Cyclodextrins: A Potential Tool for Studying the Role of Glycerolipids in Photosynthetic Membranes

Sylvie Duchêne and Paul-André Siegenthaler*

Laboratoire de Physiologie végétale, Université de Neuchâtel, CH-2007 Neuchâtel, Switzerland

ABSTRACT: A novel cyclodextrin derivative, i.e., permethylated-α-cyclodextrin (PM-α-CD), was used for removing glycerolipids from spinach thylakoid membranes and investigating their role in photosynthetic activities. A three-step selective removal of each lipid class was observed in treated membranes. Up to a concentration of 4 mM, PM-α-CD (in the presence of 75 μg chlorophyll *a*+*b*/mL), PM-α-CD displayed a marked selectivity for anionic lipids [sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG)] in comparison with galactolipids. At this concentration, half of PG and SQDG were removed. Within this range of concentration, the volume response of treated thylakoids to variation of osmolarity, an indirect mean of verifying the structural integrity of the membrane, was not altered. Similarly, neither photosystem II (PSII) nor photosystem I (PSI) activity was affected. In contrast, the low-temperature fluorescence ratio F695/F740 drastically diminished from 1.45 to about 0.7, essentially due to the decrease of PSII fluorescence. The results derived from the fast fluorescence rise expressed in the form of a spider suggest that the fraction of inactivated (non-Q_A reducing) reaction centers (RC) increases while the active (Q_A reducing) RC remained intact. Raising the concentration of PM-α-CD from 4 to 7 mM resulted in a progressive but greater diminution of the galactolipid level than that of SQDG and PG. Within this concentration range, the integrity of the membrane was not altered, nor was either PSII or PSI activity, whereas the F695/F740 ratio decreased to about 0.45 as well as the fraction of inactivated RC. At concentrations above 7 mM of PM-α-CD, the integrity of the membrane was impaired, resulting in a decrease of both electron transport activities. At all concentrations, PM-α-CD did not show any selectivity toward either the acyl chains of the lipid molecules or the molecular species of PG. The results are discussed in terms of the role of glycerolipids in thylakoid membrane function and the relationship of the chemical structure of PM-α-CD and its lipid removal capacity.

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*To whom correspondence should be addressed at Laboratoire de Physiologie végétale, Université de Neuchâtel, Rue Emile-Argand 13, CH-2007 Neuchâtel, Switzerland.
E-mail: Paul-Andre.Siegenthaler@unine.ch

Abbreviations: ABS, light absorption; CD, cyclodextrin; α-CD, α-cyclodextrin; β-CD, β-cyclodextrin; γ-CD, γ-cyclodextrin; Chl, chlorophyll; CSo, foliar cross section; DGDG, digalactosyldiacylglycerol; 2,6-DM-β-CD, 2,6-dimethyl-β-cyclodextrin; ETo, photosystem II electron transfer; F_{695}, fluorescence at 695 nm; F_{740}, fluorescence at 740 nm; LHCII, light harvesting chlorophyll *a/b* protein complex; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; PLA$_2$, phospholipase A$_2$; PM-α-CD, permethylated-α-cyclodextrin; PSI, photosystem I; PSII, photosystem II; RC, active reaction center; SQDG, sulfoquinovosyldiacylglycerol; TM, thylakoid membranes; TRo, photon trapping.

Besides proteins, acyl lipids are major components of thylakoid membranes (TM) in higher plants. They consist of four classes: monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and phosphatidylglycerol (PG). Galactolipids (MGDG and DGDG) are prominent and account for about 80% of total glycerolipids. Moreover, they are characterized by an exceptionally high content of trienoic acid, mainly α-linolenic acid (encountered in all lipid classes) and, in the so-called 16:3 plants, such as spinach, hexadecatrienoic acid (found in MGDG only). SQDG is enriched in palmitic acid (35 mol%), whereas PG contains a unique FA, *trans*-Δ^3-hexadecenoic acid [16:1 (3*t*)] (1,2). The FA composition of these acyl lipids gives rise to a great number of molecular species (3,4).

Glycerolipids display a marked transmembrane distribution in the TM. The outer monolayer is highly enriched in MGDG (5,6) and PG (7,8), whereas the inner one contains a high level of DGDG (5,6); this confirms the general sidedness of TM components. In contrast, recent analyses of glycerolipids in different domains of the membrane (e.g., intact thylakoids, grana lamellae vesicles, central core of the appressed region, grana margin vesicles, and stroma lamellae vesicles) reveal that the level of the four lipid classes, the nature of their acyl chains, and the main molecular species of PG are identical in the intact TM as well as in the four membrane domains (9).

The role of membrane lipids in the photosynthetic function have been particularly difficult to study. Although several approaches have been proposed in the literature (for a review, see Ref. 10), there is no consensus about the results obtained. Among them, the enzymatic digestion of lipids (e.g., 5,6,8,11,12), the use of specific antibodies against lipids (e.g., Refs. 13–15), the preparation of subthylakoid fractions (16,17), and the use of mutants deficient in certain lipids (18) have been extensively studied. All these techniques have advantages and drawbacks. For instance, the use of enzymatic digestion with lipolytic enzymes results in the concomitant accumulation of FFA that are known to have detergentlike properties and to displace lipids in the membrane (19,20). The use of specific antibodies against lipids is often questionable due to the difficult access of the antibody to all the lipids at the surface of the membrane, especially if they are located in membrane hollows. Although the preparation of subthylakoid fractions seemed to have been for a long time the best technique to associate structure and function, its greatest drawback comes from utilization of high concentrations of deter-

gents. This explains why a few authors have tried to obtain sub-thylakoid particles or domains without detergent by using the aqueous two-phase system (9,19,21,22). Although the mutant approach has permitted study of the effect of the degree of lipid polyunsaturation on the structure and function of the chloroplast membrane (10,18), it is extremely difficult to evaluate the side effects of specific mutations on lipid metabolism.

Recently, cyclodextrins (CD) were reported to have the capacity to remove lipids from the TM (23) and display hemolytic properties (24). CD molecules are cyclic oligosaccharides consisting of 6 (α-CD), 7 (β-CD), or 8 (γ-CD) glucopyranose units linked by α (1–4) bonds. These compounds are not perfectly cylindrical molecules but rather are somewhat cone-shaped. They adopt a torus shape and are able to bind lipids within their hydrophobic cavity to form water-soluble guest-CD inclusion complexes (25–27). So far, CD have been used mainly in the biomedical and pharmaceutical fields.

In a previous contribution, we established the conditions under which TM glycerolipids can be extracted in the presence of α-CD, β-CD, and dimethyl-β-cyclodextrin (DM-β-CD) (23). We found that the extent of lipid, protein, pigment, and plastoquinone removal depends on both CD and chlorophyll (Chl) concentrations. Simultaneous with the removal of lipids, a few photosynthetic functions were found to be altered.

In this investigation, we used a novel CD derivative, the permethylated-α-cyclodextrin (PM-α-CD). This compound was found to display properties different from those of the CD used previously. At low concentrations, it had the capacity of removing anionic lipids (SQDG and PG) without concomitant galactolipid extraction. These properties allowed us to study the relationship between the level of anionic lipids and various photosynthetic functions. Preliminary results of this investigation were presented elsewhere (28,29).

EXPERIMENTAL PROCEDURES

Materials. All the CD used in this study were obtained from Fluka (Buchs, Switzerland). The PM-α-CD was made by Dr. S. Claude (Department of Organic Chemistry, University of Neuchâtel, Switzerland). All chemicals were high-purity products from Fluka or Sigma, and Percoll was provided by Pharmacia (Uppsala, Sweden). Spinach leaves were purchased from the local market. They were washed, stored at 4°C and used within 2 d.

Preparation of TM. Spinach leaves (150 g) were ground for 10 s in 375 mL of a grinding medium containing 330 mM sorbitol, 30 mM MOPS-KOH (pH 7.8), 2 mM EDTA-Na$_2$, and 0.15% BSA by using a Waring Blender. The mixture was filtered through four layers of cheesecloth and two layers of miracloth, and the filtrate was subsequently centrifuged at $1500 \times g$ for 2 min. The supernatant was discarded by aspiration, and the intact chloroplast pellet was resuspended in the grinding medium (20 mL) and layered on two centrifuge swinging-bucket tubes containing 20 mL of Percoll 40% supplemented with the grinding medium without EDTA-Na$_2$ and BSA. After centrifugation ($2500 \times g$, 10 min), the chloroplast pellet was

resuspended in a low-osmotic medium (36 mL) containing 10 mM Tricine-KOH (pH 7.8) and 2 mM EDTA-Na$_2$ for 2 min. The osmolarity was reajusted by adding back 4 mL of 3 M sorbitol. After centrifugation at $13,400 \times g$ for 5 min, the thylakoid pellets were resuspended in a washing medium containing 300 mM sorbitol and 10 mM Tricine-KOH (pH 7.8). Fifteen milliliters of Percoll (5% Percoll in 300 mM sorbitol and 10 mM Tricine-KOH pH 7.8) were injected slowly below the suspension (20 mL) of thylakoids. The tubes were centrifuged at $20,000 \times g$ for 10 min, and the thylakoid pellets were washed in the same medium at $13,500 \times g$ for 5 min and then resuspended in the same medium at a concentration of 2 mg Chl/mL.

Incubation of thylakoids in the presence of CD. All CD were solubilized in 300 mM sorbitol and 10 mM Tricine-KOH (pH 7.8). The concentration of the stock solutions was 10 mM. CD at the desired concentrations were added to the thylakoid preparation (50–75 µg Chl/mL) and incubated at 0°C for 20–30 min. At the end of the reaction, thylakoids were centrifuged at $14,500 \times g$ for 10 min and resuspended in 300 mM sorbitol and 10 mM Tricine-KOH (pH 7.8). The concentration of Chl was readjusted to 1 or 2 mg Chl/mL depending on the fate of the preparation.

Lipid analyses. Total lipids were extracted by adding 4 mL chloroform/methanol (53:37, vol/vol) and 2 mL 0.5 M KCl to the thylakoid suspension (150 µL of thylakoids containing 1 mg Chl/mL). This resulted in a two-phase system. The lipids of the lower phase were separated by TLC with acetone/toluene/water (91:30:8) as solvents (30). Each lipid class was then hydrolyzed and esterified with 5% H$_2$SO$_4$ in methanol at 85°C for 1 h. The resulting FAME were separated and identified by GC and the molecular species of PG determined according to Xu and Siegenthaler (4). Chl concentrations were determined as described by Bruinsma (31).

Photosynthetic activities. Uncoupled photosystem II (PSII) electron flow was measured polarographically at 20°C in the presence of red actinic light (29 mW/cm^2) in a medium containing 30 mM sodium phosphate buffer (pH 6.5), 3 mM NaCl, 60 mM sucrose, 0.2 mM phenyl-*p*-benzoquinone, 2 mM NH$_4$Cl, and thylakoids corresponding to 20 µg Chl/mL, using a Clark-type oxygen electrode (evolution of O$_2$). Uncoupled photosystem I (PSI) electron flow was determined in a medium containing 40 mM sodium phosphate buffer (pH 7.4), 1 mM NaCl, 10 µM 3-(3,4-dichlorophenyl)-1,1-dimethylurea; 32 mM ascorbate, 0.3 mM, 0.12 mM methylviologen, 0.6 mM NaN$_3$, 2 mM NH$_4$Cl and thylakoids corresponding to 20 µg Chl/mL, using a Clark-type electrode (absorption of O$_2$) (8). Fluoresence studies were carried out with freshly prepared thylakoids resuspended in 300 mM sorbitol and 10 mM Tricine-KOH (pH 7.8). The low-temperature fluorescence spectra of thylakoids (diluted 1:9 in 80% glycerol) were carried out according to Rawyler and Siegenthaler (23). The fluorescence transients OJIP were measured as described by Srivastava and Strasser (32).

Response of thylakoids to osmolarity variation. The integrity of thylakoid membranes during the removal of lipids

by CD was estimated by an osmolarity test (7,33). Thylakoids were treated with various concentrations of PM-α-CD (4,6, and 9.6 mM) as indicated previously. Then, each sample (2 mg Chl/mL), including the control without CD, was suspended in 40 mM Tricine-KOH (pH 8.0) and 3 mM MgCl$_2$ and divided into four equal parts. Increasing concentrations of sucrose (40, 51, and 60 mM) were then added to each of the samples. Osmotic equilibrium was reached after 5 min, and then the thylakoid preparations were transferred to hematocrit tubes and centrifuged at $23,000 \times g$ (microcentrifuge; Christ, Zurich, Switzerland) for 5 min. The height of the thylakoid sediment in the tube was measured and compared to the total volume of the original suspension (ratio packed volume/total volume, arbitrary units).

RESULTS

Several CD (α-, β-, γ-CD) and CD derivatives were compared regarding their lipid removal capacity and selectivity. The results are presented in Table 1. α-CD [which contains six glycopyranosidic units and presents an internal diameter of the cavity of 5.2 Å (27)] and its permethylated-α-derivative (PM-α-CD) displayed a much greater preference for SQDG and PG than for galactolipids. In contrast, β-CD [which contains seven glycopyranosidic units and presents an internal diameter of the cavity of 6.4 Å (27)] removed roughly 11 mol% of the four lipid classes. The derivative of β-CD (2,6-DM-β-CD) displayed a preference for the anionic lipids (SQDG and PG) over galactolipids but to a lesser extent than the α-CD and PM-α-CD. Other β-derivatives, such as permethylated-β-, carboxylated-β−, quaternary-β-, amphiphilic-β-, and sulfated-β-CD, removed only lipids in trace amounts (data not shown). The selective capacity of γ-CD [which contains eight glycopyranosidic units and presents an internal diameter of the cavity of 8.3 Å (27)] was greater for SQDG than for all other lipid classes. But the amount of SQDG removed was rather low. A mixture of α- and β-CD removed about 30 mol% of galactolipids and 60 mol% of SQDG and PG but had no selective effect for one particular lipid class (Table 1). On

the basis of these results, we focused our attention on the effect of PM-α-CD.

The results in Figure 1 show that the removability of total lipids in the thylakoid membrane increased as a function of the concentration of PM-α-CD up to 8 mM. At this concentration, 50 mol% of the total lipids were removed. A further addition of PM-α-CD had no effect.

Figure 2 illustrates the selective removal of each lipid class as a function of PM-α-CD concentration. Three steps can be distinguished: (i) Up to a concentration of 4 mM PM-α-CD, the content in MGDG remained constant, while that of DGDG decreased slightly. In contrast, the amount in anionic lipids decreased approximately by half of its original level. (ii) Raising the concentration of PM-α-CD from 4 to 8 mM resulted in a progressive but greater diminution of the galactolipid level than that of SQDG and PG. (iii) Higher concentrations of PM-α-CD did not affect significantly the content of all four lipid classes. The concentration of PM-α-CD necessary to remove 50 mol% of each lipid was 8, 9.6, 3, and 3.5 mM for MGDG, DGDG, SQDG, and PG, respectively (see dotted lines in Fig. 2).

Figure 3 shows the relative content and composition of fatty acids in each lipid class remaining in the thylakoid membrane after a treatment with various concentrations of PM-α-CD. The PM-α-CD did not show any selectivity toward the acyl chains of the lipid molecules.

Spinach thylakoid membranes contain 10 PG molecular species (4). The three main molecular species [18:3/16:1 (3t); 18:3/16:0; 16:0/16:1 (3t)] represented 94.9 mol% of the total molecular species. The effect of PM-α-CD (0, 2, 5, and 9.6 mM) on their content in treated thylakoids was found to be 79.5 to 82.3 mol% for 18:3/16:1 (3t), 6.4 to 8.0 mol% for 18:3/16:0, and 3.2 to 5.6 mol% for 16:0/16:1 (3t). All these values presented no statistical difference between the control values and those of the different concentrations of PM-α-CD (individual data not shown). In conclusion, PM-α-CD did not show any selectivity toward the molecular species of PG, i.e., each molecular species was removed in the same proportion at each concentration of the CD.

TABLE 1
Comparison of the Removability of Lipid Classes in Thylakoids Treated by Various Cyclodextrins (CD)

CD used[a]	Lipid removal (mol%)				
	MGDG	DGDG	SQDG	PG	n
α-CD[b]	5.4 ± 4.8	5.4 ± 2.7	42.4 ± 2.1	39.1 ± 7.4	4
PM-α-CD[c]	12 ± 4	15 ± 2	64 ± 5	54 ± 5	6
β-CD[b]	13.9 ± 2.4	14.5 ± 3	8.1 ± 3.5	12.5 ± 2.6	4
2,6-DM-β-CD[b]	28.1 ± 3.5	17.8 ± 2.1	43.7 ± 6.4	38.3 ± 2.8	4
γ-CD[c]	5 ± 3	Trace	22 ± 7	8 ± 6	3
α- + β-CD[b]	27.7 ± 5.0	27.0 ± 5.5	60.0 ± 6.4	54.4 ± 5.9	6

[a]Thylakoids were incubated with 5 mM of one of the cyclodextrins in a medium containing 300 mM sorbitol and 10 mM Tricine-KOH (pH 7.8) at 0°C.
[b]50 µg chlorophyll/mL; 30 min incubation [results from Rawyler and Siegenthaler (23)].
[c]75 µg chlorophyll/mL; 20 min incubation. MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol.
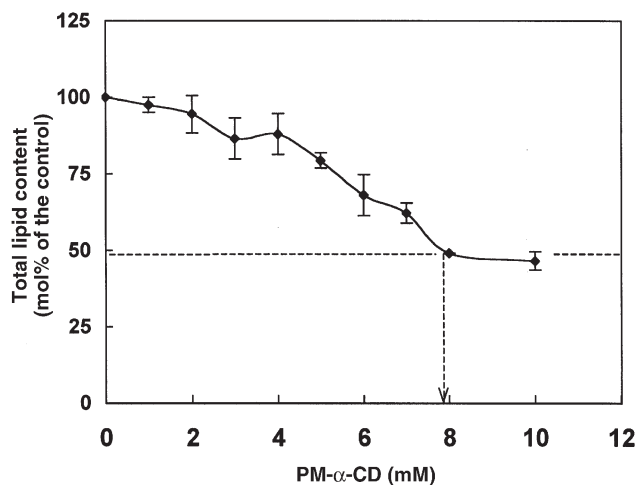
FIG. 1. Changes in the relative content of total lipids in thylakoid membranes treated with various concentrations of permethylated α-cyclodextrin (PM-α-CD). The SD was calculated on the basis of six experiments ($n = 6$). The dotted line shows that 50 mol% of the total lipids was removed in the presence of 8 mM PM-α-CD. The 100% value corresponded to 2600 ± 100 nmol/mg chlorophyll (Chl).

The effect of PM-α-CD on electron transport activity in spinach thylakoids is shown in Table 2. Up to a concentration of 7 mM PM-α-CD, neither PSII nor PSI activity was affected. However, a small stimulation of PSI activity at 5 mM PM-α-CD could be detected. At a concentration of 7 mM, 30 mol% of each galactolipid, 70 mol% of SQDG, and 60 mol% of PG were removed by this treatment (Fig. 2). A further increase of PM-α-CD (up to 9.6 mM) inhibited both activities,
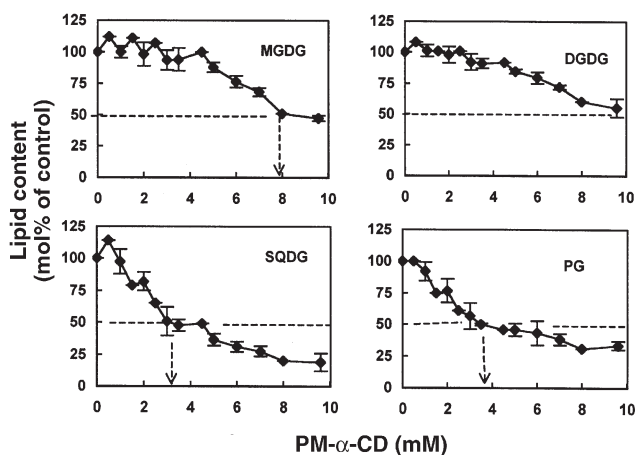

FIG. 2. Changes in the relative amount of individual diacyl lipid classes (MGDG, DGDG, SQDG, PG) in thylakoid membranes treated with PM-α-CD. The standard deviation was calculated on the basis of nine experiments ($n = 9$). The dotted lines indicate the concentration at which PM-α-CD was necessary to remove 50 mol% of each lipid class. For each of the four lipid classes, the control amount, given in nmol lipid per mg Chl, was MGDG (1400), DGDG (707), SQDG (165), and PG (227). MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDQ, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol; for other abbreviation see Figure 1.
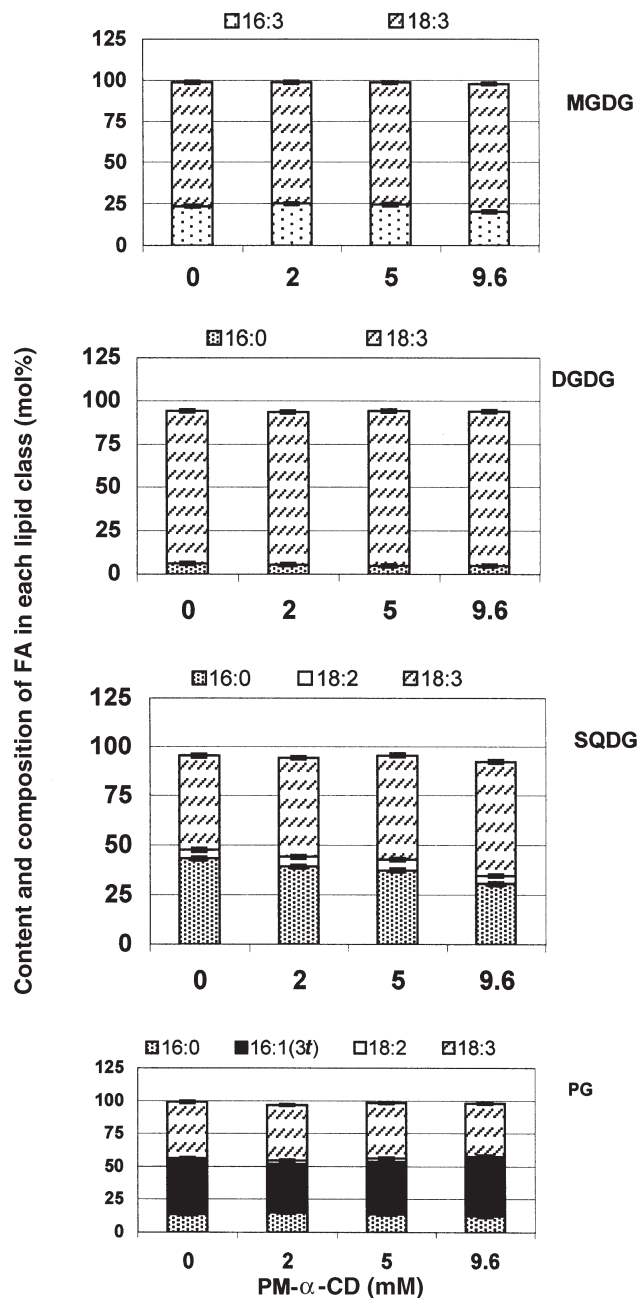

FIG. 3. Relative content and composition of FA in each lipid class remaining in the thylakoid membrane after treatment with PM-α-CD. Only the main FA of each lipid class are illustrated in this graph. This explains why some 100% values are not reached (e.g., DGDG and SQDG). The standard deviation was calculated on the basis of seven experiments ($n = 7$). The 100% values of the controls (0 mM PM-α-CD) corresponded in nmol per mg Chl to 1400 for MGDG, 707 for DGDG, 165 for SQDG, and 227 for PG. The 100% values for all the other histograms are different and can be calculated from the results of Figure 2. See Figures 1 and 2 for abbreviations.

although to different extents for PSII (35% inhibition) and PSI (56% inhibition). At this concentration, an additional treatment of the membrane with phospholipase A$_2$ (PLA$_2$)

TABLE 2
Effect of PM-α-cyclodextrin on Photosystems II and I Electron Transport Activity in Spinach Thylakoids

PM-α-CD (mM)	Photosystem II (PSII) activity (%)[a]	Photosystem I (PSI) activity (%)[b]
0	100	100
2	97 ± 11 (n = 5)	98 ± 6 (n = 5)
5	95 ± 14 (n = 5)	132 ± 24 (n = 5)
7	92 ± 12 (n = 5)	104 ± 6 (n = 5)
9.6	65 ± 11 (n = 6)	44 ± 13 (n = 7)
9.6 + PLA$_2$[c]	33 ± 6 (n = 2)	45 ± 3 (n = 2)

[a]PSII electron transport actitivy: 100% corresponded to 36 ± 12 micromol O_2/mg Chl × h (n = 7).
[b]PSI electron transport activity: 100% corresponded to 460 ± 90 micromol O_2/mg Chl × h (n = 7).
[c]PLA$_2$, phospholipase A$_2$ from porcine pancreas. After the permethylated-α-cyclodextrin (PM-α-CD) treatment (20 min, 2°C), PLA$_2$ (4 Boehringer units/mg Chl) was added to the same reaction mixture and incubated at 20°C for 40 min.

further decreased PSII activity from 65 to 33% but had no effect on PSI activity (Table 2).

It was important to verify the structural state of the membrane in PM-α-CD-treated thylakoids. This was achieved by testing the volume response of thylakoids to variations of the osmolarity (33). As shown in Figure 4, an increase in the osmolarity (from 49 to 109 Osmol) resulted in a decrease of about half of the packed volume of thylakoids. This occurred not only in the control samples but also in thylakoids treated with 4 and 6 mM PM-α-CD. However, in the presence of 9.6 mM PM-α-CD (Fig. 4) or 9.6 mM PM-α-CD + PLA$_2$ (data not shown), the osmotic response was nearly abolished.

Figure 5 shows the effect of various concentrations of PM-α-CD on low-temperature fluorescence. In the control thylakoid membrane, the ratio of fluorescence at 695 vs. 740 nm (F695/F740) was 1.45, indicating that the PSII fluorescence was greater than that of PSI. This ratio decreased progressively when the concentration of CD was raised. The extent
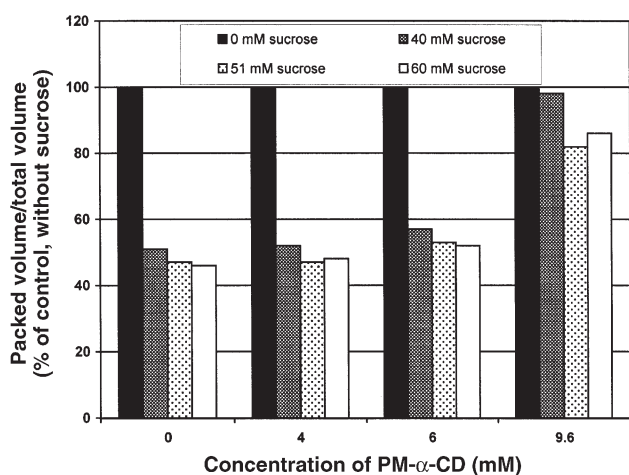
FIG. 5. Effect of lipid depletion by PM-α-CD on the low-temperature (77 K) fluorescence expressed as the ratio of the fluorescence at 695 nm (photosystem II: PSII) to the fluoresence at 740 nm (photosystem I: PSI) in thylakoid membranes. Where indicated, the SD was calculated on the basis of six experiments (n = 6). For the other ones, n = 2. Inset: fluorescence spectra at 77 K of control sample (solid line) and PM-α-CD-treated (5 mM) thylakoids (dashed line). The shift of wavelength at 695 nm between the two samples is not significant. For abbreviation see Figure 1.

of this decrease was greater at low than at high concentrations of PM-α-CD. At a concentration of 4 mM, the ratio corresponded to half of the value of the original one. As shown in the inset of Figure 5, the decrease in the F695/F740 ratio was entirely due to the decrease of the fluorescence at 695 nm in the presence of PM-α-CD.

The results derived from the fast fluorescence rise (OJIP) (32,34) of CD-treated thylakoids are illustrated as a spider-plot presentation in Figure 6. This is a multiparametric description of structure and function of each photosynthetic

FIG. 4. Osmotic response of thylakoids first pretreated by 4, 6, and 9.6 mM PM-α-CD, and then incubated in a medium containing 0, 40, 51, or 60 mM sucrose corresponding to 49, 89, 100, and 109 Osmol, respectively (other conditions as in the Experimental Procedure section). For abbreviation see Figure 1.
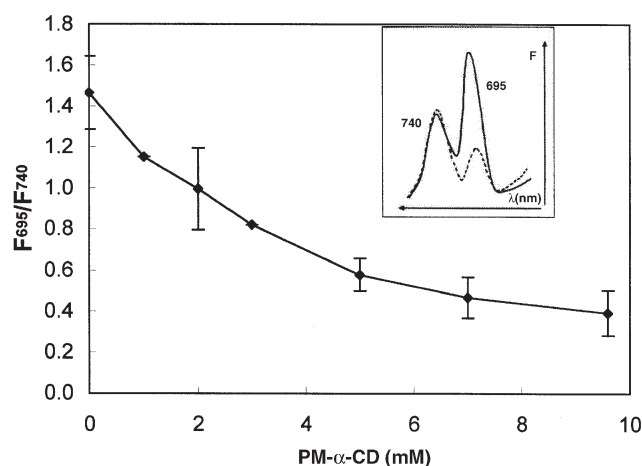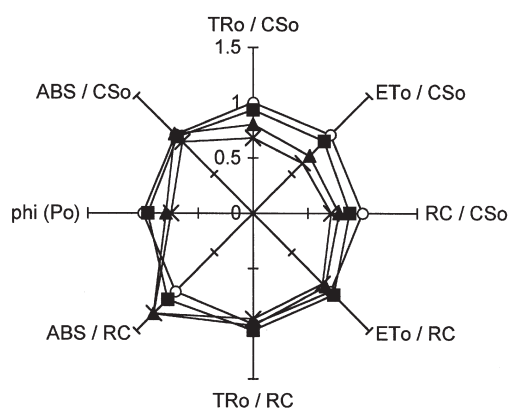
FIG. 6. Effect of lipid depletion by PM-α-CD on various parameters characterizing the photochemistry of the thylakoid membranes; ABS, light absorption; TRo, photon trapping; ETo, PSII electron transfer; RC, active reaction center; CSo, foliar cross section; phi (Po) = TRo/ABS, yield of primary photochemistry. Symbols: ○, 0 mM PM-α-CD; ■, 2 mM; ▲, 4 mM; x, 6 mM. The scale from 0 (center of the spider) to 1.5 corresponds to ratios of the various parameters of a treated sample and its control. For other abbreviation see Figure 1.

sample (control and PM-α-CD-treated thylakoids), presented by an octagonal line. This type of presentation facilitates the comparison of the effect of different treatments. The relative values (relative to the corresponding value of the control, which thus become equal to unity) of selected expressions, such as PSII electron transfer/cross-foliar section (ETo/CSo) and ETo/active reaction center (ETo/RC), etc., can be plotted using a spider-plot presentation. The fluorescence transients and the spider presentation are thoroughly described by Strasser *et al.* (34). On a sample basis (per CS), the light absorption (ABS)/CSo (corresponding to the same amount of Chl) remained constant from 0 to 6 mM PM-α-CD, whereas the photon trapping (TRo)/CSo and electron transfers (ETo/CSo) decreased, in particular at 4 and 6 mM PM-α-CD. However, the specific trapping (TRo/RC) and, to a lesser extent, electron transfers (ETo/RC) per reaction center were not affected up to about 6 mM. In contrast, the light absorption per active reaction center (ABS/RC) increased, especially from 0 to 4 mM PM-α-CD. In addition, the density of active reaction centers (RC/CSo) decreased steadily upon PM-α-CD addition, whereas the overall quantum yield phi (Po) decreased only in the presence of the highest levels (4 to 6 mM) of CD.

DISCUSSION

The results of this investigation show that CD, in particular their permethylated derivative, PM-α-CD, offer interesting properties for removing glycerolipids from the TM. When comparing the lipid removal capacity of the different CD and derivatives (Table 1), we chose to investigate further the properties of PM-α-CD for the following reasons: (i) This compound removed 50 to 70 mol% of anionic lipids (PG and SQDG) from the membrane (Fig. 2); (ii) at a concentration of 5 mM, it had almost no effect on galactolipids (Fig. 2); (iii) compared with α-CD, the permethylated derivative displays a greater cavity opening of the larger ring edge of the molecule, which might enhance its capacity for engulfing lipid molecules (35); (iv) compared with all β-CD and its derivatives, the α-CD and PM-α-CD have the advantage of displaying a greater solubility in water (27); and (v) in contrast to detergent and enzymatic treatments, PM-α-CD, up to a concentration of 6 mM, had no deleterious effect on the TM (Fig. 4), thereby avoiding special protective precautions, e.g., using high concentrations of BSA to remove FFA resulting from lipid digestion (20). All these properties make PM-α-CD a suitable compound for studying lipid composition–function relationships in TM.

The first set of results shows that, up to a concentration of 4 to 5 mM, PM-α-CD displayed a marked selectivity for the removal of anionic lipids compared to galactolipids. Within this range of concentrations (Fig. 2), the structural integrity of the membrane was preserved (Fig. 4), and neither PSII nor PSI activity was affected (Table 2). For instance, 50% removal of both SQDG and PG did not impair PSII and PSI electron flow activities. This surprising finding can be explained in view of

earlier results concerning the effect of enzymatic digestion of membrane lipids by various phospholipase treatments (7,20). As is well established, the TM is constituted of two monolayers that display an asymmetric distribution of their lipids (2). For instance, the outer monolayer contains 70 mol% PG and the inner one, 30 mol%. On the other hand, the enzymatic digestion of all the PG located in the outer monolayer did not affect PSII and PSI activities, even after the removal of the products (e.g., FA) resulting from the enzymatic digestion. In contrast, the removal of a small amount of PG in the inner monolayer drastically obliterates the activity of PSII (7).

Altogether, these experiments strongly suggest that the PG molecules that were removed by PM-α-CD originated from the outer monolayer only, since PSII and PSI activities were not altered. At a higher concentration of PM-α-CD (9.6 mM), half of both MGDG and DGDG molecules were removed from the TM by this treatment (Fig. 2). At this high concentration of PM-α-CD, the osmotic response was almost completely abolished, indicating that the structure of the TM was greatly impaired. Interestingly, *in vivo*, similar results were found in an *Arabidopsis* mutant characterized by a defective MGDG synthase gene (MGD1). The amount of MGDG in this mutant is reduced by 42% compared with the wild type. The MGDG deficiency of the mgd1 mutant is correlated with striking defects in chloroplast ultrastructure (36). A similar observation was made in an *Arabidopsis* mutant dgd1 where DGDG lipid content undergoes a 90% reduction compared to the wild type (37).

In contrast to electron flow activity, Chl fluorescence was the most sensitive photosynthetic parameter to be affected by a treatment of thylakoids with PM-α-CD. Indeed, the ratio F695/F740 decreased from 1.45 in the control to 0.4 in the presence of 9.6 mM of PM-α-CD. Interestingly, the greatest decrease in the ratio occurred only when anionic lipids were removed by the CD, i.e., at a concentration of 4 mM PM-α-CD, about 50% of SQDG and 50% of PG were removed, whereas the pools of MGDG and DGDG were essentially intact. The decrease in the ratio F695/F740 was mainly due to the diminution of the fluorescence in PSII, thus suggesting that the removal of PG alters the organization of the light-harvesting Chl *a/b* protein complex (LHCII) (see Ref. 38 for a discussion of relations between the trimerization of LHCII, grana stacking, and light energy distribution in photosynthetic membranes).

These results also argue in favor of the existence of bulk and specific lipid molecules in the TM. The characteristics of these two types of molecules have recently been discussed (9,10). Thus, most of the PG and SQDG molecules that are removed from the outer monolayer at low concentrations of PM-α-CD (up to 4–5 mM) can be considered as being essentially bulk molecules since PSII and PSI electron flow activities are not impaired. However, one cannot exclude that certain specific anionic lipids, probably PG molecules, are also removed, thus altering the molecular organization of LHCII and its related fluorescence. At higher concentrations of PM-α-CD (>4 mM), the removal of only a few specific molecules of DGDG may change the conformation of LHCII proteins. Indeed, PG molecules have been reported to be essential in

the formation of LHCII trimers and its two- and three-dimensional crystallization. Furthermore, DGDG was found to bind to peripheral sites of LHCII (four molecules of DGDG per polypeptide) and to be crucial for the three-dimensional crystallization. Thus, specific PG and DGDG are both important molecules for the maintenance of the structure and function of LHCII (38–41). According to the spider-plot representation, the electron transfer (beyond Q_A^-) decreased per cross section (ETo/CSo) and remained constant per active reaction center (ETo/RC) in PM-α-CD-treated TM. Thus, it is concluded that the fraction of reaction centers (RC) inactivated (inactive RC/active RC + inactive RC) by the removal of lipids increased while the active RC remained intact but decreased in number. A few authors (14,15) have hypothesized how reaction centers can be inactivated. Certain bound SQDG and DGDG molecules are localized as prosthetic groups at the surface of the native D_1/D_2 heterodimer, holding the dimer together, whereas PG can be considered to be essential for the orientation and stabilization of the D_1-protein. Ionic interactions and van der Waal's forces in hydrophobic pockets or clefts might be the principal binding system between D_1 and PG. For instance, after PLA$_2$ treatment (14,15) or after removal of PG by PM-α-CD, the cleft could be closed and the function of the lipoprotein complex (D_1 (Q_B) and D_2 (Q_A) in the reaction center) obliterated. These changes might correspond to an increase of the fraction of inactivated reaction centers.

The capacity of CD to engulf lipids is the result of several properties of not only the host (i.e., CD) but also the guest (i.e., lipids) molecules. Concerning the host molecule, the number of glucopyranose units, and the diameter of the cavity (26), the nature of the chemical groups (hydroxyl and methoxy groups) at the edges of the cavity (27,35), the polarity of each cyclic unit molecule, and the distribution of the charges, etc., are relevant factors in understanding the ability of CD to form water-soluble guest-CD inclusion complexes (27). On the other hand, the chemical properties of the guest molecules, such as the global charges of lipid molecules, the electrostatic properties of the lipid headgroups, and the nature of the acyl chains of the lipids (length and degree of unsaturation), are also determinative for generating this engulfing process (24). In our case, the proportion of removable lipids originating from a membrane should also depend on the specific characteristics of the membrane where lipids are inserted, namely, the transmembrane distribution of lipids, the packing pressure of the membrane, and the accessibility of lipid molecules to CD. Although this investigation was not aimed at studying the host–guest interactions, our results may reveal information about this process.

First, let us consider the host characteristics: It has been reported that the permethylation of CD enlarges the whole cavity of the molecule (35). The methyl groups introduced into the O$_3$ position extend the O$_2$–O$_3$ side of the cavity and make narrower the O$_6$ side (27), thus favoring the formation of inclusion complexes. Our results are in agreement with this observation (Table 1). The removal of all four classes of lipids was increased in the presence of PM-α-CD compared with

α-CD as well as in the presence of 2,6 DM-β-CD compared with the β-CD. When considering the guest molecule, the presence of negative charge in the molecule (SQDG and PG) allowed a better interaction with the host molecule (Table 1). Interestingly, Debouzy *et al.* (24) used liposomes (multilamellar vesicles) to study the overall sensitivity of different phospholipids for their interactions with α-CD. They found that among the most reactive phospholipids appeared PI and PS, which are both anionic lipids. However, PE, but not sphingomyelin and PC, which are characterized by a zero global charge, is also reactive toward α-CD. Obviously, the global charge of lipids appears not to be the only criterion influencing the formation of the host-guest complex. On the other hand, the nature of the acyl chains of the anionic lipids is apparently not decisive for the recognition of the guest by the host (Fig. 3). An intriguing question concerns the high concentration of PM-α-CD (up to 5 mM) necessary to initiate the removal of galactolipids from the membrane. As discussed by Bekers *et al.* (27), it may be possible that more than one molecule of the host is necessary to find complete accommodation within the inclusion complex. In addition to that, the lack of global charges in galactolipids may require higher concentrations of PM-α-CD.

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