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The main thylakoid membrane lipid monogalactosyldiacylglycerol (MGDG) promotes the de-epoxidation of violaxanthin associated with the light-harvesting complex of photosystem II (LHCII)

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ABSTRACT

In higher plants, the major part of the xanthophyll cycle pigment violaxanthin (Vx) is non-covalently bound to the main light-harvesting complex of PSII (LHCII). Under saturating light conditions Vx has to be released from its binding site into the surrounding lipid phase, where it is converted to zeaxanthin (Zx) by the enzyme Vx de-epoxidase (VDE). In the present study we investigated the influence of thylakoid lipids on the de-epoxidation of Vx, which was still associated with the LHCII. We isolated LHCII with different concentrations of native, endogenous lipids and Vx by sucrose gradient centrifugation or successive cation precipitation. Analysis of the different LHCII preparations showed that the concentration of LHCII-associated Vx was correlated with the concentration of the main thylakoid lipid monogalactosyldiacylglycerol (MGDG) associated with the complexes. Decreases in the MGDG content of the LHCII led to a diminished Vx concentration, indicating that a part of the total Vx pool was located in an MGDG phase surrounding the LHCII, whereas another part was bound to the LHCII apoproteins. We further studied the convertibility of LHCII-associated Vx in *in-vitro* enzyme assays by addition of isolated VDE. We observed an efficient and almost complete Vx conversion in the LHCII fractions containing high amounts of endogenous MGDG. LHCII preparations with low concentrations of MGDG exhibited a strongly reduced Vx de-epoxidation, which could be increased by addition of exogenous, pure MGDG. The de-epoxidation of LHCII-associated Vx was saturated at a much lower concentration of native, endogenous MGDG compared with the concentration of isolated, exogenous MGDG, which is needed for optimal VDE activity in *in-vitro* assays employing pure isolated Vx.

1. Introduction

Depending on the actual light conditions, the chlorophyll a/b protein complexes of higher plants are either responsible for efficient light-harvesting or photoprotection. The most abundant antenna complex is the light-harvesting complex of photosystem II (LHCII), which alone covers more than one third of the thylakoid membrane area [1] and binds more than 40% of the total chlorophyll in the thylakoids [2]. Under low or medium light intensities the excitation energy is efficiently absorbed by the light-harvesting pigments and transferred to the reaction center of PSII. Under high light intensities the LHCII undergoes a conformational change, which leads to the safe dissipation of excess excitation energy as heat, thereby protecting the photosynthetic apparatus from photodamage (for recent reviews see [3,4]). This enhanced heat dissipation becomes visible as a strong reduction of the chlorophyll a fluorescence and has been termed non-photochemical quenching (NPQ). The conformational change of the PSII light-harvesting system (for a recent model see [5]) is controlled by the proton gradient, the PsbS protein [6,7] and by the conversion of the light-harvesting pigment violaxanthin (Vx) into antheraxanthin (Ax) and zeaxanthin (Zx) within the so-called xanthophyll or Vx cycle (for a review of the Vx cycle see [8]). The synthesis of Ax and Zx is catalyzed by the enzyme violaxanthin de-epoxidase (VDE), which is located in the lumen of the thylakoids [9,10]. The acidification of the lumen upon strong illumination leads to the activation of the VDE, which includes the reversible binding of the enzyme to the thylakoid

Abbreviations: LHCII, light-harvesting complex of photosystem II; PSII, photosystem II; Vx, violaxanthin; Ax, antheraxanthin; Zx, zeaxanthin; VDE, violaxanthin de-epoxidase; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; CP, chlorophyll protein complex; NPQ, non-photochemical quenching of chlorophyll a fluorescence; Chl, chlorophyll; H_{II} phase, inverted hexagonal phase; DM, n-dodecyl β-D-maltoside; TX-100, Triton X-100; TLC, thin-layer chromatography; SQDG, sulfoquinovosyldiacylglycerol; DES, de-epoxidation state of the violaxanthin cycle pigment pool

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membrane [11,12], where it gains access to its hydrophobic substrates Vx and Ax.

An essential factor for Vx de-epoxidation is the presence of the main thylakoid lipid monogalactosyldiacylglycerol (MGDG), which is needed for the solubilization of Vx [13,14] and the binding of the VDE to the thylakoid membrane [15,16]. The non-bilayer lipid MGDG, furthermore, forms so-called inverted hexagonal structures (H_{II} phases), which in *in-vitro* assays [8,13,17–19] strongly enhance the Vx de-epoxidation rate. Besides its important role for Vx de-epoxidation, MGDG has been shown to support the ability of LHCII macro-aggregates to undergo light-induced structural changes [20].

Although a major part of the Vx cycle pigments is thought to be bound to the light-harvesting proteins [19,21–23], it is generally accepted that the de-epoxidation of Vx to Ax and Zx occurs in the lipid phase surrounding the antenna complexes [11,16,19,23–25]. This means that Vx has to be released from its xanthophyll binding site to become accessible by the enzyme VDE [25]. In the light-harvesting system of PSII the major part of Vx seems to be loosely bound at the peripheral V1 site of the LHCII. In the minor PSII antenna complexes CP24, CP26 and CP29, which are enriched in Vx, Vx appears to occupy the internal L2 binding site, as the V1 site is missing in these proteins [23]. To answer the question which part of the total Vx pool is convertible to Zx, Vx de-epoxidation was investigated in recombinant LHCII proteins, which were reconstituted with different combinations and concentrations of xanthophylls [26–28]. The data of these studies implies that only Vx bound to the V1 binding site, as it is the case in the LHCII, is easily accessible for the enzyme VDE [26], whereas Vx bound to the L2 site in reconstituted minor PSII antenna complexes is not or only very inefficiently convertible to Zx [28].

Further studies have shown that the release of Vx and its diffusion into the lipid phase appear to be the rate-limiting steps of Vx de-epoxidation [18,23,29]. This means that for a better understanding of Vx de-epoxidation *in-vivo* it should be analyzed which part of the total Vx pool is loosely bound to the light-harvesting proteins and which part exists as free Vx in the lipid matrix surrounding the proteins, thereby being accessible for the VDE. This question, however, is difficult to address, because Vx is easily lost during the isolation of light-harvesting proteins due to the weak pigment–protein interaction [19]. This fact is also reflected in the literature, where for different LHCII preparation methods significant differences in the concentration of bound Vx have been reported [30–32]. In general, the lowest amounts of Vx are found in reconstituted complexes, while the Vx concentration of isolated native complexes depends on the different detergents and solubilization conditions used [19]. It could also be shown [33] that the isolation procedure, and especially the concentration of the applied detergent, does not only influence the pigment composition but also the amount of lipids, which remain attached to the isolated LHCII.

In the present study we wanted to investigate in closer detail how different isolation procedures influence the composition and concentration of native, endogenous lipids and pigments associated with the LHCII, with a special focus on the Vx cycle pigment Vx and the H_{II} phase forming lipid MGDG. To achieve this, we compared LHCII fractions derived from a sucrose gradient centrifugation with LHCII complexes isolated after successive cation precipitation steps. Both preparation methods included a solubilization of thylakoid membranes with the detergents dodecylmaltoside (DM) or Triton X-100 (TX-100). To obtain information about the lipid dependence of Vx de-epoxidation in isolated antenna systems, the light-harvesting complexes containing different amounts of Vx and lipids were used for *in-vitro* de-epoxidation assays employing the isolated Vx cycle enzyme VDE. These *in-vitro* enzyme assays should answer the question, which part of the LHCII-associated Vx is convertible to Ax and Zx and how the MGDG content associated with the antenna complexes influences the de-epoxidation reaction. The present experiments with native LHCII complexes are also thought to complement the data in the literature on the Vx de-epoxidation in recombinant, Vx-binding LHCII [26].

2. Materials and methods

2.1. Preparation of spinach thylakoid membranes, violaxanthin de-epoxidase and violaxanthin

Fresh spinach leaves (*Spinacia oleracea* L.) were obtained from the local market. Intact thylakoid membranes were isolated according to the procedure described by [34]. The isolated thylakoid membranes were used for the preparation of Vx, for LHCII preparation by sucrose gradient centrifugation or for VDE isolation. VDE isolation from spinach thylakoids was done according to the procedure described in [11]. Vx was prepared from spinach thylakoids by a method described in [35]. Thylakoid membranes used for the LHCII preparation by successive cation precipitation were isolated according to [36].

2.2. Preparation of light-harvesting complexes II (LHCII) of spinach

PSII light-harvesting complexes (LHCII) were isolated by sucrose gradient centrifugation as described in [37] or by successive cation precipitation according to [36]. For sucrose gradient centrifugation isolated spinach thylakoid membranes were solubilized with different detergents and different detergent concentrations. The mild, non-ionic detergents n-dodecyl β-D-maltoside (DM) and Triton X-100 (TX-100) were used at detergent/Chl ratios of 10, 20 or 40 (w/w), corresponding to 1, 2 and 4% DM or TX-100 (w/v), respectively.

2.3. Characterization of the different LHCII fractions

Absorbance spectra were recorded with a Specord M 500 spectrophotometer (Zeiss, Germany) in the wavelength range from 350 to 750 nm with a bandpass setting of 1 nm. 77 K fluorescence emission and excitation spectra were recorded with an F-3000 fluorescence spectrophotometer (Hitachi, Japan) according to [38]. The protein composition of the isolated LHCII fractions was determined by SDS-PAGE according to [38].

2.4. In-vitro de-epoxidation assays with violaxanthin and LHCII

In-vitro de-epoxidation assays were performed following established procedures [13,16] in a reaction medium adjusted to the pH optimum of VDE (10 mM KCl, 5 mM MgCl2, 40 mM MES pH 5.2). The enzyme assay contained either 0.4 μM of pure Vx (in ethanolic solution) or isolated LHCII. When the isolated LHCII was used, the concentration of the LHCII was adjusted to reach a final concentration of LHCII-associated Vx of 0.4 μM. Pure Vx or the LHCII suspension was mixed with 11.6 μM MGDG (dissolved in methanol), incubated for 5 min and injected into the reaction medium with a Hamilton syringe, the final methanol concentration in the enzyme assay did not exceed 2% (v/v). Then VDE solution was added, either 25 μl mL^{-1} for the assays with pure Vx or 250 μl mL^{-1} for assays employing the isolated LHCII (see also the Results section). The reaction mixture was incubated at 30 °C for 5 min and then a 700 μL control sample was collected. Vx de-epoxidation was started with the addition of ascorbate (30 mM) and further samples were taken after specific time points of the reaction. During the fast de-epoxidation reaction in the enzyme assays with pure Vx, samples were collected after 0, 1, 2, 5, 10 and 20 min. During the enzyme assays with the isolated LHCII, which exhibited a slower de-epoxidation of the LHCII-associated Vx, samples were taken after 0, 30, 60, and 120 min, respectively. The collected samples were immediately transferred into Eppendorf tubes containing 700 μL of pigment extraction medium ($CHCl_3$:MeOH:NH_3 in the ratio of 1:2:0.004, v/v) and were repeatedly stirred. After a phase separation had taken place, the lower organic phase was collected, dried under a gentle stream of nitrogen and stored at −20 °C until pigment analysis by HPLC was performed.

2.5. Pigment extraction and HPLC analysis

The dried pigments were dissolved in a medium consisting of 90% methanol/0.2 M ammonium acetate (9:1, v/v) and 10% ethyl acetate, centrifuged for 2 min at 20,000×*g* and analyzed by HPLC as described in [39]. Quantification of pigments was performed according to [40].

2.6. Lipid extraction from thylakoid membranes and LHCII preparations

Total lipids were extracted from the isolated spinach thylakoid membranes and LHCII preparations by addition of $CHCl_3$:CH_3OH (2:1, v/v) to yield a final concentration of 8:4:3 (v/v) according to [41]. After the separation of the organic and the aqueous phase had taken place, the organic phase was collected and washed with 5 M NaCl at a ratio of organic solvent to NaCl solution of 60:40. The water phase was re-extracted and the organic extracts were combined and dried under a gentle stream of nitrogen. The dried lipids were then stored at −20 °C until lipid determination by thin-layer chromatography was performed.

2.7. Separation and quantification of lipids

Concentrated lipid extracts with a defined Chl a concentration were applied to HPTLC silica gel 60 plates (Merck, Germany) and developed in TLC chambers using two different eluent systems. The first system was based on a method described by [42] with methylacetate:isopropanol: CH_3Cl:MeOH:KCl (0.25%) in the ratio 25:25:25:10:4 as eluent. The second system was performed according to [43] with CH_3Cl:MeOH: acetic acid:H_2O in the ratio 75:13:9:3 as eluent. The use of two eluent systems was necessary because the lipid extracts of the LHCII preparations contained significant amounts of the employed detergents DM or TX-100, which covered different lipid spots in the two systems. A combination of both TLC eluent systems, however, enabled the exact quantification of all lipid classes of the thylakoid membrane. After the TLC separation, the lipids were stained with primuline (Direct Yellow 59) according to [44]. The spots were then semi-quantitatively assessed using a digital imaging system in combination with the program BiodocAnalyze (Biometra, Germany). The concentration of the different lipids was calculated in relation to lipid standards with a known molecular weight and concentration. The results are expressed relative to the amount of Chl a.

2.8. Chemicals

MGDG was purchased from Lipid Products (UK). The detergents n-dodecyl-β-D-maltoside (DM) and Triton X-100 (TX-100) were acquired from Roth (Germany) and from Merck (Germany), respectively.

3. Results

3.1. Characterization of the different LHCII preparations

To obtain information about the functionality of the different LHCII preparations used in the present study, absorbance (Fig. 1A) and low-temperature fluorescence spectra (Fig. 1B) were recorded. The additional analysis of the protein composition of the LHCII fractions

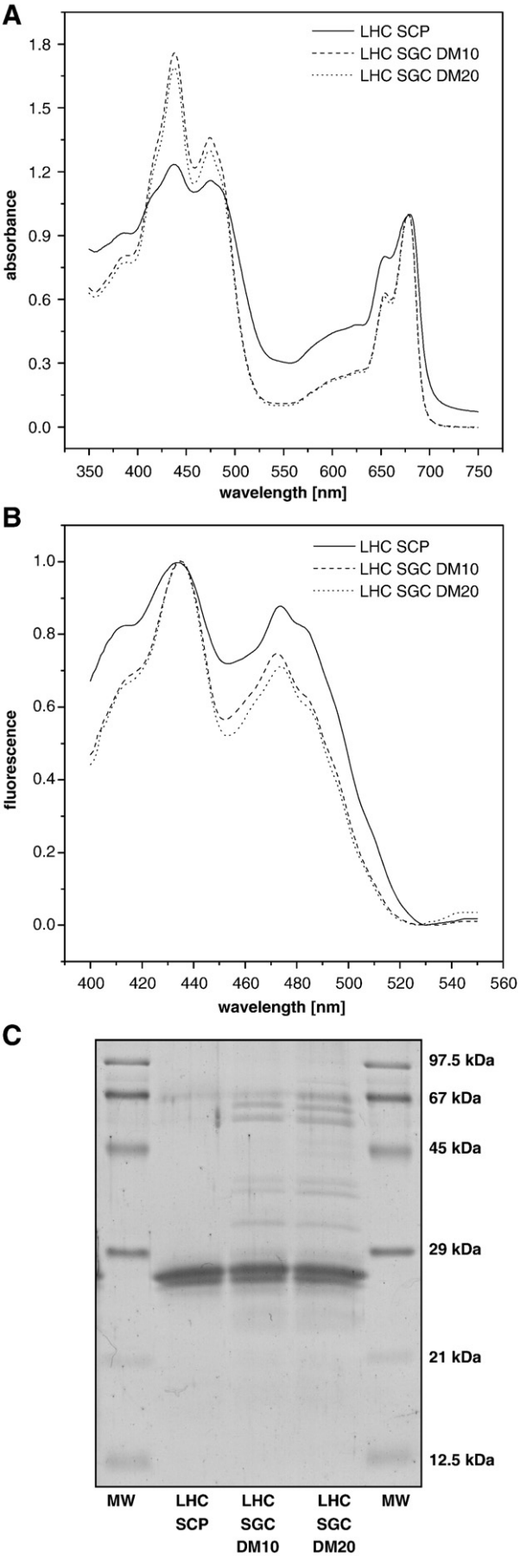

Fig. 1. Absorbance spectra (1A), 77 K fluorescence excitation spectra (B) and protein composition (C) of the different LHCII fractions used in the present study. LHCII was prepared from sucrose gradient centrifugation of thylakoid membranes solubilized with a DM/Chl ratio of 10 (LHC SGC DM10) or 20 (LHC SGC DM20) or by successive cation precipitation after solubilization of thylakoids with TX-100 (LHC SCP). The 77 K fluorescence excitation spectra were recorded with a fixed fluorescence emission wavelength of 680 nm. The protein composition was determined by SDS-PAGE and the proteins were stained with Coomassie Blue. In the lanes of the SDS gel depicted as MW molecular weight markers have been applied.

by SDS-PAGE (Fig. 1C) provided information about the purity of the isolated complexes.

The absorbance spectra of the LHCII fractions (Fig. 1A), which were normalized at the Q_Y absorption of Chl a at 680 nm, showed the typical Chl a maxima at 440 and 680 nm. The high concentration of Chl b in the LHCII fractions was visible as pronounced absorption peaks at 475 nm and 655 nm. The Chl b absorption was found to be highest in the LHCII fractions isolated by successive cation precipitation according to [36]. The additional broad shoulder at wavelengths longer than 480 nm was caused by the high concentration of xanthophylls bound to the isolated LHCII. The significantly lower absorption of the LHCII fraction isolated by successive cation precipitation in the blue region of the spectrum was caused by a higher light scattering of this LHCII preparation, which indicates that these light-harvesting complexes exist in a highly aggregated form. Macroaggregation of the LHCII isolated by successive cation precipitation was also visible in the 77 K fluorescence emission spectra as a pronounced shoulder at a wavelength of around 700 nm, which accompanied the main fluorescence emission maximum at 680 nm (data not shown). The 77 K fluorescence excitation spectra (Fig. 1B) were measured at a constant emission wavelength of 680 nm, which corresponds to the main fluorescence emission band of the LHCII. The excitation spectra, which were normalized at the Chl a maximum at 440 nm, showed that both Chl b and the xanthophylls isolated with the different LHCII fractions were functionally associated with the apoproteins and completely transferred the absorbed excitation energy to Chl a. Chl b excitation became visible as a pronounced peak at 475 nm, whereas the xanthophyll excitation was seen as a broad shoulder at wavelengths up to 510 nm. Like in the absorbance spectra, the LHCII isolated by cation precipitation was characterized by a higher Chl b and xanthophyll maximum compared to the LHCII fractions isolated by DM solubilization and sucrose gradient centrifugation. It is also noteworthy that in the 77 K fluorescence emission spectra of the isolated LHCII fractions no short-wavelength fluorescence at 670 nm was visible, which might have indicated the existence of Chl a molecules without a functional binding to the LHCII apoproteins (data not shown).

The SDS-PAGE of the isolated LHCII fractions (Fig. 1C) shows that the protein composition of all preparations was dominated by the 25 and 27 kDa LHCII apoproteins. While the LHCII isolated by cation precipitation was completely free of other proteins, the LHCII fractions prepared by sucrose gradient centrifugation contained some other protein bands. Due to their low concentration in comparison to the main LHCII bands these proteins can only be seen as very minor contaminations. However, it is of interest for the present experiments that the minor Chl a/b antenna proteins CP29 and CP26 were present in low concentration in the LHCII fractions isolated by sucrose gradient centrifugation. The presence of CP29 and CP26, which are characterized by a higher Chl a/b ratio compared with the peripheral LHCII, was also reflected in the slightly higher Chl a/b ratio of the LHCII fractions isolated by sucrose gradient centrifugation compared with the LHCII isolated by successive cation precipitation (Table 2).

3.2. Lipid and pigment composition of the different LHCII preparations

Table 1 summarizes the lipid composition and concentration of the LHCII preparations obtained after sucrose gradient centrifugation or successive cation precipitation. To compare the lipid content of the different LHCII fractions to the overall thylakoid lipid content and composition, the lipid concentration of intact spinach thylakoids is additionally presented.

The total lipid concentration of spinach thylakoid membranes was 1337 mM M^{-1} Chl a. LHCII fractions isolated with sucrose gradient centrifugation after a solubilization of thylakoids with a DM/Chl ratio of 10 still contained a high amount of lipids. The total lipid concentration of 880 mM M^{-1} Chl a was, however, reduced in comparison to that of the intact thylakoid membrane. The relatively mild solubilization increased the risk of a contamination with other PSII proteins, a fact which is reflected by a Chl a/b ratio of the LHCII of 1.47 (Table 2). Higher concentrations of DM, i.e. solubilization of thylakoids with a DM/Chl ratio of 20, led to a decrease in the total lipid content and lipid concentrations of 770 mM M^{-1} Chl a were typically found. Increases in the detergent concentration, on the other hand, enhanced the purity of the isolated LHCII as shown by the lower Chl a/b ratio of 1.33. The purest complexes with a Chl a/b ratio of 1.27 were found after successive cation precipitation according to [36]. This isolation procedure also removed a larger part of the lipids associated with the LHCII and resulted in a final lipid concentration of only 399 mM M^{-1} Chl a.

It is interesting to note that the reduction of the individual lipid classes in the isolated LHCII fractions depended on the isolation procedure and the detergent used. The galactolipids MGDG and DGDG were not as strongly affected by the solubilization with DM as the negatively charged lipids PG and SQDG and remained in higher concentrations in the LHCII fractions obtained after sucrose gradient centrifugation. After the isolation employing successive cation precipitation, on the other hand, PG and SQDG were found in approximately the same total amount as in the sucrose gradient preparations, but here the galactolipids MGDG and DGDG were affected more severely. This was especially true for MGDG, which was reduced by more than 70% compared to a reduction by only 40% in the LHCII fractions after solubilization with a DM/Chl ratio of 20 and sucrose gradient centrifugation.

The relative distribution of the lipid classes in the different LHCII preparations showed differences compared to that of the thylakoid membrane. In all preparations, MGDG represented the most abundant lipid. The contribution of MGDG to the total lipid fraction ranged between 40% and more than 50% in the thylakoid membranes and the LHCII fractions prepared by sucrose gradient centrifugation. The LHCII isolated by cation precipitation, however, showed reduced amounts of

Table 1
Lipid composition and concentration of isolated spinach thylakoids and the different LHCII preparations used in the present study. LHCII was prepared from sucrose gradient centrifugation of thylakoid membranes solubilized with a DM/Chl ratio of 10 (LHC SGC DM10) or 20 (LHC SGC DM20) or by successive cation precipitation after solubilization of thylakoids with TX-100 (LHC SCP). For further details see the Materials and methods section. The lipid composition is depicted as mM lipid M^{-1} Chl a. This table shows the mean values of three independent lipid determinations with the respective standard deviations.

	Thylakoids	LHC SGC DM10	LHC SGC DM20	LHC SCP
MGDG	548 ± 82	459 ± 45	325 ± 40	146 ± 11
DGDG	330 ± 16	211 ± 18	204 ± 12	97 ± 14
SQDG	129 ± 29	59 ± 5	60 ± 4	71 ± 17
PG	219 ± 26	118 ± 21	106 ± 18	94 ± 10
PC	70 ± 5	0	0	0
Total lipid	1337 ± 108	880 ± 35	770 ± 119	399 ± 73

Table 2
Pigment composition and concentration of isolated spinach thylakoids and the different LHCII preparations used in the present study. LHCII was prepared from sucrose gradient centrifugation of thylakoid membranes solubilized with a DM/Chl ratio of 10 (LHC SGC DM10) or 20 (LHC SGC DM20) or by successive cation precipitation after solubilization of thylakoids with TX-100 (LHC SCP). For further details see the Materials and methods section. The pigment composition is depicted as mM pigment M^{-1} Chl a. This table shows the mean values of three independent pigment determinations with the respective standard deviations.

	Thylakoids	LHC SGC DM10	LHC SGC DM20	LHC SCP
Neoxanthin	61 ± 8	105 ± 12	107 ± 13	101 ± 12
Violaxanthin	153 ± 3	101 ± 4	97 ± 2	62 ± 5
Antheraxanthin	9 ± 1	6 ± 1	5 ± 1	2 ± 1
Zeaxanthin	5 ± 1	3 ± 1	1 ± 1	4 ± 2
Lutein	214 ± 3	311 ± 28	292 ± 17	312 ± 12
Chl b	388 ± 10	682 ± 45	750 ± 2	786 ± 59
β-Carotene	83 ± 6	15 ± 3	10 ± 4	27 ± 5
Chl a/b	2.58	1.47	1.33	1.27

MGDG, and MGDG only represented about 35% of the bound lipid. The concentration of the second galactolipid DGDG was more stable and DGDG contributed around 25% to the total lipid in all different LHCII preparations, an amount which is similar to the DGDG concentration in the intact thylakoid membrane. The contribution of the negatively charged membrane lipids PG and SQDG to the overall lipid content was significantly lower than that of the galactolipids. PG and SQDG together represented about 25% of the total thylakoid membrane lipid. In the different LHCII preparations, the concentration of the two negatively charged lipids was nearly constant. This stable association together with the reduction of MGDG led to an increase of the PG and SQDG contribution to the total LHCII-associated lipid in the LHCII fractions isolated by cation precipitation (40%) compared with the LHCII fractions derived from sucrose gradient centrifugation (20%). The phospholipid PC was only found in minor amounts in the isolated spinach thylakoids and has to be seen as a contamination by the outer envelope membrane of the chloroplast [45].

With respect to the pigment composition all different LHCII preparations were characterized by a strongly reduced amount of β-carotene compared with the β-carotene content of intact thylakoid membranes (Table 2). The remaining β-carotene can be seen as a contamination of the LHCII fractions, as β-carotene normally only binds to the core proteins of PSII. The LHCII preparations were enriched in the xanthophylls neoxanthin and lutein, which were found in similar concentrations (around 100 and 300 mM pigment M^{-1} Chl a, respectively) in the different LHCII fractions. LHCII fractions prepared by DM solubilization and sucrose gradient centrifugation showed a reduced amount of Vx cycle pigments compared to the intact thylakoid membranes. This was especially obvious in the Vx concentration, which was reduced from 153 mM M^{-1} Chl a to 101 mM M^{-1} Chl a or 97 mM M^{-1} Chl a in the LHCII fractions isolated with a DM/Chl ratio of 10 or 20, respectively. The lowest concentration of Vx was found in the LHCII fractions, which had been prepared using cation precipitation and solubilization with TX-100. These LHCII preparations normally contained only around 60 mM Vx M^{-1} Chl a (Table 2). The decrease in the Vx content of the different LHCII preparations compared with the thylakoid membrane is due to the fact that the Vx cycle pigments are enriched in the minor light-harvesting complexes CP24, CP26 and CP29, which are additionally present in the thylakoids, and that the solubilization of the thylakoid membrane by detergents leads to a release of a certain part of the total Vx pool associated with the antenna proteins.

Based on our observation that the different LHCII preparations contained different amounts of lipids and Vx, we correlated the Vx content of the complexes with the concentration of LHCII-associated MGDG. Fig. 2 shows that a linear correlation exists between the content of Vx and MGDG found in the LHCII preparations (note that for Fig. 2 data from additional LHCII preparations with a higher variety of DM and TX-100 concentrations were used). LHCII preparations isolated by sucrose gradient centrifugation, which were enriched in MGDG, also contained high concentrations of Vx. LHCII fractions where the MGDG content was reduced, either by the use of higher DM concentrations or by successive cation precipitation, contained lower amounts of Vx. It is of further importance that the linear correlation between the Vx and MGDG content does not pass through the origin but cuts the ordinate at a Vx concentration of around 50 mM M^{-1} Chl a. This implies that even in the complete absence of MGDG a significant part of Vx is associated with the LHCII. It is reasonable to believe that this part of the Vx pool represents Vx, which is rather tightly bound to the respective Vx binding sites, whereas the other part of the pool is composed of Vx, which is situated in a lipid shield isolated with the LHCII apoproteins or is so loosely bound to the LHCII that it is lost with the respective MGDG. The direct correlation between Vx and MGDG led us to the question of how the different concentrations of LHCII-associated MGDG influence the de-epoxidation of native Vx by the enzyme VDE.

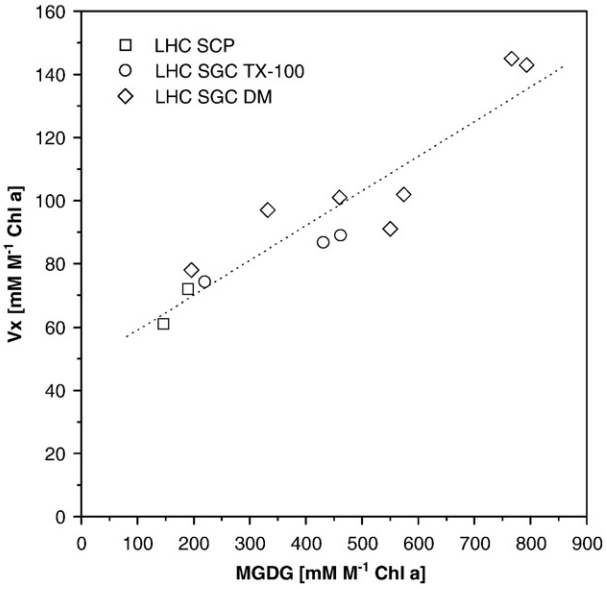

Fig. 2. Correlation between the endogenous Vx and MGDG concentration of different LHCII fractions isolated by successive cation precipitation (LHC SCP) or sucrose gradient centrifugation (for further details see the Materials and methods section). To obtain a greater variety of LHCII fractions prepared by sucrose gradient centrifugation, thylakoid membranes were solubilized with different concentrations of either dodecylmaltoside (LHC SGC DM) or Triton X-100 (LHC SGC TX-100). Data were fitted with the linear function $y = 0.11x + 48.1$, the regression coefficient is $R^2 = 0.86$. This figure shows the mean values of three independent MGDG and Vx determinations for each preparation.

3.3. Vx de-epoxidation of the different LHCII preparations without addition of exogenous MGDG

Fig. 3 shows the results from the *in-vitro* Vx de-epoxidation using the isolated LHCII fractions obtained by the different preparation methods. In these experiments no additional exogenous MGDG had been added to the enzyme assays. The addition of LHCII to the enzyme assay was adjusted in such a way that the LHCII-associated Vx reached a concentration of 0.4 μM. This concentration was similar to the final Vx concentration of *in-vitro* assays with purified Vx, which were used as control de-epoxidation assays. Preliminary tests also showed that Vx de-epoxidation using isolated LHCII fractions was significantly slower than Vx conversion in *in-vitro* assays with purified Vx (data not shown). Thus, the concentration of VDE was increased tenfold for the enzyme assays with the different LHCII fractions and the total reaction time was prolonged from 20 to 120 min (see also the Materials and methods section).

When the Vx de-epoxidation of the different LHCII preparations was compared, we observed the typical Vx de-epoxidation kinetics in the LHCII fractions isolated by DM solubilization and sucrose gradient centrifugation, which were enriched in native, endogenous MGDG. Both LHCII fractions, isolated with different DM/Chl ratios, showed a rapid de-epoxidation of the LHCII-associated Vx within the first 30 min of the de-epoxidation assay, which was followed by a slower second phase during the remaining 90 min of the enzyme assay. In both cases a high de-epoxidation state of the Vx cycle pigment pool was reached at the end of the incubation period. The highest value for the de-epoxidation state, which was typically observed, was 0.8. This, however, also indicates that a part of the Vx, which was associated with LHCII fractions resulting from the sucrose gradient centrifugation, was not convertible to Ax and Zx. In contrast to the LHCII fractions isolated by sucrose gradient centrifugation, Vx de-epoxidation was severely restricted when the LHCII prepared by successive cation precipitation and TX-100 solubilization was used. Enzyme assays with this LHCII fraction, which showed strongly reduced amounts of endogenous MGDG, exhibited a slow de-epoxidation of the native

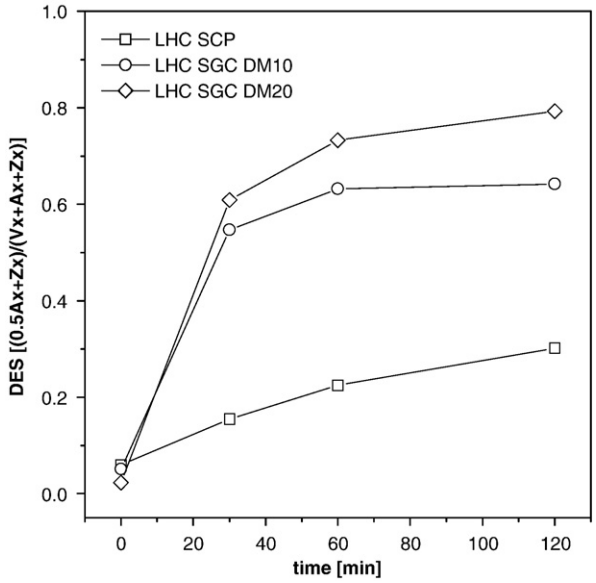

Fig. 3. Time-course of Vx de-epoxidation in *in-vitro* enzyme assays with the isolated LHCII fractions in the single presence of endogenous lipid and Vx. LHCII was prepared from sucrose gradient centrifugation of thylakoid membranes solubilized with a DM/Chl ratio of 10 (LHC SGC DM10) or 20 (LHC SGC DM20) or by successive cation precipitation after solubilization of thylakoids with TX-100 (LHC SCP). For further details see the Materials and methods section. Vx de-epoxidation is depicted as the increase in the de-epoxidation state of the Vx cycle pigment pool (DES). The enzyme assay was carried out in reaction medium (RM) pH 5.2 and the concentration of endogenous Vx was adjusted to 0.4 μM. To 1 mL of the de-epoxidation assay 250 μL of spinach VDE solution were added and the de-epoxidation reaction was started by addition of 30 mM ascorbate. This figure shows the result from a typical *in-vitro* enzyme assay, three independent experiments confirmed the data depicted in the figure.

Vx. Even after 120 min of the *in-vitro* assay, the de-epoxidation state of the Vx cycle pigment pool remained on a low value of around 0.27. It is important to note that the differences in the de-epoxidation kinetics between the LHCII fractions prepared by sucrose gradient centrifugation and successive cation precipitation cannot be explained by different detergent contents of the isolated LHCIIs. The LHCII isolated by successive cation precipitation, which exhibited the slowest and most inefficient Vx de-epoxidation, was completely free of TX-100 as demonstrated by the lipid separation and quantification by TLC (see Supplementary material). The LHCII fractions prepared by sucrose gradient centrifugation, however, still contained low concentrations of DM. It is possible that these contaminations by DM had a slightly inhibiting effect on the Vx de-epoxidation in these preparations, as *in-vitro* assays with isolated VDE and pure Vx had shown that low concentrations of DM lead to a minor decrease of the VDE activity (data not shown). The possibility of a slightly inhibiting effect of DM in the LHCII fractions prepared by sucrose gradient centrifugation, on the other hand, indicates that the difference between these MGDG-enriched complexes and the lipid-depleted LHCII prepared by cation precipitation would even be more pronounced in the complete absence of a DM contamination. The slight contamination of the LHCII fractions isolated by sucrose gradient centrifugation with DM in combination with the presence of low amounts of CP29 and CP26, which are characterized by a slower Vx de-epoxidation than the peripheral LHCII [21], may, however, provide an explanation why the de-epoxidation rates in the present experiments were lower than those in enzyme assays with the isolated VDE and pure Vx [13].

To test if the drastic difference in Vx de-epoxidation was really caused by the different MGDG concentrations associated with the LHCII, we added pure exogenous MGDG to the *in-vitro* de-epoxidation assays.

3.4. Vx de-epoxidation of the different LHCII preparations in the presence of additional exogenous MGDG

Fig. 4 shows Vx de-epoxidation using the different LHCII preparations in the absence or presence of pure, exogenous MGDG. The LHCII isolated after successive cation precipitation, which contained strongly reduced amounts of MGDG, showed a significant increase of the de-epoxidation of native Vx after addition of exogenous MGDG to the *in-vitro* assay (Fig. 4A). Addition of 11.6 μM MGDG, which resulted in a ratio of exogenous MGDG to LHCII-associated Vx of 29, led to a rapid conversion of Vx to Ax and Zx and at the end of the 120 min of the enzyme assay a DES of the Vx cycle pigment pool of 0.62 was reached. This DES was comparable to the DES exhibited by the lipid-enriched LHCII fractions isolated by sucrose gradient centrifugation in the absence of exogenous MGDG (Fig. 3). Addition of higher concentrations of MGDG, i.e. 23.2 μM MGDG, resulting in a ratio of exogenous MGDG to native Vx of 58, decreased the Vx de-epoxidation kinetics, which was especially obvious after 30 and 60 min of incubation time. However, at the end of the de-epoxidation assay a comparably high DES of the Vx cycle pigment pool with a value of 0.59 was observed.

In contrast to the positive effect on the de-epoxidation of Vx associated with the LHCII isolated by cation precipitation, additional exogenous MGDG decreased the Vx conversion in the lipid-enriched LHCII fractions prepared by sucrose gradient centrifugation (Fig. 4B and C). Both the LHCII isolated with a DM/Chl ratio of 10 and the LHCII isolated with a ratio of 20 showed a slower de-epoxidation of the native Vx in the presence of 11.6 μM MGDG. Further decreases in the Vx de-epoxidation rate were observed when the higher concentration of exogenous MGDG (23.2 μM) was employed. It is interesting to note that the LHCII preparation derived from the solubilization with a DM/Chl ratio of 10, which contained the highest amount of lipids, exhibited the strongest decrease of the Vx de-epoxidation rate after the addition of MGDG (Fig. 4B). In that case, already the lower MGDG concentration of 11.6 μM led to a 50% reduction of the DES observed after 120 min of the enzyme assay compared to the DES obtained in the absence of exogenous MGDG. The suppression of Vx conversion was then only slightly enhanced after the addition of the higher MGDG concentration of 23.2 μM.

The LHCII prepared with a higher DM/Chl ratio of 20, which contained a lower lipid and MGDG content, did not show such a strong reduction of the de-epoxidation of LHCII-associated Vx after the addition of exogenous MGDG (Fig. 4C). Here, we typically observed a reduction of the DES of the Vx cycle pigment pool of around 30% or 50% in the presence of the low and high MGDG concentration, respectively, compared to the DES obtained in the absence of exogenous MGDG.

It is of further importance that addition of increasing MGDG concentrations also led to a reduction of the Vx de-epoxidation rate in *in-vitro* enzyme assays where, instead of LHCII-associated Vx, pure Vx was used. In these assays optimal Vx de-epoxidation could be observed at an MGDG/Vx ratio of 29. MGDG concentrations of 23.2 μM, i.e. a ratio of exogenous MGDG to Vx of 58, however, led to a strong reduction of the Vx de-epoxidation kinetics resulting in a significantly lower DES at the end of the *in-vitro* incubation (data not shown).

In summary the results from Fig. 4 show that the addition of exogenous MGDG to a lipid-depleted LHCII leads to an improvement of the de-epoxidation of native Vx, whereas additional MGDG decreases the conversion of Vx to Ax and Zx when lipid-enriched LHCII preparations are used. To gain further insight into the dependence of Vx de-epoxidation on the endogenous and exogenous MGDG concentration, further LHCII preparations were examined.

3.5. Dependence of the de-epoxidation of LHCII-associated Vx on the MGDG/Vx ratio

Fig. 5 depicts the dependence of the de-epoxidation of LHCII-associated Vx on the MGDG/Vx ratio in the single presence of

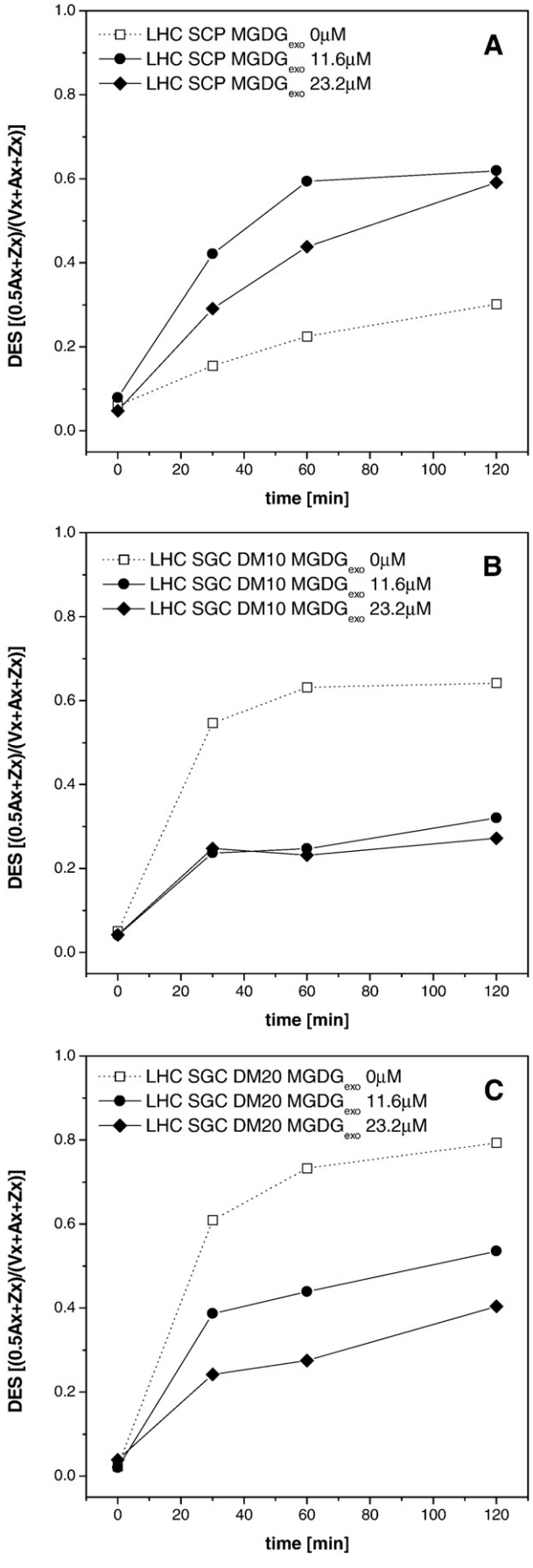

Fig. 4. Time-course of Vx de-epoxidation in *in-vitro* enzyme assays with the isolated LHCII fractions with or without addition of exogenous MGDG. Exogenous MGDG ($MGDG_{exo}$) was added at a concentration of 11.6 μM or 23.2 μM resulting in a ratio of exogenous MGDG to LHCII-associated Vx of 29 or 58, respectively. Vx de-epoxidation is depicted as the increase in the de-epoxidation state of the Vx cycle pigment pool (DES). The enzyme assay was carried out in reaction medium (RM) pH 5.2 and the concentration of endogenous Vx was adjusted to 0.4 μM. To 1 mL of the de-epoxidation assay 250 μL of spinach VDE solution were added and the de-epoxidation reaction was started by addition of 30 mM ascorbate. This figure shows the result from a typical *in-vitro* enzyme assay, three independent experiments confirmed the data depicted in the figure. Panel A: LHCII isolated by successive cation precipitation after solubilization of thylakoids with TX-100 (LHC SCP), panel B: LHCII isolated by sucrose gradient centrifugation after solubilization of thylakoids with a DM/Chl ratio of 10 (LHC SGC DM10), panel C: LHCII isolated by sucrose gradient centrifugation after solubilization of thylakoids with a DM/Chl ratio of 20 (LHC SGC DM20).

endogenous MGDG (Fig. 5A) or after the addition of exogenous pure MGDG (Fig. 5B). Fig. 5A shows that the highest de-epoxidation rates of LHCII-associated Vx with values of around 1.5 mM Vx M^{-1} Chl a min^{-1} were obtained at a ratio of endogenous MGDG to Vx between 3.4 and 5. The optimal ratio for Vx conversion did not per se depend on the LHCII isolation procedure, i.e. it was comparable for LHCII fractions obtained by either DM or TX-100 solubilization of thylakoid membranes. MGDG/Vx ratios below 3, which were typically observed in LHCII preparations isolated by successive cation precipitation, were characterized by significantly decreased Vx de-epoxidation rates. Increases of the ratio of LHCII-associated MGDG/Vx above a ratio of 5.5 also led to a pronounced decrease of the Vx de-epoxidation rate. In the present experiments, the overall lowest Vx conversion rates were observed at a ratio of endogenous MGDG to Vx of 2.4 or 6.

The data depicted in Fig. 5A also explain why the LHCII isolated with a DM/Chl ratio of 10 exhibited a slightly decreased Vx de-epoxidation rate in the absence of exogenous MGDG compared with the LHCII isolated with a DM/Chl ratio of 20 (Figs. 3 and 4B and C). Both LHCII fractions contained almost similar concentrations of native Vx (Table 2), but the LHCII prepared from a solubilization of thylakoids with a DM/Chl ratio of 10 was characterized by a higher content of endogenous MGDG (Table 1). This led to a MGDG/Vx ratio, which was slightly higher than the MGDG/Vx ratio needed for optimal Vx de-epoxidation.

Addition of exogenous pure MGDG to the lipid-depleted LHCII isolated by successive cation precipitation led to a strong shift in the optimal MGDG/Vx ratio (Fig. 5B). In these experiments ratios of exogenous MGDG to native Vx between 20 and 30 were needed to obtain the highest Vx de-epoxidation rates. These MGDG/Vx ratios were comparable to the optimal MGDG/Vx ratio of 29, which led to a high de-epoxidation rate of purified Vx in the *in-vitro* enzyme assay (data not shown).

4. Discussion

4.1. Lipid and pigment composition of the different LHCII preparations

Our results show that different isolation procedures, employing different detergents, have a strong impact on the overall concentration of LHCII-associated lipids. They also lead to significant differences in the lipid composition of the isolated LHCII fractions, with respect to the contribution of the individual lipid classes to the total bound lipid. We find that the content of the anionic lipids PG and SQDG is only slightly reduced in LHCII fractions isolated by successive cation precipitation and solubilization with TX-100, whereas the concentration of the galactolipids MGDG and DGDG is decreased more severely. MGDG and DGDG, on the other hand, are not as strongly affected as PG and SQDG when solubilization with low DM concentrations and sucrose gradient centrifugation is employed. Increases in the DM concentration, however, also lead to a stronger loss of the galactolipids, which is mainly due to a significant decrease of the MGDG

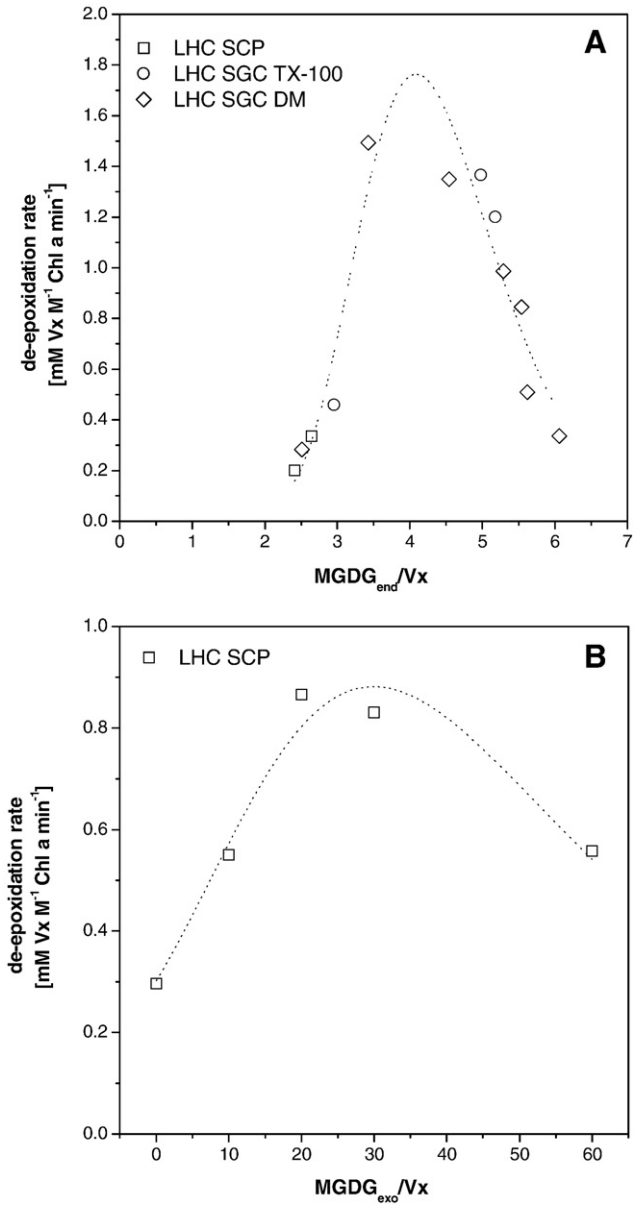

Fig. 5. Dependence of the de-epoxidation rate of LHCII-associated Vx on the MGDG/Vx ratio. Vx de-epoxidation is expressed as the Vx de-epoxidation rate, which was calculated as mM Vx M^{-1} Chl a min^{-1} using the time point 30 min of the Vx de-epoxidation assay. Panel A presents data from different *in-vitro* enzyme assays with various LHCII fractions prepared by sucrose gradient centrifugation with either DM (LHC SGC DM) or TX-100 (LHC SGC TX-100) as detergent or prepared by successive cation precipitation (LHC SCP). For details see the Materials and methods section. The *in-vitro* de-epoxidation assays depicted in panel A were carried out in the single presence of endogenous MGDG ($MGDG_{end}$), which was associated with the different LHCII fractions. No exogenous MGDG was added to the enzyme assays. Panel B depicts the results of *in-vitro* assays where the lipid-depleted LHCII prepared by successive cation precipitation (LHC SCP) was complemented with different concentrations of exogenous MGDG ($MGDG_{exo}$), resulting in different ratios of exogenous MGDG to native Vx. For all de-epoxidation assays depicted in panels A and B the concentration of the LHCII-associated Vx was adjusted to 0.4 μM. All assays were carried out in reaction medium (RM) pH 5.2 in the presence of 30 mM ascorbate.

concentration. This means that, in general, we observe a reduction of the ratio of galactolipids to anionic lipids with an overall decrease of the total lipid (Table 1). These findings are supported by a former study [46], where it was observed that increases in the DM concentration induced a marked decrease of the galactolipid/Chl ratio of the isolated LHCII, whereas the PG/Chl ratio remained nearly constant. Furthermore, it was found that successive cation precipitation in the presence of increasing TX-100 concentrations led to a slight decline of the anionic lipid content, accompanied by a stronger decrease of the galactolipid concentration [33].

The stability of lipid binding to the LHCII is in accordance with the function of the different lipids. PG and DGDG seem to interact directly with the LHCII and they are important for the formation of trimers and the higher order structures of LHCII [47]. At present it is unclear if SQDG has a comparable structural function, but studies of the lipid composition of isolated LHCII show that SQDG usually remains attached to the protein in similar or even higher relative concentrations as it is present in the thylakoid membrane [48]. MGDG does not seem to be an integral component of the LHCII but various studies have suggested that an interaction between LHCII and MGDG exists. MGDG, as well as PG and DGDG, is thought to stabilize the reconstituted LHCII against thermal denaturation [49]. It was also shown [20,50] that lipid-depleted, disordered aggregates of LHCII were able to rebind a high amount of exogenous MGDG, thereby forming large lamellar aggregates with long-range order, i.e. higher order structures of LHCII. For our present experiments it is of high importance that MGDG is found in association with the isolated LHCII, which represents the site where the majority of Vx cycle pigments is located [21].

Due to the different binding affinities of the pigments to the LHCII apoprotein, their concentration in the isolated LHCII fractions of the present study was also influenced by both the isolation procedure and the nature and concentration of the different detergents. According to the LHCII crystal structure [47], one LHCII apoprotein binds two lutein molecules at the L1 and L2 sites and one neoxanthin molecule at the N1 binding site. Both lutein and neoxanthin are characterized by a strong pigment–protein interaction, which is supported by van der Waals contacts and hydrogen bonds. The binding of violaxanthin, which is located in a hydrophobic pocket at the monomer–monomer interface at the so-called V1 binding site, is, however, relatively weak. These findings are also supported by our present results, which show that the lutein and neoxanthin content of the different isolated LHCII fractions is more or less constant. The Vx concentration, on the other hand, is highly variable. Vx is found in high concentrations in LHCII fractions isolated by sucrose gradient centrifugation after solubilization with low detergent concentrations. The concentration of Vx is, on the other hand, severely reduced in LHCII prepared by successive cation precipitation and solubilization with TX-100 (Table 2).

Based on the assumption that one LHCII trimer binds 3 molecules of PG and 24 Chl a [47], the lipid and pigment composition of the different LHCII fractions of the present study can be calculated on a molecular basis. The LHCII isolated by solubilization with a DM/Chl a ratio of 20 followed by sucrose gradient centrifugation is associated with 9 to 10 molecules of MGDG, 6 DGDG, 2 SQDG and 3 PG per LHCII trimer. It contains 3 molecules of neoxanthin, 2 to 3 violaxanthin, 7 lutein and 18 Chl b. One LHCII trimer prepared by TX-100 solubilization and successive cation precipitation is associated with only 4 to 5 molecules of MGDG, 3 DGDG, 2 SQDG, 3 PG and 2 to 3 molecules of neoxanthin, 1 to 2 violaxanthin, 7 to 8 lutein and 19 Chl b.

4.2. Correlation between the MGDG and Vx content

Based upon our observation that the different isolation conditions resulted in differences of both the total lipid content and the Vx concentration of the LHCII, we correlated the concentration of native Vx with the concentration of endogenous MGDG. This correlation was used to gain information, if and how the concentration of LHCII-associated Vx depends on the content of MGDG, which has been shown to solubilize Vx in an aqueous environment [13] and provides the inverted hexagonal phases (H_{II} phases) needed for de-epoxidation [8,13]. Further, it has been suggested that Vx has to be released from its protein binding site into the surrounding lipid phase to become accessible to the enzyme VDE [16,25]. It is reasonable to assume that

after the release from its binding site, Vx becomes dissolved in an MGDG-enriched part of the thylakoid membrane, which surrounds the LHCII. This would allow for an interaction of Vx and VDE in the close vicinity of the LHCII, which might also be essential for the fast rebinding of Ax and Zx to the LHCII apoprotein [51].

The linear correlation we find for the content of endogenous MGDG and Vx of isolated LHCII preparations (Fig. 2), is, according to our opinion, a tool to estimate, which part of the total Vx pool is bound to the LHCII apoprotein and which part is situated in the MGDG shield surrounding the antenna complexes under native conditions. In this respect it is important to note that the linear correlation starts at a Vx concentration of 50 mM M^{-1} Chl a, which implies that an LHCII preparation where all MGDG is removed, still contains a significant amount of Vx. It is reasonable to assume that this part of the native Vx pool represents Vx, which is rather tightly bound to the LHCII apoprotein at the V1 binding site. According to the correlation presented in this paper, this binding of Vx to the LHCII occurs at a Vx/Chl a ratio of 0.050, which would mean that on average one Vx molecule is bound per LHCII trimer. This ratio is lower than the maximum Vx/Chl a ratio of 0.125, which would occur under the assumption that each monomer of LHCII binds eight Chl a molecules [47] and that all three V1 binding sites of an LHCII trimer are occupied by Vx. In [21] it was shown that Vx/Chl a ratios of 0.125, implying an occupation of all three V1 binding sites, can be obtained by a gentle solubilization of thylakoids. However, one has to assume that LHCII fractions isolated under these conditions are enriched in native lipids, which would certainly accommodate a part of the total Vx pool. In our present experiments LHCII fractions with an intact shield of MGDG molecules were also characterized by a Vx/Chl a ratio higher than 0.1.

With regard to the stoichiometry of Vx binding to the LHCII, one also has to take into account that in the intact membrane there is most probably a constant exchange of Vx between the V1 binding site and the surrounding MGDG, especially during the operation of the Vx cycle. Furthermore, it has to be considered that the Vx cycle pigment pool size is highly variable, depending on the plant species and the growth light conditions [52]. It is possible that under limiting light conditions, not all of the available Vx binding sites of the LHCII apoprotein are occupied [21]. The results of the present study, furthermore, imply that an increased amount of Vx molecules, which would be typically observed in high light grown plants, can be accommodated in the MGDG shield surrounding the LHCII. It is also possible that high MGDG concentrations are needed to stabilize the binding of Vx to all three V1 binding sites of the LHCII trimer.

4.3. Vx de-epoxidation of the different LHCII preparations

In our present study we show for the first time that native Vx, associated with LHCII fractions prepared by different methods, can be de-epoxidized in *in-vitro* enzyme assays with the isolated Vx cycle enzyme VDE. According to our data the de-epoxidation of endogenous Vx strongly depends on the concentration of MGDG, which remains associated with the LHCII after the isolation (Fig. 3). LHCII fractions enriched in endogenous MGDG are characterized by a strong and fast de-epoxidation, whereas LHCII preparations with reduced amounts of MGDG have to be supplemented with pure, exogenous MGDG to achieve a reasonable Vx conversion. It is of further interest that a decrease of other lipids, which were found in association with the LHCII, also leads to a reduced Vx de-epoxidation. In the case of an MGDG reduction the negative effects on Vx de-epoxidation are most probably due to a reduced solubilization capacity for the substrate Vx [13,16] and to the smaller size/amount of the inverted hexagonal phases provided by MGDG [13,18]. A reduced solubilization capacity for Vx would lead to an aggregation of Vx molecules, which would no longer be able to serve as substrate for the VDE [13]. Furthermore, the low MGDG content might restrict the formation of correct inverted hexagonal phases that are needed to attract the VDE and ensure the interaction between the enzyme and the substrate Vx [13,18]. The additional removal of lipids other than MGDG, i.e. PG and DGDG, might have exerted a negative effect on the structure of the LHCII, thereby leading to a decreased possibility of Vx detachment and diffusion into the lipid shield surrounding the LHCII, where the actual Vx de-epoxidation is supposed to take place.

It is interesting to note that in [26] a strongly reduced Vx de-epoxidation was found in experiments with reconstituted LHCII trimers when compared to Vx containing LHCII monomers. Both the rate of Vx de-epoxidation (reduction by a factor of about 10) and the maximum Vx convertibility (50% versus 80%) were strongly affected by the trimerization. These observations are partly confirmed by our present results on isolated native LHCII trimers, where we also noticed a significantly slower de-epoxidation of native Vx compared with the Vx conversion in *in-vitro* enzyme assays using pure, isolated Vx. Increases of the concentration of the isolated VDE, however, improved Vx de-epoxidation in the enzyme assays with the different LHCII fractions, so that in the trimeric LHCII preparations a maximum conversion of 80% of the LHCII-associated Vx could be obtained.

An incomplete de-epoxidation of Vx is also observed in the natural thylakoid membrane [53,54]. The amount of convertible Vx is varying from 50 to 80% and depends on the total size of the Vx cycle pigment pool. Studies on plants with a strongly reduced antenna size indicated that the limitation of Vx de-epoxidation results from differences in the binding of Vx to the antenna proteins [54,55]. Later work with reconstituted antenna proteins led to the conclusion [19] that the multiphasic and incomplete kinetics of Vx de-epoxidation *in-vivo* can be explained by different pools of Vx in the thylakoid membrane. Their data implies that Vx bound to the V1 and N1 binding site of the antenna proteins is easily accessible for de-epoxidation, whereas Vx bound to the L2 site is only slowly convertible to Zx or not convertible at all. The data of the present study does not allow us to discriminate between Vx molecules, which are located at different LHCII xanthophyll binding sites. Our experiments, however, are in line with the existence of distinct Vx pools in the native membrane. One pool is comprised of Vx molecules, which are bound to the LHCII apoprotein, whereas the second pool consists of Vx, which is located in an MGDG shield surrounding the antenna proteins. It is reasonable to assume that the latter Vx pool is characterized by a good convertibility of Vx, once the VDE is in contact with the MGDG phase. Here our results show that higher concentrations of endogenous LHCII-associated MGDG are capable of solubilizing a higher amount of Vx, thereby increasing the pool of easily accessible Vx. Increased concentrations of endogenous MGDG, furthermore, enhance the Vx de-epoxidation rate significantly (Fig. 3). The de-epoxidation kinetics of the pool consisting of Vx molecules bound to the LHCII apoprotein will most likely be influenced by the strength of binding and the detachment and diffusion of Vx to the MGDG phase. In that case, an increased shield of MGDG molecules might facilitate the Vx detachment and diffusion and the attraction of the VDE. With respect to Vx diffusion, however, one also has to take into account that the tight packing of proteins in the grana regions of the thylakoids can be a rate-limiting step for the de-epoxidation reaction *in-vivo* [56].

With regard to the very slow and inefficient de-epoxidation of native Vx, which was observed when the lipid-depleted LHCII isolated by successive cation precipitation was used in the enzyme assays, one also has to take into account that strongly lipid-depleted macro-aggregates of LHCII tend to form disorganized structures [33]. These structural alterations might have severely restricted the conversion of Vx. It is worth to note that addition of exogenous MGDG, which restores efficient Vx de-epoxidation, also leads to the reformation of ordered LHCII macro-aggregates, which are able to undergo light-induced structural changes.

Our observation of a maximum conversion of 80% of the Vx pool is in line with data from the literature. It indicates that the LHCII binds a pool of non-convertible Vx, which could be required as structural

element for the stabilization of single antenna proteins or for the formation of the PSII antenna suprastructure [28].

Another important finding of our present study is the observation that the de-epoxidation of endogenous Vx is saturated at much lower concentrations of LHCII-associated MGDG than the conversion of pure Vx in *in-vitro* enzyme assays employing isolated MGDG. Different studies have shown that for maximum de-epoxidation of pure Vx in VDE enzyme assays a ratio of MGDG to Vx of around 30 is needed [13,18,57]. In our present experiments with different isolated LHCII fractions, we observe that the highest rate of Vx de-epoxidation is already achieved at a ratio of endogenous MGDG to Vx of around 4 (Fig. 5A). One possible explanation for this strong difference between the enzyme assay with pure Vx and the de-epoxidation experiments with isolated LHCII lies in the observation that in the LHCII fractions different pools of native Vx exist. In the enzyme assays with pure Vx the MGDG concentration has to be high enough to solubilize the total amount of Vx. Otherwise Vx will form aggregates in the aqueous reaction medium, a pigment structure which is not accessible to the enzyme VDE [13,16]. In the LHCII fractions, on the other hand, only a part of the endogenous Vx exists in the MGDG shield around the complex, whereas another part is bound to the apoprotein. It is reasonable to believe that in these experiments the MGDG phase has to accommodate lower concentrations of Vx. These would comprise the Vx pool, which is already located in the MGDG shield and the Vx, which during the de-epoxidation reaction detaches from the protein. Furthermore, one can assume that Zx, which is formed by the de-epoxidation of Vx rebinds to the V1 site of the LHCII apoprotein, thereby decreasing the concentration of xanthophylls in the MGDG phase. The rebinding of Zx to the LHCII apoprotein, which might be even stronger than the binding of Vx, is corroborated by [21].

At present it is, however, not clear why in the LHCII fractions isolated by successive cation precipitation high concentrations of exogenous MGDG are needed to restore the activity of Vx de-epoxidation (Fig. 4A). In de-epoxidation assays with these lipid-depleted LHCII fractions a comparable MGDG/Vx ratio of around 30, as in the *in-vitro* assays with pure Vx, is needed to saturate the conversion of the LHCII-associated Vx (Fig. 5B). It may be argued that the commercially available MGDG, which has been used as exogenous MGDG in both the *in-vitro* assays with pure Vx and the LHCII prepared by successive cation precipitation, is not as effective in stimulating the de-epoxidation as native MGDG. It is also unclear, if exogenous MGDG is capable of forming a lipid shield around the LHCII apoprotein, which is comparable to the native MGDG phase surrounding the antenna complexes. High concentrations of exogenous MGDG might also be needed to compensate for the inhibitory effects of the negatively charged lipids PG and SQDG on the de-epoxidation reaction [58], taking into account that PG and SQDG contribute 40% to the total lipid of the LHCII isolated by successive cation precipitation.

Addition of exogenous MGDG to LHCII fractions prepared by sucrose gradient centrifugation, which contain significant amounts of endogenous MGDG and are characterized by an efficient Vx conversion, decreases the de-epoxidation rate (Fig. 4B and C). This, however, is also observed in *in-vitro* enzyme assays with pure Vx once the MGDG/Vx ratio surpasses the saturation point. Decreases of the Vx de-epoxidation rate at high MGDG concentrations have been interpreted as being due to an increased size of the inverted hexagonal phase aggregates needed for optimum VDE activity [8,13,16,18]. A large size of the H_{II} phase would increase the diffusion time of solubilized Vx molecules to the outer regions of the H_{II} phase, which represent the docking sites of the VDE, thereby decreasing the overall Vx conversion to Zx.

4.4. Relevance of the present experimental system for the mechanism of Vx de-epoxidation in the native thylakoid membrane

According to our opinion, the results presented in this study provide a step forward for the investigation of the requirements of Vx de-epoxidation in the native thylakoid membrane. By using isolated native LHCII together with a part of its direct lipid environment we come much closer to the situation in the natural membrane than the other *in-vitro* systems, which have been investigated so far [13,14,18]. These *in-vitro* systems relied on isolated pure Vx, which was not associated with a native protein and also employed purified MGDG, which was not necessarily identical to the native MGDG found in the vicinity of the light-harvesting complexes. The present *in-vitro* system, however, has still room for further improvements. It is not unlikely that the removal of residual DM in the LHCII complexes isolated by sucrose gradient centrifugation, which might have inhibited the VDE slightly or which might have blocked the association of a small part of VDE molecules with the LHCII, leads to a significantly increased overall rate of Vx de-epoxidation.

5. Conclusion

The present results show that MGDG, which is located in the vicinity of the LHCII, strongly enhances the de-epoxidation of LHCII-associated Vx. The highest efficiency of Vx de-epoxidation is found at a ratio of native MGDG to native Vx of around 4. It is reasonable to believe that in the natural thylakoid membrane MGDG realizes the solubilization of Vx and provides the three-dimensional structures, which are needed for the binding of VDE and for the accessibility of the substrate Vx to the enzyme. The importance of MGDG for the Vx de-epoxidation *in-vivo* has also been shown recently in a study of the *mgd1-1* mutant of *A. thaliana* whose MGDG content is approximately 40% less than that of the wild type [59] and which is characterized by an inefficient operation of the xanthophyll cycle [60].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.12.011.

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