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Short Communication

O-Phosphoamino acid analysis of phosphorylated proteins by gas chromatography with flame photometric detection

HIROYUKI KATAOKA,* YUKIZO UENO and MASAMI MAKITA

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan

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Introduction

Phosphorylation of amino acids in proteins is one of the major regulatory mechanisms for the control of diverse intracellular functions [1, 2]. In eukaryotic organisms, serine, threonine and tyrosine residues are main substrates for phosphorylation by protein kinases. Measurement of these phosphorylated amino acids in proteins is important to determine the extent and nature of phosphorylation and the effect of each site of phosphorylation on the function of the altered proteins.

The quantitation of phosphorylated residues has been carried out through incorporation of [^{32}P] into the protein. The labelled protein was then hydrolysed, and the released *O*-phosphoamino acids were analysed by thin-layer chromatography [3–6], high-voltage electrophoresis [3, 7, 8] and high-performance liquid chromatography (HPLC) [9–13]. However, the analyses of protein hydrolysates after *in vivo* and *in vitro* labelling experiments tend to make errors by the possible presence of either mononucleotides, ATP, ribose phosphate or inorganic orthophosphate, which represent phosphorylated contaminants comigrating with *O*-phosphoamino acids. Furthermore, these radiotracer methods are not applicable to the analysis of endogenously phosphorylated proteins. On the other hand, nonradiolabelling analyses of *O*-phosphoamino acids in proteins have been carried out by HPLC [14–16] and immunodetection assay [17–19]. However, the former methods require a lengthy separation time or show poor resolution, and the latter involve problems about the specificity and cross-reactivity of the antibodies raised against each *O*-phosphoamino acid.

Recently, a selective and sensitive method for determining *O*-phosphoamino acids by gas chromatography (GC) with flame photometric detection (FPD) [20] has been developed, in which compounds were analysed as their *N*-isobutoxycarbonyl methyl ester derivatives. This method was available for the analysis of *O*-phosphoamino acids in the pico-mole range. The present work describes application of this method to the *O*-phosphoamino acid analysis of protein phosphorylated by protein kinase without radiolabelling.

Experimental

Reagents

O-Phospho-L-serine (P-Ser), *O*-phospho-D,L-threonine (P-Thr) and *O*-phospho-L-tyrosine (P-Tyr) were purchased from Sigma (St Louis, MO, USA). 2-Amino-4-phosphonobutyric acid (APB) and 2-amino-7-phosphonoheptanoic acid (APH) as internal standards (I.S.) were also purchased from Sigma. Each compound was dissolved in water to make up a stock solution at a concentration of 1 mM.

* Author to whom correspondence should be addressed.

Histone from calf thymus type II S, protamine sulphate from salmon sperm, albumin from bovine serum fraction V, dephosphorylated α-casein from bovine milk, protein kinase catalytic sub-unit from bovine heart, γ-ATP, prostaglandin E1 and human thrombin were purchased from Sigma. Isobutyl chloroformate was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). *N*-Methyl-*N*-nitroso-*p*-toluenesulphonamide to generate diazomethane was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical grade.

Phosphorylation of protein by protein kinase

Phosphorylation of a protein sample was performed in a 0.5 × 50-mm glass test tube. The reaction mixture (0.2 ml), containing 50 mM Hepes buffer pH 6.8, 10 mM $MgCl_2$, 20 nmol APB (I.S.), 1 mg protein, 2 mM ATP and 320 μg protein kinase catalytic sub-unit (reconstituted with dithiothreitol), was incubated at 37°C for 4 h. The reaction was terminated by addition of 10 μl of 2 M HCl, and the reaction mixture was used for the acid hydrolysis.

Phosphorylation in human platelets stimulated by thrombin

Isolation of platelets was performed using a polypropylene tube at room temperature. Human blood was collected in ethylenediamine tetraacetate (5 mM), and then centrifuged at 3000***g*** for 6 min. Prostaglandin E1 (280 nM) was added to the supernatant platelet-rich plasma (1 ml), and the mixture was centrifuged at 800***g*** for 15 min. The pellet (platelet) was resuspended in 1 ml of platelet-washing buffer (113 mM NaCl, 4.3 mM K_2HPO_4, 4.3 mM Na_2HPO_4, 24.4 mM NaH_2PO_4, 5.5 mM glucose) containing 280 nM prostaglandin E1 [21]. After centrifugation at 800***g*** for 15 min, the platelet was finally resuspended in 0.2 ml of modified Tyrode–Hepes buffer (113 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.36 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM glucose, 5 mM Hepes at pH 7.4). Stimulation of human platelet by thrombin was carried out by the method of Nakamura *et al.* [22]. Thrombin (1 unit ml^{-1}) was added to the suspended platelet and the mixture was allowed to stand for 1 min at room temperature. The reaction was terminated by addition of 10 μl of 2 M HCl, and the mixture was used for the acid and base hydrolyses.

Determination of protein concentration

Protein concentration was measured by the method of Miller [23] with bovine serum albumin as a standard.

Acid hydrolysis

Phosphorylated protein and platelet samples were placed in a 5 × 50-mm glass test tube, and 0.5 nmol APB (I.S.) was added to the platelet sample. The mixture was dried in a Model RD-41 centrifugal evaporator (Yamato Kagaku, Tokyo, Japan), and hydrolysed with 0.2 ml of 6 M HCl in the vapour phase for 2 h at 110°C with a Pico-Tag workstation (Waters Assoc., Milford, MA, USA). The resulting hydrolysate was extracted twice with 0.5 ml of distilled water, the extracts being transferred to another reaction tube (10-ml Pyrex glass tube with a PTFE-lined screw cap), and then used for the P-Ser and P-Thr analyses.

Base hydrolysis

A platelet sample was placed in a 9 × 75-mm polypropylene tube, and 0.1 nmol APH (I.S.) was added. After adding 0.25 ml of 10 M KOH, the total volume was made up to 0.5 ml with distilled water if necessary. The mixture was then hydrolysed for 1 h at 130°C in the Pico-Tag workstation. To the resulting hydrolysate was added 0.2 ml of 8 M HCl, before the solution was transferred to the reaction tube, and used for the P-Tyr analysis.

Derivatization procedure

O-Phosphoamino acids in the acid and base hydrolysates were derivatized as previously described [24]. After the hydrolysate sample had been adjusted to pH 10 with 2 M NaOH if necessary, 0.1 ml of isobutyl chloroformate was added. The mixture was shaken with a shaker set at 300 rpm (up and down) for 10 min at room temperature. The reaction mixture was then acidified to pH 1–2 with 2 M HCl and extracted with 3 ml of diethyl ether in order to remove the excess of reagent, the ethereal extract being discarded. The aqueous layer was saturated with NaCl and then extracted twice with 3 ml of diethyl ether containing 10% 2-propanol. The pooled ethereal extracts were methylated by bubbling diazomethane, generated according to the micro-scale procedure of Schlenk and Gellerman [25], through the solution until a yellow tinge became visible. After standing for 5 min at room temperature, the solvents were removed by evaporation to

dryness at 80°C under a stream of dry air. The residue was dissolved in 0.05–0.1 ml of ethyl acetate and then 1 μl of this solution was injected into the gas chromatograph.

Gas chromatography

GC analysis was carried out with a Shimadzu 14A gas chromatograph equipped with a flame photometric detector (P-filter). Fused-silica capillary columns of cross-linked DB-1701 (J&W, Folsom, CA, USA, 15 m × 0.53 mm i.d., 1.0 μm film thickness, for the P-Ser and P-Thr analyses) and DB-5 (J&W, 15 m × 0.53 mm i.d., 1.0 μm film thickness, for the P-Tyr analysis) were used. The operating conditions were as follows: column temperatures, 210°C (for P-Ser and P-Thr) and programmed at 2°C min^{-1} from 245 to 270°C (for P-Tyr); injection and detector temperatures, 260°C (for P-Ser and P-Thr) and 290°C (for P-Tyr); nitrogen flow-rate, 10 ml min^{-1}. The peak heights of *O*-phosphoamino acids and the I.S. (APB or APH) were measured, and the peak height ratios against the I.S. were calculated to construct a calibration curve.

Results and Discussion

O-Phosphoamino acids could be conveniently converted into their *N*-isobutoxycarbonyl trimethyl ester derivatives and selectively determined by the FPD–GC method as previously described [24]. By using this method, the calibration curves were linear in the range of 0.05–5 nmol for P-Ser and P-Thr, and in the range of 0.05–1 nmol for P-Tyr. The detection limits for P-Ser, P-Thr and P-Tyr were about 0.26, 0.18 and 0.30 pmol as injection amounts, respectively. The *O*-phosphate linkages of serine and threonine residues are more stable to acid than to base, but that of a tyrosine residue is more stable to base than to acid. Therefore, protein hydrolyses were carried out in acid for the P-Ser and P-Thr analyses, and in base for the P-Tyr analysis. The best hydrolysis conditions for the proteins proved to be 6 M HCl at 110°C for 2 h (for P-Ser and P-Thr), and 5 M KOH at 130°C for 1 h (for P-Tyr).

The usefulness of this FPD–GC method in identifying *O*-phosphoamino acids in protein hydrolysates was first determined using phosphorylated calf thymus histone as an example. Cyclic AMP-dependent protein kinase has been demonstrated to phosphorylate serine residue on histone [12]. As shown in Fig. 1, when histone is phosphorylated by the catalytic subunit of protein kinase, serine and threonine residues are phosphorylated (Fig. 1B). Although a trace of endogenous P-Ser residue exists in histone, phosphorylation of serine and

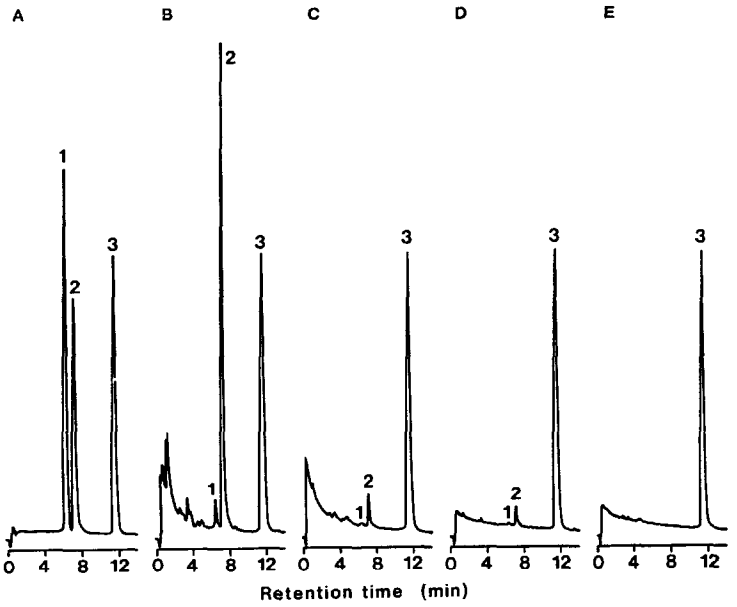

Figure 1
Gas chromatograms obtained from (A) a standard solution containing 20 nmol of each compound, and acid hydrolysates after phosphorylation assay of histone incubated (B) with complete system, (C) without protein kinase, (D) without ATP and (E) without histone. GC conditions: see Experimental. Peaks: 1=*O*-phosphothreonine, 2=*O*-phosphoserine, 3=2-amino-4-phosphonobutyric acid (internal standard).

Table 1
O-Phosphoamino acid analysis of some proteins phosphorylated by protein kinase

Protein	*O*-Phosphoamino acid	*O*-Phosphoamino acid (nmol mg^{-1} protein)*		Level of significance (*P* value)†
		−Protein kinase	+Protein kinase	
Histone	P-Ser	0.33 ± 0.03	4.24 ± 0.39	<0.001
	P-Thr	0.04 ± 0.004	0.14 ± 0.02	<0.01
Protamine	P-Ser	0.42 ± 0.03	1.39 ± 0.20	<0.01
	P-Thr	0.03 ± 0.004	0.06 ± 0.01	<0.01
Albumin	P-Ser	0.14 ± 0.01	0.58 ± 0.08	<0.001
	P-Thr	ND‡	0.06 ± 0.01	<0.001
Dephosphorylated α-casein	P-Ser	116.4 ± 5.7	161.4 ± 6.8	<0.001
	P-Thr	0.24 ± 0.01	0.56 ± 0.01	<0.001

*Mean ±SD (n = 3).
†Student's t-test was used for statistical evaluation.
‡Not detectable.

Table 2
Effects of stimulation by thrombin on the phosphorylation of human platelet proteins

O-Phosphoamine acid	Content (pmol mg^{-1} protein)*		Level of significance (*P* value)†
	−Thrombin	+Thrombin	
P-Ser	924 ± 65	1246 ± 129	<0.02
P-Thr	62 ± 9	90 ± 5	<0.01
P-Tyr	16 ± 1	25 ± 1	<0.01

*Mean ±SD (n = 3).
†Student's t-test was used for statistical evaluation.

threonine residues is not observed without protein kinase or ATP (Fig. 1C,D). By incubation without histone, it is demonstrated that interfering peaks do not arise from other sources such as protein kinase (Fig. 1E). Therefore, enzymatic phosphorylation was evaluated by subtracting the value for the non-protein kinase assay from the value for the protein kinase assay. Under the same phosphorylation conditions, protamine, albumin and dephosphorylated α-casein were also significantly phosphorylated in serine and threonine residues (Table 1).

A second application of this FPD–GC method in identifying *O*-phosphoamino acids in proteins was carried out using human platelets stimulated by thrombin. Stimulation by thrombin has been demonstrated to phosphorylate serine, threonine and tyrosine residues in platelet proteins [22, 26]. As shown in Table 2, when platelets were stimulated by thrombin, the phosphorylation of serine, threonine and tyrosine residues increased by about 1.3–1.6 times that of non-stimulated platelets. The increases in these *O*-phosphoamino acids were statistically significant.

The proposed method is reproducible and directly applicable for the analysis of the extent of both *in vivo* and *in vitro* phosphorylation without radiolabelling. Therefore, this method is considered to provide a useful tool for biochemical and biomedical research requiring *O*-phosphoamino acid analysis.

References

[1] E.G. Krebs, *Biochem. Soc. Trans.* **13**, 813–820 (1985).
[2] P. Cohen, *Proc. Roy. Soc. London*, **B234**, 115–144 (1988).
[3] M. Manai and A.J. Cozzone, *Anal. Biochem.* **124**, 12–18 (1982).
[4] W.C. Chang, M.L. Lee, C.K. Chou and S.C. Lee, *Anal. Biochem.* **132**, 342–344 (1983).
[5] E. Neufeld, H.J. Goren and D. Boland, *Anal. Biochem.* **177**, 138–143 (1989).
[6] G. Munoz and S.H. Marshall, *Anal. Biochem.* **190**, 233–237 (1990).
[7] M.P. Kamps and B.M. Sefton, *Anal. Biochem.* **176**, 22–27 (1989).
[8] E. Hildebrandt and V.A. Fried, *Anal. Biochem.* **177**, 407–412 (1989).
[9] J.C. Yang, J.M. Fujitaki and R.M. Smith, *Anal. Biochem.* **122**, 360–363 (1982).
[10] N. Morrice and A. Aitken, *Anal. Biochem.* **148**, 207–212 (1985).
[11] L. Carlomagno, V.O. Huebner and H.R. Matthews, *Anal. Biochem.* **149**, 344–348 (1985).

[12] J.C. Robert, A. Soumarmon and M.J.M. Lewin, *J. Chromatogr.* **338**, 315–324 (1985).
[13] M.C. McCroskey, J.R. Colca and J.D. Pearson, *J. Chromatogr.* **442**, 307–315 (1988).
[14] J.S. Niedbalski and D.P. Ringer, *Anal. Biochem.* **158**, 138–145 (1986).
[15] D.A. Malencik, Z. Zhao and S.R. Anderson, *Anal. Biochem.* **184**, 353–359 (1990).
[16] L.R. Murthy and K. Iqbal, *Anal. Biochem.* **193**, 299–305 (1991).
[17] J.Y.J. Wang, *Anal. Biochem.* **172**, 1–7 (1988).
[18] D. Heffetz, M. Fridkin and Y. Zick, *Eur. J. Biochem.* **182**, 343–348 (1989).
[19] R.M. Lyons, N. Stanford and P.W. Majerus, *J. Clin. Invest.* **56**, 924–936 (1975).
[20] H. Kataoka, Y. Ueno and M. Makita, *Agric. Biol. Chem.* **55**, 1587–1592 (1991).
[21] N.L. Baenziger and P.W. Majerus, *Methods Enzymol.* **31**, 149–155 (1974).
[22] S. Nakamura and H. Yamamura, *J. Biol. Chem.* **264**, 7089–7091 (1988).
[23] G.L. Miller, *Anal. Chem.* **31**, 964 (1959).
[24] H. Kataoka, N. Sakiyama and M. Makita, *J. Chromatogr.* **436**, 67–72 (1988).
[25] H. Schlenk and J.L. Gellerman, *Anal. Chem.* **32**, 1412–1414 (1960).
[26] U.P. Naik, E. Kornecki and Y.H. Ehrlich, *Biochim. Biophys. Acta* **1092**, 256–264 (1991).

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