Reconstitution by monogalactosyldiacylglycerol of energy transfer from light-harvesting chlorophyll *a/b*-protein complex to the photosystems in Triton X-100-solubilized thylakoids

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1. INTRODUCTION

Galactolipids are the major polar lipids of thylakoids, MG and DG comprising about 50% and 25% of the total polar lipids, respectively [1]. Specific functions of galactolipids in the thylakoid have not been established [2,3], although the lipids are generally assumed to form the bilayer matrix. Photochemically active chl–protein complexes containing low levels of galactolipids have been isolated but the function of the lipids as intrinsic constituents of these proteins has been questioned [4].

Thylakoids contain functionally distinct chl–protein complexes which are closely associated within the lipid matrix as evident from 77 K fluorescence. Intact membranes exhibit fluorescence of the photosystems at 695 and 734 nm and not of LHC at 681 nm indicating energy absorbed by LHC is transferred almost completely to the photosystems. When thylakoids are solubilized by detergents fluorescence from the photosystems decrease and a band near 681 nm from LHC itself appears due to separation of the chl–protein complexes. The pigment–protein complexes thus separated also can be resolved electrophoretically [7].

In [8] reconstitution of energy transfer from purified LHC to the PS complexes was reported in a mixture of the thylakoid lipids in their naturally occurring ratio. We also examined the question of reconstitution with attention to the role of specific lipids in the process and show herein that MG but not other thylakoid lipids restores energy transfer from LHC to the photosystems in Triton X-100-solubilized membranes. We suggest that in the presence of MG, chl–protein complexes reassemble to supramolecular structures similar to those in intact thylakoid membranes.

Abbreviations: chl, chlorophyll; HPLC, high-performance liquid column chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LHC, light-harvesting chl *a/b*–protein complex; PS, photosystem; Tricine, *N*-[2-hydroxy-1,1-bis (hydroxymethyl)-ethyl]-glycine; TLC, thin-layer chromatography; DG, digalactosyldiacylglycerol; MG, monogalactosyldiacylglycerol; PC, phosphatidylcholine-(lecithin); PG, phosphatidylglycerol; SL, sulfoquinovosyldiacylglycerol

Molecular species of lipids are presented by combination of abbreviated forms of fatty acids; e.g., MG (18:3/16:3), octadecatrienoyl–hexadecatrienoyl species of MG

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2. MATERIALS AND METHODS

2.1. *Thylakoids and LHC*

Deveined spinach leaves (*Spinacia oleracea* L.) were homogenized in a Waring blender for 10 s at 4°C in grinding medium composed of 300 mM sorbitol, 50 mM HEPES–Na (pH 7.5), 10 mM NaCl, 1 mM MgCl, and 1 mM EDTA. The homogenate was filtered through nylon cloth (37 μm mesh), and the chloroplasts sedimented at 3000 × *g* for 1 min, washed in grinding medium without $MgCl_2$ and EDTA, re-sedimented and finally suspended in distilled water to 2 mg chl/ml. Aliquots of 300 μl were frozen in liquid N_2, stored at −70°C and used within 1 month. LHC was isolated by isoelectric focusing of Triton X-100-solubilized thylakoids as in [7].

2.2. *Chloroplast lipids*

The preparative details for isolation of chloroplast lipids used here will be described elsewhere (in preparation). Lipids recovered from an acid-washed florisil column were further purified by reverse-phase HPLC [MG (18:3/18:3), MG (16:3/18:3) and DG (18:3/18:3)] or TLC (PG, SL). MG and DG were chlorophyll and carotenoid free and homogenous by TLC. PG contained a small amount of SL (<5%) and SL contained a small amount of PG (<5%). PC was TLC pure. Galactolipids and SL were quantitated with galactose as standard [9]. The phospholipids were assayed using the Fiske and Subbarow procedure with KH_2PO_4 as standard [10]. Chloroplast lipids were stored in $CHCl_3$ under N_2 at −70°C and were used within 1 month.

2.3. *Solubilization of thylakoids*

Thylakoids equivalent to 1.5 mg chl were solubilized in 25–30 mg Triton X-100/ml solution at room temperature for 5 min with stirring. Chl:Triton X-100 ratios were kept in the range of 1/20 (w/w) to avoid secondary effects of higher Triton concentrations on LHC [11] while being sufficient to separate LHC from the PS complexes as confirmed by isoelectric focusing [7] for 5 min.

2.4. *Exposure of solubilized membranes to exogenous lipids*

Solubilized material was diluted with 50 mM Tricine–Na buffer (pH 7.8) to a final conc. 0.5 mg chl/ml. Typically 60 μl aliquots were transferred to test tubes containing either lipids dried under N_2 (sample) or no added lipid (control), sonicated for 15 s in a waterbath sonicator (Branson B-220H) at 19°C and immediately frozen in liquid N_2.

2.5. *Fluorescence emission at 77 K*

A Perkin Elmer model 44-A fluorescence spectrophotometer with a simplified arrangement for low temperature measurements as in [12] was used. Exciting (438 nm) and emitted light were guided to and from the sample by fiber optics (Dolan-

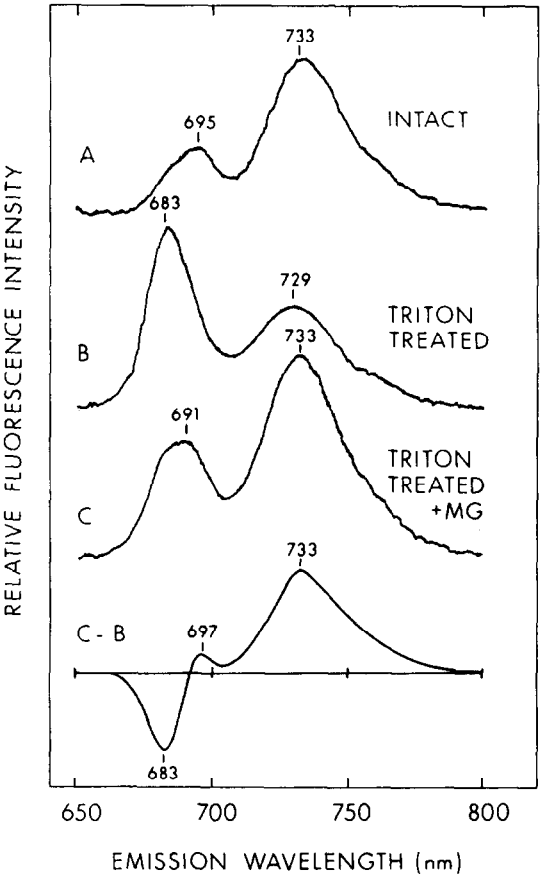

Fig. 1. The 77 K fluorescence emission of intact thylakoids (A), thylakoids solubilized with Triton X-100 (B), and solubilized thylakoids exposed to MG (C). The curve C–B is the difference fluorescence spectrum between the two treatments. Assay mixtures (60 μl) contained thylakoids equivalent to 30 μg chl and when present, 500 μg Triton X-100 and 600 μg exogenous MG. For experimental details see section 2.

Jenner, Type EK) connected to a light-collecting glass rod which was in turn placed directly on the sample under liquid N_2. Emitted light was filtered through a red cut-off filter (Corning CS 2-61, 3.3 mm optical path). Slit widths were 10 nm and scanning speed was 30 nm/min at 1.5 s response time.

2.6. *Other methods*

Absorbance spectra were obtained with a Perkin Elmer model 356 spectrophotometer. Chl was determined according to [13]. Fiske-Subbarow reducer (no. 661-8), egg-yolk lecithin (no. P-5388) and Triton X-100 (no. T-6878) were from Sigma; saturated MG (no. 4 6030) was from Supelco Inc.

3. RESULTS

Fig. 1 shows that the 77 K fluorescence-emission spectrum of intact thylakoids (A) was strongly modified on solubilization with Triton X-100 (B) but was restored to a significant degree on subsequent treatment with exogenous MG (C). The difference fluorescence spectrum between Triton-treated thylakoids exposed to MG *minus* the unexposed control (C–B) showed two effects, namely a decrease of relative fluorescence intensity at 683 nm, and increases in regions where intact membranes exhibit maximal emission; i.e., near 695 and 733 nm. MG did not similarly affect the fluorescence of LHC purified according to [7] (not shown).

The MG-exposure protocol was critical for restoring fluorescence. Restoration was optimal when sonication was 19°C and only slight at 9°C or 29°C. Sonication for 15 s yielded the results presented in fig. 1 whereas sonication for 1 or 2 min was detrimental. Solubilization time in Triton X-100 prior to treatment with MG also affected fluorescence. While some restoration was observed in thylakoids exposed to Triton X-100 for 1 h, the F-683 band from LHC was decreased only slightly when membranes were solubilized for $\geq$10 min. However, the F-733 band could still be restored under these longer solubilizations.

Table 1 shows that while MG which contained unsaturated fatty acids restored the fluorescence signal of solubilized thylakoids, saturated MG had no effect. In the case of unsaturated MG, replacing one C18:3 acid with C16:3 acid did not affect restoration.

MG was the only chloroplast lipid that restored the original fluorescence; DG and PG had little effect (fig. 2A,B). After addition of DG or PG the ratio of fluorescence intensity in the red and far-red spectral region (F_1/F_2) was 1.59 or 1.65, respectively, as compared to 1.81 for the Triton-treated control. In contrast, SL and PC further modified the fluorescence signal as seen from shifts in the position of the far-red emission maximum (fig. 2C,D). Egg lecithin caused even greater modification, reducing the far-red emission maximum to a shoulder adjacent to a 683 nm band (not shown).

Table 1

Effect of molecular species of MG on fluorescence emission spectrum at 77 K in Triton X-100-treated thylakoids

Sample	Fluorescence maximum (nm)		Ratio of fluorescence intensities at λ_1 and λ_2 (F_1/F_2)
	λ_1	λ_2	
Triton-treated membranes			
(1) no addition	683	728	1.81
(2) + MG (saturated)	683	730	1.80
(3) + MG (16:3/18:3)	691	733	0.56
(4) + MG (18:3/18:3)	691	733	0.59
Intact membranes	695	733	0.44

Triton X-100 solubilized membranes (60 μl) were sonicated in absence or presence of 600 μg MG as in section 2: chl/Triton X-100/exogenous MG, 1/16.6/20 (by wt)

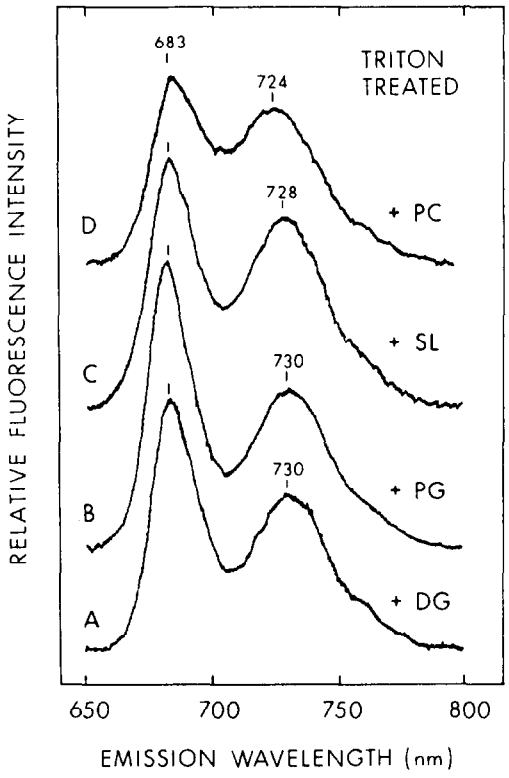

Fig. 2. The 77 K fluorescence emission of Triton X-100-treated thylakoids exposed to the indicated thylakoid lipids. Except for the species of lipid added, the conditions were as for treatment C in fig. 1.

Various mixtures of chloroplast lipids were examined. MG *plus* DG at 600 μg and 300 μg, respectively, in 60 μl also restored fluorescence of Triton-treated thylakoids. A mixture of the thylakoid lipids in their naturally occurring ratio [1] (600 μg MG, 300 μg DG, 110 μg PG, 75 μg SL and 50 μg PC per 60 μl) showed only slight restoration capacity.

Table 2 shows that in the absence of exogenous MG only 7% of total chl (column 1) from Triton-treated thylakoids could be pelleted at 37000 × g and that this pellet has an F_1/F_2 ratio of 0.64 similar to intact membranes. The supernatant (column 3) contained modified complexes as seen from its 673 nm absorbance maximum and from the F_1/F_2 ratio which was higher than in samples shortly after solubilization (table 1, line 1). The latter suggests continued action of Triton during centrifugation.

Addition of MG to Triton-treated thylakoids changes the chl distribution between the 37000 × g pellet and supernatant, increasing the chl–protein pellet 7-fold (table 2). This fraction was heterogenous and could be partially sedimented at 17000 × g (not shown). The pellet showed a restored F_1/F_2 value of 0.66, while the F_1/F_2 value of the supernatant was even higher than in the sample without MG, perhaps due to more efficient action of Triton on the chl–protein-depleted super-

Table 2

Formation of a chl–protein pellet with reconstituted energy transfer in Triton X-100-treated thylakoids exposed to exogenous MG

	37000 × g Pellet		37000 × g Supernat.	
	−MG	+MG	−MG	+MG
Chl distribution (%)	7.0	49.3	93.0	50.7
Red absorbance maximum at 25°C (nm)	678.0	676.0	673.0	762.0
Chl *a*/*b* ratio (w/w)	3.1	3.0	3.1	3.3
Fluorescence ratio F_1/F_2	0.64	0.66	4.46	6.25

Solubilized membranes (120 μl) containing 60 μg chl and 1.2 mg Triton X-100 were sonicated without added lipids (−MG) or in the presence of a mixture of 0.6 mg MG (16:3/18:3) and 0.6 mg MG (18:3/18:3) as in section 2, diluted with 3 ml 50 mM Tricine–Na buffer pH 7.8 at 0°C, and centrifuged for 20 min at 37000 × g. For 77 K-fluorescence measurements pellets resuspended in 120 μl buffer or 120 μl of supernatants were used. F_1 and F_2 are relative fluorescence intensities at the red and far-red emission maxima, respectively

natant [11]. The red-absorbance maximum of the pellet was 676 nm indicating the state of chl bound to proteins in the pellet was close to that of intact membranes. The chl *a/b* ratios in the pellet and supernatant were similar (table 2, line 3) indicating that the ratio of LHC to chl–protein complexes of the photosystems was the same in the pellet as in intact membranes. In similar experiments utilizing DG, PG, SL, or PC from chloroplasts and hydrogenated MG, only DG yielded a green pellet fraction from solubilized thylakoids (52% of total chl was recovered in the pellet); for other lipids ≥85% of total chl remained in the supernatant. Analyses of the lipid distribution between 37000 × *g* pellet and supernatant of Triton-treated membranes exposed to MG or DG showed the major part of the lipids (69% for the MG and 70% for the DG sample) was recovered in the pellet.

4. DISCUSSION

4.1. *The formation of chl–protein aggregates which exhibit intercomplex energy transfer*

The results show addition of MG to Triton X-100 solubilized thylakoids mediated the formation of aggregates (table 2) which exhibit a 77 K fluorescence emission similar to intact membranes (fig. 1). Similarities between intact membranes and the chl–protein–MG aggregates are illustrated by the difference-fluorescence spectrum between Triton-treated thylakoids with and without added MG (fig. 1C–D). This spectrum indicates the exciton densities in PS II (F-695) and in PS I (F-733) are increased while the fluorescence by LHC at 683 nm is decreased in the presence of MG. The chl *a/b* ratio (table 2) indicates that the aggregates are neither enriched nor depleted of LHC as compared to intact membranes. These observations suggest chl–protein complexes of Triton-treated thylakoids are reconstituted in the presence of MG into structures which perform intercomplex energy-transfer similar to intact membranes.

The possibility that MG might specifically quench fluorescence emission of the LHC rather than mediate energy transfer to the other chl–protein complexes seems improbable since MG did not similarly affect the 77 K fluorescence emission of isolated LHC. The path of energy migration from LHC to the photosystems has not been established for these chl–protein–MG aggregates, however analyses of fluorescence induction kinetics at 77 K will be required to characterize this system in relationship to known pathways for intact membranes [17].

4.2. *The specific effect of MG*

Various proteins isolated from chloroplast membranes have been reconstituted into lipid vesicles [18–20] composed either of lipids not endogenous to chloroplasts or of mixed thylakoid lipids [8,21]. Until now a specific lipid requirement has not been reported. Here, we show reassembly of separated chl–protein complexes into aggregates which perform intercomplex energy transfer is specific in its lipid requirement. Lecithin has no effect and MG is the only thylakoid lipid that mediates assembly (fig. 1,2).

While the nature of the aggregates formed on reconstitution with MG cannot yet be described in detail the ability of MG to mediate reconstitution appears related to several properties. Native MG is the only lipid of those examined which forms a hexagonal-II phase under hydration [23], a characteristic that has been attributed to 'cone-shaped' lipids [24]. Saturated MG which forms an extended open lamellar phase instead of the hexagonal-II phase [25] did not incorporate chl–protein complexes but precipitated as a white chl-free pellet (table 1). Although unsaturated MG which is capable of forming the hexagonal-II phase is required for reassembly of chl–protein complexes, the length of one fatty acid does not appear to be critical (table 1).

The fact that Triton X-100 has been shown to stabilize bilayer structures of 'cone-shaped' lipids [26] raises the possibility Triton X-100 and MG combine to provide a bilayer structure or other large aggregate favorable for reconstitution. If so, the formation of large protein-containing structures itself is not sufficient for reconstitution inasmuch as DG also induced formation of large aggregates but did not restore energy transfer. The unique ability of MG to mediate the assembly of chl–protein complexes reported here may reflect a property of this lipid that is required in vivo for the assembly of complexes in thylakoid membranes.

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