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PHOSPHOLIPID AND FATTY ACID COMPOSITION OF OUTER AND INNER MEMBRANES OF PLANT MITOCHONDRIA

F. MOREAU, J. DUPONT and C. LANCE

Laboratoire de Biologie Végétale IV, Université de Paris VI, Paris (France)*

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SUMMARY

1. Outer and inner membranes were isolated from cauliflower bud (*Brassica oleracea* L., CV Botrytis) mitochondria in the presence of nupercaine to prevent phospholipid hydrolysis by an endogenous phospholipase A.

2. Outer membranes are characterized by a high content of phosphatidylinositol and, to a lesser extent, of phosphatidylglycerol. They contain only very small amounts of diphosphatidylglycerol, which is specifically localized in the inner membranes.

3. Fatty acids of outer membrane phospholipids are mostly saturated, in marked contrast to those of inner membranes, which are predominantly unsaturated. This fact could account for the difference of plasticity observed between the two mitochondrial membranes.

4. Compared to outer membranes, microsomes have a much higher content in phosphatidylcholine and phosphatidylethanolamine. The degree of saturation of their fatty acids is intermediate between those of the fatty acids in inner and outer mitochondrial membranes.

INTRODUCTION

The striking dissimilarities in their biochemical, mechanical and osmotic properties displayed by the inner and outer mitochondrial membranes should find their explanation in the lipoprotein composition of each membrane. In animal mitochondria preferential locations have already been assigned to such phospholipid molecules as diphosphatidylglycerol [1, 2] or phosphatidylinositol [3–5]. In plant mitochondria, however, though the phospholipid and fatty acid compositions are now well documented [6, 7], very little is known about the distribution of these components between the two mitochondrial membranes [8, 9]. As a matter of fact, separation of both membranes for a number of plant mitochondria has been achieved only recently [10–13]. In this paper, we report on the phospholipid and fatty acid composition of the inner and outer membranes of cauliflower bud mitochondria.

* Postal address: 12, rue Cuvier, 75005 Paris, France.

Particular attention has been paid to the possible interference of an endogenous phospholipase A that could eventually alter the membrane composition [14]. Moreover, in view of the close similarities of properties between the outer mitochondrial membrane and the microsomal fraction of the cell [13], an attempt has been made to discriminate between both types of membranes on the basis of their lipid composition. A preliminary account of this work has appeared [15].

MATERIALS AND METHODS

Preparation and assay of membrane fractions

The techniques for the preparation of mitochondria from cauliflower buds (*Brassica oleracea* L., CV Botrytis) and the procedures for obtaining outer and inner membrane fractions by osmotic disruption have been described in detail in a previous paper [13]. In this paper, three mitochondrial fractions are considered: a fraction called mitochondria, consisting of mitochondria purified on a sucrose density gradient according to the procedures described [13], an inner-membrane fraction, consisting of the inner membrane plus matrix, and finally pure outer membranes. In some cases, the inner-membrane fraction was submitted to an ultrasonic disintegration (6 times 15 s, Biosonik III) and pure inner membranes were sedimented (30 min, 90 000 $\times g$) with the matrix compartment remaining in the supernatant. For comparison, microsomes were also prepared and purified as already described [13].

The only modification to the procedure previously used [13] is the addition of nupercaine to all preparative media at a concentration of 150 μM. Following the results of Scarpa and Lindsay [14], nupercaine was used in this study to effectively prevent the hydrolysis of phospholipids by an endogenous phospholipase A known to be present in these mitochondria [6].

The purity of each membrane fraction was assayed by the determination of selected marker-enzyme activities: succinate: cytochrome *c* reductase and antimycin-sensitive NADH: cytochrome *c* reductase for the inner membranes, antimycin-insensitive NADH: cytochrome *c* reductase for the outer-membrane fraction and antimycin-insensitive NADPH: cytochrome *c* reductase for the microsomal fraction. These activities were measured as already described [13].

Proteins were determined by the method of Lowry et al. [16] using pure bovine serum albumin as a standard.

Lipid analysis

The membrane fractions were inactivated in boiling ethanol for 10 min according to Douce et al. [6]. Phospholipids were then extracted following Folch et al. [17], with the substitution of ethanol instead of methanol in order to avoid the formation of phosphatidylmethanol [6]. Fractionation of total phospholipids was achieved by one-dimensional thin-layer chromatography (silica gel G, Merck). A chloroform–methanol–water mixture (65 : 25 : 4, v/v/v) was used as the developing solvent. Identification of individual phospholipids was carried out by exposure to iodine vapours, using pure phospholipid samples as reference standards. Quantitative estimations of phospholipids were made by chemical determination of phosphorus [18].

Fatty acids were released from phospholipids by methanolysis [19] (methanol–sulfuric acid; 100 : 2.5, v/v) at 85 °C for 90 min. Methyl esters were extracted by light petroleum and gas-chromatographed at 180 °C on a polar phase of diethyleneglycol–succinate [19].

Reagents

Nupercaine was purchased from Ciba, Saint-Cloud, France, and other reagents were obtained from Sigma, St. Louis, Mo., U.S.A.

RESULTS

Purity and integrity of the membrane fractions

Table I shows that the succinate:cytochrome *c* reductase and antimycin-sensitive NADH:cytochrome *c* reductase activities are specifically localized in the inner-membrane fraction, whereas the antimycin-insensitive NADH:cytochrome *c* reductase activity is mostly concentrated in the outer-membrane fraction. The microsomes also contain an antimycin-insensitive NADH : cytochrome *c* reductase but are specifically characterized by the presence of an antimycin-insensitive NADPH: cytochrome *c* reductase activity. From the levels of both the antimycin-sensitive NADH:cytochrome *c* reductase and the NADPH:cytochrome *c* reductase activities, one can conclude that there is very little contamination of the outer-membrane fraction by the inner-membrane or the microsomal fractions (less than 5%).

The beneficial effect of the addition of nupercaine to the preparative media during the isolation of the membrane fractions is shown in Fig. 1. In the 2 h following the isolation of purified mitochondria, 15% of phosphatidylcholine, which represents 40–45% of the total phospholipid content in cauliflower mitochondria (cf. Table IV), is destroyed under the action of an endogenous phospholipase A, with the concomitant formation of lysophosphatidylcholine. The same remark also applies to

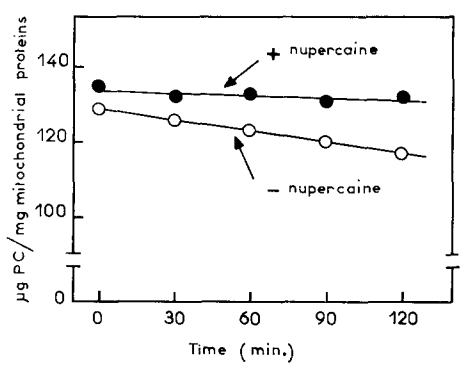

Fig. 1. Protective effect of nupercaine on the integrity of cauliflower mitochondrial membranes. Purified mitochondria were prepared with or without the presence of nupercaine (150 µM) and then kept at 4 °C for 2 h in the same medium. Hydrolysis of phosphatidylcholine (PC) was quantitatively followed by the direct determination of phosphorus after chromatography on silica gel. Note that during the time required for the preparation of purified mitochondria phosphatidylcholine has already undergone a 5% destruction in the absence of nupercaine.

TABLE I

DISTRIBUTION OF MARKER-ENZYME ACTIVITIES IN VARIOUS MEMBRANE FRACTIONS FROM CAULIFLOWER BUDS

Fraction	Specific activities (nmoles cytochrome *c* reduced/min per mg protein)			
	Succinate cytochrome *c* reductase	NADH:cytochrome *c* reductase (antimycin-sensitive)	NADH:cytochrome *c* reductase (antimycin-insensitive)	NADPH:cytochrome *c* reductase (antimycin-insensitive)
Mitochondria	360	1270	70	2
Inner membranes (plus matrix)	560	1300	25	2
Outer membranes	10	60	320	0
Microsomes	0	0	110	15

phosphatidylethanolamine, the second major phospholipid; but phosphatidylinositol and phosphatidylglycerol are strongly resistant to the action of phospholipase A, as already observed during the normal aging of these mitochondria [19]. In the presence of 150 μM nupercaine no phospholipid hydrolysis has occurred (Fig. 1) 2 h after the isolation of purified mitochondria, which is the time approximately required for the preparation of the outer- and the inner-membrane fractions.

Douce and Lance [19] have demonstrated that linolenic acid ($C_{18:3}$) is the major fatty acid released from mitochondrial phospholipids by phospholipase A during the normal aging of cauliflower mitochondria. This acid is specifically released from the 2-position in the glycerol moiety of the two major mitochondrial phospholipids [20]. The same conclusion can be drawn from the examination of the fatty acid composition of inner membranes (plus matrix) prepared in the absence or in the presence of nupercaine (Table II). In the absence of nupercaine one observes a specific decrease in the proportions of linolenic acid. On the other hand, palmitic acid, a saturated fatty acid (C_{16}), appears as the most resistant to the action of the endogenous phospholipase A.

TABLE II

PROTECTIVE EFFECT OF NUPERCAINE ON THE FATTY ACID COMPOSITION OF INNER MITOCHONDRIAL MEMBRANES

The figures give the percent fatty acid composition of inner membranes (plus matrix) isolated in the presence or in the absence of nupercaine. Fatty acids were estimated as indicated in Materials and Methods.

Fatty acid	Plus 150 μM nupercaine (percent)	Without nupercaine (percent)
C_{16}	10	16
C_{18}	1	1
$C_{18:1}$	7	9
$C_{18:2}$	13	13
$C_{18:3}$	69	61

These results show that nupercaine is an effective agent in preventing phospholipid hydrolysis during the isolation of the membrane fractions. Consequently, nupercaine was systematically added to all preparative media for all fractions.

Phospholipid distribution

As shown in Table IIIA, mitochondria and inner membranes have lower phospholipid contents than both the outer-membrane and the microsomal fractions. In addition the phospholipid content of the outer membranes is significantly higher than that of the microsomes. It should be pointed out, however, that both mitochondria and inner membranes include a matrix compartment which interferes with the quantitative determination of phospholipids in these fractions. To evaluate the amount of phospholipids in pure inner membranes cleared of their matrix compartment, the inner-membrane (plus matrix) fraction was disintegrated by sonication (cf. Materials and Methods). Under these conditions, it appears that the phospholipid content of

TABLE III

PHOSPHOLIPID COMPOSITION OF VARIOUS MEMBRANE FRACTIONS FROM CAULIFLOWER BUDS

Phospholipids were quantitatively determined as indicated under Materials and Methods. The phospholipid content of each membrane fraction was calculated assuming that the phosphorus content in most phospholipids is 4 %. Figures are mean values ± S.E. of the mean, from six experiments.

Fraction	μg phospholipid/mg protein	Percent phospholipid*
A. Mitochondria	356±5	26
Inner membranes (plus matrix)	409±21	29
Outer membranes	634±12	40
Microsomes	510±16	30
B. Inner membranes	473±19	32
Matrix	42±2	3

* $\frac{\text{mg phospholipid}}{\text{mg phospholipid} + \text{mg protein}} \cdot 100$

TABLE IV

PHOSPHOLIPID COMPOSITION OF MITOCHONDRIAL AND MICROSOMAL FRACTIONS FROM DIFFERENT SOURCES

—, not detected.

Fraction	Percent of total phospholipids				
	Cauliflower	White potato		Rat liver (ref. 5)	Guinea pig liver (ref. 3)
		(ref. 8)	(ref. 9)		
Mitochondria					
Phosphatidylcholine	44	44.3		40.5	40.0
Phosphatidylethanolamine	34	26.3		34.7	28.4
Diphosphatidylglycerol	11	16.8		14.8	22.5
Phosphatidylglycerol	4	—		—	2.3
Phosphatidylinositol	7	12.7		6.6	7.0
Inner membranes (plus matrix)					
Phosphatidylcholine	41	27.0	33	39.2	44.5
Phosphatidylethanolamine	37	29.0	33	39.9	27.7
Diphosphatidylglycerol	14	19.5	19	15.4	21.5
Phosphatidylglycerol	3	—	5	—	2.2
Phosphatidylinositol	5	24.5	7	3.6	4.2
Outer membranes					
Phosphatidylcholine	42	52.6	36	49.4	55.2
Phosphatidylethanolamine	24	25.0	64	34.9	25.3
Diphosphatidylglycerol	3	12.1	—	4.2	3.2
Phosphatidylglycerol	10	—	—	—	2.5
Phosphatidylinositol	21	10.3	—	9.2	13.5
Microsomes					
Phosphatidylcholine	50	44.3		58.7	62.8
Phosphatidylethanolamine	35	17.6		25.5	18.3
Diphosphatidylglycerol	1	19.0		1.6	0.5
Phosphatidylglycerol	8	—		—	1.1
Phosphatidylinositol	6	19.1		8.2	13.4

pure inner membranes is still markedly lower than that of the outer membranes (Table IIIB). The phospholipid content of the matrix is very low and probably results from a contamination by phospholipids released from the membranes by sonication.

Table IV gives the quantitative distribution of the individual phospholipids in these fractions. For comparison and discussion, a number of similar data from plant or animal mitochondria or microsomes have also been included [3, 5, 8, 9]. Phosphatidylcholine and phosphatidylethanolamine are the two major phospholipids of cauliflower mitochondria. Diphosphatidylglycerol represents 11% of the total. Phosphatidylglycerol and phosphatidylinositol are present only in small proportions. A similar distribution is found in the inner membranes (plus matrix), characterized, however, by a 25% increase in diphosphatidylglycerol and a similar decrease in phosphatidylinositol. Outer membranes, on the other hand, are characterized by a high proportion of both phosphatidylglycerol and phosphatidylinositol, but contain only very small amounts of diphosphatidylglycerol (3%). Since this value exceeds the value (0.7%) one can compute assuming a 5% contamination of this fraction by inner membranes (Table I), one cannot conclude that diphosphatidylglycerol is not present in outer membranes. The microsomes differ strikingly from the outer-membrane fraction by their phospholipid composition.

Fatty acid distribution

By far linolenic acid ($C_{18:3}$) is the major fatty acid present in both mitochondria and inner membranes (plus matrix) (Table V). Palmitic (C_{16}), oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids are present in the same proportions. Only traces of stearic acid (C_{18}) are found. In the outer-membrane fraction, palmitic acid is predominant, followed by oleic and linolenic acids. Microsomes have a composition somewhat intermediate between those of inner and outer membranes. Considering the ratio unsaturated/saturated fatty acids, inner membranes predominantly contain unsaturated acids whereas the reverse situation is observed in outer membranes. This is illustrated in Fig. 2 which shows the fatty acid composition of phosphatidylcholine, the major phospholipid of all these membrane fractions. It is clear from this figure

TABLE V

FATTY ACID COMPOSITION OF MITOCHONDRIAL AND MICROSOMAL FRACTIONS FROM CAULIFLOWER BUDS

Fatty acids were estimated as indicated in Materials and Methods.

Fatty acid	Percent of total fatty acids			
	Mitochondria	Inner membranes (plus matrix)	Outer membranes	Microsomes
$C_{16:0}$	15	10	50	26
$C_{18:0}$	3	1	4	2
$C_{18:1}$	10	7	20	11
$C_{18:2}$	12	13	8	10
$C_{18:3}$	60	69	18	51
Unsaturated/ saturated	4.5	8.1	0.8	2.6

that outer membranes are very different from inner membranes or microsomes in their fatty acid composition.

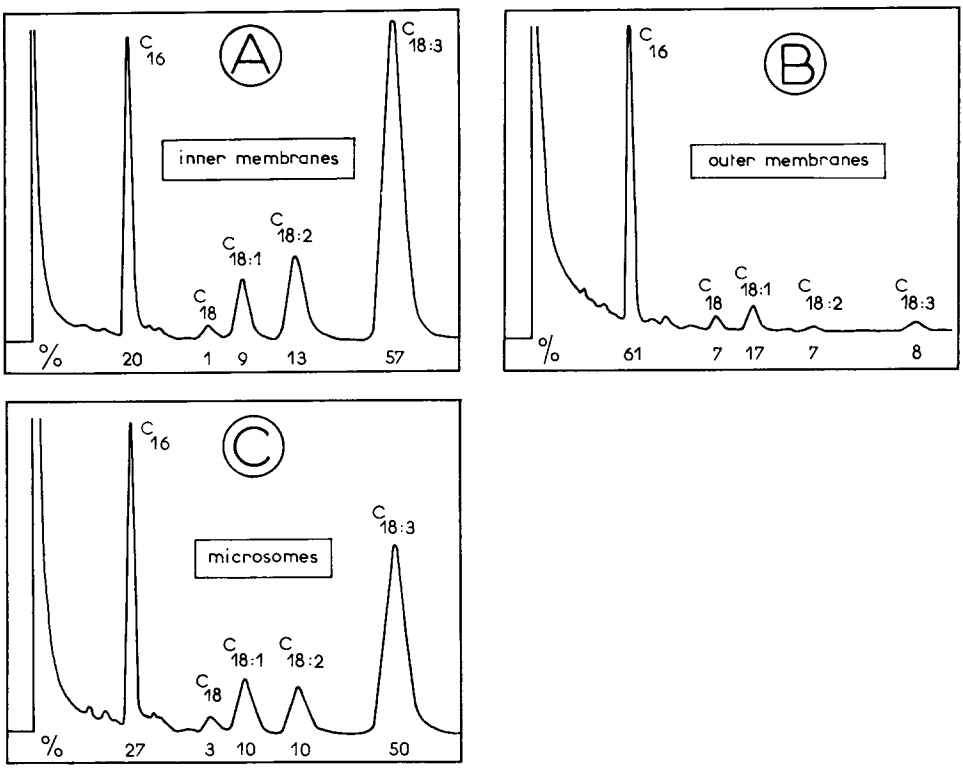

Fig. 2. Gas chromatograms of fatty acids from phosphatidylcholine in mitochondrial and microsomal fractions from cauliflower buds. Preparation of the membrane fractions and fatty acid analysis are indicated in Materials and Methods. The percent fatty acid composition is given on the chromatograms.

DISCUSSION

It has now become possible to obtain the separation of the two mitochondrial membranes through a variety of techniques [21]. Compared to the inner membrane with its numerous cristae, the outer membrane represents only a small fraction of the total mitochondrial membrane material (probably 10 to 15% in the case of cauliflower mitochondria [13]). This fact, together with the difficulty of isolation, makes it difficult to obtain outer membranes in appreciable amounts. For this reason little has been known of the biochemical composition and properties of the outer mitochondrial membrane until recently [21]. Present knowledge further indicates that the outer mitochondrial membrane shares a number of properties in common with the microsomal fraction of the cell [21].

In this work we have tried to extend to the lipid composition the characterization of specific properties that we have been able to demonstrate concerning particular

components or enzyme activities in the outer membrane of cauliflower mitochondria [13]. Our purpose was also to establish a clear discrimination between the biochemical characteristics of outer membranes and those of microsomes, which are the usual contaminants of all crude mitochondrial preparations.

Phospholipids are destroyed in preparations of plant mitochondria through the action of two phospholipases [19]: phospholipase D, associated with a light membrane fraction [22], which releases phosphatidic acid, and phospholipase A, associated with the mitochondrial membranes themselves, which produces lyso-derivatives mostly from phosphatidylcholine and phosphatidylethanolamine [23]. In order to overcome the actions of these phospholipases mitochondria were purified on sucrose density gradients to eliminate the phospholipase D present in lighter membrane fractions and nupercaine was used to inhibit phospholipase A. From the results presented here (Tables I and II, Fig. 1) it can be assumed that the mitochondria used in this study are reasonably clean on the grounds of both the purity of the fractions and the biochemical integrity of mitochondrial components.

It appears that the outer mitochondrial membrane has a higher absolute amount of phospholipids than the inner membrane. This fact, as will be discussed later, could be related to the difference in the degree of plasticity of both mitochondrial membranes. However, the main differences are found in the qualitative lipid composition of these membranes.

Conflicting reports are found in the literature over this point, particularly in the case of plant mitochondria (cf. Table IV). Phosphatidylcholine and phosphatidylethanolamine, as in most mitochondria, are the two major phospholipids of cauliflower mitochondrial membranes. Microsomes still have a higher proportion of these two compounds. Diphosphatidylglycerol is mostly found in inner membranes, probably in association with cytochrome oxidase in the respiratory chain [1]. Phosphatidylinositol seems to be rather specific to the outer membranes, as in animal mitochondria (Table IV). This phospholipid is known to be resistant to phospholipase A [19], so that the possibility exists that the relatively high content in phosphatidylinositol is due to the selective destruction of both phosphatidylcholine and phosphatidylethanolamine. In our experimental conditions, this objection is overcome by the presence of nupercaine in all media. In marked contrast to other reports [3, 5, 8, 9] the same appears to be true of phosphatidylglycerol too, because the presence of this phospholipid in outer-membrane fractions cannot be attributed to a contamination by plastids or proplastids. It is well-known that phosphatidylglycerol is a typical component of plastid membranes [6], but the same is true of galactolipids [9] (mono- and digalactosyl diglycerides) and we have never noticed the presence of galactolipids in our outer-membrane fractions.

As already observed from their fatty acid composition, inner membranes predominantly contain unsaturated fatty acids whereas outer membranes mostly contain saturated acids. This result is in accordance with similar data from animal mitochondria [5, 24], although some disagreement still exists [25]. Even if the possibility of an auto-oxidation of unsaturated fatty acids during the preparative procedures cannot be entirely ruled out, it is hard to conceive that auto-oxidation would have selectively affected unsaturated fatty acids in outer membranes only.

The differences in fatty acid content and in the degree of saturation of fatty acids could explain the difference in the mechanical properties of the two mitochon-

drial membranes. The plasticity of the mitochondrial inner membrane, which is able to undergo passive and active swelling and contraction, is related to its high content in unsaturated fatty acids. On the other hand, the relative rigidity of the outer mitochondrial membrane, which is evident on electron micrographs of osmotically contracted cauliflower mitochondria [26], can be associated with the saturated character of the fatty acids of this membrane fraction.

The results presented in this paper also establish a clear distinction between outer membranes and microsomes. Microsomes from cauliflower have a relatively higher content in phosphatidylcholine and phosphatidylethanolamine and a lower content in both phosphatidylglycerol and phosphatidylinositol than outer membranes. They also show a lower degree of saturation of their fatty acids. In this respect microsomal fatty acids show a degree of saturation that is intermediate between those of the two mitochondrial membranes.

The localization of phospholipid biosynthesis in biological membranes is still a matter of controversy [27, 28]. It is generally agreed that mitochondria are unable to synthesize their major phospholipids, phosphatidylcholine and phosphatidylethanolamine. These are probably produced in the endoplasmic reticulum and then transferred to the mitochondrial membranes. Mitochondria, however, could be able to synthesize their minor phospholipids [28, 29]. From this, it appears that mitochondrial phospholipids are the result of both an endogenous synthesis and exchange reactions between different cellular compartments [7, 30]. Even if we assume that the major phospholipids in cauliflower mitochondria originate from a common pool, they must be considered to undergo some local rearrangements in their fatty acid composition in order to explain the specificity of fatty acid distribution between the different membrane fractions.

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