ARE GALACTOLIPIDS INTEGRAL COMPONENTS OF THE CHLOROPHYLL–PROTEIN COMPLEXES IN SPINACH THYLAKOIDS?

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1. Introduction

The thylakoid membrane may be described in terms of the fluid mosaic model [1] with integral globular proteins being dispersed in a fluid lipid matrix [2]. In this membrane, the proteins comprise ~60% of the membrane material, and the polar lipids and pigments account for the remaining 40% [3,4]. The majority of the membrane protein consists of 3 types of chl–protein complexes, i. e., the photochemically active chl *a*–protein complexes, containing photocenter I or II surrounded by antennae chl, and the light-harvesting but photochemically inactive chl *a*/*b*–protein complex [5,6]. About 80% of the thylakoid polar lipids are uncharged galactolipids, the major part being MGDG [3].

When thylakoids are fractionated by detergent treatment, variable amounts of lipids are recovered in the solubilized chl–protein complexes [6]. Preparations of the light-harvesting chl *a*/*b*–protein complex obtained after gentle solubilization of spinach [7] or maize [8] thylakoids with the non-ionic detergent Triton X-100 contain significant portions of membrane lipids. Even the peripheral protein violaxanthin de-epoxidase, isolated from inner thylakoid space without detergent, has been shown to bind MGDG [9]. These findings point to the possibility that, although the bulk of the membrane lipids is organized as fluid bilayer matrix, small fractions of the lipids might be integral components of membrane proteins interacting specifically with the chl–protein complexes. Tight and specific coupling is well established to exist between the apoproteins of the chl–protein complexes and the lipophilic thylakoid pigments, chlorophylls carotenoids [10,11].

Here we show that specific bonds between galactolipids and chl–protein complexes, which might exist in intact thylakoid membranes, are not maintained when the thylakoids are fractionated by methods gentle enough to maintain the bonds between chlorophylls and the chl–protein complexes as well as their photochemical activities. Therefore, if specific interactions between chl–protein complexes and galactolipids occurred, they are not comparable with the strong and specific binding between these complexes and the thylakoid pigments.

Abbreviations: chl, chlorophyll; DPC, 1,5-diphenylcarbazide; DPIP, 2,6-dichlorophenolindophenol; IEF, isoelectric focusing; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol; TLC, thin-layer chromatography

2. Materials and methods

2.1. *Preparation of chloroplast material*

Spinach (*Spinacia oleracea* L.) was grown in the garden or purchased from a local market. Unlabelled thylakoid membranes were isolated from 20 g deveined spinach leaves, by homogenization for 5 s in a Braun mixer with 150 ml medium containing 300 mM sorbitol, 50 mM Hepes/NaOH buffer (pH 7.5), 1 mM EDTA, 10 mM NaCl and 1 mM $MgCl_2$, filtration through nylon cloth (26 μm mesh width) and centrifugation at 3000 × *g*). The pelleted membranes were washed in glass-distilled water, then in 5 mM EDTA solution (pH 7.8) [12] and once more in glass-distilled water. After centrifugation for 3 min at 6000 × *g*, the pellets were adjusted to 2 mg chl/ml.

For the preparation of [^{14}C]galactolipid-labelled thylakoids, intact chloroplasts were isolated according to [13] via Percoll gradients. O_2-evolution rates, measured as in [13], were in the range of 50 μmol O_2/mg chl × h. The chloroplasts were incubated with UDP-

[U-^{14}C]galactose for 2 h at room temperature in a reaction mixture containing betain as osmoticum [14] under conditions as in [15]. After incubation, intact chloroplasts were selected by means of a Percoll gradient and sedimented after dilution with isotonic incubation medium. The intact chloroplast fraction was osmotically shocked in glass-distilled water, followed by 2 washes in glass-distilled water, 1 wash in 5 mM EDTA solution at pH 7.8 and a final wash in glass-distilled water. The pellets were adjusted to 2 mg chl/ml. All preparation steps were at 0–4°C.

2.2. *Isoelectric focusing on agarose gel plates*

For one agarose gel plate (9 × 12 cm), 1.8 g sorbitol, 0.15 g agarose and 15 ml distilled water were heated while stirring until the agarose had melted. Then 4 ml 5% digitonin solution, 0.4 or 0.5 ml 25% ampholyte solution (pH 5–7) and 0.4 or 0.3 ml 25% ampholyte solution (pH 3.5–5) were added, and the mixture was poured on a supporting glass plate. After 5 min cooling, a sample trough was formed by placing a glass tube (diam. 1 cm) on the surface of the gel that was later removed.

Washed thylakoids were solubilized with Triton X-100 by stirring the solubilization mixtures for 10 min at 0°C. Further solubilization conditions are given in the legends of figures and tables. IEF was performed in the LKB Multiphor 2117 chamber, with a constant wattage power supply. Electrode solutions were 1 N NaOH (cathode) and 1 N H_3PO_4 (anode). Gel plates, oriented with their sample application troughs towards the cathode, were cooled to 2°C. After application of the solubilized samples, gel plates were run at 3 W/gel for 20 min, ensuring that most of the green material left the trough, then at 100 V for 10–12 h.

2.3. *Assays*

Densitometer traces at 675 nm (with 725 nm as reference) and pH-gradients of the gels were determined as in [16].

For the determination of spectral properties and photochemical activities of the chl–protein complexes, the green bands were cut from the gel and homogenized in a Potter-Elvehjem vessel with 5–10 ml 50 mM Tris–HCl buffer (pH 7.6). The resulting green solutions were separated from the gel material by centrifugation. The supernatants were analysed for chlorophyll content, spectral properties, photosystem II activity (light-induced DPIP reduction in the presence of DPC) and photosystem I activity (light-induced P700 oxidation) as in [16].

For lipid analysis, solubilized thylakoids equivalent to 2 mg chl were focused on 8 agarose gel plates and the gel layers separated at the pH 5.5 line. The gel material was homogenized and neutralized by adding small amounts of 1 M Tris–HCl buffer (pH 7.6) and the chlorophyll content of the gel suspensions determined in 80% acetone according to [17]. The aqueous suspensions were kept at −20°C until used for lipid extraction. This was accomplished by adding 5 vol. $CHCl_3$/MeOH (2/1,v/v) and thorough mixing. After clean phase separation overnight, the lipid-containing subphase was removed and evaporated in a rotatory evaporator resulting in a liquid residue. This could not be used for lipid determination after TLC separation due to contamination by substances from the gel, detergents and ampholytes. Most of them were removed by dissolving the residue in a few ml MeOH, diluting with diethyl ether (150 ml) followed by 2 washes with water (30 ml). The ether solution was evaporated, the residue dissolved in $CHCl_3$/ether/MeOH (5/5/1, by vol.) and used in aliquots (150–550 μl) for TLC separation of MGDG and DGDG using $CHCl_3$/MeOH (88/12, v/v) and $CHCl_3$/MeOH/water (70/30/4, by vol.) as solvents. Lipid spots were visualized under UV light after spraying with anilinonaphthalene sulfonic acid (0.2% in MeOH, w/v), scraped off and used for colorimetric determination by reaction with anthrone. Evaluation was based on calibration curves obtained from simultaneously run standard series of pure MGDG and DGDG as in [18].

For [^{3}H]MGDG and [^{14}C]galactolipid determination, gel slices were extracted with 3 ml methanol until colorless. Chlorophyll absorbance of the extracts at 665 nm was measured in a Zeiss spectrophotometer PMQ II. Aliquots of the methanol extracts were either mixed directly (single counting) with 5 ml scintillation cocktail (Unisolve I) or first evaporated to dryness (double counting) and then dissolved in Unisolve I. The extracted gel slices, analysed in Unisolve I, retained ~10% of the tracers. The samples were counted in a Packard model 3380 liquid scintillation counter. About 85% of the labels were recovered after IEF.

For identification of [^{14}C]galactolipids, the methanolic gel extracts (2.75 ml) were extracted by addition of 6 ml chloroform and 1.5 ml NaCl solution (0.45%). After addition of galactolipid carriers (100 μg each of MDGD and DGDG) the lipophilic subphase was removed, concentrated and chromatographed on silica-

gel G plates with chloroform/methanol/water (70/30/4, by vol.) as solvent.

2.4. *Materials*

Precoated Kieselgel G plates (0.25 mm thick) were from Merck (Mannheim). Digitonin, Serva 19550 and agarose-EF, LKB 1802-300 were used. Ampholytes, i. e. Ampholine (pH 3.5–5 and pH 5–7) were from LKB. UDP-[U-^{14}C]galactose (320 μCi/μmol) was from The Radiochemical Centre, Amersham. 1,2-Di-*O*-[9′, 10′-$^{3}H_2$]oleoyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol ([^{3}H]MGDG) was obtained via semisynthetic [19] 1,2-di-*O*-octadec-9′-ynoyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol. The two triple bonds of this compound were reduced stereospecifically with tritium gas to 9-*cis*-octadecenoyl residues by The Radiochemical Centre, Amersham, as tritium-labelling service TR 3. 9-Octadecynoic acid required for this synthesis was prepared from oleic acid as in [20]. [^{3}H]MGDG, according to its synthesis with spec. act. ~10–20 mCi/μmol, was purified by TLC immediately before use and dissolved without addition of carrier MGDG in 2.5% Triton X-100 solution.

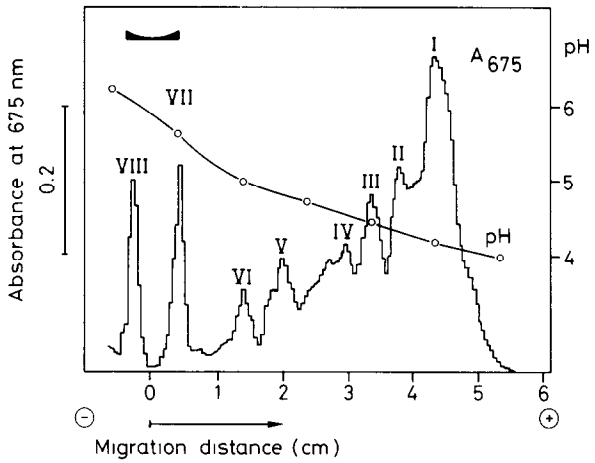

Fig.1. Densitometer trace at 675 nm and pH-gradient of an ampholyte-containing agarose gel plate loaded with Triton X-100-solubilized spinach thylakoids and subjected to IEF. For IEF, a sample of solubilized thylakoids containing 230 μg chl, 1 mM EDTA (pH 7.8) and 2.5% Triton X-100 in 250 μl final vol. was placed in the trough of a gel plate (▬) near the cathode. For further details see section 2.

3. Results

3.1. *Separation of chl–protein complexes and free lipids from spinach thylakoids*

IEF of Triton X-100 solubilized spinach thylakoids on agarose gel allowed to separate various chl–protein complexes based on their electrophoretic mobility under conditions of a pH gradient (fig.1). As shown in table 1, the sequence of complexes focusing between pH 4 and pH 5 was comparable with earlier results from bean thylakoids [16], with the light-harvesting chl *a*/*b*–protein complex (fraction I) focusing at the most acidic pH followed by complexes with an active photocenter II (fractions II–IV) and by complexes with an active photocenter I (fractions V,VI). Fraction VII occured as a sharp band at the anode-oriented rim of the sample application trough and represented mainly membrane particles not able to penetrate the gel. This fraction (~8–9% total chl) contained both photosystem I and photosystem II activity. In contrast, fraction VIII was photochemically inactive and its red absorbance maximum

Table 1

Spectral properties, chlorophyll content and photochemical activities of thylakoid fractions obtained from Triton X-100-solubilized spinach thylakoid after IEF on agarose gel (solubilization conditions and designation of fractions as in fig.1)

Fraction	Red maxima (nm)	Rel. chl-content (%)	P700 oxidation (mol P700 ox./ 100 mol chl *a*)	DPIP-reduction rate (μmol DPIP red./ mg chl × h)
I	675, 651	30.0	0	5.0
II	675, (651)	15.5	0	30.7
III	676, (651)	10.5	0.12	38.0
IV	677	10.0	0.24	26.8
V	677	7.8	0.48	10.9
VI	677	7.2	0.62	0
VII	676, (651)	8.2	0.40	24.0
VIII	670	10.8	0	0

(670 nm) showed a blue shift typical for chl–Triton micelles [21]. Therefore fraction VIII (~10% total chl) is regarded to consist of 'free pigments' perhaps separated from their protein during the isolation procedure [10].

As seen in fig.1, the separation of different chl–protein complexes was not perfect when agarose instead of acrylamide [16] was the supporting gel material. However, agarose gels were used in this study because of their better consistency as compared to the 3% acrylamide used before [16].

In contrast to proteins, uncharged galactolipids did not migrate when separately subjected to IEF under identical conditions (fig.2a). Therefore IEF was used to determine the ratio between galactolipids bound to chl–protein complexes and galactolipids not associated with chl–protein complexes after membrane solubilization. Table 2 shows that the major part of galactolipids (84–89%) was not bound to chl–protein complexes but remained as immobile fraction near the sample application trough.

3.2. *Distribution of newly synthesized galactolipids between chl–protein complexes and free lipid fractions*

The presence of galactolipids in both the immobile lipid fraction and the mobile chl–protein fractions (table 2) could suggest that galactolipids of thylakoid membranes are organized heterogenously, including galactolipids separated easily from the chl–protein complexes during membrane solubilization and others remaining bound to the complexes. We expected some clarification of this question from experiments on the distribution of newly synthesized galactolipids since in vivo and in vitro labelling of galactolipid molecular species had indicated several non-mixing galactolipid pools [22]. Therefore we investigated whether these lipids after being incorporated into thylakoid membranes occurred preferentially in the fraction of potentially free lipids or whether they became associated with chl–protein complexes. For this purpose we prepared thylakoids containing newly synthesized, ^{14}C-labelled galactolipids by incubating intact chloroplasts

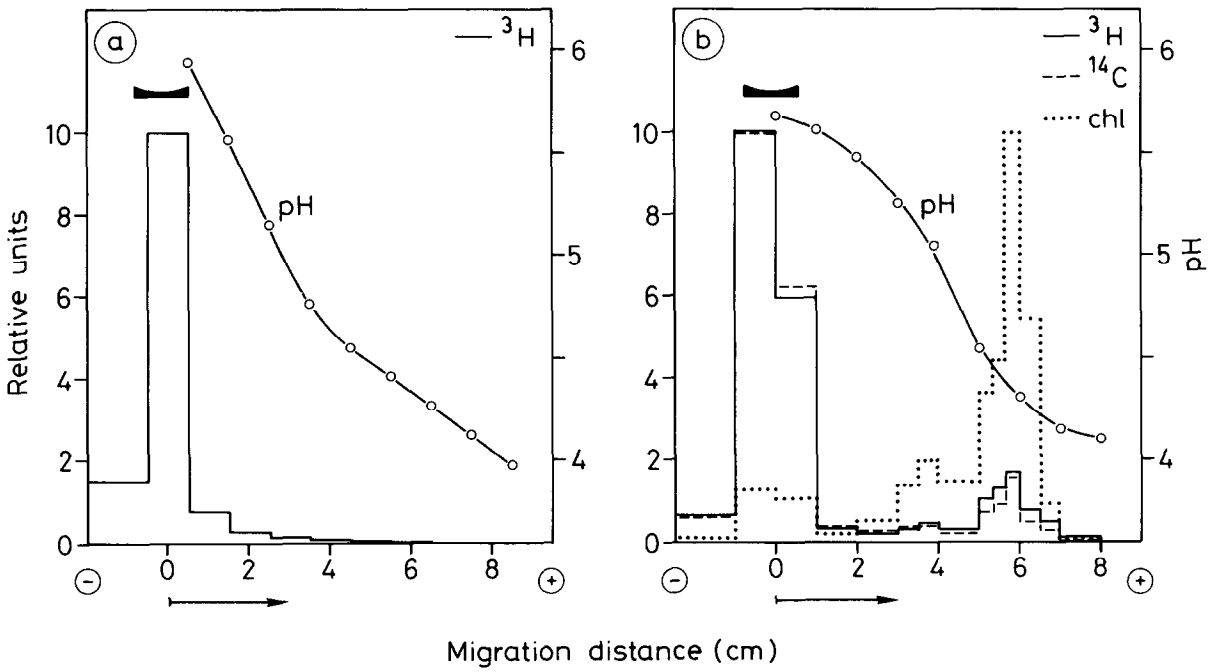

Fig.2. Distribution of chlorophyll and [^{14}C]galactolipids after IEF of solubilized thylakoids on agarose gel in comparison with synthetic [^3H]MGDG. (a) IEF of synthetic [^3H]MGDG in Triton X-100 solution (conditions as in table 3, expt. I). (b) IEF of synthetic [^3H]MGDG added to [^{14}C]galactolipid-labelled thylakoids after solubilization with Triton X-100 (conditions as in table 3, expt. III). Gel slices obtained after IEF were extracted with methanol and the extracts analyzed as in section 2. The traces are normalized in relation to their maximum, i.e., 0.71 × 10^6 dpm ^3H in (a) and 3.2 × 10^6 dpm, 0.14 × 10^6 dpm and absorbance units at 665 nm for ^3H, ^{14}C and chlorophyll, respectively, in (b). (▬) Sample application trough.

Table 2
Partial separation of chlorophyll- and lipid-containing fractions from Triton X-100-solubilized spinach thylakoids by IEF on agarose gel

Compound	Content in gel region with			
	pH 3.5 – 5.5[a] (μg)	(%)	pH ⩾ 5.5[b] (μg)	(%)
Chl	1540	81.0	360	19.0
MGDG	260	16.3	1340	83.7
DGDG	35	11.4	271	88.6

[a] Gel region containing fractions I–VI of table 1
[b] Gel region containing sample application trough and fractions VII + VIII of table1

The percentage values sum up to 100% separately for each compound in the horizontal lines

for 2 h with UDP-[^{14}C]galactose. During this time [^{14}C]galactosyl residues were incorporated into polyunsaturated galactolipids by the envelope-located galactosyl transferease [23] and subsequently transported to thylakoid membranes [15,24]. Analysis of washed thylakoids prepared from these chloroplasts showed that ~40% of the radioactive substrate was incorporated into the lipid fraction, in which galactolipids were the only compounds labelled.

Fig.2b and table 3 show that newly assembled, ^{14}C-labelled galactolipids occured mainly in the immobile fraction of free lipids, while 10–20% were observed in the mobile chl–protein fractions. This distribution resembles the distribution of unlabelled lipids (table 2). In the chl–protein fractions labelling varied in parallel to the chlorophyll content (fig.2b), but a clear stoichiometry was not evident. The labelled galactolipids occuring in the different mobile and immobile fractions were further analysed for label in individual galactolipid homologues. All fractions (I, II, III + IV, V + VI, VII + VIII) contained labelled MGDG, DGDG and TGDG in very similar ratios. When label in MGDG was set as 100, the labelling of DGDG in the different fractions varied from 14.6–16.7 and that of TGDG from 1.9–5.8.

3.3. *Distribution of exogenous MGDG between chl–protein complexes and free lipid fractions*

The various chl–protein fractions could be expected to have similar ratios of newly synthesized MGDG to DGDG, if galactolipids after release during membrane solubilization became associated unspecifically with released chl–protein complexes. To check the possibility of unspecific association we added synthetic [^{3}H]-

Table 3
Distribution between different pH ranges of synthetic [^{3}H]MGDG, [^{14}C]galactolipids and chlorophyll-containing fractions from Triton X-100-solubilized spinach thylakoids after IEF on agarose gel plates

Expt[a]	Compound	Rel. content (%) in gel region with	
		pH 3.5–5.5	pH ⩾ 5.5
I: [^{3}H]MGDG	[^{3}H]MGDG	1.2	98.8
II: Thylakoids labelled with UDP-[^{14}C]galactose	[^{14}C]Galactolipids	19.7	80.3
	Chlorophyll	86.0	14.0
III: [^{3}H]MGDG + thylakoids labelled with UDP-[^{14}C]galactose	[^{3}H]MGDG	13.8	86.2
	[^{14}C]Galactolipids	11.4	88.6
	Chlorophyll	82.9	17.1

[a] (I) Samples subjected to IEF contained [^{3}H]MGDG (1.1 × 10^{6} dpm) in 80 μl 2.5% Triton X-100; (II) Thylakoids (360 μg chl) labelled with [^{14}C]galactolipids (10^{6} dpm) synthesized from UDP-[^{14}C]galactose, solubilized in 265 μl final vol. containing 1 mM EDTA (pH 7.8) and 2.25% Triton X-100; (III) [^{3}H]MGDG (7.6 × 10^{6} dpm) plus thylakoids (350 μg chl) labelled with [^{14}C]galactolipids (0.32 × 10^{6} dpm) synthesized from UDP-[^{14}C]galactose, solubilized in 270 μl final vol. containing 1 mM EDTA (pH 7.8) and 2.25% Triton X-100

For labelling, intact chloroplasts (0.8 mg chl) were incubated with UDP-[U-^{14}C]galactose (1–2.5 μCi) in isotonic medium (1 ml) for 2 h

MGDG to solubilized thylakoids and separated the mixture by IEF (fig.2b). The [^{3}H]MGDG pattern obtained under these conditions differed significantly from the pattern obtained after IEF of pure [^{3}H]-MGDG in the absence of thylakoids (fig.2a), since now 13.8% of the ^{3}H-label migrated with the mobile fractions (table 3, expt. III). This result indicates that exogenous galactolipids added to the mixture of Triton X-100 solubilized thylakoids can indeed be bound to the chl–protein complexes in such a way that they are not removed during subsequent IEF.

This was confirmed by a double-labelling experiment. [^{3}H]MGDG was added to solubilized thylakoids which had been labelled biosynthetically with [^{14}C]-galactolipids. After IEF of the double-labelled mixture, exogenous [^{3}H]MGDG and endogenous [^{14}C]-galactolipids were recovered from the different chl–protein fractions in similar proportions as shown by the ^{3}H/^{14}C ratios which in all fractions were 23–32. The similar stability in complexation of [^{3}H]MGDG and [^{14}C]galactolipids was also shown by the following continuation of this experiment: When the chl–protein bands from the above separation, double-labelled with exogenous [^{3}H]MGDG and endogenous [^{14}C]-galactolipids, were cut from the gel plate after the first IEF and refocused on a second gel, both labels were found decreased in the refocused complexes, the decrease varying from 20–40% of either label. Thus, complexation between chl–proteins and exogenous [^{3}H]MGDG or endogenous [^{14}C]galactolipids could not be distinguished, neither by means of specific preferences for different chl–proteins nor by differences in stability.

4. Discussion

When thylakoids are fractionated by treatment with Triton X-100, >80% of their galactolipids are separated from their chl–protein complexes, i. e., from the majority of the integral thylakoid proteins. Since this fractionation allows the recovery of chl–protein complexes in a photochemically active state and releases only ~10% of the chl, it is regarded to be gentle enough to maintain the integrity of the chl–protein complexes. Therefore, the 'free' galactolipids obtained under our experimental conditions seem to originate from the lipid matrix of the thylakoid membrane.

Less than 20% of the thylakoid galactolipids are associated with chl–protein complexes, which means an average ratio of 'bound' galactolipid to chl of 1:4.5. These 'bound' galactolipids, or at least their main part, do not seem to be bound specifically to chl–protein complexes as would be expected for an integral component. Instead, they seem to participate in associations formed during membrane solubilization between released galactolipids and chl–proteins. This is concluded from the observation that [^{3}H]MGDG added to the mixture of solubilized membranes is distributed between chl–proteins and the free-lipid fraction similar to the galactolipids pre-existing in the membrane or introduced biosynthetically. The occurrence of both exogenous MGDG and endogenous galactolipids in similar ratios as 'free' and 'bound' species suggests that in the solubilization mixture pre-existing galactolipids, newly assembled MGDG and DGDG as well as exogenous MGDG are subjected to the same distribution mechanism between 'free' and 'bound' condition in the form of a dynamic equilibrium. As a consequence, galactolipids would not be fixed to a special complex but would permanently exchange between simple lipid–detergent associations and between associations formed of chl–protein, lipids and detergent. Therefore, specific associations between endogenous galactolipids and chl–protein complexes are not evident after membrane solubilization. On the other hand, the possibility that a minor part of the 'bound' galactolipids might be bound specifically to a chl–protein complex is not ruled out completely by our experiments. In mitochondrial membranes as little as 1 or 2 mol cardiolipin were found to be associated specifically with 1 mol cytochrome *c* oxidase [25].

While galactolipids might not play a significant role as integral parts of the chl–protein complexes, they represent the main constituents of the lipid matrix in thylakoid membranes. Taking into account the protein-bound status of chlorophylls these lipid domains may be regarded as non-green bilayer patches. Apart from uncharged lipids, a few charged lipids such as sulfo- and phospholipids are present in thylakoids [3]. Whether these lipids are associated more specifically to the chl–protein complexes [26] than the galactolipids requires further examination.

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