Localization of Phosphatidylcholine in Outer Envelope Membrane of Spinach Chloroplasts

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ABSTRACT We have examined the effects of phospholipase C from *Bacillus cereus* on the extent of phospholipid hydrolysis in envelope membrane vesicles and in intact chloroplasts. When isolated envelope vesicles were incubated in presence of phospholipase C, phosphatidylcholine and phosphatidylglycerol, but not phosphatidylinositol, were totally converted into diacylglycerol if they were available to the enzyme (i.e., when the vesicles were sonicated in presence of phospholipase C). These experiments demonstrate that phospholipase C can be used to probe the availability of phosphatidylcholine and phosphatidylglycerol in the cytosolic leaflet of the outer envelope membrane from spinach chloroplasts. When isolated, purified, intact chloroplasts were incubated with low amounts of phospholipase C (0.3 U/mg chlorophyll) under very mild conditions (12°C for 1 min), >80% of phosphatidylcholine molecules and almost none of phosphatidylglycerol molecules were hydrolyzed. Since we have also demonstrated, by using several different methods (phase-contrast and electron microscopy, immunochemical and electrophoretic analyses) that isolated spinach chloroplasts, and especially their outer envelope membrane, remained intact after mild treatment with phospholipase C, we can conclude that there is a marked asymmetric distribution of phospholipids across the outer envelope membrane of spinach chloroplasts. Phosphatidylcholine, the major polar lipid of the outer envelope membrane, is almost entirely accessible from the cytosolic side of the membrane and therefore is probably localized in the outer leaflet of the outer envelope bilayer. On the contrary, phosphatidylglycerol, the major polar lipid in the inner envelope membrane and the thylakoids, is probably not accessible to phospholipase C from the cytosol and therefore is probably localized mostly in the inner leaflet of the outer envelope membrane and in the other chloroplast membranes.

Chloroplast membranes, like all functional membrane systems in cells, are composed of proteins and polar lipids arranged largely in bilayer form (1). As proposed by Singer and Nicholson (2), the polar and ionic head groups of the lipid molecules together with all the ionic side chains of the amphipathic globular proteins (integral proteins) are on the exterior surfaces of the membranes. In contrast, the nonpolar side chains of the integral proteins are on the interior of the membrane, together with the hydrocarbon tails of the polar lipids.

A characteristic feature of all the plastid membranes analyzed so far is their polar lipid composition: They contain large amounts of glycolipids (galactolipids and sulfolipid) that are absent from the other cell membranes, whereas phospholipids—the major polar lipids in nonplastidial membranes—usually account for only a small part of the total plastid polar lipids. In addition, the lipids of the plastid membranes have a remarkably high degree of unsaturated acyl groups, particularly in galactolipids (for review, see reference 3). However, recent analyses of the two chloroplast envelope membranes from pea (4) and spinach (5) have led to the conclusion that large amounts of phosphatidylcholine (PC)[1] are present in the

[1] *Abbreviations used in this paper:* MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

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outer envelope membrane. Since it is now well established that biological membranes are structurally and functionally asymmetric, we decided to determine whether phospholipids, and especially PC, were distributed equally between both leaflets of the outer envelope membrane, or if their distribution was asymmetric.

A great variety of methods has been used to demonstrate the asymmetry of bilayer vesicles and cell membranes (6, 7). Phospholipases are the most widely used enzymes in assessing the distribution of polar lipids across chloroplast membranes (8). In this article, we examine the effects of phospholipase C (from *Bacillus cereus*) on the extent of phospholipid hydrolysis in envelope membrane vesicles and in intact chloroplasts. The results demonstrate a marked asymmetric distribution of PC and phosphatidylglycerol (PG) across the outer envelope membrane of spinach chloroplasts.

MATERIALS AND METHODS

Isolation of Purified Intact Spinach Chloroplasts: Chloroplasts were isolated and purified by isopycnic centrifugation in Percoll gradients, as described by Douce and Joyard (9). We then used the intact chloroplasts for envelope membrane purification and/or phospholipase C digestion experiments. The intactness of the phospholipase C–treated and untreated chloroplasts was determined by several methods. First, the ferricyanide-dependent O_2 evolution before and after an osmotic shock, under reduced irradiance (700 W/m^2), was assayed according to Lilley et al. (10). Second, the treated and untreated chloroplasts were observed by phase-contrast and electron microscopy. Third, the presence of outer envelope polypeptides in phospholipase C-treated chloroplast was controlled by immunochemical and electrophoretic analyses.

Purification of Envelope Membranes: Untreated and phospholipase C–treated chloroplasts were lysed in a hypotonic medium and the total envelope membranes purified from the lysate (swollen chloroplasts) by centrifugation through a step sucrose gradient as described by Douce and Joyard (9).

Phospholipase C Treatment of Purified Intact Chloroplasts and Isolated Envelope Membrane Vesicles: Phospholipase C (phosphatidylcholine/[glycerol] choline/[glycerol] phosphohydrolase, EC 3.1.4.3.) from *B. cereus* (grade 1, 4,000 U/ml) was purchased from Boehringer (Mannheim, Federal Republic of Germany). Intact and purified chloroplasts (final concentration, 1 mg chlorophyll/ml) were incubated at various temperatures in the following medium: 330 mM sorbitol, 10 mM tricine-NaOH (pH 7.8), phospholipase C (from 0.3 to 1 Boehringer U/ml). Control experiments were carried out under the same conditions except that no phospholipase C was added to the mixture. The digestion was terminated by the rapid cooling of the chloroplast suspension to almost 0°C; the incubation mixture was layered on top of a 40% Percoll cushion containing 330 mM sorbitol and 10 mM tricine-NaOH (pH 7.8). The tubes were centrifuged as described by Joyard et al. (11) in order to remove both the phospholipase C and the broken chloroplasts (which represent ~10% of the total chloroplasts) from the incubation mixture. We have carefully verified that no phospholipase C remained in the intact chloroplast pellet. In addition, phospholipase C is large enough (M_r 20,000 [12]) that penetration through outer envelope membrane appears unlikely. The treated intact chloroplasts were recovered as a pellet, and the envelope membranes were prepared as described above.

Isolated envelope membrane vesicles (1 mg protein/ml) were incubated at various temperatures in the following medium (suspension medium): 330 mM sorbitol, 10 mM tricine-NaOH (pH 7.8), phospholipase C (1 Boehringer U/mg protein). Control experiments were carried out under the same conditions except that no phospholipase C was added to the mixture. The digestion was terminated by addition of 4.5 ml of chloroform/methanol mixture (2:1 vol/vol) to 1.2 ml of incubation mixture. As a prerequisite, we verified that no phospholipid lysis by phospholipase C could occur in the chloroform/methanol mixture.

Immunochemical Studies of Intact Phospholipase C–treated Chloroplasts: Preparation and characterization of antibodies to envelope polypeptides E24 and E30 was previously described by Joyard et al. (11, 13). Antibodies raised against envelope polypeptides were used to probe the outer surface of phospholipase C–treated (0.3 Boehringer U phospholipase C/mg chlorophyll at 12°C for 3 min) and untreated chloroplasts as described by Joyard et al. (11).

Electrophoretic Analyses of Envelope Membrane Polypeptides: Envelope polypeptides prepared from phospholipase C–treated (0.3 Boehringer U/mg chlorophyll at 12°C for 3 min) and untreated chloroplasts were analyzed by SDS PAGE as previously described by Joyard et al. (13).

Lipid Analyses: Total envelope lipids were extracted according to Bligh and Dyer (14). We determined the lipid composition by using thin-layer chromatography to separate polar lipids and gas chromatography to analyze their fatty acids (15).

Electron Microscopy: Control and phospholipase C–treated intact chloroplasts were prepared for electron microscopy as described by Carde and Launay (16).

Chlorophyll and Protein Determination: We measured chlorophyll concentrations in 80% acetone extracts (17) and protein concentrations (18) using bovine serum albumin as a standard.

RESULTS

Phospholipase C Treatment of Isolated Envelope Membranes Vesicles

To demonstrate whether phospholipase C could be used for selective analyses of phospholipids exposed on the surface of the outer envelope membrane, we followed the degradation of PC and PG in isolated envelope membranes. The extent of hydrolysis of phospholipids is largely determined by the incubation temperature. Almost no hydrolysis of phospholipids occurred at temperatures <4°C, whereas rapid hydrolysis was observed at higher temperatures (results not shown). Table I clearly shows that only phospholipids, but not glycolipids, could be destroyed by phospholipase C treatment. In addition, the increase of the diacylglycerol content of the envelope membranes corresponded stoichiometrically to the decrease of their phospholipid content (Table I). This observation was confirmed by the analyses of the fatty acid composition of envelope diacylglycerol molecules (Table II). Before treatment, envelope diacylglycerol contained large amounts of 16:3 and 18:3 fatty acids. This composition reflected the origin of diacylglycerol molecules in the envelope, i.e., monogalactosyldiacylglycerol (MGDG), as a result of the functioning of the galactolipid/galactolipid galactosyltransferase (19; for review, see reference 20). The results obtained are in good agreement with our previous analyses of envelope lipids (20, 21). After phospholipase C treatment, the fatty acid composition of envelope diacylglycerol changed greatly: the proportions of 16:3 and 18:3 fatty acids decreased whereas the proportions of 16:1, 18:1, and 18:2 fatty acids increased (Table II), thus reflecting the phospholipid origin of the new diacylglycerol molecules. This relationship is clearly demonstrated in Fig. 1*A*; in addition, this figure also shows that during a time-course experiment, PC and PG molecules were not hydrolyzed to the same extent by phospholipase C. It is difficult, from our data, to discriminate between an enzyme specificity of the phospholipase for a given phospholipid and the availability of the different substrates (PC, PG, and phosphatidylinositol [PI]) to the phospholipase. In fact, the first hypothesis, enzyme specificity, is probably valid for PI since several studies have suggested that this phospholipid cannot be hydrolyzed by phospholipase C from *B. cereus* (22). However, the second hypothesis, substrate availability to the enzyme, cannot be excluded. Since phospholipase C has a molecular weight of ~20,000 (12), this enzyme probably cannot penetrate the membranes of isolated vesicles. To verify if such a hypothesis could explain the different behavior of PG and PC molecules during phospholipase C treatment, we sonicated envelope membrane vesicles in the presence of phospholipase C. In this case, phospholipase C would be

Table I

Lipid Composition of Envelope Membranes after Phospholipase C Treatment

	Control (a)		Phospholipase C treatment of					
			Isolated envelope (b)		Sonicated envelope (c)		Intact chloroplasts (d)	
	µg fatty acids/mg protein	%	*µg fatty acids/mg protein*	%	*µg fatty acids/mg protein*	%	*µg fatty acids/mg protein*	%
MGDG	186	15	186	15	175	14	182	16
DGDG	372	30	347	27	387	31	386	34
TDGD	43	3.5	42	3.5	42	3.5	51	4.5
TTGDG	19	1.5	13	1	19	1.5	17	1.5
SL	87	7	77	6	93	7.5	102	9
PC	248	20	29	2	6	0.5	40	3.5
PG	93	7.5	69	5.5	18	1.5	80	7
PI	31	2.5	25	3	31	2.5	23	2
DG	149	12	478	38	479	38	250	22
Total	1,228	99	1,266	100	1,250	100	1,131	99.5

We have compared the envelope lipid composition after phospholipase C treatment of isolated envelope vesicles (*b*), sonicated envelope vesicles (*c*), and intact chloroplasts (*d*), with the lipid composition of untreated envelope vesicles (*a*). In all of these experiments, the low level of MGDG, the presence of trigalactosyldiacylglycerol (*TGDG*) and tetragalactosyldiacylglycerol (*TTGDG*) among the envelope lipids are due to the functioning of a galactolipid/galactolipid galactosyltransferase (19, 20). This enzyme is located on the outer surface of the outer envelope membrane and can be destroyed by thermolysin treatment of intact chloroplasts (20). However, it is almost impossible to have a good yield of intact chloroplasts after a double incubation in presence of protease (thermolysin) and phospholipase C. Therefore, we used non-thermolysin-treated chloroplasts in all these experiments. Actually, we have clearly demonstrated that the galactolipid/galactolipid galactosyltransferase becomes active only after the osmotic shock, which is used to prepare the envelope membranes (20) and therefore does not interfere with the phospholipase C treatment of intact chloroplasts or of isolated envelopes during the short phospholipase C incubation (5 min). The incubation mixtures contained 1 Boehringer U phospholipase C/mg of envelope protein in experiments (*b* and *c*) and 0.3 Boehringer U phospholipase C/mg chlorophyll in the experiment (*d*). When the incubation was done with sonication, (*c*), the envelope vesicles were first mixed with the phospholipase C and then subjected to a 20-s sonication (Sonimass 250 T, set at 60 W). The given compositions are from 5-min incubations. The values are expressed as micrograms of fatty acids per milligram of envelope protein and as weight percent fatty acids for each given lipid. DGDG, digalactosyldiacylglycerol; SL, sulfolipid; DG, diacylglycerol.

Table II

Fatty Acid Composition of Envelope Diacylglycerol, MGDG, PC, and PG from Phospholipase C-treated and Untreated Envelope Vesicles or Chloroplasts

	16:0	16:1	16:3	18:0	18:1	18:2	18:3
Diacylglycerol from							
Untreated envelope control (*a*)	5	—	20	1	2	3	69
Treated envelope (*b*)	15	7	11	tr	6	15	45
Treated and sonicated envelope (*c*)	18	9	10	tr	8	15	40
Envelope of treated chloroplasts (*d*)	17	tr	11	tr	8	14	49
MGDG*	7	—	19	—	1	3	70
PC*	19	—	—	1	12	28	40
PG*	15	26	—	2	3	8	46

We have compared the fatty acid composition of envelope diacylglycerol after phospholipase C treatment of envelope vesicles or chloroplasts with that of envelope diacylglycerol, MGDG, PC, and PG from untreated envelope membranes (control). The incubation mixture contained 1 Boehringer U phospholipase C/mg envelope protein (samples *b* and *c*) or 0.3 Boehringer U/mg chlorophyll (sample *d*). Sonication, in sample *c*, was done as described in Table I. The given compositions are from 5-min incubations. The values are expressed as weight percent of total fatty acids in a given lipid. Note that 16:1 fatty acid, which is characteristic for chloroplast PG, is present in diacylglycerol from treated envelope vesicles (*b* and *c*). This indicates that PG can be degraded, when accessible, by phospholipase C from *B. cereus*. On the contrary, in envelope membranes from treated intact chloroplasts (*d*), diacylglycerol contains only traces of 16:1 fatty acid, which demonstrates that most of envelope PG molecules are not accessible to phospholipase C from the cytosolic side of intact chloroplasts. *tr*, traces.

* From untreated envelope (control).

present inside and outside the isolated envelope vesicles and all of the envelope PC and PG molecules would be available to hydrolysis. A time course of phospholipid decrease is shown in Fig. 1 *B*. After only 3 min, 90% of the PC and 60% of PG molecules were hydrolyzed; after 10 min no PC and only 10% of PG molecules remained in the envelope vesicles. However, as previously seen, phospholipase C did not show any detectable activity against PI. These results demonstrate that in the first experiment, which used envelope vesicles and phospholipase C without sonication, the availability of PC and PG to the enzyme strongly limited hydrolysis of both phospholipids (see also Table II). However, as shown in Fig. 1, the rate of hydrolysis is faster for PC than for PG. This difference could be because of a substrate specificity, the initial concentration of PC and PG in the membrane, or a limitation due, for instance, to an association between PG and some envelope membrane protein. In conclusion, for the experiments done with isolated envelope vesicles, it is obvious that PC and PG molecules can be totally converted (although at different velocity) into diacylglycerol by phospholipase C treatment of envelope membranes. Therefore, this enzyme can be used to probe the availability of PC and PG molecules in the cytosolic leaflet of the outer leaflet of the outer envelope membrane from spinach chloroplasts.

Phospholipase C Treatment of Intact Purified Spinach Chloroplasts

After treatment of intact chloroplasts with various concentrations of phospholipase C under mild conditions, we purified the chloroplasts on Percoll cushions (see above), isolated their envelope membranes, and analyzed the constituent polar

lipids. At low phospholipase C concentration (0.3 Boehringer U/mg chlorophyll), 55% of the total phospholipids of envelope membranes could be hydrolyzed; 85% of the PC molecules and almost none of the PG molecules were degraded (Fig. 2) without rupture of the chloroplasts. The loss of PC from phospholipase C–treated intact chloroplasts was extremely rapid (Fig. 3): ~90% of the total envelope PC was degraded within 1 min of phospholipase C treatment. In addition, we have shown that at all temperatures tested (up to 20°C) few envelope PG molecules were hydrolyzed whereas almost all envelope PC molecules were susceptible to phospholipase C degradation. Taken together, these findings provide compelling evidence that PC, the major phospholipid in the outer envelope membrane from spinach chloroplasts, is mainly in the cytosolic monolayer of the bilayer. It is clear, however, that most of the envelope PG molecules are not accessible to phospholipase C under our experimental conditions. Since most of these molecules were susceptible to hydrolysis in isolated envelope vesicles (see Fig. 1*B*), it is likely that few PG molecules are present in the cytosolic leaflet of the chloroplast outer envelope membrane.

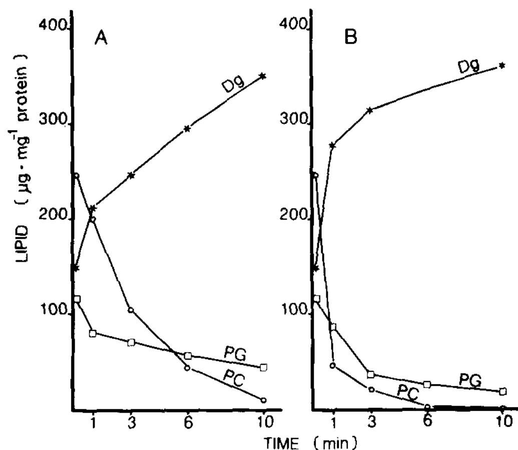

FIGURE 1 Kinetics of phospholipid hydrolysis during phospholipase C treatment of isolated envelope membrane vesicles. Envelope membranes were prepared from intact spinach chloroplasts (9). The phospholipase C concentration used was adjusted to 1 Boehringer U/mg envelope protein. (*A*) Kinetics of phospholipid hydrolysis without sonication and at room temperature; (*B*) kinetics of phospholipid hydrolysis after 20 s of sonication (Sonimass 250 T at 60 W; Ultrasons Annemasse, France) at room temperature. The reaction was stopped by addition of a chloroform-methanol mixture and the lipids were extracted and analyzed as described in *Lipid Analyses*. The values are expressed as micrograms of fatty acids in a given lipid per milligram of envelope protein.

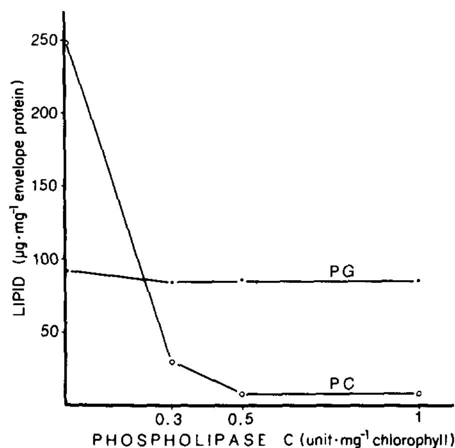

FIGURE 2 Phospholipid content of envelope membranes isolated from phospholipase C–treated intact chloroplasts. Isolated purified intact chloroplasts (1 mg chlorophyll/ml) were treated with different phospholipase C concentrations (0 to 1 Boehringer U/ml) during 5 min at 12°C. After incubation, intact treated chloroplasts were recovered as a pellet after centrifugation on a Percoll cushion. It has been verified that phospholipase C was entirely removed by centrifugation through a Percoll cushion. The envelope membranes were then prepared (9), and the lipids were extracted and analyzed as described in *Lipid Analyses*. The values are expressed as micrograms of fatty acids in a given lipid per milligram of envelope protein.

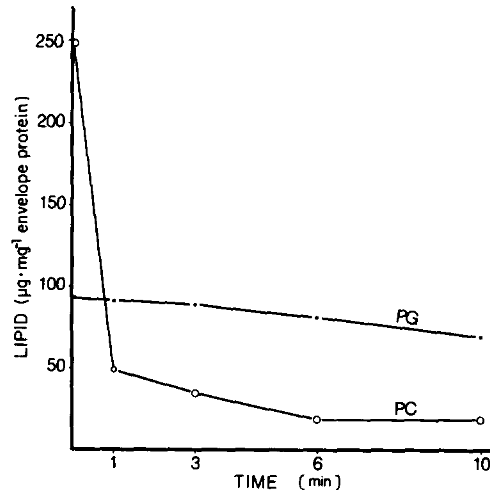

FIGURE 3 Kinetics of envelope phospholipids hydrolysis during phospholipase C treatment of intact chloroplasts. Isolated purified intact chloroplasts (1 mg chlorophyll/ml) were treated with phospholipase C (0.3 Boehringer U/ml) at 12°C for different times. After incubation, intact treated chloroplasts were recovered as a pellet after centrifugation through a Percoll cushion. The envelope membranes were then prepared (9), and the lipids were extracted and analyzed as described in *Lipid Analyses*. The values are expressed as micrograms of fatty acids in a given lipid per milligram envelope protein.

Unfortunately, phospholipase C treatment of intact chloroplasts may induce lysis or severe perturbation of envelope outer membrane structure, leading to artifactual conclusions about phospholipid distribution. In other words, the outer envelope membrane might be altered during phospholipase C treatment so as to reveal sites that were unreactive in intact chloroplasts. Therefore, the intactness of phospholipase C–treated chloroplasts must be clearly established.

Intactness of Phospholipase C–treated Spinach Chloroplasts

Since the chloroplasts used for further analyses of envelope phospholipids were purified on Percoll after incubation with phospholipase C (from 0.3 to 1 Boehringer U/mg chlorophyll), the percentage of intactness as determined by the ferricyanide assay (10) was always maintained at a high value (>80%; results not shown). However, the recovery of intact chloroplasts by Percoll purification was much higher at low temperature (from 4 to 12°C) and low enzyme concentration (up to 0.5 Boehringer U/mg chlorophyll) than at 20°C and high enzyme concentration; the figures obtained were usually

90% recovery in the former case and <10% in the latter. The ferricyanide assay is based on the permeability properties of the inner envelope membrane (10) and therefore cannot be used to probe the integrity of the outer envelope membrane.

Indeed, the integrity of the envelope membranes, and especially that of the outer membrane, after mild treatment of intact chloroplasts with phospholipase C, was verified by several techniques: phase-contrast and electron microscopy, and immunochemical and electrophoretic analyses of envelope polypeptides. Under phase-contrast microscopy, intact chloroplasts appear highly reflective and surrounded by a bright halo whereas broken chloroplasts appear dark and granular (see, for example, reference 11). No striking differences could be detected between the phospholipase C–treated and untreated chloroplasts. Clear proof of the integrity of the outer envelope membrane was provided by addition of antibodies raised against envelope polypeptides to a suspension of phospholipase C–treated and untreated chloroplasts. Incubation of the two types of chloroplast suspensions in the presence of anti-E24, an antibody raised against a polypeptide localized on the outer surface of the outer envelope membrane (11), led to a strong agglutination of intact chloroplasts, which indicates that the antigenic domain corresponding to E24 was still exposed to the outer surface of the outer envelope membrane after phospholipase C treatment. On the contrary, the antigen corresponding to E37, an inner envelope membrane polypeptide (23), was not accessible to anti-E37. Furthermore, analyses of envelope polypeptides by SDS PAGE led to the conclusion that no striking differences in the polypeptide pattern (on the basis of their Coomassie Blue staining) could be detected after phospholipase C treatment. This was particularly true for polypeptides known to be localized on the outer envelope membrane (see reference 23). Taken together, these results strongly suggest that the outer envelope membrane structure was not severely altered by the mild phospholipase C treatment used (i.e., 3 min treatment of intact chloroplasts at 12°C with 0.3 Boehringer U/mg chlorophyll of phospholipase C). An additional line of evidence was provided by electron microscopy.

As shown in Fig. 4, spinach chloroplasts were still bounded by two concentric envelope membranes after phospholipase C treatment; the outer envelope membrane was smooth in outline and ~6 nm thick, and its structure was not significantly changed after phospholipase C treatment. In addition, at high magnification the trilaminar leaflet appearance was still visible, suggesting that the bilayer structure of the outer envelope membrane was probably not strongly altered (Fig. 4*C*). On the contrary, incubation of isolated chloroplasts in more severe conditions (high phospholipase C concentration and/or high temperature) led to an extensive breakage of both the outer and the inner envelope membrane (results not shown). Thus, within the short time of enzyme treatment under mild conditions, a remarkably high proportion of the envelope PC could be hydrolyzed without disintegration of the outer membrane structure, which suggests that phospholipase C restricted its action to the cytosolic half of the outer envelope membrane.

DISCUSSION

The data reported in this article clearly show that the intactness of isolated spinach chloroplasts can be preserved during phospholipase C treatment. We reached this conclusion by using several techniques such as phase-contrast and electron microscopy, and immunochemical and electrophoretic analyses. Such a result was not really surprising since isolated intact chloroplasts can remain intact after phospholipase A and D treatment (24) or protease treatment (11). It is interesting that the formation of diacylglycerol within the chloroplast outer envelope membrane does not induce a deleterious transformation of the membrane structure, although a zwitterionic polar lipid (PC) was replaced by a neutral lipid (diacylglycerol). Actually, the diacylglycerol molecules generated within the membrane are much less amphiphilic than is their parent phospholipid. It is not known whether they remain oriented in the bilayer. It is possible that these diacylglycerol molecules could escape as small oil droplets into the interior of the membrane, as suggested by Sundler et al. (25) or be released. In addition, it is difficult to be sure whether such arrangements within the membrane alter the original lipid distribution. It is remarkable, however, that after phospholipase C treatment of intact spinach chloroplasts, which results in the essentially complete degradation of envelope PC molecules, both envelope membranes (and especially the outer envelope membrane) retained an apparent bilayer structure, as suggested by electron microscopy. As discussed for other cell membranes by Op den Kamp (26), it is very possible that the other outer envelope membrane constituents (galactolipids, prenylquinones, sterols, or proteins; for review, see reference 27) contribute significantly to maintain the integrity of the lipid bilayer. In support of this suggestion, it has been demonstrated that erythrocyte ghosts treated on both sides with phospholipases retained a bilayer structure, as shown by ^{31}P nuclear magnetic resonance (28).

The major results presented in this article concern the precise localization of PC and PG within the outer envelope membrane. It is now well established that PC is the major phospholipid in the outer envelope membrane, whereas it is PG in the inner one and in the thylakoids (4, 5). Indeed, there are from 600 to 800 μg PC/mg protein in the outer envelope membrane from spinach chloroplasts and only 200 μg PG/mg protein (5). Several lines of evidences led us to the conclusion that the distribution of PC and PG within the outer envelope membrane is asymmetric: (*a*) Under controlled and mild conditions, isolated intact chloroplasts (and especially their outer envelope membrane) remained intact during phospholipase C treatment. (*b*) All PC and PG molecules, when available to phospholipase C, i.e., after sonication of the isolated envelope vesicles in presence of phospholipase C, could be totally hydrolyzed into diacylglycerol. (*c*) PC hydrolysis in isolated intact chloroplasts occurred within only 1 min of incubation at low phospholipase C concentration (0.3 Boehringer U/mg chlorophyll) and at low temperature (4 to 12°C), whereas under the same conditions, most of the envelope PG remained in the membrane. From these data, we can conclude that most of the envelope PC (>80%) is located in the cytosolic (outer) leaflet of the outer envelope membrane. The data obtained about PG are less precise since phospholipase C specificity cannot be entirely ruled out, as the inital rate of PG hydrolysis in isolated envelope vesicles is apparently lower for PG than for PC (see Fig. 1*B*). However, it is likely that the outer leaflet of the outer envelope membrane contains few PG molecules (see Table II and Figs. 2 and 3) as compared with the other chloroplast membrane systems.

Evidence for phospholipid asymmetry in cell membranes has been accumulated. In chloroplasts all of the studies per-

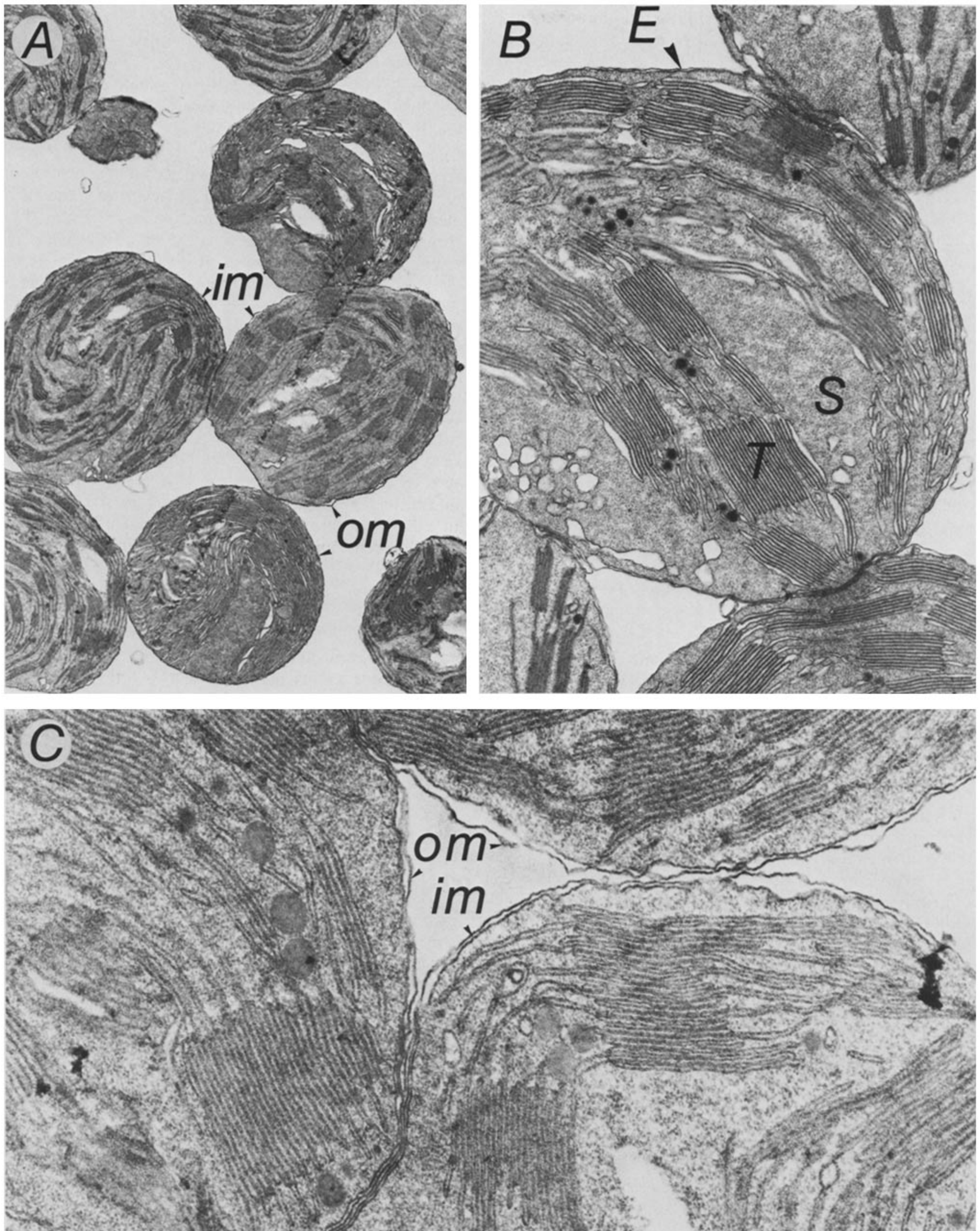

FIGURE 4 Electron micrographs of phospholipase C-treated chloroplasts. Isolated purified intact chloroplasts (1 mg chlorophyll/ml) were treated with phospholipase C (0.5 Boehringer U/ml) at 12°C for 5 min. After incubation, intact treated chloroplasts were recovered as a pellet after centrifugation through a Percoll cushion. The pellet was then prepared for electron microscopy (16, 31). *E*, envelope; *S*, stroma; *T*, thylakoids; *om*, outer membrane; *im*, inner membrane. No striking differences can be seen in the aspect of the two envelope membranes, especially at high magnification (C). *(A)* × 4,500; *(B)* × 11,000; *(C)* × 26,000.

formed so far have been done with thylakoid membranes. For example, Rawyler and Siegenthaler (29) and Unitt and Harwood (30) have demonstrated that thylakoidal PG was present mostly in the outer (stromal) leaflet of the thylakoids whereas PC was located in the inner one. However, lipolytic degradation of biological membranes may lead to contradictory conclusions about lipid topography. For intance, as discussed by Op den Kamp (7, 26), inconsistent data can be obtained due to transbilayer movements of phospholipids induced by hydrolytic experiments or to natural flip-flop. For instance, under particular experimental conditions (see, for example, reference 31), transbilayer movement of a given phospholipid can supply the outside layer with additional molecules, so the results obtained with a lipolytic enzyme do not reveal the actual distribution of this phospholipid because the molecules originally present at the inside also become available for hydrolysis at the outside (26). However, we believe that our results are not hampered by such a limitation. First, flip-flop movement, especially of PC, is usually a slow process (half-time value of 7–8 h at physiological temperatures; see references 7 and 26), whereas in our incubations, all of the envelope PC degradation occurred within 1 min of incubation of intact chloroplasts with phospholipase C. Second, the transbilayer movements discussed above usually occur at room or physiological temperature, whereas PC degradation occurs in intact chloroplast even at low temperatures (4 to 12°C) when all transmembrane movements are strongly reduced.

An intriguing question which remains to be elucidated is how PC can accumulate in the cytosolic leaflet of the outer envelope membrane. In other words, does the observed distribution of PC represent a stable configuration or only reflect a transient state? Such an asymmetry is maintained by the apparent lack of transmembrane diffusion in the outer envelope membrane. The absence of any information on lipid movements within envelope membranes does not help to provide an answer. It is interesting that the outer membrane of cellular organelles is often strongly enriched in PC. For instance, Bligny and Douce (32) demonstrated that the plant mitochondrial outer membrane contains about two-thirds PC among its polar lipids. The same is true for the peroxisomal membrane (Journet, E. P., and R. Douce, unpublished data). Therefore, these observations could be taken as an argument for the existence of a common endomembrane system including endoplasmic reticulum, nuclear envelope, etc. We think that this hypothesis is unlikely to be true, since it is difficult to understand why the outer envelope membrane contains large amounts of typical blue-green algae lipids, such as galactolipids and sulfolipid, and why its chemical composition (except for PC molecules) does not ressemble that of the extrachloroplastic membranes, which contain large amounts of phosphatidylethanolamine, a phospholipid absent from all plastid membranes and especially the outer envelope membrane (5). In addition, the hypothesis that at least only the outer leaflet of the outer envelope membrane, which contains (from the data presented in this article) ~60% of PC and only 40% of other polar lipids, could derive from extrachloroplastic membranes is also unlikely since Billecocq (33, 34) and her co-workers (35) have demonstrated that galactolipids and sulfolipid, two characteristic plastid polar lipids, are accessible to specific antibodies from the cytosolic side of the envelope of isolated intact chloroplasts. The same conclusion can be drawn from cytochemical analyses of plastid envelope membranes (36). None of these observations favors the transfer of PC by lateral diffusion within a continuous network to the outer envelope membrane. Since isolated envelope membranes cannot synthesize their own PC (37), it is possible that PC accumulation in the outer leaflet of the outer envelope membrane could be due to a direct transfer from the endoplasmic reticulum to the chloroplast, for instance via a phospholipid transfer protein (38, 39). Finally, the physiological significance of PC accumulation in the outer envelope membrane remains unclear. A number of laboratories have supported the theory that PC is the precursor of most cellular polar lipids and especially galactolipids (for reviews, see references 3, 27, 40). However, it has also been demonstrated that, owing to the presence of the enzymes involved in phosphatidic acid and diacylglycerol synthesis on the envelope membranes (41), isolated intact chloroplasts can synthesize galactolipids (42) with a 16:3/18:3 fatty acid combination (43, 44). Since sulfolipid (45) and PG (46) are also synthesized within isolated intact chloroplasts, the role of PC as the precursor of plastid polar lipids is probably restricted to that of a molecular species of galactolipids that has the 18:3/18:3 combination (43, 44). However, this postulated role of PC is not clear since it does not explain why PC accumulates in the outer leaflet of the outer envelope membrane, which does not fit with a precursor–product relationship between PC and galactolipids. We are convinced that the combination of phospholipase C treatment of isolated intact chloroplasts together with the use of phospholipid transfer protein may provide a useful tool in manipulating the PC content of the outer envelope membrane in order to study the postulated role of PC in chloroplast polar lipids biosynthesis.

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