REVISTA ESPAÑOLA DE FISIOLOGIA, 30, 43-48. 1974

Subfractions Obtained From Inner Mitochondrial Membranes Incubated With Cysteine. II. Distribution of Enzymes and Respiratory Chain Components

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(Received January 18, 1974)

J. L. SEGOVIA, N. LOPEZ MORATALLA and E. SANTIAGO. *Subfractions Obtained From Inner Mitochondrial Membranes Incubated With Cysteine. II. Distribution of Enzymes and Respiratory Chain Components.* Rev. esp. Fisiol., 30, 43-48. 1974.

Three subfractions from rat liver inner mitochondrial membranes have been obtained by differential centrifugation after incubation in the presence of cysteine; these subfractions were different with respect to their content in respiratory chain components. These subfractions would have their origin in areas of the membrane more or less altered by the peroxidation reaction induced by the cysteine according to the degree of unsaturation of their phospholipids.

It has already been established that phospholipids present in mitochondrial membranes are degraded through peroxidation of their unsaturated fatty acid constituents, when the membranes are incubated with either ascorbate or cysteine (12). These phospholipid alterations result in a decrease, or even in the complete loss of activity of a number of membrane bound-enzymes (5), and in a lysis of the membrane (13).

It has been shown (9) that three subfractions derived from areas of the inner mitochondrial membrane which differ in the degree of unsaturation of their phospholipids and in the distribution of respiratory chain components can be separated by differential centrifugation after the lysis induced by ascorbate.

WERNER and NEUPERT (16) 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.

In a previous publication (10) the lipid composition of inner membrane subfractions obtained by the action of cysteine has also been reported. In the present work the distribution of respiratory chain components and of enzyme activities in these subfractions have been studied and found to be different in each one of them.

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 (4). Isolated mitochondria were subjected to osmotic rupture following the method of PARSONS *et al.* (8). Inner mitochondrial membranes were obtained using PARSONS «low speed pellet» as starting material. In order to remove the outer membranes still present in this fraction, it was throughly washed three times by resuspending it in 0.02 M phosphate buffer, *p*H 7.4, centrifuging at 1,900 × *g* for 15 minutes and once more resuspending it in 0.25 M sucrose and centrifuging at 8,500 × *g* for 10 minutes (13).

Incubation of the inner membrane was carried out in a medium 8×10^{-4} M cysteine, 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 cysteine were incubated. The membrane fragments produced during the incubation were separated by diferential 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.* (6); cholesterol was determined by the technique of CARR and DREKTER (1).

The following components and enzyme activities were determined according to techniques already described in the literature; cytochromes a, b, c, c_1 (17), flavins (2), succinate-cytochrome c reductase (15), rotenone-sensitive NADH cytochrome c reductase (3), ATPase (11), malate dehydrogenase (7) and cytochrome oxidase (14).

Results

The values of cytochromes a, b, c_1 and c, and of flavoproteins in the different subfractions derived from the lytic action of cysteine are given in table I. It may be seen that almost the totality of all the cytochromes and flavoproteins was recovered in the subfractions which sedimented at 8,500 × *g* (Subfraction I) and in the 100,000 × *g* supernatant (Subfraction III). However, some differences in the distribution were found; cytochromes a and c_1 were liberated from the membrane, and therefore found predominantly in the 100,000 × *g* supernatant, whereas cytochrome *b* remained in a higher pro-

Table I. *Distribution of cytochromes and flavins in subfractions of inner mitochondrial membranes.*

Results (mμmoles) are referred to a suspension of inner membranes containing 100 mg protein. See text for experimental details.

	Protein recovery %	Cyt. a	Cyt. b	Cyt. c_1	Cyt. c	Flavins
Incubated inner membrane	100	35.0	30	21	25	100.0
Inner membranes incubated with cysteine	100	35.0	30	21	25	100.0
Control (Sediment of 8,500 × *g*)	80	31.0	28	18	21.8	90.4
Subfraction I	30	12.0	16.3	7.7	11.5	34.25
Subfraction II	6	2.8	3	1.7	2.5	4.9
Subfraction III	64	14.0	8.2	9.6	10	57.6

Table II. *Distribution of enzyme activities in subfractions of inner mitochondrial membranes.*
Results are referred to a suspension of inner membranes containing 100 mg protein. See text for experimental details.

	Protein recovery %	Cytochrome oxidase (μmol × min^{-1})	Succinate-cyt. reductase (μmol × min^{-1})	Rotenone-sensitive NADH cyt. c reductase (μmol × min^{-1})	ATPase (μmol Pi × min^{-1})
Incubated inner membrane	100	25.0	37.4	8.50	110.0
Inner membranes incubated with cysteine	100	16.0	16.0	4.50	57.0
Control (Sediment of 8,500 × *g*)	80	22.4	32.0	8.24	100.0
Subfraction I	30	11.0	12.9	3.90	48.0
Subfraction II	6	1.1	1.3	0.30	5.0
Subfraction III	64	3.2	1.02	—	2.6

portion in the sediment of 8,500 × *g*.

Cytochrome c oxidase, succinate-cytochrome c reductase, rotenone-sensitive NADH cytochrome c reductase and ATPase were also determined. The results are collected in table II. It may be observed that the incubation with cysteine markedly affected all those enzyme activities. The greatest loss in activity corresponded to succinate-cytochrome c reductase and the least affected activity was that of cytochrome c oxidase. The largest part of the recovered activities was found in subfraction sedimenting at 8,500 × g (Subfraction I).

Table III. *Distribution of proteins and malate dehydrogenase activity in subfractions of inner mitochondrial membranes.*
Results are referred to a suspension of inner membranes containing 100 mg protein. See text for experimental details.

	Protein recovery %	Malate dehydrogenase μmoles × min^{-1}
Incubated inner membrane	100	230.0
Inner membrane incubated with cysteine	100	230.0
Control (Sediment of 8,500 × *g*)	80	200.0
Subfraction I	30	39.0
Subfraction II	6	10.8
Subfraction III	64	166.0

Table IV. *Cholesterol content in subfractions of inner mitochondrial membranes.*
Results are referred to a suspension of inner membranes containing 100 mg protein. See text for experimental details.

	Protein recovery %	Cholesterol μg
Incubated inner membrane	100	330.0
Inner membranes incubated with cysteine	100	330.0
Control (Sediment of 8,500 × *g*)	80	300.0
Subfraction I	30	240.0
Subfraction II	6	42.4
Subfraction III	64	20.5

In a previous publication it was already shown that the inner membrane fraction contained some matrix (9); in order to determine the contamination in the different subfractions, the matrix enzyme marker malate dehydrogenase was measured in each one of them. The results are given in table III. Up to a 90 % of the activity was recovered in the sediment of 8,500 × *g*

in the incubated control. After the incubation with cysteine, 75 % activity was found in the 100,000 × *g* supernatant (Subfraction III), and the remaining 25 % was distributed between the other two subfractions.

The distribution of cholesterol in the different subfractions is given in table IV. It was recovered nearly completely in the sediment of 8,500 × *g* in the incubated controls; up to 80 % was recovered in subfractions sedimenting at 8,500 × *g* after the incubation with cysteine (Subfraction I).

Discussion

The data here presented show that the subfractions obtained by the lytic action of cysteine on the inner mitochondrial membrane are different regarding their content in components of the respiratory chain and enzyme composition. The subfraction sedimenting at 8,500 × *g* contained a high proportion of them. In a previous report (9) it had been shown that the phospholipids of that subfraction had undergone very little alteration and that they had a higher proportion of saturated fatty acids than the whole inner membrane. Tre fact that this subfraction contained a high proportion of cholesterol together with a high content in protein seem to indicate that it constitutes the core of the inner membrane from which the areas more unsaturated and with a lower cholesterol content were liberated by the action of cysteine.

Comparing this subfraction with others previously reported (9) and obtained by treatment of the membrane with ascorbate it may be concluded that this cysteine subfraction (Subfraction I) corresponds to two areas of the membrane which could be separated by the action of ascorbate: one, highly saturated without respiratory chain components and another of intermediate unsaturation and with a 50 % of the respiratory chain components. The subfraction sedimentig at 100,000 × *g* (Subfraction II) would have its origin in the area of intermediate unsaturation, slightly affected by cysteine; this assumption is based on its resemblance to a subfraction obtained after the incubation with ascorbate and corresponding to that area.

The supernatant of the 100,000 × *g* contained approximately 50 % of the flavoproteins and of the cytochromes, but with practically no activity of succinate-cytochrome c reductase, rotenone sensitive NADH-cytochrome c reductase and ATPase. The absence of these activities would be due presumably to the peroxidation of the polyunsaturated fatty acids present in the phospholipids surrounding the enzyme molecules.

The data here reported add new evidence in support of the hypothesis that a heterogeneous distribution of lipids and proteins exists in the inner mitochondrial membrane.

ACKNOWLEDGEMENTS

The expert technical assistance of Mrs. Mercedes Preciado is gratefully acknowledged. N. L.-M. and J. L. S. were recipients of scholarships from «Consejo Superior de Investigaciones Científicas» and from the Spanish Ministry of Education and Science respectively. This work was supported by a grant from the Spanish Ministry of Education and Science.

Resumen

La incubación de membranas internas mitocondriales en presencia de cisteína seguida de centrifugación diferencial, ha permitido separar tres subfracciones que difieren por su contenido en componentes de la cadena respiratoria. Estas subfracciones procederían de zonas de la membrana más o menos alteradas según el grado de insaturación de sus fosfolípidos por la reacción de peroxidación inducida por la cisteína.

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