Lipid polymorphism of the marginal region of plant thylakoid membranes

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Abstract

Thylakoid membranes (TMs) of vascular plants exhibit a highly organized architecture comprising two main distinct functional domains which accommodate virtually all components of the photosynthetic machinery responsible for the light reactions: the stacked, appressed granum membranes and the unstacked stroma lamellae. A domain with distinct structural, compositional, and functional characteristics, the marginal region of grana (grana margin, GM) has also been identified, which includes the highly curved region at the periphery of the granum vesicles that is enriched in CURT1 proteins. It is also well established that plant TMs, in addition to the bilayer (or lamellar, L) phase, contain at least two isotropic (I) lipid phases and an inverted hexagonal (H_{II}) phase. The I phases were shown to participate in membrane fusion and to be associated with the activity of the photoprotective water-soluble enzyme, the violaxanthin de-epoxidase (VDE); the H_{II} phase, for its trypsin sensitivity, was proposed to emerge from stroma-exposed lipid-protein assemblies. Considering the location of CURT1 in GM and that it possesses trypsin-sensitivity, it was hypothesized to participate in the formation of the H_{II} phase. The aim of the present study was to test this hypothesis – via fingerprinting the lipid phase behavior of GM particles isolated from spinach leaves. Our ^{31}P-NMR spectroscopy reveals that the GM fraction of TMs does not contain H_{II} phase signal; it exhibits an isotropic signature. This non-bilayer, isotropic lipid structure, together with CURT1 proteins, thus appear to promote the formation of the highly curved region of TMs.

Keywords: ^{31}P-NMR, CURT protein, granum margin, non-bilayer lipid phase, thylakoid membrane

Introduction

The thylakoid membranes (TMs) exhibit a complex architecture that accommodates the main components performing the light reactions of oxygenic photosynthesis. In chloroplasts they form extended multilamellar networks of flattened vesicles, separating the inner and outer aqueous phases, the lumen and the stroma, respectively. In vascular plants, TMs are organized into two main domains: the granum, consisting of tightly stacked membrane discs, and the stroma lamellae, which are unstacked membranes wounding around the grana in a quasi-helical manner [1, 2]. The protein composition in plant TMs displays lateral heterogeneity. Photosystem II (PSII) and its associated light-harvesting antenna complex (LHCII) are predominantly located in the appressed membranes, while Photosystem I (PSI) and its light-harvesting antenna, LHCI, along with the ATP synthase, reside in the stroma lamellae. The cytochrome *b6f* complexes are distributed uniformly throughout the membrane system [3].

In addition to these two basic building units of TMs, a third domain, the granum margin (GM), has been proposed to be a compositionally, structurally, and functionally distinct region [4-6]. The precise nature and extent of GM is still under debate. Certain electron microscopy (EM) studies identify the GM as highly curved areas at the periphery of grana, comprising roughly 5-7% of the TM area [7, 8]. Using ultrasonic fragmentation, a margin annulus of about 60 nm width was isolated, a region with large contact area between the PSII and the PSI domains [5]. GM obtained by digitonin fragmentation has been shown to contain three types of membrane fragments: curved membranes enriched in CURT proteins, fret-like protrusions and short planar membranes; this GM was found to function as degradation and disassembly zone for photo-damaged PSII [9]. Highly purified CURT1-enriched domain, CR fraction, has been identified using low concentration of digitonin (0.4%), a preparation (aka loose pellet) that is largely devoid of chlorophyll-binding protein complexes [10]. This CR fraction thus represents a subdomain of GM, distinct from the region where PSII-LHCII-PSI interactions occur. It is noteworthy that GM possesses approximately three times more lipids relative to chlorophyll (Chl) and about twice as much relative to protein than TMs [9]. This suggests that the generation of the highly curved membrane region of TMs, besides CURT1, depends on the organization of the lipid molecules.

The overall lipid composition of TMs is primarily composed of mono- and di-galactosyldiacylglycerol (MGDG and DGDG), which account for about 50% and 30% of the total lipid content, respectively, with smaller amounts of sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG). The bilayer organization of these lipids is thought to be crucial for the generation of the proton motive force, and its utilization for ATP synthesis. However, MGDG is a non-bilayer forming lipid; these lipids are inclined to form non-bilayer structures such as inverted hexagonal (H_{II}), isotropic (I), and cubic phases [11, 12]. In natural and artificial membranes containing phospholipids, the phase behavior of the bulk lipid molecules can be characterized by ^{31}P-NMR spectroscopy [13-15]. In TMs, ^{31}P-NMR spectroscopy tracks the motion of bulk PG molecules in the membrane; serving as a sensitive internal probe of the lipid phase behavior of TMs [16, 17]. This is justified by the findings that PG displays no lateral heterogeneity among the bulk phase of TM domains [18] and is homogenously distributed in TM lipid mixtures [19]. ^{31}P-NMR studies on various plant TMs have revealed three distinct phases in addition to the bilayer or lamellar (L) phase: an inverted hexagonal (H_{II}), and at least two isotropic (I) lipid phases. Extensive studies estimated that only about 40% of bulk lipids are present in the bilayer, with an equal amount in the H_{II} phase, and the remaining 20% in I phases [12]. It has also been clarified that the lipid polymorphism in isolated granum and stroma TMs is very similar to that in TMs, showing that the strikingly different protein composition of these TM domains does not bring about different lipid polymorphism [20].

The effects of lipases and proteases revealed that the non-bilayer lipid phases are found in different subdomains of TMs, distinct from the bilayer domains enriched in PSI and PSII supercomplexes. I phases were found in the lumenal compartment (VDE:lipid assembly) [21], and were proposed to be involved in membrane fusions, as inferred from the lateral fusion of PSII (BBY) membranes [22] (VDE, violaxanthin de-epoxidase). Trypsin-digestion experiments have unveiled that lipids assuming H_{II} phase are associated with stroma-exposed proteins or polypeptides [23]. Earlier, MGDG was proposed to promote inverted micellar structures around hydrophobic proteins and be responsible for generating the concave curvature region in the margin [24]. However, as clarified later, the high curvature in this region is warranted by CURT1 proteins [6].

Because of the location of CURT1 in the highly curved region of GM and the trypsin sensitivity of this protein [6], it was hypothesized to participate in the formation of such H_{II} structures [23]. In this work we tested this hypothesis and show that CR preparations do not produce ^{31}P-NMR signal with H_{II} phase signature, rather they exhibit isotropic signals – suggesting that the highly curved region is generated by CURT1:lipid assemblies with isotropic lipid arrangement.

Materials and Methods

Isolation of the curvature region

To isolate the curvature region (CR), or loose pellet we followed the step-by-step protocol described by [25]. The spinach leaves were homogenized in ice-cold B1 buffer (50 mM HEPES/KOH (pH 7.5), 330 mM sorbitol, 5 mM $MgCl_2$, 2.5 mM ascorbate, 0.05% (w/v) BSA), then filtered through two layers of nylon mesh and centrifuged at 5,000 × g at 4 °C for 4 min. Then, we suspended the resulting sediment in 30-40 mL of B2 buffer (50 mM HEPES/KOH (pH 7.5), 5 mM sorbitol, 5 mM $MgCl_2$) and centrifuged again at 5,000 × g for 4 min. We suspended the sediment in 30-40 mL of B4 buffer (50 mM HEPES/KOH (pH 7.5), 100 mM sorbitol, 5 mM $MgCl_2$), which was followed by another centrifugation with the same parameters, then we suspended again the sediment in a small amount of B4 buffer. The volume was adjusted to 0.5 mg Chl mL^{-1} with B3 buffer (15 mM Tricin (pH 7.8), 100 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$) and digitonin (Sigma-Aldrich, Burlington, MA, USA) at a final concentration of 0.4% (w/v). The suspension was stirred for 8 min with a magnetic stirrer at room temperature and then centrifuged at 1000 × g for 3 min. The supernatant was centrifuged for 30 mins at 10,000 × g to separate the core of grana; the supernatant was centrifuged at 40,000 × g for another 30 min. Following these steps, the supernatant was centrifuged at 144,000 × g for another 1 h to separate the solid sediment (containing the stroma lamellae) and the loose pellet containing the CR. The fractions collected were suspended in B4 buffer; and the suspension was at -80 °C until use.

Enzymatic treatments with wheat germ lipase and trypsin

Isolated CR particles were treated with wheat germ lipase (WGL) – a substrate nonspecific general tri-, di-, and monoglyceride hydrolase/lipase [26]. WGL was purchased from Sigma-Aldrich (Burlington, MA, USA) and was applied in 20 U mL^{-1} concentration; the treatments and incubations were performed at 5 °C. Earlier, using thin layer chromatography, we have verified that WGL digests the main TM lipid species, MGDG [22, 23].

For trypsin (T8003; trypsin from bovine pancreas; Sigma-Aldrich, Burlington, MA, USA) treatment, a stock of 300 mg mL^{-1} was dissolved in demineralized water, and 33 µL per mL of sample was added for a total concentration of 10 mg mL^{-1}. The suspension was thoroughly mixed and kept at 5 °C until the start of the measurements.

^{31}P–NMR spectroscopy

^{31}P-NMR spectroscopy was performed as described in [20]. Briefly, the spectra were recorded at 5 °C, using an Avance Neo 600 MHz NMR spectrometer (Bruker, Billerica, MA, USA) with a BBFO SmartProbe that was tuned to the P-atom frequency. The sample was loaded into 5 mm diameter NMR tubes. For spectra acquisition, 40° RF pulses with an inter-pulse time of 0.5 s were applied without ^{1}H-decoupling, as in earlier experiments [27]. As an external chemical-shift

reference 85% solution of H_3PO_4 in water was used. In the saturation transfer (ST) experiments, we employed RF pulses with low power at the designated frequency for a duration of 0.3 s, followed by 40° pulses with an acquisition time of 0.2 s and a repetition time of 0.5 s. The intensity of the pre-saturation pulse was adjusted based on the intensity of the saturated peak. For the pre-saturation RF pulses, the field strength was set to 40 Hz.

CR samples from two batches, with very similar features, were averaged to improve the signal-to-noise ratio. For ^{31}P-NMR data processing, TopSpin software (Bruker, Billerica, MA, USA) was used. The figures were plotted using MATLAB R2020b (MathWorks, Inc., Portola Valley, CA, USA).

Fourier-transform infrared spectroscopy

To determine the lipid-to-protein ratios in TMs and CR, we used FTIR spectroscopy (see Supplementary Material). Membrane fraction suspensions were washed in D_2O-based PBS (phosphate-buffered saline) solution for complete H_2O to D_2O exchange. The sample was layered between CaF_2 windows, separated by an aluminum spacer, and placed in a Bruker Vertex70 FTIR spectrometer. Spectra were recorded at 5 °C between 4,000 and 900 cm^{-1}, 512 interferograms were collected for each spectrum, the spectral resolution was 2 cm^{-1}. The infrared absorption spectrum of the samples was calculated from the background and sample of single beam spectra with Opus software of Bruker. For the analysis of the structural properties of the membrane, the ester C=O plus the amide I region, which in this paper will be referred to as 'Ester + Amide I' region was used between 1,800 and 1,595 cm^{-1}. To obtain the relative intensities of the 'ester' and the 'amide I' bands in the 'Ester + Amide I' region, a 3rd order polynomial was fitted and subtracted as baseline. The area under the curves were calculated by using in-built MATLAB functions, after trimming the spectral regions corresponding to the 'ester' and 'amide I' bands.

Scanning electron microscopy

The membrane fractions were fixed in 2.5% glutaraldehyde for 2 hours, settled on poly-L-lysine – coated polycarbonate filter for 45 min. After post-fixation in 1% OsO_4 for 50 min, the samples were dehydrated in aqueous solutions of increasing ethanol concentrations, critical point dried, covered with 5 nm gold by a Quorum Q150T ES (Quorum Technologies, Lewes, UK) sputter and observed in a JEOL JSM-7100F/LV (JEOL SAS, Croissy-sur-Seine, France) scanning electron microscope.

Results

Morphology of curvature region

For the structural characterization of CR, scanning electron microscopy (SEM) was performed. In line with our expectation, CR consisted of bent, crescent-shaped small membrane particles with a tendency to self-aggregate – in good agreement with the findings of [9]. It is worth mentioning that our isolation protocol was particularly aiming at yielding high-purity CURT1-enriched curvature fraction from the TMs.

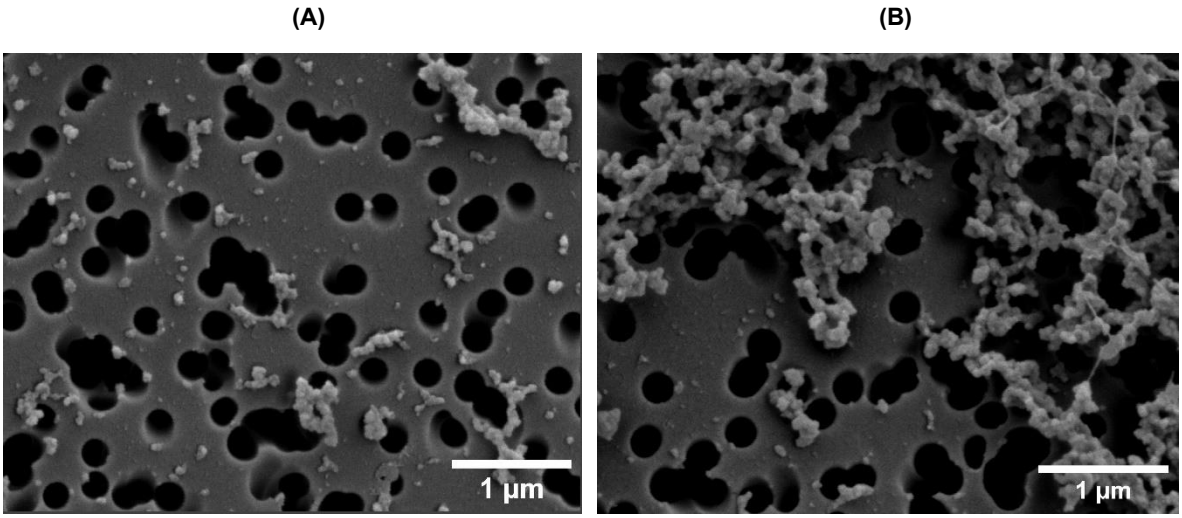

*Figure 1: SEM micrographs of CR fractions showing crescent-shaped membrane particles (**A**), which often coagulate (**B**).*

Lipid phase behavior of CR particles

As evidenced by the ^{31}P-NMR spectra shown in Figure 2, the CR particles do not contain L and H_{II} phases, which are present in intact TMs and the granum and stroma subchloroplast membrane particles isolated with the same procedure. Instead, the spectra of CR preparations are dominated by well-discernible I phases with peak positions at around 0.15 and 2.7 ppm. Saturation transfer experiments confirmed the presence of these two strongly overlapping phases, although other resonances with smaller amplitudes could also be found in the band structure.

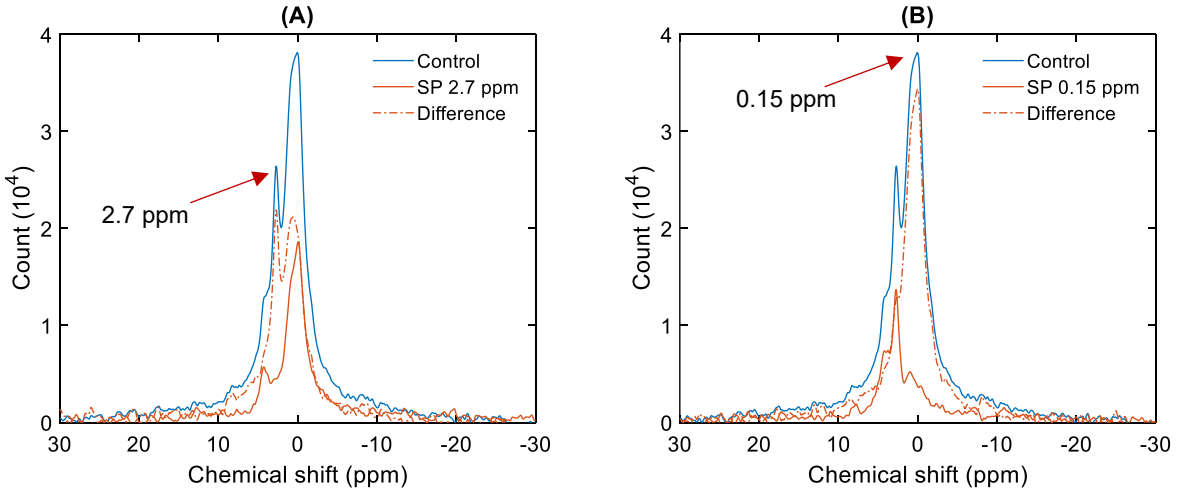

*Figure 1: ^{31}P-NMR spectra of CR under control conditions, displaying also the effects of saturation pulses (SP) at 2.7 ppm (**A**) and 0.15 ppm (**B**). The number of scans was 12,800.*

The resonance bands that originate from the lipid phases were sensitive to WGL digestion (Figure 2A) similarly to what has been observed in TMs and the granum and stroma subchloroplast particles [20, 23]. This shows the character of the I phases in CR closely resembles those in intact TM and in the digitonin-fragmented grana and stroma lamellae.

In contrast, trypsin treatment did not elicit any sizeable effect on the spectra (Figure 2B). As we have previously shown [23], trypsin treatment selectively degraded the ^{31}P-NMR detectable H_{II} phase of TMs, which revealed that this lipid phase is associated with proteins or residues found in the stromal aqueous side of TMs. Although CURT1 has been shown to be susceptible to trypsin digestion, the absence of H_{II} phase and the trypsin-insensitive nature of the detected non-bilayer lipid phases suggests that these proteins do not contribute to the formation of the H_{II} phase.

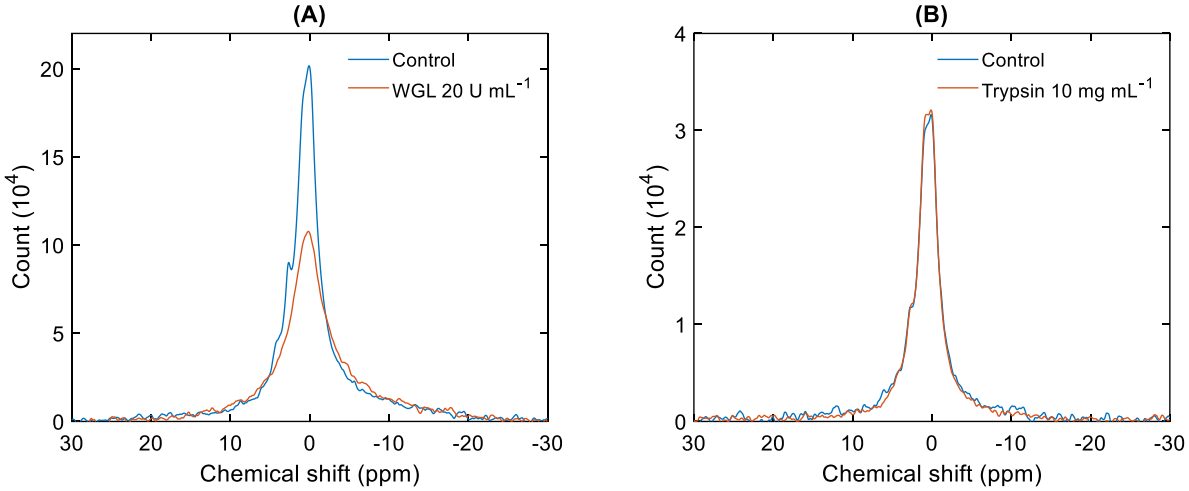

*Figure 2: ^{31}P-NMR spectra of untreated (Control) and WGL-treated (20 U mL^{-1}, 25,600 scans) (**A**) and trypsin-treated (10 mg mL^{-1}, 19,200 scans) (**B**) CR samples.*

Conclusions

Our ^{31}P-NMR spectroscopy data unveiled that the CR fraction of TMs displays merely an intense and a weaker isotropic lipid phase. The lack of the lamellar phase in CR preparations is evidently the consequence of the deficiency of PSII and PSI supercomplexes, which in TMs stabilize the bilayer. The spectral features of the CR fraction strongly suggest that the lipids around the CURT1 protein are organized into isotropic arrays; and thus the H_{II} phase in TMs must originate from a different region containing stroma-exposed proteins that are surrounded by lipid molecules. Identification of the proteins or protein subunits that are encapsulated by lipid molecules requires further experiments.

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Conflict of interest

The authors have no conflict of interest to declare.

Author contributions:

The study was conceptualized by G.G., K.B. and V.Š.; TM and CR preparations were isolated by O. Zs. and K.B.; ^{31}P NMR spectroscopy measurements were performed by U.J., supervised by J.P.; data analyses were performed by K.B. and O.D., with the help of U.J., supervised by G.G.; spectral characterizations of the membranes were performed by K.B., O.D., supervised by V.Š.; FTIR measurements were carried out and analyzed by K.B.; SEM experiments were performed by I.D.; The paper was written by G.G. and K.B., with all authors contributing to the writing.

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