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Reversed-phase high-performance liquid chromatographic separation of synthetic phosphopeptide isomers

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ABSTRACT

Selectively phosphorylated synthetic peptides corresponding to the human neurofilament protein middle-sized subunit, H-Lys–Ser–Pro–Val–Pro–Lys–Ser–Pro–Val–Glu–Glu–Lys–Gly-OH, and its analogues were separated by reversed-phase high-performance liquid chromatography of mixtures consisting of the non-phosphorylated, the diphosphorylated and the two different monophosphorylated isomers. Application of the algorithm for the expected retention times to 4–9 amino acid-long peptide fragments revealed the correct elution order of the monophosphorylated isomers. According to circular dichroism studies, this elution order is also compatible with the possibility of induced conformational orientation on the surface of the bonded phase. Chromatographic analysis of the synthetic phosphorylation reaction indicates that the reaction rates of the two structurally different monophosphorylated peptides are similar, which is in contrast to the *in vivo* site-directed reaction.

INTRODUCTION

Numerous cellular functions are regulated through covalent phosphorylation of proteins[1]. Although protein sequences deduced from cDNA clones have led us to

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understand several functions of the unmodified proteins, post-translational modifications, *e.g.*, phosphorylation and glycosylation, can be followed only by isolating phosphorylated protein fragments obtained by chemical or enzymatic cleavage methods, or by using synthetic peptides phosphorylated at the proposed phosphorylation sites. Several phosphoproteins contain more than one phosphorylation site within a short sequence[2,3]. Reversed-phase separation is the method of choice for the purification of both synthetic[4] and isolated[5] phosphopeptides; however, two-dimensional thin-layer chromatography was suggested to have advantages for the separation of phosphorylated insulin-receptor tryptic fragments[6]. Phosphorylated peptides have been shown to elute with decreased retention times compared with their parent non-phosphorylated analogues from reversed-phase high-performance liquid chromatographic (HPLC) columns[7], but the separation of monophosphorylated isomers of the same sequence has not been accomplished[8,9].

Earlier we reported the synthesis and conformation of a perphosphorylated peptide using polyphosphoric acid[10] corresponding to the repeat of the human neurofilament protein middle-sized subunit H–Lys–Ser–Pro–Val–Pro–Lys–Ser–Pro–Val–Glu–Glu–Lys–Gly–OH, where both serine residues carried covalently bound phosphate groups. The peptide, and three repeats thereof in addition to analogues, were used to identify[11] and characterize[12] the multi-phosphorylation repeats of mammalian neurofilaments. Most recently, we reported the solid-phase synthesis of this peptide by site-directed phosphorylation[13], where the serines were phosphorylated individually and together, to result in one diphosphorylated and two monophosphorylated isomers. Purification of the peptides was carried out by reversed-phase HPLC and, on the basis of the observed differences in the retention times, it was suggested that this method allows the separation of the phosphopeptide isomers obtained by a single post-synthetic phosphorylation reaction. In this paper we report the achievement of that separation for the first time.

EXPERIMENTAL

Peptide synthesis

The peptides were synthesized on *p*-alkoxybenzyl alcohol–glycine resin and peptide chain assembly was made with Fmoc-amino acid symmetrical anhydrides[14]. After cleavage with trifluoroacetic acid (TFA) and precipitation with diethyl ether, the peptides were dialyzed briefly in 1000 mol.wt. cut-off tubing (Spectrum, Los Angeles, CA, U.S.A.) against water and then lyophilized. Purity was tested by reversed-phase HPLC, amino acid analysis and positive ion fast atom bombardment mass spectrometry (FAB-MS).

Phosphorylation was carried out with polyphosphoric acid, made *in situ* from P_2O_5 and 85% H_3PO_4[10]. The reaction mixtures were stirred for 12–20 h under vacuum. Warm 2 *M* hydrochloric acid was added to the solidified product and the mixtures were shaken until they redissolved. The solutions were neutralized with 5 *M* sodium hydroxide solution and extensively dialyzed (nine solvent changes in three days) in a 1000 mol.wt. cut-off tubing against doubly distilled water and finally were lyophilized.

Chromatography

The chromatographic system consisted of two Beckman 110A pumps, regulated by a 421A controller, an Altex Ultraphere ODS 25 cm × 10 mm I.D. column, a Beckman 160 fixed-wavelength detector operating at 214 nm, 0.1 a.u.f.s., and a Shimadzu C-R6A integrator. Solvent A was 0.1% aqueous TFA, solvent B was 0.1% TFA in acetonitrile. The samples were loaded in 5% solvent A. The flow-rate was 3 ml/min throughout. A linear gradient of 1.33%/min of solvent B was used for analytical and 1%/min for preparative applications. The analytical loads were 10–100 μg and the preparative loads 2–5 mg. Fractions were collected manually.

Circular dichroism (CD) spectra were taken on a Jobin-Yvon Mark V spectrograph. Spectrograde trifluoroethanol (TFE) and water were used as solvents. All measurements were made in 0.02-cm cells. The peptide concentration was *ca.* 0.1 mg/ml. Details of the CD studies will be reported elsewhere. ^{31}P NMR spectra were taken on a Bruker AM 500 instrument. FAB-MS spectra were taken on a ZAB-E instrument at the Chemistry Department of the University of Pennsylvania by Dr. J. Dykins.

RESULTS

Three sets of peptides were synthesized and phosphorylated by the methods outlined under Experimental. A 13 amino acid-long peptide, H-Lys–Ser–Pro–Val–Pro–Lys–Ser–Pro–Val–Glu–Glu–Lys–Gly-OH (HNFM 1–13), corresponds to the repeat unit found six times in the middle-sized subunit of human neurofilament protein. A fragment four amino acids longer, H-Glu–Glu–Lys–Gly–Lys–Ser–Pro–Val–Pro–Lys–Ser–Pro–Val–Glu–Glu–Lys–Gly-OH (HNFM 1–17), was made to obtain stronger binding to anti-neurofilament antibodies. To verify the structure-determining effect of Lys^6 in HNFM 1–13 through forming intra- and intermolecular salt bridges[10], the lysine residue was replaced with Leu; this resulted in the third peptide, H-Lys–Ser–Pro–Val–Pro–Leu–Ser–Pro–Val–Glu–Glu–Lys–Gly-OH (1–13 L). After phosphorylation with polyphosphoric acid for less than 1 day, the reaction mixtures contained the monophosphorylated peptide, the diphosphate and the monophosphate isomers. The double phosphorylated forms are termed PP; the Ser^2 non-phosphorylated, Ser^7 phosphorylated peptides are termed nPP; the Ser^2 non-phosphorylated, Ser^7 nonphosphorylated peptides are termed PnP; and the HNFM 1–13 and 1–13L peptides without any phosphate are termed nPnP. In HNFM 1–17 the symbols refer to Ser^6 and Ser^{11}, respectively. With site-directed phosphorylation (when phosphorylation was carried out on the resin with dibenzyl phosphochloridate to unprotected serine residues, while the serines not to be phosphorylated were properly protected) we have shown[13] that on reversed-phase HPLC the elution order of phosphorylated and non-phosphorylated HNFM 1–13 and HNFM 1–17 peptides is PP, nPP, PnP, nPnP. Fig. 1 shows the gradient fraction of the preparative chromatogram when the post-synthetic phosphorylation mixture of HNFM 1–13 was loaded. The four peaks, (a) PP, (b) nPP, (c) PnP and (d) nPnP, were identified by comparison of the retention times with those of the corresponding selectively phosphorylated peptides and by undistinguishable binding of the phosphopeptides of different origin to anti-neurofilament antibodies that selectively recognize the different phosphate isomers. These monoclonal antibodies were raised to purified neuro-

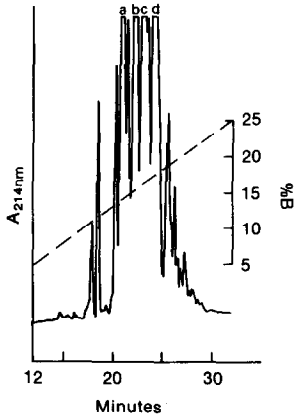

Fig. 1. Preparative reversed-phase chromatography of HNFM 1–13 phosphopeptides. Peaks were later identified as follows: a, PP, b, nPP; c, PnP; d, nPnP. P and nP refer to the presence or absence of the phosphate group on Ser^2 and Ser^7, respectively. Chromatographic conditions as described under Experimental.

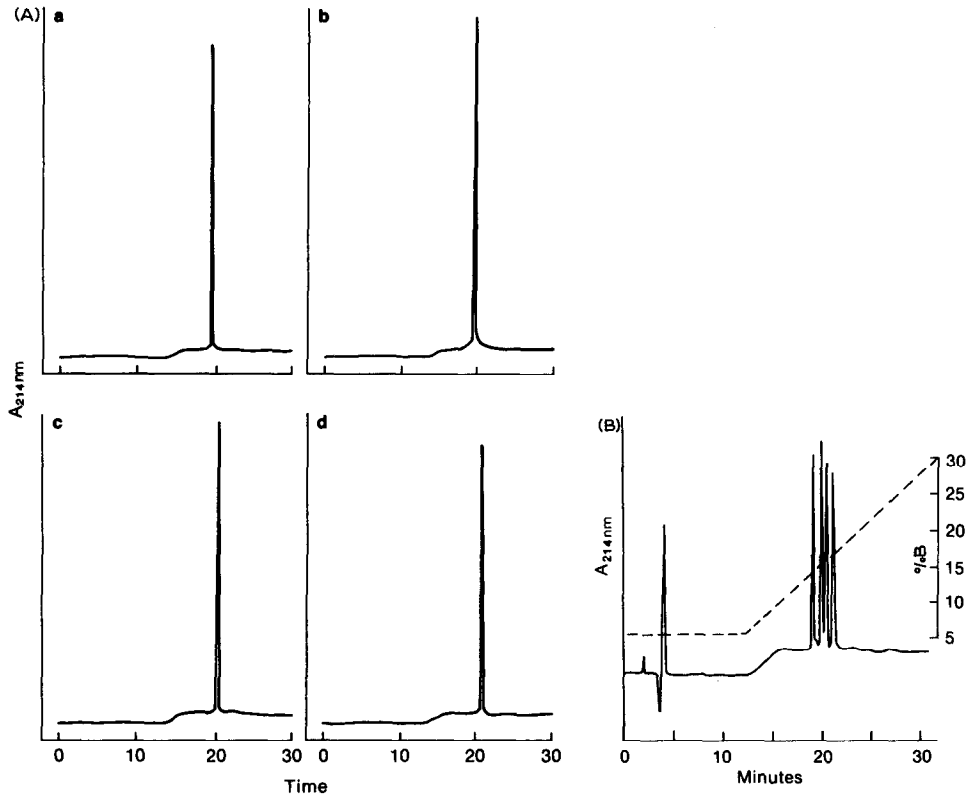

Fig. 2. (A) Chromatographic re-run of each individual peak fraction of HNFM 1–13 phosphopeptide isomers obtained from the preparative chromatography in Fig. 1. (B) Chromatographic re-run of 25 μg of each peak fraction injected together.

TABLE I

METHODS USED FOR VERIFYING THE STRUCTURES OF SEPARATED PHOSPHOPEPTIDES[a]

Peptide	*Retention time (min)*	*Phosphate analysis (%)*[b]	*FAB-MS (m/z)*	*^{31}P NMR (ppm)*	*Other*[e]
HNFM 1–13 a (PP)	19.2	102	M: 1542	n.d.[d]	ELISA[e]
HNFM 1–13 b (nPP)	20.0	96	M+H: 1463	n.d.[d]	ELISA
HNFM 1–13 c (PnP)	20.5	98	M+H: 1463	n.d.[d]	ELISA
HNFM 1–13 d (nPnP)	21.1	–	M: 1382		ELISA
HNFM 1–17 a (PP)	19.5	92	N/A[c]	1.19; 1.21	ELISA
HNFM 1–17 b (nPP)	20.4	106	M: 1905	1.28	ELISA
HNFM 1–17 c (PnP)	20.8	107	M: 1905	1.22	ELISA
HNFM 1–17 d (nPnP)	21.5	–	M: 1825		ELISA
1–13 L a (PP)	23.6	91	M+Na:1550	n.d.[d]	
1–13 L b (nPP)	24.2	109	M: 1447	n.d.[d]	
1–13 L c (PnP)	24.6	101	M: 1447	n.d.[d]	
1–13 L d (nPnP)	25.2	–	M−H: 1366		

[a] All peptides were subjected to amino acid analysis and CD.

[b] Calculation based on theoretical bound phosphate content; method as described[15].

[c] Diphosphorylated peptides sometimes fail to give any FAB-MS spectrum, possibly because of their highly hydrophilic character and intramolecular salt-bridge formation.

[d] Not determined.

[e] ELISA = Enzyme-linked immunoadsorbent assay; peptide recognition by anti-neurofilament antibodies was different, regulated by the location and number of the phosphate group(s).

filament protein subunits (the method of production was described previously[11,12]) and were selected based on their crossreactivity with synthetic peptides. Fig. 2 shows the HPLC profile of an analytical chromatographic re-run of a mixture consisting of 25 μg of each peak fraction. The HNFM 1–17 phosphopeptides were characterized as above. The analogy derived from the previous two sets of peptides was applied to fractions b and c of 1–13 L. Table I reports all parameters analyzed to verify the structure of the three sets of peptides.

To gain an insight into the kinetics of the post-synthetic phosphorylation reaction, we compared the peak integration values of four individual phosphorylations of HNFM 1–17 peptide (Table II). The percentages of the two monophosphorylated isomers were similar and reproducible.

TABLE II

PERCENTAGE OF DIFFERENTIALLY PHOSPHORYLATED HNFM 1–17 PEPTIDES OBTAINED BY FOUR PHOSPHORYLATION REACTIONS DETERMINED FROM REVERSED-PHASE HPLC PEAK-AREA INTEGRATION VALUES

Experiment No.	*PP (%)*	*nPP (%)*	*PnP (%)*	*nPnP (%)*
1	47	24	19	10
2	27	19	15	39
3	20	20	16	44
4	36	21	16	27

CD studies were conducted to characterize the conformation of the peptides carrying the phosphate groups on structurally different serine residues. In water the peptides showed unordered CD spectra. In TFE the phosphorylated fragments of HNFM 1–13 (fractions a, b and c, Fig. 1) exhibited spectra reflecting the equilibrium of unordered and repeating turn conformations, similarly to the CD curve of the non-phosphorylated peptide 1–13 nPnP (studies on the conformation of HNFM 1–13 nPnP have been reported previously[10]).

DISCUSSION

This study is a continuation of our efforts to characterize the conformation of neurofilament fragments important for their recognition by anti-neurofilament antibodies and to reveal the possible effect of neurofilament conformation in neurofibrillary tangle deposition[12]. Our selective phosphorylation procedure, in which the phosphorylation is carried out on the solid-phase synthetic support, provided HNFM 1–13 phosphate isomers in good yield[13] but longer peptides were needed for strong antibody binding and for conformational studies. Similar site-directed phosphopeptide syntheses for HNFM 1–17 = H-Glu–Glu–Lys–Gly–Lys–Ser–Pro–Val–Pro–Lys–Ser–Pro–Val–Glu–Glu–Lys–Gly-OH = Glu–Glu–Lys–Gly–1–13 resulted in low yields, probably owing to steric hindrance on the resin. Earlier, we reported a post-synthetic phosphorylation procedure by which fully phosphorylated peptides are obtained after exposure to polyphosphoric acid for 3 days[10]. On reversed-phase HPLC the resultant perphosphorylated HNFM fragment eluted with the starting 5% acetonitrile in 0.1% aqueous TFA. We obtained large amounts of both sets of peptides when we stopped the post-synthetic phosphorylation reaction after 1 day, when all four phosphate forms (PP, nPP, PnP and nPnP) were present.

Recently we found that the selectively phosphorylated phosphopeptides bound metal ions[16]. Extensive dialysis was needed to remove all residual sodium ions (introduced at neutralization after the phosphorylation reaction) in order to obtain the same retention times in reversed-phase HPLC (11–15% acetonitrile) of phosphopeptides prepared by polyphosphoric acid and selective phosphorylation, respectively. The phosphopeptides separated by reversed-phase HPLC were free from all other peptides with different phosphate forms. Application of the algorithm of Browne *et al.*[17] (based on the algorithm of Meek[18]) to 4–9 amino acid residue-long fragments of HNFM 1–17 surrounding the two structurally different serines revealed correct elution order of the two monophosphorylated isomers, where nPP was eluted with a lower acetonitrile concentration than PnP. The larger decrease in the retention time of nPP than PnP compared with nPnP is probably due to the break of an extended, relatively hydrophobic region around Ser^{11} involving the Pro–Val–Pro–Lys–Ser–Pro–Val segment by the incorporation of the hydrophilic phosphate group on the serine residue (the longest similar hydrophobic segment around Ser^{6} is Gly–Lys–Ser–Pro–Val–Pro). The decrease in retention times due to phosphate incorporation at the two positions is fairly additive: PP was eluted 2.0 min earlier than nPnP, and addition of the difference for the two monophosphorylated peptides compared with nPnP resulted in a 1.8-min retention time (Table I).

A possible induction of an ordered secondary structure of unoriented peptides on the surface of the stationary reversed-phase is the focus of many studies now[19,20].

Peptides, with a tendency to assume both β-pleated sheets[19] and α-helices[20], were reported to change their conformation during reversed-phase separation. Ostresh *et al.*[20] found that although the secondary structural prediction[21] revealed an amphipathic α-helix, CD studies in water showed a random structure. The observed increase in retention time compared with Meek's prediction[18] suggested induction of an ordered conformation. None of the four HNFM 1–13 isomers exhibited a tendency to form an ordered structure in water. In TFE the CD spectrum of PnP was similar to that of nPnP but the band intensities of the former spectrum were higher. PP exhibited a transitional spectrum between type C and that of unordered polypeptides[22]. Peptide nPP showed a C-like spectrum[10] with low band intensities. Type C spectra are similar to those of an α-helix, except that their band intensities are significantly lower. Type C spectra were measured recently for β-turns with established type I (III) character[23]. Based on our CD data, PnP shows a more pronounced tendency to assume an ordered conformation in TFE than does nPP. The stronger binding of PnP to the bonded phase may reflect this conformational orientation during the reversed-phase separation or just a result of the break of a smaller hydrophobic surface, as we discussed earlier.

The separation of the phosphate isomers by reversed-phase HPLC depends on the sequence around the phosphoserine residue[24]. We observed smaller differences in the retention times between PP and nPnP (1.6 min compared with 1.9 min for HNFM 1–13 and 2.0 min for HNFM 1–17) on peptides 1–13 L (the Lys^6 in HNFM 1–13 was replaced with Leu), when the overall retention times were higher.

Finally, we have some clues to the kinetics of the chemical post-synthetic phosphorylation reaction. Peak integration values of four individual phosphorylation reactions of HNFM 1–17 (Table II) revealed variable amounts for PP and nPnP [the solidification time of the reaction mixture varied (12–20 h), based on the amount of the polyphosphoric acid used and the strength of the vacuum; there were also small differences in the solubility of the lyophilized peptides in the phosphorylating reagent]. The very similar nPP and PnP production suggests reactions in which the concentration of the intermediate products is constant for the examined period. The ratio of the reaction rates for the two monophosphorylated isomers, k_{nPP}/k_{PnP}, is 1.27. We observed that, *in vivo*, phosphorylation of Ser^2 precedes that of Ser^7 in HNFM 1–13[25]. As the chemical phosphorylation rates do not suggest conformation in the solid state, where none of the serines is more accessible than the others to the polyphosphoric acid, the *in vivo* sequence of the phosphorylation is possibly regulated in solution by recognition of a certain part or particular conformation of the neurofilament molecule by the neurofilament-directed kinase(s).

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