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Synthesis and Fluorescence Properties of a Substrate for a Continuous Fluorimetric Assay of Protein Tyrosine Phosphatases

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Abstract: A phosphotyrosine containing peptide, H-Thr-Glu-Pro-Glu-Tyr(PO_3H_2)-Gln-Pro-Gly-Glu-NH_2, has been synthesized to study the effect of phosphate substitution on the intrinsic fluorescence of the free tyrosine-peptide. The observed difference in fluorescence between the phosphorylated and unphosphorylated peptide at 300 nm upon excitation at 275 nm can be applied for continuous fluorimetric assays of protein-tyrosine phosphatases.

Enzyme-catalyzed phosphorylation-dephosphorylation of tyrosyl residues in proteins is an important regulatory system.[1] Studies of the mode of action and substrate specificity of protein tyrosine kinases and phosphatases would be facilitated by efficient continuous spectrophotometric methods to monitor the enzymatic activity of these enzymes. Our interest in the kinetics of inhibition of the tyrosine phosphatase activity of calcineurin[2] led us to develop a fluorescent assay for protein tyrosine phosphatases (PTPases) as an alternative to the existing, labor intensive assays.[3] Fluorescence of peptides and proteins arises from the tryptophanyl and tyrosyl contributions,[4] which can vary with structural, environmental, and energy transfer phenomena. We posited that the fluorescence of phosphotyrosine-containing and the corresponding free tyrosine peptides would differ and that this change in fluorescence could be used to follow enzyme catalyzed dephosphorylation. To test this idea, the oligopeptide H-Thr-Glu-Pro-Glu-Tyr(PO_3H_2)-Gln-Pro-Gly-Glu-NH_2 [pp60$^{c\text{-}src}$(pY527)], which has been reported to be a substrate of several PTPases,[5] was synthesized as a model peptide. Recently, a series of new PTPases assays[6] was reported, which prompts us to disclose in this letter our concurrent work to develop a continuous fluorimetric assay for PTPases based on the fluorescence properties of pp60$^{c\text{-}src}$(pY527) and pp60$^{c\text{-}src}$(Y527) peptides.

Synthesis of the phosphorylated peptide was carried out on solid-phase by post-assembly phosphitylation and oxidation.[7] By phosphorylating a portion of the resin-bound peptide, the simultaneous synthesis of both phosphorylated and unphosphorylated peptide is achieved. The incorporation of Glu(O^{t}Bu), Gly, Pro, and Gln was accomplished by standard Fmoc-methodology[8] using 1,3-diisopropylcarbodiimide (DIPCDI) in the presence of 1-hydroxybenzotriazole (HOBT) as coupling agent. Unprotected tyrosine and the subsequent amino acids were coupled using [(1*H*-benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate (BOP),[9] following a minimal side-chain protection strategy proposed by Felix and *coworkers*[10] for the incorporation of

unprotected aromatic or aliphatic hydroxyamino acids. Phosphitylation of the resin-bound peptide was carried out using bis(benzyloxy)(diisoproylamino)phosphine, and oxidation was performed using the mild oxidant, *tert*-butylhydroperoxide. Once chain assembly was completed, the two peptide-resins were cleaved and the amino acid side chains simultaneously deprotected with trifluoroacetic acid (TFA) and water as a carbocation scavenger. Purification was accomplished by high pressure liquid chromatography (HPLC) and the purified products, the phosphorylated pp60$^{c\text{-}src}$(pY527) and unphosphorylated pp60$^{c\text{-}src}$(Y527) peptides, were characterized by analytical reversed-phase HPLC and fast atom bombardment mass spectrometry (FABMS).[11] The mass spectra of the phosphorylated peptide indicated that it was still partially protected with one benzyl group. Complete removal of the benzyl protecting group required further treatment with TFA:H_2O (95:5, v/v). After completion of the reaction (4 h), the crude peptide was purified by HPLC (19-33 % overall yield from the peptide-resin). The observed molecular mass of the purified phosphorylated peptide was found to agree with the calculated value.[11]

Representative activation and emission spectra for the phosphorylated and unphosphorylated peptides are shown in Figure 1. The spectra were recorded at 20° C in a 40 mM tris(hydroxymethyl)aminomethane buffer (pH= 7.4) containing 0.1 M NaCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.5 mM dithiothreitol, which give a high background signal (Figure 1).[12] The phosphate group attached to the hydroxyl functionality of the tyrosyl residue decreases the fluorescence by 34 % relative to the unphosphorylated peptide. This result is in agreement with reported data on the fluorescence of phosphotyrosine.[6b,c,13] Fluorescence linearly correlates with the phosphorylated and unphosphorylated peptide concentration in the micromolar range (Figure 2), and it linearly increases by decreasing the concentration of the phosphorylated peptide in a mixture of both (data not shown). At higher concentrations we expect a loss in linearity due to the inner filter effect.[14] A similar limitation results from the presence of tryptophan, which greatly increases the fluorescence background[15] limiting the range of substrate concentration, although it does not alter the difference in fluorescence between the phosphorylated and unphosphorylated peptide (data not shown).

Finally, we studied the dephosphorylation of pp60$^{c\text{-}src}$(pY527) by calcineurin. Unfortunately, the substrate was not hydrolyzed by the enzyme as determind by the absence of changes in the fluorescence spectra and in the analytical reversed-phase HPLC chromatogram. We also found that both peptides were stable under the assay conditions, and the phosphorylated peptide did not undergo spontaneous dephosphorylation.

In summary, we have shown that the difference in fluorescence between a tyrosine and phosphotyrosine containing peptide can potentially be used in a continuous fluorimetric assay for PTPases. Recently, Dixon and *coworkers* have reported the use of a similar approach for enzymological studies of a Yetsina (Yop51) and mammalian (PTP1U323) PTPases.[6c] Our results point out that the use of fluorogenic assays for PTPases based on the intrinsic fluorescence of tyrosine containing peptides can be limited by the experimental conditions (background fluorescence) and the presence of tryptophanyl residues. These assays will probably be more suitable for substrates with Michaelis-Menten constants in the micromolar range. The syntheses and enzymological studies of other phosphorylated substrates are currently under investigation.

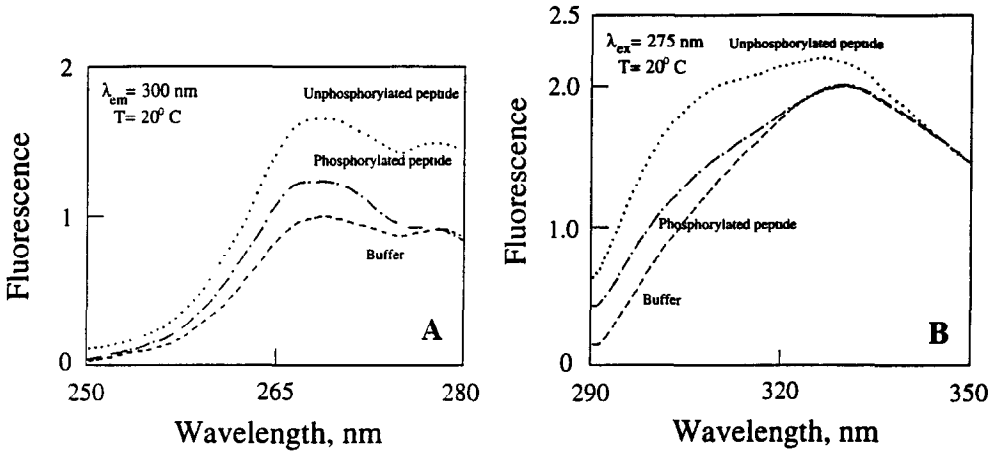

Figure 1. Activation (**A**) and emission (**B**) spectra of the peptide H-Thr-Glu-Pro-Tyr(OY)-Gln-Pro-Gly-Glu-NH_2 (Y= H or PO_3H_2; 3.0 μm). Fluorescence was recorded using a Perkin-Elmer fluorescence spectrometer model MPF-4 equipped with a Xenon Power Supply 156 .

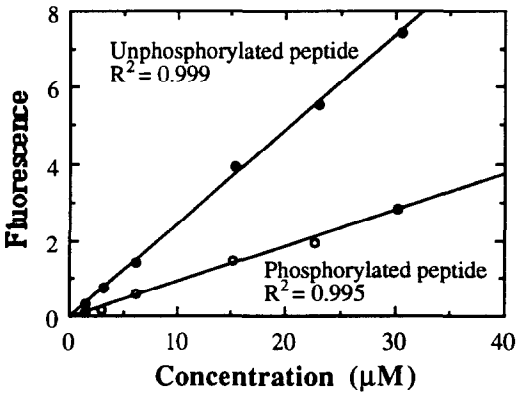

Figure 2. Linear correlation between fluorescence and peptide H-Thr-Glu-Pro-Glu-Tyr(OY)-Gln-Pro-Gly-Glu-NH_2 (Y= H or PO_3H_2) concentration. Fluorescence was recorded at 300 nm upon excitation at 275 nm. T= 20° C. The fluorescence values account for the fluorescence of the peptides after substration of the buffer background fluorescence.

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8. The synthesis of the peptides was carried out manually, starting with an Fmoc-PAL-Nle-MBHA-polystyrene-resin (0.6 g, 0.35 mmol /g; MilliGen/Biosearch) to provide the *C*-terminal amide [Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R.I.; Hudson, D.; Barany, G. *J. Org. Chem.* **1990**, 55, 3730-3743] and using N^{α}-Fmoc-amino acids. Cycles for incorporation of Fmoc-amino acids comprised deprotection with piperidine-*N,N*-dimethylformamide (3:7, v/v; 1 x 2 min + 1 x 8 min) and single coupling (3.0 fold; 90 min) mediated by DIPCDI in the presence of HOBT (3.0 fold) or BOP (3.0 fold)/DIEA (6.0 fold) in *N,N*-dimethylformamide; all couplings were ninhydrin [Kaiser, E.; Colescott, R.L.; Bossinger, C.D.; Cook, P.I. *Anal. Biochem.* **1970**, 34, 595-590] or chloranil [Christensen, T. Acta Chem. Scand. B 1979, 33, 763-766] negative within two hours.

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11. The final products were evaluated by analytical HPLC on a Vydac C-18 column (4.6 x 250 mm), linear gradient over 20 min of CH_3CN-0.036 % TFA and H_2O-0.045 % TFA from 1:19 to 4:1, flow rate 1.2 mL/min, detection at 214 and 254 nm; single peak at t_R= 11.2 min (partially protected phosphorylated peptide); t_R= 9.0 (unphosphorylated peptide); t_R= 8.4 (phosphorylated peptide). The integrity of the purified peptides was determined by FABMS; observed [M+H$^+$] molecular mass for partially protected phosphorylated peptide, 1218; unphosphorylated peptide, 1048; phosphorylated peptide, 1128.

12. It has been reported (ref. 2b) that the phosphatase activity of calcineurin is activated by divalent cations. For the enzyme assay, we also used a 40 mM Tris buffer (pH= 7.4), which contained 0.1 M NaCl, and 1 mM $NiCl_2$. The minimun phosphorylated peptide concentration required for having a fluorescence above the buffer background is 1.5 μM.

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15. Addition of tryptophan (5-fold excess) to the phosphorylated or unphosphorylated peptide solution caused a 1.5-fold increased in the relative fluorescence.