INTRAMITOCHONDRIAL LOCALIZATION OF PHOSVITIN KINASE AND PHOSVITIN PHOSPHATASE

G. CLARI, L.A. PINNA, V. MORET and N. SILIPRANDI
Institute of Biological Chemistry,
University of Padova and Centro per lo studio della Fisiologia dei Mitocondri,
Padova, Italy

Received 8 June 1971

1. Introduction

It was previously found that phosvitin kinase and phosvitin phosphatase, i.e. the two enzymes apparently responsible for the turnover of phosphate in phosphoprotein, have a different distribution within the cell. Although significant activity has been detected in mitochondria, most of the phosvitin kinase is present in the cytosol [1]. On the other hand, the phosvitin phosphatase activity is exclusively present in mitochondria [2].

In the present paper the compartmentation of phosvitin kinase and phosphatase within liver mitochondria is reported. For this investigation two different procedures have been followed: isolation of inner and outer mitochondrial membranes by digitonin treatment [3] and by osmotic shock [4]; incubation of intact mitochondria in the presence of pronase or trypsin, assuming that the proteolytic enzyme would inactive preferentially the most exposed enzymes, i.e., externally located ones.

2. Results and discussion

The distribution of phosvitin kinase and phosvitin phosphatase among the three subfractions obtainable by the digitonin procedure is reported in table 1.

It can be observed that phosvitin kinase activity is predominant in the outer membrane and in the soluble intermembrane phase while it is almost negligible in the inner membrane.

On the contrary, the phosvitin phosphatase activity is predominant in the more internal compartments (inner membrane and soluble fraction).

Such a conclusion is strengthened by the circumstance that the inner membrane is by far the least contaminated by aspecific acid phosphatase of lysosomal origin, which in the outer fractions might lead to an overestimation of protein phosphatase activity.

Very similar results have been obtained by also using the fractionation procedure based on osmotic shock, described by Sottocasa et al. [4].

The more internal localization of phosvitin phosphatase in respect to phosvitin kinase was better evidenced by exposing intact mitochondria to increasing concentrations of pronase* (fig. 1).

From the results shown in fig. 1 it appears that pronase does not penetrate the space between the outer and the inner membrane, since concentrations up to 20 μg/mg of this enzyme were ineffective on the adenylate kinase, a typical marker enzyme for the intermembrane space [13]. The finding that MAO activity, which is located in the outer membrane, was also insensitive to pronase treatment suggests that such an enzyme is located on the "inner side" of the outer membrane, in agreement with previous reports from other laboratories [13–15].

Similar results were obtained by replacing pronase with nagarse and trypsin.

* Pronase P, as well as nagarse and soya been trypsin inhibitors were from Serva; Trypsin puriss. was from Fluka AG.

North-Holland Publishing Company – Amsterdam

Table 1
Intramitochondrial localization of phosvitin kinase and phosphatase.

Digitonin fractions	Proteins (%)	Marker enzymes					Phosvitin kinase		Phosvitin phosphat.	
		Acid phosphatase μg Pi released in 15 min/mg prot.[a]	MAO units/mg prot.[b]	Adenylate kinase nmoles ADP/mg prot./min	Succinic dehydrogenase nmoles/mg prot./min	Malic dehydrogenase nmoles/mg prot./min	cpm/mg	total activity (%)	μg Pi/mg	total activity (%)
Outer membranes (high speed pellet)	7.2	6.14	128	155	40	1250	2481	35.5	13.7	9.3
Soluble intermembrane fraction (high speed supernatant)	29.9	7.00	4	524	0.00	534	960	49.9	23.2	65.4
Inner membrane plus matrix (low speed pellet)	62.8	0.0	4	111	202	7200	134	14.6	4.3	25.3

Subfractionation of rat liver mitochondria by digitonin treatment was performed according to Schnaitman and Greenawalt [3]. The three mitochondrial subfractions obtained were directly tested for their enzymatic activities. Phosvitin kinase was determined by incubating at 37° for 30 min, the mitochondrial subfractions (1–2 mg) in 1 ml of a medium containing: 100 mM tris-HCl buffer; 2 mM $MgCl_2$; 0.5 mM KH_2PO_4; oligomycin (5 μg/ml protein); 1 mM ^{32}P-ATP prepared according to Glynn and Chappel [5] containing 5 μC as terminal ^{32}P; 1 mg phosvitin, prepared according to Mecham and Olcott [6] (omitted in control experiments). Incubation was stopped by addition of 0.3 ml of 50% trichloroacetic acid. The precipitates were washed 3 times with 5 ml of 10% trichloroacetic acid at room temperature and once at 100° for 10 min before being transferred to stainless steel planchets and counted in a thin window Geiger counter. Phosvitin phosphatase was determined as Pi released from 1 mg phosvitin upon 30 min incubation at 37° in the presence of 1 mg mitochondrial subfractions, at pH 5.9, as previously described [2]. Monoamine oxidase (MAO) waa determined according to Schnaitman et al. [7]. Adenylate kinase was determined by measuring enzymatically the ADP formed in the presence of 0.1 mM ATP and AMP [8]. Malic dehydrogenase was determined as described in [4]. Acid phosphatase was determined following Shibko and Tappel [9]. Succinic dehydrogenase was measured according to King [10]. Protein was determined according to Gornall et al. [11].

[a] Acid phosphatase of lysosomes isolated on sucrose gradient centrifugation was 17.64 γP/mg protein.
[b] Units are defined according to Tabor et al. [12].

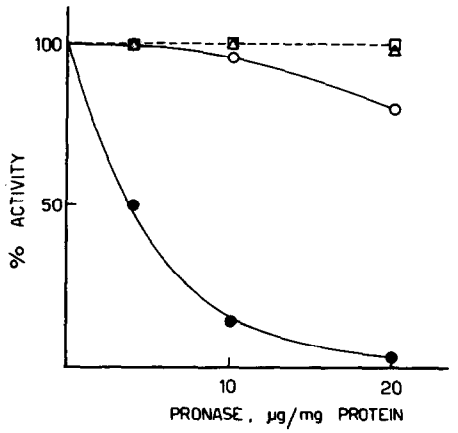

Fig. 1. The effect of preincubation with pronase on some mitochondrial enzymes. Rat liver mitochondria were isolated according to Sottocasa et al. [4] and suspended in 0.25 M sucrose containing 10 mM tris-HCl buffer pH 7.4 in order to have 5 mg protein/ml. Aliquots of the mitochondrial suspension were incubated at 2° for 15 min in the presence of pronase concentrations ranging from zero to 100 µg/ml. At the end of incubation mitochondria were recovered by centrifugation, washed once more in a large excess of 0.25 M sucrose and finally suspended in a few ml of 10 mM tris-HCl buffer pH 7.4. After four freezing and thawing MAO, adenylate kinase phosvitin kinase and phosvitin phosphatase of mitochondria were determined as described in table 1. □—□ MAO; △—△ adenylate kinase; ○—○ phosvitin phosphatase; ●—● phosvitin kinase.

It is remarkable that the mitochondrial protein phosphatase, although mostly located inside the outer membrane, is active on the cytosol phosphoproteins, while the cytosol protein phosphatase proved to be ineffective on such substrates [16].

Therefore it can also be concluded that the mitochondrial outer membrane cannot prevent the cytosol phosphoproteins from reaching the mitochondrial dephosphorylation sites.

Acknowledgement

We wish to thank Miss Carla Munari for valuable technical assistance.

References

[1] L.A. Pinna, B. Baggio, V. Moret and N. Siliprandi, Abstracts 5th FEBS Meeting, Praha 1968.
[2] V. Moret, L.A. Pinna and G. Magni, Ital. J. Biochem. 19 (1970) 204.
[3] C. Schnaitman and J.W. Greenawalt, J. Cell Biol. 38 (1968) 158.
[4] G.L. Sottocasa, B. Kuylenstierna, L. Ernster and A. Bergstrand, in: Methods in Enzymology, Vol. 10 (1967) p. 448.
[5] J.M. Glynn and J.B. Chappel, Biochem. J. 90 (1964) 147.
[6] D.K. Mecham and H.S. Olcott, J. Am. Chem. Soc. 71 (1949) 3670.
[7] C. Schnaitman, V.G. Erwin and J.W. Greenawalt, J. Cell Biol. 32 (1967) 719.
[8] C.R. Rossi and D.M. Gibson, J. Biol. Chem. 239 (1964) 1694.
[9] S. Shibko and A.L. Tappel, Biochim. Biophys. Acta 73 (1963) 76.
[10] T.E. King, Methods in Enzymology, Vol. 10 (1967) p. 323.
[11] A.G. Gornall, C.J. Bardawill and M.M. David, J. Biol. Chem., 177 (1949) 751.
[12] C.W. Tabor, H. Tabor and S.M. Rosenthal, J. Biol. Chem. 208 (1954) 645.
[13] B. Kuylenstierna, D.G. Nicholls, S. Hovmoller and L. Ernster, European J. Biochem. 12 (1970) 419.
[14] J.W. de Jong and W.C. Hulsmann, Biochim. Biophys. Acta 197 (1970) 127.
[15] S.V. Pande and M.C. Blanchaer, Biochim. Biophys. Acta 202 (1970) 43.
[16] L.A. Pinna, G. Clari and V. Moret, Biochim. Biophys. Acta 236 (1971) 278.