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Cyclodextrins: a new tool for the controlled lipid depletion of thylakoid membranes [1]

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Abstract

Cyclodextrins (CDs) have been used in a controlled lipid depletion of thylakoid membranes avoiding the use of either detergents or lipolytic enzymes. Spinach thylakoid membranes were first treated with different CDs under various conditions. After removal of the CDs by washing, the amounts of mono- and digalactosyldiacylglycerol (MGDG and DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG), protein, pigment and plastoquinone remaining in the membranes were determined. The main results, obtained with α-CD and heptakis-(2,6-di-*O*-methyl)-β-CD (DM-β-CD), were as follows. (1) Acyl lipids were removed from thylakoid membranes by both CDs (DM-β-CD being more efficient than α-CD); the extent of removal depended on both CD and chlorophyll concentrations. (2) α-CD presented a higher selectivity towards lipid classes than did DM-β-CD, but in both cases the removal order was SQDG > PG > MGDG > DGDG. (3) α-CD showed a preference for those lipids containing saturated 16-carbon acyl chains whereas DM-β-CD was essentially insensitive to the fatty acid composition of the lipids. (4) The protein, chlorophyll and carotenoid contents of thylakoids were not affected by CD treatments. (5) Plastoquinones were removable but in small amounts only and with a low efficiency (DM-β-CD > α-CD). (6) For all lipid classes, the extent of lipid removal was higher at 0° than at 20°C. (7) The presence of $MgCl_2$ reduced the removal of PG and SQDG but did not affect galactolipid depletion levels. (8) Stable lipid depletion levels in thylakoid membranes were reached after 5–10 min of CD treatment at 0°C. (9) Of the four CDs tested, only three (α-CD, β-CD, and DM-β-CD) promoted lipid depletion whereas one (hydroxypropyl-β-CD) failed completely to do so. It is concluded that CD-mediated lipid removal provides a valuable and versatile tool to achieve controlled and specific lipid depletions in biological membranes. A few examples of the consequences of a CD-induced lipid depletion on fluorescence and electron transport properties of thylakoids are given to show the usefulness of CDs in the investigation of structure–function relationships in photosynthetic membranes.

Keywords: Cyclodextrin; Lipid depletion; Thylakoid membrane

1. Introduction

The multiple functions carried out by thylakoid membranes (light capture and distribution, charge separation, redox reactions of the electron transport chain, generation of a protonmotive force, ATP synthesis) are supported by a very complex array of protein, lipid and pigment molecules. Within this array, protein–protein and lipid–protein interactions play important roles. A key factor in lipid–protein interactions is the membrane lipid to protein ratio, as it determines the proportion of the total membrane lipids which may be in close contact and interaction with membrane proteins.

The study of the molecular organization of acyl lipids and of their involvement in thylakoid membrane function calls for the availability of suitable (bio)chemical tools aimed at altering the lipid content and the lipid class and fatty acid composition of these membranes. Classical approaches, designed for lipid depletion, include (i) solvent extraction of (freeze-dried) membranes [1,2] (ii) mild solu-

Abbreviations: CD, cyclodextrin; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; DM-β-CD, heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin; HP-β-CD, hydroxypropyl-β-cyclodextrin; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; SDS, sodium dodecylsulfate; SQDG, sulfoquinovosyldiacylglycerol; Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; acyl chains are noted *n*:*x* where *n* = number of carbon atoms and *x* = number of double bonds; 16:1(3*t*), 3-*trans*-hexadecenoic acid.

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[1] This article is dedicated to the memory of Laurens L.M. van Deenen, with gratitude for his outstanding achievements and contributions to lipid and biomembrane sciences.

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bilisation of membrane components by detergents [3–6], both often followed by reconstitution with synthetic or native lipids, and (iii) controlled lipolytic treatment of thylakoids by specific enzymes (phospho- and galactolipases, acyl hydrolases), followed by removal of hydrolysis products by defatted bovine serum albumin ([7] and references therein; [8–13]). Other approaches involve (iv) lipid enrichment of thylakoids by fusion with liposomes of known composition [14–16] and (v) lowering the unsaturation of acyl chains by in situ catalytic hydrogenation of membrane lipids [17–20].

These techniques have various drawbacks. For instance, solvent extraction is restricted to relatively non-polar lipids because the solvents usually employed are themselves non-polar. Detergent treatment suffers from a lack of specificity since it invariably removes proteins and pigments in addition to membrane lipids; moreover, undesirable detergent molecules are substituted for lipids within the membrane. Lipolytic enzymes not only decrease the amount of a given lipid class but also change the qualitative membrane lipid composition by generating new products (e.g., free fatty acids, lysolipids and diacylglycerols). These products, which are difficult to remove, can lead to changes in the remaining lipid–protein interactions. Liposome-mediated lipid enrichment of thylakoids is not easily controllable and prone to back-extraction of thylakoid lipids into liposomes. Catalytic hydrogenation in situ always leaves some catalyst molecules within the membrane. Also, the decrease in the double bond index is often accompanied by the formation of artifactual *trans*-unsaturated isomers. In practice, there is no technique currently available which removes lipid molecules from thylakoid membranes without introducing foreign compounds in and/or altering the pigment and protein content of these membranes.

This has prompted us to explore the possibility of altering the lipid composition of thylakoid membranes by using CD compounds. These molecules are cyclic oligosaccharides consisting of 6, 7 or 8 glucopyranose units linked by $\alpha(1 \rightarrow 4)$ bonds. They adopt a torus shape and are able to bind a range of small guest molecules of poor water solubility within their hydrophobic cavity to form a water-soluble guest-CD inclusion complex [21–24]. A variety of lipid molecules have been shown to form such complexes with CDs [25–29]. Though widely used in the biomedical and galenic fields ([22–24] and references therein; [30–35]), CDs have not yet been examined as lipid complexing agents for lipid depletion purposes in structural and functional studies of plant membranes. In this paper, we analyse the lipid depletion potential of some common CDs towards spinach thylakoid membranes. The effect of various physico-chemical parameters on lipid removal and the consequences of such depletions on thylakoid functions have also been studied. Our results indicate that CDs are likely to be very useful tools in photosynthetic membrane research.

2. Material and methods

2.1. Materials

The α-, β-, and DM-β-CD were obtained from Fluka; HP-β-CD (with an average molar substitution of 0.9) was from Aldrich. All other chemicals were high purity products from either Fluka or Sigma. Spinach leaves were purchased from the local market. They were washed, stored at 4°C and used within 3 days.

2.2. Methods

Thylakoid membranes were prepared from highly intact chloroplasts by osmotic shock and then purified essentially as described in [12]; they were suspended in ice-cold medium I (300 mM sorbitol/10 mM Tricine-KOH, pH 7.8) or medium II (medium I + 10 mM $MgCl_2$) and used immediately.

Unless otherwise stated, CD treatments were carried out as follows. Thylakoid membranes (75 μg chlorophyll/ml in medium I or II) were incubated for 10 min at 0°C in the presence of the desired CD and pelleted (still at 0°C) at $8000 \times g$ for 5 min. The supernatants (containing free CD and water-soluble lipid-CD complexes) and the pellets were carefully separated and analysed according to need.

Acyl lipids were extracted, separated by thin-layer chromatography, recovered and their sugar or phosphorus content determined [36]. Alternatively, the amount of acyl lipids was determined after separation of their fatty acid methyl esters by gas chromatography on a 30×0.53 mm capillary column coated with 1 μm crosslinked FFAP (Hewlett-Packard). Arachidic acid was used as an internal standard.

Spectrophotometric techniques were used for the quantitative determination of plastoquinones [37], pigments [38] and proteins [39]. Polyacrylamide gel electrophoretic separation of thylakoid polypeptides in the presence of SDS was carried out as in [40].

Electron transport activities through photosystems II + I and through photosystem I alone were measured as in [8]. The 77 K fluorescence emission spectra of thylakoids were determined according to [41]. The kinetics of the Mg^{2+}-induced fluorescence increase were measured at 20°C essentially as in [42]. Additional details can be found in the legends to the figures and tables.

3. Results

Preliminary experiments, together with solubility considerations [24] led us to select α-CD and DM-β-CD for further investigation. In a few cases, other CDs were also used.

3.1. Lipid removal by CDs: general characteristics

When spinach thylakoid membranes were incubated with increasing concentrations of α-CD or DM-β-CD at 0°C for 5 min, lipid removal occurred (Fig. 1). The efficiency of this removal depended on the CD type and concentration, and also on the nature of the lipid extracted.

Both CDs removed acyl lipids, but the efficiency of the process (measured by the initial slope of the curve) was approximately three times higher with DM-β-CD (Fig. 1B) than with α-CD (Fig. 1A). In the case of α-CD, plots of concentration dependence of the removal of acyl lipids displayed a slightly sigmoidal character and reached a depletion value of 61% for an α-CD concentration of 20 mM (Fig. 1A). In the case of DM-β-CD, the efficiency of removal of acyl lipids was also sigmoidal, growing steadily with concentration up to 10 mM, then levelled off progressively to reach a maximum value of 80% between 30 and 50 mM (Fig. 1B).

Chlorophylls and carotenoids were not extracted from thylakoids by either CD (Fig. 1). Plastoquinones were removed by DM-β-CD, but with a low efficiency: 6% of the total plastoquinones were taken up at 10 mM and a maximal depletion of about 30% was reached at 35 mM (Fig. 1B). Minimal amounts ($\leq 5\%$) of plastoquinone were released by α-CD (Fig. 1A).

As the chlorophyll content of the membrane remains unaltered by the CD treatments, the effect of CD-induced lipid depletions on the protein content of thylakoids can readily be estimated by comparing the resulting protein/chlorophyll weight ratio in control and treated membranes. This ratio was 6.2 ± 0.7 ($n = 15$) in control membranes and 6.1 ± 0.8 ($n = 18$) in thylakoids treated with up to 20 mM α- or DM-β-CD, indicating that little or no protein loss occurs. This was confirmed by examining the effect of CD-induced lipid depletion on the polypeptide patterns of control and treated membranes. Thylakoid membranes were first treated either with α-, β- or DM-β-CD. Their polypeptides were then separated by SDS-polyacrylamide gel electrophoresis. No differences were detected after staining the gels with Coomassie Blue. Densitometric gel scanning followed by image processing provided quantifiable profiles from which we selected data relating to two intrinsic polypeptides belonging to the main light-harvesting complex (LHC_2-a and LHC_2-b, [43]) and two extrinsic polypeptides (α- and β-subunits of the coupling factor CF_1 complex [44]). As illustrated in Fig. 2, the relative proportions of these four polypeptides were similar in control and treated membranes, irrespective of the type of CD used and the extent of lipid removal achieved.

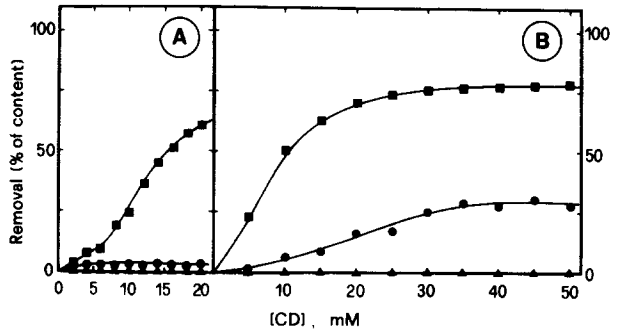

Fig. 1. Removal of thylakoid membrane components by increasing α-CD (A) or DM-β-CD (B) concentrations. Thylakoids were suspended in medium I. After treatment (see Section 2), membranes were extracted and analysed for total acyl lipids (■), plastoquinones (●) and chlorophyll + carotenoid content (▲). Removal was expressed as percentage of the content in control membranes. Total lipid and plastoquinone amounts in control thylakoid membranes were 2500 and 100 nmol/mg chlorophyll, respectively.

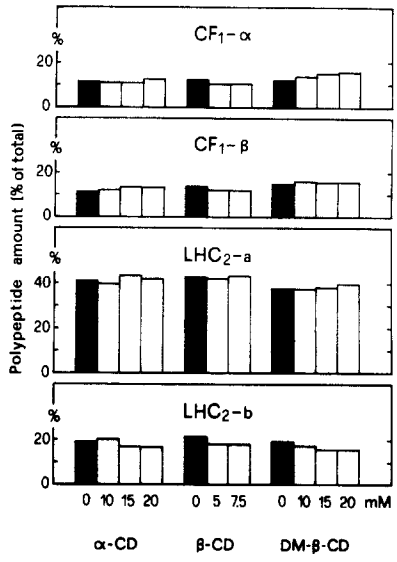

Fig. 2. Effect of different CD treatments of thylakoids on the relative proportions of two main extrinsic (α- and β-subunits of CF_1) and two main intrinsic (LHC_2-a and LHC_2-b) polypeptides. Thylakoid membranes (50 μg chlorophyll/ml in medium I) were incubated at 0°C for 30 min in the presence of α-CD, β-CD or DM-β-CD at the specified concentrations. Control and treated membranes were then analysed for total lipid content and polypeptide pattern (see Section 2). Quantitative densitometry of the Coomassie Blue-stained gels was done with the Millipore BioImage System. The amount of each polypeptide is expressed as percentage of the total protein amount in the corresponding lane. The extent of lipid removal was (in order of increasing concentrations) 0, 33, 53 and 60% for α-CD; 0, 20 and 35% for β-CD and 0, 53, 65 and 71% for DM-β-CD.

It was expected that the extent of lipid removal should depend on the CD/chlorophyll ratio. Thylakoids were treated with DM-β-CD over the same concentration range but at different membrane (chlorophyll) concentrations. After washing and centrifugation, the lipid content of pellets was measured and the amount of CD-solubilised lipids was calculated. Two typical lipid solubilisation curves are presented in Fig. 3. As might be expected, the concentration of DM-β-CD required for maximal lipid extraction was higher at higher membrane concentrations. The relative amount of solubilised lipids was appreciably greater at low than at high membrane concentration. At the low membrane concentration, up to 80% of the lipids (equivalent to 2080 nmol lipids/mg chlorophyll) were

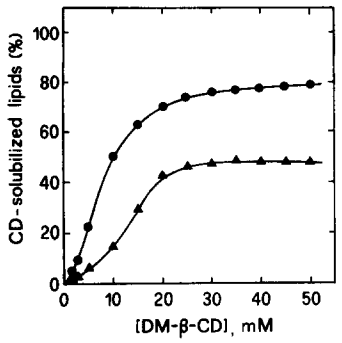

Fig. 3. Influence of the CD/chlorophyll ratio on the relative amount of solubilised lipids from thylakoid membranes treated with increasing DM-β-CD concentrations. Thylakoids were suspended in medium I to a concentration of 75 (●) or 500 (▲) μg chlorophyll/ml and incubated at 0°C for 30 min with the specified DM-β-CD concentration. The suspension was washed by centrifugation and the lipid content of pellets was measured. The amount of solubilised lipids was then calculated and expressed as percentage of the lipid content of control membranes.

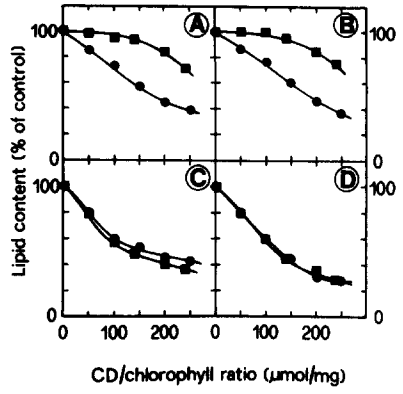

Fig. 5. Changes in the relative amounts of individual diacyl lipid classes of CD-treated thylakoid membranes. Thylakoids were suspended in medium I and incubated at 0°C for 30 min in the presence of various amounts of α-CD (■) or of DM-β-CD (●). The chlorophyll concentration was 50 μg/ml with α-CD and 75 μg/ml with DM-β-CD. Lipids were extracted from treated and control membranes, separated into lipid classes and their amount determined by gas chromatography. For each of the four lipid classes, the control amount is given (nmol lipid/mg chlorophyll). A, MGDG (1300); B, DGDG (685); C, PG (260); D, SQDG (206).

removed. In contrast, at the high membrane concentration, the final depletion level was only 48% (equivalent to 1240 nmol lipids/mg chlorophyll).

The time dependence of lipid removal in thylakoid membranes treated with either 15 mM α-CD or DM-β-CD is presented in Fig. 4. In both cases, the total lipid content of thylakoids declined dramatically and the depletion (55% with α-CD and 68% with DM-β-CD) was essentially completed after 5–10 min of CD treatment. Similar removal kinetics were obtained both at lower and higher CD concentrations, except that the final levels reached differed accordingly (not shown).

3.2. Lipid class specificity

Different CDs showed different efficiencies for the extraction of the different thylakoid membrane lipids. Data illustrating how increasing concentrations of α- or DM-β-CD change the relative amounts of the lipids remaining in such membranes are presented in Fig. 5. Using α-CD, MGDG (Fig. 5A) and DGDG (Fig. 5B) removal started only when the CD/chlorophyll ratio exceeded 50. Both galactolipids were then removed with progressively increasing efficiency as the CD/chlorophyll ratio was increased. No significant difference was observed for MGDG and DGDG. In both cases, 25–30% of these lipids were removed at a final CD/chlorophyll ratio of 240. Under the same conditions, the anionic lipids PG (Fig. 5C) and SQDG (Fig. 5D) were easily extracted from thylakoid membranes even at the lowest CD/chlorophyll ratio employed. In this case, a biphasic removal pattern was observed, consisting of an initial highly efficient depletion, followed by a second phase of decreasing efficiency. No differences between PG and SQDG were seen in the first phase of the extraction process (up to a CD/chlorophyll ratio of 100), but the final extent of lipid removal was consistently greater for SQDG (70–75%) than for PG (58–63%).

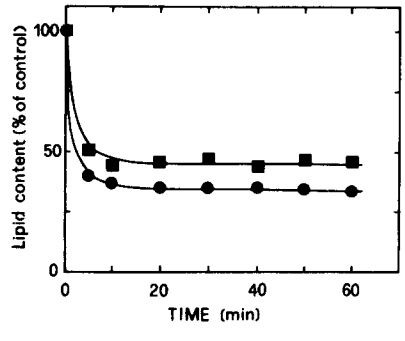

Fig. 4. Kinetics of CD-induced lipid depletion in thylakoid membranes (50 μg chlorophyll/ml in medium I) incubated at 0°C in the presence of 15 mM α-CD (■) or DM-β-CD (●). In both cases, the CD/chlorophyll ratio was 300 μmol/mg.

If thylakoid lipid depletion was carried out using DM-β-CD, the galactolipids were efficiently removed, even at low CD/chlorophyll ratios. The overall delipidation extents at a CD/chlorophyll ratio of 250 were also appreciably higher than for α-CD. Again, no noticeable differences were observed between the removal curves of the two galactolipids. The removal curves of PG and SQDG were very similar to those obtained with α-CD.

The lipid removal capacity and selectivity of various CDs were also compared, alone or in combination. A single incubation condition was selected and the extent of lipid depletion after treatment of thylakoid membranes with α-CD, β-CD, DM-β-CD, HP-β-CD and by the couple (α-CD + β-CD), all individually present at a CD/chlorophyll ratio of 100, was determined. The lipid removal capacity results are listed in Table 1 and a com-

Table 1
A comparison of the removabilities of lipid classes in thylakoids treated by various CDs under the same conditions

CD used	Lipid removal (mol%)				n
	MGDG	DGDG	PG	SQDG	
α-	5.4±4.8	5.4±2.7	39.1±7.4	42.4±2.1	(4)
β-	13.9±2.4	14.5±3.0	12.5±2.6	8.1±3.5	(4)
DM-β-	28.1±3.5	17.8±2.1	38.3±2.8	43.7±6.4	(4)
HP-β-	tr	tr	tr	tr	(1)
α-+β-	27.7±5.0	27.0±5.5	54.4±5.9	60.0±6.4	(6)

Thylakoid membranes (50 μg chlorophyll/ml) were incubated in medium I at 0°C for 30 min, each CD being individually present at 5 mM, thus giving a CD/chlorophyll ratio of 100. The amount of each lipid class removed by CD treatment is expressed as percentage of the corresponding lipid class content of control membranes. Data are given as the mean value of n experiments ± standard deviation. tr, traces(≤ 1%).

parison of the lipid class composition of the thylakoid membranes after CD treatment performed under these conditions is presented in Table 2. It can be seen that α-CD and, to a lesser extent, DM-β-CD displayed a preference for PG and SQDG over galactolipids (see also Fig. 5). In contrast, β-CD removed all lipid classes to a comparable extent. A striking feature of these results was the failure of HP-β-CD to promote lipid depletion in thylakoid membranes. Interestingly, when α- and β-CD were used together, their effect was additive for PG and SQDG, but synergic for galactolipids (Table 1). However, the relative preference of α- and DM-β-CD for PG and SQDG, though real, did not bring about significant changes in the relative lipid composition of CD-treated thylakoids (Table 2).

The influence of temperature and of $MgCl_2$ on the extent of lipid removal from thylakoid membranes were

Table 2
Lipid class composition of thylakoid membranes after the CD treatments performed as described in Table 1

CD used	Removal extent (%) [a]	Lipid composition of treated membranes (mol%)			
		MGDG	DGDG	PG	SQDG
None	0	53.1	28.0	10.6	8.3
α-	12	57.1	30.0	7.3	5.4
β-	14	53.3	27.9	10.9	7.9
DM-β-	6	52.8	31.8	9.0	6.4
HP-β-	0	53.1	28.0	10.6	8.3
α-+β-	33	57.3	30.5	7.3	4.9

Each lipid composition has been recalculated as follows. (1) The percentage of each lipid class remaining in the membrane after the CD treatment (P_{rem}) was obtained by subtracting its removal percentage (Table 1) from 100%. (2) The P_{rem} value, multiplied by the absolute amount (nmol/mg chlorophyll) of this lipid in control membranes (given in the legend to Fig. 5) gave the absolute amount A_{rem} of this lipid remaining in the membrane after CD treatment. (3) The A_{rem} values were calculated for each lipid class, summed up to give the total lipid amount T_{rem} and expressed as percentages of T_{rem}.

[a] Percent of the total lipid content of control membranes.

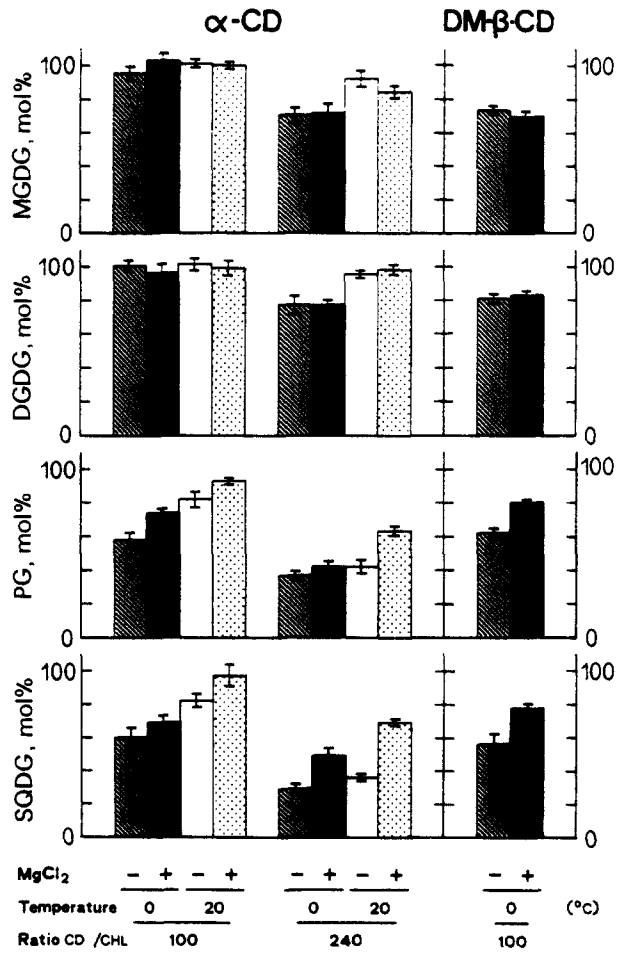

Fig. 6. Effect of temperature and of $MgCl_2$ on the amount of lipids remaining in thylakoids treated with α-CD with by DM-β-CD. Thylakoids (75 μg chlorophyll/ml) were suspended in medium I (−$MgCl_2$) or II (+10 mM $MgCl_2$) and incubated at 0° or 20°C for 30 min in the presence of α-CD or DM-β-CD at the specified CD/chlorophyll ratios. Thylakoids were spun down and the amount of their residual lipid classes was quantitatively determined and expressed in mol% of the corresponding amount in control membranes. The four graphs are, from top to bottom, those of MGDG, DGDG, PG and SQDG. Bars indicate the mean value of n experiments ± standard deviation and correspond to the following conditions: 0°C in the absence (hatched bars) and in the presence (black bars) of $MgCl_2$; 20°C in the absence (open bars) and in the presence (dotted bars) of $MgCl_2$. The number n of experiments carried out was (from left to right) 7, 3, 4, and 3 for α-CD at both ratios; for DM-β-CD, it was 2 and 2.

studied. Selected data from these studies are presented in Fig. 6. When α-CD was used at the relatively low CD/chlorophyll ratio of 100, galactolipid removal was negligible and no influence of either temperature or $MgCl_2$ was observed. However, the removal of both PG and SQDG was significantly decreased both when temperature was increased and when $MgCl_2$ was present. These two effects were complementary to each other. For instance, at the α-CD/chlorophyll ratio of 100, less than 10% of PG and almost no SQDG could be extracted at 20°C in the presence of $MgCl_2$. When α-CD was used at the higher ratio of 240, appreciable galactolipid removal occurred.

Table 3
Fatty acid composition of total lipids in thylakoid membranes before and after two CD treatments which promote a comparable lipid depletion

Fatty acid	mol% of total fatty acids in the presence of		
	none	α-CD	DM-β-CD
16:0	5.8±0.2	3.8±0.2	5.8±0.2
16:1(3*t*)	4.1±0.3	2.7±0.2	4.1±0.3
16:3	15.1±0.5	13.8±0.5	14.1±0.7
18:0	0.6±0.1	0.6±0.2	0.8±0.2
18:1	1.0±0.7	1.1±0.7	tr
18:2	1.3±0.1	1.3±0.2	1.3±0.2
18:3	72.8±0.2	76.7±0.4	73.6±0.3

Thylakoid membranes were suspended (75 μg chlorophyll/ml) in medium I and incubated for 5 min at 0°C with 10 mM α-CD or with 5 mM DM-β-CD. The subsequent depletion extents (in mol%) were respectively 25±2 and 24±4. The overall fatty acid composition of the remaining lipids was determined by gas chromatography. Data are reported as the mean value of four separate experiments ± standard deviation and are expressed in mol% of total fatty acids. tr, traces (< 0.5 mol%).

The extent of extraction was insensitive to $MgCl_2$ and depended only on the temperature, less galactolipids being removed at 20° than at 0°C. The extraction efficiency of PG and SQDG, in contrast, was relatively insensitive to temperature when $MgCl_2$ was absent from the incubation medium. In the presence of $MgCl_2$, PG and SQDG depletion was reduced at the higher temperature. If DM-β-CD was used instead of α-CD, the presence of $MgCl_2$ had no effect at 0°C on the extent of galactolipid removal, but the removal of PG and SQDG was again reduced.

3.3. Fatty acyl chain specificity

An attempt was made to see whether CD-induced lipid depletion results in the preferential removal of lipid molecules containing particular acyl chains. The total fatty acid compositions of spinach thylakoid membranes were compared before and after delipidation. The fatty acid composition of each individual lipid class was then determined. In each case, the thylakoids were treated with either α-CD or DM-β-CD under conditions which yielded comparable depletion extents in terms of total lipids. In order to avoid any limitation in lipid availability due to the different transmembrane distributions of the lipids [9], care was taken to keep this depletion well below the 50% level. Table 3 shows that after a CD-induced lipid depletion of about 25%, small but significant changes in the overall fatty acid composition of thylakoid membranes were observed after α-CD treatment, but not after DM-β-CD treatment. These changes consisted of a small increase in 18:3 at the expense of 16:0 and of 16:1(3*t*). Studies of the fatty acid composition of individual thylakoid lipid classes (results not shown) revealed no change other than a small decrease in the molar ratio of 16- to 18-carbon fatty acids in MGDG, PG and SQDG, after α-CD treatment.

3.4. Functional implications

The CD-induced lipid depletion of thylakoid membranes has important implications for their function. This will be the topic of a forthcoming paper, however, a few preliminary examples of such effects are presented in Fig. 7. Spinach thylakoids were treated with a mixture of α- and β-CD (5 mM each at 0°C for 10 min), washed and analysed. In spite of a 35% depletion of their total lipids, the treated thylakoids showed an unaltered polypeptide profile with respect to control membranes (results not shown).

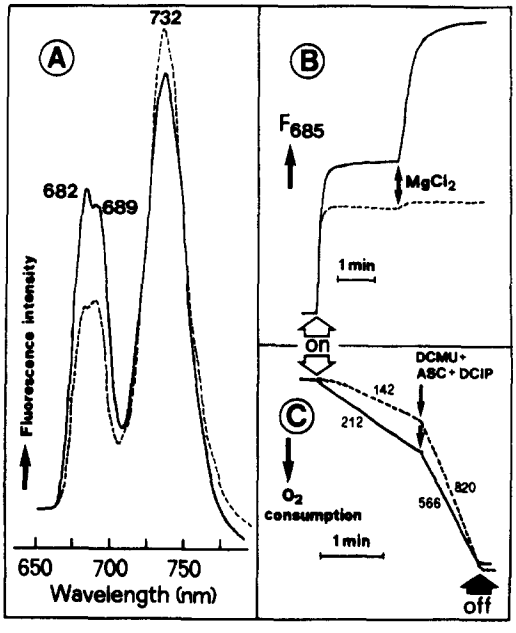

Fig. 7. Effect of a treatment of thylakoid membranes by a mixture of α- and β-CD on (A) the 77 K fluorescence emission spectrum, (B) the kinetics of the Mg^{2+}-induced increase in chlorophyll fluorescence (F_{685}) at 20°C and (C) the electron transport activities through both photosystems II+I or through photosystem I alone. The thylakoids were suspended in medium I (50 μg chlorophyll/ml) and incubated at 0°C for 10 min in the absence (————) or in the presence (---) of both α-CD and β-CD together (5 mM each). Thylakoids were washed and resuspended in medium I. Aliquots were used for lipid class analysis and determination of the fluorescence and electron transport properties. Under these conditions, the lipid removal (expressed as mol % of each lipid class) was for MGDG (30), for DGDG (32), for PG (46) and for SQDG (58); this corresponds to a depletion of 35% of the total lipids. In (A), membrane samples equivalent to 2 μg chlorophyll were diluted in 25 μl 80% glycerol and frozen in liquid nitrogen. The spectra were recorded as uncorrected responses of the photomultiplier. In (B), the stirred reaction mixture contained thylakoids (5 μg chlorophyll/ml) and 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in 3 ml of medium I. $MgCl_2$ was then added to a final concentration of 10 mM. In both (A) and (B), fluorescence was excited at 480±10 nm. In (C), electron transport activity through both photosystems II+I (uncoupled by 10 mM NH_4Cl) was measured by the methylviologen-catalysed oxygen consumption with water as electron donor; photosystem I activity was measured in the same system but in the presence of 5 μM DCMU (to inhibit photosystem II) and of the couple Na^+-ascorbate (ASC, 3 mM)+2,6-dichlorophenolindophenol (DCIP, 0.4 mM) as electron donor. Numbers on the curves express the oxygen consumption rates in μmol (mg chlorophyll)$^{-1}$ h^{-1}.

The 77 K fluorescence spectrum of the treated sample, however, showed a significant increase of the $F_{732}/F_{682-689}$ emission ratio from 1.4 in control to 2.4 in treated membranes, and the relative height of the F_{682} and F_{689} peaks was reversed (Fig. 7A). Moreover, the intensity of fluorescence emission at 20°C measured in the presence of DCMU as a function of time was characterised by a lower level of the initial fluorescence signal (F_{685}) and by the subsequent failure of Mg^{2+} to increase the F_{685} value in the delipidated membranes (Fig. 7B). Finally, the behaviour of the electron flow activity was also affected by the lipid depletion, as illustrated in Fig. 7C. The first phase of the trace, reflecting the linear, NH_4Cl-uncoupled H_2O → methylviologen electron flow activity through both photosystems II + I, showed an inhibition (−33%) in lipid-depleted thylakoids whereas the second phase, reflecting photosystem I-specific electron transport, showed a stimulation (+45%).

4. Discussion

The data presented in this paper indicate that CD treatment of thylakoid membranes results in acyl lipid removal. However, in contrast to earlier work carried out on unilamellar vesicles prepared with a total thylakoid lipid extract [45], neither chlorophylls nor carotenoids were removed from the native membranes (Fig. 1). This tends to confirm the view that these pigments are closely associated with the intrinsic polypeptides of the thylakoid membranes. The protein/chlorophyll ratios and the relative proportions of two main extrinsic (CF_1-α and CF_1-β subunits) and intrinsic (LHC_2-a and LHC_2-b) polypeptides (Fig. 2) were unchanged by CD treatment. Our results indicate that removal of as much as 70% of the membrane lipids leads to no significant losses of protein from these membranes. This is in marked contrast to methods using detergents or lipolytic enzymes [46,47]. While there is evidence of some loss of plastoquinone, particularly following DM-β-CD treatment, their extraction was of much lower efficiency than that of the acyl lipids (Fig. 1). This reflects the fact that formation of the plastoquinone-CD complex is likely to be limited for steric reasons and/or because the water-soluble CDs have a restricted accessibility to the apolar domain of membranes [32].

The formation of inclusion complexes between CDs and hydrophobic molecules such as lipids is an equilibrium process [21,22]. It was therefore expected that the highest relative extent (in mol%) of lipid removal would occur when a high CD concentration was used in combination with a low membrane concentration. This has been verified using DM-β-CD (Fig. 3). The upper limit of removal was 80%. This value could not be increased by using lower membrane concentrations (results not shown). The residual lipids (about 20%) which escaped DM-β-CD action under these conditions probably correspond to molecules experiencing strong interactions with other membrane components such as proteins, or to lipid molecules which are unable to undergo transbilayer movement [10,11].

The lipid solubilisation curve at high membrane concentration (Fig. 3, black triangles) cannot be explained in these terms. If lipid availability was the only factor that limits the extent of lipid removal, one would have expected this curve to go on rising and approaching asymptotically the 80% value, instead of stopping at a value of 48%. We interpret this curve as reflecting a condition under which the maximal solubility of the lipid-DM-β-CD complexes has been reached. Thus, at high membrane concentration, lipid molecules are still being extracted from the membranes above 20 mM DM-β-CD, leading to an effective depletion extent that should come close to the 80% value; however, when the concentration of soluble inclusion complexes reaches its limit value, complexes in excess of this value precipitate and cosediment with thylakoid membranes. This will artifactually lower the removal extent to an apparent level which depends only on the membrane concentration.

The data presented in Fig. 3 indicate that a maximum of 1240 nmol of acyl lipids/mg chlorophyll can be solubilised under our conditions by DM-β-CD. Given a sample volume of 2 ml, this corresponds to 620 nmol of complexed lipids per ml, which is equivalent to 0.62 mM. Thylakoids usually contain about 2500 nmol of acyl lipids/mg chlorophyll [9]. This means that for the lipid depletion level of 80% to be achieved with DM-β-CD, the concentration of the thylakoidal suspension needs to be kept below 0.3 mg chlorophyll/ml.

Our results indicate that CD treatment of thylakoids brings about a compositional change in these membranes which is remarkably specific for acyl lipids. The method has the advantage that unlike the lipid depletions achieved using detergents [3–6] or lipolytic enzymes [10,46,47], it does not involve the introduction of foreign lipid molecules into the membrane. A further advantage of CDs is that they show no tendency to bind to biological membranes [32]. Another very favorable aspect of the CD-induced lipid removal is the short time (5–10 min) required to reach an equilibrium between depletion extent and formation of the lipid*CD inclusion complexes (Fig. 4). This value is similar to that reported for β-CD-mediated cholesterol removal from human erythrocytes [32,33]. This compares with incubation times of 30–90 min required for detergent or lipolytic treatments ([7] and references therein). An attractive feature of the method is that a given depletion extent can be preselected and obtained simply by working at the appropriate CD/membrane ratio, as explained above. This is particularly valuable when studying the consequences of very low depletion extents, which are difficult to control in a reproducible way when detergents or lipolytic enzymes are used.

CDs are capable of extracting all four main lipid classes (MGDG, DGDG, PG and SQDG) of thylakoid membranes

(Fig. 5 and 6, Table 1). Some element of specificity can, however, be introduced by choice of the most suitable CD and appropriate manipulation of the extraction conditions. Both the cavity diameter and the nature of the substituent group bound to the hydroxyl oxygen atoms of CD molecules play key roles in determining the specificity of lipid extraction (Table 1).

We have observed that the extent (and specificity) of CD-induced lipid depletion depended on the temperature (Fig. 6). The main effect of increasing the extraction temperature was to cause a general reduction in the extent of lipid depletion. This probably reflects the normal influence of temperature on complex formation equilibria [22,24]. This effect may be used to decrease galactolipid removal by α-CD and thus to increase its lipid class selectivity for PG and SQDG. This, however, can only be achieved at the expense of a substantial loss in removal efficiency. On the other hand, working at low temperature offers the advantage of a higher lipid removal efficiency combined with an increased stability of membrane structure and function.

The other variable studied in this investigation was the effect of $MgCl_2$. Addition of $MgCl_2$ decreases the extent of removal of the anionic lipids PG and SQDG by both α- and DM-β-CD (Fig. 6). This effect does not appear to reflect a general reduction in the accessibility of membrane lipids to CDs due to the $MgCl_2$-induced stacking of thylakoid membranes, as the efficiency of the removal of the galactolipids remains unaltered. The effect of Mg^{2+} is more likely to be due to an electrostatic effect of the divalent cation Mg^{2+} on the negatively charged headgroups of PG and SQDG. Ionic bridges can be formed between a Mg^{2+} cation and two anionic lipids; alternatively, individual PG and SQDG molecules can be bridged to negatively charged amino acid residues of membrane polypeptides by a Mg^{2+} cation. In either case, the increased size of the resulting aggregates will protect them from CD action.

How may the structural properties of thylakoid membranes be affected by such lipid removal? Contrary to depletions achieved with lipolytic enzymes [9,11,12], CD-induced lipid depletion does not greatly alter the general balance between bilayer-forming (DGDG, PG, SQDG) and nonbilayer-forming (MGDG) lipids in thylakoid membranes, as the relative proportions of residual lipid classes were almost unaffected (Table 2). CD treatment may however change the barrier properties of membranes, as it removes (part of) their sealing material. In addition, the fluidity of the thylakoid membrane may be altered by CD treatment, as the lipid/protein ratio (believed to control the fluidity of thylakoid membranes [48–50]) is decreased. Also, lipid depletion is expected to decrease the relative distances between the supramolecular complexes of thylakoid membranes by bringing them in closer proximity.

Removal of lipid molecules from thylakoid membranes has major effects on the function of these membranes, as illustrated in Fig. 7. The changes in the fluorescence parameters both at low temperature (Fig. 7A) and room temperature (Fig. 7B), as well as the changes in electron transport rates (Fig. 7C), indicate that the distribution of excitation light between the two photosystems has been markedly altered by the CD treatment. These changes are likely to occur as a consequence of the structural modifications mentioned above.

In conclusion, we have shown that CDs can be used as tools for the specific delipidation of thylakoid membranes, without altering either their pigment or their protein content. Depending on the concentration range and on the type of CD used, a certain degree of class selectivity in the removal of acyl lipids can be expected. Because the use of detergents tends to solubilise all membrane components, lipid depletion of biological membranes has been usually achieved by lipolytic treatments or solvent extraction of (freeze-dried) membranes. The introduction of CD-induced lipid depletion will valuably complement these earlier techniques whenever rapidity, simplicity and reasonable selectivity are required. Increased selectivity may still be expected by modulating the complexing potential of CDs via the preparation of suitable derivatives (using the many free hydroxyl groups of CDs) by appropriate chemistry. Promising applications of these new tools may be envisaged, e.g., in membrane sidedness studies, in the fine control of the degree of membrane lipid depletion for functional studies, in the preliminary delipidation before solubilisation of membrane proteins or also in the transfer/exchange of lipid molecules between membranes and/or artificial lipid vesicles.

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