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PHOSPHOLIPIDS AND POLYPEPTIDES IN THE OUTER MEMBRANE OF MAIZE MITOCHONDRIA

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Abstract—The outer membrane from mitochondria of etiolated maize epicotyls was isolated by a shrinkage–swelling–shrinkage procedure. The polypeptide pattern of the outer membrane revealed five porin isoforms (M_rs 33 000–34 000) in the p*I* region between 6.7 and 7.5. The phospholipid composition of the outer membrane was characterized by a very high phosphatidylcholine/phosphatidylethanolamine ratio, a significant amount of phosphatidylinositol and a low double bond index value. Analysis of molecular species by HPLC showed that 16:0/18:2 molecules of phosphatidylcholine and phosphatidylethanolamine were more specifically associated with the outer membrane, while 18:2/18:2 molecules were preferentially localized in the inner membrane. The results are discussed in relation to the biogenesis of mitochondrial lipids. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Mitochondria are surrounded by a double membrane (an outer and an inner membrane) containing a specific mix of lipids and protein required for membrane function [1]. As in animal mitochondria, the polypeptide pattern of the outer membrane of plant mitochondria is quite distinct from that of inner membrane [2]. The main protein components of the outer membrane are polypeptides of M_r of *ca* 30 000, which form large aqueous channels corresponding to porin, freely permeable to molecules smaller than 10 000 [3–5]. Recently, a Ca^{2+}-dependent protein kinase and multiple other phosphoproteins have also been reported in the outer membrane of mitochondria isolated from potato tubers [2]. Likewise, differences exist in lipid composition between the outer and inner membranes of plant mitochondria [6, 7]. The outer membrane is relatively enriched in phosphatidylcholine (PC) and phosphatidylinositol (PI), in contrast to the inner membrane, which is enriched in phosphatidylethanolamine (PE) and is the preferential site of diphosphatidylglycerol (DPG) [8–10]. Using both phospholipase A2 and chemical probes, Cheesbrough and Moore [11] have also shown asymmetric distribution of phosphoglycerides in mitochondrial membranes from castor bean endosperm; more PI was located on the outer leaflet of the outer membrane, while PE was concentrated in the outer leaflet of the inner membrane [7]. As in animals [12], higher saturation was found in phospholipids from the outer membrane than those in the inner membrane of plant mitochondria [8, 10, 13]. However, no data was available concerning the distribution of molecular species of phospholipids between the outer and inner membranes of plant mitochondria, especially in the case of PC and PE, the main components of mitochondrial membranes. Most mitochondrial phospholipids (PC, PE and PI) are synthesized in the endoplasmic reticulum and then imported into mitochondria *via* a mechanism which still remains unexplained [14]. In the present paper, the polypeptide pattern and the distribution of molecular species of these phospholipids have been investigated in the outer membranes from *Zea mays* mitochondria using two-dimensional electrophoresis and HPLC, respectively.

RESULTS AND DISCUSSION

Isolation and purity of outer mitochondrial membrane fractions

Outer membrane fractions were obtained from Percoll-purified mitochondria isolated from etiolated maize epicotyls by a successive shrinkage–swelling–shrinkage treatment. In a first step, mitochondria were contracted in hypertonic medium containing EDTA, in order to increase the susceptibility of the mitochondrial

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Table 1. Distribution of enzyme marker activities in inner and outer membranes from maize epicotyl mitochondria

Fractions	NADH-cytochrome *c* reductase		
	Ant-sensitive	Ant-insensitive	Ant-insensitive/ant-sensitive ratio
Mitochondria	463±29	293±97	0.63
Inner membrane (+matrix)	634±100	233±73	0.36
Outer membrane	77±29	621±158	8.06

Specific activities are given in nmol cytochrome *c* reduced mg protein^{-1} min^{-1}. Results are mean values with S.D. from four experiments. Ant, antimycin.

outer membrane to osmotic responses [5]. Then, rupture of outer membranes was achieved by quick immersion into a weakly hypotonic medium and subsequent shrinkage of mitoplasts (inner mitochondrial membrane plus matrix) by addition of a hypertonic solution containing $MgCl_2$. The presence of $MgCl_2$ makes the outer membrane less susceptible to osmotic changes and improves the separation of this membrane from the mitoplast fraction [5].

Based on the antimycin-insensitive NADPH-cytochrome *c* reductase activity [10], contamination of maize mitochondria purified on the Percoll gradient by microsomal membranes was extremely low (less than 5%). In addition, as estimated by the galactolipid content, contamination by proplastidial membranes did not exceed 10% of the total. On the other hand, comparison between the antimycin-sensitive NADH cytochrome *c* reductase activity (marker for inner membrane) and antimycin-insensitive NADH cytochrome *c* reductase activity (marker for outer membrane) in plant mitochondria [2, 10], showed that the purity of the outer membrane fractions isolated from maize epicotyl was higher than 90% (Table 1). The ratio between marker activities of the outer and inner membranes increased by *ca* 12-fold as compared with intact mitochondria. This result is consistent with recent data obtained by Pical *et al.* [2] on mitochondrial membranes isolated from potato tubers.

Polypeptide pattern

Two-dimensional-PAGE analysis of the polypeptide composition of maize mitochondria revealed a very complex pattern (Fig. 1). An average of 350–400 polypeptidic spots could be observed; several of these spots may represent different isoforms of the same protein. After isolation of the outer mitochondrial membrane, separation of the matrix compartment from mitoplasts and isolation of the inner membrane, electrophoretic analysis showed striking differences in the polypeptide composition of these fractions. However, it must be emphasized that the level of sensitivity is such that many of these spots may be due to contaminants arising principally from the inner membrane or to the trapping of newly synthesized polypeptides during their import through the membrane. However, some spots barely detectable in intact mitochondria, and strongly enriched in the pattern of outer membranes, could be attributed to specific polypeptides of these membranes (the most representative are indicated by arrows in Fig. 1). Among them, the most abundant polypeptides of the outer membrane, are represented by a group of five polypeptides numbered from 1 to 5 (Fig. 1) with an apparent M_r of 34 000 and apparent p*I*s of 6.8, 7.0, 7.2, 7.3 and 7.5, respectively. On the basis of molecular mass analogy with mitochondrial porins [3, 4] these polypeptidic groups, which make up about half of the outer membrane proteins, may be considered as corresponding to different porin isoforms. It should be noted that three different isoforms have already been characterized in rat liver mitochondria [15] and potato mitochondria [4], whereas the possible existence of porin isoforms has not been investigated in the outer membrane of corn mitochondria [3]. The present data indicate that the presence of porin isoforms is probably a common feature of both monocotyledons and dicotyledons.

Phospholipid distribution between mitochondrial membranes

As in other plant materials [7], the main phospholipids of mitochondrial membranes from maize (PC and PE) together represent 80–90% of the total phospholipids (Table 2). In addition, diphosphatidylglycerol (DPG) was specifically associated with the inner membrane (*ca* 10% of total content), whereas PI was preferentially localized in the outer membrane (*ca* 14 and 6% of total content in outer and inner membranes, respectively). Moreover, in agreement with previous data obtained for either potato [13], cauliflower [10] or mung bean epicotyl mitochondria [8], mitochondrial membranes from maize exhibit a PC/PE ratio which is two- or three-fold higher in the outer membrane than that in the inner membrane.

Fatty acid distribution in phosphatidylcholine and phosphatidylethanolamine from both mitochondrial membranes

The fatty acid composition of PC and PE in mitochondrial membranes of maize is reported in Table 3. Linoleic acid (18:2) was the major fatty acid of phospholipids from both membranes, with a maximum value in the inner membrane (81 and 88% of total

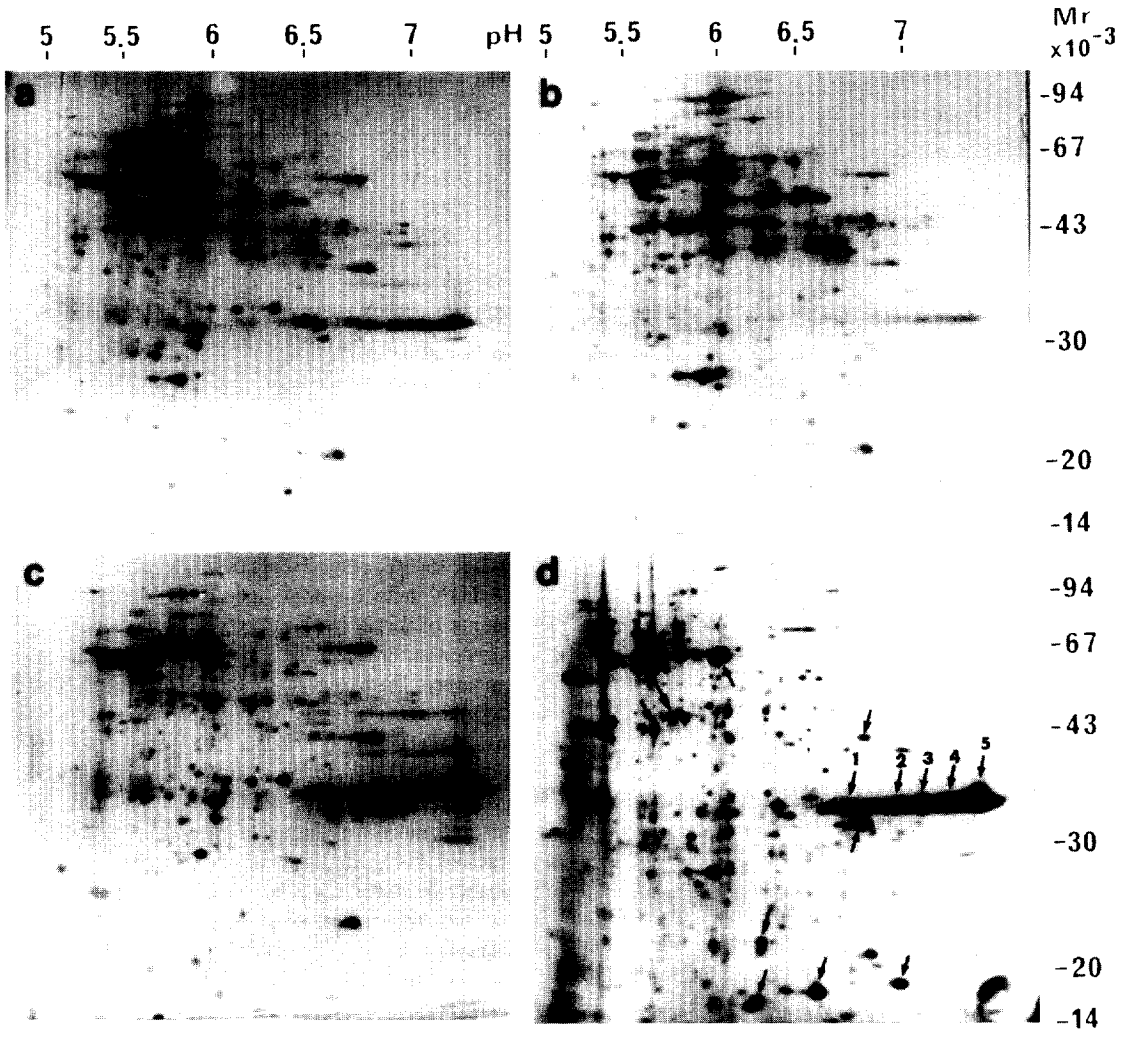

Fig. 1. Polypeptide composition of different compartments isolated from maize mitochondria: (a) intact purified mitochondria; (b) matrix compartment; (c) inner membrane; (d) outer membrane. Two-dimensional isoelectric focusing/SDS–PAGE followed by silver-staining.

content for PC and PE, respectively). In addition, both phospholipids (PC and PE) of the outer membrane were clearly enriched in palmitic acid (16:0). As a consequence, the double bond index (DBI) was significantly lowered in the outer membrane, especially in the case of PE, indicating that the outer membrane from maize was more saturated than the inner membrane, as has been reported for other plant materials [8, 10, 13].

Table 2. Comparison between phosphoglyceride content of inner and outer membranes of maize epicotyl mitochondria

	Phosphoglycerides (mol%)					
Fractions	DPG	PC	PE	PG	PI	PC/PE ratio
Mitochondria	8±0.4	49±2.5	34±1.7	3±0.2	6±0.2	1.4
Inner membrane (+matrix)	10±0.5	47±2.4	35±1.8	2±0.1	6±0.3	1.3
Outer membrane	t	61±4.1	22±2.1	3±0.3	14±1.0	2.8

DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; t = traces. Data are mean values with S.D. from four experiments.

Table 3. Fatty acid composition (mol%) of phosphatidylcholine and phosphatidylethanolamine in the inner (IM) and outer (OM) membrane of maize epicotyl mitochondria

	Phosphatidylcholine		Phosphatidylethanolamine	
Fatty acids	IM	OM	IM	OM
16:0	12.9±0.52	31.0±1.58	9.2±0.46	27.8±1.40
18:0	0.5±0.03	1.4±0.07	0.2±0.05	nd
18:1	1.8±0.09	2.8±0.14	1.1±0.45	nd
18:2	81.1±3.24	62.5±2.50	87.6±4.40	72.2±3.32
18:3	3.7±0.19	2.3±0.10	1.9±0.90	nd
DBI*	3.5	2.7	3.6	1.4

16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; nd, not detected. Data are mean values with S.D. from four experiments.

*Unsaturation of lipids estimated from their double bound index (DBI).

Molecular species in phosphatidylcholine and phosphatidylethanolamine from both mitochondrial membranes

The molecular species of PC and PE present in mitochondrial membranes isolated from maize epicotyls are presented in Table 4. Three main molecular species were found in both outer and inner mitochondrial membranes, namely, the 18:2/18:2, 16:0/18:2 and linolenic acid (18:3)/18:2 combinations. However, differences occurred between these two mitochondrial membranes. The 18:2/18:2 molecular species of either PC or PE was more specifically associated with the inner membrane, whereas the outer membrane was somewhat enriched in 16:0/18:2 molecules. As a consequence, the ratio of molecular species of 18:2/18:2 to 16:0/18:2 molecules was markedly lower in the outer membrane than in the inner one, *viz.* 1.3 and 1.9 in the outer membrane and 5.2 and 6.6 in the inner membrane, for PC and PE, respectively (Table 4). In comparison, the same analysis performed on mitochondrial membranes isolated from potato tubers provided comparable results despite the relative higher content of 18:3/18:2 molecules found in this material (*ca* 13% of total content) (data not shown).

Data reported by Justin *et al.* [16] for microsomal membranes (essentially endoplasmic reticulum) isolated from different plant tissues show that the ratio of molecular species of 18:2/18:2 to 16:0/18:2 molecules ranged between either 0.8 and 1.3, for PC, or 0.5 and 1.3, for PE, respectively. By comparison it appears that both mitochondrial membranes exhibit higher amounts of 18:2/18:2 molecules of PC or PE and a lower content in 16:0/18:2 molecules than microsomal membranes. With regard to the biosynthesis of mitochondrial phospholipids, this result raises some questions. Indeed, mitochondria are devoid of CDP-cholinephosphotransferase activity and, consequently, transfer of PC from the endoplasmic reticulum to the outer and inner membrane of mitochondria have been suggested [14]. Similarly, the endoplasmic reticulum probably produces the majority of PE in the plant cell. However, the presence of a phosphatidylserine decarboxylase activity synthesizing PE from PS in mitochondria can tentatively be suggested, since this enzyme has been found in the inner mitochondrial membrane of mammalian cells and yeast [6]. Accordingly, the differences in PC/PE ratio could result from this additional pathway for PE biosynthesis being present only in the inner mitochondrial membrane. On the other hand, assuming that PC and PE found in mitochondria originate exclusively from the endoplasmic reticulum,

Table 4. Molecular species composition (mol%) of phosphatidylcholine and phosphatidylethanolamine from inner (IM) and outer (OM) membranes from maize epicotyl mitochondria

	Phosphatidylcholine		Phosphatidylethanolamine	
	IM	OM	IM	OM
18:3/18:3	nd	nd	nd	nd
18:2/18:3	4.2	4.0	4.7	4.0
18:2/18:2	79.0	50.0	77.5	58.3
16:0/18:3	1.8	2.7	1.8	2.0
18:1/18:3	nd	nd	0.5	nd
16:0/18:2	12.0	39.0	15.0	31.4
18:1/18:2	1.6	nd	0.5	1.6
18:0/18:3	0.1	0.3	nd	nd
18:0/18:2	0.6	2.0	nd	0.8
16:0/18:1	0.6	1.6	0	1.9
18:1/18:1	0.1	0.4	nd	nd
$\frac{18:2/18:2}{16:0/18:2}$ ratio	6.6	1.3	5.2	1.9

Data are mean values of two experiments; nd, not detected.

results reported in this paper suggest that specific molecular species of PC might be preferentially transferred to mitochondrial membranes and possibly more intensively to the outer than to the inner membrane. Alternatively, it can be speculated that the differences observed in the distribution of molecular species of PC and PE might result from acyltransferase activities displaying selectivity for acyl groups. Indeed, such enzymes have already been reported in plant mitochondria [17]. However, further studies are required in order to discriminate between these hypotheses.

EXPERIMENTAL

Plant material. Seeds of maize (*Z. mays* L., cv. Mona), a gift from Pioneer France-Maïs (France), were grown on moist vermiculite in the dark at 30°. Etiolated epicotyls were collected after 6 days.

Prepn of mitochondria. Crude mitochondria were isolated from etiolated maize epicotyls essentially as described in ref. [18]. Further purification was performed on a discontinuous Percoll gradient (consisting of 3, 13, 8 and 3 ml of 18, 23, 40 and 80% Percoll supplemented with 0.3 M mannitol and 10 mM MOPS, pH 7.4) [19]. After centrifugation at 39 000 *g* for 40 min, pure mitochondria were collected at the interface 23/40% Percoll. Percoll was removed by centrifugation at 7000 *g* for 20 min in wash medium without EDTA containing 0.3 M mannitol and 10 mM MOPS (pH 7.3).

Sepn and purification of mitochondrial inner and outer membranes. Purified mitochondria were osmotically disrupted using an improved procedure based on a swelling–shrinkage method [5, 10]. The concd (80–100 mg protein) purified mitochondria (in 1 ml of wash medium) were shrunk by adding 1 ml of 2 M sucrose and kept on ice for 1 hr. The final osmolarity was *ca* 1 Osm. Rupture of the outer membrane was achieved by quickly injecting with a syringe the shrunk mitochondria into 5 mM K-P_i buffer (pH 7.2) containing 2 mM EDTA; the final osmolarity was *ca* 25 mOsm. The membrane suspension was kept at 0–4° for 20 min under gentle stirring. Subsequent addition of 2 M sucrose and 5 mM $MgCl_2$ induced shrinkage of mitoplasts; the final osmolarity was 300 mOsm. After gentle stirring at 0–4° for 20 min, the mitoplast fr. was pelleted by centrifugation at 30 000 *g* for 35 min. The outer membrane fraction was collected by centrifugation of the mitoplast supernatant at 245 000 *g* for 60 min. Both mitoplast and outer membrane pellets were resuspended in 5 mM K-P_i buffer (pH 7.2).

Enzyme assays. Purity and integrity of mitochondria were controlled by measurement of cytochrome *c* oxidase activity (EC 1.9.3.1) [19]. Antimycin-insensitive NADH-cytochrome *c* reductase activity (outer membrane marker) and antimycin-sensitive NADH-cytochrome *c* reductase activity (inner membrane marker) were assayed as described in ref. [10].

Gel electrophoresis. Mitochondria and submitochondrial frs were stored at 20° in 80% (v/v) acetone before solubilization in a soln containing 9 M urea, 2% (w/v) CHAPS, 5% (v/v) 2-mercaptoethanol and 1.2% (w/v) ampholytes (Pharmacia) of p*I* range 5–8 and 0.4% (w/v) of p*I* range 3–10; 50 μl of solubilization soln were used for *ca* 1 mg of mitochondrial protein. Proteins were analysed by 2D isoelectric focusing/SDS–polyacrylamide gel electrophoresis (IEF/SDS–PAGE) as previously described [20]. Proteins were detected by silver-staining according to ref. [21]. Gel analyses were performed by computerized image analysis.

Lipid analysis. Lipids were extracted according to ref. [22]. A minimum of 4 mg protein was used to separate individual polar lipids either on silica gel plates (Merck) by 2D-TLC [23] or by HPLC on a column packed with silicic acid (7.8 × 300 mm Waters Millipore) [24]. HPLC elution was performed for 50 min with a linear gradient of two mixed solvents, running from 100% of soln A: iso-PrOH–hexane (4:3) to 100% of soln B: iso-PrOH–hexane–H_2O (8:6:1.5). The flow rate was 1.5 ml min^{-1} and lipids were detected at 205 nm. A Bondapak C18 column (3.9 × 300 mm) were used for separation of the molecular species of each class of lipids using MeOH–H_2O–MeCN (90.5:7:2.5) at a flow rate 1.5 ml min^{-1} [25]. Frs collected at the exit of the HPLC column were extracted according to ref. [26]. For quantification on a mass basis, an appropriate amount of an int. std (heptadecanoic acid) was added to the pooled eluate representing each peak. Fatty acid Me esters from lipid classes were prepd by *trans*-methylation [27] and analysed by GC at 210° with a fused-silica capillary column (50 m × 0.3 mm) coated with Carbowax 20 M.

Protein determination. Protein measurements were performed as described in ref. [28] using BSA as standard.

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