RAT LIVER MITOCHONDRIAL POLYNUCLEOTIDE PHOSPHORYLASE

Yew Phew SEE and P.S. FITT
Department of Biochemistry, University of Ottawa, Ottawa 2, Ontario, Canada

Received 2 April 1971

1. Introduction

We have recently isolated, partially purified and demonstrated the specificity of a polynucleotide phosphorylase from crude guinea-pig liver nuclei [1, 2]. The enzyme was associated with membrane lipids and catalysed the phosphorolysis of polyribonucleotides to ribonucleoside diphosphates. However, the animal enzyme did not appear to catalyse the synthesis of polyribonucleotides, unlike the polynucleotide phosphorylases isolated from a wide variety of bacteria [3].

Examination of subcellular fractions of rat liver has shown that mitochondria also contain polynucleotide phosphorylase. In the present paper, we describe: (i) the evidence that this enzyme catalyses phosphorolysis of poly A and poly C to ADP and CDP respectively; (ii) the demonstration that it is a mitochondrial enzyme, and not associated with lysosomes or peroxisomes; (iii) the localization of the enzyme in the inner mitochondrial membrane.

2. Methods

Rat liver mitochondria were prepared according to Schnaitman and Greenawalt [4], using 0.25 M sucrose, 0.01 M tris-HCl buffer, pH 7.4 instead of the isolation medium mentioned in the reference. Submitochondrial fractions were isolated by the digitonin procedure of Schnaitman and Greenawalt [4, 5] using 1.1 mg digitonin/10 mg mitochondrial protein.

The distribution of polynucleotide phosphorylase between mitochondria, lysosomes and peroxisomes was studied by the sucrose–glycogen gradient centrifugation method of Beaufay et al. [6], using their gradient Gh(10) prepared with oyster glycogen. The centrifuge tubes were cut at 0.45 cm intervals (average vol/fraction: 0.22 ml) using a tube-slicer (Model I-3600, Microchemical Specialties Co., Berkeley, Calif., USA).

Polynucleotide phosphorylase was assayed by the standard polyribonucleotide phosphorolysis procedure [2]. The assay medium (0.1 ml) contained: 0.1 M tris-HCl buffer, pH 7.8; 6 mM $MgCl_2$; 1.0 mM 2-mercaptoethanol; 10 mM $K_2H^{32}PO_4$ (100,000 cpm/μmole); 0.1% (v/v) Triton X-100; poly A, 1.5 mg/ml; enzyme. Incubation was at 37° for 1 hr in a water-bath shaker. The reaction was stopped with a charcoal–$HClO_4$ suspension and the charcoal washed, dried and counted as previously described [2].

3. Results and discussion

The results in fig. 1 show that the mitochondrial inner membrane fraction catalysed phosphorolysis of poly A and poly C to ADP and CDP respectively in the conditions of the standard polyribonucleotide phosphorylase assay. The products were almost exclusively nucleoside diphosphates and no significant amount of the corresponding monophosphates or triphosphates was detectable. No significant formation of ^{32}P-labelled ribonucleoside diphosphates occurred in the conditions of the phosphorolysis assay in the absence of polyribonucleotides or when the latter were replaced by ribonucleoside monophosphates or triphosphates. Polynucleotide phosphorylase is therefore present in this fraction. The evidence that this was the inner membrane of the rat liver mitochondria is as follows.

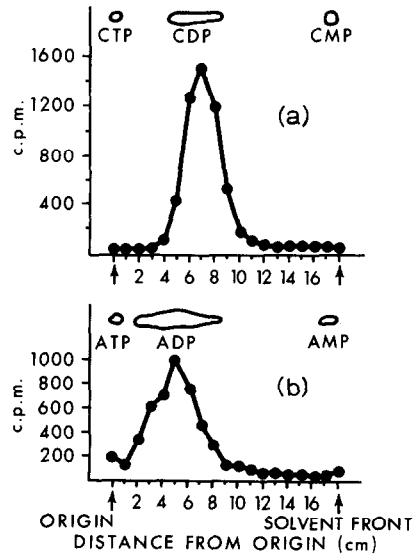

Fig. 1. Product specificity of rat liver mitochondrial polynucleotide phosphorylase. The standard phosphorolysis assay mixture (see Methods) was scaled-up three-fold, with either (a) poly C or (b) poly A as substrate. Incubation was for 2 hr at 37° in the presence of mitochondrial inner membranes (0.47 mg protein). The reaction was stopped with a charcoal–$HClO_4$ suspension and the nucleotides were eluted from the charcoal and separated on DEAE-cellulose TLC plates as previously described [1]. Successive areas (1 cm long) of DEAE-cellulose were then scraped from the plates and counted by liquid scintillation.

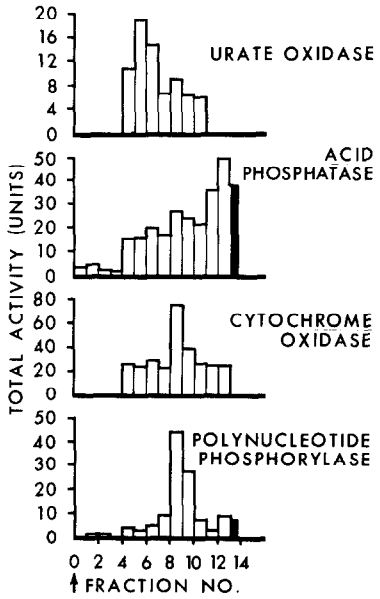

Fig. 2. Distribution of polynucleotide phosphorylase activity after isopycnic centrifugation of rat liver mitochondria. Sucrose–glycogen gradient centrifugation was carried out as described in Methods. Enzymes were assayed by the following procedures: urate oxidase according to Schneider and Hogeboom [8] in 1 ml and at 37°; cytochrome oxidase according to Wharton and Tzagoloff [9]; acid phosphatase according to Linhardt and Walter [10], using $\frac{1}{3}$ of the original scale. Polynucleotide phosphorylase activity was determined by the standard assay with the following modifications: incubation was for 3 hr, the pH was 7.8, and 6 mM $MgCl_2$ was used. All assays were performed in the presence of 0.1% (v/v) Triton X-100. The shaded areas represent the pellets at the bottom of the tubes.

A detailed study was made of the distribution of the polynucleotide phosphorylase activity relative to known marker enzymes after isopycnic centrifugation of mitochondria in a sucrose–glycogen gradient. The distribution of the marker enzymes, urate oxidase (peroxisomes), acid phosphatase (lysosomes) and cytochrome oxidase (mitochondria), is shown in fig. 2 and was very similar to that reported by Beaufay et al. [6]. The polynucleotide phosphorylase activity had the same distribution as cytochrome oxidase and was therefore present in the mitochondria and not in lysosomes or peroxisomes.

Mitochondria were separated into outer membrane and inner membrane–matrix fractions by digitonin treatment followed by differential centrifugation [4, 5]. Rotenone-insensitive NADH–cytochrome *c* reductase and succinate–cytochrome *c* reductase were used as marker enzymes for the outer and inner membranes, respectively [4, 7]. Table 1 shows the intra-mitochondrial distribution of polynucleotide phosphorylase: it can be seen that its specific activity was highest in the inner membrane–matrix fraction. Table 2 shows that 91% of the total polynucleotide phosphorylase activity was in this fraction. The latter was then separated into matrix and inner membrane fractions by sonication followed by high-speed centrifugation, and 89% of the total activity of the crude extracts (corresponding to almost 100% of the activity in the inner membrane–matrix fraction) was recovered in the inner membrane pellet. This final fraction also contained 86% of the total succinate–cytochrome *c* reductase activity. These results show that rat liver mitochondrial polynucleotide phosphorylase is located in the inner membrane of the organelle.

Table 1
Intra-mitochondrial distribution of polynucleotide phosphorylase.

Fractions	Marker enzymes		
	NADH–cyt. *c* reductase (rotenone insensitive)	Succinate–cyt. *c* reductase	Polynucleotide phosphorylase
	(μmoles cyt. *c* reduced/min/mg protein)		(nmoles ^{32}P-Pi incorp./hr/mg protein)
Whole mitochondria	0.30	0.063	119
9,500 *g* pellet	0.17	0.144	210
40,000 *g* pellet	0.44	0.022	10
100,000 *g* pellet	1.46	0.0004	0
100,000 *g* supernatant	0.14	0.000	15

Mitochondria were fractionated by digitonin treatment and differential centrifugation [4, 5] to yield the following fractions: 9,500 *g* pellet (inner membrane-matrix); 40,000 *g* pellet (inner membrane fragments); 100,000 *g* pellet (outer membrane); 100,000 *g* supernatant. Rotenone-insensitive NADH–cytochrome *c* reductase and succinate–cytochrome *c* reductase were assayed according to Sottocasa et al. [7] at 37° in a volume of 1 ml, using 5 μM rotenone where appropriate [4]. Polynucleotide phosphorylase was assayed as described in Methods.

Table 2
Polynucleotide phosphorylase activity in submitochondrial fractions.

Fraction	Total units (nmoles ^{32}P-Pi incorporated/hr)	Recovery (%)
Whole mitochondria	13902	100
9,500 *g* pellet	12640	91
40,000 *g* pellet	112	1
100,000 *g* pellet	0	0
100,000 *g* supernatant	225	2
Fractionation of 9,500 *g* pellet		
inner membrane (pellet)	12375	89
matrix (supernatant)	135	1

Rat liver mitochondria were fractionated as described in table 1. Sonication of the inner membrane–matrix fraction (9,500 *g* pellet) followed by centrifugation at 100,000 *g* for 1 hr gave the matrix (supernatant) and inner membrane (pellet) fractions. Polynucleotide phosphorylase activity was determined by the standard assay (Methods).

Rat liver nuclei also contain a polynucleotide phosphorylase similar to the guinea-pig liver enzyme previously described [1, 2]. Further studies (to be reported elsewhere) showed that the nuclear fraction contained less than 3% of the total liver mitochondria and that less than 5% of the activity of the crude extract of rat nuclei or less than 0.6% of the activity of the partially-purified particulate nuclear enzyme could arise from such contaminating mitochondria. It therefore appears that rat nuclei and mitochondria both contain membrane-associated polynucleotide phosphorylases and further detailed comparison of these enzymes will be of interest.

Acknowledgements

We thank the Medical Research Council of Canada for an operating grant (to P.S.F.), the Province of Ontario for a Graduate Fellowship (to Y.P.S.) and Dr. A. D'Iorio for the use of his centrifuge tube slicer.

References

[1] P.S.Fitt and Y.P.See, Biochem. J. 116 (1970) 309.
[2] Y.P.See and P.S.Fitt, Biochem. J. 119 (1970) 517.
[3] M.Grunberg-Manago, Prog. Nucleic Acid Res. 1 (1963) 93.
[4] C.Schnaitman and J.W.Greenawalt, J. Cell. Biol. 38 (1968) 158.
[5] C.Schnaitman, V.G.Erwin and J.W.Greenawalt, J. Cell. Biol. 32 (1967) 719.

[6] H.Beaufay, P.Jacques, P.Baudhain, O.Z.Sellinger, J. Berthet and C. de Duve, Biochem. J. 92 (1964) 184.
[7] G.L.Sottocasa, B.Kuylenstierna, L.Ernster and A.Bergstrand, J. Cell. Biol. 32 (1967) 415.
[8] W.C.Schneider and G.H.Hogeboom, J. Biol. Chem. 195 (1952) 161.
[9] D.C.Wharton and A.Γzagoloff, Methods in Enzymology, Vol. 10 (Academic Press, New York, 1967) p. 245.
[10] K.Linhardt and K.Walter, in: Methods of Enzymatic Analysis, ed. H.U.Bergmeyer (Verlag Chemie, Weinheim, 1963) p. 783.