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Homogeneity of the Outer Mitochondrial Membrane. Studies on the Fragments Obtained Through Lysis by Ascorbate

N. López-Moratalla, P. Pérez, J. Eugui and E. Santiago

Department of Biochemistry
University of Navarra
Pamplona (Spain)

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Outer mitochondrial membranes from rat liver undergo a structural lysis when incubated in the presence of 1 mM ascorbate. The fragments produced can be separated by differential centrifugation. Three fractions were obtained, one sedimenting at 22,000 × g, another at 100,000 × g and a supernatant from this last centrifugation. The lipid composition and the distribution of enzyme markers monoamine oxidase and rotenone-insensitive NADH-cyt. c reductase in these three fractions has been studied. The supernatant fraction was composed of solubilized proteins accompanied by some altered phospholipids and negligible amounts of cholesterol. The other two fractions were rather similar in distribution of enzymes and lipid composition. An interpretation of the results presented indicate that the outer mitocondrial membrane is rather homogeneous in what refers to distribution of their lipid and protein components, contrary to what has been established for the inner membrane.

Several authors have studied the effect of ascorbate on mitochondria. OTTOLENGHI (11) has shown that formation of lipid peroxides generated from unsaturated fatty acids. HUNTER *et al.* (5) have reported that mitochondria incubated in the presence of ascorbate undergo a process of lysis concomitant with the formation of peroxides. They suggested that the peroxidation reactions would lead to alterations of the membrane phospholipids causing a desintegration of the membrane, due probably to the peroxidation of polyunsaturated fatty acids.

In previous work from our laboratory (6, 13) it has been found that during the incubation of either isolated inner or outer mitochondrial membranes in the presence of ascorbate some phospholipids are degraded, precisely those with polyunsaturated fatty acids present in their molecules. This has permitted to establish the presence of different molecular species of phospholipids with regard to the nature

of their fatty acid constituents. The fragments obtained by the lytic action of ascorbate on isolated inner membranes were different in morphology, chemical composition and in distribution of enzyme activities, being this an indication of a heterogeneous distribution both of lipids and proteins (7, 14).

It seemed interesting to use the same approach to study the isolated outer mitochondrial membrane with the purpose of determining whether the distribution of its different components is also heterogeneous. The results here reported point out that, contrary to what happens with the inner membrane, the outer mitochondrial membrane is rather homogeneous with regard to the distribution of phospholipids, degrees of unsaturation, and of the enzyme markers rotenone insensitive NADH-cyt. c reductase and monoamine oxidase.

Materials and Methods

Male Wistar rats weighing approximately 200 g were used in all the experiments. After decapitation of the animals, livers were rapidly removed and mitochondria isolated according to the method of HOGEBOOM (4). The microsomal fraction was obtained by centrifuging at 100,000 × g during 1 hour the supernatant resulting from the homogenate after removing the mitochondria. Outer mitochondrial membranes were prepared as described by PARSONS *et al.* (12). Inner membranes were obtained bye the method of PARSONS *et al.* (8) with the modifications described (14).

Incubation of the membranes was carried out in a medium containing 1 mM ascorbate, 20 mM Tris-HCl buffer, *p*H 7.4, and 0.25 M sucrose, at 30° C. Simultaneously, membranes were incubated as controls in a similar medium without ascorbate. Incubation times were 60 minutes or 120 minutes.

The membrane fragments produced during the incubation were separated by differential centrifugation. The suspensions were centrifuged at 22,000 × g during 10 minutes and a first fraction was obtained, which will be referred to as F_1C (control) and F_1A (with ascorbate). The resulting supernatants were centrifuged at 100,000 × g during 60 minutes and sediments F_2C (control) and F_2A (with ascorbate) were obtained. Supernatants of this second centrifugation will be referred to as fractions F_3C (control) and F_3A (with ascorbate).

Proteins were determined by the method of LOWRY *et al.* (8). Lipid phosphorus was determined by the method of BARTLETT (1). Phospholipids were extracted as previously described (15) and separated by thin layer chromatography (10). Methyl esters of fatty acids in the total lipid extract and in the individual phospholipids were prepared by direct methylation following the procedure of MORRISON and SMITH (9), and analyzed by gas chromatography as previously described (6). Methyl esters of fatty acids present in the lipids which remained bound to protein after the usual extraction procedure were prepared as previously described (6). Enzymes monoamine oxidase and rotenone-insensitive NADH cytochrome c reductase were used as markers of the outer mitochondrial membranes and determined by the methods of WEISBACH (7) and HATEFI and RIESKE (3) respectively. Glucose-6-phosphatase was used as marker of the microsomal fraction and determined according to SWANSON (17). As marker of the inner mitochondrial membrane cytochrome oxidase was used and determined by the methods of SOTTOCASA *et al.* (16).

Since ascorbate would interfere with the measurement of the enzyme activity of rotenone insensitive NADH cytchrome c reductase it was necessary to oxidize previously the ascorbate present in the suspension with the enzyme ascorbate oxidase (7). Cholesterol was determined following the method of CARR and DREKTER (2). Mem-

branes were examined by electron microscopy using negative staining techniques as already described (14).

Results

Specific enzyme activities of the outer membrane preparations are shown in Table I. It can be observed that they had only a very slight contamination of microsomes and inner mitochondrial membranes.

After the incubation with ascorbate, the outer membranes were examined by electron microscopy. The structure was rather preserved, although the membranes presented small erosions on the surface exhibiting a moth-eaten aspect. Some granular material was also observed. The examination of the fractions obtained by differential centrifugations showed that the disaggregation was greated going from fraction F_1C to F_3C; the only difference consisting in the size of the fragments. Fractions F_1A and F_2A showed the structure of outer membrane fragments, with eroded surfaces. Fraction F_3A was made up of granular metarial more or less aggregated.

Table II shows the ratios of μg of lipid P/mg protein, μg of P bound to protein/mg protein and μg of cholesterol/mg protein of the suspension of outer mitochondrial membranes incubated in the presence of ascorbate. In the presence of ascorbate a decrease in extractable lipid P took place, which corresponded to an increase in P bound to protein.

Table III shows the percent distribution of the proteins in the fractions obtained by differential centrifugation after 60 and 120 minutes of incubation, as well as their ratios of μg of lipid P/mg protein and μg of cholesterol/mg protein, and the percent distribution of P bound to protein.

It can be seen that the desintegration of the membranes increases with the incubation time and with the presence of ascorbate. Up to 23 % of the proteins were found in fraction F_3A and only 8 % for the corresponding control membranes.

Table I. *Enzyme activities of different subcellular fractions.*
(See text for experimental details.)

Fraction	Glucose-6-phosphatase (mμmoles PI/min/mg protein)	Cytochrome oxidase (μmoles cyt. c oxidized/min/mg protein)	Monoamine oxidase (mμmol Kynurenine oxidized/mg protein/min)	Rotenone insensitive NADH-cyt. c red. (mμmol cyt. c reduced/min/mg protein)
Microsomes	150	—	—	—
Mitochondria	10	210	20	100
Inner membranes	n. d.	305	n. d.	n. d.
Outer membranes	14	30	100	560

n. d.: Not detected.

Table II. *Phospholipid and cholesterol content and P bound to protein in outer mitochondrial membranes.*
(See text for experimental details.)

	μg P lipid/mg protein		μg P bound to protein/mg		μg cholesterol/mg protein
	60 min	120 min	60 min	120 min	120 min
Incubated outer membranes	20	20	4	4	33.5
Outer membranes incubated with ascorbate	14.5	13	9.5	11	33.5

Table III. *Phospholipid and cholesterol content and P bound to protein in subfractions of outer mitochondrial membranes.*
Subfractions were obtained by differential centrifugation, after incubating outer mitochondrial membranes as controls (F_1C, F_2C, F_3C) or in the presence of 1 mM ascorbate (F_1A, F_2A, F_3A). F_1C, F_1A, sediments after 22,000 × g, 10 min; F_2C, F_2A, sediments after 100,000 × g, 60 min; F_3C, F_3A, supernatants of the last centrifugation. (See text for other experimental details.)

Fraction	Protein (%)		μg P lipid/mg protein		P bound to protein (%)	μg cholesterol/mg protein
	60 min	120 min	60 min	120 min	120 min	120 min
F_1C	71	64	20.5	20.5	55	33.3
F_2C	20	28	20	19	35	33.5
F_3C	8	8	*	*	10	12
F_1A	60	40	18	14.5	47	35.5
F_2A	22	37	14.3	12	33	39
F_3A	18	23	4	9	20	14

* No lipid P was detected in this fraction.

fraction F_3C, after two hours of incubation.

Lipid P/protein ratio was similar in fractions F_1C and F_2C obtained from the incubated controls. In fraction F_3C no lipid P could be found. In fractions F_1A, F_2A and F_3A obtained from the membranes incubated with ascorbate the values of this ratio were much lower, and corresponding the lowest value to the supernatant F_3A.

The percent distribution of P bound to protein was rather parallel to the protein distribution in each fraction. The phospholipid composition of fractions F_1C, F_2C, corresponding to the controls, and of fractions F_1A and F_2A obtained from the membranes incubated with ascorbate is given in Table IV.

Table IV. *Percent distribution of phospholipids in different subfractions of the outer mitochondrial membrane.*
See text for experimental details.
Abbreviations: See Table III.

	F_1C	F_2C	F_1A	F_2A
Phosphatidylserine + sphingomyeline	3.1	3	*	*
Phosphatidylinositol	14	16.5	*	*
Phosphatidylcholine	40	40.5	27.5	29
Phosphatidylethanolamine	29	27	15	13
Cardiolipin	14.3	11	11	8

* Real values of P lipid in these phospholipids after incubation with ascorbate were not obtained because of the interference of P present in degraded phospholipids.

It can be seen that only minor differences were found between fractions F_1C and F_2C. In fractions F_1A and F_2A an appreciable decrease in phosphatidylcholine, phosphatidylethanolamine, and cardiolipin, was found; but the differences F_1A and F_2A were also very small.

The distribution of fatty acids present in the total lipid extracts prepared from each fraction, as well as that of the fatty acids which remained bound to the proteins, after being subjected to the lipid extraction procedure, are given in Table V.

The ratios of the areas of the chromatographic peak of each fatty acid per mg of protein were lower for fractions sedimenting at 100,000 × g (F_2C and F_2A) than for those sedimenting at 22,000 × g (F_1C and F_1A). It can be also observed that there was a decrease in the values of these ratios in the fractions obtained from the membranes incubated with ascorbate (F_1A and F_2A) when compared to those of the corresponding controls (F_1C and F_2C). This decrease was more marked for the

Table V. *Distribution of fatty acids in the lipid extract and P bound to protein in subfractions of outer mitochondrial membranes.*
See text for experimental details. Abbreviations: See Table III.

Fatty acids	Fatty acids in lipid extract (area/mg protein)				Fatty acids bound to protein (area/mg protein)			
	F_1C	F_2C	F_1A	F_2A	F_2C	F_2C	F_1A	F_2A
16 : 0	2500	2250	1800	1287	130	110	690	600
18 : 0	2000	1620	1400	940	97	90	470	400
18 : 1	860	686	540	286	47	40	200	170
18 : 2	600	510	490	215	19	10	30	30
20 : 4	1600	1260	800	386	20	—	20	10

unsaturated fatty acids. It can be seen that a high proportion of palmitic, stearic and oleic acids which had disappeared in the lipid extract of the fractions obtained from the membranes incubated with ascorbate were recovered as bound to proteins. However, polyunsaturated fatty acids, linoleic and arachidonic acids were not recovered.

Table VI. *Monoamine oxidase and rotenone — insensitive NADH — cyt. c reductase activities in outer mitochondrial membranes and in subfractions obtained by ascorbate induced lysis.*
See text for experimental details.
Abbreviations: See Table III.

Fraction	Monoamine oxidase (mμmol Kynurenine oxidize/mg protein/min)	Rotenone insensitive NADH-cyt. c red. (mμmol cyt. c reduced/mg protein/min)
Incubated outer membrane	94	530
Incubated outer membrane with ascorbate	80	290
F_1C	94	520
F_2C	94	520
F_3C	n. d.	n. d.
F_1A	92	300
F_2A	96	290
F_3A	n. d.	80

n. d.: Not detected.

Table VI shows the rotenone-insensitive NADH-cyt. c reductase and monoamine oxidase activities both in the total suspensions of outer membranes and in their different fractions. The rotenone-insensitive NADH-cyt. c reductase became partly inactivated during the incubation with ascorbate, whereas monoamine oxidase was practically unaffected. The distribution of the enzyme activity of monoamine oxidase was very similar in fractions F_1C and F_2C and in the total suspension of the incubated controls. Their values were also quite close for the corresponding fractions of the membranes incubated with ascorbate. The rotenone insensitive NADH-cyt. c reductase was also uniformly distributed in fractions F_1C, F_2C, F_1A and F_2A.

Discussion

The study of the different fragments obtained by differential centrifugation of outer mitochondrial membranes incubated in the presence of ascorbate seems to indicate that the structural disaggregation is due partly to the fact of the incubation at 30° C, since some disaggregation took also place in the incubated controls, and mainly to the peroxidation reactions on the polyunsaturated fatty acids of the phospholipids. However, the lytic effect of ascorbate on the outer membrane is more limited than on the inner membrane (4) due to

a much smaller proportion of polyunsaturated fatty acids in the phospholipids of the outer membrane.

On the basis of a homogeneous distribution of lipids and proteins in the outer membrane it is easily understood that after 60 minutes of incubation predominated the larger fragments, i.e. those sedimenting at 22,000 × g (fractions F_1C and F_1A) (Table III). The decrease in the ratio of μg lipid P/mg protein was very small in fraction F_1A with respect to the value in the total incubated control. This decrease was slightly higher for fraction F_2A. After 120 minutes the only changes in protein distribution consisted in an increase in fraction F_2A at the expense of fraction F_1A, whereas fraction F_3A remained practically the same at 60 and 120 minutes. This could be interpreted as a further alteration of the lipids present in the larger fragments, which is reflected in the values of the ratios of lipid P/proteins in fractions F_1A and F_2A after 120 minutes of incubation. The lipid P, as well as the palmitic, stearic and oleic acids which disappeared in the lipid extracts prepared from these two latter fractions were recovered bound to the extracted proteins (Tables III and V). The fact that this lipid P and fatty acids recovered in the proteins are uniformily distributed in fractions F_1A and F_2A can be taken as an indication that the different molecular species of the phospholipids according to the degree of unsaturation of their fatty acids are homogeneously distributed in the outer membrane. Fraction F_3A would be constituted by proteins released from the membrane, together with some altered phospholipids and carrying only a very small proportion of cholesterol, and therefore the ratio of μg cholesterol per mg protein would increase in fractions F_1A and F_2A.

The inactivation of the rotenone insensitive NADH-cyt. c reductase can be interpreted as a consequence of the alteration of its accompanying phospholipids. The remaining activity of this enzyme and that of the monoamine oxidase were uniformly distributed between fractions F_1A and F_2A.

This homogeneity of the outer mitochondrial membrane would not necessarily imply that each enzyme protein would not be specifically associated with certain phospholipids.

Resumen

Las membranas externas mitocondriales aisladas del hígado de rata sufren una desagregación estructural cuando se incuban en presencia de ascorbato 1 mM. Los fragmentos producidos pueden ser separados por centrifugación diferencial. Se obtuvieron tres fracciones; una que sedimenta a 22.000 × g; otra a 100.000 × g, y una tercera constituida por el sobrenadante de esta última centrifugación. Se ha estudiado la composición lipídica y la distribución de los enzimas marcadores monoamina oxidasa y NADH-citocromo c reductasa insensible a la rotenona en cada una de estas fracciones. La fracción sobrenadante está compuesta por las proteínas solubilizadas acompañadas por algunos fosfolípidos alterados y una pequeña cantidad de colesterol. Las otras dos fracciones resultaron ser muy similares en la distribución de enzimas y composición lipídica. La interpretación de los resultados presentados indica que la membrana externa mitocondrial es muy homogénea en lo que se refiere a la distribución de sus lípidos y proteínas en contraste con lo que se ha establecido para la membrana interna.

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