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STUDIES ON THE LOCALIZATION OF RAT LIVER MITOCHONDRIAL 5'-ENDONUCLEASE

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SUMMARY

Isolated rat liver mitochondria exposed to an hypotonic medium hydrolyzed poly A at a faster rate than did those incubated under isotonic conditions, indicating that the former medium causes unmasking of a substantial part of the 5'-endonuclease activity. Most of the unmasked activity is released in a soluble form in the incubation medium. The digitonin technique has been used to separate rat liver mitochondria into three fractions: inner membrane *plus* matrix, outer membrane, and a soluble fraction containing proteins localized between the membranes. The distribution of the 5'-endonuclease has been compared with those of four other enzymes: monoamine oxidase, cytochrome oxidase, malate dehydrogenase and adenylate kinase. The endonuclease was found in both the outer membrane and the soluble fraction: this suggests a dual localization. Attempts to measure the penetration of poly A into mitochondria were unsuccessful because this polymer was adsorbed on the mitochondrial membranes, resulting in a distribution space much larger than that of water.

INTRODUCTION

Previous work[1] from this laboratory reported the presence in rat liver of an enzyme able to hydrolyze poly A forming oligonucleotides with a 5'-phosphomonoester end group. Studies on the intracellular distribution of this enzyme have revealed that the ribonuclease was mainly localized in the mitochondria[2]; the localization was ascertained by the use of polyadenylic acid as a substrate, for alkaline ribonuclease does not hydrolyze this polymer[3,4]. Furthermore, it was observed that the purified enzyme hydrolyzed denatured DNA[5,6], and could thus be considered as a 5'-endonuclease. LINN AND LEHMAN[7] and CURTIS *et al.*[6], respectively, reported the presence of a similar enzyme in *Neurospora crassa* and rat liver mitochondria.

That mitochondria possess and can synthetize DNA[9–11] and RNA[12–14] has been suggested by autoradiographic and biochemical studies *in vivo*, and has been proved conclusively by biochemical studies on isolated mitochondria. Therefore, it was of interest to determine whether or not the 5'-endonuclease was involved in the metabolism of the mitochondrial nucleic acids. As a first approach to this problem, it was decided to look at the intramitochondrial localization of the enzyme. Evidence is

presented in this report indicating that the endonuclease is localized in both the outer membrane and the soluble fraction isolated from mitochondria treated with digitonin[15–16].

MATERIALS AND METHODS

Preparation of the mitochondrial fractions

Wistar male rats of an average weight of 175 g were sacrificed by decapitation. A liver homogenate was prepared as previously described[1]. The homogenate was centrifuged at 2200 rev./min for 10 min in the Lourdes Betafuge model A centrifuge with rotor 9RA. The sediment was discarded and the supernatant centrifuged at 9000 rev./min for 10 min (6500 × *g* average) in the same centrifuge. The supernatant and the fluffy layer were discarded and the pellet washed twice by resuspension, with a Potter–Elvehjem homogenizer, in one-fourth the original volume of 0.25 M sucrose followed by centrifugation at 9000 rev./min for 10 min. The mitochondrial pellet was stored at 1° for not more than 2 h before use.

To a suspension of mitochondria in 0.25 M sucrose was added a solution of 2% (w/v) digitonin in 0.25 M sucrose until a final ratio of 1.4 mg of digitonin per 10 mg of mitochondrial protein was reached. From this mixture, the inner and outer mitochondrial membrane fractions were isolated by differential centrifugation essentially as described by SCHNAITMAN *et al.*[16]: the inner membrane *plus* matrix fraction was sedimented by centrifugation at 9500 × *g* for 10 min and the resulting supernatant was used as the outer membrane fraction. This fraction was, on some occasions, further centrifuged at 144000 × *g* for 1 h, giving a pellet (P) and a supernatant (S). The 9500 × *g* and 144000 × *g* pellets were resuspended in a 0.125 M ice-cold sucrose solution containing mercaptoethanol (0.001 M). The 144000 × *g* supernatant fraction was rendered 0.125 M with respect to sucrose and 0.001 M in mercaptoethanol.

Enzymatic and chemical assays

All enzymic activities were determined with a Beckman DU spectrophotometer. Absorbance measurements were made in a final volume of 3 ml in a cuvette with a 1-cm light path, at room temperature. The reactions were initiated by the addition of either substrate, electron acceptor, or enzyme.

Cytochrome oxidase activity was measured by following the oxidation of reduced cytochrome *c* at 550 nm[17]. Monoamine oxidase was assayed by following the formation of benzaldehyde at 250 nm[18]. Malate dehydrogenase was measured by following the oxidation of NADH at 340 nm [19]. Adenylate kinase was assayed by following the reduction of $NADP^+$ at 340 nm[20]. 5′-Endonuclease was assayed as previously described[2].

The concentration of protein was determined by either the biuret[21] or the LOWRY *et al.* methods[22] using bovine serum albumin for reference standard.

Estimation of total and extramitochondrial water

To 2.3 ml of a reaction mixture containing 50 μmoles of Tris–HCl buffer, pH 7.5, 0.4 μmole of $MgCl_2$, 28 μmoles of K_2HPO_2, 2.5 μmoles of mercaptoethanol, 625 μmoles of sucrose and 11 mg of mitochondrial protein was added 0.2 ml of various concentrations of poly[^{14}C]A or [^{14}C]carboxydextran. The mixture, after standing

2 min at 1 or 23° was centrifuged at 15000 rev./min for 15 min in the Spinco model L ultracentrifuge using rotor 50. The total and extramitochondrial water was then measured essentially as described by ALLMANN *et al.*[23].

Materials

The following chemicals were obtained from Sigma Chemical Co.: cytochrome *c*, NADH, $NADP^+$, ADP, hexokinase and glucose-6-phosphate dehydrogenase. Oxaloacetic acid and the potassium salt of poly A were purchased from Calbiochem. [^{14}C]Carboxydextran (specific activity, 0.05 mC/38.5 mg) and poly[^{14}C]A (specific activity, 0.21 mC/μmole of polynucleotide phosphorus) were from New England Nuclear Corporation and Schwarz Bioresearch, respectively.

RESULTS

Hydrolysis of poly A by the mitochondrial fraction of rat liver

The mitochondria isolated from rat liver and incubated in an isotonic medium hydrolyze poly A (Table I). When they are incubated in an hypotonic medium (sucrose replaced by water), the degradation of the polymer is more than doubled suggesting that part of the poly A is not accessible to the enzyme in the isotonic medium used. The possibility that sucrose might inhibit the endonuclease activity and thus be responsible of the smaller activity measured, was ruled out because addition of sucrose to hypotonically treated mitochondria or to purified 5'-endonuclease[5] was without effect on the activity. Mitochondria incubated in the isotonic sucrose medium undergo swelling which is nearly complete after 30 min. Preincubation of the mitochondria under these conditions followed by their sedimentation gives a clear supernatant containing less than 15% (0.045 as compared to 0.359) of the original enzymatic activity (Table I), indicating that most of the 5'-endonuclease activity had sedimented with the mitochondria. Preincubation of the mitochondria in the hypotonic medium, however, released about 45% (0.393 as compared to 0.849) of the original endonuclease activity.

When the isotonic and hypotonic preincubation media are submitted to high

TABLE I

HYDROLYSIS OF POLY A BY MITOCHONDRIA

The isotonic incubation medium contains in 2.5 ml, the following: 50 μmoles of Tris–HCl buffer, pH 7.5, 0.4 μmole of $MgCl_2$, 28 μmoles of K_2HPO_4, 2.5 μmoles of mercaptoethanol, 0.5 mg of poly A, 625 μmoles of sucrose, and 0.70 mg of mitochondrial proteins. The mixture is incubated at 23° for 30 min. At the end of the incubation, the mixture is treated as previously described[2]. Hypotonic medium: the sucrose is replaced by water. Supernatant I: the mitochondria are preincubated 30 min at 23° in the isotonic medium without poly A and then centrifuged at 10000 rev./min for 10 min. The clear supernatant is used as source of enzyme. Supernatant II: same as Supernatant I, except that the mitochondria are preincubated into the hypotonic medium. The data presented are the average of three different experiments.

Incubation medium	*Absorbance at 260 mμ*
Isotonic	0.359
Hypotonic	0.849
Supernatant I	0.045
Supernatant II	0.393

speed centrifugation, the percentages endonuclease activity present in the clear supernatants are similar to those reported into Table I, suggesting that the enzyme is rather solubilized than present into small vesicles. The data in Table II show that the release of the activity in the isotonic incubation medium is a slow process reaching 18% of the original activity after 60 min while in the hypotonic one nearly all the solubilized activity (35%) is already released after 30 min of incubation.

TABLE II

PERCENT 5′-ENDONUCLEASE RELEASED INTO THE MEDIUM DURING PREINCUBATION

The isotonic medium composition is the same as described under Table I, excepted that 1.05 mg of mitochondrial proteins are used. At intervals, the media are centrifuged at 50000 rev./min in the Spinco model L ultracentrifuge rotor 50, for 30 min. The clear supernatants obtained are incubated with poly A for 30 min at 23° and treated, thereafter, as previously described[2]. Hypotonic medium: same as the isotonic medium, except that the mitochondria are preincubated into the hypotonic medium and centrifuged, at intervals, at 42000 rev./min for 30 min. The absorbance measured at 260 nm after a 30-min incubation of the mitochondria in presence of poly A is taken as 100. The data presented are the average of five different experiments.

Time (*min*)	*5′-Endonuclease released* (%)	
	Isotonic	*Hypotonic*
15	8.0	26.6
30	12.2	34.9
45	16.0	37.2
60	18.0	35.5

Uptake of poly A by mitochondria

It was of interest to know whether or not poly A penetrated the outer membrane of the mitochondria incubated in isotonic medium. It was thought that if poly A did not penetrate the mitochondria, then the endonuclease had to be present in the mitochondrial outer membrane since the enzymatic activity released in the isotonic medium during incubation could not account for the amount of poly A hydrolyzed (Table I). On the other hand, if poly A did penetrate into the mitochondria, then the enzymatic activity measured could be provided by the endonuclease present either in the inner membrane *plus* matrix fraction or solubilized between the inner and outer membrane. The penetration experiments were first carried out by adding the labeled polymer (0.01 mg per ml of incubation mixture) to a mitochondrial suspension kept at 0° followed by immediate centrifugation of the resulting mixture. It was found that the uptake of the label by the mitochondria was such that it gave a "distribution space" much larger than water. This finding suggested that poly A was adsorbed by these subcellular particles. If, indeed, adsorption occurred, it was then expected that the use of lower concentrations of labeled poly A would yield increasing estimates of the extraparticulate fluid. The results reported in Table III show that such was the case. Labeled poly A was added to three samples of mitochondrial suspension in final concentrations of 0.07, 0.035 and 0.007 mg/ml. The percent total water penetrable by this polymer was respectively increasing from 73.8 to 217.9%. Control experiments were run concurrently with labeled carboxydextran, a polymer of glucose which neither penetrates the mitochondria nor is adsorbed on their surfaces[24]. In fact, in our experimental conditions, the percent water penetrable by this labeled poly-

TABLE III

ADSORPTION OF POLY A AND CARBOXYDEXTRAN ON RAT LIVER MITOCHONDRIA

The incubation medium composition just as the determination of the total water content and the water equivalence of the polymers in the pellets are described under MATERIALS AND METHODS. Labeled polymers were added to the mitochondrial suspension kept at 0°.

Conc. of labeled poly A (mg/ml)	*Concn. of labeled carboxy-dextran (mg/ml)*	*Total water in pellet (µl water per mg protein)*	*Water equivalence of poly A in pellet (µl water per mg protein)*	*Water equivalence of carboxy-dextran in pellet (µl water per ml protein)*	*% Total water* penetrable by poly A*	*% Total water* penetrable by carboxy-dextran*
0.07	—	3.28	2.42	—	73.8	—
0.035	—	3.37	3.17	—	94.1	—
0.007	—	3.24	7.06	—	217.9	—
	0.6	3.37	—	1.15	—	34.1
	0.3	3.21	—	1.05	—	32.7
	0.06	3.06	—	1.00	—	33.7

* The percent total water penetrable by the polymers is obtained by dividing the water equivalence of the polymers in pellet by the total water in pellet.

glucose was similar whichever the concentrations used, and is assumed[25,26] to represent the extraparticulate water. Similar uptake of poly A or carboxydextran was obtained when the labels were added to a mitochondrial suspension kept at 23°.

Intramitochondrial localization of the 5'-endonuclease

To localize the 5'-endonuclease it became necessary to isolate the mitochondrial subfractions. Table IV shows the enzymic activities of two mitochondrial subfractions prepared by digitonin treatment: the inner and outer membrane fractions containing, respectively, the matrix and the soluble proteins localized between the membranes[27]. Four enzyme activities were used as markers of these two fractions: cytochrome oxidase and malate dehydrogenase for the inner membrane[16,28] and monoamine oxidase and the adenylate kinase for the outer membrane[16,19]. The results show that 73.6 and 65.9% of the cytochrome oxydase and malate dehydrogenase activities, respectively, are localized in the inner membrane fraction while 104.6 and 93.1% of the monoamine oxidase and the adenylate kinase activities, respectively, are found in the outer membrane fraction. Similar results have been previously observed[29,30]. The 5'-endonuclease has a distribution similar to the later enzymes since 93.1% of the activity is found in the outer membrane fraction. Comparable results were obtained when we prepared the mitochondrial subfractions by large-amplitude swelling of the mitochondria according to the method described by PARSONS *et al.*[31].

To differentiate between the outer membrane and the soluble proteins localized between the inner and outer membranes, the outer membrane fraction obtained after digitonin treatment of the mitochondria was further centrifuged. Table V shows that the monoamine oxidase is an outer membrane enzyme while the adenylate kinase is a soluble protein localized between the inner and outer membranes. The distribution of the 5'-endonuclease is rather different since equal activity is found in both the outer membrane and the soluble fraction. Similar results, however, were recently reported by SCHNAITMAN AND GREENAWALT[20] for the nucleoside diphosphokinase

TABLE IV

ENZYMIC ACTIVITIES OF MITOCHONDRIAL FRACTIONS PREPARED BY DIGITONIN TREATMENT

Mitochondrial fractions: see MATERIALS AND METHODS. Outer membrane fraction contains the soluble proteins localized between the inner and outer mitochondrial membranes. Control mitochondria: unfractionated mitochondria (digitonized). Malate dehydrogenase and adenylate kinase are expressed, respectively, in μmoles of NADH and NADPH oxidized per min per mg protein using an extinction coefficient of $6.9 \cdot 10^6$ cm^2·mole^{-1}. Monoamine oxidase is expressed in nmoles benzaldehyde produced per min per mg protein using an extinction coefficient of $12.8 \cdot 10^6$ cm^2·mole^{-1}. 5′-Endonuclease is expressed in nmoles of adenylic acid produced per min per mg protein using an extinction coefficient of $14.2 \cdot 10^6$ cm^2·mole^{-1}. Cytochrome oxidase is expressed in μmoles of cytochrome *c* oxidized per min per mg protein using an extinction coefficient of $19.6 \cdot 10^6$ cm^2·mole^{-1}. The data presented are the average of two different experiments. Percentages: as compared to control mitochondria.

Mitochondrial fractions	*Protein*		*Cytochrome oxidase*		*Malate dehydrogenase*		*Monoamine oxidase*		*Adenylate kinase*		*5′-Endonuclease*	
	mg	%	*Specific activity*	%	*Specific activity*	%	*Specific activity*	%	*Specific activity*	%	*Specific activity*	%
Inner membrane + matrix 9500 × *g* (P)	156.3	62.1	0.98	73.6	1.73	65.9	0.77	6.0	0.021	4.6	9.0	12.9
Outer membrane	105.3	41.7	0.42	21.2	1.11	28.7	19.71	104.6	0.628	93.1	97.4	93.1
Control mitochondria	252.5	100	0.83	100	1.62	100	8.02	100	0.282	100	44.0	100
Recovery		103.7		94.7		94.6		110.6		97.7		106.1

TABLE V

LOCALIZATION OF ENZYMES IN THE MITOCHONDRIAL OUTER MEMBRANES FRACTIONS* PREPARED BY DIGITONIN TREATMENT

144000 × *g* (P) and 144000 × *g* (S): the mitochondrial outer membrane fractions were centrifuged at 144000 × *g* for 1 h. A pellet (P) and a supernatant (S) were obtained. Control mitochondria: unfractionated mitochondria. See Table IV. The specific activities are expressed as described under Table IV. Percentages: as compared to control mitochondria. The data presented are the average of two different experiments. Recovery: as compared to outer membrane fractions.

Mitochondrial fractions	*Protein*		*Monoamine oxidase*		*Adenylate kinase*		*5′-Endonuclease*	
	mg	%	*Specific activity*	%	*Specific activity*	%	*Specific activity*	%
144000 × *g* (P)	54.8	21.8	29.18	82.2	0.052	4.1	97.0	47.4
144000 × *g* (S)	50.25	19.9	9.56	24.9	1.254	89.0	100.0	44.0
Control mitochondria	252.5	100.0	8.02	100.0	0.282	100.0	44.0	100.0
Recovery		99.7		102.4		100.0		98.2

* This fraction contains the soluble proteins localized between the inner and outer mitochondrial membranes. See also Table IV.

suggesting a dual localization of this enzyme or a loose association of this enzyme with the outer mitochondrial membrane.

DISCUSSION

The results presented in this paper show that rat liver mitochondria exposed to an hypotonic medium hydrolyzed poly A at a faster rate than did those incubated under isotonic conditions, indicating that the former medium causes "unmasking" of a substantial part of the 5'-endonuclease activity. These results can be compared to those of LEHNINGER[32] and OKAMOTO *et al.*[33], where added NADH or NADPH are also oxidized faster by mitochondria hypotonically treated. Most of the unmasked 5'-endonuclease activity is released in the incubation medium and accounted for 35–40 % of the original activity. In contrast, a similar hypotonic procedure releases full activity of glutamate and malate dehydrogenases[34,35] which exist in rather soluble form in the mitochondrial matrix[36].

Since 60–65 % of the total 5'-endonuclease activity sedimented after high speed centrifugation of the hypotonically treated mitochondria, the data in Table II suggest a dual localization of this enzyme, that is to say partly soluble and partly associated with the mitochondrial particulate material (inner and outer membranes). The results obtained after digitonin treatment of the mitochondria reinforced this suggestion. It was found that 61.2 % of the 5'-endonuclease activity was localized in the mitochondrial particulate material and 44.8 % in the proteins which are soluble between the inner and outer membranes[27]. Most of the mitochondrial particulate material activity is present in the outer membrane. It should be pointed out, however, that the results obtained can not exclude the possibility that some of the 5'-endonuclease activity might be loosely associated with the inner or outer membrane, so that it is extracted by hypotonic or digitonin treatments of the mitochondria. Recently, SCHNAITMAN AND GREENAWALT[20] reported that the enzyme nucleoside diphosphokinase was found in both the outer membrane and the soluble fraction of rat liver mitochondria, suggesting a dual localization.

In the experimental isotonic conditions described in this paper, the macromolecule poly A is adsorbed on the mitochondria rendering difficult a quantitative measure of the penetration of this polymer into the mitochondria. However, the results indicate that poly A is hydrolyzed by the 5'-endonuclease localized in the outer membrane fraction. Such localization suggests that the enzyme is not directly involved in the metabolism of intramitochondrial RNA and DNA and raises the possibility that it protects the mitochondria against "invasion" by extramitochondrial RNA or DNA. It is of some interest in this connection that an enzymatically active component, capable of degrading infectious RNA, has been found associated with cell wall of Krebs ascites cells[37]. It should be noted, however, that PINCHOT AND HORMANSKI[38] have found that a polynucleotide is necessary for oxidative phosphorylation in *Alcaligenes feacales* extracts: the 5'-endonuclease of mitochondria has been shown to produce small oligonucleotides[5,39].

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