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Heterogeneity of the Inner Mitochondrial Membrane Through the Unequal Distribution of Phospholipid Unsaturation

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Three different subfractions from rat liver inner mitochondrial membranes have been obtained by differential centrifugation after the lysis of the membranes induced by ascorbate, through a peroxidation reaction on their phospholipid constituents.

The evidence presented in this communication supports the view, that these three subfractions derive from areas of the inner membrane, which differ in the degree of unsaturation of their phospholipids and in the distribution of respiratory chain components. A first subfraction would derive from an area of the membrane containing predominantly the molecular species of phospholipids with saturated fatty acids, and lacking respiratory chain components; a second subfraction would have its origin in a region containing the molecular species of phospholipids with one saturated and one unsaturated fatty acid; and a third subfraction would originate from an area containing the molecular species of phospholipids with their fatty acids unsaturated, plus part of the phospholipids with both saturated and unsaturated fatty acids in their molecules. Cytochromes, flavins and coenzyme Q were found in these two latter subfractions.

It has been previously shown (12) that the lysis process which inner mitochondrial membranes undergo when they are incubated in the presence of ascorbate is the result of alterations of certain phospholipids through peroxidation reactions on the unsaturated fatty acids which might be present in their molecules. These alterations render those phospholipids unextractable by the usual organic solvents and remain bound to the neighboring membrane proteins. The submitochondrial particles derived from the lysis of the rat liver inner mitochondrial membrane were separated by differential centrifugation and shown to be different in morphology and in their lipid to protein ratios. These results suggest a heterogeneous distribution of the phospholipids within the membrane in what concerns the degree of unsaturation of their fatty acid constituents. Based on these findings a model for the mito-

chondrion was proposed; according to this model the inner membrane would be constituted by a tubular component with the projecting subunits, and a lamellar structure devoid of such subunits (12). WERNER and NEUPERT (17) using a combined technique of swelling, shrinking and sonication have also separated submitochondrial fragments which differed in morphological aspect, chemical composition, electrophoretic behaviour of the proteins and enzyme distribution; they could distinguish the cristae membrane, where all the enzymatic complexes of the respiratory chain would be contained, and an inner peripheral membrane in close apposition with the outer membrane.

New data on the characteristics of the molecular species of the phospholipids and of the distribution of the components of the respiratory chain within each submitochondrial fraction prepared by lysis with ascorbate have been obtained in our laboratory. According to the findings here reported, it has been concluded that the inner mitochondrial membrane has a heterogeneous distribution of the molecular species of the phospholipids and of the respiratory chain components.

Materials and Methods

Male Wistar rats weighing approximately 200 g were used in all the experiments. Livers were homogenized in 0.25 M sucrose and mitochondria isolated according to the method of HOGEBOOM (3). Inner mitochondrial membranes were prepared following the method of PARSONS *et al.* (8) with the modifications described by SANTIAGO *et al.* (10). The inner mitochondrial fraction had a slight contamination of outer membranes, amounting to less than 5 %, and a rather high content of matrix (11). In order to make the necessary corrections, so that the results could be referred to proteins of the inner membrane, the soluble proteins of the matrix present in this fraction were removed essentially as described by WERNER and NEUPERT (17), subjecting it to extensive sonication and centrifugation at 165,000 g during 90 minutes; the membrane proteins were recovered in the sediment and the soluble proteins of the matrix in the supernatant.

Incubation of the inner membrane plus matrix fraction was carried out in a medium 1 mM ascorbate, 0.02 M tris-HCl buffer, *p*H 7.4, and 0.25 M sucrose, at 30° C, during one hour; in every experiment controls without ascorbate were incubated. The membrane fragments produced during the incubation were separated by differential centrifugation. The suspensions were centrifuged at 8,500 × g during 10 minutes, and a first subfraction (subfraction I) was obtained. The resulting supernatant was centrifuged at 100,000 × g during 60 minutes and a sediment (subfraction II) was obtained; supernatant of this second centrifugation will be referred to as subfraction III.

Proteins were determined by the method of LOWRY *et al.* (4). Phospholipids were extracted from the samples, after precipitation with $HClO_4$ to give a final concentration of 0.3 N, as described previously (13) and separated by thin layer chromatography according to NESKOVIC *et al.* (6). Lipid phosphorus was determined by the method of BARTLETT (1). Methyl esters of the fatty acids of the different phospholipids present in the lipid extract were prepared through direct methylation with 14 % BF_3 in methanol according to MORRISON and SMITH (5). Methyl esters were then analyzed with a Beckman GC4 gas chromatograph, using a double column, with a 1/8 inch diameter and a 6 ft length; the liquid phase was 20 % DEGS, and the solid phase, Chromosorb W; particle size, 42/60 μ diameter; hydrogen and air flows were respectively 50 cc/min. and 250 cc/min.; column temperature, 160° C, and that of the detectors, 280° C.

Methylation of fatty acids still bound to proteins after lipid extraction was carried out as follows: 6 ml of 96 % ethanol and

0.4 ml of 50 % NaOH were added to the protein precipitate amounting up to 20 mg, and heated in a water bath during 30 minutes, checking that the medium remained alkaline. The hydrolyzate was acidified with HCl and the fatty acids extracted with 3 ml of petroleum ether and methyl esters prepared as described above.

The following components and enzyme activities were determined according to techniques already described in the literature: cytochromes a, b, c, c_1 (18), flavins (2), coenzyme Q (9), monoamine oxidase (16), malate dehydrogenase (7) and cytochrome oxidase (15).

Results

Table I shows the values of the activities of malate dehydrogenase and cytochrome oxidase, and of lipid P in the inner membrane fraction still containing matrix, and also in the sediment and in the supernatant after sonication and centrifugation at 165,000 g. It can be seen that malate dehydrogenase appeared exclusively in the supernatant, whereas cytochrome oxidase was present only in the sediment. These results indicate that 40 %

Table I. *Enzyme markers and lipid P in inner membrane plus matrix fraction and their distribution after sonication and centrifugation.* The inner membrane plus matrix fraction was prepared as previously described (10). It was subsequently subjected to extensive sonication and centrifugation at 165.000 g, at 0° C as described by WERNER and NEUPERT (17). The results are referred to 20 mg of protein in the original inner membrane plus matrix fraction.

	Protein mg	Malate dehydrogenase mmol. min^{-1}	Cytochrome oxidase μmol. min^{-1}	Lipid P μg
I. M.*	20	48	6,100	100
Sediment	12	—	6,000	98
Supernatant	8	45	—	2

* I. M., Inner membrane plus matrix.

Table II. *Distribution of protein and enzyme markers in subfractions obtained from the inner membrane plus matrix fraction.* Inner membrane plus matrix fraction was incubated in a medium 1 mM Ascorbate, 20 mM Tris-HCl buffer, *p*H 7.4, 0.25 Sucrose, at 30° C during one hour. Protein concentration was adjusted to 0.8 mg/ml. The results are referred to 20 mg protein. At the incubation time the suspensions were centrifuged at 8.500 g during 10 minutes to obtain Subfraction I. The resulting supernatant was centrifuged 100,000 g during 60 minutes obtaining a sediment, Subfraction II, and a supernatant, Subfraction III.

	Protein mg	Malate dehydrogenase mmol. min^{-1}	Monoamine oxidase mmol. min^{-1}
I. M.*	20	48	40
Subfraction I	1.8	—	4
Subfraction II	3.5	—	29
Subfraction III	14.7	44	3

* I. M., Inner membrane plus matrix.

Table III. *Distribution of respiratory chain components in subfractions obtained from the inner membrane plus matrix fraction.* The inner membrane plus matrix fraction was prepared as previously described (10). Subfractions I. II and III were obtained as indicated in Table II. The corrected values of membrane protein have been obtained subtracting the amount of protein corresponding to the matrix (See Tables I and II).

	I. M.*	Subfraction		
		I	II	III
Membrane protein, mg (corrected value)	12	1.6	3.4	7
Coenzyme Q, μmol	40	0.14	10	23
Flavins, mμmol	20	0.6	8	11
Cytochrome a, mμmol	7.1	0.3	3	3.5
Cytochrome b, mμmol	6	0.15	2.8	3
Cytochrome c, mμmol	4.2	0.1	2	2
Cytochrome c_1, mμmol	5.1	0.1	2.5	2.5

* I. M., inner membrane plus matrix.

Table IV. *Phospholipid composition in subfractions obtained from inner mitochondrial membrane plus matrix fraction.*

The inner membrane plus matrix fraction was prepared as previously described (10). Subfractions I, II and III were obtained as indicated in Table II. The results are referred to 20 mg of protein in the original inner membrane plus matrix fraction.

	I.M.*	I.M.+ Ascorbate	Subfraction I	Subfraction II	Subfraction III
			μg of lipid P		
Phosphatidylcholine	40	11	4	4	2.5
Phosphatidylethanolamine	33	8	2.5	4	1.5
Cardiolipin	15	1.5	0.7	0.5	0.3

* I. M., Inner membrane plus matrix fraction.

of the protein in the inner membrane plus matrix fraction corresponded to the matrix, allowing thus to introduce this correction, when referring to inner membrane proteins. Table I also shows that the phospholipids are exclusively confined to the membrane.

Table II shows the distribution of proteins, malate dehydrogenase and monoamine oxidase activities in subfractions I, II and III obtained by differential centrifugation after the lysis of the inner membrane plus matrix fraction with ascorbate. The small amount of outer membranes contaminating the inner membrane plus matrix fraction was practically recovered in subfraction II as indicated by the enzyme marker monoamine oxidase (14). The proteins of the matrix were completely recovered in fraction III as

Table V. *Fatty acid distribution in the total lipid extract and in the different phospholipids present in subfractions obtained from inner membrane plus matrix fraction.*

The inner membrane plus matrix fraction was prepared as previously described (10). Subfractions I, II and III were obtained as indicated in Table II. The results are expressed in μg and referred to 20 mg of protein in the original inner membrane plus matrix fraction.

	TOTAL LIPID EXTRACT					PHOSPHATIDYLCHOLINE				
Fatty acids	I. M.*	I. M. + Ascorbate	Subfractions I	Subfractions II	Subfractions III	I. M.	I. M. + Ascorbate	Subfractions I	Subfractions II	Subfractions III
Total	2,000	500	230	180	82	800	210	80	78	48
16 : 0	380	160	70	65	25	210	65	25	23	14
18 : 0	440	140	60	50	26	180	60	24	20	12
18 : 1	200	55	30	23	10	70	20	7	12	10
18 : 2	460	60	30	20	10	120	16	7	5	3
20 : 4	410	30	16	6	4	200	10	6	2	2
	PHOSPHATIDYLETHANOLAMINE					CARDIOLIPIN				
Fatty acids	I. M.	I. M. + Ascorbate	Subfractions I	Subfractions II	Subfractions III	I. M.	I. M. + Ascorbate	Subfractions I	Subfractions II	Subfractions III
Total	660	160	47	80	29	300	35	15	10	7
16 : 0	142	45	14	20	9	24	8	2	3	2
18 : 0	225	46	16	20	9	33	7	2	2	2
18 : 1	55	15	5	6	2	38	5	3	—	—
18 : 2	90	10	4	4	1	140	6	5	—	—
20 : 4	152	6	3	2	1	28	—	1	—	—

* I. M., inner membrane plus matrix fraction.

Table VI. *Lipid P and fatty acids bound to protein after incubation of the membranes with ascorbate in subfractions obtained from the inner membrane plus matrix fraction.* The inner membrane plus matrix fraction was prepared as previously described (10). Subfractions I, II and III were obtained as indicated in Table II. The results are expressed in μg and referred to 20 mg of protein in the original inner membrane plus matrix fraction.

	P, μg	FATTY ACIDS, μg					
		Total	16 : 0	18 : 0	18 : 1	18 : 2	20 : 4
I. M.* + Ascorbate	46	530	185	200	100	20	10
Subfraction I	3	25	9	10	—	—	—
Subfraction II	12	240	80	85	40	5	—
Subfraction III	28	250	90	100	45	7	2

* I. M., inner membrane plus matrix fraction.

judged by the activity of the enzyme marker malate dehydrogenase (15).

The distribution of membrane protein and of coenzyme Q, flavins and cytochromes a, b, c and c_1 is shown in Table III. The values of membrane protein have been corrected subtracting the soluble proteins of the matrix. It can be observed that all these components of the respiratory chain were concentrated in subfractions II and III.

Table IV gives the distribution of the different major phospholipids, phosphatidylcholine, phosphatidylethanolamine and cardiolipin, in the inner membrane plus matrix fraction before and after the incubation with ascorbate, as well as in subfractions I, II and III. It can be seen that the phospholipids remaining without alteration after the incubation with ascorbate were not evenly distributed among these three subfractions, and also that the ratio of phospholipid to protein decreased in this order: subfraction I > subfraction II > subfraction III.

The fatty acid composition in the total lipid extract from the inner membrane, and from subfractions I, II and III, and the distribution of the major phospholipids, phosphatidylcholine, phosphatidylethanolamine and cardiolipin, in each one of them are given in Table V. It can be noticed that high losses of fatty acids, mainly linoleic and arachidonic acid, took place after the incubation with ascorbate. The concentration of the remaining unaltered fatty acids was found to decrease in this order: subfraction I > subfraction II > subfraction III.

The values of fatty acids and lipid P bound to protein after incubation with ascorbate are given in Table VI. It can be seen that the values for lipid P and for total fatty acids increased in this order: subfraction I > subfraction II > subfraction III. However, the ratio of total fatty acids to lipid P had its highest value in subfraction II. It should be also noticed that linoleic and arachidonic acids were practically absent in subfractions I, II and III. In the incubated controls the fatty acids bound to protein were negligible.

The controls were not affected by the incubation, and total protein, all the enzyme activities, and phospholipids could be recovered in the sediment of the centrifugation at 8,500 g during 10 minutes.

Discussion

It has already been described the existence of three types of molecular species of the phospholipids present in the inner mitochondrial membrane with respect to the nature of their fatty acid constituents.

A group of phospholipids would contain only saturated fatty acids; another group constituted by both saturated and unsaturated fatty acids; and a third group containing exclusively unsaturated fatty acids (11).

The study of the submitochondrial fractions obtained by differential centrifugation after lysis with ascorbate shows that subfraction I had a high proportion of phospholipids, which were not affected by the peroxidation reaction. These phospholipids, in turn, had a high content in saturated fatty acids; and besides, the amount of lipid P and of fatty acids bound to protein in this subfraction was negligible. It seems reasonable to assume that this subfraction has originated from an area of the membrane with phospholipids predominantly saturated. It should be also noticed that only a very small amount of the respiratory chain components are found in it. This would be in agreement with the findings of WERNER and NEUPERT (17) who have described the existence of a fraction of the inner membrane with a low content in cytochromes and in succinate-cytochrome c reductase.

Subfraction II would have its origin in an area of the membrane with molecular species of phospholipids containing one saturated and one unsaturated fatty acid. This appears as a logical conclusion, since through the action of ascorbate most of the phospholipids with an unsaturated fatty acid will be peroxidized, and consequently the saturated fatty acid, as well as the lipid P would become bound to the protein. More than 40 % of the respiratory chain components were found in this subfraction.

Subfraction III should derive from the area most sensitive to the peroxidation reaction, since it was the most highly disaggregated. A high amount of lipid P together with a comparatively small amount of fatty acids bound to protein was present in this subfraction. This would indicate that the predominant molecular species of the phospholipids originally present in the area from which this subfraction has derived would be those containing only unsaturated fatty acids, which are consequently destroyed. The fatty acids which still appear bound to protein in this subfraction would belong to molecular species of phospholipids containing one saturated or monounsaturated and one polyunsaturated fatty acid. More than 50 % of the respiratory chain components were also recovered in this fraction.

Resumen

Tres subfracciones diferentes han sido obtenidas a partir de membranas internas mitocondriales de hígado de rata por centrifugación diferencial, después de la lisis de las membranas inducida por ascorbato a través de una reacción de peroxidación sobre sus fosfolípidos.

La evidencia presentada en esta comunicación apoya la opinión de que estas tres subfracciones deriven de áreas de la membrana interna que difieren en el grado de insaturación de sus fosfolípidos. Una primera subfracción derivaría de un área de la membrana que contendría predominantemente las especies moleculares de los fosfolípidos con ácidos grasos saturados y carecería de componentes de la cadena respiratoria; una segunda subfracción tendría su origen en una región que contendría las especies moleculares de fosfolípidos con un ácido graso saturado y otro insaturado; y una tercera subfracción se originaría a partir de un área en que estarían presentes especies moleculares de fosfolípidos con sus ácidos grasos insaturados, además de una parte de los fosfolípidos con ácidos grasos saturados e insaturados en la misma molécula. Los citocromos, flavinas y coenzima Q estaban presentes en estas dos últimas fracciones.

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