CHARACTERIZATION OF CYTOSOL PHOSPHOPEPTIDES

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1. Introduction

In a previous paper the isolation from rat liver cytosol of a phosphoprotein fraction has been described [1]. Such a phosphoprotein fraction, containing both phosphorylserine and phosphorylthreonine, was found to have a phosphate content approaching 1%.

However the feeling that such a preparation was still contaminated by non-phosphorylated proteins prompted us to improve the purification. In the present paper a new procedure involving polyacrylamide gel electrophoresis is described leading to a phosphorylated fraction which is apparently free of non-phosphorylated proteins.

The average molecular weight of such a purified fraction, evaluated by gel filtration, is about 2000. Its P content is about 4%, the highest ever reported for any phosphoprotein except phosvitin. The amino acid composition indicates a very acidic molecule with about 30% of glutamic and aspartic acid residues, and virtually all its serine and threonine as phosphorylated residues. Moreover quite remarkable amounts of bound iron are constantly present.

2. Experimental

2.1 *^{32}P-labelling of 'Cytosol Phosphoprotein'*

Thirty to 40 mg of a crude preparation of cytosol phosphoprotein obtained by DEAE-cellulose column chromatography as previously described [1] were incubated in 12 ml of a medium containing: 0.1 M Tris–HCl buffer pH 7.5; 6 mM ATP containing 0.4 mCi as [γ–^{32}P] ATP; 0.5 ml of cytosol phosvitin kinase purified as previously described [2]. After 2 hr incubation at room temperature the labelled protein was separated from [^{32}P] ATP by filtration through a Sephadex G-25 column (1.9 × 140 cm) equilibrated with 0.05 M Tris–HCl buffer pH 7.5 containing 0.1 M NaCl. The radioactive protein fraction eluted at the void volume was concentrated by ultrafiltration through Diaflo UM 2 membranes to a volume of about 5 ml.

2.2 *Sephadex G-200 gel filtration of [^{32}P] proteins*

Concentrated samples of ^{32}P-labelled phosphoprotein were submitted to gel filtration through a Sephadex G-200 column (2.7 × 50 cm) equilibrated with 0.05 M Tris–HCl, pH 7.5, containing 0.7 M NaCl and 1.0 mM mercaptoethanol. Four ml fractions were collected. The two radioactive peaks eluted from the column were concentrated by ultrafiltration to a volume of about 5 ml. Finally these two [^{32}P] protein fractions were desalted by Sephadex G-25 filtration prior to further purification.

2.3 *Gel electrophoresis of [^{32}P] proteins*

Both the radioactive protein fractions obtained by the above procedure were submitted to electrophoresis in 7.5% polyacrylamide gel, pH 8.9 [3]. The spacer gel was replaced by 40 mg of Sephadex G-25 including 0.2 ml of protein sample. At the end of the run (4 mA per tube for about 60 min at room temperature) the gel columns were sliced in 2 mm segments which were transferred to stainless steel planchettes and counted in a thin window Geiger

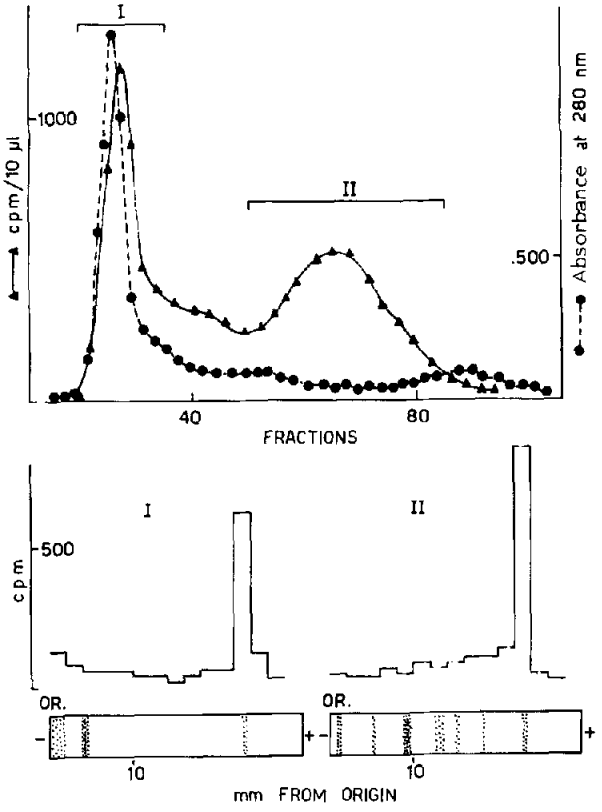

Fig. 1. Isolation of [^{32}P]phosphopeptides from cytosol 'phosphoprotein fraction'. Top figure: Sephadex G-200 gel filtration of ^{32}P-labelled cytosol phosphoprotein fraction eluted from DEAE-cellulose by 0.2 M NaCl [1]; Bottom figures: polyacrylamide gel electrophoresis profiles of peak I and II from top figure. Experimental conditions are described in the Experimental section. (●–●–●) Protein, absorbance at 280 nm; (▲–▲–▲) protein bound ^{32}P, as cpm/10 µl.

counter in order to localize the radioactive bands. A parallel gel column was stained in 0.05% Coomassie blue in 12% trichloroacetic acid in order to evidence proteins.

2.4 *Chemical analysis of purified phosphorylated fraction*

Fairly large amounts of ^{32}P-labelled peptides (0.1–0.3 mg) were prepared by pooling together the radioactive segments obtained from 30 to 40 gel-electrophoresis columns and by eluting them through electrophoresis in 25 ml of 10 mM Tris–HCl, pH 8.5, in an electrolytic cell (40 mA at about 10°C); by keeping the gel segments near the cathode, the labelled phosphopeptides in a few minutes migrate out of the gel toward the anode. The sample, once concentrated by ultrafiltration, was filtered through a Sephadex G-25 column (1.9 X 50 cm) equilibrated with distilled water and finally lyophilized.

Protein concentration was determined by the Folin and Ciocalteau procedure as modified by Lowry et al. [4].

Alkali labile P was determined as P_i liberated in 1 N NaOH at 100°C in 15 min, according to the Martin and Doty procedure [5].

Total P was determined according to Wagner [6] after digestion of the dried sample in 0.4 ml of 70% $HClO_4$.

Iron was determined spectrophotometrically as Bathophenanthroline complex extracted into isoamyl alcohol [7].

Amino acid analysis was carried out in a Geol Gacylc 5AH apparatus on 50–100 µg samples of purified phosphopeptides previously hydrolyzed in 3 ml of 6 N HCl at 105°C for 24 hr in sealed tubes under nitrogen atmosphere.

The molecular weight of the purified cytosol phosphorylated fraction was evaluated by gel filtration of 25–50 µl aliquots (equivalent to 60 000 cpm as protein bound ^{32}P) through a 0.9 X 70 cm Sephadex G-50 superfine column equilibrated with 10% acetic acid. 0.5 ml fractions were collected and 0.25 ml from each fraction were counted in a Packard mold 3375 liquid scintillator.

2.5 *Materials*

Gel filtration materials were from Pharmacia. All the reagents for gel electrophoresis and protein staining were from Serva. Horse heart cytochrome *c* was from Sigma. Salmin and insulin were from BDH. [γ^{-32}P] ATP was from the Radiochemical Centre, Amersham. Diaflo UM2 ultrafiltration membranes were from Amicon. Other reagents were from Merck. The synthetic octapeptide was a gift of Professor F. Marchiori, Istituto di Chimica Organica, University of Padova.

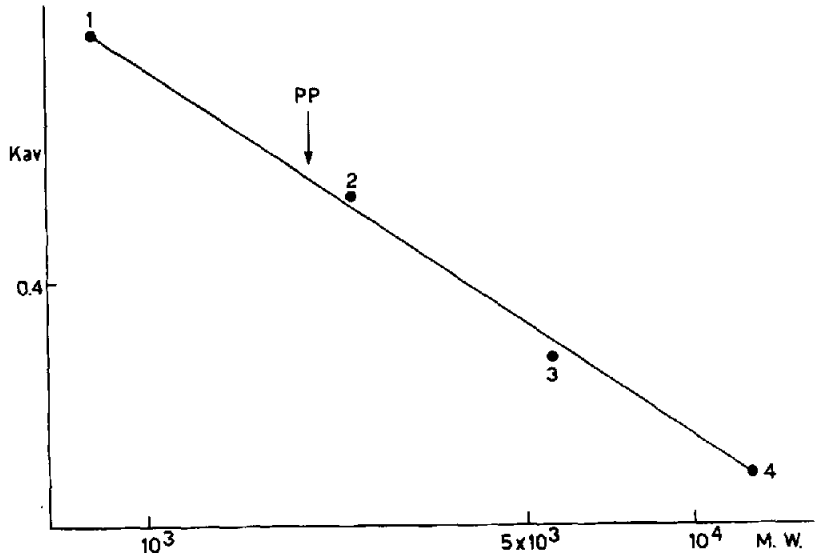

Fig. 2. Molecular weight evaluation of cytosol phosphopeptides by Sephadex G-50 gel filtration. General conditions are described in the experimental section. The column was calibrated with Dextran blue (to determine the void volume) and with: i) RNAase synthetic octapeptide 13–20 (mol. wt 800); ii) insulin A chain [9] (mol. wt 2400; iii) salmine sulfate (mol. wt 5 500 [8]); iv) horse heart cytochrome *c* (mol. wt 12 400).

3. Results

3.1 *Purification of cytosol phosphopeptides*

The crude phosphorylated fraction obtained by DEAE-cellulose chromatography was labelled by incubation with purified protein kinase in the presence of [^{32}P] ATP and then submitted to a purification procedure consisting of Sephadex G-200 gel filtration followed by gel electrophoresis in 7.5% polyacrylamide pH 8.9 (see fig. 1). As previously reported [1], gel filtration resolves the labelled fraction into two radioactive peaks with sharply different specific radioactivities (expressed as cpm/mg protein). Both peaks however, once submitted to gel electrophoresis, give rise to a single major radioactive band exhibiting the same specific radioactivity independently of the G-200 peak from which it is derived. Such a band runs near the front, quite well separated from several non-radioactive proteins which either are retarded or do not even enter the gel. The polyacrylamide pattern would indicate that both Sephadex G-200 peaks contain the same or very similar phosphorylated components which are tightly bound to unlabelled proteins of larger molecular weight. Apparently gel electrophoresis is more effective than gel filtration in separating the labelled components from the unlabelled ones.

3.2 *Molecular weight estimation*

The great mobility on polyacrylamide gel electrophoresis suggested that the purified phosphorylated fraction had a very low molecular weight. After several attempts a satisfactory evaluation of its molecular weight was attained by gel filtration through Sephadex G-50 in 10% acetic acid. Under these conditions the ^{32}P-labelled material is eluted as a rather broad peak slightly more retarded than insulin A chain; its average mol.wt, calculated at the top of the peak, results as slightly more than 2000 (fig. 2).

3.3 *Chemical composition*

The purified ^{32}P-labelled fraction obtained by polyacrylamide gel electrophoresis was found to con-

Table 1
P and Fe content of crude phosphorprotein fraction and of phosphopeptides purified from it.

	μg/mg protein	
	P	Fe
Crude phosphoprotein fraction	1.05	0.39
Purified phosphopeptides	47.50	18.72

Conditions are described in the experimental section.

tain more than 4% phosphate, virtually all alkali labile, and variable, but always appreciable, amounts of bound iron. As shown in table 1, where the data from a typical preparation are reported, both P and Fe increase in a parallel way while passing from the crude DEAE-cellulose fraction to the purified one, thus suggesting a close relationship between these two constituents. In table 2 the amino acid composition of purified cytosol phosphopeptide fraction is reported together with that of nuclear phosphoprotein [10].

Table 2
Aminoacid composition of cytosol phosphoperptides.

	Moles/100 moles of amino acids	
	Cytosol phospho-peptides	Nuclear phosphoproteins [10]
Asp	11.7	9.5
Glu	19.8	13.5
Lys	5.5	8.0
His	2.4	2.2
Arg	3.5	9.8
Ser	9.3	10.3
Thr	5.0	4.3
Pro	5.5	7.1
Gly	11.6	7.6
Ala	7.2	6.4
Cys	-	0.3
Val	5.5	5.1
Met	-	1.6
Ile	2.6	3.2
Leu	6.1	6.5
Tyr	-	2.1
Phe	2.6	2.7
Basics/acidics	0.36	0.87
Phosphorous	4.3%	1.14%

The data reported are the average of six analyses carried out on different phosphopeptide preparations obtained from Sephadex G-200 peak II.

4. Discussion

The purification procedure described in the present paper provided the demonstration that the two ^{32}P-labelled peaks obtained by Sephadex G-200 gel filtration of rat liver cytosol phosphorylated crude fraction [1] contain the same or very similar phosphorylated components, which, according to their very low molecular weight, must be regarded as large phosphopeptides rather than phosphoproteins. Such a fraction is characterized by high phosphate content – over 4% – the highest ever reported for any phosphoprotein but phosvitin. Assuming that all the phosphate present is esterified with serine and threonine – which is expectable for its alkali lability – and calculating a hydrolytic loss of about 20% for these amino acids, it must be concluded that nearly all the serine and threonine residues are phosphorylated. By the way this would also indicate that any appreciable contamination from non-phosphorylated proteins or peptides is unlikely unless one assumes the absence of both serine and threonine from such hypothetical contaminants. A similar situation has been found in phosvitin [11]. Another similarity between cytosol phosphopeptides and phosvitin is the enzyme specificity, i.e. both are phosphorylated by cytosol protein kinase and dephosphorylated by mitochondrial protein phosphatase. Moreover both contain conspicuous amounts of protein-bound iron. On the contrary the cytosol phosphopeptides differ from histones and protamines, which also must be regarded as phosphorylated protein [10, 12], for their acidic nature, high phosphate content and enzyme specificity [1].

A comparison between the amino acid composition of cytosol phosphopeptides and nuclear phosphoproteins is of some interest. One major discrepancy is due to the ratio of the basic to acidic amino acids, which, in the cytosol phosphopeptides, is about three times lower than in nuclear phosphoproteins. Moreover the phosphate content of cytosol phosphopeptides is three times higher than that of nuclear phosphoproteins. These two discrepancies would indicate that cytosol phosphopeptides and nuclear phosphoproteins

are not identical, unless one assumes that cytosol phosphopeptides are generated during their isolation and purification by the proteolytic cleavage of larger phosphoproteins. The finding however that such phosphorylated peptides can be isolated also from rat liver cytosol prepared in the presence of 10^{-6}/M Diisopropylphosphofluoridate (DEP), which is expected to inhibit proteases, apparently rules out such a possibility. It is still possible however that, on the other hand, nuclear phosphoproteins are contaminated by histones, which could account for both the low phosphate content and the relatively high basic to acidic amino acid ratio.

The clarification of this point might be of great relevance for the understanding of the role of such acidic phosphopeptides. Although this problem is still open, at present our knowledge about the presence of iron in cytosol phosphopeptides, the small size of their molecules and the intracellular localization of the enzymes involved in their phosphorylation and dephosphorylation [13] suggest that cytosol phosphopeptides might be involved in iron translocation across the metochondrial membrane. Experiments supporting such an hypothesis have been already reported [14].

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