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Regulation of LHCII aggregation by different thylakoid membrane lipids

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ABSTRACT

In the present study the influence of the lipid environment on the organization of the main light-harvesting complex of photosystem II (LHCII) was investigated by 77K fluorescence spectroscopy. Measurements were carried out with a lipid-depleted and highly aggregated LHCII which was supplemented with the different thylakoid membrane lipids. The results show that the thylakoid lipids are able to modulate the spectroscopic properties of the LHCII aggregates and that the extent of the lipid effect depends on both the lipid species and the lipid concentration. Addition of the neutral galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) seems to induce a modification of the disorganized structures of the lipid-depleted LHCII and to support the aggregated state of the complex. In contrast, we found that the anionic lipids sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) exert a strong disaggregating effect on the isolated LHCII. LHCII disaggregation was partly suppressed under a high proton concentration and in the presence of cations. The strongest suppression was visible at the lowest pH value (pH 5) and the highest Mg^{2+} concentration (40 mM) used in the present study. This suggests that the negative charge of the anionic lipids in conjunction with negatively charged domains of the LHCII proteins is responsible for the disaggregation. Additional measurements by photon correlation spectroscopy and sucrose gradient centrifugation, which were used to gain information about the size and molecular mass of the LHCII aggregates, confirmed the results of the fluorescence spectroscopy. LHCII treated with MGDG and DGDG formed an increased number of aggregates with large particle sizes in the μm-range, whereas the incubation with anionic lipids led to much smaller LHCII particles (around 40 nm in the case of PG) with a homogeneous distribution.

1. Introduction

Plant photosynthesis, i.e. the conversion of carbon dioxide into organic compounds, relies on the absorption of sunlight by the so-called light-harvesting complexes (LHC). These Chl a/b protein complexes are either responsible for efficient light-harvesting or photoprotection, depending on the actual light conditions. Under low or medium light intensities light is captured by the photosynthetic pigment molecules and transferred as excitation energy to the reaction centers. Under high light conditions light-harvesting complexes play a role in the dissipation of excessive light energy as heat which could otherwise damage the photosynthetic apparatus. This enhanced heat dissipation is measureable as a decrease of the Chl a fluorescence and has been termed non-photochemical quenching (NPQ). Although the exact mechanisms which lead to NPQ are still controversially discussed, there seems to be consensus that a conformational change of the light-harvesting system of photosystem II (PSII) is involved (for a recent model see [1]). It appears that changes in the intra-molecular organization of the pigments and their exact positioning to each other within the Chl a/b protein complexes play an important role in the switch from light-harvesting state to the energy dissipating state and vice versa. Most of the studies on the importance of pigment orientation [2–5] were performed with the main light-harvesting complex of photosystem II (LHCII), which is the most abundant antenna complex and binds more than 40% of the total chlorophyll in the thylakoids [6]. In the native state LHCII forms stable trimers composed of Lhcb1 and Lhcb2 gene products [7] which have a strong tendency to form aggregates *in vitro* [e.g. 4,8,9]. *In vitro* aggregation of LHCII is accompanied by a decrease of the Chl fluorescence yield, thus indicating an increased rate of energy dissipation [10,11]. It is known that the *in vitro* aggregation of LHCII

Abbreviations: Chl, chlorophyll; CMC, critical micelle concentration; DGDG, digalactosyldiacylglycerol; DM, n-dodecyl β-D-maltoside; H_{II} phase, inverted hexagonal phase; LHCII, light-harvesting complex of photosystem II; MGDG, monogalactosyldiacylglycerol; NPQ, non-photochemical quenching of chlorophyll a fluorescence; PCS, photon correlation spectroscopy; PG, phosphatidylglycerol; PSII, photosystem II; SGC, sucrose gradient centrifugation; SQDG, sulfoquinovosyldiacylglycerol

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can be induced or increased by the addition of cations [2,3]. Kirchhoff et al. [4] correlated the salt-induced aggregation of LHCII with the quenching of Chl a fluorescence and observed two distinct transitions. The first transition at low salt concentrations depends on the temperature and the detergent used at concentrations below the critical micelle concentration (CMC). The second transition at higher salt concentrations is independent of both these factors. The first transition was explained by a lateral micro-organization of the LHCII and the second by a stacking of small LHCII sheets (about 100 nm in diameter) to form larger aggregates of approximately 3 μm in diameter. In several studies it was shown that the *in vitro* aggregation of LHCII and the observed decrease of the Chl fluorescence yield are reversible upon the addition of different detergents [e.g. 4,5,12,13]. It was further suggested that the lipid environment plays a role in the regulation of the LHCII function and macro-organization *in vivo* [4]. Indeed, it was shown that the lipid content, which remained attached to an LHCII preparation, has a strong influence on the ultrastructure of the isolated LHCII macro-aggregates and their ability to undergo light-induced reversible structural changes [14]. Furthermore, it was illustrated that the structural flexibility is lost in strongly delipidated LHCII, but that it can be recovered by addition of isolated thylakoid lipids [15]. Depending on the nature of the added lipid, different alterations of the disorganized structure of the delipidated LHCII can be observed.

The thylakoid membrane of vascular plants consists of four main classes of lipids. The main thylakoid lipid is monogalactosyldiacylglycerol (MGDG) which accounts for about 50% of the total lipid content [16]. MGDG is a non-bilayer lipid which forms the so-called inverted hexagonal structures (H_{II} phases) in an aqueous medium [17,18]. Even in mixtures with bilayer lipids a lamellar formation can be realized only at low MGDG concentrations, i.e. in a binary mixture of MGDG and DGDG the MGDG contribution has to be lower than 20% [19]. For the native thylakoid membrane it is generally assumed that the formation of a lamellar phase is imposed by the high concentration of membrane-integral and associated proteins. Estimates based on biochemical and ultrastructural data predict that proteins occupy 70% of the area in thylakoid membranes [20]. Simidjiev et al. [18] proposed that especially LHCII, which accounts for half of the thylakoid protein content [6], inhibits the formation of H_{II} phases by limiting the space available for MGDG. The second most abundant thylakoid lipid is the bilayer forming digalactosyldiacylglycerol (DGDG), with about 30% of the total thylakoid lipid content [16]. The remaining lipids of the thylakoid membrane are the negatively charged sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG). The negative charge of SQDG is derived from the presence of a sulfite group whereas PG contains a phosphate group.

It has to be emphasized that, besides their hypothetical role in supporting structural changes of the LHCII, thylakoid lipids are essential for the native structure of the LHCII. LHCII trimers are stabilized by a tightly bound PG molecule. The contact between adjacent LHCII trimers is mediated by a peripherally bound molecule of DGDG which also allows for the formation of 2D and 3D crystals [21]. In the stacked grana regions of the thylakoid membrane LHCII trimers are organized into structures of higher order whose occurrence is bound to the presence of the main thylakoid lipid MGDG [22]. The role of the fourth thylakoid lipid, SQDG, is at present unclear, although it is found in the isolated LHCII in similar or even higher relative concentrations as it is present in the thylakoid membrane [14,23,24].

Based on the lack of detailed information on the effect of different membrane lipids on thylakoid protein structures, the influence of the neutral galactolipids MGDG and DGDG and the negatively charged lipids SQDG and PG on the higher order structure of the LHCII was analyzed in closer detail. For that purpose LHCII was prepared in a state of a high aggregation and incubated with the different purified lipids. Changes in the LHCII aggregation state at different lipid concentrations, pH values and in the presence of Mg^{2+} ions were analyzed by 77K fluorescence spectroscopy. Additionally, photon correlation spectroscopy and sucrose gradient centrifugation were used to gain information about the size and molecular mass of the LHCII samples after treatment with the different lipid species.

2. Materials and methods

2.1. Preparation of LHCII of spinach

Fresh spinach leaves (*Spinacia oleracea* L.) were obtained from the local market. PSII light-harvesting complexes (LHCII) were isolated by successive cation precipitation according to Krupa et al. [25]. The isolated LHCII was stored in 50 mM Tricine–NaOH buffer (pH 7.8), containing 50% (w/v) glycerol, in the dark at −20 °C until further use.

2.2. Incubation of LHCII with lipids

For the incubation with lipids, 100 μL LHCII suspension with a Chl concentration of 50 μg mL^{-1} was mixed with 5 μL lipid solution using a Hamilton syringe. The lipids MGDG, DGDG, SQDG and PG (Lipid Products, UK) were dissolved in methanol and prepared as stock solutions of 4, 8 and 16 mg mL^{-1}, respectively. The LHCII lipid mixtures were incubated for 15 min in the dark at a temperature of 20 °C. LHCII suspended in Tricine buffer without lipids served as a control. The effect of the addition of 5 μL methanol (as the lipid solvent) on the fluorescence spectra of the LHCII was also regularly checked and no influence was observed. For the measurements of the 77 K fluorescence spectra the 50 mM Tricine–NaOH buffer (pH 7.8) and glycerol were added to the different LHCII samples in order to achieve a final glycerol concentration of 60% (v/v) and a Chl concentration of 5 μg mL^{-1}. The final lipid/Chl ratios of the LHCII lipid mixtures were 4, 8 and 16, respectively.

The measurements analyzing the pH-dependence of the lipid effects were performed with the following modifications: Before the addition of lipids, the LHCII samples were carefully centrifuged and resuspended into either 40 mM MES buffer adjusted to the pH values of 5, 5.5, 6 and 6.5, or 40 mM HEPES buffer adjusted to a pH of 7, 7.5 and 8.

The measurements used to investigate the influence of Mg^{2+} on the lipid effects were done with the following modifications: Before the addition of lipids, the LHCII samples were incubated for 15 min in the dark at 20 °C in 50 mM Tricine–NaOH buffer, pH 7.8 with 40 mM $MgCl_2$.

2.3. Spectroscopy

77K fluorescence spectra were recorded with a FluoroMax-3 spectrofluorimeter (Jobin Yvon Horriba, France) equipped with a 77K measuring cuvette and DataMax software. Fluorescence emission spectra were recorded in the wavelength range from 600 to 800 nm with an excitation wavelength of 440 nm and an excitation and emission bandwidth of 2 and 1 nm, respectively. For the sampling of fluorescence excitation spectra, the constant emission wavelength was set to the Chl a fluorescence emission maximum (typically 680 nm). Excitation spectra were recorded in the wavelength range from 400 to 550 nm with settings of the excitation and emission bandwidths of 5 and 2 nm, respectively. For each spectrum at least three scans were averaged.

Photon correlation spectroscopy was done with a Zetasizer Nano ZS (Malvern Instruments Ltd, United Kingdom). The viscosity was adjusted to 7.55 cP and the refractive index to 1.402. All analyses were performed at 20 °C and after an equilibration time of 5 min.

2.4. Sucrose gradient centrifugation

For the determination of the molecular mass of the LHCII lipid particles by sucrose gradient centrifugation (SGC) the following modifications with regard to the incubation of LHCII with lipids were performed: The volume of the LHCII suspension was increased to 1 mL

with a Chl concentration of 100 μg mL^{-1}. To achieve a lipid/Chl ratio of 8, the LHCII suspensions were mixed with 50 μL of a methanolic lipid stock solution (MGDG, DGDG, SQDG or PG), which contained a lipid concentration of 16 mg mL^{-1}. After incubation (15 min, 20 °C, dark), the LHCII lipid mixtures were loaded onto a linear sucrose gradient from 0.1 to 1.5 M sucrose (w/v, in 50 mM Tricine–NaOH, pH 7.8) and were centrifuged for 18 h at 110,000 *g* (Optima C-90K, Beckman Coulter) using a swing-out rotor (SW 28, Beckman Coulter). As a reference spinach thylakoids were solubilized with n-dodecyl-β-D-maltoside (DM) at a final detergent/Chl ratio of 20 (for details see [26]) and separated into their different pigment–protein complexes by SGC as described above. Note that in the case of the solubilized spinach thylakoids the sucrose gradient was complemented with 0.02% DM to keep the pigment–protein complexes in a solubilized state. DM, however, was completely omitted from the sucrose gradients that contained the LHCII lipid mixtures, since control measurements had shown that even very low concentrations of DM had a strong disaggregating effect on the LHCII, which would have masked the effects of the different lipids on the aggregation state of the light-harvesting complex (see the Supplementary Material).

3. Results

3.1. Effect of different lipids on LHCII structure

In the present study we examined the effect of different thylakoid membrane lipids on the macrostructure of the LHCII. For this purpose we decided to use a strongly delipidated LHCII isolated by successive cation precipitation according to Krupa et al. [25]. A detailed characterization of this LHCII preparation with regard to its protein, pigment and lipid composition can be found in Schaller et al. [24]. They showed that the total lipid content of the LHCII preparation was reduced by approximately 70% in comparison to that of the intact thylakoid membrane. The absorption spectra of the LHCII fraction isolated by successive cation precipitation exhibited a significantly lower absorption in the blue region of the spectrum when compared to LHCII preparations with higher lipid content. This indicates that the LHCII isolated according to the method of Krupa et al. [25] existed in a highly aggregated form and that the aggregated particles shielded each other from the measuring light, which in turn led to a strongly increased “sieve effect” or “particle flattening” [27].

This strong macro-aggregation was also visible in the 77K fluorescence emission spectra of the untreated sample (Fig. 1, LHCII in buffer) where the LHCII was excited at the blue Chl a absorption maximum of 440 nm. The main fluorescence emission band at 680 nm (F680) was accompanied by a pronounced shoulder at a wavelength of around 700 nm (F700) which is characteristic for aggregated LHCII trimers [4,5,12]. It is common to use the ratio of F700 to F680 to characterize the level of aggregation. In our present experiments the highest ratio of the long to the short wavelength fluorescence emission (F700/F680 = 1.05, see Fig. 1) was observed for the untreated LHCII fraction.

The addition of lipids to the delipidated LHCII changed the spectroscopic properties of the bound pigments significantly. Fig. 1 shows the influence of the different thylakoid lipids on the LHCII structure via changes in the Chl a fluorescence spectra at pH 7.8. In general, the thylakoid lipids led to a decrease or even loss of the long wavelength shoulder at 700 nm, which was in most cases accompanied by an increase of the fluorescence emission signal at 680 nm (Fig. 1A). Accordingly, the F700/F680 ratio was reduced after the addition of lipids, indicating structural changes and in the case of the anionic lipids a disaggregation of the LHCII trimers. It has to be emphasized that the addition of detergent up to concentrations at the critical micelle concentration (CMC) led to an even more pronounced increase of the fluorescence emission and reduction of the F700/F680 ratio. DM concentrations of only 0.02% DM (w/v), which were shown

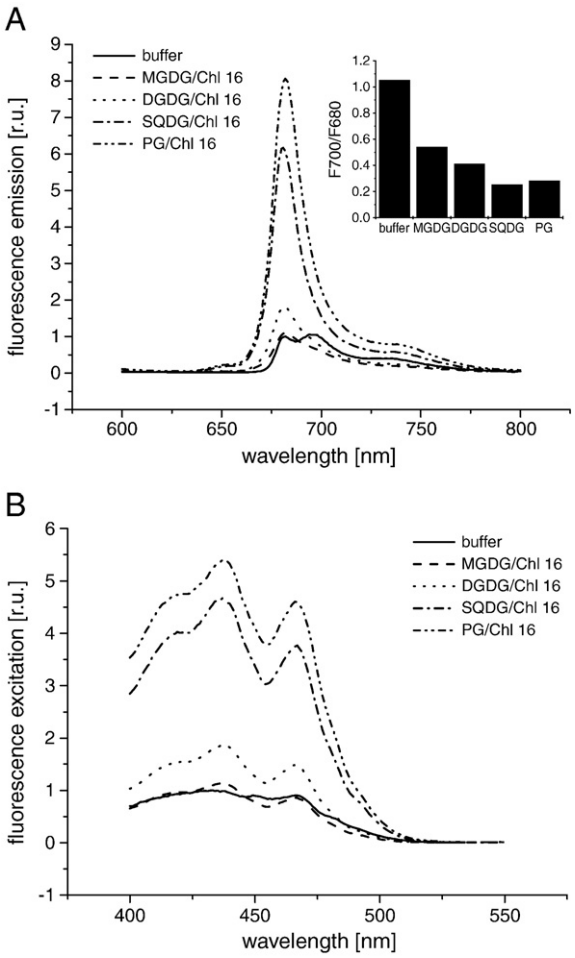

Fig. 1. 77K fluorescence emission (A) and excitation spectra (B) of LHCII samples after incubation with the galactolipids MGDG or DGDG or the anionic lipids SQDG or PG in comparison to a control sample in Tricine buffer without lipids. The final concentration of the samples was adjusted to 5 μg Chl mL^{-1} and the lipid/Chl ratio was 16. All measurements were performed at pH 7.8 in the absence of additional $MgCl_2$. For the emission spectra the samples were excited at 440 nm, the excitation spectra were measured at a constant emission wavelength of typically 680 nm corresponding to the Chl a fluorescence emission maximum. All spectra are shown in relation to the normalized maximum fluorescence emission value of the control sample (which was normalized to the value 1). Insert: Changes of the ratio of the fluorescence emission at 700 nm to the emission at 680 nm (F700/F680) of LHCII incubated with the different lipids in comparison to the control LHCII without lipids. Fig. 1 shows the result from a typical measurement, three independent experiments confirmed the data depicted in the figure.

to disassemble LHCII aggregates into trimers [13, see also the Supplementary Material], led to a drastic increase of the 680 nm emission and to a complete loss of the 700 nm shoulder in the present experiments (data not shown).

Addition of lipids also led to significant changes of the Chl a fluorescence excitation spectra (Fig. 1B) which were measured at a constant emission wavelength of 680 nm corresponding to the main fluorescence emission band of the LHCII. The fluorescence excitation signal of untreated delipidated LHCII was low and the Chl a and b maxima at 440 and 475 nm, as well as the xanthophyll excitation, which was seen as a broad shoulder at wavelengths up to 510 nm, were not much pronounced. After addition of lipids to the LHCII, higher fluorescence excitation signals were observed and the band structure of the different excitation maxima was much more pronounced. This indicates that the lipid addition did not disturb the excitation energy transfer between the accessory pigments and Chl a and that the light-scattering, which was caused by the high aggregation state of the delipidated LHCII, was significantly reduced.

It is an important result of the present study that the different thylakoid lipids varied markedly in the strength of their effect. We observed that at the same lipid concentration the anionic lipids SQDG and PG led to a much stronger increase of the fluorescence emission at 680 nm than the neutral galactolipids MGDG and DGDG (Figs. 1A and 2, for a detailed description see the next section). This was accompanied by a change of the F700/F680 ratios (Fig. 1A). At a lipid/Chl ratio of 16, the neutral galactolipids MGDG and DGDG reduced the F700/F680 ratio by approximately 50%, whereas SQDG and PG led to a stronger reduction of approximately 75%, in comparison to the untreated LHCII (Fig. 1A).

To further elucidate the effect of the lipids on the LHCII structure we systematically examined the effect of different lipid concentrations, pH values and the presence of Mg^{2+}.

3.2. Influence of different lipid concentrations

Fig. 2 shows the change of the LHCII fluorescence emission at 680 nm after lipid treatment in comparison to a control LHCII in Tricine buffer pH 7.8. Three different lipid concentrations were applied, resulting in final lipid/Chl ratios of 4, 8 and 16. For all lipids we observed an increase in the fluorescence emission at 680 nm with increasing lipid concentration. This was especially pronounced in the case of the anionic lipids. Treatment of the LHCII with PG induced a three times higher emission signal at the lowest lipid concentration and an increase of the fluorescence yield by a factor of eight at the highest concentration. In contrast to that, MGDG did not increase the fluorescence signal at the lowest concentration, and at the highest MGDG concentration an increase of the emission by a factor of only 1.52 was found (Fig. 2).

The extent of the Chl a fluorescence increase was again largely dependent on the lipid species. At all applied lipid concentrations addition of the anionic lipids SQDG and PG resulted in a much stronger increase of the fluorescence emission at 680 nm than the neutral galactolipids MGDG and DGDG. At the highest lipid concentration MGDG and DGDG increased the fluorescence yield by a factor of about 1.5 and 3.4, respectively, whereas SQDG and PG led to an enhancement of the 680 nm emission by a factor of about 5.9 and 8.8. This is a further indication that the neutral galactolipids rather lead to rearrangements within the LHCII aggregates, while the anionic lipids induce a strong disaggregation of the complexes (see also below).

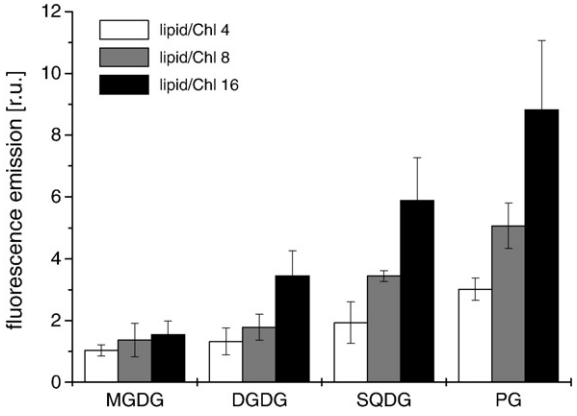

Fig. 2. Influence of different concentrations of the lipids MGDG, DGDG, SQDG and PG on the 77K fluorescence emission maximum of the LHCII samples at pH 7.8 in the absence of additional $MgCl_2$. The final concentration of the LHCII samples was adjusted to 5 μg Chl mL^{-1} and the lipids were used at lipid/Chl ratios of 4, 8 and 16. The maximum fluorescence emission value of the control LHCII without lipids was normalized to 1 and the relative amplitudes of the emission maxima of lipid incubated LHCII samples are shown in relation to the normalized control value. Fig. 2 shows the mean values of three independent experiments, the standard deviations are depicted as error bars.

3.3. Influence of different pH environments

The measurements shown above, which were performed in a Tricine buffer adjusted to a pH of 7.8, revealed that the anionic lipids SQDG and PG exert a significantly stronger effect on the fluorescence properties of delipidated LHCII than the neutral galactolipids MGDG and DGDG. To analyze if the negative charge of the anionic lipids is responsible for the pronounced changes in fluorescence emission, the influence of the pH environment on the lipid-induced increase of the LHCII fluorescence was examined. It is assumed that due to the high proton concentration at a low pH, negatively charged side chains or head groups of molecules are protonated and (partially or completely) lose their negative charge. On the one hand, it is possible that the negative charge of the anionic lipids is directly affected or that, on the other hand, negatively charged amino acid side chains of the membrane proteins become protonated so that an interaction with the anionic lipids is restricted. In consideration of the pKa values of the phosphate group of PG and the sulfonic acid group of SQDG, which lie in the range of pH 2, a compensation of the negative charge of the anionic lipids at pH 5 is not as likely as a shielding of a part of the negative surface charge of the LHCII. It is of further interest for the experiments with different pH environments that previous studies indicated that LHCII changes its aggregation state at low pH values [28] which might also lead to an altered interaction with the different membrane lipids. Taking into account these facts, the effect of lipids on the LHCII was tested in reaction media buffered to different pH values in a physiological range from pH 5 to pH 8.

Fig. 3 depicts the change of the LHCII fluorescence emission signal at 680 nm after treatment with the thylakoid lipids at different pH values in comparison to a control LHCII in Tricine buffer pH 7.8. When the pH dependence of the lipid-induced fluorescence increase was compared between the neutral galactolipids and the anionic lipids, we observed significant differences. In line with our assumption that high proton concentrations might be able to shield negative charges, the anionic lipids SQDG and PG exhibited a strongly decreased ability to enhance the LHCII fluorescence yield at low pH values. The strong increase of the fluorescence emission, which was visible at pH 7.8, was reduced by more than 90% at pH 5 in the case of PG and around 85% in the case of SQDG (Fig. 3A). On the other hand, the fluorescence of the LHCII treated with galactolipids at low pH values was higher than the fluorescence caused by these lipids in a neutral or slightly basic pH environment (Fig. 3B). It is not unlikely that a protonation of the LHCII apoproteins takes place at low pH values which leads to an increased hydrophobic interaction with MGDG and DGDG, thereby stimulating a better interaction with LHCII and an increase of the fluorescence emission. However, the results show clearly that the compensation of the negative charge of SQDG and PG blocks the ability of the lipids to disturb the macro-aggregation of the delipidated LHCII.

3.4. Influence of Mg^{2+}

To further investigate if the negative charge of the anionic lipids is responsible for their strong effect on the LHCII fluorescence, the high proton concentration was substituted by another positively charged ion which should also shield the negative charge of the anionic lipids or the surface areas of the membrane domains of LHCII which might interact electrostatically with the anionic lipids. Additionally, it was shown in former studies that cations, and especially Mg^{2+}, play a role in LHCII aggregation [4].

Fig. 4A shows the 77K fluorescence emission spectra of LHCII samples which were treated with different concentrations of $MgCl_2$, resulting in final concentrations of 0, 10, 20, 40 and 80 mM, respectively. In general, the treatment with Mg^{2+} led to a decrease of the fluorescence emission signal at 680 nm and to a slight increase of the 700 nm shoulder (Fig. 4A). This was also reflected in the F700/F680 ratio which was slightly increased from 0.98 in the LHCII

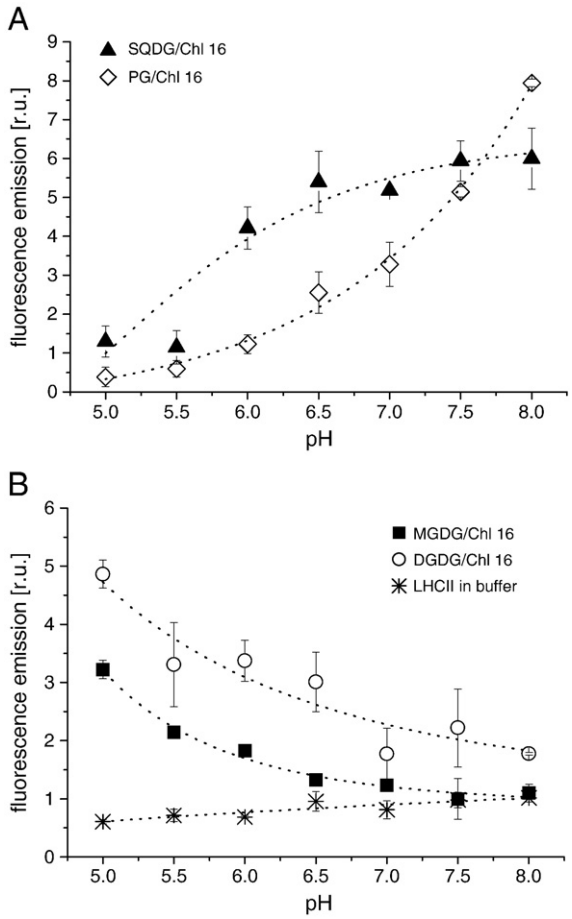

Fig. 3. Influence of different pH values on the 77K fluorescence emission maximum of the LHCII samples after incubation with the anionic lipids SQDG or PG (A) or the galactolipids MGDG or DGDG (B). Additionally, in Fig. 3B untreated LHCII in buffer after incubation with different pH values is depicted. These measurements were performed in the absence of additional $MgCl_2$. The final concentration of the LHCII samples was 5 μg Chl mL^{-1} and the lipids were used at a lipid/Chl ratio of 16. The maximum fluorescence emission value of the control LHCII without lipids was normalized to 1 and the relative amplitudes of the emission maxima of lipid incubated LHCII samples are shown in relation to the normalized control value at pH 7.8. Each data point shows the mean value of three independent experiments, the standard deviations are depicted as error bars.

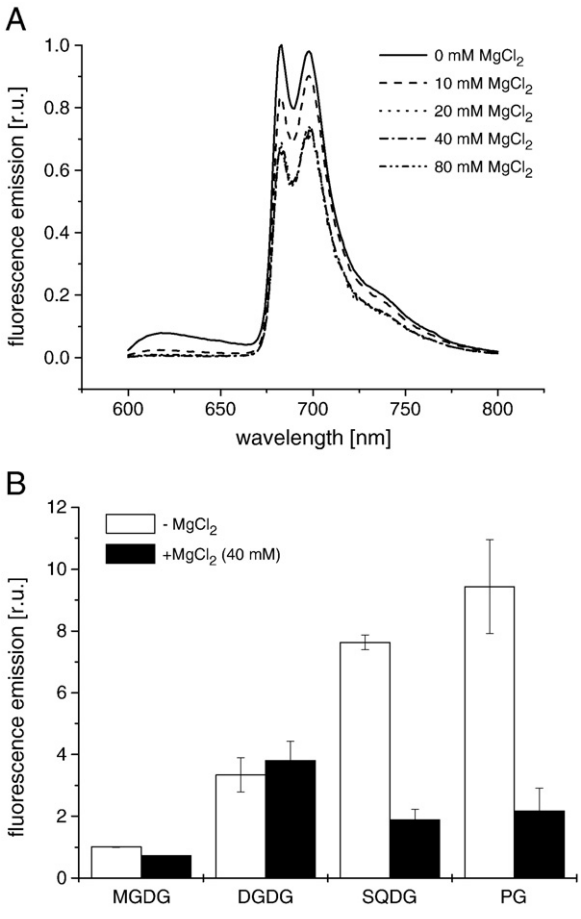

Fig. 4. A) 77K fluorescence emission spectra of LHCII samples in the absence or presence of $MgCl_2$ which was added at the final concentrations of 10, 20, 40 and 80 mM, respectively. The pH was kept constant at a pH of 7.8. The spectra are shown in relation to the normalized maximum fluorescence emission value of the control sample without $MgCl_2$ (which was normalized to the value 1). Fig. 4A shows the result from a typical measurement, three independent experiments confirmed the data depicted in the figure. B) Influence of the addition of 40 mM $MgCl_2$ on the 77K fluorescence emission maximum of the LHCII samples incubated with MGDG, DGDG, SQDG and PG at pH 7.8. The fluorescence maxima were compared to lipid incubated LHCII samples without addition of $MgCl_2$. The final concentration of the LHCII samples was 5 μg Chl mL^{-1} and the lipids were used at a lipid/Chl ratio of 16. The maximum fluorescence emission value of the control LHCII without lipids was normalized to 1 and the relative amplitudes of the emission maxima of lipid incubated LHCII samples are shown in relation to the normalized control value. Fig. 4B shows the mean values of three independent experiments, the standard deviations are depicted as error bars.

incubated in Mg^{2+}-free Tricine buffer to 1.10 in the LHCII fractions in the reaction media with the highest $MgCl_2$ concentrations of 20, 40 and 80 mM. The fluorescence emission spectra of LHCII samples treated with the higher $MgCl_2$ concentrations were almost identical, which indicates that a $MgCl_2$ concentration of 20 mM is sufficient to induce the highest possible aggregation state of the LHCII. This data supports former studies which ascribe Mg^{2+} an aggregating effect on the LHCII which is opposite to the disaggregating effect of the anionic lipids observed in the present study.

After the investigation of the Mg^{2+} effect on the LHCII emission spectrum, the influence of the thylakoid lipids on the LHCII aggregation was analyzed in the presence of 40 mM $MgCl_2$ (Fig. 4B), a concentration which had led to further aggregation of the LHCII in comparison to the control LHCII in buffer (see Fig. 4A). It was obvious that the LHCII samples incubated with the neutral galactolipids MGDG and DGDG did not show significant differences in the presence or absence of 40 mM $MgCl_2$. The negatively charged lipids SQDG and PG, on the other hand, exhibited obvious differences in their effect on the fluorescence emission when they were either used in the Mg^{2+}-containing or Mg^{2+}-free Tricine buffer. Addition of Mg^{2+} to the medium strongly decreased the LHCII disaggregating effect of SQDG and PG and the increase of the LHCII fluorescence yield, which was observed in the absence of Mg^{2+}, was reduced by approximately 75%. This is a good indication that the compensation of the negative charge of the anionic lipids and/or the membrane domains of LHCII alters the way of interaction between lipids and aggregated delipidated LHCII.

3.5. Estimation of the size of lipid treated LHCII samples

The previously presented data obtained by 77K fluorescence spectroscopy indicated a disaggregation of the delipidated LHCII after incubation with the anionic lipids SQDG and PG. To complement the 77K fluorescence spectra and to further characterize the disaggregating effect of the lipids, photon correlation spectroscopy (PCS) and sucrose gradient centrifugation (SGC) were employed. Both methods were used to estimate the size of the delipidated LHCII in Tricine buffer in the presence or absence of the various thylakoid membrane lipids.

Fig. 5 shows the distribution of particle sizes measured by photon correlation spectroscopy (PCS) of the different LHCII lipid mixtures in comparison to a control LHCII in Tricine buffer without lipids and an LHCII which was solubilized with 0.02% DM. PCS is normally used to estimate the size of homogeneous particles in solutions. Since the

different LHCII complexes exhibited a certain degree of heterogeneity after the incubation with the different lipids and DM, we chose to show not only the average particle size but the minimal and maximal particle sizes as well. This way information about differences in the heterogeneity of the LHCII complexes treated with the different lipids can be depicted. PCS is based on the fact that particles in dispersion cause the intensity of scattered light to fluctuate as a function of time. This information can then be analyzed in different ways. In the present case a multiple exponential was fitted to the correlation function to obtain the distribution of particle sizes. According to our opinion, the easiest way to present the PCS data is to depict the parameter "number" where the different light scattering intensities are recalculated to the number of particles of a certain size which cause this light scattering. This type of analysis takes into account that larger particles will scatter the light much stronger than a smaller particle. The exact values of the PCS measurements are summarized in Table 1. Delipidated LHCII suspended in Tricine buffer without lipids formed heterogeneous aggregates with particle sizes in the range between 170 and 590 nm. In addition, very large particles with a size of around 4 μm were observed. The particle sizes of the LHCII, which

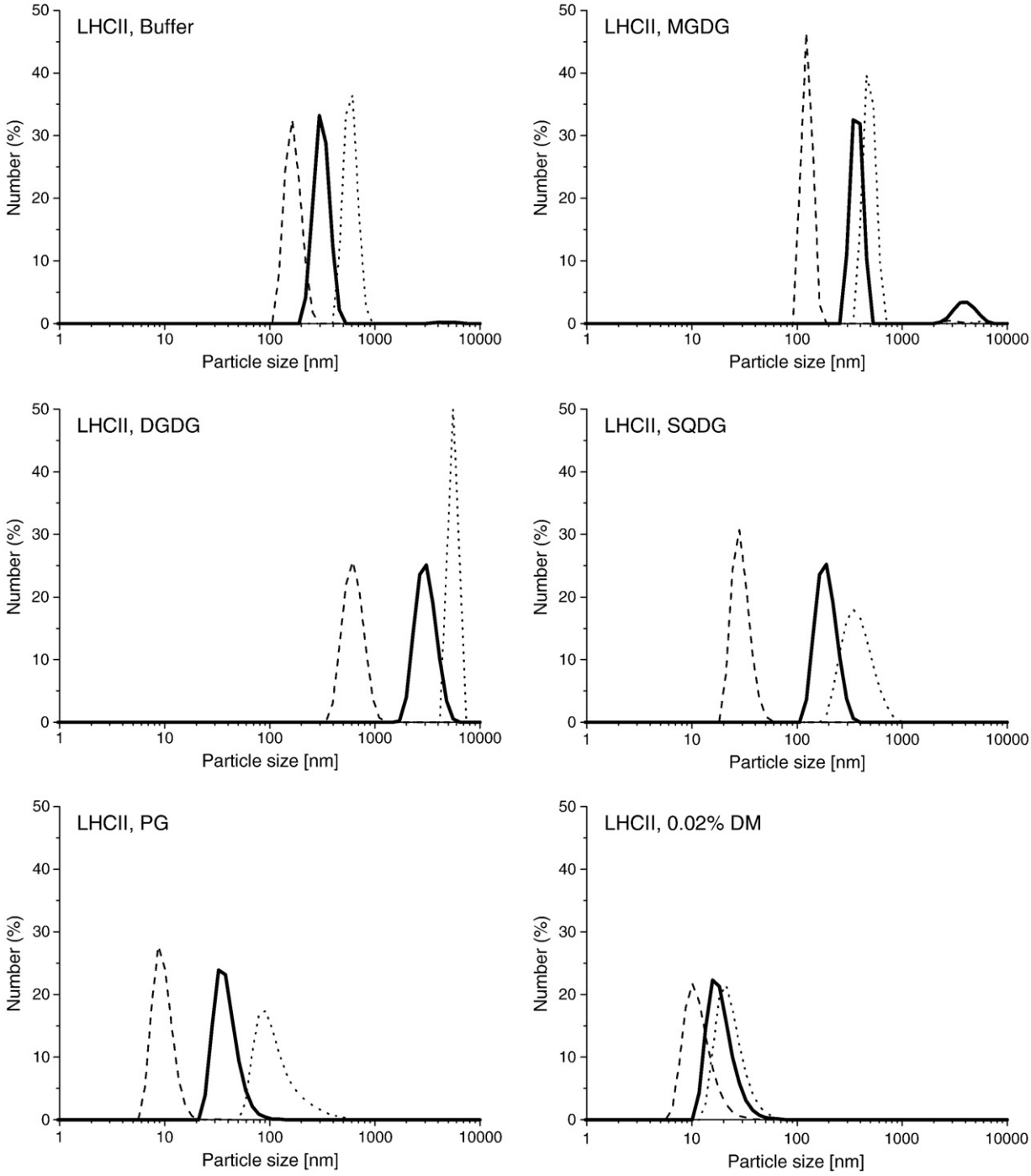

Fig. 5. The distribution of particle sizes [nm] of LHCII in Tricine buffer, lipid incubated LHCII samples and LHCII solubilized with 0.02% DM, at pH 7.8 and in the absence of additional MgCl2, measured by photon correlation spectroscopy (PCS). For incubation the lipids MGDG, DGDG, SQDG and PG were used at a lipid/Chl ratio of 16. The final concentration of the LHCII samples was 5 μg Chl mL^{-1}. Fig. 5 shows the results of three independent experiments where each sample was measured 6–12 times, depending on the reproducibility of the measurement and heterogeneity of the sample. The bold lines show the average particle sizes, dashed and dotted lines stand for the minimal and maximal measured particle sizes, respectively.

Table 1
Particle size and molecular mass of isolated LHCII in the absence and presence of different thylakoid membrane lipids and dodecyl-β-D-maltoside (DM) determined by photon correlation spectroscopy (PCS). For detailed information about the measurement conditions see the Materials and methods and Results sections. For PCS the LHCII samples were used at a final Chl concentration of 5 μg mL^{-1}. The table presents the minimum, maximum and mean particle sizes of the samples with or without thylakoid lipids and DM. Since some of the samples showed a heterogeneous particle distribution the percentage of the different particles sizes is additionally depicted. For PCS three independent measurements were done and a minimum of 18 spectra were recorded for each sample.

Sample	Particle size of isolated LHCII estimated by photon correlation spectroscopy [nm]			
pH 7.8	%	Minimum	Maximum	Mean
LHCII, Buffer	99.5%	168	592	293
	0.5%	2999	5590	4351
LHCII, MGDG	89.6%	125	491	300
	10.4%	2294	5590	4000
LHCII, DGDG	8.0%	165	297	231
	92.1%	634	5590	3270
LHCII, SQDG	100%	30	388	223
LHCII, PG	100%	10	124	41
LHCII, 0.02% DM	100%	12	24	19

was incubated with MGDG, did not differ much from the untreated LHCII, but the percentage of the very large particles in the μm-range was higher (10.4% instead of 0.5%). The formation of very large particles was strongest in LHCII samples which were incubated with DGDG where more than 90% of the LHCII lipid particles were found in the μm-range. In contrast to the neutral galactolipids we found an enhanced formation of smaller LHCII lipid particles when the negatively charged lipids SQDG and PG were added to the LHCII. SQDG reduced the average particle size from around 300 nm to 220 nm, while the effect of PG was even stronger and particles of only 40 nm were observed. With respect to the disaggregation of LHCII aggregates, the anionic lipids behave like a detergent at low concentrations and their effect can be compared to DM which in our present experiments reduced the particle sizes of the delipidated LHCII to approximately 20 nm.

A comparison of the relative particle sizes of LHCII treated with the different lipids and of the control LHCII in the presence and absence of DM was additionally performed by sucrose gradient centrifugation (SGC) using a linear gradient from 0.1 to 1.5 M sucrose (for pictures of the gradients with the different LHCII fractions see the Supplementary Material).

With regard to the size determination by SGC it is important to note that SGC does not only depend on the molecular mass but also on the density of a particle. Despite the fact that proteins and lipids have different densities and thus exert a different mobility in the gradient, we think that a relative comparison between the different lipid-treated LHCII complexes is reasonable, taking into account that for all incubations the ratio of lipids to proteins was kept constant. Additionally, the lipid LHCII complexes were compared with the different pigment–protein complexes of solubilized spinach thylakoids (DM per Chl ratio of 20 details see [29]).

The analysis of the LHCII complexes treated with the different lipids was based on the assumption that different aggregation states of the lipid protein complexes would be visible as different bands in different regions of the sucrose gradient. However, the extremely high aggregation state of the delipidated control LHCII was not visible as a defined band in the gradient but occurred as solid pellet after the ultra-centrifugation. This means that the particle size and the density of the aggregated control LHCII was too high to be separated within the range of sucrose concentrations used in the present study. Interestingly, the presence of 0.02% DM in the sucrose gradient was already sufficient to solubilize the aggregated LHCII during the centrifugation and a single protein band was obtained slightly below the region where the LHCII trimer derived from solubilized spinach thylakoids was visible. As mentioned above, this indicates that very low concentrations of DM induced an almost complete disaggregation of the LHCII aggregates into trimers [13].

In line with the PCS measurements and the results of the 77K fluorescence spectroscopy, there was a notable difference between the LHCII complexes treated with neutral galactolipids and the complexes incubated with the anionic lipids in the band patterns of the sucrose gradients. The main part of the LHCII which was incubated with DGDG was found as a pellet and two minor bands were additionally observed. While the lower band was composed of LHCII lipid particles with a lower particle size/density than the main particles in the pellet, the upper band solely contained lipids, i.e. DGDG which did not interact with the LHCII proteins. LHCII treated with MGDG also formed a pellet and a major band located at high sucrose concentrations directly above the pellet, as well as a minor band which was visible in the upper part of the gradient in the region of the LHCII trimer band of solubilized spinach thylakoids. In contrast to these results, LHCII treated with the anionic lipids was observed as defined bands which were restricted to one region of the gradient, indicating a more homogenous distribution of the lipid protein particles. Furthermore, these bands were located at significantly lower sucrose concentrations in higher regions of the gradient than the bands obtained after incubation with the galactolipids, which is in line with a lower particle size and density of the complexes. Like in the PCS measurements PG was the lipid which was most effective in the disaggregation of the LHCII and induced the formation of the smallest LHCII lipid particles.

As it was already observed in the fluorescence spectroscopic measurements high proton concentrations, i.e. pH 5, and addition of Mg^{2+}, can partially compensated the disaggregating effect of the negatively charged lipids (Fig. S3 in the Supplementary Material). For both SQDG and PG, incubation in the presence of pH 5 and $MgCl_2$ led to a shift of the LHCII lipid bands towards regions of higher sucrose concentration, indicating higher particle sizes and densities of these LHCII–lipid complexes.

4. Discussion

4.1. Effect of different lipids on the LHCII structure

The aim of this study was to gain some insight into the mechanisms that regulate the organization of the LHCII within the thylakoid membrane with a special focus on the lipid environment. To study the effect of thylakoid lipids on the LHCII organization it was decided to use highly delipidated LHCII preparations which could reproducibly be supplemented with the different thylakoid membrane lipids. The results indicate that the addition of exogenous thylakoid lipids is able to modulate the spectroscopic properties of the isolated LHCII aggregates and that the extent of the lipid effect depends on the lipid species employed. In our present experiments we found that the untreated, delipidated LHCII was highly aggregated, as shown by a very low Chl fluorescence at 680 nm [10,11] and the appearance of a pronounced shoulder at around 700 nm [4,5,12]. Addition of thylakoid lipids to the aggregated LHCII led to a diminishment or even loss of the 700 nm shoulder and in most cases to an increase of the fluorescence emission signal at 680 nm. According to Simidjiev et al. [15], this means that the highly delipidated LHCII, whose aggregates prefer to form disorganized structures, experiences an alternation of its ultrastructure whose extent depends on the lipid species added to the protein complex. This view is also supported by the present results which showed that the neutral galactolipids and the anionic lipids exhibit pronounced differences in their effect on the LHCII fluorescence and aggregation. The anionic lipids SQDG and PG induced a much stronger increase of the Chl fluorescence than the galactolipids MGDG and DGDG which indicates a strong disaggregation of the LHCII in the presence of SQDG

and PG while MGDG and DGDG lead to rearrangements within the LHCII aggregates or even stabilize them (see below).

It is important to note that the effect of the lipids, even at the highest lipid concentration used in the present experiments (lipid/Chl ratio of 16), was much lower than the impact of detergents, used at the critical micelle concentration (CMC). Above the CMC, detergents lead to the disruption of membranes and solubilization of membrane proteins which, at high detergent concentrations, may even result in the loss of pigments and the disruption of functional LHCII trimers [5]. The results of the present study indicate that treatment of the LHCII with lipids is relatively mild and does not destroy the native pigment–protein–lipid structure and function. This is also supported by the fluorescence excitation spectra which showed that the pigments are still functionally bound to the LHCII proteins after the treatment with the different lipids. The effect of the thylakoid membrane lipids on the LHCII seems to be comparable to a detergent treatment with detergent concentrations below the CMC, which leads to changes in the arrangement of membrane proteins but does not disturb the functionality of the proteins. The lipid per Chl a ratio of the native thylakoid membrane shows a value of around 1.4 and is, therefore, lower than the lipid concentration used in the present experiments [24]. However, the distribution of the lipids within the membrane is at present still not absolutely clear so that a local enrichment of special lipid classes within certain membrane areas cannot be excluded. It should also be mentioned that, although the majority of the present experiments were done with a lipid per Chl ratio of 8 or 16, the disaggregating effects of the negatively charged lipids were already visible at significantly lower lipid concentrations.

With respect to the structural changes caused by the addition of the membrane lipids there are examples in the literature that show that protein-surrounding lipids may influence protein–protein interactions and the state of protein aggregation in biological membranes [30]. Depending on the lipid species different effects are possible which can be (partially) explained by the lateral pressure profile [31,32]. The lateral pressure is the result of the balance between the interactions of the lipid acyl chains and the lipid headgroups which interact with each other as well as among each other. The apolar acyl chains and the polar headgroups exhibit a strong hydrophobic tension at their interface which results in a large negative pressure. This negative pressure is balanced by a positive lateral pressure due to the repulsion between the hydrocarbon chains in the acyl chain region. Additional components, which influence the lateral pressure, are caused by interactions in the headgroup region, e.g. in the presence of charged headgroups, which result in either repulsion or attraction [31,33]. Calculations show that the conformational state or the oligomeric structure of membrane proteins can be influenced by changes in the membrane lateral pressure [31,34]. Non-bilayer lipids, like MGDG, with their overall conical shape, decrease the headgroup pressure and increase the chain pressure in comparison to a membrane which consists of only bilayer lipids. Due to this effect it is expected that non-bilayer lipids support the membrane binding of peripheral membrane proteins and stabilize the oligomeric structure of integral membrane proteins [32]. A similar role was already proposed for MGDG which induces the transformation of isolated, disordered macroaggregates of LHCII into stacked lamellar aggregates with a long-range chiral order of the complexes [18]. In the present study it could also be shown that MGDG supports or stabilizes the formation of higher order structures of the LHCII. According to the 77K fluorescence measurements even the highest concentrations of MGDG did not lead to a disaggregation of the aggregated LHCII which indicates only minor structural changes concerning the pigment orientation. The determination of the LHCII lipid particle sizes by PCS and SGC measurements showed that the percentage of larger LHCII particles was increased after the addition of MGDG. The formation of larger LHCII aggregates was even more pronounced in the presence of the second galactolipid DGDG which is in line with the observation that DGDG induces a lateral aggregation of isolated, delipidated LHCII, but does not lead to the formation of stacked, granum-like structures [18,21]. A comparable mechanism, but with opposite effect, was reported by Kirchhoff et al. [4] for the detergent Triton TX-100 which seemed to influence only the lateral aggregation, but not the stacking of LHCII. A possible explanation could be that detergent or neutral lipid molecules interact mainly with the hydrophobic protein domains, which become sequestered upon LHCII sheet formation. While Kirchhoff et al. [4] concluded that the three-dimensional stacking of LHCII trimers is determined predominantly by protein–protein interactions, this explanation does not exclude the possibility that lipids play an indirect role by mediating these interactions. The promotion of protein–protein interactions by lipids could be made possible by a coverage or exposure of charged groups of the membrane proteins, thus masking or enhancing electrostatic interactions. Such a mechanism may be important for LHCII aggregation *in vitro* where electrostatic interactions seem to play a major role. The binding of cations does not directly influence the aggregation but mainly helps to overcome the electrostatic repulsion that prevents the formation of higher order structures of LHCII [4]. Taking into account these observations, the anionic lipids, which possess a strong negative charge, are likely candidates for a modulation of the LHCII aggregation *in vivo*. The results of the present study are in line with the notion that anionic lipids have a significant impact on the structure of LHCII macroaggregates.

Further studies suggested that the anionic membrane lipids, in particular SQDG, and the galactolipids have antagonistic effects. Sakai et al. [35] proposed that SQDG may occupy a position in the opposite leaflet of the thylakoid membrane compared with MGDG to counteract the formation of inverted hexagonal phases and to stabilize the orderly arrangement of other molecules functioning in photosynthesis. Quinn et al. [36] also observed that charged lipids can suppress the formation of non-bilayer structures by imposing a bilayer arrangement on MGDG-enriched preparations. In the light of our present results, it could be possible that a balance between anionic lipids and neutral galactolipids is not only needed to regulate the membrane properties in general but also the organization of membrane proteins, and in particular the structure of the LHCII. This assumption is in line with a recent study by Apostolova et al. [37] who observed that in cyanobacteria PG depletion, despite its small contribution to the overall lipid content, has an influence on both the thylakoid structure and the macro-organization of the membrane-integral photosynthetic complexes (see next chapter).

4.2. The importance of LHCII organization for the thylakoid membrane architecture and function

The data of the present study suggests that the nature of the lipid interaction with the LHCII modulates the oligomeric state of the protein complex which in turn has a strong influence on the structural organization of the thylakoid membrane and the photosynthetic function. MGDG and DGDG seem to induce a modification of the disorganized structures of the lipid-depleted LHCII and support the aggregated state of the LHCII, while SQDG and PG lead to a disaggregation of the antenna. The interaction of the lipids with the LHCII is not a static but a dynamic process and may be fine-tuned by the establishment of the proton gradient and variations in the Mg^{2+}-concentration, which both suppress the LHCII disaggregation by the anionic lipids. In this respect it is of importance that PG seems to be more sensitive to changes of the pH-value than SQDG.

In the native thylakoid membrane the neutral galactolipids MGDG and DGDG may be needed to stabilize the PSIIα complexes, which are present in the grana membranes. PSIIα forms dimers and is associated with a large antenna consisting of four LHCII trimers per monomer

(see references in [38]). The negatively charged lipids SQDG and PG, on the other hand, may be important for the formation of the monomeric structures of the PSIIβ-centers, which are located in the stroma lamellae and are characterized by a significantly smaller antenna. The existence of different PSII subpopulations is important for the mechanism of PSII turnover: Both the degradation of the damaged PSII protein complexes and their replacement by newly synthesized PSII subunits take place in the stroma exposed membranes, after a migration of the damaged monomeric PSII complex from the grana regions to the stroma lamellae [39].

The grana formation itself is also regulated by the PSII content. PSIIα forms a supercomplex together with the LHCII which supports the grana formation by molecular contacts to other PSII supercomplexes located on the opposite grana membrane. The membrane ultrastructure of the granum is additionally stabilized by higher order LHCII structures which span several membranes. These LHCII macroaggregates, which are important for the dynamics and functioning of the native membrane [40–42], depend on the presence of MGDG. In *in vitro* experiments it was shown that MGDG supports the ability of the isolated, aggregated LHCII to undergo light-induced structural changes, i.e. to behave in a similar way as in the intact thylakoid membrane [15]. The importance of MGDG for the LHCII structure is confirmed by the results of the present study which show that both galactolipids, MGDG and DGDG, have a more stabilizing effect than the anionic lipids.

Further studies have shown that the organizational state of the LHCII influences the electric surface properties of the thylakoid membrane [43–45], which are important for the regulation of grana formation through electrostatic interactions [41,42]. It seems that larger aggregates of LHCII decrease the negative surface charge density of the thylakoid membrane while smaller LHCII units lead to an enhanced negative charge on the membrane surface thereby increasing the repulsion of opposite membranes. These changes of the electric membrane properties result in structural modifications and rearrangements of the different pigment–protein complexes which become visible as changes in 77K fluorescence spectra.

The data of Apostolova et al. [45] showed that LHCII disaggregation leads to an increased energy transfer from PSII to PSI. Furthermore, the authors observed that in conjunction with a decrease of LHCII oligomerization the proportion of functionally active PSIIα centers diminishes while the concentration of PSIIβ increases, which in turn leads to a reduced oxygen evolution. Changes in the oligomerization state of the LHCII are thought to play a role in the protection against destructive UV-A radiation. Such a notion was supported by Ivanova et al. [46] who also reported that the susceptibility of energy transfer and oxygen evolution to UV-A radiation depends on the higher order structure of the LHCII. It is important that our present study shows that the aggregation or disaggregation of the LHCII may be triggered by different thylakoid lipids. According to our results the LHCII disaggregation, which seems to be important for the modulation of UV protection, depends on the interaction of LHCII with the negatively charged SQDG and PG.

Aggregation of the LHCII, as it is needed for the process of NPQ, might be enabled or at least stabilized in the presence of MGDG or DGDG. According to the latest models [1], this aggregation would facilitate the formation of a special Chl a–Chl a homodimer or a Chl a–lutein heterodimer representing the quenching center within the LHCII which dissipates a large part of the excessive excitation energy as heat. It is noteworthy that a pH value of the thylakoid lumen of 5, which efficiently triggers the conformational change of the LHCII and leads to a full activation of the enzyme violaxanthin de-epoxidase, was shown to suppress the disaggregating effect of PG and SQDG on the LHCII in our present experiments. It is not unlikely that in the non-energized thylakoid membrane, which is characterized by an LHCII in the light-harvesting state, PG and SQDG located in the vicinity of the LHCII exert a partially disaggregating effect. In the energized state of the membrane, the high proton gradient, maybe in conjunction with a high Mg^{2+}-concentration, has to compensate the disaggregating effect of the anionic lipids, thereby enabling complete LHCII aggregation and efficient NPQ. The importance of Mg^{2+} for the process of NPQ has been shown recently by Goss et al. [47]. In this study the authors analyzed the NPQ capacity of isolated spinach thylakoids in the presence and absence of $MgCl_2$. Thylakoid membranes without Mg^{2+} were unstacked and exhibited a dramatically decreased NPQ in comparison to thylakoid membranes which, due to the presence of Mg^{2+}, showed the normal grana stacking and were able to induce efficient NPQ. The authors concluded that Mg^{2+} is needed for the macro-aggregation of the LHCII which forms the mechanistic basis of NPQ. With respect to the native plastidic Mg^{2+} concentration, Ishijima et al. [48] estimated the internal concentration of the chloroplast stroma to be 0.5 mM with an increase up to 2 mM upon illumination. This Mg^{2+} concentration is lower than the $MgCl_2$ concentrations used in the present experiments. However, in the native chloroplast it may still be sufficient to compensate the effects of the negatively charged lipids, taking into account that the lipid concentration of the native thylakoid membrane calculated on a Chl a basis is also reduced compared with the lipid concentration used in the present study.

In conclusion, our data implies that the thylakoid lipids are an important regulatory element for the thylakoid structure and function by influencing the higher order organization of the main protein of the membrane, the LHCII.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2010.12.017.

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