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Note

Dansylation and high-performance liquid chromatographic separation of phosphoserine, phosphothreonine and phosphotyrosine from ^{32}P-labeled protein hydrolyzates

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The role of protein phosphorylation as the major general mechanism for the control of cellular activity is now widely recognized[1]. Further investigations on the specificity of phosphorylation require a careful analysis of the phosphorylation sites in peptides or proteins. Although the most common phosphorylation sites in proteins are the amino acids serine and threonine, tyrosine can also be phosphorylated[2–6]. Thyrosine phosphorylation was initially discovered in cells infected with RNA tumor viruses[2–4] but can also take place in normal cells[5,6]. The analysis of ^{32}P-labeled phosphoamino acids from protein hydrolyzates was traditionally accomplished by electrophoresis. In this communication we describe a new method of analysis for labeled phosphoamino acids which is fast and does not require autoradiography of electrophoretic plates.

Our method consists on the dansylation of the amino acids present in protein hydrolyzates with dansyl chloride (Dns, 5-dimethylaminonaphthalene-1-sulphonic chloride). The dansylated products are then separated using reversed-phase C_{18} columns or diphenyl columns. This method has been applied for the analysis of the phosphoamino acids of proteins labeled with ^{32}P by two different techniques. First, *in vitro* labeling of cell cultures with ^{32}phosphate. And second, *in vitro* labeling of enzyme preparations (RNA polymerase II) using ATP as a source of labeled phosphate.

MATERIALS AND METHODS

Materials

Distilled deionized water utilized for high-performance liquid chromatography (HPLC) was passed through C_{18}-silica cartridges (Waters Assoc.) and filtered through Millipore 0.22-μm filters. Acetonitrile (HPLC grade) was from Fisher and trifluoroacetic acid (TFA) from BDH or Baker. Dns and HCl (constant boiling point) were from Pierce. O-Phospho-L-serine, O-phospho-DL-threonine and O-phospho-DL-tyrosine were obtained from Sigma. O-phosphotyrosine was also synthesized according to the method of Rothberg *et al.*[7]. DNA-dependent RNA polymerase II from calf thymus was obtained from Bethesda Research Laboratories (BRL, batches 1811

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and 2111). ^{32}P-Phosphoric acid (carrier free) and [γ-^{32}P]ATP (2300–2900 Ci/mmol) were from New England Nuclear.

Labeling of phosphoproteins

$40 \cdot 10^6$ fetal rat liver cells were incubated for 1 h at 37°C in 1 ml modified F12 medium suplemented with albumin and transferrin[8] and containing 800 mCi ^{32}P-phosphate. After incubation the cells were washed 5 times with Hank's balanced salt solution. The cells were extracted with a mixture of HCl, formic acid and TFA[9] and the supernatant subjected to reversed-phase extraction using a Waters Sep-Pak C_{18} cartridge[9]. The Sep-Pak eluate was applied to a reversed-phase C_{18} column (μBondaPak C_{18}, Water Assoc.) and eluted with a linear gradient of 0–80 % acetonitrile in 0.1 % TFA in water for 45 min at a flow-rate of 1.7 ml/min. One fraction of the eluted compounds containing a protein of 15,000 daltons according to its mobility on acrylamide gels[10] was evaporated under a stream of nitrogen and used as a test of the method of dansylation to be described below. As an example of the application of the same method of dansylation to proteins labeled in vitro with ATP we incubated 350 units of calf thymus DNA dependent RNA polymerase II for 10 min at 37°C with 20 μCi [γ-^{32}P]ATP in 60 μl 0.05 *M* Tris–HCl buffer (pH 7.5) containing 5 m*M* $MgCl_2$ and 10 μ*M* 2-mercaptoethanol. The reaction was stopped with cold trichloroacetic acid containing phosphoric acid, adenosine and pyrophosphate[11]. 50–100 μg albumin and 100 μg tRNA were added as carriers and the samples were centrifuged, washed 2 times with 0.75 *M* trichloroacetic acid and hydrolyzed with hot trichloroacetic acid[12]. The proteins were centrifuged, washed with ether and dried under a stream of nitrogen.

Hydrolysis and dansylation

Proteins were hydrolyzed for 2 h at 110°C in 0.5 ml concentrated HCl (Pierce). This short time of hydrolysis is essential to prevent degradation of phosphoamino acids[7]. The samples were evaporated under a stream of nitrogen, suspended in 100 μl 0.2 *M* Na_2CO_3 and evaporated again. Then the samples were suspended in 100 μl water and 100 μl of a freshly prepared solution of 2.5 mg Dns[13] per ml acetone and incubated in the dark for 1 h at 37°C. Water and acetone were removed under a stream of nitrogen and the samples were dissolved in 100 μl of 0.5 % TFA or larger volumes of 0.2 % TFA and used for HPLC. The same method of dansylation was used to prepare the Dns-derivatives of phosphoserine, phosphothreonine and phosphotyrosine. Volumes of 200 μl of 20 m*M* solutions of the phosphorylated amino acids were treated with 200 μl of Na_2CO_3, 200 μl water and 200 μl Dns as indicated above. The phosphoamino acids (4–12 μl of 20 m*M* solutions) can also be added directly to the sample before acid hydrolysis.

HPLC with C_{18} columns

The apparatus utilized for HPLC consisted of a Laboratory Data Control minipump (LDC, Milton Roy) connected with a guard or pre-column of C_{18} silica (Whatman No. 6561-403) and an analytical column (μBondaPak C_{18}, Waters Assoc.). The column was washed with water–acetonitrile–TFA (925:75:1) for 15 min at a flow-rate of 1.7 ml/min and then the samples were applied and separated isocratically using the same water–acetonitrile–TFA mixture. The absorbance at 280 nm was

monitored with a Perkin-Elmer LC15 detector and 0.85 ml fractions were collected and counted using a scintillation counter and Formula 947 (New England Nuclear) as scintillation fluid. The column was washed for 10 min with water–acetonitrile (1:4) and then with the water–acetonitrile–TFA mixture described before. If the column is heavily contaminated with labeled phosphorylated compounds the column can be washed with a linear gradient of 20 ml of water–TFA (1000:1) and 20 ml of water–acetonitrile–TFA (200:800:1). All linear gradients described in this paper were prepared by adding the finishing solution at a rate of 0.85 ml/min into a mixing beaker with a magnetic stirrer and containing the appropriate amount of starting solution (in this case 20 ml). The mixture was withdrawn from the beaker at a speed of 1.7 ml/min and injected into the columns with the minipump. Samples containing labeled phosphotyrosine require the use of two analytical columns in series or the use of diphenyl columns as described below.

HPLC using diphenyl columns

We used the same apparatus as described above but the analytical C_{18} column was replaced with a Protesil 300 diphenyl column from Whatman (Cat. No. 4250-111). The column was washed with water–acetonitrile–TFA (970:30:1) for 15 min at a flow-rate of 1.7 ml/min. Alternatively, the column may be washed with water–TFA (1000:1) in samples containing large amounts of polar labeled compounds to obtain a better separation of these compounds from phosphoserine. The dansylated protein hydrolyzate was applied to the column and eluted with a linear gradient of 30 ml water–acetonitrile–TFA (970:30:1) and 30 ml water–acetonitrile–TFA (900:100:1). The linear gradient was prepared with the two pumps at different speeds as indicated above. Polar labeled compounds can be washed out of the column by eluting them with water–TFA (1000:1) before starting the gradient. After every run the columns were washed for 10 min with water–acetonitrile (1:4). If the columns are contaminated with large amounts of labeled compounds they can be further washed with the gradient of acetonitrile in water as indicated for the C_{18} columns.

RESULTS AND DISCUSSION

Fig. 1A shows the chromatogram of a mixture of dansylated phosphoserine (peak 1), serine (peak 2), phosphothreonine (peak 3) and threonine (peak 4) separated with a C_{18} μBondapak column as indicated in the Materials and methods section. This time the column was washed with water–TFA (1000:1) for 15 min. Then the sample was applied and eluted with the same solution for 10 min. This previous washing without acetonitrile is useful in samples containing large amounts of free phosphoric acid and other polar labeled compounds, because they can be better separated from the phosphorylated amino acids this way than using the standard water–acetonitrile–TFA mixture described in the Materials and methods. The elution with water–acetonitrile–TFA was started at the position of the arrow. The first absorbance peak eluting from the column in this figure and the small peak eluting between this first peak and dansylated phosphoserine in other figures are degradation products of the large excess of Dns used for the dansylation of the amino acids. They probably correspond to dansic acid and Dns-amide. The method of separating Dns-phosphoserine and -phosphothreonine indicated in Fig. 1A will be useful with a

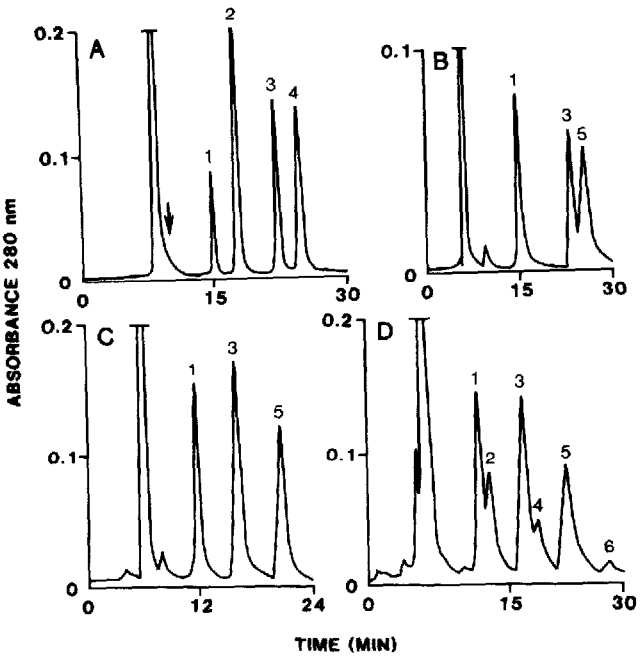

Fig. 1. HPLC from the Dns-derivatives of phosphorylated serine, threonine and tyrosine using C_{18} columns (A and B) and diphenyl columns (C and D). (A) separation of dansylated phosphoserine, phosphothreonine, serine and threonine using one analytical C_{18} column. (B) Separation of Dns-phosphoserine, -phosphothreonine and -phosphotyrosine using two analytical C_{18} columns attached in series. (C) Separation of the three phosphorylated, Dns-amino acids using a diphenyl column. (D) Phosphoserine, phosphothreonine and phosphotyrosine were heated at 110°C for 2 h. Then the mixture was dansylated and applied to a diphenyl column. The chromatographic conditions are indicated in the text. The numbers indicate the elution positions of the Dns-derivatives of phosphoserine (1), serine (2), phosphothreonine (3), threonine (4), phosphotyrosine (5) and tyrosine (6).

vast majority of samples, because the third phosphorylated amino acid (phosphotyrosine) represents only a small proportion of the total phosporylated amino acids of normal cells[1]. If phosphotyrosine is present, only the attachment of two analytical columns in series (Fig. 1B) results in a reasonable separation of phosphothreonine (peak 3) and phosphotyrosine (peak 5). However, the detection of small amounts of labeled phosphotyrosine in the presence of large amounts of labeled phosphothreonine would be almost impossible using this particular method of C_{18} column. For this reason it is better to use Diphenyl columns for samples containing all three phosphorylated amino acids (Fig. 1C). In this case the resolution between phosphothreonine (peak 3) and phosphotyrosine (peak 5) is excellent. In some cases it may be useful to calculate the recovery of the phosphoamino acids after acid hydrolysis. This can be done by adding non-labeled phosphotyrosine, phosphoserine and phosphothreonine to the protein sample before hydrolysis. Fig. 1D shows the separation of the three phosphorylated amino acids dansylated after an acid hydrolysis for 2 h at 110°C. Note the small amounts of (Dns) serine (peak 2), threonine (peak 4) and tyrosine (peak 6) present in the sample. From this chromatogram it would be easy to quantitate the degradation of phosphotyrosine. The degradation of phosphoserine

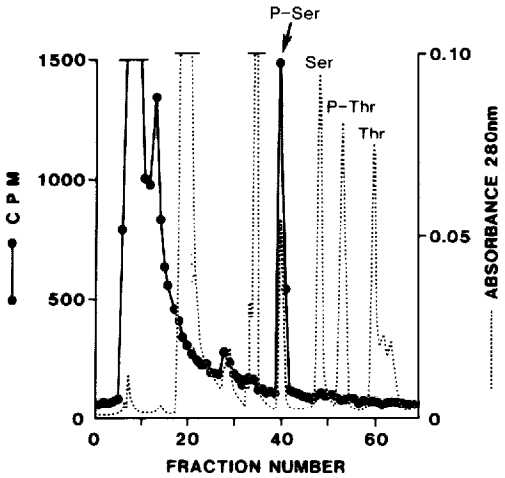

Fig. 2. HPLC using a C_{18} column of the phosphorylated and Dns-amino acids from a protein isolated from cultures of rat liver cells. Dns-phosphoserine, -phosphothreonine, -serine and -threonine were added as standards. There are other absorbing materials in the sample analyzed.

and phosphothreonine could be better observed using C_{18} columns as indicated in Fig. 1A, because these columns separate better serine and threonine from their phosphorylated derivatives. This method for calculating recoveries can not be applied to hydrolyzates of samples with large amounts of protein, because other Dns-amino acids might interfere with the identification and quantitation of the phosphorylated amino acids. It should be pointed out that many peptides can be hydrolyzed enzymatically with pronase[14]. This method eliminates the problem of partial hydrolysis in concentrated HCl.

Fig. 2 shows the analysis of the labeled phosphoamino acids present in a protein isolated from fetal rat liver cells after a 1-h incubation with ^{32}-P-phosphate. It is evident that the only major phosphorylated amino acid is phosphoserine. This column was run for 10 min with water–TFA (1000:1) before the application of the gradient of water–acetonitrile–TFA as indicated in the Materials and methods section. This example indicates that this method may be useful for the analysis of other labeled phosphorylated proteins obtained from cells incubated with labeled phosphate.

Fig. 3 shows the analysis of the labeled phosphoamino acids present in a RNA polymerase II preparation of calf thymus after a 10 min incubation with [γ-^{32}P]ATP. The chromatographic conditions were identical to those of Fig. 2. Note that the main phosphorylated amino acids are phosphoserine and phosphothreonine, as has been previously shown for the phosphorylation sites of RNA polymerase II labeled *in vitro* with casein kinase II[15]. Since purified calf thymus RNA polymerase II does not contain subunits structurally related to casein kinases I and II[16] one may conclude that the autophosphorylation of the commercial polymerase preparation used here may be due to the presence of some contaminating casein kinase or other kinase activity not as yet identified. It could be similar to the protein kinase NII, obtained during the purification of RNA polymerase I from Morris hepatoma cells and which has been suggested to play a role on the regulation of the RNA polymerase II[17]. The

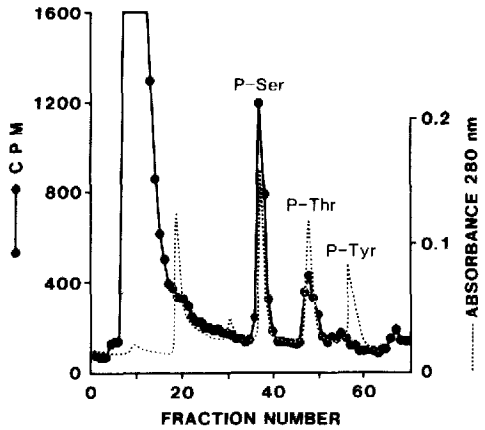

Fig. 3. HPLC with a diphenyl column of the phosphorylated and Dns-amino acids present in a RNA polymerase II preparation labeled with [γ-^{32}P]ATP as indicated in the next. The absorbance at 280 nm indicates the positions of the markers for Dns-phosphoserine, -phosphothreonine and -phosphotyrosine run immediately after the chromatography shown in this figure.

method described in this paper could then be useful to identify the phosphorylation sites of endogeneous protein kinase activities during the preparation of RNA polymerase.

Note that the small peak of radioactivity eluting after phosphothreonine in Fig. 3 does not correspond with the elution of phosphotyrosine in this chromatographic run using diphenyl columns. However, it did elute together with phosphotyrosine in the system shown in Fig. 1B (result not shown). This result indicates the importance of diphenyl columns for the analysis of samples suspected to have phosphorylated tyrosine. It should be pointed out that in proteins linked with RNA[7] partial acid hydrolysis can give nucleotidyl–(P–O) amino acids (or peptides) instead of the free phosphorylated amino acid. Phosphotyrosine can be cleaved from these compounds after micrococcal nuclease treatment[7]. Therefore, a micrococcal (or staphylococcal) nuclease treatment should be included in protein samples which may be transiently linked[18] or form stable bonds[7] with nucleic acids.

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