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Reassembly of solubilized chlorophyll-protein complexes in proteolipid particles – comparison of monogalactosyldiacylglycerol and two phospholipids

Dorothea Siefermann-Harms [a], Helga Ninnemann [a] and Harry Y. Yamamoto [b]

[a] *Institut für Chemische Pflanzenphysiologie der Universität, Tübingen (F.R.G.)*
and [b] *Department of Plant Molecular Physiology, University of Hawaii at Manoa, Honolulu, HI (U.S.A.)*

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Formation and properties of chlorophyll-proteolipid particles assembled from Triton-X-100-solubilized spinach thylakoid membrane proteins and monogalactosyldiacylglycerol (MG), or phosphatidylcholine (PC), or phosphatidylethanolamine (PE), were compared. MG particles differed from phospholipid particles in three respects. (1) Chlorophyll proteins were more readily embedded into MG particles than into PE or PC particles. (2) MG particles contained chlorophyll proteins and cytochromes in their in vivo ratios, whereas PE or PC particles were enriched in the light-harvesting chlorophyll *a*/*b*-protein complex (LHC). (3) As judged by 77 K fluorescence emission spectra and P-700 oxidation kinetics, energy transfer from LHC to reaction center I which was lost in solubilized thylakoids, was restored almost completely in MG particles. In contrast, energy transfer was restored only partially or not at all in proteolipid particles formed with PE or PC, respectively. These unique properties of MG might be linked to its role as major lipid in thylakoid membranes.

Introduction

While lipid bilayers of mitochondrial and plasma membranes are composed of phospholipids [1], galactolipids are the main lipid components of chloroplast membranes [2]. In spinach thylakoids, for example, MG and DG comprise about 50% and 25% of he total polar lipids, respectively [2]. The fact that galactolipids are also the major constituents of the lipid bilayers in cyanobacteria [3] supports the endosymbiotic hypothesis [4,5] that chloroplasts were derived from these organisms. The question, however, why chloroplasts have largely maintained their specific lipid pattern throughout evolution of the plant kingdom is still unanswered. Most impressive is the fact that, while the lipid pattern has been conserved during the course of plant evolution, the pathway for galactolipid synthesis has changed from the more simple 'prokaryotic' to the complex 'eukaryotic' pathway [6,7].

Specific properties of galactolipids required for

Abbreviations: Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; LHC, light-harvesting Chl *a*/*b*-protein complex; DG, digalactosyldiacylglycerol; MG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, Photosystem; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine

Correspondence: D. Siefermann-Harms, Kernforschungszentrum Karlsruhe GmbH, Institut für Genetik und Toxikologie, Postfach 3640, 7500 Karlsruhe 1, F.R.G.

the assembly and function of the thylakoid membrane may explain the conservative lipid pattern of thylakoids. Indeed, galactolipids are required to optimize the function of various chloroplast proteins, although a strict galactolipid specificity is seldom seen. Krupa and Baszynski [8] were able to restore PS I activity of heptane-extracted thylakoids with either MG or DG. Yamamoto and Higashi [9] showed that violaxanthin de-epoxidase, a water-soluble enzyme acting on membrane-bound violaxanthin, binds MG and requires this lipid for activity. The intrinsic membrane protein cytochrome *b*-559 is converted from its native high-potential form into low-potential forms when it is extracted from the membrane with detergents. Matsuda and Butler [10] restored about one-half and one-third of the high-potential form of cytochrome *b*-559 when they incorporated the protein into DG and MG liposomes, respectively. According to Gounaris et al. [11], oxygen evolution of a Triton-X-100-derived PS II preparation was strongly enhanced by DG. PC was nearly as effective as DG in activating oxygen evolution, but MG had no effect.

In Ref. 12 we examined the ability of isolated lipids to restore the 77 K fluorescence spectrum of intact thylakoids in solubilized membranes. When the mixture of total solubilized membrane proteins was combined with purified thylakoid lipid species only MG was able to partially restore the 77 K fluorescence. We interpreted this observation as evidence for reconstitution of energy transfer from LHC into the photosystems following solubilization. In Ref. 13 we applied more specific assays to demonstrate reconstitution of energy transfer in the Chl-protein-MG particles. Variable fluorescence at 695 nm and velocity of light-induced P-700 oxidation were used to examine energy migration from LHC into PS II or PS I, respectively. Here we compare the ability of MG and the phospholipids, PC and PE, to form proteolipid particles, to incorporate thylakoid proteins, and to restore energy transfer from LHC to PS I. Our results show further unique properties of MG that may be related to its function in the assembly and function of thylakoid membranes (part of this work was presented at the Workshop on Plant Lipids in Freiburg, F.R.G., in September 1985).

Materials and Methods

Preparation of unstacked thylakoids. Chloroplasts were isolated from spinach, *Spinacia oleracea* L., purchased from a local market. All procedures were performed at 4°C. Deveined leaves were homogenized in a Braun blendor for 10 s in grinding medium composed of 300 mM sorbitol, 50 mM Hepes-NaOH (pH 7.5), 10 mM NaCl, 1 mM $MgCl_2$, and 1 mM EDTA. The homogenate was filtered through nylon cloth (37 μm mesh), and the chloroplasts of the filtrate were sedimented by centrifugation at $3000 \times g$ for 1 min, washed in grinding medium without $MgCl_2$ and EDTA, and resuspended. The washed chloroplasts were incubated under gentle stirring for 20 min in 1 mM Hepes-NaOH (pH 7.5) in order to break their envelopes and to unstack grana membranes [14,15]. The membrane suspension was centrifuged at $200 \times g$ for 5 min to remove cell debris and at $27000 \times g$ for 5 min to collect the membranes which were then resuspended in glass-distilled water at 2 mg Chl/ml. Aliquots of 500 μl were frozen in liquid N_2 and stored at 77 K until used. All experiments reported in this communication were performed with the same thylakoid preparation. The Chl/protein ratio of the preparation was 1/7.8 (w/w) comparable to the value of 1/6.8 reported in Ref. 2.

MG purification. About 250 g spinach leaves were ground in 250 ml buffer containing 300 mM sorbitol, 50 mM Mops-NaOH (pH 7.2) and 50 mM NaCl, filtered through nylon cloth (37 μm mesh) and centrifuged at $3000 \times g$ at 0°C for 30 min. The resulting chloroplast pellet was resuspended in 120 ml of the same buffer then extracted according to Bligh and Dyer [16]. The extract was dried on a rotary evaporator, taken up in 25 ml $CHCl_3$ and resolved into lipid classes on a 23 × 250 mm column packed with 50 g hydrated florisil (15% w/w) as a $CHCl_3$ slurry. The elution schedule was (A) 300 ml 15:85 acetone/$CHCl_3$, followed by 500 ml each of (B) 1:1 acetone/$CHCl_3$, (C) acetone, and (D) anhydrous methanol. MG was eluted in solvent B, DG in solvent C, and phospholipids plus sulfolipid in solvent D. Butylated hydroxytoluene and butylated hydroxyanisole were added to the acetone-containing and methanol fractions, respectively. All fractions were

dried, taken up in 10 ml $CHCl_3$, and stored under N_2 at $-70\,^{\circ}C$ until further purified.

MG isolated on florisil usually contained traces of pigment contaminant and was therefore further purified by HPLC. The sample was dried, taken up in 4 ml methanol/water (92 : 8), and injected in 2 ml aliquots onto a preparative ODS column (Alex ultrasphere, 10 mm × 250 mm). Separation was isocratic with methanol/water (95 : 5) at 4.0 ml/min. Refractive index and 220 nm absorption were used in tandum for detection. The two major components MG_1 (18 : 3/16 : 3, retension 25 min) and MG_2 (18 : 3/18 : 3, retension 38 min) were combined and stored in $CHCl_3$ under N_2 at $-20\,^{\circ}C$ until used. MG thus prepared was free of pigments and butylated hydroxytoluene. Other minor MG species also were resolved but they were not used.

Phospholipids. PC (L-α-phosphatidylcholine from soybean, Type III-S, No. P-6263) was from Sigma, and, according to the manufacturer contained the following fatty acids: 63.3% $C_{18:2}$, 14.4% $C_{16:0}$, 13.2% $C_{18:1}$, 5% $C_{18:3}$ and 4.1% $C_{18:0}$.

PE (L-α-phosphatidylethanolamine from soybean, Type IV, No. P-4513) was from Sigma, and, according to the manufacturer, contained the following fatty acids: 64.3% $C_{18:2}$, 17.6% $C_{16:0}$, 8.5% $C_{18:1}$, 6.2% $C_{18:3}$ and 2.6% $C_{18:0}$.

Solubilization of thylakoids. Thylakoid samples of 50 µl (100 µg Chl) were mixed with 10 µl Triton X-100 (2 mg Triton, pH 6.0) and solubilized at room temperature in the dark for 5 min with stirring. A Chl-Triton ratio of 1/20 (w/w) was used to minimize secondary effects of higher Triton concentrations [17]. Solubilized samples were diluted to 333 µl with 50 mM Tricine-NaOH (pH 7.5) and used immediately for experiments.

Preparation of Chl-proteolipid particles. Typically 100-µl aliquots of solubilized membranes (30 µg Chl, 600 µg Triton X-100) were transferred to test tubes containing 600 µg lipid which had been dried on the glass walls under N_2 from $CHCl_3$ solutions. The samples were sonicated for 15 s in a water bath sonicator (Bransonic 221) at 19 °C [12], then treated in one of two ways. They were either mixed immediately with 900 µl 50 mM Tricine-NaOH (pH 7.5), or they were frozen in liquid N_2, placed on ice and, after addition of 900 µl Tricine buffer, slowly thawed (77 K/0 °C treatment, modified from Ref. 18). The resulting suspensions were transferred into 1.5-ml centrifuge tubes (Eppendorf) and centrifuged at $37000 \times g$ (at tip of the tubes) and 4 °C for 10 min. Determination of lipids remaining in the sonication tubes indicated that 30–40% of the PE, 15–25% of the MG and 5–10% of the PC remained on the glass walls (together with traces of Chl and Triton) and were not suspended. The Chl-proteolipid particles sedimented at $37000 \times g$ and the supernatants were diluted with 50 mM Tricine-NaOH (pH 7.5) to desired concentrations and assayed for components, P-700 oxidation kinetics and 77 K fluorescence.

Assays. Chl was determined in 80% acetone according to Jeffrey et al. [19]. Protein was determined according to Lowry et al. [20] with bovine serum albumin as standard. For lipid determinations of the aqueous suspensions were extracted according to Bligh and Dyer [16] and the extracts were dried on a boiling water bath. The phospholipids were digested for 10 min in boiling 60% perchloric acid and assayed following the Bartlett phosphate determination [21] with KH_2PO_4 as standard. MG was quantitated according to Ref. 22 with galactose as standard. Triton X-100 was quantitated spectrophotometrically at 275 nm (A-275) on samples diluted to Triton concentrations less than 0.025%. The contribution of protein to the total absorbance at 275 nm was estimated by A-310 × 1.4 and subtracted from A-275 of the sample. Absorbance spectra were monitored with a Perkin Elmer Model 356 double-wavelength double-beam spectrophotometer using 1 nm slit widths and 1 cm optical path.

Cytochromes. In samples of Triton-treated thylakoids (± exposure to lipids) cytochromes were determined by difference spectra (530–580 nm) of dithionite-reduced minus ferricyanide-oxidised cytochromes. Dithionite and ferricyanide were added as crystals. For intact thylakoids the different cytochromes were determined by difference spectra of hydroquinone vs. ferricyanide, sodium ascorbate vs. hydroquinone, and dithionite vs. ascorbate-treated samples according to Ref. 10. Difference extinction coefficients used were as in Ref. 23.

P-700 oxidation. P-700 oxidation was measured

at room temperature. Measuring and actinic light beams were arranged at right angle and their optical paths within the samples were 1 cm each. Distance between photomultiplier and sample was 3.5 cm to minimize interference from sample fluorescence. The photomultiplier was protected against actinic light with a dark-red color filter (Schott RG 695). The Model 356 spectrophotometer was operated in the Dual mode with sample beam at 700 and reference beam at 725 nm. Slit width was 3 nm. Actinic light came from light source KL 150 B (Schott) equipped with a flexible light pipe and was filtered through interference filters (Balzers Filtraflex B-40) with maximal transmittence at 436 nm (routine determinations of P-700 content) or at 470 nm (P-700 oxidation kinetics at variable fluence rates). Reduced fluence rates were obtained with neutral-glass filters at 50 and 25% transmission (Schott, NG series). Fluence rates of actinic light were measured with a YSI-Kettering Model 65 A radiometer. P-700 content was quantitated using an extinction coefficient for P-700 at 700 nm of 64 $mM^{-1} \cdot cm^{-1}$ according to Ref. 24.

77 K fluorescence spectroscopy. Fluorescence emission spectra were monitored with a Yobin Ivon spectrofluorometer Model JY3C (with near-infrared sensitive photomultiplier R777-01) combined with an arrangement for low-temperature measurements as in Ref. 25. Exciting (470 ± 10 nm) and emitted light were guided to and from the top of the frozen samples by bifurcated fiberoptics (Schott). Samples (0.1 ml; layer thickness, approx. 2 mm) and terminal part of the light pipe were placed in liquid N_2. Fluence rate of the exciting light on top of the samples was 1.4 W/m^2; the band width of the emission monochromator was 4 nm. The spectra are not corrected for spectral response of the photomultiplier.

Results

Comparative properties of Triton X-100 treated and intact thylakoids

Treatment of unstacked thylakoids with low amounts of Triton X-100 (Chl/Triton = 1/20) disrupted the membranes, yielding protein mixtures that remained in the supernatant after centrifugation at $37000 \times g$. The physical separation of LHC from other Chl proteins was confirmed by isoelectric focusing of the Triton-treated membranes on polyacrylamide gels according to Ref. 26. The LHC fraction penetreated the gels within 5 min whereas other Chl proteins followed more slowly. Examination of the Chl proteins after isoelectric focusing showed that the PS-I supracomplex of P-700 binding core protein and associated light-harvesting protein [27–29] had not been split. Use of unstacked rather than stacked thylakoids appears to be critical for membrane solubilization with low amounts of Triton X-100. Solubilization of stacked thylakoids with grana stabilized in the presence of Mg^{2+} requires higher Chl/Triton ratios of 1/100 to 1/200 [30].

Fig. 1 shows normalized initial rates of P-700 oxidation in intact (curve A) and Triton-treated (curves B_1, B_2) thylakoids excited at 470 nm under suboptimal fluence rates. The Chl concentration

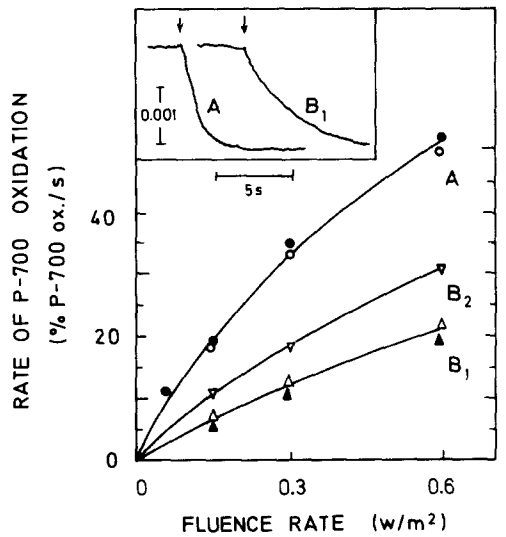

Fig. 1. Effect of Triton X-100 on the initial rate of P-700 oxidation of spinach thylakoids for excitation at 470 nm and suboptimal fluence rates. The initial rate of P-700 oxidised per s is expressed as % of total P-700 present in the sample. Inset: Kinetics of P-700 oxidation under illumination at 0.6 W/m^2 (arrows: light on) monitored as absorbance decrease at 700 nm. A: Intact thylakoids, 15 μg Chl/ml, absorbance at 470 nm (A-470): 0.68/cm; B_1: Triton-treated thylakoids, 15 μg Chl/ml, A-470: 0.88/cm; B_2: Triton-treated thylakoids, 11.6 μg Chl/ml, A-470: 0.68/cm. The 2.5-ml samples further contained 0.2 mM sodium ascorbate, 60 μM methyl viologen, 50 mM Tricine-NaOH (pH 7.5), and in case of the thylakoid sample 6 μM DCMU. For Triton treatment (Chl/Triton = 1/20; w/w) and other experimental details see Materials and Methods.

for curves A and B_1 was identical, but the 470-nm absorbance for the Triton-treated sample was 20% higher than for intact thylakoids. Curve B_2 was obtained for Triton-treated membranes with a 470 nm absorbance identical to that of membranes in curve A. The difference between curves B_1 and B_2 is due to differences in the actinic light gradient passing through samples with differing optical densities. Hence, all subsequent studies of P-700 oxidation compared samples with identical absorbance at 470 nm to maintain similar actinic light exposure.

Fig. 1, curves A and B_2 show that the initial rate of P-700 oxidation in Triton-treated membranes was inhibited 40% relative to intact thylakoids. The difference in rates of P-700 oxidation was not due to availability of electron acceptors on the reducing side of P-700, since varying the artificial acceptor methyl viologen between 1 and 60 μM did not affect the oxidation rate. Instead, the slow P-700 oxidation in Triton-treated thylakoids appears to be due to decreased absorptive cross-section of PS I caused by the physical separation of PS I and adjacent LHC.

The 77 K fluorescence emission spectra of our intact and Triton-treated thylakoids (Fig. 3) agreed with observations by others [30,31]. After Triton treatment the long-wavelength emission band at 735 nm was decreased and shifted to 730 nm and the short-wavelength emission bands at 685 and 695 nm were superimposed by a strong emission band at 681–683 nm. An additional emission band at 653 nm due to Chl *b* emission was small indicating that energy transfer from Chl *b* to Chl *a* within individual Chl proteins was almost completely retained in the Triton-treated sample.

Fluorescence emission near 730 nm of intact thylakoids is considered to be from Chl *a* in both, the light-harvesting protein of PS I [32,33] and the core protein of PS I [28,31]. The strong fluorescence near 681 nm of Triton-treated thylakoids is mainly emitted from Chl *a* located in solubilized LHC [34], but some 681-nm fluorescence may also originate from Chl *a* of PS-I core proteins which have been modified by Triton X-100 [31]. The decrease of 730-nm fluorescence and increase of 681-nm fluorescence of the Triton-treated membranes appears to be caused mainly by interruption of energy transfer from LHC to PS I due to physical separation of both Chl proteins.

Sample concentration significantly affected the shape of the fluorescence emission spectrum of Triton-treated thylakoids: the 730-nm emission band was less intense at lower sample concentration, but the 681-nm band was enhanced by dilution (up to 15 μg Chl/ml; data not shown). Evidently, increasing the distance between solubilized LHC molecules increased LHC emission at 681 nm due to the lowered re-absorption of emitted light by neighbor molecules. For intact thylakoids the distance between Chl proteins within a membrane is not altered by dilution, and therefore the shape of the emission spectrum showed little change, as observed before by others [35,36].

Incorporation of Triton X-100-solubilized thylakoid proteins into proteolipid particles

Previously we showed [12,13] that when Triton-treated thylakoids were exposed to dry films of MG under gentle sonication, the 77 K fluorescence of the sonicated mixture resembled that of intact thylakoids more closely than that of Triton-treated thylakoids. About 50% of the Chl in the sonicated mixture was incorporated into Chl-protein-MG particles which could be sedimented at $37\,000 \times g$ [12]. As shown in Table I, when PE or PC was used most of the Chl was recovered in the supernatant and did not show 77 K fluorescence comparable to that previously observed with MG. In contrast, subjecting the sonicated mixtures of phospholipids and solubilized proteins to a freeze-thaw step following the procedure of Kasahara and Hinkle [18] resulted in recovery of more than 50% of the green material in the $37\,000 \times g$ pellet (Table I). In the following, Chl proteolipid particles sedimenting at this gravity were compared for the different lipids.

Components of the Chl proteolipid particles

The absorbance spectrum of pellets and supernatants obtained after the above treatments differed depending on the lipid used. As shown in Fig. 2 (left) pellets from phospholipid exposure had increased absorbance at 650 nm as compared to their respective supernatants, indicating an enrichment in Chl *b*. The spectra of pellets and supernatants obtained after exposure to MG (freeze-thaw step omitted) did not differ in the 650-nm range.

TABLE I

INCORPORATION OF Chl PROTEINS INTO PROTEOLIPID PARTICLES SEDIMENTING AT 37000 × *g*

Treatment of Triton X-100-solubilized thylakoids			Chl content (%)		Lipid content (%)	
lipid added	sonication	77 K/0 °C treatment	pellet	supernatant	pellet	supernatant
no	+	–	4.8	95.2	–	–
+ MG [a]	+	–	49.8	50.2	69.0	31.0
+ PE	+	–	4.5	95.5	62.9	37.1
+ PC	+	–	12.5	87.5	52.3	47.7
+ PE	+	+	59.6	40.4	61.5	38.5
+ PC	+	+	52.3	47.7	52.9	47.1

[a] From Ref. 12.

The reduced-minus-oxidised difference absorbance spectra of the three pellets are shown in Fig. 2 (right). The absorbance difference in the 560-nm range of cytochromes was most pronounced in the MG pellet and much less in the PE and PC pellets. In contrast, the 560-nm absorbance difference in the PE and PC supernatants was enhanced, whereas the absorbance difference of the MG supernatant was comparable to that of the MG pellet (data not shown). The shape of the difference spectra for all three pellets was similar to intact or Triton-treated thylakoids indicating that the ratio of the various cytochromes had not changed. These results suggest all three chloroplast cytochromes were less efficiently incorporated into phospholipid than into MG particles.

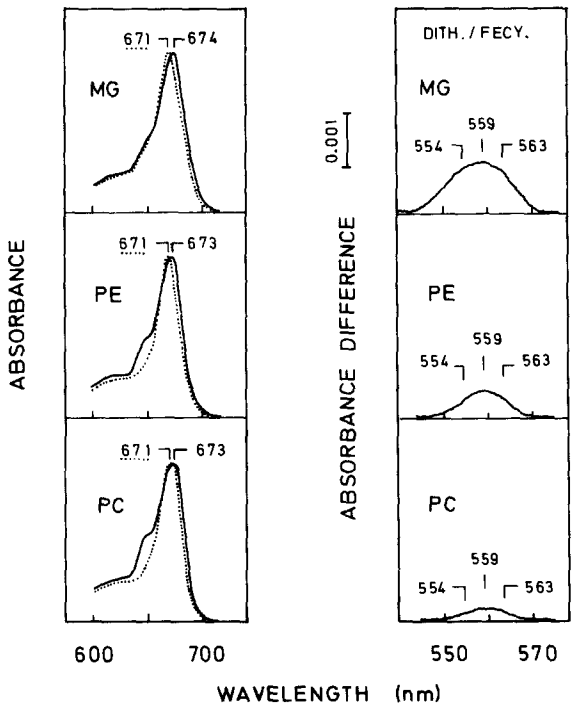

Fig. 2. Chlorophyll and cytochrome absorbance of Chl proteolipid particles. Left: absorbance spectra of particles sedimented at 37000 × *g* (———) and of supernatants (·····). The spectra are normalized for maximal Chl *a* absorbance at the indicated wavelengths. Right: Difference absorbance spectra of particles with their cytochromes in the dithionite-reduced (dith.) vs. ferricyanide-oxidised (fecy.) state. Particle suspensions contained 6.1 μg Chl *a*/ml; their Chl *b* content per ml was 2.2 μg (MG particles), 2.7 μg (PE particles) and 3.2 μg (PC particles).

Table II compares Chl, P-700 and cytochrome content of thylakoids and the three types of Chl proteolipid particles. The ratio of these components in MG particles was almost identical to that in thylakoids but significantly different in PE and PC particles. The Chl *a*/*b* ratios of the phospholipid particles were low indicating an enrichment in LHC. The P-700 content of PE particles was slightly higher and that of PC particles was lower compared to MG particles. The relative enrichment in LHC and P-700 content in PE particles suggests a lower amount of PS II. The Chl *a*/*b* ratio and P-700 content of PC particles indicates an enrichment of LHC and a lower amount of PS I. PS II appears to represent only a minor component of the PC particles, since the cytochrome content, including PS-II associated cytochrome *b*-559 [37], was low (Fig. 2, right).

The lipid content of the Chl proteolipid particles varied somewhat depending on preparation

TABLE II

COMPARISON OF Chl PROTEOLIPID PARTICLES AND THYLAKOIDS

	Thylakoids, intact or Triton-treated	MG particles	PE particles	PC particles
Preparation from Triton-treated thylakoids plus indicated lipid by				
sonication	–	+	+	+
77 K/0 °C treatment	–	–	+	+
Pigment ratio (mol/mol)				
Chl *a*/Chl *b*	2.8	2.8	2.3	1.9
Components (mol/100 mol Chl *a*)				
Chl *b*	35.70	35.50	43.50	52.60
P-700	0.24	0.25	0.26	0.21
cytochromes	1.51	1.48	0.82	0.48

and was strongly affected by the lipid/Chl ratio used during particle preparation. With our standard procedure we obtained particles containing 20–30 mol lipid per mol Chl. The particles also contained Triton X-100 at Chl/Triton (w/w) ratios in the range of 1/10, as compared with the supernatants with ratios in the range of 1/30. Washing of the particles lowered this ratio to about 1/5.

Fluorescence properties of the Chl proteolipid particles

Fig. 3 compares the 77 K fluorescence emission spectra of the Chl proteolipid particles with those of intact and Triton-treated thylakoids. In the proteolipid preparations the short-wavelength emission band near 683 nm was lower than in Triton-treated thylakoids but higher than in intact membranes. The long-wavelength emission band near 730 nm was slightly increased for PC and PE particles compared to Triton-treated thylakoids. For PC particles the emission maximum was shifted to 727 nm. A shift to shorter wavelengths has been observed before [12] when PC isolated from spinach thylakoids was used for reconstitution. Only MG particles had long-wavelength emission intensities comparable to intact thylakoids, although the maximum was shifted slightly

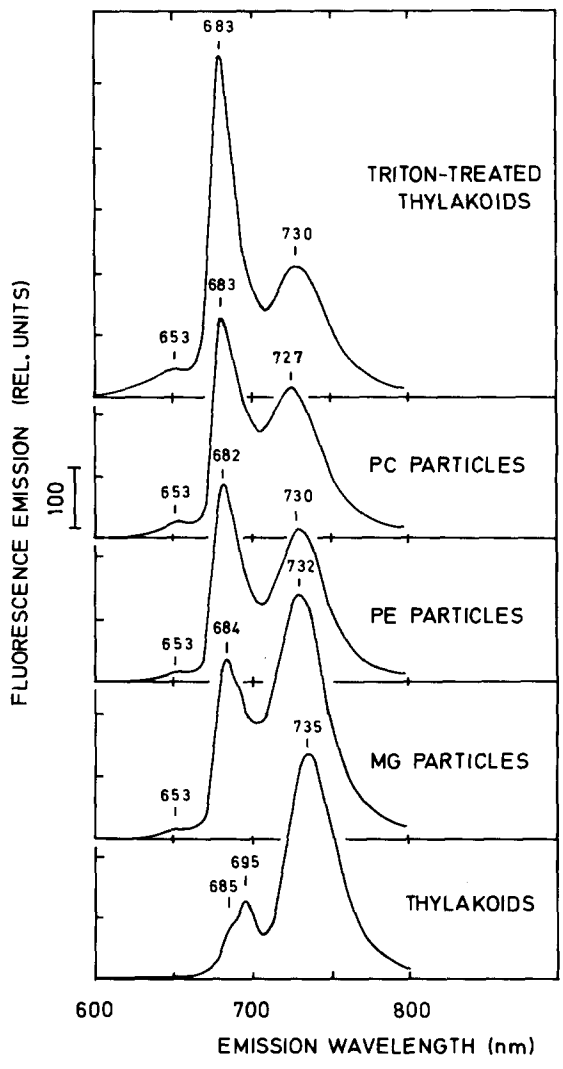

Fig. 3. 77 K fluorescence emission spectra for excitation at 470 nm of spinach thylakoids, Chl-proteolipid particles prepared with MG, PE, or PC, respectively, and Triton-treated thylakoids. The 100 μl samples contained 61 μg Chl/ml.

to shorter wavelengths. Washing procedures that partially removed Triton from the proteolipid particles did not affect their fluorescence properties.

In a recent study Murphy [38] prepared proteoliposomes by sonicating mixtures of Triton-treated thylakoids and preformed liposomes. The 77 K fluorescence emission spectrum of his PC particles was almost identical to the spectrum emitted by our PC particles and also showed the hypsochromic shift of the long-wavelength band. MG particles prepared in the same way, however, did not show an emission band in the 730-nm range, thus differing significantly from our MG particles. The difference might be due to different techniques applied for particle preparation and to different MG species used. While Murphy worked with commercial MG, about 80% of the fatty acids being $C_{18:2}$, we used highly unsatured MG from spinach thylakoids (see Materials and Methods).

As noted in the first subsection of Results, the shape of the emission spectrum of Triton-treated membranes but not of intact membranes was strongly affected by sample concentration. The ratio of short-wavelength to long-wavelength emission can be used to quantitate the observed effects. We used this ratio to compare the concentration-dependent fluorescence properties of Chl proteolipid particles with those of intact and Triton-treated membranes. As shown in Fig. 4 a, the emission ratio of intact thylakoids was 0.2 for the highest Chl concentration tested and increased by a factor of 2 when the sample was diluted 16-fold. In contrast, the emission ratio of Triton-treated thylakoids was 2.0 for the highest Chl concentration tested and, upon dilution, increased by a factor of 4. The Chl proteolipid particles did not show the strong changes in fluorescence emission observed for Triton-treated membranes. Instead, their emission ratio increased with dilution in a manner comparable to intact thylakoids. This behavior is consistent with the arrangement of their Chl proteins in supramolecular structures.

Fig. 4b compares intensities of the long-wavelength fluorescence band of intact and Triton-treated thylakoids and of Chl proteolipid particles under conditions of increasing sample concentration. Since this fluorescence is emitted by PS I [33,39], sample concentrations are expressed on the basis of P-700 content. For any sample the

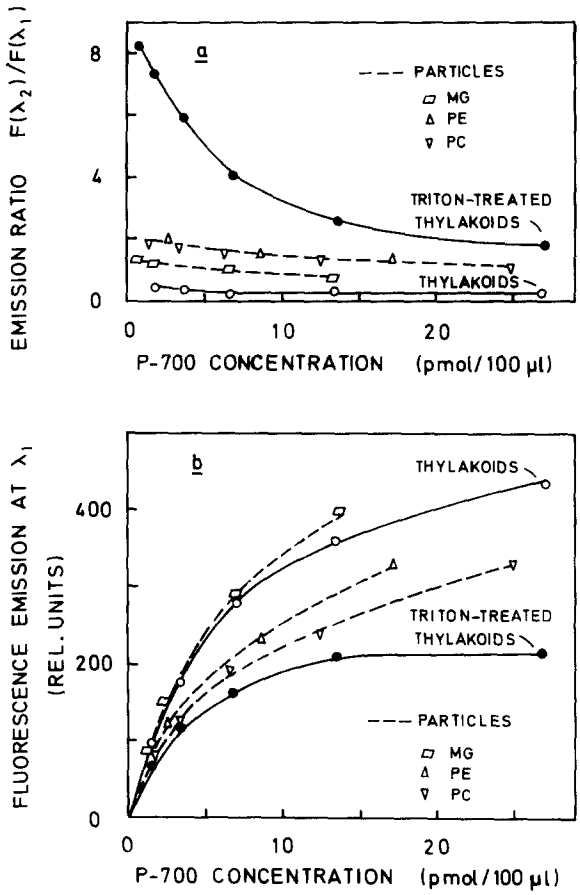

Fig. 4. Effect of Chl protein concentration on the properties of 77 K fluorescence excited at 470 nm in thylakoids, Triton-treated thylakoids and Chl-proteolipid particles. P-700 content of the different samples was used as a relative measure for Chl-protein concentration. (a) Variations in the ratio of fluorescence intensities of emission bands near 730 nm (λ_1) and near 684 nm (λ_2). (b) Variations in fluorescence intensity of the emission band near 730 nm (λ_1). For actual band position see Fig. 4. 100-µl samples were used. Samples at 10 pmol P-700/100 µl contained 5.2 µg Chl (thylakoids ± Triton treatment), or 5.0 µg Chl (MG and PE particles), or 6.6 µg Chl (PC particles).

emission increased with increasing sample concentration until saturation. For all P-700 concentrations tested, the emission of PE and PC particles was higher than for Triton-treated thylakoids, but lower than for intact membranes. Only the emission of MG particles was as high as that of intact membranes.

TABLE III

P-700 OXIDATION IN INTACT AND TRITON-TREATED THYLAKOIDS AND IN Chl PROTEOLIPID PARTICLES

Samples were excited at 470 nm and 0.6 W/m^2 fluence rate. In addition to thylakoid material they contained 60 μM methyl viologen, 3 mM sodium ascorbate, 50 mM Tricine-NaOH (pH 7.5) and, in case of intact thylakoids, 2.5 μM DCMU. Only samples with similar absorbance at 470 nm should be compared (see Fig. 1).

Sample	Absorbance at 470 nm (A-470/cm)	Initial rate of P-700 oxidation (P-700 ox./s in % of total P-700)
Thylakoids	0.76	52.6
Triton-treated thylakoids	0.73	28.6
MG particles	0.75	47.4
Thylakoids	0.64	57.5
Triton-treated thylakoids	0.65	29.6
PE particles	0.64	39.0
Thylakoids	0.89	49.6
Triton-treated thylakoids	0.88	20.0
PC particles	0.87	14.0

P-700 oxidation in the Chl proteolipid particles

The enhanced emission near 730 nm for Chl proteolipid particles observed above may indicate an increased absorptive cross-section of PS I. The initial rate of P-700 oxidation of the Chl proteolipid particles for excitation at suboptimal fluence rate was examined to check whether enhanced fluorescence occurred together with increased energy transfer into reaction center I. As shown in Table III, the velocity of P-700 oxidation in MG particles was significantly higher than for Triton-treated thylakoids and nearly as high as for intact membranes. Whereas PE particles showed somewhat higher P-700 oxidation than Triton-treated thylakoids, PC particles oxidised P-700 at a somewhat lower rate than Triton-treated thylakoids. Thus, energy transfer into reaction center I was restored to varying degrees in the MG and PE particles but not in PC particles.

Discussion

The galactolipid MG as well as the two phospholipids PE and PC examined in this study are able to incorporate solubilized Chl proteins into Chl proteolipid particles (Table I; Fig. 4a). However, MG differs from the other two lipids in several ways: (1) MG incorporates solubilized Chl proteins much more readily than phospholipids do (Table I). (2) MG incorporates Chl proteins and cytochromes in ratios similar to those in intact thylakoids, whereas the phospholipids preferentially incorporate LHC and partially exclude cytochromes and PS II (Fig. 2; Table II). (3) The intensity of the long-wavelength fluorescence emission band (near 730 nm) at 77 K, which is low in solubilized thylakoids, is restored to intact thylakoid levels in MG particles but only partially in phospholipid particles (Fig. 3 and 4b). (4) The low velocity of P-700 oxidation in solubilized thylakoids (Fig. 1) is restored almost to intact-thylakoid levels in MG particles, to a lesser degree in PE particles, and not at all in PC particles (Table III).

Our observations that lipids differ in their ability to incorporate thylakoid components is in line with the results of other authors. Ikegami [40] reconstituted Chl *a* into Chl-depleted PS-I particles using MG. Reconstitution of Chl *a* into Chl-depleted particles was also accomplished in the presence of PC but not with PE, suggesting specific actions of the different lipids. Sprague et al. [41] used freeze-fracture electron microscopy to demonstrate the ability of DG to embed both purified LHC and PS-II core complex into proteoliposomes. They also showed that in contrast with DG, PC incorporated LHC more efficiently than PS-II core complexes. The preferential incorporation of LHC into PC particles observed by us (Table II) is consistent with the latter result. On the other hand, Murphy et al. [42] used PC to assemble purified LHC and PS-II complexes in proteoliposomes although yields were not reported.

The P-700 oxidation kinetics and 77 K fluorescence at 730 nm of proteolipid particles were used to examine restoration of energy transfer from LHC to PS I. We started with thylakoids treated under low-salt conditions [14,15] to obtain unstacked membranes wherein Chl protein complexes are distributed randomly [15] and energy transfer from LHC to the supracomplex of PS I is optical [43,44]. Triton treatment of the unstacked membranes reduced the P-700 oxidation rate

(Fig.1, Table III) and the 77 K fluorescence emission near 730 nm (Figs. 3 and 4b) by 40–50% indicating that PS I lost about half of its absorptive cross-section at 470 nm due to separation from adjacent LHC. Energy transfer from LHC into PS I appears to be restored to some degree in all of our newly assembled proteolipid particles as suggested by the enhanced fluorescence emission near 730 nm (Fig. 4b). In MG and PE particles, the P-700 oxidation rate was also enhanced (Table III) indicating energy transfer into the reaction center of PS I. As judged from the P-700 oxidation kinetics, energy transfer from LHC to the reaction center of PS I was restored almost completely in MG particles, and partially or not at all in particles formed with PE or PC, respectively.

Our results with PC particles appear to be in contrast to observations by Murphy [38]. This author treated pea thylakoids with Triton X-100 at a Chl/Triton ratio of 1/7 followed either by dialysis or by exposure to preformed PC liposomes, sonication of the mixture and dialysis. Intact control thylakoids, Triton-treated thylakoids, and proteoliposomes thus formed were examined for electron transport from 2,6 dichlorophenolindophenol/ascorbate to methyl viologen through PS I at increasing intensities of actinic light. Under low light electron-transport rates of the three samples were almost identical; under high light, however, the electron-transport rate continued to increase for Triton-treated thylakoids but reached saturation for intact thylakoids and proteoliposomes. These data were taken as evidence for reconstituted energy transfer in the proteoliposomes. The fact, however, that electron-transport rates under low light were not affected by Triton treatment does not favor this conclusion. Rather it appears that Triton X-100 at the low level used did not efficiently solubilize the membrane while improving access of the hydrophilic electron-donor couple to PS I.

As shown in the first paper of this series [12], MG is unique among thylakoid lipids in its ability to restore partially the 77 K fluorescence emission of intact thylakoids when combined with Triton-treated membranes. Here we substantiate our working hypothesis that restoration by MG of the fluorescence signal is due in part to reconstituted energy transfer from LHC to PS I. Compared to the phospholipids PE and PC, MG is also unusual in its ability to embed Chl proteins and cytochromes of the thylakoid membranes in their in vivo ratio rather than selectively. The unique properties of MG are not easily explained. Recently we speculated [12] whether the 'cone shape' of native MG [45,46] might be responsible. PE, a lipid also characterized by its 'cone shape' [47], is shown here to restore energy transfer only partially (Table III) and to embed thylakoid proteins differently from MG (Tables I and II). Whether similarities in the behavior of MG and PE can be explained by similarities in their molecular shape, and differences by either their different fatty acid content or uncharged (MG) versus charged (PE) headgroups remains to be established.

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