King et al., 1990), was used for deprotection and cleavage from the resin. The cleavage cocktail (5 mL) was prepared freshly, degassed with N_2, and added to the dry protected peptide–resin (0.5 g). After 4 h at 25 °C, the cleavage mixture was filtered, and the cleaved resin was washed with additional reagent K (2 × 2 mL). The peptide was precipitated from the combined filtrates with diethyl ether (30 mL) at 0 °C, collected by low-speed centrifugation, washed with cold ether (5 × 10 mL), dissolved in H_2O (20 mL), lyophilized, and purified.

Using the general procedures described, two phosphopeptides were prepared: the tetrapeptide H-Asp-Tyr(PO_3^{2-})-Met-Lys-NH_2 and the decapeptide H-Ser-Ser-Glu-Asn-Phe-Asp-Asp-Tyr(PO_3^{2-})-Met-Lys-NH_2. Hydrolysis and amino acid analysis of the tetrapeptide–resin showed the expected ratios [Asp, 0.95; Met, 0.67; Tyr, 0.51; Orn, 1.09; Lys, 1.00] and final substitution level (0.19 mmol of amino sites/g of peptide–resin). After cleavage, the crude tetrapeptide (77 μmol, 96% cleavage yield) was applied to semipreparative HPLC (see General section). Fractions with the correct peptide were pooled and lyophilized to provide a white powder (18 μmol, 26% isolated yield for preparative chromatography), which was pure by HPLC (Figure 1B) and CZE (Figure 3A). Hydrolysis and amino acid analysis of the peptide showed the expected ratios: [Asp, 1.06; Met, 0.68; Tyr, 1.17; Lys, 1.00]. The purified phosphopeptide was characterized further by FABMS (thioglycerol matrix): calculated monoisotopic mass of $C_{24}H_{39}N_6O_{10}SP$ 634.22, positive spectrum m/z 657.4 [$(M + Na)^+$], 635.4 [MH^+]; negative spectrum m/z 655.3 [$(M - 2H + Na)^-$], 633.3 [$(M - H)^-$], and ^{31}P NMR (D_2O) [referenced to external H_3PO_4] δ 1.43 (s).

Hydrolysis and amino acid analysis of the decapaptide–resin showed the expected ratios [Asp, 2.67; Ser, 1.02; Glu, 0.85; Met, 0.90; Tyr, 0.45; Phe, 0.83; Orn, 1.06; Lys, 1.0] and final substitution level (0.14 mmol of amino sites/g of peptide–resin). After cleavage, the crude decapeptide (27 μmol, 97% cleavage yield) was purified by semipreparative HPLC to provide a white powder (5 μmol, 19% isolated yield) which was pure by HPLC (Figure 2B) and CZE (Figure 3B). Hydrolysis and amino acid analysis of the peptide showed the expected ratios: [Asp, 2.19; Ser, 1.17; Glu, 0.72, Phe, 0.79; Tyr, 0.75; Met, 0.49; Lys, 1.00]. Further evidence for the structure was from FABMS (thioglycerol matrix): calculated monoisotopic mass of $C_{52}H_{76}N_{13}O_{23}SP$ 1313.46, positive spectrum m/z 1336.2 [$(M + Na)^+$], 1314.2 [MH^+]; negative spectrum m/z 1334.3 [$(M - 2H + Na)^-$], 1312.4 [$(M - H)^-$], and ^{31}P NMR (D_2O) [referenced to external H_3PO_4] δ 0.02 (s).

Documentation of a Novel Side Reaction upon Use of a Literature Procedure for Synthesis of Phosphotyrosine-Containing Peptides. Prior to the development of the preferred procedure just given, we explored postsynthetic approaches that involve phosphoramidite chemistry and an oxidation step. All aspects of chain assembly were the same as already given (0.3–0.4 g of starting resin; NMM used as base throughout for single 1-h couplings), except that tyrosine was incorporated as its N^{α}-Fmoc derivative (free phenol side chain). The completed peptide–resin retaining the N-terminal Fmoc was dried in vacuo overnight, and aliquots were phosphitylated as follows [reaction volumes are cited for 50-mg scale; in one case 300-mg scale was used]: under argon, ^1H-tetrazole (50 equiv) from a 1 M solution in *dry* tetrahydrofuran (THF) (0.52 mL) was added, followed by di-*tert*-butyl *N,N*-diethylphosphoramidite (30 μL, 10 equiv). The reaction was stirred for 1 h at 25 °C, following which the resin was filtered and washed with THF (5 × 5 mL) and chilled to 0 °C. Next, 70% aqueous *tert*-butyl hydroperoxide (30 μL, 20 equiv of oxidizing agent) was added for a 45-min reaction at 0 °C. The resultant phosphorylated peptide–resin was then washed with THF (5 × 5 mL) and anhydrous ether (5 × 5 mL) and dried for 24 h in vacuo (1 mm). Fmoc removal, cleavage with reagent K, workup, HPLC analysis, and (in one case) semipreparative HPLC were carried out in the same way described previously. For the two target phosphopeptides of this work, HPLC of the crude products (Figure 4) showed a major peak coeluting with the correct structure and a second trailing peak (14–16%). FABMS of the crude tetrapeptide gave not only the expected ion at m/z 635.6 [MH^+] but also a significant ion at m/z 619.2. Similarly, FABMS of the crude decapeptide gave ions at m/z 1314.7 as well as m/z 1298.7. In the latter case, the byproduct was isolated pure and showed only the FABMS ion at m/z 1298.5. Further evidence for the assignment of the byproduct as the H-phosphonate peptide came from experiments in which the oxidation step was

intentionally *not* carried out. In these cases, HPLC of the crude cleavage products showed single major peaks corresponding to the elution positions of the byproducts in the synthesis that included the oxidation steps.

Purification of Rat Brain Protein Tyrosine Phosphatase and Human Adipocyte Acid Phosphatase. Rat brain PTPase (Guan et al., 1990; Guan & Dixon, 1991) and acid phosphatase (Shekels et al., 1992) were expressed in *Escherichia coli* JM109 and purified as glutathione S-transferase (GST) fusion protein, as described by Smith and Johnson (1988). Briefly, overnight cultures harboring the recombinant plasmid were diluted 1:10 and allowed to grow for 1 h at 37 °C. Expression of the fusion protein was induced by addition of IPTG to a final concentration of 0.1 mM. The culture was allowed to grow for 3–6 h, after which time cells were collected by centrifugation and resuspended in a solubilization buffer containing 20 mM sodium phosphatate, pH 7.3, 150 mM NaCl, and 1% (v/v) Triton X-100. Cells were lysed by sonication, and bacterial debris was removed by centrifugation. The supernatant fraction was mixed with glutathione–agarose, and the fusion protein was allowed to bind at 4 °C for 15 min. The resin was collected in a column and washed with 10 mM sodium acetate, pH 6.0, 5 mM EDTA, and 10% (v/v) glycerol. The GST fusion protein was then eluted from the resin with 5 mM glutathione in the same buffer. Protein concentration was determined by the method of Bradford (1976), and phosphatase activity was followed throughout the purification using *p*-nitrophenyl phosphate (pNPP) as substrate. The purity of the eluted fusion proteins was assessed by SDS–PAGE (Laemmli, 1970) followed by Coomassie blue staining and was generally ≥95% pure. The specific activity of the purified acid phosphatase fusion (GST-HAAPβ) was typically 10–20 units/mg; for the PTPase (GST-PTPU323), it was 20–30 units/mg.

Phosphatase Assays: (*A*) *pNPP as Substrate.* Activity was measured at 37 °C in 50 mM sodium acetate buffer, pH 6.0. Either GST-HAAPβ or GST-PTPU323 (typically 1 μg) was incubated with a given concentration of pNPP (50–1000 μM) for 10 min in a final volume of 0.5 mL. Reactions were quenched by addition of 1 N NaOH (1 mL), and the amount of *p*-nitrophenol released was measured at 405 nm using a molar extinction coefficient of 18 000 M^{-1} cm^{-1} (Zhang et al., 1990). Kinetic parameters were calculated from double-reciprocal plots as described previously (Shekels et al., 1992).

(*B*) *Phospho-L-tyrosine as Substrate.* The phosphatases (1–2 μg) were incubated for 5 min in a final volume of 0.5 mL with substrate (0.5–50 mM) in 50 mM sodium acetate buffer, pH 6.0. Dephosphorylation was stopped by addition of 1 N NaOH (1 mL), and formation of tyrosine was followed by measuring absorbance at 293 nm, using a molar extinction coefficient of 2381 M^{-1} cm^{-1} (Zhang et al., 1990).

(*C*) *Phosphotyrosine-Containing Protein as Substrate.* Homogeneous phosphoALBP was prepared as described by Buelt et al. (1992). Briefly, ALBP (1–2 mg) was incubated at 37 °C for 6–12 h in a buffer containing 25 mM Tris-HCl, pH 7.4, 4 mM $MnCl_2$, and 100 μM γ-[^{32}P]ATP (30 Ci/mol), with the autophosphorylated soluble kinase domain of the human insulin receptor (Cobb et al., 1989). Following ALBP phosphorylation, the unreacted ATP was removed by Excellulose GF-5 desalting chromatography (Pierce). The proteins were applied to DEAE-Sephadex resin in the same buffer, and phosphoALBP and ALBP were recovered in the non-binding fractions, free of the catalytic domain of the insulin receptor. Separation of phosphoALBP from ALBP was achieved by anti-phosphotyrosine–agarose immunoaffinity chromatography. The bound phosphoALBP was eluted from the resin with 10 mM pNPP in 25 mM Tris-HCl, pH 7.4. The protein was concentrated and the buffer was changed to 50 mM sodium acetate, pH 6.0, by Centricon-10 microconcentration (Amicon). The concentration and purity of the phosphoprotein was determined by a combination of spectrophotometry, scintillation counting, and analytical isoelectric focusing (Buelt et al., 1992). The catalytic efficacies of the two phosphatases toward phosphoprotein substrate were assessed by addition at 37 °C with phosphoadipocyte lipid binding protein (100 nM) in 50 mM sodium acetate buffer, pH 6.0. Typically 1–2 μg of phosphatase was incubated in a final volume of 250 μL with 100 nM phosphoALBP (~2000 dpm/μg) and at various times (0–60 min) aliquots of the reaction (20 μL) were removed, mixed with an equal volume of sample buffer (Laemmli, 1970), and heated for 3 min at 95 °C to quench the reaction. The extent of dephosphorylation was followed by SDS–PAGE and autoradiography and quantitated by direct scintillation counting of the radioactive protein band excised from the dried polyacrylamide gel.

(*D*) *Phosphotyrosine-Containing Peptides as Substrates.* The desired concentration of synthetic phosphopeptide (10 μM–2 mM) was established in a final reaction volume of 0.5 mL from a 4 mM aqueous stock in 50 mM sodium acetate buffer, pH 6.0. The reactions were initiated by the addition of 1–2 μg of enzyme and were incubated at 37 °C for either 10 min for GST-PTPU323 or for 45 min with GST-HAAPβ. In preliminary experiments, phosphate release was shown to be linear under these conditions with time and amount of enzyme (results not shown). The reaction was stopped with the addition of 0.5 mL of 3% aqueous trichloroacetic acid. Color was developed by the addition of 0.2 mL of 2% ammonium molybdate tetrahydrate and 0.3 mL of 14% ascorbic acid in 50% aqueous TCA (Black & Jones, 1983). One minute later, 1 mL of a solution containing 2% trisodium citrate dihydrate, 2% sodium arsenite, and 2% acetic acid was added. After 10 min, the absorbance at 700 nm was measured. Nanomoles of inorganic phosphate (P_i) released was calculated by comparison to a standard curve. All kinetic parameters were calculated from double-reciprocal plots as described previously (Shekels et al., 1992).

RESULTS

Synthesis and Purification of Phosphotyrosine-Containing Peptides. The substrates for this study were a tetrapeptide and decapeptide corresponding to the tyrosine phosphorylation site of murine adipocyte lipid binding protein (Buelt et al., 1991). Solid-phase synthesis with Fmoc chemistry used BOP/HOBt protocols for couplings [Hudson, 1988; Albericio et al. (1990) and references cited therein]. The base used was NMM for the first part of the synthesis and DIEA for the incorporation of Fmoc-phospho-L-tyrosine (unprotected side chain) and *all subsequent* amino acids. The final substitution levels observed were in accord with expectation for quantitative chain assembly and no loss of chains from the support. Detachment of the linear sequences from the support, and concurrent removal of all side-chain protecting groups, occurred readily upon treatment with freshly prepared reagent K (King et al., 1990). Cleavage yields were uniformly high (90–97%), as judged by amino acid ratios with respect to Orn "internal reference" in the recovered cleaved resins. Moreover, the analytical reversed-phase high-performance liquid chromatography (HPLC) traces of crude material suggested good

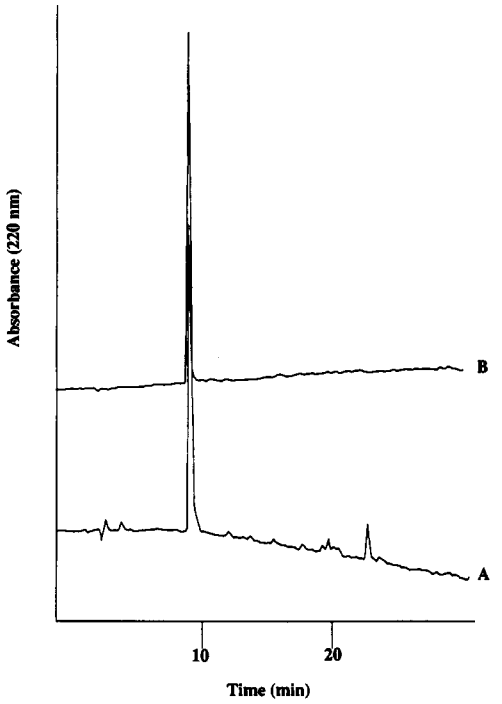

FIGURE 1: Analytical HPLC of H-Asp-Tyr(PO_3^{2-})-Met-Lys-NH_2. See Experimental Procedures for details about HPLC conditions. (A) Crude peptide directly after cleavage; (B) purified synthetic peptide after semipreparative HPLC. The desired peptide elutes at 9.7 min; the corresponding dephosphorylated peptide elutes at 18.7 min (determined from separate synthesis). A partially oxidized (methionine sulfoxide) phosphopeptide would be assumed to elute several minutes earlier upon HPLC, based on experience with other peptides.

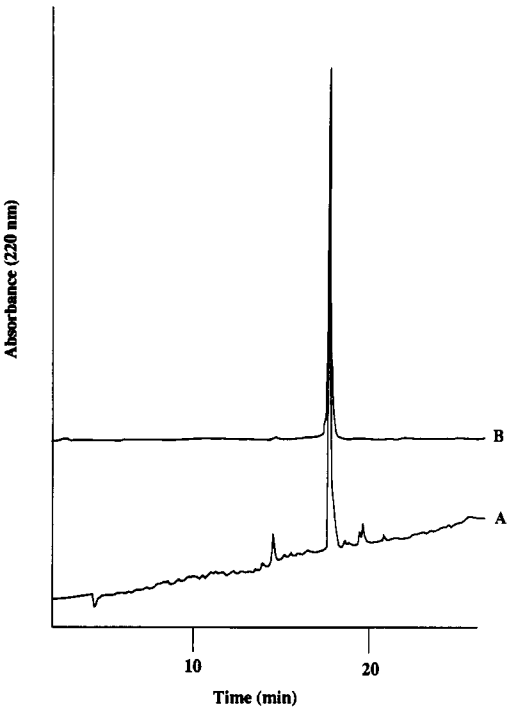

FIGURE 2: Analytical HPLC of H-Ser-Ser-Glu-Asn-Phe-Asp-Asp-Tyr(PO_3^{2-})-Met-Lys-NH_2. See Experimental Procedures for details about HPLC conditions. (A) Crude peptide directly after cleavage; (B) purified synthetic peptide after semipreparative HPLC. The desired peptide elutes at 18.3 min; the corresponding dephosphorylated peptide elutes at 20.8 min (determined from separate synthesis). A partially oxidized (methionine sulfoxide) phosphopeptide would be assumed to elute several minutes earlier upon HPLC based on experience with other peptides.

purity for both the tetra- and decapeptide directly from the cleavages (traces A, Figures 1 and 2). The synthetic phosphopeptides were purified by high-performance liquid chromatography (HPLC) and characterized by amino acid analysis (AAA), capillary zone electrophoresis (CZE) (Figure 3), fast atom bombardment mass spectrometry (FABMS), and ^{31}P nuclear magnetic resonance (^{31}P NMR). The various analytical criteria revealed that synthetic phosphopeptides were free (<1%) of potential contaminating peptides due to either dephosphorylation (authentic synthetic samples available for comparison) or methionine oxidation.

H-Phosphonate Byproducts upon Use of a Literature Postphosphorylation Approach. Prior to our demonstration that Fmoc-phospho-L-tyrosine is an ideal intermediate for phosphopeptide synthesis, we explored the approach of first incorporating Fmoc-L-tyrosine (free side chain). Once the peptide sequence was assembled fully on the support, the desired phosphorylation was carried out by phosphoramidite chemistry (Bannworth & Trzeciak, 1987; de Bont et al., 1990; Andrews et al., 1991; Kitas et al., 1991; Perich et al., 1991; Staerker et al., 1991). HPLC analysis of the crude products after cleavage (Figure 4) showed significant byproducts which were absent from chromatograms of the corresponding stage of the preferred synthetic procedure (traces A, Figures 1 and 2). FABMS revealed that these byproducts had a molecular weight 16 amu less than the desired phosphopeptide. The byproducts also become the major species when the oxidation step was omitted intentionally. We conclude that the byproducts should be assigned the H-phosphonate structure ROP(H)(OH)(=O) [P^{5+} valence state], which are the favored tautomers over $ROP(OH)_2$ [P^{3+} valence state] (Corbridge et al., 1966). The H-phosphonate byproducts evidently arise from incomplete oxidation of the phosphitylated intermediate

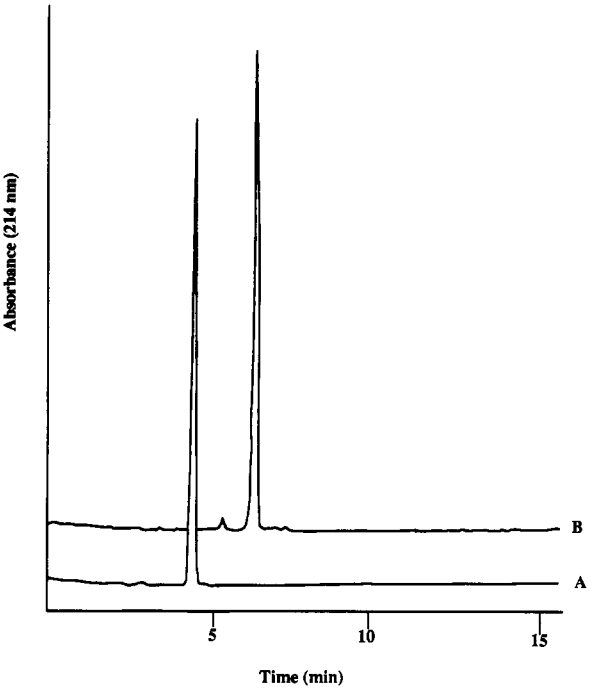

FIGURE 3: Capillary zone electrophoresis (CZE) of purified synthetic peptides after semipreparative HPLC. See Experimental Procedures for details about CZE conditions. (A) H-Asp-Tyr(PO_3^{2-})-Met-Lys-NH_2 elutes at 4.8 min; (B) H-Ser-Ser-Glu-Asn-Phe-Asp-Asp-Tyr(PO_3^{2-})-Met-Lys-NH_2 elutes at 5.5 min.

[P^{3+} level]; however, they could not be eliminated by changes in oxidation conditions such as variety in reagents and/or solvents, longer reaction times, and increased or decreased temperatures.

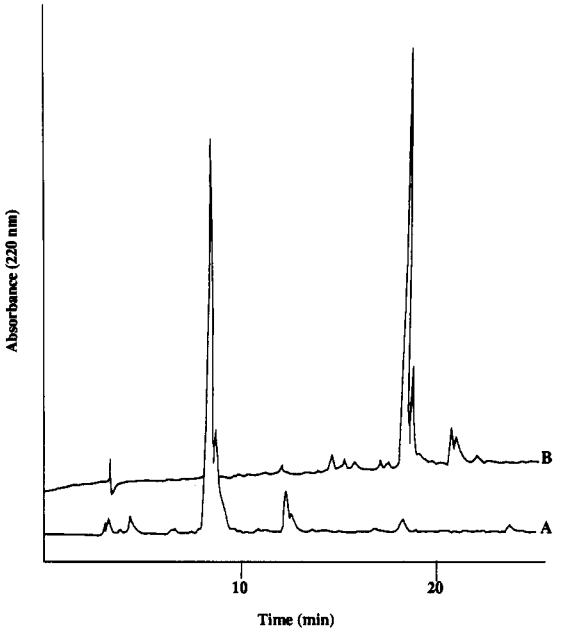

FIGURE 4: Analytical HPLC of crude phosphopeptides prepared by phosphitylation/oxidation approach. (A) H-Asp-Tyr(PO_3^{2-})-Met-Lys-NH_2; (B) H-Ser-Ser-Glu-AsnPhe-Asp-Asp-Tyr(PO_3^{2-})-Met-Lys-NH_2. In each case, the major peak coelutes with the correct product (compare to Figures 1 and 2). The significant byproducts, respectively at 0.4 min (A) and 0.5 min (B) after the desired phosphopeptide, became the *major* products when the oxidation step was *intentionally omitted*.

Table I: Comparison of the Catalytic Properties of GST-HAAPβ and GST-PTPU323[a]

	GST-HAAPβ	GST-PTPU323
	pNPP (n = 5)	
K_m (μM)	57	120
V_{max} [μmol min^{-1} (mg of enzyme)$^{-1}$]	20	32
k_{cat}/K_m (s^{-1} M^{-1})	2.6×10^5	2.8×10^5
	phosphotyrosine (n = 3)	
K_m (mM)	9.1	1.2
V_{max} [μmol min^{-1} (mg of enzyme)$^{-1}$]	10.2	14.2
k_{cat}/K_m (s^{-1} M^{-1})	8.2×10^2	1.2×10^4
	phosphoALBP (100 nM, n = 2)	
velocity [μmol min^{-1} (mg of enzyme)$^{-1}$]	1.2×10^{-5}	2.0×10^{-3}

[a] The kinetic constants for GST-HAAPβ and GST-PTPU323 were determined at pH 6 and 37 °C as described in Experimental Procedures. Kinetic constants were calculated from double-reciprocal plots as described previously (Shekels et al., 1992). Results presented are from one experiment repeated n times.

Characterization of Rat Brain PTPase and Human Adipocyte Acid Phosphatase. Both enzymes were evaluated first for their ability to hydrolyze in vitro the two small substrates, *p*-nitrophenyl phosphate and phospho-L-tyrosine. Dephosphorylation of pNPP by GST-HAAPβ revealed a K_m of 57 μM and a V_{max} of 20 μmol min^{-1} mg^{-1}, whereas GST-PTPU323 activity on pNPP yielded a K_m of 120 μM and a V_{max} of 32 μmol min^{-1} mg^{-1}. Therefore, k_{cat}/K_m values for hydrolysis of pNPP by the two phosphatases are similar (Table I). Using phospho-L-tyrosine as substrate, GST-HAAPβ dephosphorylated the amino acid with a K_m of 9.1 mM and a V_{max} of 10.2 μmol min^{-1} mg^{-1}. Comparable affinity (14 mM) for phospho-L-tyrosine was observed previously for bovine heart phosphatase (Zhang et al., 1990). The kinetic parameters for the rat brain PTPase GST-PTPU323 were also determined for phospho-L-tyrosine and contrasted with GST-HAAPβ: the rate of dephosphorylation (14.2 μmol min^{-1} mg^{-1}) was similar, but affinity (K_m = 1.2 mM) was almost 10-fold higher (Table I). Calculation of k_{cat}/K_m values reveals that GST-PTPU323 hydrolyzes the phosphoamino acid about 15 times more effectively than the acid phosphatase.

Despite the limited availability of phosphotyrosine-containing proteins, we were able to evaluate the efficacy of the two enzymes toward phosphoALBP dephosphorylation at a single concentration of substrate. PhosphoALBP (100 nM) was dephosphorylated by the acid phosphatase with a velocity of 1.2×10^{-5} μmol min^{-1} mg^{-1}. In contrast, the rat brain PTPase exhibited a much greater rate of hydrolysis ($V = 2.0 \times 10^{-3}$ μmol min^{-1} mg^{-1}) than that observed for GST-HAAPβ (Table I).

A more complete characterization of the two enzymes was conducted using pure synthetic phosphopeptides of lengths 4 and 10 residues. The K_m and k_{cat}/K_m values for both phosphopeptides were measured by the colorimetric assay developed by Black and Jones (1983), which measures the release of P_i by formation of a blue molybdate complex. This method has the advantage of allowing an easy and accurate determination of kinetic constants, without requiring the use of scarce ^{32}P-labeled peptides or proteins. Both peptides were dephosphorylated by the two enzymes, but with grossly different rates and affinities. The rat brain PTPase incubated with the tetrapeptide at pH 6.0 exhibited a K_m of 68 ± 9 μM and a V_{max} of 38 μmol min^{-1} mg^{-1}, giving a k_{cat}/K_m of 4.9×10^5 s^{-1} M^{-1}. The decapeptide had a slightly higher binding affinity with a K_m of 42 ± 11 μM and a similar V_{max} of 34 μmol min^{-1} mg^{-1}, giving a k_{cat}/K_m of 6.9×10^5 s^{-1} M^{-1}. These results show that both peptides were efficiently dephosphorylated by the rat brain PTPase. In contrast, when assayed with the human adipocyte acid phosphatase at pH 6.0, both peptides showed nonsaturating linear kinetics up to 2 mM phosphopeptide, indicating very low binding affinity and catalytic efficacy. These results indicate that the acid phosphatase preferentially dephosphorylates small molecules and that phosphopeptides can clearly be discriminated by the two enzymes.

DISCUSSION

The goal of this investigation was to synthesize and characterize phosphotyrosine-containing peptides that could be used to assess the catalytic efficacy of tyrosine phosphatases. Despite the utility of polymerase chain reaction techniques to isolate large numbers of new clones corresponding to phosphatases, the methodology to assess the catalytic properties of these enzymes is lacking. In addition, strategies to purify tyrosine phosphatases specific for a given phosphorylation site require the availability of sufficient quantities of homogeneous substrates. The ensuing discussion considers in turn the applicability and usefulness of low molecular weight phosphoorganic, large phosphoprotein, and intermediate-size phosphopeptides as substrates to address these issues.

This work examines two phosphatases which exhibit protein tyrosine phosphatase activity in vitro but are clearly of different classes. The rat brain PTPase belongs to that family of enzymes widely acknowledged to participate in intracellular protein tyrosine phosphatase hydrolysis of substrates that have been phosphorylated by growth factor receptors, oncogenes, or protooncogenes (Alexander, 1990). In contrast, the acid phosphatases (which share no sequence similarity to the PTPases but also catalyze protein tyrosine phosphatase activity in vitro) dephosphorylate a wide variety of small molecules including flavin 5′-mononucleotide (Fuchs et al., 1992).

Examination of the kinetic properties of the rat brain PTPase and the acid phosphatase indicates that both enzymes are able to readily dephosphorylate the small molecule pNPP. While pNPP hydrolysis is a convenient assay method frequently used to follow the progress of tyrosine phosphatase purifications (Liao et al., 1991), it clearly does not discriminate between classes of phosphatases. The use of phospho-L-tyrosine affords a somewhat greater degree of specificity when dealing with different phosphatases (i.e., a 15-fold difference in k_{cat}/K_m). However, the discrimination is not significant when considering just the velocity of the reaction at saturating substrate (Table I), a condition routinely utilized during protein purification.

Clearly, homogeneous phosphoproteins are the ideal substrates either for characterization of a cloned phosphatase or for purification of new activities. Unfortunately, the required amounts of such substrates are often difficult or impossible to obtain. In addition, common procedures for phosphorylation of proteins with tyrosine kinases often result in less than stoichiometric phosphorylation and must be followed by extensive purification using anti-phosphotyrosine antibodies in an immunoaffinity chromatography procedure (Buelt et al., 1992). We were able to isolate sufficient pure phosphoALBP to allow its testing at a single concentration (100 nM) as substrate for the two phosphatases under investigation. An approximately 165-fold velocity difference was found, with the PTPase having the far greater hydrolysis rate.

Phosphorylated peptides such as Raytide or phosphoangiotensin I have been used successfully as substrates for PTPases (Pot et al., 1991). For such cases, peptides must first be phosphorylated in vitro by tyrosine kinases and subsequently purified. Therefore, such techniques are limited in terms of the numbers and complexities of phosphopeptides that can be generated for study. The ready availability of synthetic phosphopeptides for use as substrates would alleviate these problems.

The underlying rationales behind prior approaches for synthesis of phosphopeptides have been covered in the introduction. Our approaches focused on Fmoc chemistry with the tris(alkoxybenzyl)amide (PAL) anchor [Albericio et al. (1990) and references cited therein] and side-chain protecting groups which are all cleaved in a final, relatively mild step with TFA/scavenger mixtures, specifically reagent K [King et al. (1990) and references cited therein]. To obviate a new side reaction first discovered as part of this research for the phosphitylation/oxidation approach, we opted to leave the side-chain phosphate on tyrosine *unprotected*. We are aware of only a single short report, lacking experimental details, on the use of Fmoc-Tyr(PO_3H_2); this derivative was said to provide relatively homogeneous products when incorporated near the amino terminus, but problems were noted in other cases and the protected phosphate approach was recommended (Chatterjee et al., 1992). Boc-Tyr(PO_3H_2)-OH has been applied with success, the key being the use of the sodium salt of this derivative and addition of HOBt to catalyze coupling (Zardeneta et al., 1990). In the Fmoc methodology of the present paper, the potent BOP/HOBt/NMM coupling protocol was found to provide sufficient activation to achieve incorporation of phosphotyrosine while avoiding potential side reactions at the unprotected phosphate side chain. This concurs with our recent demonstration that BOP/HOBt/DIEA serves to introduce unprotected tyrosine sulfate in Fmoc chemistry (Bontems et al., 1992). The phosphopeptide syntheses reported here are noteworthy for the high initial purities of the crude cleaved products (traces A, Figures 1 and 2), in particular, the avoidance of both inadvertent dephosphorylation and oxidation at sensitive methionine residues. Such problems have been reported with several literature methodologies (Kitas et al., 1989, 1990; Andrews et al., 1991).

The new procedure was utilized to prepare tyrosine phosphorylated peptides of lengths 4 and 10 residues based on the phosphoALBP sequence. These phosphopeptides were used to test the generality of the result already noted on discrimination of two phosphatase enzymes on the full phosphoALBP substrate. Both enzymes were able to dephosphorylase the synthetic peptide substrates, albeit at grossly different rates. The rat brain PTPase hydrolyzed each of the phosphopeptides with simple Michaelis–Menten kinetics. The k_{cat}/K_m value of approximately 10^5 for the peptides is about the same as that using pNPP as the substrate, but far greater than that for phospho-L-tyrosine. Hence, the synthetic phosphopeptides are excellent substrates. In contrast, dephosphorylation of both the 4- and 10-residue phosphopeptides by the acid phosphatase exhibited linear kinetics with little if any detectable saturation at concentrations up to 2 mM. Therefore, the synthetic peptides represent pure substrates that are available in quantitiy which can be used efficiently to assay and discriminate between protein tyrosine phosphatases.

Tyrosine-containing phosphopeptides may have applications beyond their use as substrates for protein tyrosine phosphatases. Attention has been drawn recently to molecules that associate noncatalytically with domains on growth factor receptors containing phosphotyrosine residues (Mayer et al., 1991). Several molecules implicated in cell signaling, such as phosphatidylinositol-3-kinase, phospholipase C-γ, the *ras* GTPase activator protein (GAP) and the *crk* oncogene [reviewed by Koch et al. (1991)] possess sequences termed *src* homology 2 (SH2) domains, i.e., 100-residue domains responsible for phosphotyrosine binding. The availability of phosphotyrosine-containing peptides will allow for a more extensive biochemical examination of SH2 domains, particularly with regard to their association with regulatory regions on signaling molecules containing phosphotyrosine.

CONCLUSIONS

This paper reports a novel and convenient methodology for the synthesis of phosphotyrosine-containing peptides. Such peptides can be prepared in sufficient quantity and acceptable purity to be used as substrates for enzymes catalyzing phosphotyrosine hydrolysis. Kinetic analysis of such enzymes reveals that while PTPases efficiently catalyze phosphotyrosine dephosphorylation, acid phosphatases function poorly for this purpose. These findings are consistent with the idea that while acid phosphatases carry out protein tyrosine phosphate dephosphorylation in vitro, they are more likely to function as flavin mononucleotide phosphatases in vivo. We anticipate future use of synthetic phosphotyrosine-containing peptides for biochemical examination of a number of processes involving tyrosine phosphorylation/dephosphorylation cycles related to growth control, differentiation, and oncogenesis.

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