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FURTHER COMPARATIVE CHARACTERIZATION OF RAT LIVER MITOCHONDRIAL AND CYTOSOL PROTEIN PHOSPHATASE

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SUMMARY

Mitochondrial and cytosol phosphoprotein phosphatases (phosphoprotein phosphohydrolase, EC 3.1.3.16) have been purified 452 and 156 times, respectively.

At such a stage of purification they appear to be two chromatographically distinct enzyme proteins, completely free of ATPase and of other phosphomonoesterase activity.

The two enzymes dephosphorylated two phosphoproteins (casein and phosvitin) to different extents: in particular the mitochondrial protein phosphatase was very active on phosvitin, whereas the cytosol enzyme was ineffective on this phosphoprotein.

INTRODUCTION

The activity of some enzyme systems, such as phosphorylase[1-3], glycogen synthetase[4,5] and pyruvate dehydrogenase complex[6,7], is regulated through a phosphorylation and dephosphorylation process catalysed by kinases and phosphatases. On the contrary, the physiological role of cellular phosphoproteins, apparently devoid of enzymic activity, is still an open problem. An experimental approach is provided by the study of intracellular localization of the enzymes responsible for the turnover of their protein-bound phosphate: namely protein kinase and phosphoprotein phosphatase.

Previously[8], it was shown that in the rat liver cells most of the protein kinase (phosvitin kinase) is located in the cytosol. On the other hand, in the same cells two different phosphatases have been evidenced: one free in the cytosol and the other located in the mitochondria[9].

The present paper deals with the purification of mitochondrial and cytosol phosphoprotein phosphatases (phosphoprotein phosphohydrolase, EC 3.1.3.16) up to a stage at which they appear as two chromatographically distinct enzyme proteins. Both purified enzymes appear to be specific for phosphoproteins and do not exhibit any activity towards other phosphoester substrates or ATP.

METHODS AND MATERIALS

Enzymic activity determinations

The protein phosphatase activity was assayed with casein as substrate. The incubation medium contained in a final volume of 2 ml: 100 μmoles of succinate buffer (pH 5.9), 12 μmoles of $MgCl_2$, 6 μmoles of cysteine, and casein equivalent to 5 μmoles of alkali-labile phosphate[10]. The reaction was started by the addition of enzyme preparation. After incubation for 20 min at 37° the reaction was stopped by the addition of 0.25 ml of 50% trichloroacetic acid and 0.75 ml silicotungstic acid. After centrifugation an appropriate aliquot of the supernatant was taken for determination of inorganic phosphate by the method of CHEN *et al.*[11].

Other substrates were also used in concentrations corresponding to 5 μmoles of phosphate.

A unit of activity is defined as the amount of enzyme that releases from the substrate 1 nmole of P_i in 1 min. Protein content was measured according to GORNALL[12].

Phosvitin was prepared from egg yolk, according to MECHAM AND OLCOTT[13].

Hammarsten casein was from Merck. All other substrates were from Sigma.

Purification of mitochondrial enzyme

Mitochondria were prepared from livers of Wistar strain albino rats (100–200 g) of either sex by the procedure of SCHNEIDER AND HOGEBOOM[14]. Mitochondrial pellets were twice extracted with water at 0–4° and then three times with 0.2 M acetate buffer (pH 5.4) containing 0.5 M NaCl. The ionic extract was heated at 70° for 3 min and the precipitate discarded. Protamine (2%) was added to the supernatant to a final concentration 0.2%, and the precipitate discarded.

The supernatant was treated with ammonium sulphate to a final 0.80 saturation. The precipitate, retaining about 80% of the original protein phosphatase activity, was dissolved in 0.002 M acetate buffer (pH 5.4) containing 0.005 M NaCl, and chromatographed on a carboxymethyl cellulose (CM-cellulose) column (1.5 cm × 18 cm) previously equilibrated with the same buffer. Stepwise elution was carried out with 0.002 M acetate buffer (pH 5.4) containing increasing concentrations of NaCl: 0.1, 0.15 and 0.2 M.

All operations were carried out in a cold room at 0–4°.

Purification of cytosol enzyme

The cytosol, prepared by centrifuging for 60 min at 105 000 × *g* the post-mitochondrial supernatant of liver cell homogenate in 0.25 M sucrose, was submitted to acetone fractionation[15]. Cold acetone (at −20°) was slowly added with mechanical stirring to a final concentration of 50% (v/v) and the precipitate discarded by centrifugation. The acetone concentration of the supernatant was then raised to 55% and the precipitate discarded. Finally the acetone concentration of the supernatant was rapidly raised to 90% (v/v) and the suspension kept for 1 h at −20° before centrifugation. The precipitate, containing 50–55% of the original cytosol protein phosphatase activity, was dissolved in a small volume of 0.002 M acetate buffer (pH 5.4) containing 0.005 M NaCl and chromatographed on a carboxymethyl-cellulose column (1.5 cm × 18 cm) previously equilibrated with the same buffer.

TABLE I

PURIFICATION OF MITOCHONDRIAL PROTEIN PHOSPHATASE

Units are defined in METHODS AND MATERIALS.

Stage	*Description*	*Activity (total units)*	*Protein (mg)*	*Specific activity (units/mg protein)*	*Purification factor*
I	Mitochondria	1495	1650	0.90	1
II	Acetate–NaCl extract	860	122	7.05	7.7
III	Heat at 70°	846	51.2	16.52	18.2
IV	Protamine sulphate treatment and 80% $(NH_4)_2SO_4$ precipitation	676	43.7	15.40	16.9
V	Fractionation on CM-cellulose column	554	1.35	41.03	452.8

Stepwise elution was carried out with 0.002 M acetate buffer (pH 5.4) containing increasing concentrations of NaCl: 0.1, 0.15, 0.2 M.

All operations were carried out in a cold room at 0–4°. Fractions, about 3 ml per tube, were collected and assayed for enzymic activity and for protein content.

RESULTS AND DISCUSSION

Mitochondrial protein phosphatase

The results of the various steps of the purification procedure are shown in Table I. The phosphoprotein phosphatase activity was enriched 452 times as compared with the original mitochondrial preparation. The highest degree of purification was obtained by chromatography on CM-cellulose by which the enzyme was strongly retained, being eluted with 0.2 M NaCl (Fig. 1).

The peak containing 82% of the original phosphoprotein phosphatase activity was completely free of ATPase and of any other tested phosphomonoesterase activity

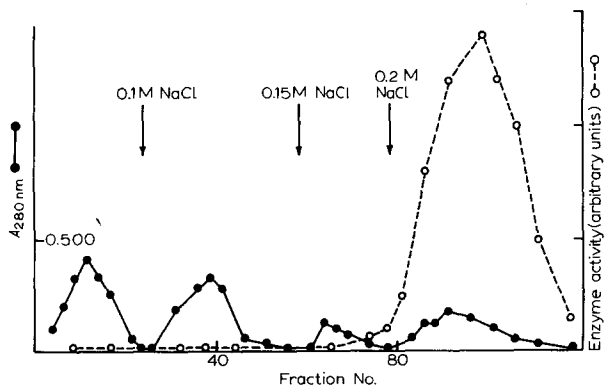

Fig. 1. CM-cellulose chromatography of the mitochondrial phosphoprotein phosphatase, under conditions described in METHODS AND MATERIALS. Fractions, about 3 ml per tube, were collected and assayed for enzymic activity and for protein content. ●—●, absorbance at 280 nm; ○---○, casein phosphatase activity (units/ml).

TABLE II

SUBSTRATE SPECIFICITY OF THE ENZYME EXTRACT FROM MITOCHONDRIA WITH WATER AND WITH SALINE MEDIUM

Mitochondria were twice extracted with water and then three times with 0.2 M acetate buffer (pH 5.4) containing 0.5 M NaCl. The enzymic activity of the two extracts was determined as described in METHODS AND MATERIALS. Casein was present in a final concentration corresponding to 2.5 μmoles/ml of alkali-labile phosphate[10]. Other substrates were present in a final concentration corresponding to 2.5 μmoles/ml of organic phosphate.

Substrate	*Mitochondria*	*Water-extract*		*Acetate–NaCl extract*	
	Activity (units)	*Activity (units)*	*Recovery (%)*	*Activity (units)*	*Recovery (%)*
Casein	1 760	176	10.0	910	51.8
ATP	13 500	3850	28.5	150	1.2
AMP	8 500	3900	46.0	250	3.0
Phenyl phosphate	8 650	3750	43.5	565	6.5
Phosphoserine	1 085	257	23.7	37.5	3.4
Glucose 6-phosphate	16 500	7514	45.5	1065	6.5

present in the mitochondria. These latter activities, unlike casein phosphatase, were removed to a large extent by washing the mitochondria twice with water, as described in METHODS AND MATERIALS (see Table II).

Cytosol protein phosphatase

The cytosol enzyme was purified 156 times as compared with the starting material (cytosol) (Table III). The acetone fractionation step was of critical importance to avoid the inactivation of the enzyme that takes place quickly with the

TABLE III

PURIFICATION OF CYTOSOL PROTEIN PHOSPHATASE

Units are defined in METHODS AND MATERIALS.

Stage	*Description*	*Activity (total units)*	*Protein (mg)*	*Specific activity (units/mg protein)*	*Purification factor*
I	Cytosol	2 040	1087	1.87	1
II	55–90% (v/v) acetone	1 225	120	10.20	5.4
III	Fractionation on CM-cellulose column				
	Peak I	419	1.44	291.00	156
	Peak II	165	0.87	189.6	101

crude cytosol both in the presence of buffer at 0° and through $(NH_4)_2SO_4$ fractionation. Raising the acetone concentration from 50 to 55% led to removal of most of the phosphomonoesterase activities originally present in the cytosol. The casein phosphatase activity was found in the fraction precipitated between 55% and 90% of acetone. This fraction, unlike the cytosol, retained its activity for at least two

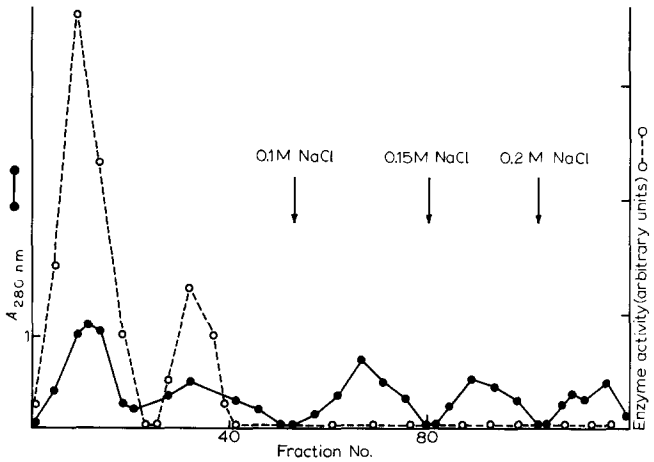

Fig. 2. CM-cellulose chromatography of the cytosol protein phosphatase, under conditions described in METHODS AND MATERIALS. ●—●, absorbance at 280 nm; ○---○, casein phosphatase activity (units/ml).

weeks at 0°, thus allowing a further purification through CM-cellulose chromatography (Fig. 2).

Fig. 2 shows that cytosol protein phosphatase, unlike the mitochondrial one, was weakly retained by the column, being eluted into two peaks with the equilibrating buffer. Therefore the cytosol phosphatase appears to be a more acidic protein than the mitochondria enzyme. The two peaks, into which the cytosol protein phosphatase was resolved, like the purified mitochondrial enzyme, lacked any detectable phosphomonoesterase activity.

Dephosphorylation extent of casein and phosvitin

The purified mitochondrial and the cytosol protein phosphatases differed from

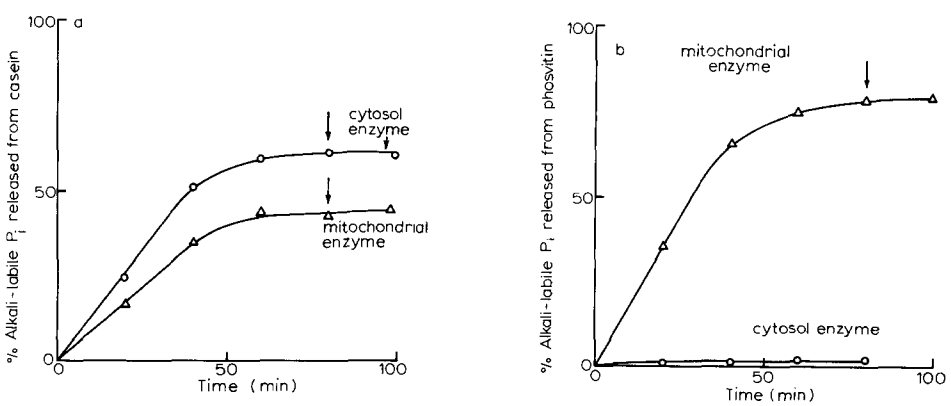

Fig. 3. Time-course of the release of inorganic phosphate from casein (a) and from phosvitin (b) by purified mitochondrial and cytosol protein phosphatase. General conditions as described in METHODS AND MATERIALS. At the time indicated by the arrow, fresh enzyme or fresh phosphoprotein were added. ○—○, cytosol enzyme; △—△, mitochondrial enzyme.

each other not only in chromatographic behaviour but also in their activity towards the same phosphoprotein substrate.

The time-course of the dephosphorylation of casein and of phosvitin by the two protein phosphatases is shown in Figs. 3a and 3b, respectively. Neither enzyme released all the phosphate from the tested phosphoprotein. The inability of the enzymes to dephosphorylate completely the substrates was not due to their inactivation during the incubation: in fact a further addition of substrate after 60 min of incubation resulted in a dephosphorylation of the new aliquot. On the contrary a further addition of the tested fresh enzyme was without effect on the partially dephosphorylated phosphoprotein. Moreover the figures show that the mitochondrial protein phosphatase dephosphorylated the casein to a lesser extent than the cytosol enzyme.

On the contrary, the mitochondrial protein phosphatase was very active on the phosvitin, whereas the cytosol enzyme was almost ineffective. The ineffectiveness of cytosol protein phosphatase towards phosvitin was observed over a large pH range (from 4.0 to 7.0) and at different phosvitin concentrations.

It is remarkable that phosvitin, which is phosphorylated by a cytosol protein kinase[8] can be dephosphorylated only by a protein phosphatase located in the mitochondria. Further, the endogenous phosphoproteins isolated from rat liver cytosol are dephosphorylated, like phosvitin, by the mitochondrial preparation, but not by the cytosol one[16].

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