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Protein-mediated transfer of phosphatidylcholine from liposomes to spinach chloroplast envelope membranes

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We have demonstrated that an active transfer of phosphatidylcholine from liposomes towards spinach chloroplast envelope was catalyzed by a phospholipid-transfer protein purified from spinach leaves. The transfer is actually a complex process. During the first 10 min of the incubation, the exchange of phosphatidylcholine between liposomes and isolated envelope vesicles was predominant, as shown by the equilibration of phosphatidylcholine specific activity to the same level in both the liposomes and the envelope vesicles. Further incubation led to a 35% increase of the phosphatidylcholine content of envelope membranes, thus corresponding to a net transfer of phosphatidylcholine from liposomes towards envelope vesicles. After incubation of intact chloroplasts and liposomes in the presence of purified phospholipid-transfer protein, most of the radioactive phosphatidylcholine transferred to intact chloroplasts was recovered with the envelope membrane fraction. In addition, a mild phospholipase C treatment of intact chloroplasts after phosphatidylcholine transfer has demonstrated that all the radioactive phosphatidylcholine remained in the cytosolic leaflet of the outer envelope membrane and was not redistributed towards internal chloroplast membranes. Such a result, which mimics the in vivo situation, suggests that the phospholipid-transfer protein might be partly responsible (together with the apparent lack of transmembrane lipid diffusion) for the different lipid composition of the outer envelope membrane (when compared with the other plastid membranes) and for the asymmetrical distribution of phosphatidylcholine within this membrane.

Introduction

In contrast with all other plant cell membranes, plastid membranes contain only small amounts of phosphatidylcholine [1,2]. In addition, phosphatidylcholine is not uniformly distributed among plastid membranes: thylakoids and the inner envelope membrane contain only trace amounts of this phospholipid whereas most (if not all) of the chloroplast phosphatidylcholine is located in the outer envelope membrane [3,4], concentrated in the membrane leaflet facing the cytosol [5]. Furthermore, since isolated envelope membranes are apparently devoid of choline phosphotransferase [6], they are probably unable to synthesize phos-

Abbreviation: Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)-ethyl)glycine.

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phatidylcholine. This hypothesis is strengthened by the observations that: (a) chloroplast phosphatidylcholine has a typical 'eukaryotic' structure [7], (b) the envelope Kornberg-Pricer pathway [8], responsible for the synthesis of glycerolipids having a 'prokaryotic' structure (such as monogalactosyldiacylglycerol, phosphatidylglycerol or sulfolipid), is unable, up to now, to form glycerolipids having the 'eukaryotic' structure. In contrast, phosphatidylcholine synthesis has been demonstrated in several membrane systems, such as the endoplasmic reticulum [2] or Golgi vesicles [9]. Therefore, the simplest explanation for the unique distribution of phosphatidylcholine within chloroplast membranes is that this phospholipid is transferred from its site of synthesis (an extraplastidial membrane such as endoplasmic reticulum) to the outer envelope membrane. The discovery of phospholipid-transfer proteins in photosynthetic tissues provided evidence for a protein-mediated process [10–12]. Such a protein has been purified from spinach leaves [13] and from maize seedlings [14]. This protein is able to catalyze not only the transfer of phosphatidylcholine, but also that of phosphatidylinositol and phosphatidylethanolamine [12].

The purpose of this study was to analyze whether phosphatidylcholine could be transferred from liposomes to spinach chloroplast envelope membranes in the presence of a highly purified phospholipid-transfer protein isolated from spinach leaves.

Experimental procedures

Isolation of purified, intact spinach chloroplasts. Chloroplasts were isolated from 3–4 kg spinach leaves. Deveined leaf sections were cut into chilled medium (2.5 l/kg leaves) containing 330 mM mannitol, 15 mM tetrasodium pyrophosphate and 0.1% bovine serum albumin; pH was adjusted to 7.8 with HCl. A crude chloroplast pellet was obtained as described by Douce et al. [15] and purified further by isopycnic centrifugation in Percoll gradients [16]. The intact chloroplasts were then used either for phosphatidylcholine-transfer studies and/or for envelope membrane purification.

Purification of chloroplast envelope membranes. Total envelope membranes were prepared after lysis of intact chloroplasts in a hypotonic medium (10 mM Tricine-NaOH (pH 7.8)/4 mM $MgCl_2$) as described by Douce et al. [15]. Membrane fractions enriched in outer or inner envelope membranes were prepared after incubation of intact chloroplasts in a hypertonic medium (0.6 M mannitol/4 mM $MgCl_2$/10 mM Tricine-NaOH (pH 7.8)) and separation of both membranes with a Yeda press, as described by Block et al. [4]. All envelope membrane fractions were suspended in a minimal volume of medium containing 0.3 M sucrose and 10 mM Tricine-NaOH (pH 7.8).

Purification of phospholipid-transfer protein from spinach leaves. The phospholipid-transfer protein was purified 195-fold from 5 kg of mature spinach leaves by gel filtration and ion-exchange chromatography as described by Kader et al. [13]. The protein, exhibiting a unique band with Coomassie blue staining after SDS-polyacrylamide gel electrophoresis [13], was stored at 4 °C in a medium containing 10 mM sodium phosphate, 10% glycerol, 3 mM sodium azide and 8 mM 2-mercaptoethanol (pH 7.2) (buffer A) at a protein concentration of 50 μg/ml and used within a month.

Preparation of liposomes. [^{3}H]Phosphatidylcholine (486 Bq/mol) was prepared from potato slices incubated with methyl[^{3}H]choline chloride (2850 Bq/mol, Amersham) as previously described [13]. (^{3}H/^{14}C)-labelled liposomes were prepared by mixing 260 nmol of egg yolk phosphatidylcholine, 10 nmol of [^{3}H]phosphatidylcholine (28 000 dpm, prepared as described above) and 0.2 nmol of cholesteryl [1-^{14}C]oleate (25 000 dpm, 2110 Bq/mol, Amersham). The solvent was evaporated and 0.5 ml of buffer A (see above) was added. The suspension was then sonicated for 30 min at about 15 °C under nitrogen. In some experiments, liposomes containing [^{14}C]phosphatidylcholine were used. The radioactive phosphatidylcholine was prepared, as described [13], from potato slices incubated in presence of [1-^{14}C]acetate (1.89 Bq/mmol, CEA). The ^{14}C-labelled liposomes were prepared, as described above for ^{3}H/^{14}C-labelled liposomes, by mixing 260 nmol of egg yolk phosphatidylcholine and 8 nmol of [^{14}C]phosphatidylcholine (30 000 dpm) to a final volume of 0.5 ml in buffer A.

Transfer assays with isolated envelope membranes. The incubation mixture contained 0.5 ml of the liposomes suspension, various amounts of envelope membranes and phospholipid transfer protein (up to 100 μg) and buffer A to reach a total volume of 4 ml. Control experiments were performed in the absence of the phospholipid-transfer protein. Envelope membranes were then recovered after 10–30 min incubation at 30 °C by centrifugation through a 0.45 M sucrose cushion (30 min, 113000 × *g*, rotor SW 50, Beckman). The rapid cooling (an ice) of the mixture together with the loading on top of a precooled (4 °C) sucrose cushion were used to retard the transfer at the end of the incubation. However, it was not possible to stop completely the transfer between the end of the incubation and the separation of the membranes by centrifugation. The radioactivity in the pellet (containing envelope membranes) and in the supernatant (containing the liposomes, but also some small envelope fragments as contaminants) was determined, using an ACS scintillation mixture (Amersham). The percentage of ^{3}H and ^{14}C activity in each sample was calculated taking the initial activity in the liposomes as a reference. The transfer activity was determined as described previously [10,13] by calculating the percentage of ^{3}H from liposomes incorporated into the envelope fraction, corrected for the contamination (usually negligible) of the envelope pellet by liposomes. The level of contamination was estimated by determination of the ^{14}C radioactivity (due to cholesteryl [^{14}C]oleate, a non-transferable tracer) into the envelope pellets. The total amount (in nmol) of phosphatidylcholine transferred from liposomes to envelope membranes was then determined from the corrected percentage of [^{3}H]phosphatidylcholine recovered in the envelope pellets.

Since the level of contamination of envelope pellets by liposomes during centrifugation was extremely low, we also used ^{14}C-labelled liposomes for experiments needing an accurate determination of phosphatidylcholine specific activity. The experimental procedure was identical to that previously described, but the envelope and liposome fractions were extracted according to the method of Bligh and Dyer [17] for further analysis of phosphatidylcholine.

Transfer assays with intact chloroplasts. Isolated intact spinach chloroplasts (21 mg chlorophyll, about 320 mg protein) were incubated in buffer A in the presence of ^{14}C-labelled liposomes (3 ml) and 300 μg phospholipid transfer protein in a total volume of 30 ml. The incubation was done for 30 min at 20 °C, instead of 30 °C, to prevent chloroplast breakage during the experiment. Control experiments were done without phospholipid-transfer protein in the incubation medium. The experiment was stopped by centrifugation at 4 °C through a 40% Percoll cushion containing 0.3 M sucrose, as described by Joyard et al. [18]; this procedure also allows the selection of the chloroplasts which remained intact after the experiment. The chloroplasts were then resuspended in a small volume of suspension medium and separated into two parts. Half of the preparation was treated with phospholipase C (0.3 units/mg chlorophyll) for 3 min at 12 °C, as described by Dorne et al. [5]. Again, the chloroplasts were centrifuged through a Percoll cushion, as above, in order to stop the digestion by phospholipase C and select only intact chloroplasts. Total envelope membranes, stroma and thylakoids were then prepared from non-treated and phospholipase C-treated intact chloroplasts, as described by Dorne et al. [5] and extracted according to the method of Bligh and Dyer [17] for phosphatidylcholine preparation and analysis.

Analyses of phosphatidylcholine. After extraction of the different membrane fractions, the polar lipids were separated by two-dimensional thin-layer chromatography on silica-gel 60 plates (Merck), using chloroform/ methanol/ water (65 : 25 : 4, v/v) in the first development and chloroform/ acetone/ methanol/ acetic acid/ water (100 : 40 : 20 : 20 : 10, v/v) in the second development [5]. The areas corresponding to phosphatidylcholine were scraped into scintillation vials and either directly counted for radioactivity or transesterified for fatty acids analysis and quantitative determination.

Quantitative analyses of fatty acids were performed after transesterification in 4 ml of methanol/ sulfuric acid/ benzene (100 : 5 : 5, v/v). The vials were bubbled with argon and placed in an oven at 70 °C for 2 h [5]. Fatty acid methyl esters were extracted with hexane and chromato-

graphed with an Intersmat gas chromatograph (IGC 131) equipped with a hydrogen flame ionisation detector and with an Intersmat integrator as described by Dorne et al. [5].

Chlorophyll and protein determination. Chlorophyll concentration in chloroplast suspensions were measured according to the method of Bruinsma [19] in 80% acetone extracts. Proteins were determined according to the method of Lowry et al. [20] for membranes and according to the method of Bradford [21] for phospholipid-transfer protein (other methods seemed to overestimate the amount of enzyme).

Determination of radioactivity. Radioactivity in the different samples was measured after addition of ACS scintillation mixture (Amersham) using a liquid scintillation counter (SL 4000, Intertechnique).

The experiments presented in this article have been reproduced at least three times. The results presented in figures and tables are from representative experiments.

Results

Transfer of phosphatidylcholine towards chloroplast envelope membranes

After 30 min incubation of isolated envelope membranes in presence of ($^3H/^{14}C$)-labelled liposomes, ^3H label was recovered in the envelope pellet only when phospholipid-transfer protein was present in the incubation mixture. In addition, as shown in Fig. 1, we observed that the percentage of ^3H recovered in the envelope pellet was proportional (up to 20 μg protein) to the amount of phospholipid-transfer protein present in the mixture; a plateau (corresponding to about 30% of the ^3H label present in the liposomes) was reached when the incubation mixture contained about 25 μg phospholipid-transfer protein. A parallel decrease of the ^3H radioactivity in the liposomes fraction was observed (Fig. 1). On the contrary, very little ^{14}C label (up to 2%) was recovered in the envelope pellet, even with high amounts of phospholipid-transfer protein (Fig. 1). Since ^3H and ^{14}C label were due to phosphatidylcholine and cholesteryl oleate (a non-transferable tracer), respectively, these results demonstrate that during the experiment, almost no fusion and/or co-precipitation of liposomes with envelope vesicles occurred, whereas a protein-dependent phosphatidylcholine transfer (or exchange) between lipo-

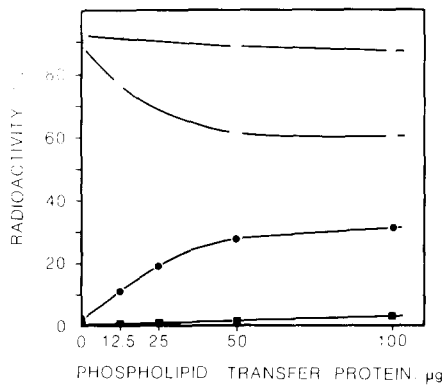

Fig. 1. Protein-dependence of phosphatidylcholine transfer from liposomes towards isolated envelope vesicles. $^3H/^{14}C$-labelled liposomes containing 270 nmol [^{3}H]phosphatidylcholine (28000 dpm) and 0.2 nmol cholesteryl [^{14}C]oleate (25000 dpm) were incubated together with envelope vesicles (corresponding to 370 μg proteins) in the presence of different amounts of phospholipid-transfer protein purified from spinach leaves. After incubation for 30 min at 30 °C, the liposomes and envelope vesicles were separated by centrifugation, as described in Experimental procedures. The ^3H radioactivity in the envelope pellet (●) and in the supernatant (○), the ^{14}C radioactivity in the envelope pellet (■) and in the supernatant (□) are expressed as % of the initial radioactivity of liposomes.

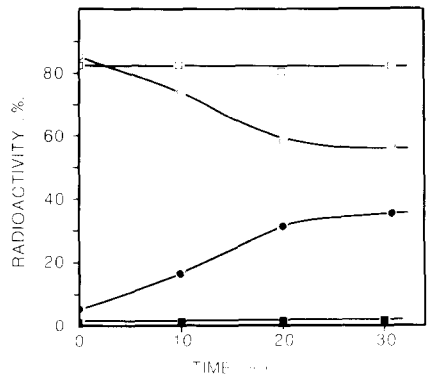

Fig. 2. Time-course transfer of phosphatidylcholine from liposomes towards chloroplast envelope vesicles. The experimental conditions were similar to those of Fig. 1, except that each assay contained 50 μg purified phospholipid-transfer protein and the incubation time was from 0 to 30 min. The ^3H radioactivity in the envelope pellet (●) and in the supernatant (○), the ^{14}C radioactivity in the envelope pellet (■) and in the supernatant (□) are expressed as % of the initial radioactivity of liposomes.

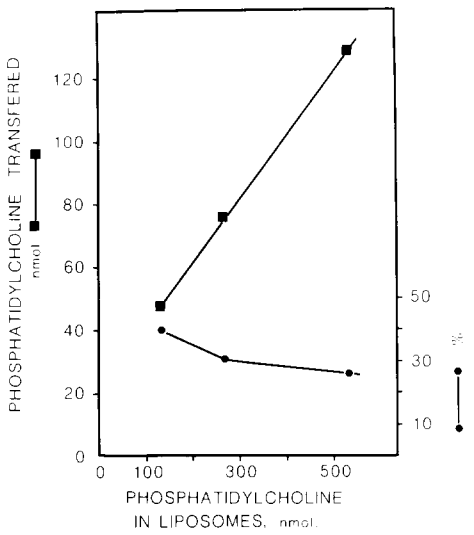

Fig. 3. Effect of the amount of liposomes on the transfer of phosphatidylcholine from liposomes towards chloroplast envelope vesicles. The experimental conditions were similar to those described in Fig. 1, except that the incubation mixture contained 135, 270 and 540 nmol of [^{3}H]phosphatidylcholine and 50 μg purified phospholipid-transfer protein from spinach leaves. Transfer is expressed as nmol of phosphatidylcholine (■) and as % of the radioactivity (●) present in liposomes at the beginning of the experiment. The values are corrected for the contamination of the envelope pellet by liposomes (as estimated by the amount of cholesteryl [^{14}C]oleate present in the pellet).

somes and envelope vesicles was obvious. The transfer (or exchange) of phosphatidylcholine was time dependent: after 30 min incubation, the percentage of [^{3}H]phosphatidylcholine recovered in the envelope pellet was maximal (Fig. 2). Similar results were obtained with a membrane fraction, prepared as described by Block et al. [4], and strongly enriched in outer envelope membrane (results not shown).

The total amount of [^{3}H]phosphatidylcholine recovered in the envelope pellets was shown to be strongly dependent on the amount of liposomes present in the incubation mixture, but the efficiency decreased slightly with large amounts of liposomes (Fig. 3). To analyse whether the efficiency of transfer was dependent upon the phosphatidylcholine content of envelope vesicles, we prepared envelope membranes from non-treated and phospholipase C-treated intact chloroplasts. As described by Dorne et al. [5], such a treatment led to the hydrolysis of almost all the envelope

TABLE I

EFFECT OF THE PHOSPHATIDYLCHOLINE CONTENT OF ENVELOPE MEMBRANES ON THE EFFICIENCY OF PHOSPHATIDYLCHOLINE TRANSFER FROM LIPOSOMES TOWARDS ENVELOPE VESICLES

The amount of phosphatidylcholine in envelope membranes was reduced by phospholipase C treatment of isolated intact chloroplasts according to the method of Dorne et al. [5]. Envelope membranes (corresponding to 350 μg protein), prepared from non-treated (control) or phospholipase C-treated intact chloroplasts were then incubated with ^{3}H/^{14}C-liposomes in the presence of purified phospholipid-transfer protein (100 μg), as described in Experimental procedures. Phosphatidylcholine transfer was expressed as % of the ^{3}H radioactivity in the envelope pellets and corrected for contamination by liposomes (estimated by the amount of cholesteryl [^{14}C]oleate present in the pellets).

Envelope membranes	Phosphatidylcholine content of envelope membranes (mg fatty acid/mg protein)	Phosphatidylcholine-transfer activity (% of liposome radioactivity)
From non-treated chloroplasts	0.285	32.1
From phospholipase C-treated chloroplasts	0.060	29.2

phosphatidylcholine. In good agreement with these data, we found that envelope membranes prepared from phospholipase C-treated chloroplasts contained only 0.06 mg phosphatidylcholine/mg protein, instead of 0.285 in envelope membranes from non-treated chloroplasts (Table I). These two preparations were then incubated with (^{3}H/^{14}C)-labelled liposomes. We found that, regardless of the phosphatidylcholine content of the envelope vesicles, almost the same amount of [^{3}H]phosphatidylcholine was recovered in each envelope pellet (Table I). Such a result suggests strongly, therefore, the possibility of a net transfer reaction.

To discriminate between a simple exchange process (between liposomes and envelope vesicles) and a net transfer (to envelope vesicles) of phosphatidylcholine, we have incubated envelope vesicles and liposomes containing [^{14}C]phosphatidylcholine in the presence of phospholipid-transfer protein. After extraction of the envelope (and liposome) lipids and purification of phosphatidylcholine by two-dimensional thin-layer

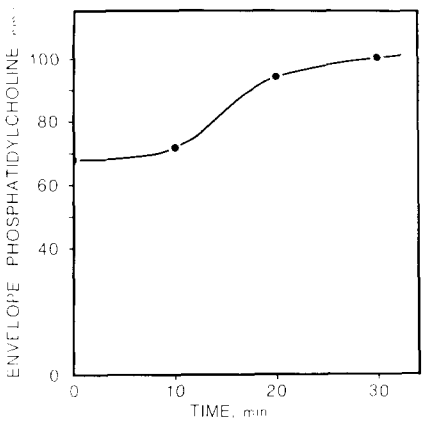

Fig. 4. Time-course evolution of the phosphatidylcholine content of envelope membranes during the protein-mediated transfer of phosphatidylcholine from liposomes towards chloroplast envelope vesicles. The experimental conditions for the incubation were identical to those of Fig. 2, except that [^{14}C]phosphatidylcholine was used. After incubation, the envelope membranes (370 μg) were extracted according to the method of Bligh and Dyer [17], and their total phosphatidylcholine content determined as described in Experimental procedures.

chromatography, we determined the amount of phosphatidylcholine present in each sample, together with its specific radioactivity. Fig. 4 demonstrates that the total amount of phosphatidylcholine in envelope vesicles (on a protein basis) increased by 35% after 30 min incubation in the presence of phospholipid-exchange protein, thus demonstrating a net transfer of phosphatidylcholine from liposomes to envelope vesicles. However, during the first 10 min of incubation, the envelope phosphatidylcholine content was stable (Fig. 4), although our previous experiments (see Fig. 2) have demonstrated that a significant percentage of the radioactivity was recovered with the envelope pellet, thus suggesting an exchange reaction between liposomes and envelope vesicles prior to the net transfer of phosphatidylcholine. This hypothesis was supported by analyses of the fatty acid composition of envelope phosphatidylcholine (Table II): fatty acids from potato phosphatidylcholine (which was used to make liposomes) have modified the fatty acid pattern of envelope phosphatidylcholine. Palmitic (16 : 0) and oleic (18 : 1) acids increased in envelope phosphatidylcholine during the incubation whereas the proportion of linolenic acid (18 : 3), which is only a minor component in potato tubers [10], decreased. The reverse observation was made in the liposome fraction. This result demonstrates that new phosphatidylcholine molecules from liposomes have been added to envelope membranes. The specific activity of phosphatidylcholine increased in envelope vesicles during the first 10 min of incubation, to reach a value of 700–800 dpm/μg fatty acid, i.e., half of the original value in the liposomes (1400 dpm/μg fatty acid). After 10 min incubation, no further increase of specific activity was measured (Table II). We believe that this corresponds to an equilibration of phosphatidylcholine specific activity between liposomes and envelope vesicles, due to the exchange reaction. This was indeed the case after 10 min incubation, as shown in Fig. 5.

Together, these experiments demonstrate a rather complex series of events. First, when incubated together in the presence of phospholipid-transfer protein, liposomes and envelope vesicles exchange their phosphatidylcholine. This reaction was no more visible when the specific activities of phosphatidylcholine in liposomes and envelope

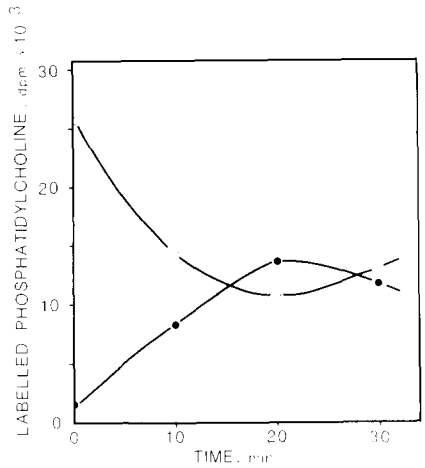

Fig. 5. Time-course evolution of the [^{3}H]phosphatidylcholine content of liposomes (○) and envelope vesicles (●) during the transfer of phosphatidylcholine from liposomes towards chloroplast envelope vesicles. The experimental conditions were similar to those of Fig. 2. The amounts of [^{3}H]phosphatidylcholine (in nmol) present (after incubation) in envelope vesicles and in the liposomes are almost identical, thus suggesting the equilibration of [^{3}H]phosphatidylcholine between both fractions.

TABLE II

EVOLUTION OF THE FATTY ACID COMPOSITION AND SPECIFIC ACTIVITY OF ENVELOPE PHOSPHATIDYLCHOLINE DURING THE INCUBATION OF ENVELOPE VESICLES WITH LIPOSOMES IN THE PRESENCE OF PURIFIED PHOSPHOLIPID-TRANSFER PROTEIN

Envelope membranes (corresponding to 350 μg proteins) were incubated with liposomes (containing [^{14}C]phosphatidylcholine) in the presence of purified phospholipid-transfer protein (50 μg) for 0–30 min, as described in Experimental procedures. The experimental conditions for phosphatidylcholine analyses are identical to those described in Fig. 4. The time indicated represents only the incubation time; however, the separation of envelope vesicles from liposomes is not an instantaneous procedure (see Experimental procedures). This is most important for time 0: in this experiment, the mixture, containing the envelope vesicles, the radioactive liposomes and the phospholipid-transfer protein, was directly layered on top of the precooled (4° C) Percoll cushion and centrifuged: therefore, some transfer was possible between the end of the incubation and the actual separation of the envelope vesicles from the liposomes. This explains why some radioactive phosphatidylcholine was recovered in the envelope pellet for time 0. We have controlled that this was not due to contamination by liposomes. In addition, although the separation procedure allows the preparation of envelope vesicles almost devoid of contamination from liposomes, this is not the case for the liposomes which are contaminated by small pieces of envelope vesicles, and therefore cannot be analyzed for their fatty acid content after incubation in the presence of envelope membranes.

Time (min)	Fraction	Specific activity (dpm/μg fatty acid)	Fatty acid composition (% total fatty acid)			
			16:0	18:1	18:2	18:3
	Liposomes	1400	35	33	27	4
0	Envelope	170	20	10	25	45
10	Envelope	770	25	16	26	33
20	Envelope	820	22	19	26	31
30	Envelope	720	24	21	25	29

vesicles were the same, i.e., after 10 min incubation. Then, the amount of phosphatidylcholine in envelope vesicles increased (on an envelope protein basis), due to a net transfer of this phospholipid to envelope vesicles (as shown in Fig. 4) catalyzed by the phospholipid-transfer protein.

Transfer of phosphatidylcholine towards intact chloroplasts

All the experiments described above were performed using isolated envelope membranes. A more physiological situation is the use of isolated intact chloroplasts, which allows transfer studies towards membranes that have kept their normal topology. When isolated intact chloroplasts are incubated together with liposomes in the presence of phospholipid-transfer protein, a significant part of the radioactive phosphatidylcholine was recovered in the chloroplast pellet, in good agreement with the results of Kader et al. [13]. To determine whether phosphatidylcholine remained in the envelope membranes or was transferred inside the chloroplast to thylakoids, we have fractionated intact chloroplasts after incubation into stroma, thylakoids and envelope membranes, according to the method of Douce et al. [15], and analyzed the distribution of the radioactivity due to [^{14}C]phosphatidylcholine. Obviously, the specific activity was much higher in the envelope fraction (50–100-times) than in the stroma or the thylakoids (Table III), suggesting that radioactive phosphatidylcholine was mostly located in the envelope membranes. However, since envelope membranes correspond to about 2% of the total chloroplast proteins, our data suggest that about 55–60% of the radioactivity was recovered with the envelope fraction whereas a significant part of the radioactivity was recovered in the stroma (10%) and thylakoid (30%) fractions. A possible explanation for such a result was an intraplastidial transfer of phosphatidylcholine towards thylakoids. However, this hypothesis was ruled out by the following experiment: after incubation with liposomes and phospholipid-transfer protein, the intact chloroplasts (selected by Percoll purification) were incubated in the presence of phospholipase C, as described by Dorne et al. [5], and (after a new Percoll purification) fractionated into stroma,

TABLE III

DISTRIBUTION OF [^{14}C]PHOSPHATIDYLCHOLINE WITHIN CHLOROPLASTS AFTER INCUBATION OF INTACT CHLOROPLASTS AND LIPOSOMES IN THE PRESENCE OF PURIFIED PHOSPHOLIPID-TRANSFER PROTEIN

Isolated intact chloroplasts (corresponding to 21 mg chlorophyll) were incubated with liposomes (containing [^{14}C]phosphatidylcholine, 712000 dpm) in the presence of purified phospholipid-transfer protein (3000 μg), as described in Experimental procedures. After purification of intact chloroplasts through a Percoll cushion, the chloroplasts were separated into two parts, to be incubated in presence or absence of phospholipase C, as described. After incubation and purification (through a Percoll cushion) of non-treated and phospholipase C-treated chloroplasts, both samples were fractionated into envelope, stroma and thylakoid fractions. The lipids in the different fractions were then extracted and phosphatidylcholine analyzed.

Chloroplast fractions	[^{14}C]Phosphatidylcholine (dpm/mg protein)	
	non-treated chloroplasts	phospholipase C-treated chloroplasts
Envelope	3600	0
Thylakoids	76	0
Stroma	30	21

thylakoids and envelope fraction. Surprisingly, analyses of radioactive phosphatidylcholine among the different chloroplast fractions demonstrated that no radioactivity could be detected in both the envelope and thylakoid fractions (Table III). This is in good agreement with the previous experiments of Dorne et al. [5] demonstrating that almost all the envelope phosphatidylcholine is concentrated in the cytosolic leaflet of the outer envelope membrane, since the phospholipase treatment was mild enough to preserve the integrity of both envelope membranes [5]. Therefore, all the radioactive phosphatidylcholine transported from liposomes to chloroplasts remained on the cytosolic side of the outer envelope (since it was totally accessible to phospholipase C) and was not transferred further from envelope membranes to thylakoids. The presence of about 30% of the chloroplast radioactive phosphatidylcholine in the thylakoid fraction from non-treated chloroplasts (Table II) only reflects the contamination of this fraction by (outer) envelope vesicles. This conclusion is in agreement with previous observations [1,5,16].

Discussion

The results presented above demonstrate that a protein-mediated transfer of phosphatidylcholine from liposomes to envelope membranes is possible, either by using isolated envelope vesicles or intact chloroplasts. In addition, our experiments provide a physiological significance for such a transfer.

Using isolated envelope vesicles, we have demonstrated that phosphatidylcholine transfer from liposomes is a complex process. During the first 10 min of incubation, an exchange of phosphatidylcholine between envelope and liposomes occurs, since (a) the specific activity and the total amount of radioactive phosphatidylcholine equilibrate between envelope and liposomes, (b) the fatty acid composition of the envelope phosphatidylcholine is modified, whereas little change in the total amount of phosphatidylcholine was observed. The ability of the purified phospholipid-transfer protein for phospholipid exchange is consistent with other experiments performed with plant mitochondria [22] or with animal phospholipid-transfer proteins [22–24]. However, a simple phospholipid exchange cannot be responsible for the formation of new membranes. Indeed, the observation that a net transfer of phosphatidylcholine to envelope vesicles was possible (Fig. 4) is most important. For instance, we have shown that phosphatidylcholine transfer is possible in envelope membranes almost devoid of endogenous phosphatidylcholine (i.e., after phospholipase C treatment of intact chloroplasts). In our normal experimental conditions (see Fig. 4), after 30 min incubation, 75 nmol of phosphatidylcholine from liposomes were added to envelope vesicles corresponding to 1 mg protein; this value represents about 35% of the envelope phosphatidylcholine present in the mixture (210 nmol). This is surprisingly high and has important physiological significance: the enzyme purified from spinach leaves can be responsible for the in vivo transfer of phosphatidylcholine from its site of synthesis to its site of accumulation. The concept of net trans-

fer was also proposed for animal phospholipid-transfer proteins [22–24]. For instance, non-specific proteins isolated from bovine liver, which are (to some extent) similar to the spinach protein, are able to increase the phosphatidylcholine content of microsomal membranes incubated with liposomes [22–24].

Obviously, the outer surface of the chloroplast is the structure involved in the interaction with the phospholipid exchange protein, as clearly demonstrated in the experiments with intact chloroplasts. Our experiments demonstrate that in the presence of purified phospholipid-transfer protein, phosphatidylcholine is transferred from liposomes to the outer surface of chloroplast outer envelope and is not redistributed to internal chloroplast membranes. This experiment suggest that phospholipid-transfer protein is probably partly responsible for the establishment of outer envelope membrane asymmetry; indeed, the addition of radioactive phosphatidylcholine to outer envelope membrane mimics a situation close to that found in vivo. All the other outer envelope membrane components are typical from prokaryotes such as cyanobacteria or *Prochloron* [25]. For instance, this membrane contains large amounts of galactolipids and sulfolipid [3,4] which are accessible to specific antibodies from the cytosolic side of chloroplasts [26–28]. Furthermore, the outer envelope membrane contains specific plastid components, such as carotenoids [4] and prenylquinones [29]. These findings support cytochemical analyses demonstrating that the outer envelope membrane shares with all the other plastid membranes characteristic components responsible for the specific staining of plastid membranes [30,31]. Interestingly, the polar lipid composition of cytoplasmic and outer membranes from cyanobacteria strongly resemble that of envelope membranes (for a review, see Ref. 25), thus giving some experimental support to the endosymbiotic theory of chloroplast origin. However, a major difference between cyanobacteria and chloroplasts is the lack of phosphatidylcholine in almost all cyanobacteria. Therefore, one can suggest that during the course of the integration of the prokaryote within the protoeukaryotic ancestor, one of the possible adaptations was the addition of phosphatidylcholine (a typical eukaryotic lipid) into the outer leaflet of the outer membrane of the prokaryote. In fact, cyanobacteria [32], like all Gram-negative bacteria [33], have a highly asymmetric outer membrane which contains much less lipids in the outer leaflet than in the inner one. Such a situation would highly favor integration of additional lipids in the outer leaflet. Therefore, it is possible that within the plant cell phospholipid-transfer proteins might be responsible for the addition of eukaryotic lipids, such as phosphatidylcholine, into the outer membrane of cellular organelles, thus explaining, together with the apparent lack of transmembrane lipid flip-flop, the asymmetry of the outer envelope membrane. Interestingly, the presence of high amounts of phosphatidylcholine in the outer membrane is not restricted to chloroplasts: the same is true for mitochondria [34] and peroxisomes [5], but it is not known whether these membranes have an asymmetric distribution of phosphatidylcholine. A question which remains to be elucidated is why in vivo only phosphatidylcholine, and not phosphatidylethanolamine (a major eukaryotic lipid in extraplastidial membranes), is transported to envelope membranes, since these membranes, like all plastid membranes, are almost devoid of phosphatidylethanolamine [1,2]. It is possible that different phospholipid-transfer proteins, specific for each phospholipid and/or organelle, could be present in the cytosol and responsible for such a difference, but this remains to be demonstrated. The situation is strikingly different for phosphatidylglycerol: in plastids, this phospholipid has a unique structure [35] typical for prokaryotic lipids, and is synthesized within chloroplasts [36] by enzymes concentrated in the inner envelope membrane [37].

Finally, our results provide additional evidence for the very low level (if not the lack) of phosphatidylcholine in thylakoids. This is in agreement with previous experiments by Dorne et al. [5]. Experiments with intact chloroplasts also suggest the lack of intrachloroplastic transfer of phosphatidylcholine from the outer leaflet of the outer envelope membrane to thylakoids. In fact, the chloroplast stroma is probably devoid of phospholipid-transfer activity [38], although Nishida and Yamada [39] have detected galactolipid-transfer activity in this fraction. Unfortunately, almost nothing is known about the different mechanisms

(involving transfer proteins or fusion of vesicles with growing thylakoids, etc.) which could be responsible for the transfer of plastidial components (such as glycerolipids) from the envelope membranes, where most of them are synthesized [1], to thylakoids, where the bulk of these compounds accumulate.

We are convinced that such experiments, combining the use of phospholipase C-treated chloroplasts together with phospholipid-transfer proteins, will provide a useful tool in manipulating the phosphatidylcholine composition of the outer envelope membrane. As discussed by Dorne et al. [5], such experiments, using liposomes containing well-characterized phosphatidylcholine molecular species, would allow studies of the postulated role of phosphatidylcholine in providing the C_{18}/C_{18} backbone [40] characteristic of the eukaryotic structure of plastid glycerolipids. Such studies are presently being carried out.

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