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SEPARATION OF TRYPTIC PHOSPHOPEPTIDES OF RIBOSOMAL ORIGIN BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Phosphorylation sites for cyclic AMP-dependent kinase in ribosomal proteins and their synthetic analogues were converted to tryptic phosphopeptides and analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) using gradients of acetonitrile in water and 0.1% trifluoroacetic acid. Tryptic variants differing by only NH_2-terminal basic amino acid residues or phosphoryl groups were not always well resolved under these conditions. The different phospho forms could be resolved by RP-HPLC in phosphate buffers at pH 7.0. A combination of gel permeation chromatography, RP-HPLC and thin-layer cellulose mapping was found to be the most effective strategy for the absolute purification of tryptic phosphopeptides from crude tryptic digests.

INTRODUCTION

A prominent early event following the stimulation of mammalian cells with various polypeptide growth factors is the phosphorylation of ribosomal protein S6 at multiple sites [1—8]. Identification of the growth-associated S6 kinases requires knowledge of their specific phosphorylation sites. The sites can be selectively cleaved from whole ribosomes with trypsin [8—11]; this strategy has led to the isolation and characterisation of the sites for cyclic AMP-dependent protein S6 kinases [9, 11] (for other S6 kinases see ref. 12). In rat liver ribosomes, these and other sites appeared to be clustered within the sequence Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala-Ser-Thr-Ser-Lys-Ser-Glu-Glu-Ser-Gln-Lys [11]. The first serine is the preferred site for cyclic AMP-dependent protein kinase, with the second and third serines being phosphorylated at high concentrations of the kinase in vitro [9, 11].

Tryptic digestion of S6 phosphorylated in this region generates a complex mixture of structurally related phosphopeptides [9, 11]. The variety of peptides originating from the same region is attributable to the clustering of sites within the region, the presence of several potential tryptic sites and the inhibi-

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tory influence of neighbouring phosphoryl groups on certain tryptic cleavages [11].

The isolation and characterisation of each of the tryptic phospho-derivatives are necessary steps in the interpretation of phosphorylation patterns for the different S6 kinases. An important purification step has been reversed-phase high-performance liquid chromatography (RP-HPLC) using C_{18}-bonded microsilica columns [13—16] eluted with gradients of acetonitrile in water containing the hydrophobic ion-pairing reagent trifluoroacetic acid (TFA) [15, 16]. Under these conditions, the order of elution of small peptides (<20 residues) is closely correlated with their amino acid compositions [16]. Predicted retention coefficients for individual residues [16—18] suggest that it should be possible to resolve peptides of the types encountered in tryptic digests of phosphorylated S6 that differ in structure by only a single basic or phosphorylated amino acid residue. However, the statistically determined retention coefficients do not allow for interactions within sequences and the overall conformational features of peptides that might also influence retention.

Here we investigate the potential of RP-HPLC for resolving structurally related tryptic variants of S6. The elution characteristics of synthetic peptide analogues of the region of S6 containing the phosphorylation sites for cyclic AMP-dependent kinase were also studied. The effects of phosphoryl groups on column retention were investigated using material phosphorylated with cyclic AMP-dependent protein kinase in vitro.

EXPERIMENTAL

Phosphopeptides of ribosomal origin

Liver ribosomes were prepared as in ref. 9 from 10—20 weeks old male Wistar rats which had been starved overnight. The ribosomes were phosphorylated with [γ-^{32}P]ATP and the purified catalytic subunit of cyclic AMP-dependent protein kinase from beef heart (a gift from Dr. B.E. Kemp) under conditions resulting in the transfer of approximately 2 mol phosphate per mol S6 [11]. Partial tryptic peptides containing the phosphorylation sites were selectively cleaved from whole ribosomes by mild tryptic digestion [9, 11] and the peptides fractionated according to their size by gel permeation chromatography on Sephadex G-25 in 1 *M* acetic acid [9].

Synthetic peptides

The peptides were synthesised by B.E. Kemp using the Merrifield solid phase synthesis procedure [20] in a Beckman 990 synthesiser. Peptides S6-2 and KS6-2 were prepared as the COOH-terminal amide form whereas S6-1 and KS6-1 and S6-Y were in the free COOH-terminal form. The general properties of the peptides and their abilities to serve as substrates for cyclic AMP-dependent kinase will be detailed separately [19]. The stoichiometry of peptide phosphorylation was determined by binding to phosphocellulose papers and counting the radioactivity by liquid scintillation spectrometry [21]. The assignment of phosphorylation sites was made on the basis of isoelectric focusing properties of tryptic peptides [9, 11] and data obtained from automated sequence analysis [22]. Tryptic digestions of phosphorylated peptides were performed in 0.1 *M* NH_4HCO_3 for 16 h at 30°C (trypsin, 50 μg ml^{-1}).

HPLC

Peptides were analysed by RP-HPLC using a Varian delivery system and UV monitor set at 214 nm with a μBondapak C_{18} column (Waters Assoc.). In standard analyses (Table I), the column was eluted with a linear gradient of aqueous acetonitrile containing 0.1% trifluoroacetic acid (TFA), with the acetonitrile concentration increasing at a rate of 1% min^{-1}. The flow-rate was constant at 1 ml min^{-1}. Samples were injected 1 min prior to the commencement of the gradient. ^{32}P-radioactivity associated with phosphorylated peptides was monitored by Cerenkov counting of HPLC fractions (1 ml) in a liquid scintillation spectrometer. The elution position of each peptide was expressed as retention time (t_R). Determinations of t_R were performed in triplicate; the S.E. in each case was less than 0.1 min. The recoveries of phosphopeptides in fractions eluted from HPLC columns were generally > 90%. However, the overall yields of the more hydrophobic phosphopeptides (e.g. the Peak II compared with the Peak I peptides described in Fig. 1) were appreciably lower due to selective losses (up to 80%) during lyophilisation steps.

TABLE I

RP-HPLC OF RIBOSOMAL TRYPTIC PEPTIDES AND THEIR SYNTHETIC ANALOGUES

Peptide	Sequence***	t_R (min)
Ribosomal*		
$T2_A$	RRLS(P)S(P)LR	20.0
$T2_B$	RLS(P)S(P)LR	22.5
$T2_C$	RLS(P)SLR	22.0**
$T1_A$	RLS(P)S(P)LRAS(P)TSK	22.5
$T1_C$	RLS(P)S(P)LRASTSKSEESQK	19.0
Synthetic*		
KS6-2	KRRLSSLRASTSKS(NH_2)	20.8
	KRRLS(P)SLRASTSKS(NH_2)	20.8
S6-2	RRLSSLRASTSKS(NH_2)	20.7
KS6-1	KRRLSSLRA	22.6
S6-1	RRLSSLRA	23.2
	RRLS(P)SLRA	22.9
	RRLS(P)SLR	22.0
	RLS(P)SLR	22.3**
	LSS(P)LR LSSLR	19.4
S6-Y	KRRASSLKA	19.5

*5 nmol peptide analysed with a linear gradient of acetonitrile in aqueous 0.1% TFA increasing at an acetonitrile concentration of 1% min^{-1}.

**Different μBondapak columns were used for the analyses of ribosomal and synthetic peptides, hence the reason for the slight difference in t_R for the RLS(P)SLR species.

***Amino acid residues are designated by the single-letter code of Dayhoff [26]; the positions of phosphoryl serine residues are denoted by S(P).

Different phosphorylated forms of peptides were separated by RP-HPLC using a μBondapak C_{18} column eluted with gradients of acetonitrile in aqueous potassium phosphate buffer at pH 7.0. Solution A was 50 m*M* potassium phosphate. The percentage of solution B was increased as described in the legends to Figs. 6 and 7.

Other analytical procedures

Two-dimensional mapping of phosphopeptides was carried out on thin-layer cellulose plates (Macherey-Nagel) using electrophoresis at pH 4.4 [23] as the first dimension and chromatography in butanol—acetic acid—water—pyridine (15:3:12:10) as the second dimension [24]. Amino acid analyses were performed on an LKB Biochrom 4400 amino acid analyser and automated Edman sequence analyses on an Applied Biosystems Model 470A gas—liquid phase sequencer.

RESULTS

Isolation of tryptic peptides of ribosomal origin

RP-HPLC using a Waters C_{18} μBondapak column with gradients of acetonitrile in water and 0.1% (v/v) TFA was only partially effective for resolving tryptic peptides of ribosomal origin [9, 11]. The major problems encountered related to the difficulty of separating individual species within families of structurally related tryptic variants containing multiple phosphorylation sites. This is illustrated with the case of the larger phosphopeptides released during mild

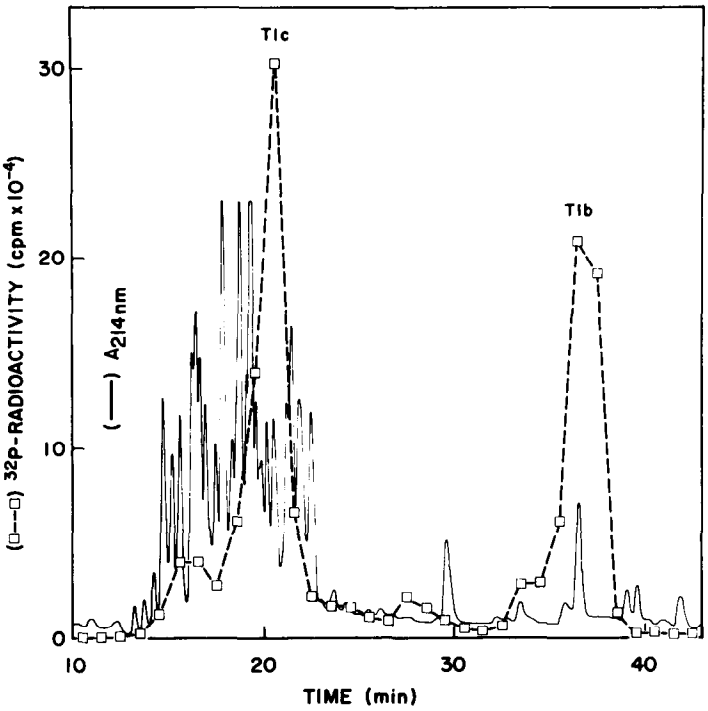

Fig. 1. Fractionation of phosphopeptides selectively cleaved from ribosomes by mild tryptic digestion. Phosphorylated ribosomes (50 nmol) were digested with trypsin for 20 sec, and the large phosphopeptide fraction (V_e/V_0 = 1.2—1.35) obtained by gel filtration on Sephadex G25 as described in ref. 11. The phosphopeptides were analysed by RP-HPLC using a discontinuous gradient of acetonitrile in 0.1% TFA; the concentration of acetonitrile increased at 1.5% min^{-1} from 0 to 10 min after injection and at 0.5% min^{-1} from 10 to 50 min. The phosphopeptide fractions denoted as T1c and T1b in the figure are referred to as the Peak I and Peak II fractions, respectively, in the text.

tryptic digestion of ribosomes phosphorylated with cyclic AMP-dependent protein kinase.

The tryptic peptides were sized by gel chromatography on Sephadex G-25. The larger peptides (V_e/V_0 = 1.20—1.35) [11] were analysed by RP-HPLC using a gradient of acetonitrile increasing at 0.5% min^{-1} in water—0.1% TFA (Fig. 1). Two major peaks of ^{32}P-labelled peptides were eluted with retention times of 24 min (Peak I) and 33 min (Peak II) (cf. the retention times quoted in Table I which were determined with acetonitrile gradients increasing at 1% min^{-1}).

The peak I fraction has been shown previously to contain three species of phosphopeptides [11]. These were only partially resolved by further HPLC using a discontinuous gradient of acetonitrile in aqueous 0.1% TFA (Fig. 2). Complete resolution of the three phosphopeptides was eventually achieved by two-dimensional mapping on thin-layer cellulose plates (Fig. 3a). The thin-layer cellulose step also resolved the radioactive species from several non-phosphorylated species which coeluted with the phosphopeptides during RP-HPLC (Fig. 3a). Structural analyses showed that the three phosphorylated species represented the mono-, di- and triphospho derivatives of the sequence Arg-Leu-Ser-Ser-Leu-Arg-Ala-Ser-Thr-Ser-Lys-Ser-Glu-Glu-Ser-Gln-(Lys) originating from ribosomal S6 [11].

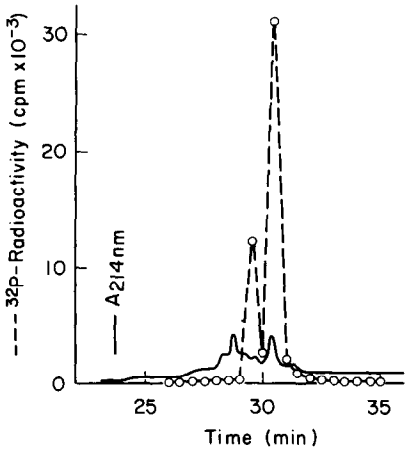

Fig. 2. The Peak I (T1c) phosphopeptides (sample size ca. 2 nmol) described in Fig. 1 were analysed by RP-HPLC using a discontinuous gradient of acetonitrile in aqueous 0.1% TFA; the acetonitrile concentration increased from 0 to 12% during the period from 0 to 10 min after injection, remained constant at 12% from 10 to 20 min, increased from 12 to 15% from 20 to 23 min and, thereafter, was held constant at 15%.

The Peak II phosphopeptides eluted as a single broad peak during further RP-HPLC analyses using various forms of discontinuous gradients of acetonitrile, water and 0.1% TFA (not illustrated). However, thin-layer cellulose mapping showed that several species of phosphopeptides were present in the Peak II fraction (Fig. 3b).

The major species (spot 4, Fig. 3b) was only recovered in sufficient quantities for microsequence analysis by automated Edman degradation. The first

13 residues were identified as Ala-Ile-Thr-Gly-Ala-Ser-Leu-Ala-Asp-Ile-Met-Ala-Lys. Several observations showed that the other species were structurally related tryptic variants of the spot 4 sequence. First, the number of species was reduced to two by further tryptic digestion (50 μg trypsin per millilitre for 8 h at

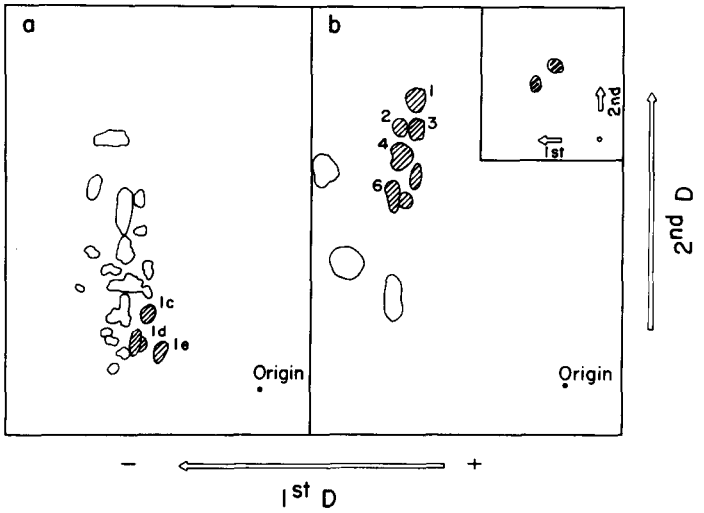

Fig. 3. Thin-layer cellulose mapping of ribosomal tryptic peptides. a and b, Peaks I and II phosphopeptide fractions, respectively, as defined in Fig. 1; insert in b, Peak II peptide fraction subdigested with trypsin as described in the text. The open areas describe peptide spots stained with fluorescamine whereas the hatched-in areas represent ^{32}P-labelled phosphopeptides detected by autoradiography.

TABLE II

COMPOSITIONS OF THE TRYPTIC DERIVATIVES OF PEAK II PHOSPHOPEPTIDES

Hydrolyses were done in 5.7 *M* HCl—5 m*M* phenol for 24 h at 110°C. Serine and threonine were corrected for 10% and 5% destruction, respectively. Results were expressed as mol amino acid per mol peptide after assuming that T1b and T1b′ represented 13- and 14-residue peptides, respectively. Impurities <0.2 mol are omitted. The values in parentheses represent the composition of T1b reported previously [9].

Amino acid	Peptide*	
	T1b	T1b′
Asp/Asn	0.7 (1.4)	1.3
Thr	1.0 (1.1)	0.7
Ser	1.5 (1.2)	1.4
Glu/Gln	0.5 (0.9)	0.6
Gly	1.7 (1.2)	1.9
Ala	2.8 (3.6)	2.7
Val	0.7 (0.7)	0.6
Met	1.2 (0.2)	1.0
Ile	1.0 (1.6)	0.9
Leu	0.7 (1.1)	1.3
Lys	1.2 (1.0)	1.7

*Peak II peptides isolated by RP-HPLC (Fig. 1) were subdigested with trypsin and the resulting phosphopeptides isolate by thin-layer cellulose mapping (Fig. 3b, insert).

30°C) of the original Peak II peptides. The two species could not be resolved by RP-HPLC but were clearly resolved by thin-layer cellulose mapping (Fig. 3b, insert). While the amino acid compositions of the peptides indicated that they were still not pure, it was apparent that the two species were closely related probably differing by only a single lysine residue (Table II). The compositions also showed that the two species were related to the spot 4 sequence identified in the undigested Peak II fraction (Fig. 3b) and to the previously isolated peptide T1b [9].

Elution characteristics of synthetic analogues of ribosomal S6

The investigation described above has provided examples of two problems often encountered during the purification of tryptic derivatives of ribosomal phosphoproteins by RP-HPLC. The problems relate to the unsatisfactory resolution of structurally related peptides differing only by number of phosphoryl groups or by terminal basic amino acid residues. To gain a further understanding of these problems, the chromatographic properties of a range of structurally related synthetic peptide analogues of the region of ribosomal S6 giving rise to the Peak I tryptic peptides (see Fig. 1 and Table I) were investigated. In some cases, the peptides were phosphorylated with the cyclic AMP-dependent protein kinase under conditions in which the first serine residue in the peptides was fully phosphorylated [19].

Terminal basic residues

The influence of additional NH_2-terminal lysine residues was investigated by comparing the elution characteristics of the pairs of related synthetic peptides S6-1/KS6-1 and S6-2/KS6-2 using linear gradients of acetonitrile in water and

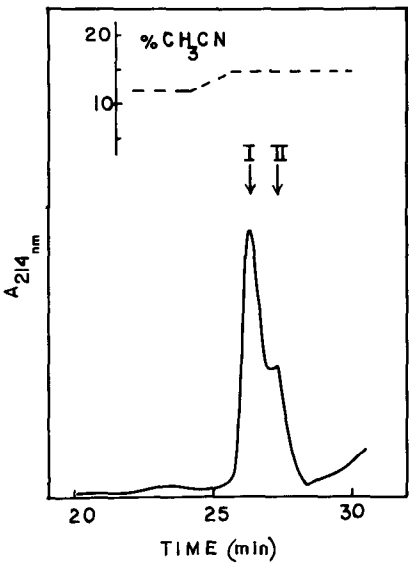

Fig. 4. Resolution of synthetic peptides KS6-2 and S6-2 by RP-HPLC using the discontinuous gradient of acetonitrile (in 0.1% TFA) described in the figure. The order of elution was S6-2 (10 nmol injected) followed by KS6-2 (5 nmol).

0.1% TFA (Table I). The addition of a lysine residue to the S6-1 sequence (to make KS6-1) decreased the t_R by 0.4 min. However, the addition of lysine to the larger S6-2 sequence slightly increased the t_R (by 0.1 min). The influence of the additional lysine in the latter case was more apparent when a mixture of the peptides was partially resolved using a discontinuous gradient of acetonitrile in water and 0.1% TFA (Fig. 4).

The inconsistent effects of NH_2-terminal basic residues was even more apparent with smaller phosphopeptides generated by tryptic digestion of phosphorylated S6-1 (Fig. 5, Table I). In particular, the Leu-Ser-Ser(PO_4)-Leu-Arg and Arg-Leu-Ser(PO_4)-Ser-Leu-Arg species were well resolved (t_R values differed by 2.9 min). However, the presence of a second NH_2-terminal arginine to make the Arg-Arg-Leu-Ser(PO_4)-Ser-Leu-Arg species actually decreased the t_R by 0.3 min.

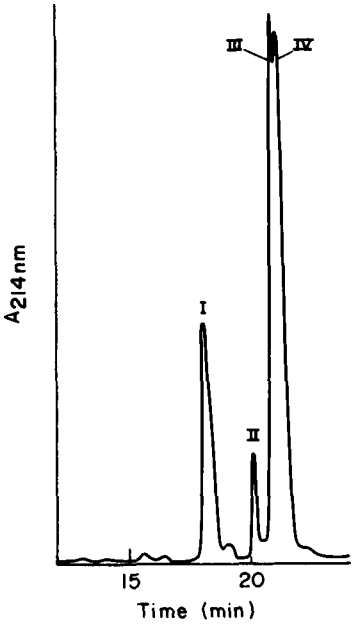

Fig. 5. Tryptic digest of phosphorylated synthetic peptide S6-1 (10 nmol) analysed by RP-HPLC in a discontinuous gradient of acetonitrile and aqueous 0.1% TFA; the acetonitrile concentration increased at 2% min^{-1} during the initial 5 min and at 0.5% min^{-1} thereafter. The ^{32}P-radioactivity was associated with Peaks I, III and IV which were found by automated Edman degradation analyses to have the amino acid sequences Leu-Ser-Ser-Leu-Arg, Arg-Arg-Leu-Ser-Ser-Leu-Arg and Arg-Leu-Ser-Ser-Leu-Arg, respectively. Isoelectric focusing analysis in polyacrylamide gels [9] showed that the phosphopeptides were predominantly in the monophosphorylated forms; the position of the phosphate was assigned to the first serine residue on the basis of the appearance of ^{32}P-radioactivity and serine adducts in the cleavage products at each cycle of the Edman degradation [22].

Effects of phosphoryl groups in the TFA system

The phosphorylation of synthetic peptides either slightly decreased or had no effect on the t_R values during RP-HPLC using acetonitrile gradients in 0.1% TFA (Table I). For example, the t_R for the phospho form of S6-1 was just

0.3 min less than that for unphosphorylated S6-1 whereas with the larger S6-2 and KS6-2 species the phosphorylated and unphosphorylated forms appeared to coelute. In what appears to be an unusual case, the t_R for the ribosomal peptide Arg-Leu-Ser(PO_4)-Ser(PO_4)-Leu-Arg was actually greater by 0.5 min than that for the monophospho form of the same peptide [9] (Table I).

Resolution of phosphopeptides using phosphate buffers of pH 7

The different phosphorylated and unphosphorylated forms of peptides were resolved by RP-HPLC on a C_{18} column using acetonitrile gradients in aqueous buffers at pH 7.0 to maximise the negative influence of charged phosphoryl groups (Figs. 6 and 7). Both potassium phosphate and triethylammonium phosphate (TEAP) buffers [13] (at 50 m*M*) have been employed successfully although the peptide peaks under these conditions were generally broader than those eluted with 0.1% TFA conditions. TEAP buffers gave sharper peptide peaks particularly with late-eluting peptides; however, potassium buffers were preferred because of the relative absence of impurities which interfered with UV monitoring at high sensitivity settings. Peptides isolated in potassium phosphate gradients were desalted as required by re-chromatography using aqueous acetonitrile gradients in 0.1% TFA.

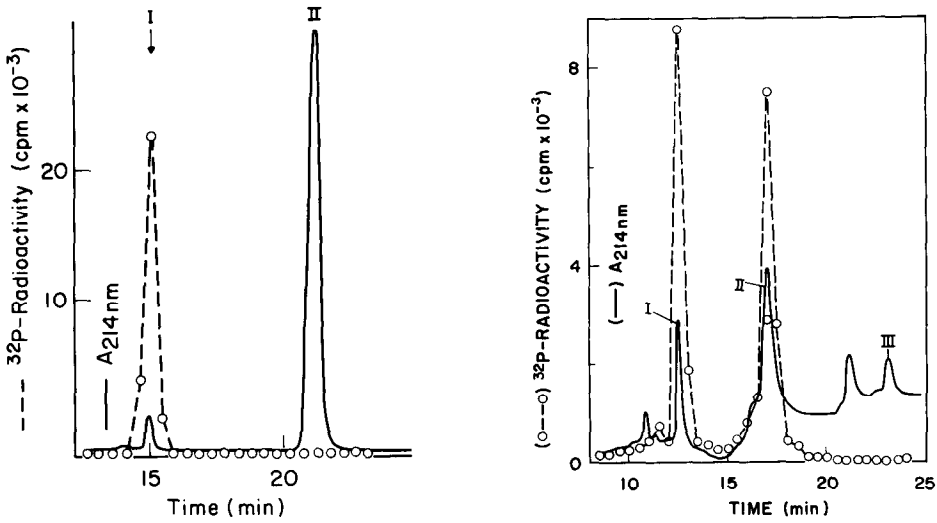

Fig. 6. Separation of phosphorylated and unphosphorylated forms of a tryptic derivative of S6-1 by RP-HPLC. The peaks containing the Leu-Ser-Ser-Leu-Arg peptides (Peak I; Fig. 4) were resolved using a gradient of acetonitrile in aqueous 50 m*M* potassium phosphate, pH 7.0. The acetonitrile concentration increased at 1% min^{-1} for the initial 7.5 min and at 0.25% min^{-1} therafter. The flow-rate was held constant at 1 ml min^{-1}. Both the peptides resolved (Peaks I and II) were found to have the amino acid sequence Leu-Ser-Ser-Leu-Arg by automated Edman degradation analyses.

Fig. 7. Analysis of phosphorylated synthetic peptide S6-Y (Table I) by RP-HPLC using phosphate-buffered gradients of acetonitrile gradients. The stoichiometry of phosphorylation of the peptide was 1.4 mol phosphate per mol peptide. The acetonitrile concentration increased at 1.5% min^{-1}. The flow-rate was constant at 2 ml min^{-1}.

The effectiveness of the procedure, using 50 m*M* potassium phosphate buffer with a gradient of acetonitrile increasing at 0.25% min^{-1}, is illustrated for the monophosphorylated and unphosphorylated forms of the pentapeptide Leu-Ser-Ser-Leu-Arg generated during trypsin digestion of phosphorylated S6-1 (Fig. 6). Mono- and diphospho forms of peptides were also well resolved. For example, preparations of the peptide S6-Y (Table I) phosphorylated with the cyclic AMP-dependent protein kinase (stoichiometry, 1.4 mol phosphate per mol peptide), yielded two peaks of phosphopeptides (Fig. 7), which were identified as the monophospho (early eluting peak) and diphospho (late eluting) forms according to their specific radioactivities. In this experiment, the rate of increase of the acetonitrile gradient was increased to 1.5% min^{-1}, to counter the problem of decreasing peak sharpness encountered with larger and more basic peptides.

DISCUSSION AND CONCLUSIONS

RP-HPLC has been used effectively in the purification of tryptic phosphopeptides originating from ribosomal S6. However, structurally similar derivatives differing only by single basic residues or phosphoryl groups were not always well resolved under the TFA buffer conditions employed.

Phosphoryl groups have been found previously to appreciably decrease retention of small peptides using TFA [16] as well as heptafluorobutyric acid [16] and *n*-hexanesulphonic acid [25] as ion-pairing reagents. Similarly, the association of sulphate groups with tyrosine residues has been observed to appreciably decrease the retention of peptides in RP-HPLC under acidic conditions [17]. The relatively small influence of phosphoryl groups on retention of ribosomal peptides may be at least partly due to intramolecular ion pairing between phosphoryl and closely situated guanidinium groups. Such an interaction could explain the apparently anomalous case where the diphospho-hexapeptide Arg-Leu-Ser(PO_4)-Ser(PO_4)-Leu-Arg was retained more than the monophospho derivative of the same sequence (Table I) [9].

The minimal effects of basic residues on retention were puzzling in view of the potential ion pairing between these residues and TFA ions leading to increased retention. Meek and Rossetti [17] have suggested that a concentration of 0.1% TFA is insufficient for efficient ion pairing between basic residues and TFA ions. However, the addition of arginine at the NH_2-terminus of the monophosphorylated Leu-Ser-Ser-Leu-Arg peptide increased retention appreciably (Table I) as might have been expected if ion pairing had occurred. In contradiction, the extension of the same sequence with a second NH_2-terminal arginine residue slightly decreased retention suggesting that ion pairing with TFA ions may be less effective in the region of adjacent arginine residues. A similar phenomenon could explain the minimal effects of lysine residues added to the NH_2-terminal of the S6-1 and S6-2 synthetic peptide analogues of S6 (Table I).

The results of this investigation show that RP-HPLC with acetonitrile gradients in TFA and phosphate buffers can be used to resolve many of the tryptic variants likely to be generated from phosphorylated S6. However, the difficulties in resolving certain peptides differing only in single basic amino acid and

phosphoryl residues has led to the use of thin-layer cellulose mapping (following the RP-HPLC step) to check sample purity and, where required, as the final step in the purification of ribosomal tryptic phosphopeptides.

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REFERENCES

1 I.G. Wool, Ann. Rev. Biochem., 48 (1979) 719.
2 D.P. Leader, in P. Cohen (Editor), Molecular Aspects of Cellular Regulations, Vol. 1, Elsevier, Amsterdam, 1980, p. 203.
3 J.A. Traugh, in G. Litwack (Editor), Biochemical Actions of Hormones, Vol. III, Academic Press, New York, 1981, p. 167.
4 S.M. Lastick and E.H. McConkey, Biochem. Biophys. Res. Commun., 95 (1980) 917.
5 G. Thomas, M. Siegmann, A.-M. Kubler, J. Gordon and L. Jimenez de Asua, Cell, 19 (1980) 1015.
6 J.C. Chambard, A. Franchi, A. Le Cam and J. Pouyssegur, J. Biol. Chem., 258 (1983) 1706.
7 R.E.H. Wettenhall and G.J. Howlett, J. Biol. Chem., 254 (1979) 9317.
8 R.E.H. Wettenhall, C.N. Chesterman, T. Walker and F.J. Morgan, FEBS Lett., 162 (1983) 171.
9 R.E.H. Wettenhall and P. Cohen, FEBS Lett., 140 (1982) 263.
10 R.E.H. Wettenhall, P. Cohen, B. Caudwell and R. Holland, FEBS Lett., 148 (1982) 207.
11 R.E.H. Wettenhall and F.J. Morgan, J. Biol. Chem., 259 (1984) 2084.
12 T.H. Lubben and J.A. Traugh, J. Biol. Chem., 258 (1983) 13992.
13 J.E. Rivier, J. Liquid Chromatogr., 1 (1978) 343.
14 W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding and M.T.W. Hearn, Science, 200 (1978) 1168.
15 H.P.J. Bennett, A.M. Hudson, C. McMartin and G.E. Purdon, Biochem. J., 168 (1977) 9.
16 C.A. Browne, H.P.J. Bennett and S. Solomon, Anal. Biochem., 124 (1982) 201.
17 J.L. Meek and Z.L. Rossetti, J. Chromatogr., 211 (1981) 15.
18 S.J. Su, B. Grego, B. Niven and M.T.W. Hearn, J. Liquid Chromatogr., 4 (1981) 1745.
19 B. Gabrielli, R.E.H. Wettenhall, B.E. Kemp and L. Bozinova, FEBS Lett., in press.
20 R.S. Hodges and R.B. Merrifield, Anal. Biochem., 65 (1975) 241.
21 B.E. Kemp, E. Benjamini and E.G. Krebs, Proc. Nat. Acad. Sci. U.S., 73 (1976) 1038.
22 R.E.H. Wettenhall, in preparation.
23 H. Lindemann and B. Wittmann-Liebold, Hoppe-Seyler's Z. Physiol. Chem., 358 (1977) 843.
24 A. Aitken, T. Bilham, P. Cohen, D. Aswad and P. Greengard, J. Biol. Chem., 256 (1981) 3501.
25 B. Fransson, U. Ragnarsson and Ö. Zetterqvist, Anal. Biochem., 126 (1982) 174.
26 M.D. Dayhoff, Atlas of Protein Sequence and Structure, Vol. 5, National Biomedical Research Foundation, Washington, DC, 1972.