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Lipid dependence of diadinoxanthin solubilization and de-epoxidation in artificial membrane systems resembling the lipid composition of the natural thylakoid membrane

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Abstract

In the present study, the solubility and enzymatic de-epoxidation of diadinoxanthin (Ddx) was investigated in three different artificial membrane systems: (1) Unilamellar liposomes composed of different concentrations of the bilayer forming lipid phosphatidylcholine (PC) and the inverted hexagonal phase (H_{II} phase) forming lipid monogalactosyldiacylglycerol (MGDG), (2) liposomes composed of PC and the H_{II} phase forming lipid phosphatidylethanolamine (PE), and (3) an artificial membrane system composed of digalactosyldiacylglycerol (DGDG) and MGDG, which resembles the lipid composition of the natural thylakoid membrane. Our results show that Ddx de-epoxidation strongly depends on the concentration of the inverted hexagonal phase forming lipids MGDG or PE in the liposomes composed of PC or DGDG, thus indicating that the presence of inverted hexagonal structures is essential for Ddx de-epoxidation. The difference observed for the solubilization of Ddx in H_{II} phase forming lipids compared with bilayer forming lipids indicates that Ddx is not equally distributed in the liposomes composed of different concentrations of bilayer versus non-bilayer lipids. In artificial membranes with a high percentage of bilayer lipids, a large part of Ddx is located in the membrane bilayer. In membranes composed of equal proportions of bilayer and H_{II} phase forming lipids, the majority of the Ddx molecules is located in the inverted hexagonal structures. The significance of the pigment distribution and the three-dimensional structure of the H_{II} phase for the de-epoxidation reaction is discussed, and a possible scenario for the lipid dependence of Ddx (and violaxanthin) de-epoxidation in the native thylakoid membrane is proposed.

Keywords: Bilayer lipid; Non-bilayer lipid; Inverted hexagonal phase; Thylakoid membrane; Diadinoxanthin; Xanthophyll cycle

1. Introduction

The xanthophyll cycles of vascular plants and algae are photoprotective mechanisms located in the chloroplast. At present, three different cycles are known: the violaxanthin (Vx) cycle of vascular plants and green and brown algae [1,2], the lutein epoxide cycle that has been detected in a limited set of vascular plants [3,4], and the diadinoxanthin (Ddx) cycle of the algal classes Bacillariophyceae, Chrysophyceae, Xanthophyceae, Haptophyceae and Dinophyceae [5]. The de-epoxidation reaction of the Ddx cycle comprises one step from the mono-epoxy xanthophyll Ddx to the epoxy-free diatoxanthin (Dtx), whereas the Vx cycle consists of a forward reaction with two de-epoxidation steps, and which transforms the di-epoxy-xanthophyll Vx into the epoxy-free zeaxanthin (Zx) via the intermediate antheraxanthin (Ax). Both de-epoxidation sequences take place when vascular plants or algae are illuminated with high actinic light intensities. Low light or darkness

Abbreviations: LHC/FCP, light-harvesting complexes of vascular plants or diatoms, respectively; DDE, diadinoxanthin de-epoxidase; VDE, violaxanthin de-epoxidase; Ddx, diadinoxanthin; Dtx, diatoxanthin; Vx, violaxanthin; Zx, zeaxanthin; MGDG, monogalactosyldiacylgycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine

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stimulates the back reaction of the cycles, in which the de-epoxidized xanthophylls are reverted to the respective epoxidized forms [for a review see 6].

The de-epoxidation step in the Ddx cycle is catalyzed by the enzyme Ddx de-epoxidase (DDE), which is located in the thylakoid lumen and becomes activated by a decreasing lumenal pH due to light-driven photosynthetic electron transport. DDE is optimized in various ways compared with the xanthophyll cycle enzyme of vascular plants, the Vx de-epoxidase (VDE). DDE activation can be observed at almost neutral pH values [7,8], whereas for VDE activation more acidic pH values are needed [9]. Further, DDE exhibits a significantly lower K_M value for its co-substrate ascorbate compared with the VDE of vascular plants [8].

Like VDE, DDE requires the presence of the major thylakoid membrane lipid MGDG for activity [10–13]. MGDG comprises about half of the total thylakoid membrane lipid, the second most abundant thylakoid lipid is the bilayer-forming DGDG which amounts to about 30% of the thylakoid lipids [14]. The overall lipid composition of thylakoid membranes of vascular plants and diatoms is comparable and MGDG is also the main thylakoid lipid of diatoms. MGDG and DGDG from diatoms may, however, contain long chain fatty acids with up to 22 carbon atoms and as many as six double bonds [for a review on diatom lipids see 15]. Due to a strongly increased solubility of Ddx in MGDG compared with Vx, the MGDG concentrations for optimal DDE activity are much lower compared to those needed for full activation of VDE [13]. In addition to its role in pigment solubilization, MGDG provides another essential feature needed for xanthophyll de-epoxidation. MGDG belongs to the so-called non-bilayer lipids which, due to their small headgroup area and critical packing parameter value higher than one, form inverted hexagonal structures (H_{II} phases) in an aqueous medium [16,17]. These three-dimensional structures have been shown to be required for efficient Vx de-epoxidation in artificial liposomes composed of the lipids phosphatidylcholine (PC) and MGDG [11,12,18]. Latowski et al. [11] proposed that the H_{II} phases enable the binding of VDE to the thylakoid membrane after the pH-dependent activation of the enzyme has taken place. Inverted hexagonal structures which may also be formed by the phospholipid phosphatidylethanolamine (PE) and which support Vx de-epoxidation [12], seem to be essential for Ddx de-epoxidation, as well [13]. However, the data of Goss et al. [13] were derived from undefined lipid/water systems. Moreover, experiments showing the influence of H_{II} phase forming lipids on Ddx de-epoxidation in liposome systems resembling the natural thylakoid membrane are still lacking.

Although the existence of H_{II} phases in thylakoid membranes has been indicated by various methods [19,20], their precise arrangement in artificial lipid/water systems or in the natural thylakoid membrane is still unclear. It has been suggested that the non-bilayer lipids are forced into a bilayer structure in the native thylakoid membrane due to interactions with the main light-harvesting complex of photosystem II, the LHC II [21,22]. Garab et al. [22] also proposed, that under certain conditions, such as structural rearrangements of proteins or even protein degradation, non-bilayer lipids can be sequestered from the membrane and form H_{II} phases which are attached to the surface of the thylakoid bilayer. These inverted hexagonal phases stay in direct contact with the bilayer, so that rapid and direct interactions between the two lipid phases are possible.

In the present study, we have investigated, in more detail, the lipid dependence of Ddx de-epoxidation. To overcome the limitations of the undefined MGDG/water system we chose to use artificial membrane systems that more closely resemble the natural thylakoid membrane. The influence of inverted hexagonal structures on Ddx de-epoxidation was studied in PC liposomes that were supplemented with increasing concentrations of either of the H_{II} phase forming lipids MGDG or PE. In a third membrane system, a combination of the bilayer lipid DGDG and the non-bilayer lipid MGDG was used. In addition to the kinetic analysis of Ddx de-epoxidation, solubilization of Ddx in liposomes or bilayer/non-bilayer lipids was measured to obtain information about the distribution of xanthophyll cycle pigments in bilayer versus inverted hexagonal phases. The data of the present study are discussed with respect to their relevance for the lipid dependence of de-epoxidation in artificial membrane systems and in the natural thylakoid membrane.

2. Materials and methods

2.1. Plant material

The diatom *Cyclotella meneghiniana* (strain 1020-1a) was obtained from the Culture Collection of Algae, Göttingen (SAG, FRG) and was grown as batch culture in silica-enriched ASP medium according to Lohr and Wilhelm [23]. The PPFD during cultivation was set to 50 μmol m^{-2} s^{-1} with a light/dark regime of 16/8 h. The temperature of the growth chamber was adjusted to 20 °C. For the preparation of DDE, Ddx and Dtx, algal cultures with a chlorophyll concentration of 10 mg Chl *a/c* L^{-1} were used.

2.2. Preparation of DDE

DDE was prepared from dark-adapted cells of *C. meneghiniana* according to Goss et al. [13]. After the preparation, thylakoid lumen extracts enriched in DDE were frozen in liquid nitrogen and stored at −80 °C until use in the in vitro de-epoxidation assays.

2.3. Preparation of pigments

Ddx and Dtx were extracted from illuminated cells of *C. meneghiniana* and purified by HPLC, essentially as described by Goss et al. [13] and Grouneva et al. [8]. Ddx and Dtx from several HPLC separations were pooled, dried and stored at −80 °C until further use in the DDE enzyme assays.

2.4. Preparation of liposomes

Liposomes were prepared from three different combinations of lipids: the bilayer forming lipid phosphatidylcholine (PC) was combined with (1) the inverted hexagonal structure forming lipid monogalactosyldiacylglycerol (MGDG) or (2) another H_{II} forming lipid, phosphatidylethanolamine (PE). In a third combination of lipids, the bilayer forming lipid digalactosyldiacylglycerol (DGDG) was combined with MGDG. For all different combinations, ratios of 85/15, 70/30 and 50/50 mol% of bilayer versus H_{II} forming lipids were prepared. This corresponds to final lipid concentrations of 32.9/5.8, 27.1/11.6 and 19.35/19.35 μM, respectively. The mixture of lipids in chloroform was complemented with Ddx, subsequently evaporated under a stream of nitrogen, and further dried under vacuum for 1 h. The dried lipid/pigment mixture was dissolved in ethanol and injected slowly with a Hamilton syringe into the reaction medium of the de-epoxidation assay (10 mM KCl, 5 mM $MgCl_2$ and 40 mM MES, pH 5.2) under continuous bubbling with nitrogen. The final concentration of ethanol in the

enzyme assay did not exceed 1.25%. The final lipid concentration in all liposome suspensions was 38.7 μM, irrespective of the different ratios of bilayer versus H_{II} forming lipids. The Ddx concentration was 0.4 μM.

MGDG and DGDG were purchased from Lipid Products (UK), PC was obtained from Sigma-Aldrich (USA) and PE from Fluka (Switzerland).

2.5. In vitro de-epoxidation assay

The standard de-epoxidation assay contained the liposome suspensions prepared as described above. Before the start of the de-epoxidation reaction, 100 μL of DDE solution was added to a 3 mL assay. After addition of the DDE solution, the reaction mixture was incubated at 30 °C for 5 min, then a 700 μL control sample (corresponding to time point 0 of the de-epoxidation kinetics) was taken. De-epoxidation of Ddx was then initiated by addition of ascorbate (30 mM final concentration) to the reaction mixture and samples were collected after de-epoxidation times of 5, 10 and 20 min. In the 700 μL samples, de-epoxidation was stopped by mixing with 50 μL of 1 N KOH solution, thereby increasing the pH of the reaction mixture to basic pH-values and inactivating the xanthophyll de-epoxidases.

The samples were put on ice for 10 min and afterwards centrifuged at 13000 g for 5 min (Centrifuge 5417 C, Eppendorf, FRG). The supernatant was removed and the pellet containing the Ddx cycle pigments was stored at − 80 °C until use for pigment analysis by HPLC.

2.6. Pigment extraction and HPLC analysis

Pigments were extracted from the frozen pellets and analyzed by HPLC as described in Goss et al. [13]. Pigments were quantified according to Lohr and Wilhelm [23].

2.7. Determination of Ddx solubility by absorption spectroscopy

To determine the solubility of Ddx in the different lipids employed in the present study, 0.4 μM Ddx was mixed with MGDG, DGDG, PE or PC (for concentrations see Results), respectively. The pigment/lipid mixture was then injected into the reaction medium pH 5.2, which was adjusted to a temperature of 30 °C. Furthermore, spectra of Ddx incorporated into liposomes composed of PC/MGDG, PC/PE and DGDG/MGDG with different lipid ratios (see above) were determined.

To gain information about the distribution of Ddx in bilayer versus hexagonal phases of the liposomes, liposomes composed of 85/15 and 50/50 mol% PC/MGDG were prepared and the solubility of Ddx in these liposomes was compared with the solubility in pure MGDG. In contrast to the liposome systems described above, the total lipid concentration of the liposomes was now varied and adapted to the concentrations of pure MGDG. The actual MGDG concentrations in these measurements were: 1, 2, 3, 4 and 6 μM. In the 50/50 mol% PC/MGDG liposomes, 1, 2, 3, 4 and 6 μM PC was additionally present. In the 85/15 mol% PC/MGDG liposomes MGDG was supplemented with 5.6, 11.3, 17, 22.6 and 34 μM PC. The Ddx concentration was 0.4 μM.

Absorption spectra of Ddx were recorded on a Specord M 500 photometer (Zeiss, FRG) in a wavelength range between 300 and 600 nm with a bandpass setting of 1 nm. As a parameter depicting the solubility of Ddx in the different lipids and liposomes, III/II values of the respective Ddx spectra were calculated as the ratio of the third absorption peak at 485 nm to the second absorption peak at 455 nm with the minimum between the two peaks at 470 nm serving as a baseline. The III/II values of the Ddx spectra at different lipid concentrations were then normalized to the maximum III/II value achieved after complete solubilization of the pigment in the respective lipid.

In addition to measurements of the Ddx solubility, the solubility of Dtx in MGDG was determined.

3. Results

Fig. 1 shows the Ddx de-epoxidation in PC liposomes complemented with increasing concentrations of the inverted hexagonal phase forming lipid MGDG. In liposomes containing

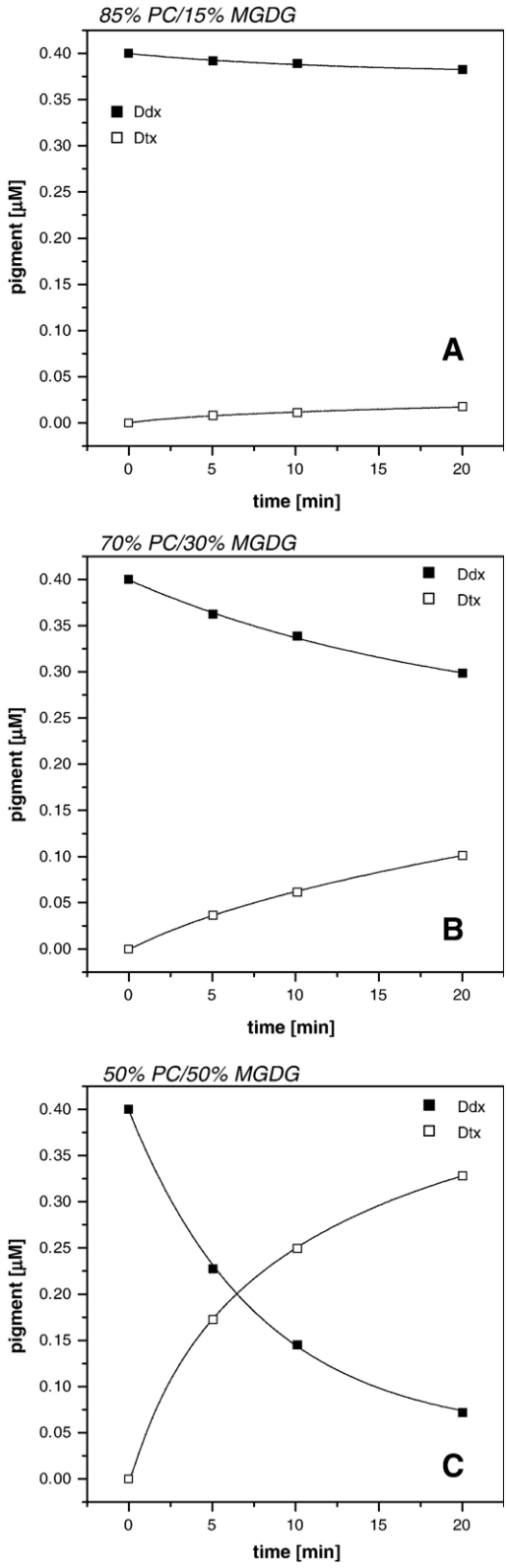

Fig. 1. The time-course of Ddx de-epoxidation by *C. meneghiniana* DDE in liposomes composed of the bilayer lipid PC and the H_{II} phase forming lipid MGDG. The reaction was carried out in reaction medium (RM) pH 5.2, the Ddx concentration was 0.4 μM; 30 mM ascorbate was added to start the de-epoxidation. (A) Liposomes composed of 85 mol% PC (32.9 μM) and 15 mol% MGDG (5.8 μM). (B) Liposomes composed of 70 mol% PC (27.1 μM) and 30 mol% MGDG (11.6 μM). (C) Liposomes composed of 50 mol% PC and 50 mol% MGDG (19.35 μM each). This figure shows the mean values of three independent experiments with SD<10%.

Table 1
Ddx conversion rate in the artificial membrane systems used in the present study

Bilayer/non-bilayer lipid (mol%)	Ddx conversion rate (nM Ddx min^{-1}) in different artificial membrane systems		
	PC/MGDG	DGDG/MGDG	PC/PE
100/0	0.4	0.3	0.4
85/15	1.7	1	0.8
70/30	7.3	5	3
50/50	34.8	25.9	16

Ddx de-epoxidation rates of the *C. meneghiniana* DDE in the three different artificial membrane systems with different ratios of bilayer (PC, DGDG) versus non-bilayer lipids (PE, MGDG), for actual lipid concentrations see Fig. 1. The de-epoxidation rate is depicted as nM Ddx min^{-1} and was calculated 5 min after the start of the in vitro de-epoxidation assay. The total lipid concentration was 38.7 μM in all membrane systems. The Ddx concentration at the beginning of the measurement was 0.4 μM; the de-epoxidation reaction was started with the addition of 30 mM ascorbate. This table shows the mean values of three independent experiments with SD<10%.

15 mol% of MGDG (Fig. 1A), a slow de-epoxidation of Ddx was observed, typically leading to the conversion of only 5% of the total Ddx to Dtx within a timespan of 20 min. The de-epoxidation rate (Table 1) was consequently low with a value of only 1.7 nM Ddx de-epoxidized per minute. In the liposome system composed of 70 mol% PC and 30 mol% MGDG (Fig. 1B) a more pronounced de-epoxidation of Ddx to Dtx was detected, and after 20 min of the in vitro enzyme assay about 25% of the Ddx pool had been converted to Dtx. The de-epoxidation rate was also significantly increased, and showed a value of 7.3 nM Ddx min^{-1}. A fast Ddx de-epoxidation accompanied by an almost complete conversion of Ddx to Dtx was found in liposomes which were composed of equal amounts of both the bilayer forming lipid PC and the H_{II} inducing lipid MGDG (Fig. 1C). In this liposome system Ddx de-epoxidation proceeded with a high rate of 34.8 nM Ddx min^{-1} (Table 1), a value that is more than 20 times higher than the de-epoxidation rate in liposomes complemented with 15 mol% MGDG. The fast de-epoxidation reaction led to the conversion of half of the Ddx pool to Dtx within 5–6 min.

The dependence of the enzymatic reaction on the concentration of MGDG in the liposomes was not caused by a limited solubility of Ddx in the liposomes with lower amounts of MGDG. From the absorbance spectra in Fig. 2, it is clear that in all different PC liposome systems containing 85/15, 70/30 and 50/50 mol% PC/MGDG, respectively, Ddx was completely solubilized. In all liposomes, a clear Ddx spectrum, indicating complete solubilization, was visible. Aggregation of Ddx molecules in the aqueous reaction medium in the absence of lipids led to a loss of the typical xanthophyll absorption maxima in the wavelength range 400–500 nm accompanied by the rise of a broad absorption peak at 385 nm (Fig. 2).

Although all different combinations of PC and MGDG with a total lipid concentration of 38.7 μM led to complete solubilization of Ddx, Fig. 3 shows that Ddx exhibited a much better solubility in the inverted hexagonal phase forming lipid MGDG compared with the bilayer inducing lipid PC. For a complete solubilization of 0.4 μM Ddx, MGDG concentrations of only 2.9 to 5.8 μM were needed (Fig. 3A), corresponding to a lipid per pigment ratio of about 7. This is illustrated by Fig. 3C, where the lipid dependence of Ddx solubility (depicted as normalized III/II values) is shown. Normalized III/II values of 1, which indicate complete solubilization of Ddx, were achieved when Ddx was solubilized in 5.8 μM MGDG. However, much higher concentrations of PC were necessary to completely solubilize Ddx (Fig. 3B). At PC concentrations of 5.8 and 11.6 μM the Ddx spectrum was distorted, indicating that a significant amount of pigment was still in an aggregated state. The incomplete solubilization of Ddx at these PC concentrations is also visible as normalized III/II values which are significantly lower than 1 (Fig. 3C). A further increase of PC enhanced the Ddx solubility until at a final PC concentration of 38.7 μM complete pigment solubilization was accomplished. The lipid per pigment ratio for the complete solubilization of Ddx in PC was found to be around 90 and was more than ten times higher than the ratio for the solubilization of Ddx in MGDG. However, since all PC/MGDG liposome systems employed in the present study contain a total lipid concentration of 38.7 μM complete solubilization of Ddx was ensured, irrespective of the different percentages of PC and MGDG in the liposome (see Fig. 2).

However, the distribution of Ddx between the PC and MGDG phases in the different liposome types can be expected to differ significantly. It is reasonable to assume that in the liposomes composed of 85 mol% PC and 15 mol% MGDG a significant part of Ddx will be solubilized in the PC phase, whereas with increasing MGDG concentration more and more Ddx will be concentrated in the MGDG phases. Due to the higher solubility of Ddx in the H_{II} forming lipid MGDG, liposomes composed of 50 mol% PC and 50 mol% MGDG will finally contain the largest part of the total Ddx in the MGDG phases. This assumption is corroborated by the results depicted in Fig. 4 where the dependence of Ddx solubility on the MGDG concentration in liposomes composed of 85/15 and 50/50 mol% PC/MGDG is

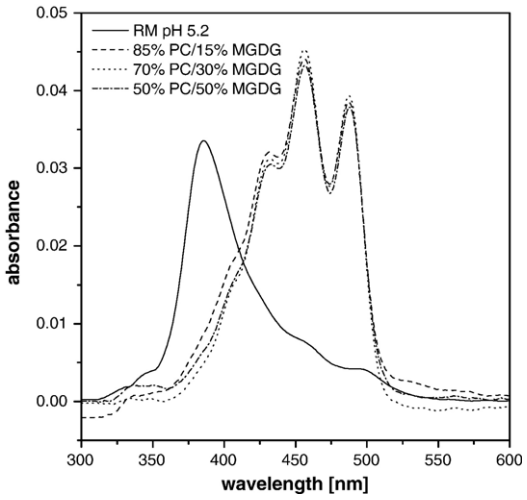

Fig. 2. Absorption spectra of Ddx in RM pH 5.2 and in liposomes with different concentrations of PC and MGDG. The mol percentages of the lipids in the liposomes were: 85/15, 70/30 and 50/50 mol% PC/MGDG, respectively (for actual lipid concentrations see legend of Fig. 1). The Ddx concentration was 0.4 μM.

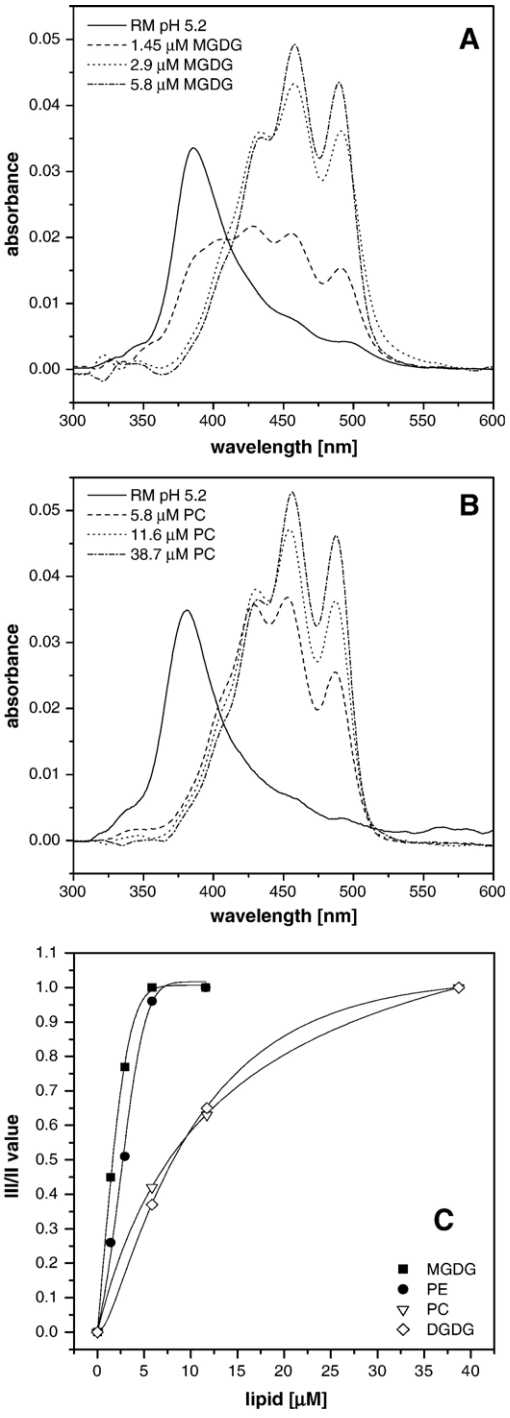

Fig. 3. Absorption spectra of Ddx in RM pH 5.2 in the presence of different concentrations of the inverted hexagonal phase forming lipid MGDG (A) or the bilayer forming lipid PC (B). The Ddx concentration was 0.4 μM. In (C) the dependence of the normalized III/II value of the Ddx spectrum on the concentration of the respective bilayer or inverted hexagonal phase forming lipids is shown.

compared to the solubility in pure MGDG. In contrast to the other liposome systems used in the present study, the total lipid concentration was not held constant in this case, and the different MGDG concentrations used for Ddx solubilization were supplemented with the respective PC concentrations (see Materials and methods). Fig. 4 shows that the MGDG dependence of Ddx solubility in liposomes composed of 50 mol% PC and MGDG was similar to the Ddx solubility in pure MGDG. This indicates that in these liposomes, Ddx was almost completely located in the MGDG phases. In the liposomes containing high amounts of PC, i.e., the 85/15 mol% PC/MGDG liposomes, Ddx was completely solubilized at significantly lower MGDG concentrations than in pure MGDG. The shift of complete Ddx solubilization towards lower MGDG concentrations can only be explained taking into account that now a significant part of Ddx is present in the bilayer phases of the liposomes formed by PC.

In addition to the liposome system composed of PC and MGDG, two additional artificial membrane systems were studied, namely membranes composed of the bilayer forming lipid DGDG in combination with MGDG, and liposomes composed of PC and another hexagonal phase forming lipid PE. The results of all three liposome systems are summarized in Fig. 5 and Table 1. From Fig. 5 it becomes visible that efficient Ddx de-epoxidation in the PC/MGDG liposomes was dependent on the concentration of MGDG in the artificial membranes. Increasing concentrations of MGDG led to significant increases in the de-epoxidation rate. It is important to note that the increase in the MGDG concentration from 30 to 50 mol% (from 11.6 to 19.3 μM in Fig. 5) induced a particularly drastic acceleration of the de-epoxidation. Similar observations were made for the DGDG/MGDG system, where de-epoxidation rates comparable to the PC/MGDG system were noted. Again, the de-epoxidation rate correlated with the amount of MGDG in the artificial membranes, and the strongest enhancement of Ddx conversion was observed when the MGDG concentration was changed from 30 mol% to 50 mol% (see also Table 1). The same result was obtained for Ddx de-epoxidation in liposomes composed of PC and PE. Increases in the concentration of the H_{II} forming lipid PE led to increases in the de-epoxidation rate. The overall rates of Ddx de-epoxidation in the PC/PE system were, however, lower than those in the PC/MGDG and DGDG/MGDG systems (Table 1). This is due in part to the less efficient Ddx solubilization in PE compared with

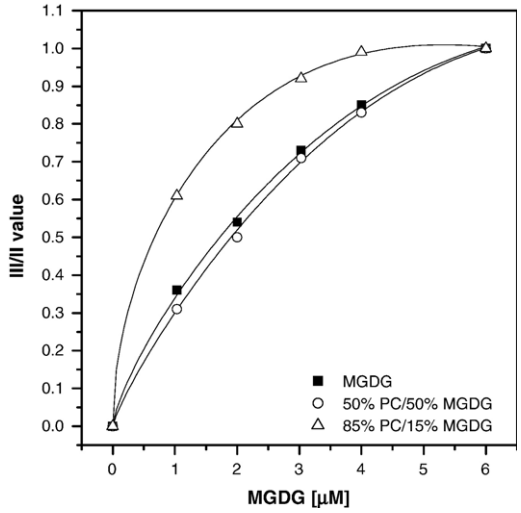

Fig. 4. Dependence of Ddx solubility (depicted as normalized III/II values) on the MGDG concentration in pure MGDG or in liposomes composed of 85/15 or 50/50 mol% PC/MGDG, for actual lipid concentrations see Materials and methods. The Ddx concentration was 0.4 μM.

MGDG (Fig. 3C and see below) but may also be influenced by a smaller size of the inverted hexagonal phases formed in the presence of PE (see Discussion).

Complete solubilization of Ddx was observed in all liposomes with different ratios of DGDG/MGDG and PC/PE at a total lipid concentration of 38.7 μM, and resulted in Ddx absorption spectra similar to those depicted in Fig. 2 (data not shown). We also tested the solubility of Ddx in DGDG and PE (Fig. 3C) and found that Ddx had a much better solubility in PE than in DGDG. The PE concentrations needed for Ddx solubilization were comparable to those of the other H_{II} phase forming lipid MGDG, although slightly higher PE concentrations were necessary to achieve complete pigment solubilization. DGDG, like the other bilayer forming lipid PC, was much less suited for Ddx solubilization and a similarly high lipid/pigment ratio as observed for PC was found for DGDG. Taking into account that Ddx exhibits a higher solubility in all H_{II} forming lipids, one can assume that the situation outlined above for the distribution of Ddx in the different phases of PC/MGDG liposomes also holds true for the DGDG/MGDG and the PC/PE system.

In addition to the determination of Ddx solubility in MGDG we also analyzed the solubility of Dtx in the H_{II} forming lipid (Fig. 6). Our data show that the de-epoxidized xanthophyll cycle pigment Dtx exhibited a significantly decreased solubility in MGDG compared with the epoxidized Ddx (Fig. 6). In our present experiments, 11.6 μM of MGDG were needed for the complete solubilization of Dtx whereas Ddx solubilization was already achieved at MGDG concentrations between 2.9 and 5.8 μM as indicated by normalized III/II values of around 1. The lipid/pigment ratio for complete Dtx solubilization was found to be around 30 and was four times higher than the ratio for the complete solubilization of Ddx.

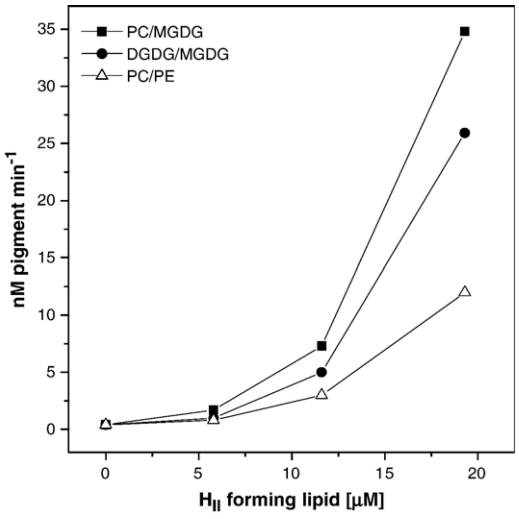

Fig. 5. Dependence of the Ddx conversion rate of the *C. meneghiniana* DDE on the concentration of H_{II} phase forming lipids in three artificial membrane systems: (1) liposomes composed of PC and MGDG, (2) membranes composed of DGDG and MGDG and (3) liposomes composed of PC and PE. For actual concentrations of lipids and corresponding mol percentages see Materials and methods. The Ddx concentration at the beginning of the de-epoxidation reaction was 0.4 μM; the reaction was started with 30 mM ascorbate. Ddx de-epoxidation rates were calculated from the values 5 min after the start of the enzyme assay. This figure shows the mean values of three independent experiments with SD<10%.

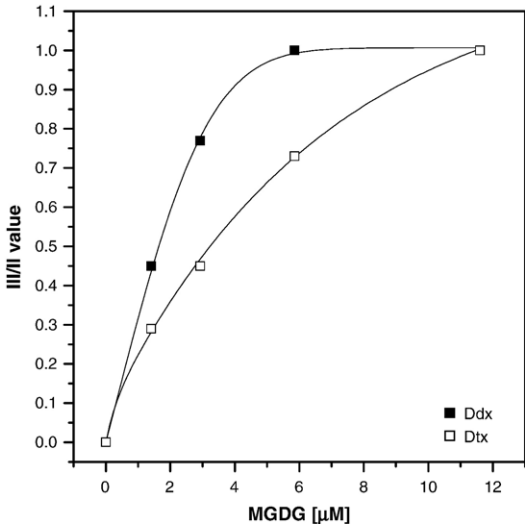

Fig. 6. Comparison of Ddx and Dtx solubility (depicted as normalized III/II values) in MGDG. The pigment concentration was 0.4 μM.

4. Discussion

4.1. Dependence of Ddx de-epoxidation on the concentration of H_{II} forming lipids

The results of the present study show that Ddx de-epoxidation by the xanthophyll cycle enzyme DDE depends on the concentration of inverted hexagonal structure forming lipids in artificial membrane systems. The nature of the lipid providing the inverted hexagonal phases, however, is not important. In other words, both the H_{II} forming galactolipid MGDG and the phospholipid PE are capable of strongly stimulating Ddx de-epoxidation. These results are in agreement with data from the literature showing that Vx de-epoxidation by the VDE of vascular plants depends on the concentration of MGDG in artificial membrane systems [11]. Our present data provide the additional information that PE can play the same role as MGDG in liposomes composed of PC, most probably by providing similar three-dimensional structures necessary for DDE activity. We also show that in a membrane system composed of DGDG and MGDG, which closely resembles the lipid composition of the natural thylakoid membrane, efficient Ddx de-epoxidation likewise depends on the concentration of the H_{II} forming MGDG. In liposomes composed solely of the bilayer forming lipids PC or DGDG, i.e. in the absence of inverted hexagonal phases, Ddx de-epoxidation is almost completely inhibited (Table 1). The extremely low de-epoxidation rates observed in these liposomes were most probably due to very low concentrations of residual MGDG molecules in the DDE enzyme preparations. Our present results indicate that low amounts of H_{II} forming lipids are not able to significantly stimulate Ddx de-epoxidation, and that a certain threshold concentration of these

lipids must be reached to achieve efficient pigment conversion. This is most likely due to the size of the inverted hexagonal phases in the liposome system, which is suggested to be much smaller compared to the aggregate size when injecting similar amounts of H_{II} lipids in an aqueous medium [11]. This assumption is corroborated by structural data derived from a binary mixture of DGDG and MGDG which show that at high DGDG/MGDG ratios MGDG is not able to form H_{II} phases of significant size [24,25]. Under these conditions MGDG is even forced into a bilayer structure by the surplus of DGDG. With increasing amounts of MGDG, it is then sequestered from the DGDG bilayer and forms inverted spherical or hexagonal phases attached to the bilayer. The localization of the H_{II} phases in artificial and natural membrane systems is still a matter of debate. Garab et al. [22] proposed that the inverted hexagonal phases are located outside the lipid bilayer, but stay in close contact with the membrane bilayer and act as reservoirs for membrane lipids and as the sites of specific physiological reactions. We propose that in the artificial membrane systems used in the present study, the H_{II} phases created by MGDG or PE are either located outside of the bilayer or are laterally separated from the bilayer composed of PC or DGDG. However, in both cases the H_{II} phases would form a continuum with the remaining membrane bilayer. Based on our finding that the inverted hexagonal phases are essential for efficient Ddx de-epoxidation, we propose that both possible localizations of the H_{II} phases facilitate the fast binding of activated DDE to the inverted hexagonal phases. In this scenario, the number of DDE molecules which is able to bind to the H_{II} phases would be limited by the size and number of the inverted hexagonal structures. With respect to the mode of DDE binding to the H_{II} phases there is, in our opinion, a plausible explanation. Due to the small headgroup size of the MGDG molecule, the packing density at the membrane–water interface in the H_{II} phases will be strongly reduced compared to the rest of the membrane consisting of bilayer-lipids where the headgroups cover a significantly larger area. MGDG and other non-bilayer lipids are therefore able to create hydrophobic insertion sites for membrane associated proteins [for a review see 26]. The pH-dependent activation of DDE/VDE probably leads to the protonation of charged amino acid residues of the enzyme followed by a conformational change [27,28]. Especially the protonation of four histidine residues which are located within the lipocalin region of the enzyme [28] may lead to an exposure of hydrophobic protein regions, including the catalytic site of the enzyme. We propose that activated DDE/VDE is then able to bind to the hydrophobic insertion sites of the H_{II} phases, thereby gaining access to the hydrophobic membrane domains where the xanthophyll cycle pigments are located. Furthermore, we suggest that due to the dense packing at the membrane–water interface in membranes or membrane regions composed solely of bilayer lipids, penetration of the enzymes catalytic site into the membrane is impossible and xanthophyll de-epoxidation is inhibited. According to our assumptions, the efficient binding and insertion of a high number of DDE molecules to the H_{II} phases is likely one of the key components regulating Ddx de-epoxidation. The second key factor may be differences in the solubility of the xanthophyll cycle pigments in bilayer and H_{II} forming lipids.

4.2. Dependence of Ddx de-epoxidation on pigment solubilization in the artificial membrane systems

Our data show that, in all different liposome systems containing a total lipid concentration of 38.7 μM, complete solubilization of 0.4 μM Ddx was achieved. However, the drastic difference in pigment solubilization in either non-bilayer or bilayer forming lipids, as well as the data for the MGDG dependence of Ddx solubility in liposomes and pure MGDG, indicate that the Ddx distribution in the liposomes composed of different percentages of bilayer and non-bilayer lipids is not uniform. In liposomes with high concentrations of bilayer forming lipids, a significant part of the total Ddx must be located in the lipid bilayer, especially when taking into account that the H_{II} phases are relatively small in these artificial membranes. This means that, apart from restrictions in DDE binding to the inverted hexagonal phases, Ddx de-epoxidation will be limited by the necessity of a large amount of Ddx molecules to diffuse from the bilayer to the H_{II} phases. This diffusion is supposed to be slow and has also been described by Latowski et al. [11] as the time-limiting step of Vx de-epoxidation in PC/MGDG liposomes containing high amounts of PC. With increasing concentrations of H_{II} lipids in the artificial membranes, increasing Ddx is situated in the inverted hexagonal phases due to the significantly better solubilization of this pigment in non-bilayer lipids until in liposomes composed of equal amounts of bilayer and non-bilayer lipids the majority of Ddx molecules is located in the inverted hexagonal phases. This enrichment of Ddx molecules, together with an efficient binding of a high number of DDE molecules to the H_{II} phases, leads to the strong increase in the Ddx de-epoxidation rate observed upon increasing the ratio of non-bilayer forming lipids from 30 to 50 mol% in the liposome suspensions. We conclude that the number and size of the inverted hexagonal phases must pass a certain threshold level, such that both a high concentration of Ddx molecules can be present inside the H_{II} phases and a high amount of DDE can attach to the outside of the H_{II} phases.

4.3. Lipid dependence of Ddx/Vx de-epoxiation in the natural thylakoid membrane

We believe that the data of the present study provide valuable informations for the lipid dependence of xanthophyll de-epoxidation in vivo and propose the following scenario for the situation in the native thylakoid membrane (please note that this working model is intended to provide ideas for future measurements and that significant aspects addressed below still await experimental verification).

In the thylakoids of vascular plants and diatoms light-harvesting complexes (LHC, FCP) enriched in xanthophyll cycle pigments [29–31] are incorporated into a lipid bilayer. In vascular plants these LHC proteins were shown to be surrounded by a high concentration of the non-bilayer lipid MGDG [32], which is forced into a bilayer structure by the respective antenna proteins [21]. In the dark-adapted plant or alga, the LHC/FCP is in a non-aggregated state and Ddx or Vx are bound to the antenna complexes. Upon high light illumination, the light-harvesting proteins become protonated by the increase of the proton concentration in the

thylakoid lumen and aggregate [for a review see 33]. After the aggregation a part of the MGDG, which was stabilized by the light-harvesting proteins, may be segregated from the bilayer to form a lipid phase resembling the H_{II} phases in the artificial liposome system used in the present study. Although, at present, there exist no direct experimental data showing a light-induced MGDG-segregation, the experiments of Simidjiev et al. [21,34] provide evidence that the amount of MGDG which can be incorporated into a bilayer structure depends on the conformation of the LHC proteins and that additional MGDG molecules form H_{II} phases which remain closely associated with the LHC/MGDG bilayer. Based on these experimental findings and the proposals made by Garab et al. [22] the assumption of an MGDG segregation from the bilayer upon the light-induced LHC/FCP aggregation seems to be justified. According to our opinion this segregation could either take place into the thylakoid lumen or as a lateral segregation within the plane of the thylakoid membrane. After the formation of the MGDG phases Ddx/Vx might disconnect from the LHC and migrate preferentially into these MGDG enriched regions which in both cases still form a continuous space with the rest of the membrane bilayer. The proposed release of xanthophyll cycle pigments from the LHC/FCP proteins is supported by various studies which show that during high-light illumination a dynamic equilibrium exists between protein-associated and lipid matrix-localized xanthophylls [35–37]. The MGDG phases would also represent the docking site for the xanthophyll cycle enzymes DDE or VDE, which have been activated by the pH-drop in the thylakoid lumen caused by photosynthetic electron transport [35]. In our view, segregation of the non-bilayer lipids is required to create hydrophobic insertion sites for the xanthophyll de-epoxidases. Without segregation of MGDG from the bilayer phase of the thylakoid membrane, the MGDG molecules would not be able to reduce the packing density at the membrane–water interface, due to their strong interactions with the membrane proteins. In this case, binding of VDE/DDE to MGDG molecules incorporated into the membrane bilayer would not be possible. After the binding of DDE or VDE to the segregated MGDG phases, Ddx or Vx are de-epoxidized to Dtx or Zx which then would rebind to the LHC/FCP to induce the thermal dissipation of excess excitation energy [38,39]. The rebinding of Dtx and Zx to the light-harvesting proteins may be facilitated by the different chemical properties of the de-epoxidized xanthophyll cycle pigments which in the case of Dtx lead to a decreased solubility in MGDG compared with Ddx. Although the solubility of both pigments in the bilayer forming lipid PC is comparable (data not shown), the difference in Dtx solubility between the MGDG and the PC phase is much less pronounced than that of Ddx solubility, thereby supporting the re-entrance of Dtx into the bilayer phase of the membrane incorporating the light-harvesting proteins. The differences in solubility between epoxidized and de-epoxidized xanthophyll cycle pigments may also be responsible for the increase in membrane rigidity observed upon the light-induced conversion of Vx to Zx in higher plants [40]. It should be emphasized that the hydrophobic area of the segregated MGDG phases should form a continuity with the hydrophobic area of the membrane bilayer and the integral membrane proteins. Such an arrangement would minimize the diffusion times of the xanthophyll cycle pigments between their protein binding sites and the sites of de-epoxidation, leading to the fast rebinding of de-epoxidized xanthophylls necessary for efficient photoprotection.

We conclude that the role of MGDG in the de-epoxidation sequence is twofold: (1) MGDG provides the special lipid phases needed for pigment solubilization and enzyme attachment, and (2) MGDG, due to its association with the light-harvesting proteins, specifically targets the xanthophyll de-epoxidases to the sites of the thylakoid membrane enriched in xanthophyll cycle pigments.

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