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Synthesis of a Tri-phosphorylated Peptide Corresponding to the Major Autophosphorylation Site in the Insulin Receptor : Conformational Comparison with its Non-Phosphorylated Analogue

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Abstract : Using FmocTyrO(PO_3Bzl_2)OH, we synthesized and purified a triphosphosphorylated peptide corresponding to the major autophosphorylation site of the insulin receptor. By CD spectroscopy, conformational comparison of the phosphorylated peptide with its non-phosphorylated analogue showed a rigidification of the peptide backbone when the aromatic side chains bear negatively charged groups.

Phosphorylation or dephosphorylation of endogenous sites represents a common regulatory mechanism in molecular activation of serine and tyrosine kinases [1, 2]. The insulin receptor is a multisubunit ($\alpha_2\beta_2$) tyrosine specific protein kinase which, upon insulin binding to the α-subunit, undergoes a rapid phosphorylation at several endogenous tyrosine sites of the β-subunit [3]. The autophosphorylation of at least one major site, including tyrosines 1146, 1150 and 1151, leads to the increase of the V_{max} of the substrates. The mechanism of such an activation is not yet elucidated. Indirect evidence suggested that the β-subunit of the receptor undergoes a conformational change during activation. In this context we addressed the question whether a peptide corresponding to the endogenous site can undergo conformational changes during phosphorylation.

In order to assess this hypothesis, we compared the conformation of peptide fragment 1137-1157 of the proreceptor, in its phosphorylated and unphosphorylated states. Thus, we synthesized a 21 amino-acid peptide related to the major autophosphorylation domain of the β-subunit, with tyrosine residues 1146, 1150, 1151 phosphorylated or not :

GDFGMTRDIYETDYYRKGGKG peptide 1

GDFGMTRDI(P)YETD(P)Y(P)YRKGGKG peptide 2

Peptide synthesis. Solid phase peptide syntheses were carried out on aminated polyacrylic resin (Expansin^{TM} from Expansia, Aramon, France), by fluorenemethoxycarbonyl (Fmoc) strategy on an automatic apparatus (9050 Pepsynthesiser, Milligen). The coupling reagent was TBTU and the side chain protections used were: Arg, 2,2,5,7,8-pentanemethylchroman-6-sulfonyl (Pmc); Asp, tertio-butyl (But); Glu, (But); Lys, t-butyloxycarboyl (Boc); Thr, (But); Tyr (But).

For the synthesis of phosphorylated analogue we used FmocTyrO(PO3Bzl2)OH which was prepared from BocTyrO(PO3Bzl2)OH (Peninsula Laboratories, Merseyside, England) using the method developed by P.W. Seale (Glaxo Group Research Limited, personal communication). The Boc group was cleaved by TFA/DCM (40/60) during 1 hour. TFA and solvents were evaporated under vacuum without heating. The residue was dried overnight under high vacuum in the presence of P_2O_5. The TyrO(PO3Bzl2)OH (5.54 mM) was treated by 9-fluorenylmethylsuccinimidilcarbonate (6.1 mM) overnight in 50ml acetone/H_2O (50/50) containing two CO3HNa equivalent (pH 9). The FmocTyrO(PO3Bzl2)OH was extracted by ethyl acetate and used for synthesis without any purification. The overall yield of the reaction was about 90 %.

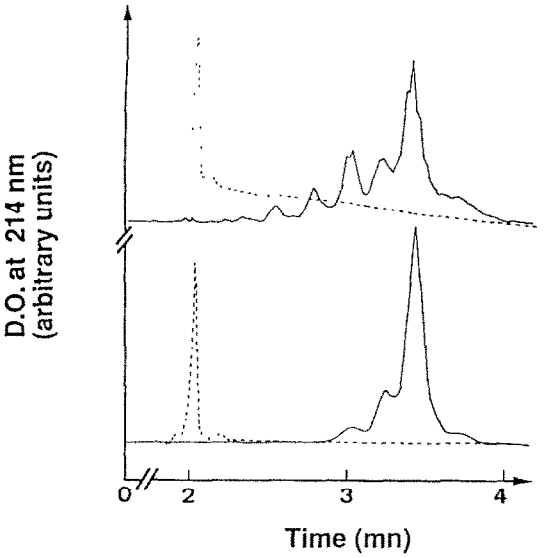

Figure 1:
High Performance Capillary Electrophoresis (HPCE)
i) Upper: unpurified peptide 1 (dash line) and 2 (solide line)
ii) Lower: purified peptides

The C-terminal of the first amino acid was linked to the resin with glycolamidic ester group [4]. After the completion of the synthesis, the side chain blocking groups were deprotected with peptides still attached to the resin, using reagent K (82.5% TFA, 5% phenol, 5% H_2O, 5% thioanisole, 2,5% ethanedithiol) [5] for 2 hours at room temperature. Peptide cleavage was then performed by saponification [6,7]. After freeze-drying, the crude peptide was desalted on Sephadex G-10 column and then lyophilized. The crude peptide was analysed by HPCE (P/ACE system 2000, Beckman) and the profiles are depicted in Figure 1, upper panel.

Peptide purification; The peptide 1 was purified by RP-HPLC (Nucleosil C8 5μ, 20 mm x 250 mm) using a linear gradient of 0 to 90% acetonitrile in 0.1% TFA. The peptide 2 was purified by anion-exchange chromatography on DEAE-5PW (LKB, 7,5x75 mm) with a linear gradient of 0 to 40% NaCl followed by RP-HPLC in the same condition as for peptide 1. The HPCE profiles of purified peptides are depicted on Figure 1, lower panel. After hydrolysis of peptides with 6N HCl in evacuated sealed tubes at 110°C for 24 h, the amino acid analysis was performed on 6300 Beckman Apparatus monitored by the Gold System (Beckman) using norleucine as internal standard. The amino acid analysis of non-phosphorylated peptide 1 showed Asp 3 (3), Thr 2.58 (3), Glu 1.1 (1), Gly 5.88 (6), Arg 1.95 (2), Met 0.97 (1), Ile 0.96 (1), Tyr 3.05 (3), The 0.98 (1), Lys 2 (2) and that of the phosphorylated peptide 2 Asp 2.9 (3), Thr 2.6 (3), Glu 1.09 (1), Gly 5.90 (6), Arg 2.0 (2), Met 0.89 (1), Ile 0.90 (1), Tyr 3.07 (3), Phe 1.05 (1), Lys 1.94 (2).

The mass spectrometry of the two purified peptides were in agreement with the expected structure. When partially purified insulin receptor kinase [8] was incubated with two peptides in the presence of (γ^{32}P)ATP and Mn^{2+} cofactor, phosphate incorporation increased in an insulin-dependent manner in peptide 1 whereas highly purified peptide 2 was not radioactively labeled, thus confirming the phosphorylated state of the peptide.

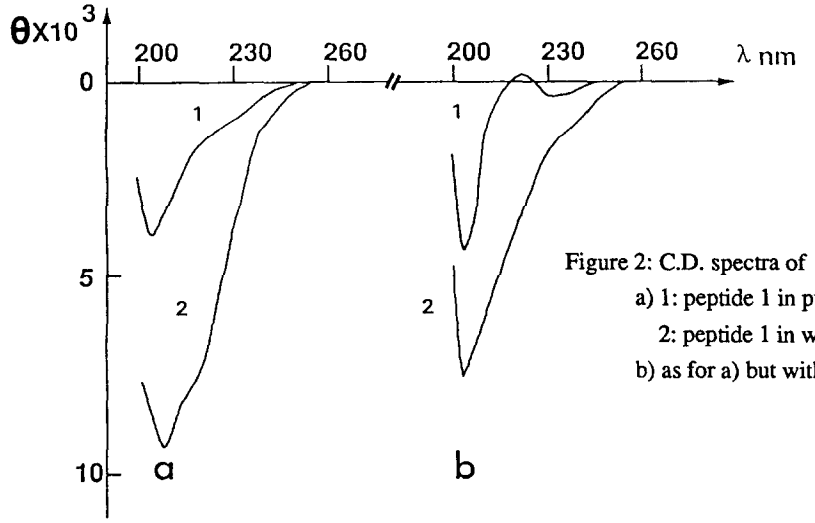

Figure 2: C.D. spectra of
a) 1: peptide 1 in pure water
2: peptide 1 in water/TFE (50/50)
b) as for a) but with peptide 2

c = 20 μg/200 μl, cell path 1 mm, θ is given in deg.cm^2 mole^{-1} in peptide unit.

Structural analysis. In pure water, the far U.V. circular dichroism (CD) spectrum of both peptides is characterized by a negative band at 205 nm, indicative of a non-ordered structure or the presence of β-turns. The shoulder or the band at 230-235 nm can be probably attributed to the aromatic side chain of tyrosines[9,10].

The main difference between peptide 1 and 2 lies in their ability to undergo a coil (or β-turn) to helix transition upon addition of TFE or acetonitrile in the medium. This transition could be induced for the non-phosphorylated compound (Figure 2, panel a), while only a slight modification in the side chain absorption region (230 nm) occurs for the phosphorylated peptide (Figure 2, panel b). This difference of behaviour may reflect a rigidification of the peptide backbone, when the aromatic side chains bear negatively charged groups.

Although these peptides were too short to reflect this behaviour in the whole receptor, this observation suggest that upon phosphorylation, at least the 1137-1157 fragment can undergo a conformational transition or is unable to take some conformation. We conclude from these data that it is possible to synthesize easily tri-phosphorylated peptides using the FmocTyrO(PO_3)Bzl_2)OH residue and a continuous flow technique. Comparison of the CD spectrum of both peptides, which represents the first evidence of a conformational transition in a peptide upon phosphorylation, reinforced the hypothesis that activation (or regulation) or a kinase may be due to a conformational change induced following phosphorylation.

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TBTU : 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TFE : trifluorethanol