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Phase behavior of phosphatidylglycerol in spinach thylakoid membranes as revealed by ^{31}P-NMR

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Abstract

Non-bilayer lipids account for about half of the total lipid content in chloroplast thylakoid membranes. This lends high propensity of the thylakoid lipid mixture to participate in different phases which might be functionally required. It is for instance known that the chloroplast enzyme violaxanthin de-epoxidase (VDE) requires a non-bilayer phase for proper functioning in vitro but direct evidence for the presence of non-bilayer lipid structures in thylakoid membranes under physiological conditions is still missing.

In this work, we used phosphatidylglycerol (PG) as an intrinsic bulk lipid label for ^{31}P-NMR studies to monitor lipid phases of thylakoid membranes. We show that in intact thylakoid membranes the characteristic lamellar signal is observed only below 20 °C. But at the same time an isotropic phase is present, which becomes even dominant between 14 and 28 °C despite the presence of fully functional large membrane sheets that are capable of generating and maintaining a transmembrane electric field. Tris-washed membranes show a similar behavior but the lamellar phase is present up to higher temperatures. Thus, our data show that the location of the phospholipids is not restricted to the bilayer phase and that the lamellar phase co-exists with a non-bilayer isotropic phase.

Keywords: Non-bilayer lipid phases; Isotropic lipid phase; Phosphatidylglycerol; ^{31}P-NMR; Thylakoid membranes

1. Introduction

In the thylakoid membranes, lipids constitute ~20% of the dry mass and the protein content of thylakoids is likewise high (~80%); the non-bilayer lipid monogalactosyl diacylglycerol (MGDG) is the major lipid constituent, accounting for about half of the total lipid content of the thylakoid membrane [1]. The rest of the thylakoid lipids are bilayer-prone lipids; in higher plant chloroplasts, digalactosyl diacylglycerol DGDG (30%), sulfoquinovosyl diacylglycerol SQDG (5–12%) and phosphatidylglycerol PG (5–12%). The high content of non-bilayer lipids lends high non-bilayer propensity to the whole lipid mixture, which thus can readily participate in different lipid phases [1].

The relative amounts of non-bilayer lipids change readily in response to variations in environmental conditions [2] but it is generally believed that in biological membranes non-lamellar phases are not present in sizeable quantities and for substantial time periods [1,3]. However, it has been shown that total lipid extracts from thylakoid membranes and inner membrane chloroplast envelope form non-lamellar structures in dilute salt solutions [1,4]. It has also been established that non-bilayer phases can be formed in the thylakoid membrane at low pH [5], in the presence of high concentrations of metal cations [6], phospholipase A2 [7], at high temperature [8] and in the presence of co-solutes [9].

In addition to the well established functional roles in the activities of various membrane proteins [3,10,11] non-bilayer

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lipids have been hypothesized to play important roles in the bulk phase. It has been proposed that they exert high lateral packing pressure on intrinsic membrane proteins, thereby influencing their functional activity [12]. The ability of the lipid mixture to segregate into bilayer and non-bilayer phases has been proposed to regulate the protein content of the membranes, and it was suggested that the non-lamellar phase might be closely associated with the membrane and might exist in a dynamic equilibrium with the bilayer phase, thus contributing to the structural flexibility of the membranes [13]. It was shown that the major thylakoid protein, the main light-harvesting complex of photosystem II, LHCII, can force purified MGDG to form bilayer structures [14]. LHCII–lipid macroassemblies were also obtained from the association of freshly prepared lamellar aggregates of LHCII and different purified thylakoid lipids. They exhibited a remarkable structural flexibility, being capable of undergoing light-induced reversible structural rearrangements, which closely resembled the analogous transients in the native membranes [15]. These reorganizations were enhanced by the addition of lipids, with the largest enhancement obtained with MGDG [16].

It was shown that MGDG [17] and non-bilayer phases in general [18] are needed for the operation of VDE, an enzyme that is involved in the photoprotection of plants [19]. It was proposed that in the thylakoid membrane MGDG serves to solubilize the xanthophyll cycle pigments and furthermore provides inverted hexagonal phase (H_{II}) structures associated with the membrane bilayer, which are essential for efficient xanthophyll de-epoxidase activity [20]. Recently, Szilágyi et al. [21] proposed that the operation of VDE requires negative curvature elastic stress in the thylakoid lipid bilayer. These data show that non-bilayer lipids and non-lamellar phases play important roles in the operation of VDE in thylakoid membranes. Their role, however, is evidently not confined to this particular enzymatic function of the thylakoid membrane: e.g. cyanobacteria lack VDE but the lipid composition of their thylakoid membranes is essentially the same as in higher plants. Also, all energy converting membranes contain high amounts of non-bilayer lipids, which has been suggested to play a role in safe-guarding their high protein content and enhancing their structural flexibility [13].

Despite the relatively low concentration of PG in thylakoid membranes (about 10% of the total lipid content) and association of a number of PG molecules with pigment-protein complexes this lipid species is present in the bulk; one can estimate that the PG which is not associated with proteins represents nearly 90% of the total PG content [22]. As estimated from electron paramagnetic resonance measurements, about 30% of the PG is part of the solvation shell of membrane proteins and hence motionally restricted and 70% of PG can be rapidly exchanged by laterally diffusing membrane lipids [23]. In ^{31}P-NMR experiments all PG molecules with the exception of the insufficiently mobile ones (e.g. protein-bound) do contribute to the signal. Furthermore, the inorganic phosphorus content of isolated thylakoid membranes is low; with the exception of a possible inclusion of some inorganic phosphate in the lumen, it can be washed out together with the stroma liquid. Thus, PG is part of the bulk lipid mixture and hence can be used as a reporter of variations in the bulk lipid phase behavior of thylakoid membranes. Moreover, although PG is a bilayer forming lipid, it can also participate in non-bilayer structures, which were detected by ^{31}P-NMR, upon neutralization of its negative surface charge [24] and also in total lipid extract of thylakoid membranes [25] and chloroplast envelope membranes [4].

^{31}P-NMR studies of thylakoid membranes have been performed before. However, the membranes were washed in 0.8 M Tris (pH 8.0) in order to remove the loosely bound Mn, which might broaden the ^{31}P-NMR signal [26], and were used either as a suspension or were freeze-dried and subsequently rehydrated with D_2O [26,27] in order to ensure better proton decoupling. These treatments, however, perturb the functional activity of membranes: they loose the oxygen-evolving capability [28] and lead to changes in the macroorganization of the membrane proteins [29], also observed as a loss of the psi-type circular dichroism signal originating from LHCII-containing chiral macrodomains (Krumova, S.B. and Garab, G., unpublished data). Hence, these treatments might affect the hydration shell of the lipids, and thus the physical state of the lipid phase. To our knowledge, intact thylakoid membranes (capable of oxygen evolution and retaining the macroorganization of the complexes) have not been studied with ^{31}P-NMR. In our experiments freshly isolated thylakoid membranes were suspended in isotonic medium with an ionic strength optimum for their functions, and the measurements were performed within a few hours during which time the membranes retained their structural integrity and functional activity, including their electrical impermeability. Our results revealed isotropic motion of the phospholipids in the temperature range 7–40 °C, in both intact and Tris-washed thylakoids, thus suggesting the presence of non-bilayer structures in these membrane preparations.

2. Materials and methods

2.1. Isolation and treatment of thylakoid membranes

Dark-adapted spinach leaves were homogenized in a medium containing 50 mM Tricine (pH 7.5), 400 mM sorbitol, 5 mM $MgCl_2$ and 5 mM KCl; the suspension was filtered through 4 layers of cheese cloth and centrifuged for 4 min at 4000 × *g*. The chloroplasts were osmotically shocked in a hypotonic medium containing 50 mM Tricine (pH 7.5), 5 mM $MgCl_2$ and 5 mM KCl, and centrifuged for 5 min at 6000 × *g*. After washing in the same medium supplemented with 400 mM sorbitol, the pellet was resuspended in this isotonic medium.

Electrochromic absorbance changes, ΔA515, induced by saturating single-turnover flashes, were measured at 515 nm, the maximum of the transients in thylakoid membranes, in a set-up described earlier [30]. The samples were thermostated; the time constant was set to 100 μs; 32 kinetic traces were collected with a repetition rate of 1 s^{-1} and averaged.

Oxygen-evolving activity of thylakoid membranes upon illumination was measured polarographycally using a Clark-type oxygen electrode (Hansatech Instruments, King's Lynn, UK) in the presence of 5 mM potassium ferricyanide. The samples were illuminated with saturating white light provided by a KL1500 lamp source (Schott, Germany). The temperature was controlled by a circulating water bath during the measurements.

Thermoluminescence measurements were carried out in a home-built apparatus described by Demeter et al. [31]. The samples were excited at −40 °C by two

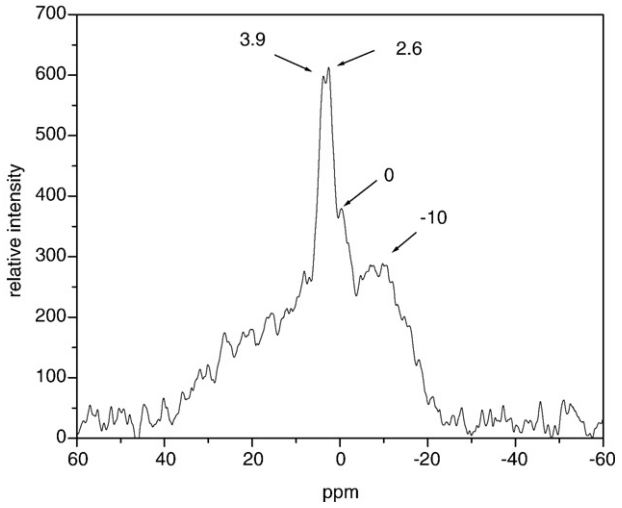

Fig. 1. ^{31}P-NMR spectrum of thylakoid membranes at 7 °C. 6700 free induction decays were accumulated, the line-broadening was 100 Hz. The positions (in ppm) of the different resonances are denoted by arrows.

saturating single-turnover flashes and the glow curves were recorded at a heating rate of 20 °C/min.

For ΔA515, oxygen evolution and thermoluminescence experiments, the thylakoid membranes were pre-incubated at 7, 20 or 30 °C at a concentration of 5 mg chl/ml. They were subsequently diluted to 60 and 200 μg chl/ml for ΔA515 and oxygen evolution, and thermoluminescence measurements, respectively.

For Tris-washing, the thylakoid membranes were isolated as described above and treated according to [26] with some modifications — the Tris-treatment was only 1 h and after washing with the same buffer, the sediment was resuspended in non-deuterated buffer 0.8 M Tris (pH 8.0).

In all our experiments, the leaves used for isolation of thylakoid membranes were dark-adapted for prolonged periods (1–8 h), which results in dephosphorylation of the membrane proteins [32], hence significant contribution of protein-bound phosphate can be neglected under our experimental conditions.

2.2. ^{31}P-NMR Measurements

^{31}P-NMR spectra were recorded with an AMX300 wide-bore spectrometer (Bruker, Germany) tuned at the resonance frequency of the ^{31}P nucleus (121.500 MHz, 7 T). 20 mm outer diameter tubes were used containing 15 ml of concentrated thylakoid suspension (~5 mg chl/ml). The temperature was controlled within 0.1 °C; spectra were recorded using a 40° pulse, an interpulse time of 0.5 s and no ^{1}H-decoupling was applied. Chemical shifts were scaled by referencing against a capillary containing methylene diphosphonic acid pH 8.9 (0.2 M) providing a resonance at 16.92 ppm.

For saturation transfer experiments, 0.3 s saturation irradiation (power 40 dB and frequency — 1800 Hz) was applied before switching on the measuring pulse (acquisition time 0.2 s).

Since we wanted to record the ^{31}P-NMR spectra within the shortest possible time, all spectra were collected for only 1 h. In this way the signal to noise ratio remained low, but the characteristic features for the different phases were still clearly discernible. All experiments were repeated at least 3 times and the observed tendencies were always the same. Stirring of the sample had no noticeable effect on the spectra, indicating that no significant magnetic orientation of membranes occurred. For the determination of the chemical shift position of isotropic di-myristoyl PG and inorganic phosphate, PG micelles formed with excess amounts of SDS in 20 mM tricine buffer were used; inorganic phosphorus was added to the suspension at a concentration of 1 mM. It was established that isotropic di-myristoyl PG resonates at +1 ppm and the inorganic phosphate at 2.6 ppm at pH 7.5.

3. Results and discussion

3.1. Line shape analysis of ^{31}P-NMR spectra of isolated intact thylakoid membranes at 7 °C

The ^{31}P-NMR spectrum of isolated thylakoid membranes (Fig. 1) detected at 7 °C shows that the signal is originating from several different chemical environments of the phosphorous nucleus. The inorganic phosphate content of washed thylakoid membranes, isolated from dark-adapted leaves, is largely reduced because their phosphoproteins are dephosophorylated and the stroma liquid is washed out (cf. Materials and methods). Thus, the observed resonances originate from PG and possibly from inorganic phosphate in the lumen.

As expected, the contribution of the lamellar phase could clearly be seen — with the high intensity peak at high field at about −10 ppm and the low intensity shoulder at low field around 30 ppm. This lamellar signal is broader than found in model systems [33], which is probably due to a larger heterogeneity in the dimensions of the lamellae. Besides this signal, we also observed a pronounced resonance at 4 ppm and weaker resonances at 2.6 ppm and 0 ppm (Fig. 1). It was established that the resonance at 2.6 ppm, which appears only as a shoulder on the 4 ppm signal, originates from inorganic phosphate (probably in the lumen), since it increased after the addition of potassium phosphate (data not shown). This resonance also dominates the ^{31}P-NMR spectrum of unbroken chloroplasts (data not shown), due to the high amount of phosphate in the stroma.

In order to determine the isotropic resonance position of the PG molecules constituting the thylakoid, the membranes were solubilized with SDS. After this treatment the spectra are dominated by the isotropic peak (Fig. 2), centered at about 0 ppm, coinciding with the low intensity resonance in unsolubilized membranes around 0 ppm (Fig. 1) and similar to the resonance frequency of di-myristoyl PG in SDS micelles (1 ppm,

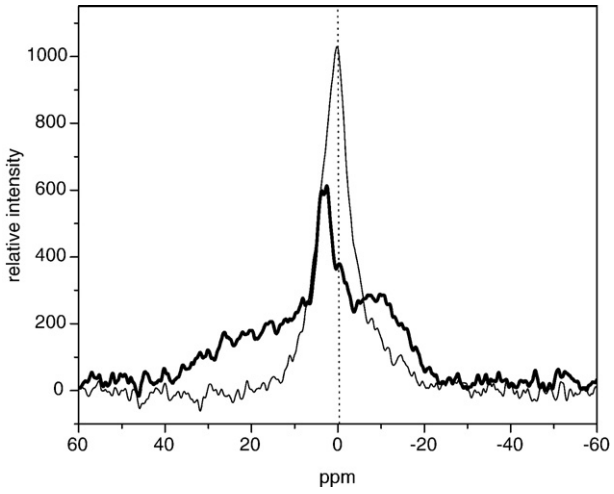

Fig. 2. ^{31}P-NMR spectra of thylakoid membranes before (thick line) and after (thin line) solubilization with 3% SDS. For each spectrum 6700 free induction decays were accumulated, the line-broadening was 100 Hz. The spectrum of non-solubilized membranes was recorded at 7 °C and the one of solubilized membranes at 14 °C. The dashed line denotes the isotropic position of PG determined after solubilization of the thylakoid membranes by SDS.

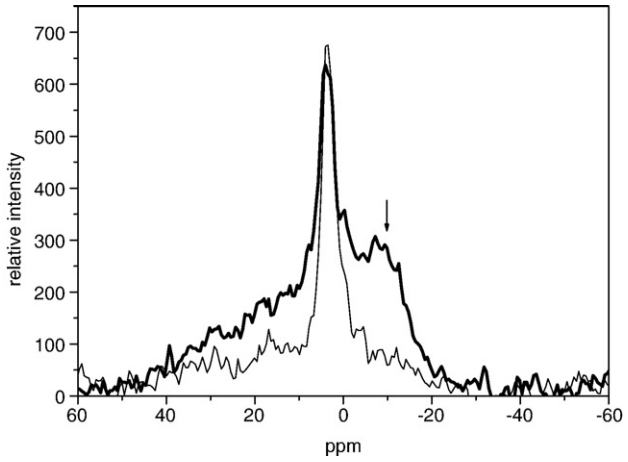

Fig. 3. ^{31}P-NMR spectra of thylakoid membranes without (thick line) and with (thin line) saturation at 7 °C. For the saturation transfer, irradiation with 0.3 s pulses of power 40 dB at -1800 Hz (-10 ppm, designated by arrow) was applied. For each spectrum 6700 free induction decays were accumulated, the line-broadening was 100 Hz.

see Materials and methods). At small line-broadening the 4 ppm resonance can be seen as a shoulder (data not shown).

Since the resonance at around 4 ppm appears close to the position where the high intensity edge of the H_{II} phase is expected, we checked whether this resonance possesses the asymmetrical shape characteristic for the H_{II} phase [33]. The spectral shape of this 4 ppm resonance can be obscured by its overlap with the resonance of the lamellar phase. To probe its spectral shape, we performed saturation transfer experiments (Fig. 3), where the irradiation frequency was set at −10 ppm, i.e., at the high intensity peak of the lamellar phase. This resulted in sufficient elimination of the lamellar resonance and revealed that the remaining 4 ppm peak is isotropic in nature. Moreover, its intensity was not decreased, suggesting that under these experimental conditions, at 7 °C, there is no considerable magnetization transfer between the lipids experiencing isotropic motion and the ones motionally restricted in the bilayer. This indicates that at 7 °C there is little or no exchange (on the ^{31}P-NMR timescale) between the phospholipids giving rise to these two resonances. In order to avoid irradiation of the high field shoulder of the potentially present H_{II} phase, we also performed saturation transfer experiments applying strong pulses at frequencies between 2500 and 800 Hz (10–30 ppm), where no contribution of the H_{II} signal can be expected. The lamellar signal was eliminated upon irradiation at 800 Hz, applying 30 dB pulse power. The intensity of the 4 ppm peak was also strongly reduced but its shape was rather symmetric (data not shown), thus giving no indication that it originated from H_{II} phase. An isotropic peak at this position was also observed in total lipid extracts of wheat leaves [25] and was attributed to cubic phase. However, we have shown that the isotropic position of thylakoid PG is at about 0 ppm, and hence the cubic phase should emerge at this resonance position. Thus the nature of the peak at 4 ppm remains unclear. Due to the low intensity of the 0 ppm peak in this preparation it is difficult to judge whether the saturation of the lamellar signal affects the isotropic peak at 0 ppm. However, this is clearly demonstrated in Tris-washed membranes (see section 3.3 and Fig. 5).

3.2. Thermally induced changes in the lipid phase behavior of intact thylakoid membranes

The heat-induced changes in the ^{31}P-NMR spectra are presented in Fig. 4A. With the increase of the temperature from

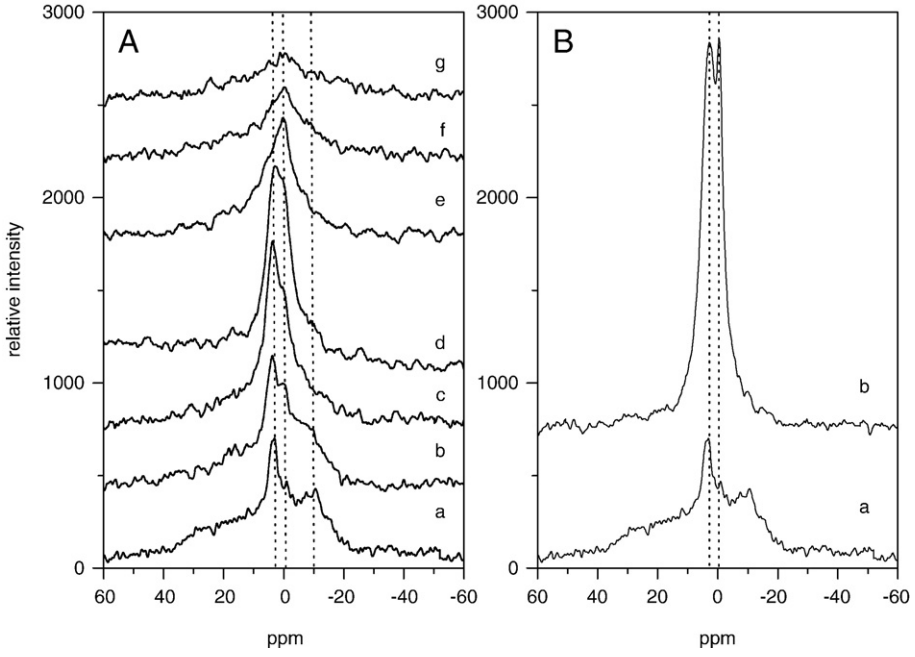

Fig. 4. Temperature dependence (A) of the ^{31}P-NMR spectra of thylakoid membranes: (a) 7 °C, (b) 14 °C, (c) 21 °C, (d) 28 °C, (e) 35 °C, (f) 42 °C, (g) 49 °C and comparison (B) of the spectra at 7 °C obtained before (a) and after (b) heating of the sample to 49 °C. For each spectrum 6700 free induction decays were accumulated, the line-broadening was 100 Hz. The dashed lines indicate the positions of the different resonances.

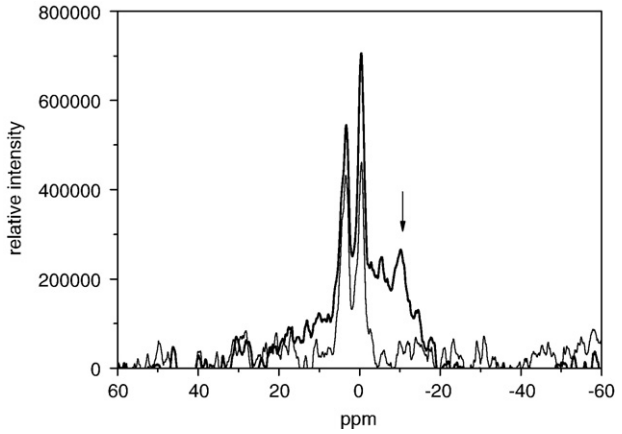

Fig. 5. ^{31}P-NMR spectra of Tris-washed thylakoid membranes without (thick line) and with (thin line) saturation at 7 °C. For the saturation transfer, irradiation with 0.3 s pulses of power 35 dB at −1800 Hz (−10 ppm, designated by arrow) was applied. For each spectrum 6700 free induction decays were accumulated, the line-broadening was 100 Hz.

7 °C to 21 °C the lamellar signal gradually disappears. The intensities of the 4 and 0 ppm peaks increase, both resonances start to broaden and the weak resonance at 2.6 ppm is no longer detectable. At 35 °C only one isotropic peak (0 ppm) remains, whereas the intensity of the 4 ppm resonance is obscured. At higher temperatures also the 0 ppm peak is decreasing in intensity, probably due to an increased T1 relaxation time (longer than the interpulse time of 0.5 s used in our experiments). Upon cooling of the samples (Fig. 4B) the contribution of the lamellar phase could not be recovered, however, two high intensity peaks at 0 ppm and 2.9 ppm were detected.

Since isotropic signals originate from regions which allow the motion of phopsholipid molecules in all directions, one might think that the marginal regions of the thylakoids could give rise to such resonances, since the margins are strongly curved, protein free areas ([34] and references therein). However, the marginal regions are much smaller in size as compared to the granal and stromal surfaces (at most 5% of the total volume of thylakoid membranes, [34]) and moreover their contribution is largely reduced in unstacked (Tris-washed and heat-treated) thylakoids. Thus, the isotropic peaks can at most only partly be due to the margin regions.

Alternatively, the isotropic signals might be generated upon destruction of the membrane and formation of small micelles. In order to test the integrity of the membranes, we recorded electrochromic absorbance changes at 515 nm (ΔA515) induced by single-turnover flashes. The amplitude of this absorbance transient measures the capability of membranes to generate a transmembrane electric potential gradient and its decay kinetics probes the permeability of the membranes [35]. Our results showed that 1 h incubations of the membranes at 7, 20 and 30 °C did not affect the magnitude of transmembrane electrical field; the membrane permeability was also retained at 7 and 20 °C, with a temperature dependence similar to that of freshly isolated intact chloroplasts [36]. Substantial deteriorations of these membrane functions and of the overall ultrastructure, revealed by electron microscopy, were observed only in samples incubated at and above 30 °C (data not shown). We have also determined that the oxygen-evolving capability, and the charge separation and stabilization in photosystem II (as measured by thermoluminescence) were preserved up to 30 °C; moreover, there was no substantial lipid peroxidation as indicated by the very low intensity (data not shown) of the high-temperature thermoluminescence band at around 75 °C [37] (data not shown). All these complementary experiments show that the integrity and functionality of the thylakoid membranes are retained between 7 and 28 °C, hence under conditions where

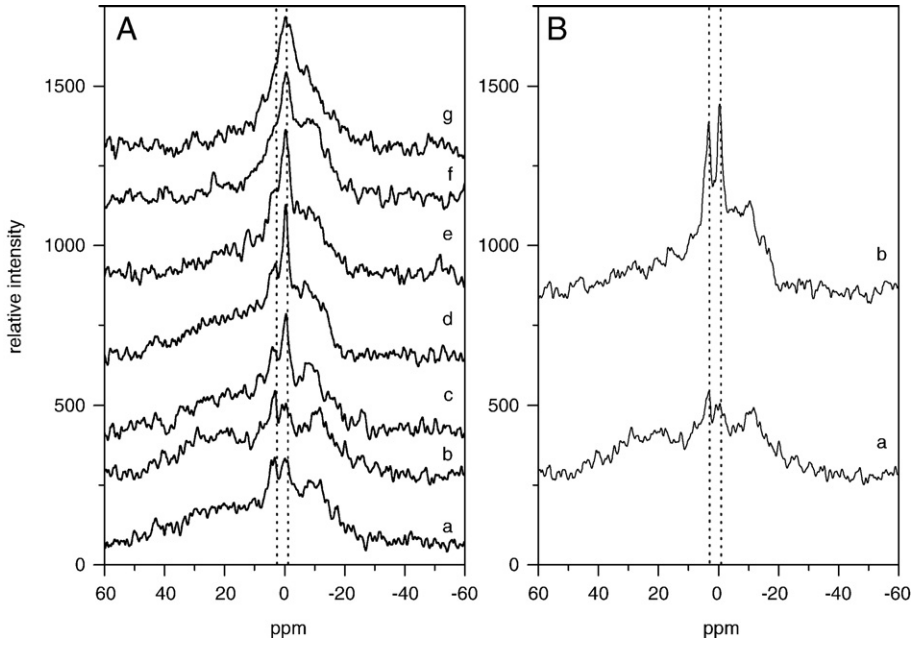

Fig. 6. Temperature dependence (A) of the ^{31}P-NMR spectra of Tris-washed thylakoid membranes: (a) 7 °C, (b) 14 °C, (c) 21 °C, (d) 28 °C, (e) 35 °C, (f) 42 °C, (g) 49 °C and comparison (B) of the spectra at 7 °C obtained before (a) and after (b) heating of the sample to 49 °C. For each spectrum 6700 free induction decays were accumulated, the line-broadening was 150 Hz.

isotropic peaks in the ^{31}P-NMR spectrum were observed. Thus, these resonances can not be attributed to degradation and vesicularization of the membrane.

This apparent contradiction (loss of the lamellar signal but presence of intact bilayer) can be explained only if the motion of PG molecules is not restricted to the bilayer, i.e., they are either released from the existing bilayer and form small vesicles or micelles or they are allowed to exchange with other non-bilayer structures, which results in averaging of the ^{31}P-NMR signal. The first possibility is highly unlikely taking into account that under our experimental conditions the large sheets of granal thylakoids and stromal lamellae are preserved and they do contain PG — it was shown [38] that the relative distribution of the four lipid classes, as well as the relative amount of the different PG molecular species was identical in intact thylakoids and isolated granal, stromal and marginal membrane fragments. It seems more likely that with the increase of temperature fast exchange of lipids between the bilayer and non-bilayer structures occurs which leads to averaging of the ^{31}P-NMR signal. These non-bilayer structures which, in isolated thylakoids are probably located in the lumen, must be rather small and without long-range order since their presence cannot be detected with small angle X-ray scattering (Holm, J.K., Kovács L., Garab G. and Posselt D., unpublished data).

3.3. Thermally induced changes in the lipid phase behavior of Tris-washed thylakoid membranes

Tris is known to remove the proteins of the oxygen-evolving complex from the thylakoid membrane [28]. Moreover, it was shown that Tris-treatment results in changes in the macroorganization of the membrane proteins [29]; it eliminates for instance the psi-type circular dichroism signal originating from the LHCII-containing chiral macrodomains (Krumova, S.B. and Garab, G., unpublished data, cf. [39]). Previously ^{31}P-NMR spectra of Tris-washed wheat thylakoid membranes, accumulated at different temperatures, were reported [26]. Here we show the ^{31}P-NMR spectra of Tris-washed spinach thylakoids and compare them with intact (non-Tris-washed) membranes of the same plant species. The ^{31}P-NMR spectrum of Tris-washed spinach thylakoid membranes at 7 °C (Fig. 5) is very similar to the one obtained for non-washed membranes (cf. Fig. 1). A clear lamellar signal (with a high intensity shoulder at −10 ppm) is present and two isotropic peaks at about 0 and 4 ppm are superimposed on it. Saturation-transfer experiments (applying 35 dB at −1800 Hz) resulted in elimination of the lamellar signal and showed that similarly to non-washed membranes, the isotropic peaks at 4 ppm and 0 ppm are magnetically decoupled from the bilayer lipids giving rise to the lamellar ^{31}P-NMR signal (Fig. 5).

The heat-induced changes in Tris-washed thylakoids (Fig. 6A) followed a similar trend as the ones observed for similar preparations (see Materials and methods) from wheat [26]. The differences between the data of Harańczyk et al. [26] and our experiments are possibly due to the different plant species used and the slightly modified preparation protocol (see Materials and methods). Our data show that the trend of the events observed for Tris-washed membranes is similar to the one for non-washed samples (Fig. 4A), however, the lamellar signal was preserved until 42 °C. Similarly to non-washed membranes only a broad isotropic signal at 0 ppm could be detected at 49 °C. However, its intensity was much higher as compared to the one in non-washed samples. Cooling of the Tris-washed membranes from 49 °C back to 7 °C resulted in a recovery of the lamellar phase observed in the spectrum of non-heated samples. However, the intensity of the isotropic peaks was higher in comparison with non-heated membranes (Fig. 6B). These data clearly show that the lamellar-to-isotropic phase transition could be reversed under certain conditions, but only in Tris-washed thylakoids. The exact reason for the differences in the phase behavior of PG in non-washed and Tris-washed thylakoids is not clear; possibly it is due to the specificity of the lipid-protein interactions in these two preparations.

4. Conclusions

In this work we have used the non-invasive ^{31}P-NMR technique, in which the phosphate nucleus of the membrane phospholipids serves as an internal label and hence reporter of the lipid phase behavior. Both the intact and Tris-washed thylakoid membranes show complex lipid phase behavior — the ^{31}P-NMR lineshapes reveal the co-existence of lamellar and isotropic signals. Similar isotropic resonances were detected also in other native [40] and model membranes [41,42], however their nature is still obscure. Our data show that isotropic motion (isotropic peak at 0 ppm) of the PG molecules occurred at all temperatures below 40 °C, where only the lamellar phase was expected. We attribute this isotropic signal to a separate pool of PG molecules involved in non-bilayer structures. These non-bilayer structures remain in contact with the membrane and the exchange of lipids between them and the lamellae is temperature-dependent; it is increased at temperatures higher than 14 °C which results in disappearance of the lamellar spectrum and averaging of the ^{31}P-NMR signal while the integrity of the membrane is preserved.

Acknowledgments

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