Occurrence of an acyl-CoA:1-acylglycerophosphorylcholine acyltransferase in plant mitochondria

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Abstract In the presence of oleoyl-CoA, purified and intact mitochondria from potato tuber formed phosphatidylcholine from labeled lysophosphatidylcholine. The labeled oleoyl moiety of the acyl-CoA was also incorporated in the absence of exogenous lysolipids, such incorporation being largely increased by the addition of exogenous lysophosphatidylcholine. In the presence of various other lysophospholipids, no synthesis of the corresponding phospholipids was observed, suggesting a high specificity of the acyltransferase towards the acyl acceptor. This enzyme was chiefly located in the outer membrane of mitochondria. These results indicate that any acylglycerophosphorylcholine transferred from the endoplasmic reticulum to mitochondria may be acylated to phosphatidylcholine.

Key words: Plant; Mitochondria; Lipid; Phosphatidylcholine; Lysophosphatidylcholine; Acyltransferase; 1-Acylglycerophosphocholine 0-acyl-transferase (EC 2.3-1.23)

1. Introduction

The lipid composition of mitochondria differs strongly from that of other cellular membranes. The major lipids in these organelles are phosphatidylcholine, phosphatidylethanolamine and cardiolipin, which account for about 80% of total phospholipids (for reviews, see [1,2]).

The origin of cardiolipin, a mitochondrially specific lipid, has recently been elucidated by Schlame et al. [3], and further studied by Frentzen and Griebau [4]. Plant mitochondria, like mitochondria from all eukaryotes, contain a cardiolipin synthase catalyzing the transfer of a phosphatidyl residue from CDP-DAG to PG. However, plant mitochondria are not able to synthesize phosphatidylcholine, the major phospholipid of their membranes. In plant cells, this lipid is exclusively synthesized in the endomembranous system, mainly the endoplasmic reticulum, and then transferred to organelles (mitochondria and plastids).

Various mechanisms of lipid transfer from a membrane compartment to another have been proposed. They involve either (1) transfer of lipids during direct contact of membranes, (2) membrane trafficking or vesicular flow and (3) phospholipid transfer proteins (for review, see [5]).

Recently, a new mechanism was proposed [6]: 1-acyl-*sn*-glycerophosphocholine, formed in the ER, partitions between ER, cytosol and plastids. This physical partition resulted in a net transfer of lyso-PC to chloroplasts which was further acylated to phosphatidylcholine by an acyl-CoA:1-acylglycerophosphorylcholine acyltransferase present in the envelope. This acyl-CoA:lyso-PC acyltransferase was very specific: even in the presence of other lysolipids, lyso-PC was the only acyl acceptor, in good agreement with the absence of phosphatidylethanolamine and phosphatidylserine in the chloroplast envelope [6].

To check whether a similar mechanism could be involved in the accumulation of PC in plant mitochondria, we tested for the presence of an acyl-CoA:lyso-PC acyltransferase within these organelles. We report the first demonstration that an acyl-CoA:1-acylglycerophosphorylcholine acyltransferase is present in plant mitochondria.

2. Materials and methods

2.1. Materials

Thin-layer chromatography plates were HPTLC silica gel 60 plates (Merck 60 F 254). Autoradiography was performed using Hyperfilms MP (Amersham). 1-[1-^{14}C]Palmitoylglycerophosphocholine (57 Ci/mol) and [1-^{14}C]oleoyl-CoA (58 Ci/mol) were purchased from NEN (Dupont de Nemours). All other reagents were from Sigma Chemical Co.

2.2. Isolation of mitochondria

'Washed' mitochondria were isolated from potato tubers according to Douce et al. [7], and were purified as follows (adapted from [8]): washed mitochondria were layered onto a discontinuous Percoll gradient (20 ml of 15% Percoll and 5 ml of 30% Percoll) and after centrifugation at 40 000 × *g* for 45 min (rotor SS 34, Sorvall), mitochondria were collected at the 15%/30% interface and resuspended in buffer A (0.3 M sucrose, 1 mM EDTA, 0.1% BSA (w/v), 3 mM 2-mercaptoethanol, 20 mM phosphate buffer, pH 7.4). The Percoll was removed by two centrifugations at 12 000 × *g* for 15 min and the mitochondrial pellet was resuspended in buffer A.

2.3. Isolation of submitochondrial fractions

As noted above, intact and purified mitochondria were routinely obtained using a method adapted from that in [8]. For further isola-

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Abbreviations: CDP-DAG, cytidine 5′-diphospho-*sn*-glycerol-1,2-diacylglycerol; ER, endoplasmic reticulum; HPTLC, high-performance thin-layer chromatography; Lyso-PC, 1-acyl-*sn*-glycerophosphocholine; Lyso-PE, 1-acyl-*sn*-glycerophosphoethanolamine; Lyso-PG, 1-acyl-*sn*-glycerophosphoglycerol; Lyso-PI, 1-acyl-*sn*-glycerophosphoinositol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phos-phatidylinositol.

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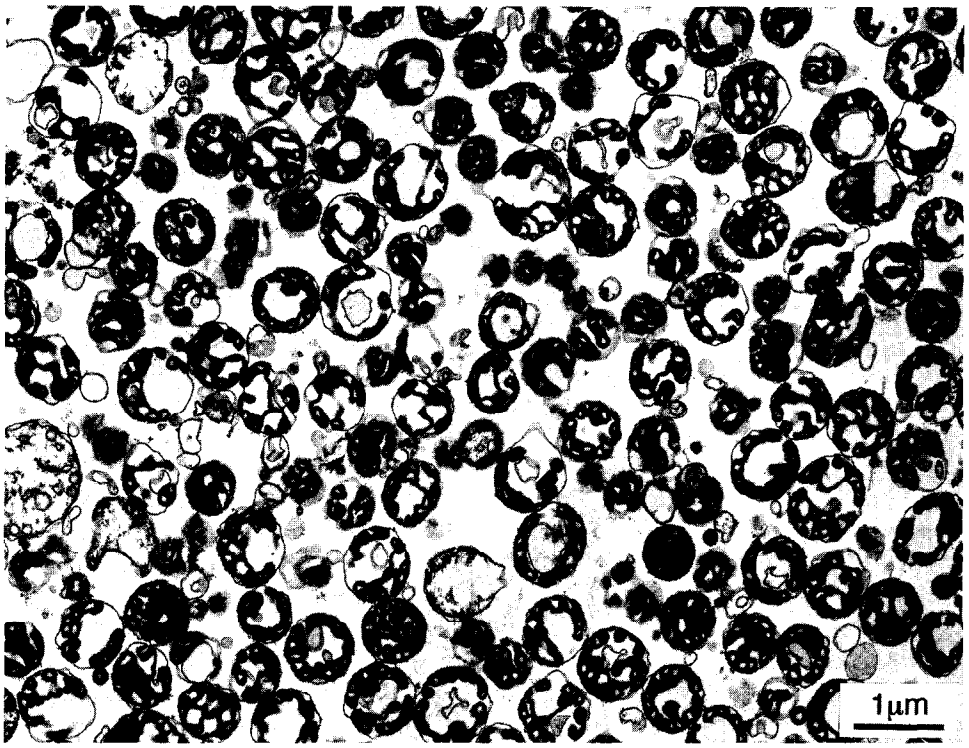

Fig. 1. Mitochondrial fraction of purified potato tubers. ×14000; other conditions as in Section 2.

tion of submitochondrial fractions, we used a slight modification of the method of [9]. Purification was performed on a discontinuous Percoll gradient (20, 23, 40 and 80% Percoll) supplemented with 0.3 M mannitol and 10 mM MOPS, pH 7.4. After centrifugation at 39000×*g* for 40 min (rotor SW 25.1 Ti, Beckman), mitochondria were collected at the 23%/40% Percoll interface. The Percoll was removed by centrifugation at 7000×*g* for 20 min in a wash medium (0.3 M mannitol and 10 mM MOPS, pH 7.3). Purified mitochondria were osmotically disrupted using an improved procedure based on a swelling-shrinkage method as described [10]. The concentrated (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 h. The final osmolarity was about 1000 mOsm. Rupture of the outer membrane was achieved by quickly injecting the shrunk mitochondria with a syringe into 5 mM phosphate buffer containing 2 mM EDTA. The final osmolarity was 20 mOsm. The membrane suspension was kept at 4°C for 20 min under gentle stirring. Shrinkage of mitoplasts was achieved after subsequent addition of 2 M sucrose and 5 mM $MgCl_2$. The final osmolarity was 300 mOsm. After 20 min the mitoplast fraction was recovered by centrifugation at 30000×*g* for 35 min. The outer membrane fraction was collected by centrifugation of the mitoplast supernatant at 245000×*g* for 60 min.

Antimycin-insensitive NADH-cytochrome *c* reductase activity (outer membrane marker) and antimycin-sensitive NADH-cytochrome *c* reductase activity (inner membrane marker) were measured according to Moreau and Lance [10].

2.4. Electron microscopy

Pellets were fixed for 2 h in 2.5% glutaraldehyde Na-cacodylate (0.1 M) pH 7.5, washed in the same buffer and post-fixed in OsO_4 1%/Na-cacodylate (0.1 M) pH 7.5 at 4°C, then washed in distilled water. To enhance membrane contrast, they were stained en bloc in 1% uranyl acetate in distilled water, then dehydrated and embedded in Epon. Thin sections were stained and examined in a CM-10 Philips electron microscope.

2.5. Lipid analysis

2 ml of $CHCl_3/CH_3OH$ (2:1) were added to intact and purified mitochondria (50 µg of protein). The volume of the aqueous phase was made up to 500 µl and, after vortexing, tubes were heated to 60–70°C for 30 min. The organic phase was isolated and the aqueous phase was washed with $CHCl_3$ (2 ml). Lipid extracts were evaporated to dryness and resuspended in 50 µl $CHCl_3/CH_3OH$ (2:1). Polar lipids were resolved by one-dimensional TLC using the solvent system described by Vitiello and Zanetta [11]. Lipids were visualized as described in [12] and identified using pure phospholipid samples as standards and quantitated by densitometry using a Camag 76510 photodensitometer. Lipids were also subjected to two-dimensional TLC, using $CHCl_3/CH_3OH/H_2O$ (65:25:4) as second solvent system.

2.6. Acyltransferase assays

Intact mitochondria or submitochondrial fractions were incubated with various amounts of substrates as indicated in the table and figure legends. Incubations were routinely carried out for 30 min at 30°C, in 100 µl of buffer A. After incubation, 50-µl mixtures were placed immediately in 2 ml $CHCl_3/CH_3OH$ (2:1) in order to stop the metabolism. The lipids were extracted and separated by TLC as described above. Individual lipids were scraped from the plates and their radioactivity was determined by liquid scintillation counting.

Table 1
Glycerolipid composition of potato tuber mitochondria

	This study	[1]
Phosphatidylcholine	39.7, 37.3	33–44
Phosphatidylethanolamine	30, 29.2	26–33
Cardiolipin[c]	14.3, 15.3	17–19
Phosphatidylinositol	6.6, 6.7	7–13
Phosphatidylglycerol[c]	5, 5	5
Digalactosyldiacylglycerol	2.3, 1.8	
Monogalactosyldiacylglycerol	nd, nd	
Lysophospholipids[a]	tr, tr	3
CDP-DAG 'like'[b]	2.3, 4.2	

Results obtained from two separate analyses are shown. They are expressed as percent of total glycerolipids. nd, not detected; tr, traces.
[a]Exclusively lysophosphatidylcholine in this study.
[b]Lipid migrating with the same R_f as CDP-diacylglycerol.
[c]The sum of cardiolipin and PG was 19.3 and 20.3. After analysis by two-dimensional TLC, PG was estimated to be 5%.

2.7. Protein determination

The protein concentration in mitochondrial fractions was determined according to Bradford [13], using BSA (1 mg/ml) as the standard.

3. Results and discussion

3.1. Purity, integrity and phospholipid content of mitochondria

Fig. 1 shows a thin section of the mitochondrial pellet isolated as indicated in Section 2. Typical mitochondria were mainly in the condensed configuration and little, if any, contamination was observed. In addition, mitochondria exhibited intact membranes and a dense matrix.

In good agreement with these results, the phospholipid content of the fraction under study (Table 1) was clearly similar to that already reported for plant mitochondria ([1] for review): the major phospholipids were phosphatidylcholine, phosphatidylethanolamine and cardiolipin (about 80% of total phospholipids). Importantly, no monogalactosyldiacylglycerol and only a few percent of digalactosyldiacylglycerol were detected, clearly demonstrating that the mitochondria were not contaminated by amyloplast envelope membranes. In addition, a lipid migrating like CDP-DAG was detected. This lipid represented 2–3% of the total lipids from the mitochondria. Only trace amounts of lysophosphatidylcholine were detected, in marked contrast with the case of plant ER reported (see [6] for discussion).

3.2. Synthesis of phosphatidylcholine from labeled lysophosphatidylcholine by purified mitochondria from potato tubers and specificity of the acyl-CoA:lyso-PC acyltransferase

We first studied the ability of potato tuber mitochondria to synthesize PC from labeled lyso-PC and unlabeled oleoyl-CoA as a function of pH, time and amount of protein. Between pH 6.5 and 7.5 the activity did not significantly vary. As a function of time, the formation of PC from 0.3 nmol of labeled lyso-PC and 5 nmol of oleoyl-CoA increased linearly for 10 min and reached 1.3 nmol/mg in 30 min (Fig. 2, inset). This value is representative of the activities found under the same conditions using mitochondria from different preparations (1.3 ± 0.5 nmol/mg per 30 min, $n = 5$).

The increase in activity was not linear as a function of the amount of protein (Fig. 2), and the specific activity of the enzyme under study slightly decreased when the protein amount increased. This phenomenon, already described for membrane-bound enzymes using amphiphilic substrates [14,15], could reflect the decrease in 'density' of the substrates in membranes [15].

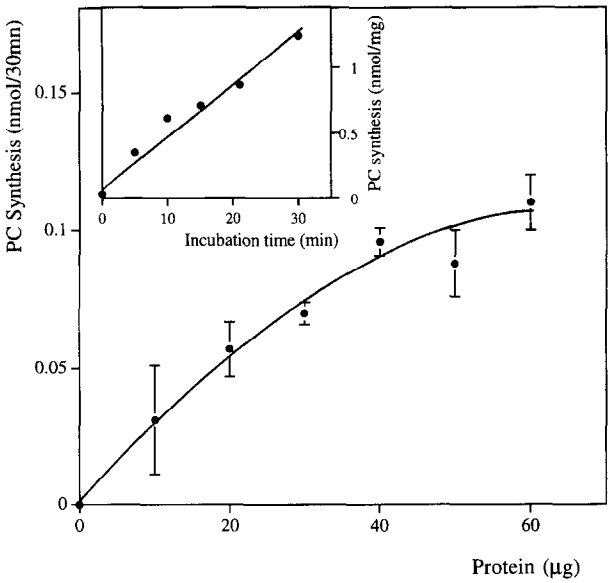

Fig. 2. Acyltransferase activity as a function of time and of protein amount. Activity was determined by incubating various amount of mitochondrial proteins at 30°C (final volume 100 µl) with oleoyl-CoA (5 nmol) and labeled lysophosphatidylcholine (0.3 nmol) for 30 min. The bars represent the mean values ± S.D. of three analyses carried out using different batches of mitochondria obtained in different periods. (Inset) Activity was determined by incubating 50 µg of mitochondrial proteins at 30°C (final volume 100 µl) with oleoyl-CoA (5 nmol) and labeled lysophosphatidylcholine (0.3 nmol) for various times. After incubation, the experimental mixture was placed immediately in 2 ml $CHCl_3/CH_3OH$ (2:1) in order to stop all metabolism and to start lipid extraction; other conditions as in Section 2.

The effect of the concentration of the acyl acceptor on activity was further studied with mitochondria incubated for 10 min with six different amounts of labeled lyso-PC (from 0.05 to 2 nmol; i.e. 0.5 to 20 µM). The results are given in Fig. 3: the maximum rate (V_m) was 0.125 nmol mg^{-1} min^{-1} and the affinity of the acyltransferase for lyso-PC was high (a K_m of 1.7 µM could be estimated from Fig. 3). A similar analysis was carried out by varying the oleoyl-CoA concentration, an affinity below 5 µM (corresponding to 0.5 nmol/assay) being determined.

The specificity of the acyl-CoA-lysolipid acyltransferase was further investigated. Purified intact mitochondria were incubated with labeled oleoyl-CoA for 30 min, in the absence or presence of various lysolipids (2 nmol).

In the absence of exogenous lysophospholipids, 50 µg of purified mitochondria incorporated 0.3 nmol/mg per 30 min of labeled oleoyl moiety of oleoyl-CoA into PC. This activity, corresponding to 0.015 nmol of PC synthesized/30 min per assay, could be due to the presence of endogenous lysophosphatidylcholine in the mitochondria. Under these conditions, neither PG, PE nor PS was labeled.

Various lysolipids (2 nmol per assay) were further added to the incubation mixture. When lyso-PC was added, incorporation of the oleoyl moiety of oleoyl-CoA into PC increased from 0.3 to 1.4 nmol/mg per 30 min but incorporation into other phospholipids was not stimulated. In contrast to the addition of lyso-PC, (Table 2), the addition of 2 nmol of lyso-PE, lyso-PG or lyso-PS resulted in a very small increase

Table 2
Effect of lysolipid addition on oleoyl incorporation

Lysolipid added	Increase in radioactivity incorporation (nmol/mg per 30 min) into:			
	PC	PE	PS	PG
Lyso-PC	1.1	0	0	0
Lyso-PE	0	0.11	0	0
Lyso-PS	0	0	tr	0
Lyso-PG	0	0	0	0.17

2 nmol of unlabeled lysophospholipid were incubated at 30°C for 30 min with 50 µg proteins from mitochondria and 5 nmol of labeled oleoyl-CoA (final volume: 100 µl). The values of oleoyl incorporation obtained in the absence of lyso-PC (0.28 ± 0.05 nmol/mg per 30 min into PC ($n = 4$) and around 0.03 nmol/mg per 30 min into other lipids) have been deduced. tr, traces.

Table 3
Sublocalization of lysophosphatidylcholine acyl-CoA acyltransferase activity

Fractions	NADH-cytochrome *c* reductase		Lysophosphatidylcholine:acyl-CoA acyltransferase (nmol/mg per 30 min)
	Antimycin-sensitive	Antimycin-insensitive	
Fraction 1	19 ± 16	197 ± 54	15.8
Fraction 2	618 ± 175	44 ± 32	2.2

Specific activities of NADH-cytochrome *c* reductase are given as nmol reduced cytochrome *c* min^{-1} (mg protein)$^{-1}$. Results are mean values ± S.D. from 6 experiments. For the sublocalization of lysophosphatidylcholine acyl-CoA acyltransferase activity, 10 μg proteins of fraction 1 or 2 were incubated for 30 min at 30°C with 5 nmol oleoyl-CoA and 0.3 nmol labeled lysophosphatidylcholine. Other experimental conditions as described in Section 2.

in incorporation (below 0.2 nmol/mg per 30 min) of the acyl moiety of oleoyl-CoA in the corresponding phospholipid. In similar experiments, lyso-PI or glycerophosphorylcholine were used and no labeled PI or lyso-PC was detected (data not shown). The differences in oleoyl incorporation when lyso-PC and other lysolipids were used are highly significant, since lyso-PC was used at saturating concentration. In other words, by using lower amounts of lysolipid per assay (0.5 nmol for example), the stimulation of oleoyl incorporation into PC by the addition of lyso-PC would be of the same order, whereas no stimulation by other lysolipids would be detected. Taken together, these results show that the acyl-CoA-lysolipid acyltransferase located in mitochondria from higher plants presents a high specificity for lyso-PC, in addition to a high affinity for this lysolipid.

Lyso-PC is probably the major lysolipid – if not the only one – observed in plant ER (see [6] for discussion), and as in plastids, is the only one significantly acylated in mitochondria. However, in contrast with the plastid envelope, plant mitochondria contain phospholipids other than PC-PE, for example. The origin of this latter lipid in mitochondria remains unknown. It would be of interest to investigate whether the presence of PE in these organelles results from the transfer of PS followed by decarboxylation in mitochondria, as described in yeast and animal cells [16–19].

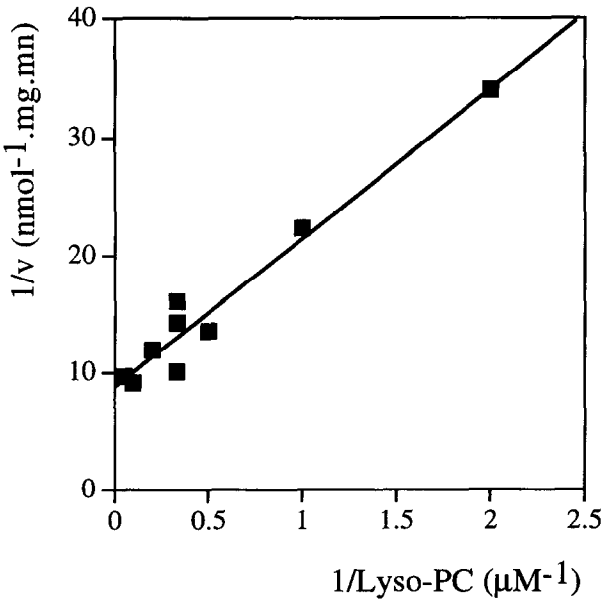

Fig. 3. Acyltransferase activity as a function of lyso-PC concentration. 50 μg of mitochondrial proteins were incubated for 10 min at 30°C (final volume 100 μl) in the presence of oleoyl-CoA (5 nmol) and various amounts of labeled lysophosphatidylcholine. At each incubation time, the experimental mixture was placed immediately in 2 ml $CHCl_3/CH_3OH$ (2:1) in order to stop all metabolism and to start lipid extraction; other conditions as in Section 2.

3.3. *Sublocalization of lysophosphatidylcholine:acyl-CoA acyltransferase*

To sublocalize the acyl-CoA:lyso-PC acyltransferase activity, purified mitochondria were osmotically disrupted using an improved procedure based on a swelling-shrinkage method as described [10], and two fractions were further isolated. Given the comparison between antimycin-sensitive NADH cytochrome *c* reductase activity (marker of the inner membrane) and antimycin-insensitive NADH cytochrome *c* reductase activity (marker of the outer membrane), it appears that fraction 2 is enriched in the inner membrane, whereas the specific activity of the outer membrane marker is around 5-times greater in fraction 1 than in fraction 2 (Table 3).

10 μg of proteins from each of these fractions were further used to measure their acyl-CoA lyso-PC acyltransferase activity. The results are given in Table 3: the specific activity of PC synthesis was much higher in fraction 1 than in fraction 2. Using 10 μg mitochondrial proteins from fraction 1, synthesis reached 15 nmol/mg per 30 min, and similar to the activity of the outer membrane marker, it was 7-fold higher than that found in fraction 2. Some PC synthesis was associated with fraction 2, probably because the outer membrane proteins remained associated with the mitoplasts.

The results shown in Table 3 strongly suggest that an acyl-CoA lyso-PC acyltransferase activity is associated with the outer membrane of plant mitochondria. Consequently, if any transfer of lyso-PC from endoplasmic reticulum to mitochondria occurs in plant cells, then PC synthesis in these organelles does not require the translocation of lyso-PC through the outer membrane and the intermembrane space.

In addition, the present data suggest that the activity of the acyltransferase allows synthesis of the mitochondrial PC within 10–15 h.

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