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Comparative study of mitochondrial and soluble rat liver protein kinase

The presence of a protein kinase which transfers the terminal phosphate from ATP to phosphoproteins in mitochondria has been described by several authors[1–6].

In a previous paper from this laboratory[7], it was reported that two forms of protein kinase are present in liver mitochondria preparations: (1) a fraction tightly bound to the mitochondrial structure, and (2) a form readily soluble in water upon sonication of mitochondria.

It was suggested that the latter enzyme activity could be accounted for by cytoplasmic contamination, since a very active protein kinase was found to be present in soluble liver cytoplasm.

The present paper deals with a comparison between liver cytoplasmic and mitochondrial protein kinases under comparable conditions, made possible by removing, almost quantitatively, ATPase activity from both fractions. The low ATPase activity still present in both fractions does not interfere critically with the rate of protein kinase reaction, since ATP breakdown at the end of incubation was never found to exceed 10%.

As can be seen in Table I, the protein kinase activity, tested both with phosvitin and with casein, is much higher (up to 80-fold) in the soluble cytoplasm than in the mitochondria.

The relatively low protein kinase activity in mitochondria cannot be completely released by sonication and several washings with water[7]. However, the activity can be extracted by ionic solutions without any previous disorganization of the lipid–protein structure of mitochondrial membranes (Table II).

TABLE I

PROTEIN KINASE ACTIVITIES IN RAT LIVER MITOCHONDRIA AND CYTOPLASM

Mitochondria were prepared following the SCHNEIDER[8] procedure, and they were washed twice in 0.25 M sucrose before extraction. Extraction was carried out overnight on the acetone powders of mitochondria with 0.1 M phosphate buffer, pH 6.8, containing 0.1 mM EDTA (10 ml buffer per 0.5 g acetone powders). More than 90% of the phosvitin kinase activity becomes soluble under these conditions. Soluble cytoplasm was prepared by centrifuging the 25 000 × *g* post-mitochondrial supernatant at 105 000 × *g* for 1 h. Both mitochondrial extracts and cytoplasm were dialyzed in 0.05 M Tris containing 0.1 mM EDTA, pH 7.00, as described by RODNIGHT AND LAVIN[9]. Protein kinase activity was tested by incubating 1 mg of phosphoprotein (phosvitin from Sigma, "Hammarsten casein" from Merck and Co.) for 1 h in 1 ml of a medium containing: $MgCl_2$, 12 μmoles; Tris–HCl buffer, pH 7.5, 100 μmoles; ATP, 1 μmole containing 1–2 μC as [γ-^{32}P]ATP, prepared according to the method of GLYNN AND CHAPPEL[10] and 1 mg of enzyme protein. Incubation was stopped by addition of 0.3 ml trichloroacetic acid 50% and the precipitate washed 4 times with 5 ml of 10% trichloroacetic acid. Finally the precipitate was transferred in a stainless steel planchet and counted in a thin-window Geiger counter. ATPase activity was estimated by measuring the inorganic and organic ^{32}P present at the end of the incubation in the trichloroacetic acid supernatants following the procedure described by WADKINS AND LEHNINGER[11].

	Protein kinase activity			
	mμmoles ^{32}P transferred per mg enzyme protein per h		*mμmoles ^{32}P transferred per g tissue per h*	
	Phosvitin	*Casein*	*Phosvitin*	*Casein*
Mitochondrial extract	1.47	0.56	14.56	5.61
Cytoplasm	32.64	9.60	1376.60	403.21

TABLE II

SOLUBILIZATION OF PHOSVITIN KINASE FROM RAT LIVER MITOCHONDRIA

Mitochondria from 5 rat livers were submitted to the following extractions: (1) with 10 ml distilled water; (2) again with 10 ml distilled water ;(3) with 10 ml 0.7 M NaCl; (4) again with 10 ml 0.7 M NaCl. Extractions were carried out for 1 h at 1–2°. After each extraction mitochondria were recovered by centrifugation and submitted to the successive extraction. After the 4th extraction mitochondria suspended in the minimal volume of 0.1 M NaCl were quickly dried in a 100 times larger volume of −20° cold acetone. The acetone powders were submitted to a 5th extraction with 8 ml of 0.1 M phosphate, pH 6.8, for 6 h. All the extracts were dialyzed in 3 changes of 0.05 M Tris *plus* 0.1 mM EDTA, pH 7.00, and their phosvitin kinase activities were tested as described in Table I.

Extractant medium	*Total phosvitin kinase activity in the extracts (counts/min per h incorporated into phosvitin)*
1. Water, 1st	9 850
2. Water, 2nd	624
3. 0.7 M NaCl, 1st	17 888
4. 0.7 M NaCl, 2nd	5 922
5. 0.1 M phosphate, pH 6.8, on acetone-dried mitochondria	0

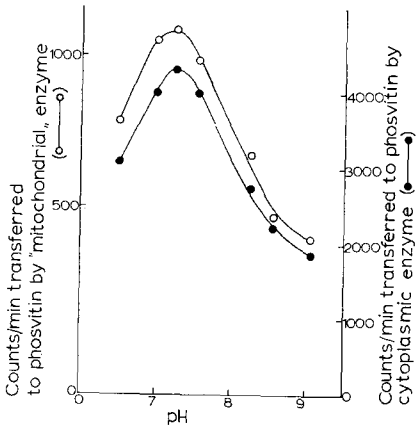

Fig. 1. pH dependence of phosvitin kinase activity. "Mitochondrial" and "cytoplasmic" phosvitin kinase were prepared as described in Table I and purified according to the method of RODNIGHT AND LAVIN[9] until the ammonium sulfate precipitation. The activity was tested as described in Table I, by using 65 μg of "cytoplasmic" enzyme and 500 μg of "mitochondrial" enzyme. pH 6.5–7.5 buffers were Tris–acetate, pH 7.5–9.1, buffers were Tris–HCl.

This finding suggests the possibility that the protein kinase activity present in the usual mitochondrial preparations might be due to the binding of the soluble cytoplasmic enzyme to mitochondrial membranes. This is also supported by the identical behaviour of "mitochondrial" and "cytoplasmic" protein kinases toward the inhibitory

TABLE III

BINDING OF CYTOPLASM PHOSVITIN KINASE BY MITOCHONDRIAL MEMBRANES AND STRUCTURAL PROTEIN

Mitochondrial membranes were prepared by extracting mitochondria twice in cold distilled water and twice in 0.7 M NaCl. Mitochondrial membranes so prepared are lacking phosvitin kinase activity. Mitochondrial structural protein, lacking enzymic activity, was prepared according to RICHARDSON, HULTIN AND FLEISCHER[12]. Cytoplasmic protein kinase was prepared and purified as described in the legend of Fig. 1. 10 mg of structural protein or membranes were incubated at 1° for 20 min in 2 ml of a medium containing: 7.5 mg of partly purified cytoplasmic protein kinase having a specific activity of 104 mμmoles P transferred to phosvitin per mg per h; 12 mM Tris, pH 7.00, and 0.012 mM EDTA. In control experiments, the addition of phosvitin-kinase preparation was omitted. After incubation the insoluble structural protein or membranes were recovered by centrifugation and washed by resuspending in 10 ml water. A second washing with 1 ml water and a following extraction with 1 ml of 0.7 M NaCl, pH 6.8, were tested for their phosvitin kinase activities as described in Table I.

		Phosvitin kinase activity (counts/min incorporated into phosvitin)
Structural protein	NaCl extract	68
Structural protein preincubated with cytoplasm protein kinase	Water extract	114
	NaCl extract	11 688
Mitochondrial membranes	NaCl extract	170
Mitochondrial membranes preincubated with cytoplasm protein kinase	Water extract	670
	NaCl extract	6 853
	NaCl extract (phosvitin omitted)	48

effects of 300 mM NaCl and of unlabelled GTP, and by the fact that they demonstrate identical curves of activity as a function of pH (Fig. 1).

Further experimental support for this hypothesis is given by the ability of mitochondrial membranes and structural protein to bind the soluble cytoplasmic phosvitin kinase.

As shown in Table III, when mitochondrial membranes or structural protein are previously incubated with soluble cytoplasmic protein kinase, they tightly bind the enzyme to give an insoluble complex from which the enzyme can be removed by extraction with NaCl solutions but not with water (see Table III).

In conclusion, the results reported strongly indicate that the protein kinase present in liver mitochondria[1,3–6] represents a fraction of the cytoplasmic soluble enzyme bound to the membranes, possibly to structural protein, through electrostatic forces. Since the liver protein kinase is mainly present in the soluble cytoplasm it is very likely that the phosphorylation of cytoplasmic proteins mediated by mitochondria in the presence of $^{32}P_i$, as reported by LIVANOVA[4], is due to an increased [^{32}P]ATP concentration rather than to a direct supply of protein kinase.

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