ISOLATION AND ENZYMATIC PHOSPHORYLATION OF RAT LIVER CYTOSOL PHOSPHOPROTEINS

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1. Introduction

We have previously reported that in rat liver cells the protein kinase activity, tested on phosvitin and casein, is located mostly (up to 90%) in the cytosol [1]. This finding prompted us to try the isolation and identification of the cytosol phosphoprotein which might be the natural substrate for the protein kinase.

2. Experimental

Phosvitin was prepared from egg yolk according to Mecham and Olcott [2]. Hammarsten casein was from Merck. Protamine was "salmine" from B.D.H. Ion exchange resins were from Serva. All the other chemicals were from Sigma.

Cytosol was prepared by centrifuging for 60 min at 105,000 × *g* the 0.25 M sucrose homogenate after nuclei and mitochondria had been previously discarded by differential centrifugations [3]. The clear supernatant was dialysed against water at neutral pH, lyophilized and dissolved in the minimal volume of 0.05 M Tris-HCl pH 7.5. Small amounts of insoluble proteins were discarded by centrifugation.

5 ml of the clear supernatant fluid, containing 300–500 mg of cytosol proteins were chromatographed through DEAE cellulose column (2 cm × 9 cm) equilibrated with 0.05 M Tris-HCl pH 7.5. A stepwise elution was carried out with 0.05 M Tris-HCl pH 7.5 containing the following increasing concentrations of NaCl: 0.10 M, 0.20 M, 0.25 M, 0.30M, 0.40 M, 0.75 M, 1.00 M; the column was finally eluted with 0.5 M acetate pH 6.0 containing 1.0 M NaCl and with 0.5 M NaOH. The protein peaks eluted from the column were dialysed against water and concentrated by liophylization.

The phosphorylation of the protein fractions was tested by incubating 1–2 mg protein, previously heated for 5 min at 65°, in 1 ml of a medium containing: Tris-HCl buffer pH 7.5, 100 μmoles; $MgCl_2$, 6 μmoles; ATP, 0.5 μmoles containing 1–3 μC of [γ-^{32}P] ATP, prepared according to Glynn and Chappel [4]. The reaction was started by addition of 1–2 units of phosvitin kinase *, or 0.5 mg of protamine kinase. The incubation, carried out at 37° for 30 min, was stopped by addition of 0.3 ml of 50% trichloroacetic acid. The precipitated proteins were washed 4 times with 10% trichloroacetic acid, transferred on a stainless planchet and counted in a thin window Geiger counter. When the phosphorylation of protamine was tested 0.25 ml of Silicotunstic acid solution, prepared as described in ref. [5], was also added besides trichloroacetic acid as precipitating agent.

The amount of protein-bound ^{32}P-phosphorylserine was determined by paper electrophoresis of the hydrolysate (2N HCl at 100° for 10 hr) of the labelled protein, as previously described [6].

* Since the protein concentration of the enzyme solutions was too low for being accurately determined by O.D. measurements, a conventional phosvitin kinase unit has been defined as that amount of enzyme which catalyzes the transfer of 1 mμmole of ^{32}P from [γ-^{32}P] ATP to phosvitin in 10 min at 37°, pH 7.5, and in the presence of 6 mM $MgCl_2$.

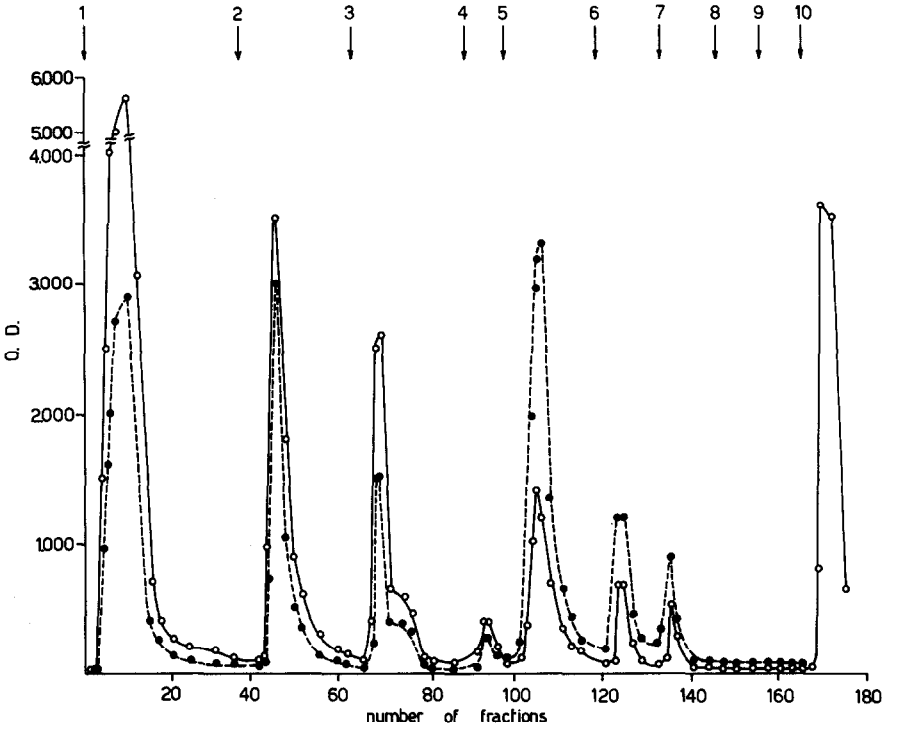

Fig. 1. DEAE-cellulose chromatography of rat liver cytosol. General conditions were described in the "experimental" section. At the arrows the column was eluted by the following buffers: 1) 0.05 M Tris-HCl pH 7.5; 2–8) 0.05 M Tris-HCl buffer pH 7.5 containing 0.10, 0.20, 0.25, 0.30, 0.40, 0.75, 1.00 M NaCl respectively; 9) 0.5 M acetate buffer pH 6.0 containing 1.00 M NaCl; at arrow 10 the column was washed with 0.5 M NaOH. The flow rate of the column was 50 ml per hr and 5 ml fractions were collected. o——o Optical density at 280 mμ; •-----• Optical density at 260 mμ.

Phosvitin kinase was purified from rat liver cytosol by following two different procedures:

A – The previously described preparation of phosvitin kinase [7] was further purified by chromatography on DEAE cellulose column equilibrated with 0.05 M Tris-HCl, pH 9.00 and eluted by the same buffer containing the following increasing concentrations of NaCl; 0.1, 0.20, 0.25, 0.30, 0.40 M. The fraction eluted by 0.20 M NaCl was employed.

B – Phosvitin kinase completely free of protamine kinase activity was prepared by submitting the crude cytosol to ion exchange chromatography through Phosphate-cellulose (P-cellulose) columns equilibrated with 0.05 M Tris-HCl pH 7.5, and by eluting with the same buffer containing the following increasing concentrations of NaCl: 0.10, 0.20, 0.25, 0.30, 0.40 and 0.75 M. Phosvitin kinase eluted by 0.40 M NaCl has been utilized for our experiments.

Protamine kinase free of any phosvitin kinase activity was prepared by lowering the pH of crude rat liver cytosol to 4.7 with 10% acetic acid, and discarding the precipitate. The supernatant was neutralized by addition of NaOH and the precipitate formed was again discarded. Finally the clear supernatant fluid was fractionated by $(NH_4)_2SO_4$ precipitation: the fraction precipitated between 0.5 and 0.9 saturation was dissolved in the minimal volume of 0.05 M Tris-HCl pH 7.0 containing 0.1 mM EDTA and dialysed for 48 hr against the same buffer.

3. Results and discussion

Crude cytosol from rat liver was fractionated on DEAE cellulose column (see fig. 1) and the fractions

separately submitted to phosphorylation by ATP^{32}, in the presence and absence of added partially purified "phosvitin kinase". In order to minimize phosphorylation not dependent on added phosvitin kinase, the single fractions were preliminary heated at 65° for 5 min. The results, reported in table 1, indicate that the proteins able to be phosphorylated by phosvitin kinase were present in the two fractions eluted at 0.20 and 0.25 M NaCl, the remaining 6 fractions being completely inactive as substrates for this enzyme. It is interesting that the main phosphoprotein fraction, eluted by 0.20 M NaCl, in spite of the preliminary heating, still retained a detectable endogenous protein kinase activity.

In fig. 2 the time course of ^{32}P incorporation into the "0.20 M NaCl" phosphoprotein fraction is reported. It can be seen that under our conditions a maximum level of phosphorylation corresponding to about 1.5–2.0 mμmoles of ^{32}P incorporated per mg protein was reached within 15–30 min incubation.

It must be mentioned that the "phosvitin kinase" preparations used in the above experiments were contaminated by "protamine kinase" *. In further experiments the phosphorylation of the "0.20 M NaCl" cytosol phosphoprotein fraction has

Table 1

Phosvitin kinase dependent phosphorylation of cytosol proteins fractionated by DEAE cellulose column chromatography. General conditions are described in the "experimental" section. Phosvitin kinase was prepared following the procedure A.

DEAE fraction eluted by:	Protein bound ^{32}P-phosphorylserine (CPM/mg protein)	
	Phosvitin kinase omitted	Plus phosvitin kinase
0.05 M Tris	0.00	0.00
+ 0.1 M NaCl	18	15
+ 0.20 M NaCl	176	850
+ 0.25 M NaCl	31	292
+ 0.30 M NaCl	11	0.00
+ 0.40 M NaCl	6	3
+ 0.75 M NaCl	0.00	0.00
+ 1.00 M NaCl	0.00	0.00

* For convenience the name "protamine kinase" will be used to indicate the protein kinase which phosphorylates protamine and histones but is inactive on phosvitin [8].

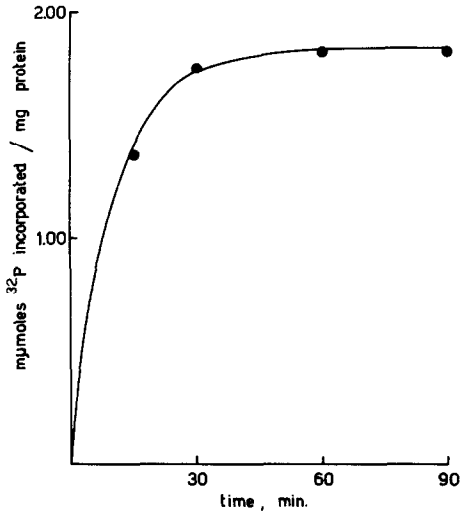

Fig. 2. Time course of ^{32}P incorporation into cytosol phosphoprotein. General conditions are described in the "experimental" section. The phosphoprotein fraction eluted from DEAE cellulose with 0.20 M NaCl was used. Phosvitin kinase was prepared following procedure A.

Table 2

Comparative phosphorylation of cytosol phosphoprotein by phosvitin kinase and protamine kinase.

	CPM incorporated into the protein by:	
	Phosvitin kinase	Protamine kinase
Phosvitin	8300	0.00
Casein	4750	0.00
Cytosol phosphoprotein	1115	260
Protamine	7	3870

General conditions are described in the "experimental" section. Protein additions were: 1 mg of phosvitin or protamine; 2 mg of casein and 1.5 mg of cytosol phosphoprotein fraction eluted from DEAE column with 0.20 M NaCl. Phosvitin kinase was prepared following the procedure B.

been studied in the presence of phosvitin kinase preparations completely free of any protamine kinase activity (preparation B: see Methods). The results, reported in table 2, show that such a preparation was still active on the cytosol phosphoprotein, while it is inactive on protamine. Table 2 also shows that a lower but significant labelling of the cytosol phosphoprotein was catalyzed by a preparation of protamine kinase

inactive both on phosvitin and casein. It is possible that some histones bound to the phosphoprotein fraction might be responsible for such a minor ^{32}P incorporation observed in the presence of protamine kinase.

No conclusion can be drawn at present about the identity between the cytosol phosphoproteins described in this paper and the phosphoproteins purified from nuclei by Langan [9] and Kleinsmith and Allfrey [10]. However the rather high ionic strength used by these authors for solubilizing the phosphoproteins from nuclei, would indicate that, unlike the cytosol phosphoproteins, the nuclear ones are probably bound to some insoluble structures.

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