Role of Hexagonal Structure-Forming Lipids in Diadinoxanthin and Violaxanthin Solubilization and De-Epoxidation†

Reimund Goss,*,‡ Martin Lohr,§ Dariusz Latowski,||,⊥ Joanna Grzyb,|| Astrid Vieler,‡ Christian Wilhelm,‡ and Kazimierz Strzalka||

Institute of Biology I, Plant Physiology, University of Leipzig, Johannisallee 21-23, 04103 Leipzig, Germany, Institute of General Botany, Johannes Gutenberg-University Mainz, Saarstrasse 21, 55099 Mainz, Germany, and Faculty of Biotechnology, Jagiellonian University, ul. Gronostajowa 7, 30-387 Krakow, Poland

Received December 3, 2004; Revised Manuscript Received January 12, 2005

ABSTRACT: In this study, we have examined the influence of different lipids on the solubility of the xanthophyll cycle pigments diadinoxanthin (Ddx) and violaxanthin (Vx) and on the efficiency of Ddx and Vx de-epoxidation by the enzymes Vx de-epoxidase (VDE) from wheat and Ddx de-epoxidase (DDE) from the diatom *Cyclotella meneghiniana*, respectively. Our results show that the lipids MGDG and PE are able to solubilize both xanthophyll cycle pigments in an aqueous medium. Substrate solubilization is essential for de-epoxidase activity, because in the absence of MGDG or PE Ddx and Vx are present in an aggregated form, with limited accessibility for DDE and VDE. Our results also show that the hexagonal structure-forming lipids MGDG and PE are able to solubilize Ddx and Vx at much lower lipid concentrations than bilayer-forming lipids DGDG and PC. We furthermore found that, in the presence of MGDG or PE, Ddx is much more solubilizable than Vx. This substantial difference in Ddx and Vx solubility directly affects the respective de-epoxidation reactions. Ddx de-epoxidation by the diatom DDE is saturated at much lower MGDG or PE concentrations than Vx de-epoxidation by the higher-plant VDE. Another important result of our study is that bilayer-forming lipids DGDG and PC are not able to induce efficient xanthophyll de-epoxidation. Even in the presence of high concentrations of DGDG or PC, where Ddx and Vx are completely solubilized, a strongly inhibited Ddx de-epoxidation is observed, while Vx de-epoxidation by VDE is completely absent. This indicates that the inverted hexagonal phase domains provided by lipid MGDG or PE are essential for de-epoxidase activity. We conclude that in the natural thylakoid membrane MGDG serves to solubilize the xanthophyll cycle pigments and furthermore provides inverted hexagonal structures associated with the membrane bilayer, which are essential for efficient xanthophyll de-epoxidase activity.

The violaxanthin cycle of higher plants and green algae (*1*, *2*) consists of a forward reaction comprising two de-epoxidation steps, in which the di-epoxy xanthophyll violaxanthin (Vx)[1] is converted to the epoxy-free zeaxanthin (Zx). Both de-epoxidation steps differ greatly in their kinetic parameters (*3*). The intermediate product of this reaction sequence is antheraxanthin (Ax), which contains one epoxy group. The conversion of Vx to Ax and Zx takes place when higher plants are illuminated with high actinic light intensities. Low light intensities or darkness stimulates the backward reaction of the violaxanthin cycle, which reverses the reaction sequence of the forward reaction and leads to the conversion of Zx back to Vx. The de-epoxidation sequence of the violaxanthin cycle is catalyzed by the enzyme violaxanthin de-epoxidase (VDE); the epoxidation of Zx to Vx is carried out by the enzyme zeaxanthin epoxidase (*2*).

A second major xanthophyll cycle, which comprises only one de-epoxidation step, is the diadinoxanthin (Ddx) cycle of the algal classes Bacillariophyceae, Chrysophyceae, Xanthophyceae, Haptophyceae, and Dinophyceae (*4*, *5*). Like in the Vx cycle of higher plants, the reaction sequence includes the conversion of an epoxy xanthophyll (Ddx) into an epoxy-free carotenoid (diatoxanthin, Dtx) and vice versa. The enzymes catalyzing the diadinoxanthin cycle are diadinoxanthin de-epoxidase (DDE) and diatoxanthin epoxidase. Both xanthophyll cycles play a major role in the photoprotection mechanisms of higher plants and algae (for a recent review, see ref *6*). De-epoxidation of Vx to Zx and Ddx to Dtx leads to an enhanced dissipation of excess excitation energy in the antenna complex of photosystem II (PS II), thereby preventing an overexcitation of the PS II reaction center (*7*−*12*).

† This work was financed from sources of the Polish Minister of Science for the years 2004−2007 as Ordered Research Project PBZ-KBN-110/P04/19 and was also supported by the Centre of Excellence grant from the European Union (Contract Bier ICAT-CT-2000-70012) and a grant from the German Academic Exchange Service (DAAD, D/03/44638).

* To whom correspondence should be addressed. Telephone: +49 341 9736873. Fax: +49 341 9736899. E-mail: rgoss@rz.uni-leipzig.de.

‡ University of Leipzig.

§ Johannes Gutenberg-University Mainz.

|| Jagiellonian University.

⊥ Present address: Department of Chemistry, Pedagogical University, ul. Podchorazych 2, 30-084 Krakow, Poland.

[1] Abbreviations: Ax, antheraxanthin; Chl, chlorophyll; DDE, diadinoxanthin de-epoxidase; Ddx, diadinoxanthin; DGDG, digalactosyldiacylglycerol; Dtx, diatoxanthin; LHC, light-harvesting complex; MGDG, monogalactosyldiacylglycerol; PPFD, photosynthetic photon flux density; PC, phosphatidylcholine; PE, phosphatidylethanolamine; VDE, violaxanthin de-epoxidase; Vx, violaxanthin; Zx, zeaxanthin.

10.1021/bi047464k CCC: $30.25
Published on Web 02/12/2005

Violaxanthin de-epoxidase (VDE) of higher plants is located in the thylakoid lumen (*13*). The enzyme requires reduced ascorbate as a cosubstrate to reduce the epoxy group, which is then eliminated as H_2O (*14*). The enzyme has a pH optimum at pH 5.2 (*14*, *15*) and is, under in vivo conditions, activated by the drop of the lumenal pH caused by the light-driven photosynthetic electron transport. Decreasing pH values in the thylakoid lumen induce the binding of the enzyme to the lumenal side of the thylakoid membrane (*13*). Besides the decrease in the lumenal pH, VDE activity also requires the presence of the galactolipid MGDG (*16*). MGDG has been proposed to facilitate the solubilization of the substrate Vx and/or to represent a functional component of the enzyme VDE itself (*16*). Recent studies, employing a lipid bilayer system resembling the natural thylakoid membrane, have shown that the de-epoxidation of Vx preferentially takes place in inverted hexagonal phase domains associated with the lipid bilayer, which are formed by higher concentrations of MGDG (*17*, *18*). High concentrations of MGDG in the liposome system shorten the average distance between violaxanthin molecules localized in the bilayer domains and the associated H_{II} domains, thereby significantly enhancing the kinetics of the first de-epoxidation step from Vx to Ax (*18*).

In comparison to the VDE of higher plants, much less is known about the diadinoxanthin de-epoxidase (DDE), the enzyme catalyzing the forward reaction of the Ddx cycle. The DDE of the diatom *Phaeodactylum tricornutum* exhibits characteristic differences with the higher-plant VDE with respect to the pH-dependent enzyme activation (*19*). DDE becomes activated at a significantly higher pH than the higher-plant VDE, which leads to a de-epoxidation of Ddx in the dark caused by the weak lumen acidification due to chlororespiratory electron flow. With regard to the lipid requirement of Ddx de-epoxidation, there exist, at present, no experimental data.

In this study, we address the question of how different galacto- and phospholipids influence Vx and Ddx de-epoxidation by the enzymes VDE and DDE, respectively. We compare hexagonal structure-forming lipids MGDG and PE with bilayer-inducing lipids DGDG and PC with respect to their solubilization efficiency for substrates Vx and Ddx and their influence on the Vx and Ddx de-epoxidation reaction. We also focus on the important question of how solubilization of the xanthophyll cycle pigments in general is achieved and if there are differences in the solubilization efficiency between Vx and Ddx.

EXPERIMENTAL PROCEDURES

Plant Material. Cyclotella meneghiniana (strain 1020-1a) was obtained from the Sammlung von Algenkulturen Göttingen (SAG) and was grown as a batch culture in silica-enriched ASP medium according to ref *20* with the modifications introduced in ref *21*. The PPFD during cultivation was set to 50 μmol m^{-2} s^{-1} with a 16 h−8 h light−dark regime. The temperature of the growth chamber was adjusted to 20 °C. For the preparation of DDE and Ddx, dark-adapted algal cultures with a chlorophyll concentration of 10 mg of Chl *a*/*c* L^{-1} were used.

VDE was prepared from 7-day-old wheat (*Triticum aestivum*) leaves. Vx was prepared from daffodil (*Narcissus poeticus*) petals, from flowers obtained at the local market.

Preparation of VDE and DDE. VDE isolation started with the preparation of thylakoid membranes from 7-day-old wheat leaves according to the procedure described in ref *13*. DDE was isolated from thylakoid membranes of *C. meneghiniana*. For this thylakoid preparation, 500−1000 mL of an algal culture was harvested by centrifugation at 2500*g* for 10 min at 4 °C (Varifuge, Heraeus, Germany). The pellet containing the concentrated cells was then resuspended in 10 mL of isolation buffer [1 M sorbitol, 2 mM KCl, 2 mM $MgCl_2$, 40 mM MES (pH 5.5), and 0.2% BSA] and put on a glass fiber filter (GF 6, Schleicher & Schüll). The cells were then broken by mechanical disruption in a glass bead homogenizer (MSK, Braun) for 60 s and centrifuged at 2500*g* for 4 min at 4 °C. The pellet containing the algal chloroplasts was osmotically shocked by a 2 min incubation in 10 mL of shock medium [2 mM KCl, 2 mM $MgCl_2$, and 40 mM MES (pH 5.5)]. The broken chloroplasts were centrifuged again (2500*g* for 4 min at 4 °C), and the pellet containing the released intact thylakoid membranes was resuspended in 1 mL of VDE preparation buffer [5 mM $MgCl_2$ and 20 mM Tris (pH 7.5)].

The subsequent preparation steps followed the VDE isolation method introduced in ref *13*. Freshly prepared thylakoids from *C. meneghiniana* and wheat were adjusted to a total chlorophyll content of 2 mg/mL with the VDE preparation medium. The thylakoid membranes were then frozen in liquid nitrogen and afterward immediately thawed in a water bath set to 30 °C. After the completion of seven freeze−thaw cycles, the broken thylakoid membranes were centrifuged for 20 min at 60000*g* and 4 °C (Allegra 64R, Beckman Instruments). If necessary, centrifugation was repeated until a clear, colorless supernatant containing the VDE or DDE had been obtained. The VDE-containing supernatant was collected and precipitated with ammonium sulfate in the range of 40−85% and centrifuged for 1 h at 40000*g* at 4 °C (K-24, Janetzky). The obtained pellet was resolved in 50 mM HEPES-NaOH buffer (pH 7.1) and dialyzed against the same buffer for 24 h. Finally, the extracts enriched in VDE and DDE were frozen in liquid nitrogen and stored at −80 °C until they were used in the in vitro de-epoxidation assays.

Preparation of Pigments. Violaxanthin (Vx) was prepared from daffodil petals by a method described in ref *22*. The total pigments were extracted with acetone, and, after evaporation to dryness, saponified in an ethanol/NaOH solution (6%). The saponified pigments were transferred to ether, dried, resuspended in a mixture composed of hexane and acetone (4:1), and purified on a silica gel column. Purity was controlled spectrophotometrically (Cary UV/Vis Bio50, Varian) and by HPLC analysis. Pure Vx was collected, dried, and stored for later use in the VDE enzyme assays. Diadinoxanthin (Ddx) was isolated from cells of *C. meneghiniana*. Batches of algal cells (250 mL) were harvested as 50 mL aliquots on glass fiber filters (GF 6, 55 mm diameter, Schleicher & Schüll), immediately frozen in liquid nitrogen, and freeze-dried for 24 h. Pigments from several filters of dried algal cells were then extracted with HPLC extraction medium composed of 90% methanol/0.2 M ammonium acetate (9:1, v/v) and 10% ethyl acetate by mechanical disruption in a glass bead homogenizer (MSK, Braun) for 20 s. The pigments were then transferred to diethyl ether, evaporated, and saponified using the same conditions

as described above. After saponification, carotenoids were separated by HPLC using a Nucleosil ET 250/8/4, 300-5, C-18 column (Macherey & Nagel) on a Waters 600-MS chromatography system equipped with a Waters 717 autosampler and a Waters 996 photodiode array detector with the eluents and gradient program described in ref *23*. Ddx from several HPLC separations was pooled, dried, and stored at −80 °C for further use in the DDE enzyme assays.

In Vitro De-Epoxidation Assay. The in vitro de-epoxidation assay is based on a method described in ref *16*. The standard assay mixture contained 0.4 μM Vx or Ddx, different concentrations of MGDG, DGDG, PE, or PC (see the Results for concentrations), 30 mM sodium ascorbate, and 5% ethanol (v/v). The enzyme assay was carried out in a reaction medium consisting of 10 mM KCl, 5 mM $MgCl_2$, and 40 mM MES (pH 5.2). A 3 mL enzyme assay typically contained 100 μL of DDE solution. In the case of the wheat VDE, 20 μL of the thylakoid lumen extracts was used to achieve similar de-epoxidation activities as with the *C. meneghiniana* DDE suspensions. The reaction mixture was prepared by mixing the ethanolic pigment solution with lipid, and injecting the pigment/lipid mixture into the reaction buffer. After addition of the VDE/DDE solution, the reaction mixture was incubated at 30 °C for 5 min before a 700 μL control sample (corresponding to time point 0 of the de-epoxidation kinetics) was taken. De-epoxidation of the respective pigment was started by addition of ascorbate to the reaction mixture, and samples were collected after de-epoxidation times of 2, 5, and 10 min. In the samples, de-epoxidation was stopped by mixing 700 μL of a reaction assay with 50 μL of 1 N KOH, thereby increasing the pH of the reaction mixture to neutral pH values and inactivating the xanthophyll de-epoxidases.

All samples were centrifuged at 13000*g* for 5 min (Centrifuge 5417 C, Eppendorf); the supernatant was removed, and the pellet containing the xanthophyll cycle pigments was stored at −80 °C until it was used for pigment analysis by HPLC.

Pigment Extraction and HPLC Analysis. Pigments were extracted from the frozen pellets by addition of 200 μL of HPLC extraction medium (see above). Pigment recovery by this method was usually higher than 90% (direct extraction of pigments with diethyl ether from control samples without prior centrifugation resulted in a substantially lower pigment recovery, although the stoichiometries of epoxidized and de-epoxidized pigments were the same for both methods). The pigment extracts were analyzed using the Nucleosil ET 250/8/4, 300-5, C-18 column (Macherey & Nagel) on the Waters HPLC system described above. Pigments were quantified according to the methods of refs *21* and *24*. The detection wavelength for the xanthophyll cycle pigments was set to 480 nm, with the exception of Vx, which was quantified at a detection wavelength of 440 nm.

Determination of Vx and Ddx Solubility by Absorption Spectroscopy. Vx and Ddx (0.4 μM each) were mixed with ethanol and lipid MGDG, DGDG, PE, or PC (for concentrations, see the Results), respectively. The pigment/lipid mixture was then injected into the reaction medium at pH 5.2, which was adjusted to a temperature of 30 °C. Absorption spectra of Vx and Ddx were recorded on a Specord M 500 photometer (Zeiss) in a wavelength range between 300 and 750 nm with a band-pass setting of 1 nm.

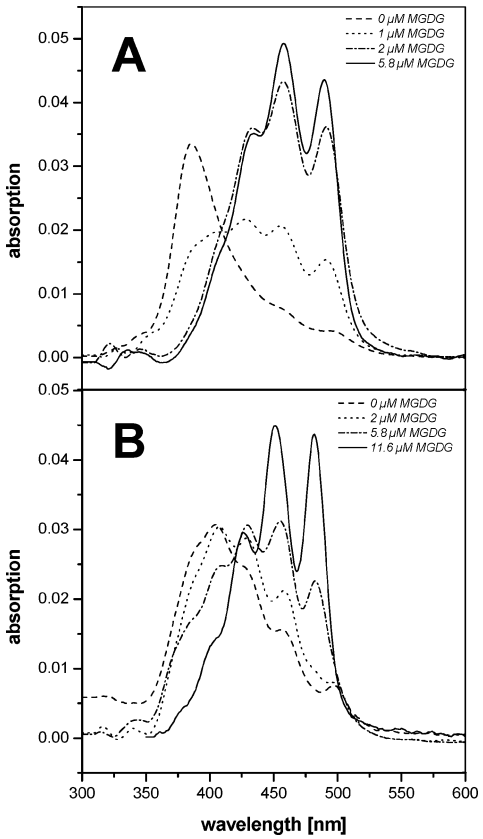

FIGURE 1: (A) Absorption spectra of Ddx in reaction medium at pH 5.2 in the presence of different concentrations of hexagonal structure-forming galactolipid MGDG. The Ddx concentration is fixed at 0.4 μM. (B) Absorption spectra of Vx in reaction medium at pH 5.2 in the presence of different concentrations of MGDG. The Vx concentration is fixed at 0.4 μM.

Chemicals. MGDG and DGDG were purchased from Lipid Products. PC was obtained from Sigma-Aldrich and PE from Fluka.

RESULTS

Solubility of Ddx and Vx in the Hexagonal Structure-Forming Lipids MGDG and PE. Figure 1 shows the absorption spectra of the xanthophyll cycle pigments Ddx and Vx in reaction medium at pH 5.2 in the presence of varying concentrations of the galactolipid MGDG.

In the absence of MGDG, Ddx exhibits an absorption maximum in the UV region of the spectrum at 385 nm (Figure 1A). The three-peak absorption spectrum, which is characteristic for solubilized carotenoid molecules in organic solvents, is not visible. The presence of increasing concentrations of hexagonal structure-forming lipid MGDG in the aqueous buffer restores the typical Ddx absorption spectrum and shifts the absorption maximum to 458 nm. Already at the lowest MGDG concentration of 1 μM the Ddx absorption spectrum shows the typical three-peak structure, although the absorption peak at 385 nm is still visible. MGDG concentrations of 2 μM, which correspond to a lipid:pigment ratio of only 5 (given the fixed pigment concentration of 0.4 μM), lead to an almost complete restoration of the typical Ddx spectrum. Further increases in the MGDG concentrations improve the spectral fine structure only slightly. It should be noted that addition of MGDG after mixing of lipid-free ethanolic pigment solution with buffer does not result

in pigment solubilization, since the absorbance spectra remain unchanged.

The absorption spectrum of Vx in the absence of MGDG (Figure 1B) is comparable to that of Ddx in MGDG-free reaction medium. However, the absorption maximum is located at longer wavelengths and peaks in the blue region of the spectrum at 405 nm. Besides the main absorption maximum, the Vx spectrum exhibits three prominent shoulders, which correspond to the main peaks of the Vx spectrum at higher MGDG concentrations. In contrast to Ddx, much higher concentrations of MGDG are needed to fully solubilize Vx at the given concentration of 0.4 μM. At an MGDG concentration of 2 μM, which almost completely restores the characteristic Ddx spectrum, the Vx absorption spectrum is still dominated by the short wavelength absorption maximum. Complete restoration of the Vx spectrum, i.e., complete solubilization of Vx, is finally achieved with an addition of MGDG to a final concentration of 11.6 μM, which corresponds to a lipid:pigment ratio of 29. The absorption maximum of solubilized Vx in reaction medium at pH 5.2 is found at a wavelength of 452 nm.

To confirm that the spectral shift of both Vx and Ddx in aqueous buffer in the absence of MGDG is a result of pigment aggregation, we also recorded absorbance spectra of pigments in aqueous 33% ethanol. In accordance with observations described in ref *25*, we were able to mimic the spectral changes, which occur in the presence of lipid, by varying the temperature of the ethanolic pigment suspension. Cooling of the samples to 5 °C resulted in absorbance spectra very similar to pigment spectra in buffer without lipid, while a temperature increase to 50 °C almost completely restored absorbance spectra which can be observed in lipid-containing buffer or pure ethanol (data not shown).

When Ddx and Vx are diluted in the aqueous reaction medium at pH 5.2 in the presence of varying concentrations of phospholipid PE, a dependence of pigment solubilization on the lipid concentration similar to that with the galactolipid MGDG can be observed (Figure 2).

PE, although structurally different from MGDG, forms the same inverted hexagonal phase domains (*26*). Like in the experiments with MGDG, the characteristic Ddx spectrum is visible in the presence of PE concentrations of 2−5.8 μM (Figure 2A). However, PE seems to be slightly less efficient in Ddx solubilization than MGDG, as can be seen from the lower spectral fine structure at lipid concentrations of 1 and 2 μM PE or MGDG. Higher concentrations of PE are needed to fully solublize Vx, whose normal spectral fine structure can be seen at PE concentrations of 11.6 μM (Figure 2B). This is again comparable to the results obtained with MGDG and indicates that Vx in general is not as readily solubilizable as Ddx. The spectral fine structure and the absorption maxima of Ddx and Vx dissolved in PE are similar to those of the pigments solubilized in MGDG.

Ddx and Vx De-Epoxidation in the Presence of MGDG and PE. The different solubility of Ddx and Vx in MGDG or PE is reflected by respective differences in the de-epoxidation efficiency (Figures 3 and 4). Ddx de-epoxidation by the *C. meneghiniana* DDE, which proceeds very slowly in the absence of MGDG (Figure 3A), is saturated at an MGDG concentration of 2 μM. This corresponds well with the observation that Ddx is almost completely solubilized

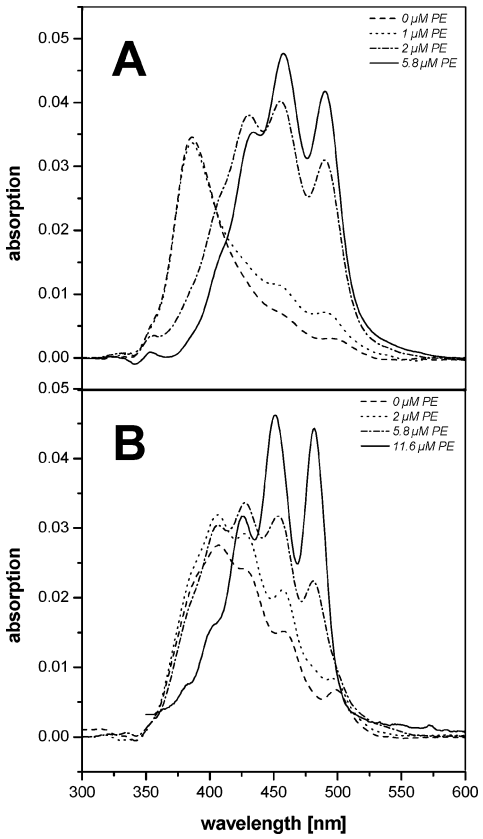

FIGURE 2: (A) Absorption spectra of Ddx in reaction medium at pH 5.2 in the presence of different concentrations of hexagonal structure-forming phospholipid PE. The Ddx concentration is fixed at 0.4 μM. (B) Absorption spectra of Vx in reaction medium at pH 5.2 in the presence of different concentrations of PE. The Vx concentration is fixed at 0.4 μM.

in the presence of 2 μM MGDG. Further increases in MGDG concentrations do not enhance the Ddx de-epoxidation kinetics.

An MGDG concentration of 11.6 μM, on the contrary, leads to a slight reduction in the Ddx de-epoxidation rate. MGDG concentrations of 38.7 μM reduce the de-epoxidation rate even further (data not shown). This is most probably due to a decreased concentration of Ddx molecules located in the periphery of the now larger sized MGDG aggregates. These Ddx molecules represent the substrate molecules, which are directly accessible by the DDE, whereas the Ddx molecules located inside the MGDG aggregates have to diffuse to the periphery of the aggregates, before they can be de-epoxidized by the enzyme. However, it has to be noted that the de-epoxidation rate at 38.7 μM MGDG is still very high and that the enzyme activity is reduced by only ~20% compared to the activity at 5.8 μM MGDG.

Vx de-epoxidation by the wheat VDE is almost completely suppressed in the absence of MGDG (Figure 3B). Stepwise increases in the MGDG concentration induce concomitant increases in the Vx de-epoxidation rates. In the case of Vx, however, 11.6 μM MGDG is needed to achieve saturation of the de-epoxidation reaction. This is again in agreement with the data from Vx solubility, which show that at an MGDG concentration of 5.8 μM only a part of the total Vx is solubilized and that 11.6 μM MGDG is needed to fully dissolve Vx. These results clearly indicate that at low concentrations of MGDG the solubility of the xanthophyll

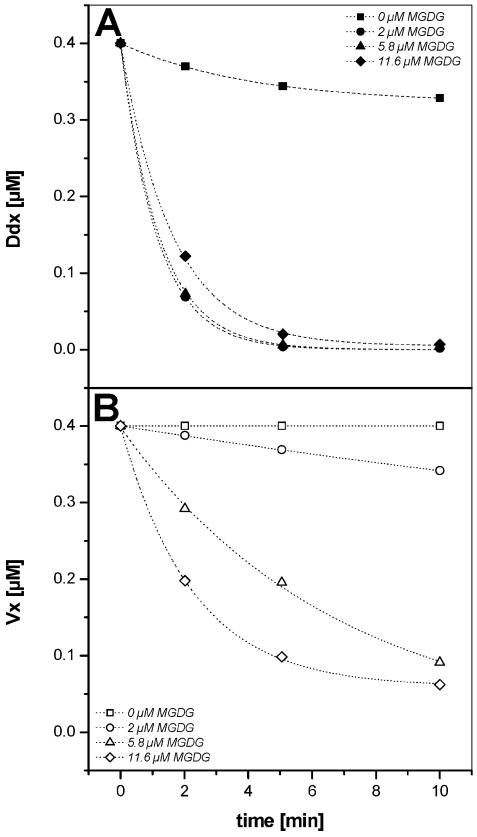

FIGURE 3: (A) Time course of Ddx de-epoxidation by the *C. meneghiniana* DDE in reaction medium at pH 5.2 in the presence of different concentrations of MGDG. The Ddx concentration is 0.4 μM, and ascorbate (final concentration of 30 mM) is added to the reaction medium to induce Ddx de-epoxidation. (B) Time course of Vx de-epoxidation by wheat VDE in reaction medium at pH 5.2 in the presence of different concentrations of MGDG. The Vx concentration is 0.4 μM, and ascorbate (final concentration of 30 mM) is added to the reaction medium to induce Vx de-epoxidation. Mean values of three (A) and five (B) independent experiments with standard deviations <9% for panel A and <5% for panel B.

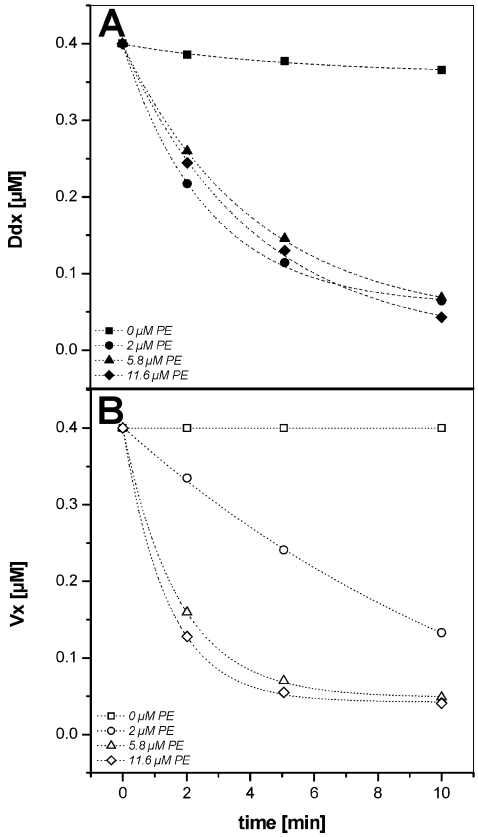

FIGURE 4: (A) Time course of Ddx de-epoxidation by *C. meneghiniana* DDE in reaction medium at pH 5.2 in the presence of different concentrations of PE. The Ddx concentration is 0.4 μM, and ascorbate (final concentration of 30 mM) is added to the reaction medium to induce Ddx de-epoxidation. (B) Time course of Vx de-epoxidation by wheat VDE in reaction medium at pH 5.2 in the presence of different concentrations of PE. The Vx concentration is 0.4 μM, and ascorbate (final concentration of 30 mM) is added to the reaction medium to induce Vx de-epoxidation. Mean values of three (A) and five (B) independent experiments with standard deviations of <8% for panel A and <3% for panel B.

cycle pigments limits the efficiency of the respective de-epoxidation reaction.

Ddx and Vx de-epoxidation in the presence of the other hexagonal structure-inducing lipid, PE, follows the same general pattern as it does in the presence of MGDG. Ddx de-epoxidation by the *C. meneghiniana* DDE is saturated at a lipid concentration of 2 μM PE (Figure 4A), which corresponds to the almost complete solubilization of Ddx at that lipid concentration. The importance of lipids capable of forming inverted hexagonal phases for xanthophyll de-epoxidation is confirmed by an almost complete suppression of Ddx conversion in the absence of PE. In contrast to Ddx de-epoxidation in reaction medium containing higher concentrations of MGDG, small decreases in the de-epoxidation rate at PE concentrations of 11.6 μM are not visible.

Again, Vx de-epoxidation by wheat VDE (Figure 4B) is saturated at significantly higher PE concentrations than Ddx de-epoxidation. PE concentrations of 5.8−11.6 μM are needed to achieve reasonably fast de-epoxidation rates. This is again in good agreement with the lower solublization efficiency in the case of Vx, where a PE concentration of 11.6 μM is needed to completely solublize the xanthophyll cycle pigment of the higher plants. The importance of the hexagonal structure-forming lipids for Vx de-epoxidation is confirmed by the complete absence of Vx de-epoxidation in reaction medium at pH 5.2 where PE has been omitted.

Decreased Solubility of Ddx and Vx in Bilayer-FormingLipids PC and DGDG. Figure 5 shows the absorption spectra of Ddx and Vx in reaction medium at pH 5.2 in the presence of varying concentrations of bilayer-forming lipid PC. Complete solubilization of both xanthophyll cycle pigments requires significantly higher lipid concentrations compared with those of hexagonal structure-forming lipids MGDG and PE. The typical Ddx absorption spectrum appears at a PC concentration of 11.6 μM (Figure 5A), although the spectral fine structure is still slightly distorted due to an increased contribution of the short wavelength absorption to the overall absorption spectrum. A PC concentration of 38.7 μM is needed to completely solubilize Ddx and to fully restore the characteristic Ddx absorption spectrum. This PC concentration corresponds to a lipid: pigment ratio of almost 100 taking into account the fixed pigment concentration of 0.4 μM. This means that the PC concentrations needed for a complete solubilization of Ddx are more than 10 times higher than the respective MGDG or PE concentrations. A similar conclusion can be derived from the shape of the Vx absorption spectra at the different

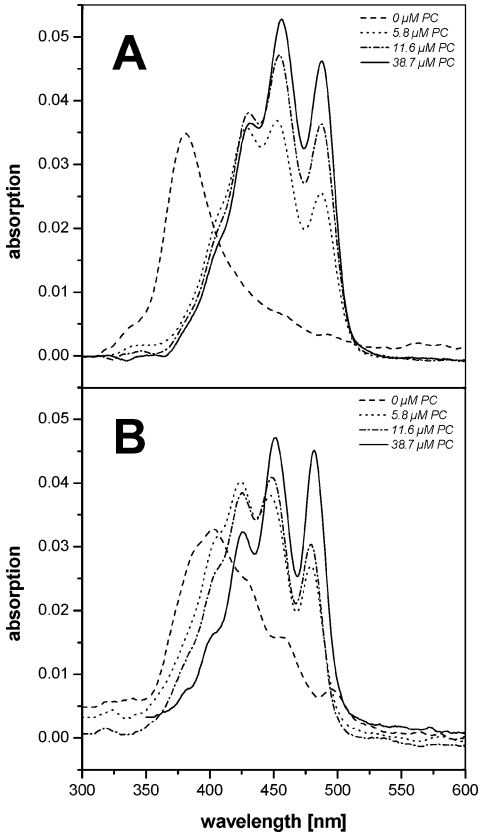

FIGURE 5: (A) Absorption spectra of Ddx in reaction medium at pH 5.2 in the presence of different concentrations of bilayer-forming phospholipid PC. The Ddx concentration is fixed to 0.4 μM. (B) Absorption spectra of Vx in reaction medium at pH 5.2 in the presence of different concentrations of PC. The Vx concentration is fixed to 0.4 μM.

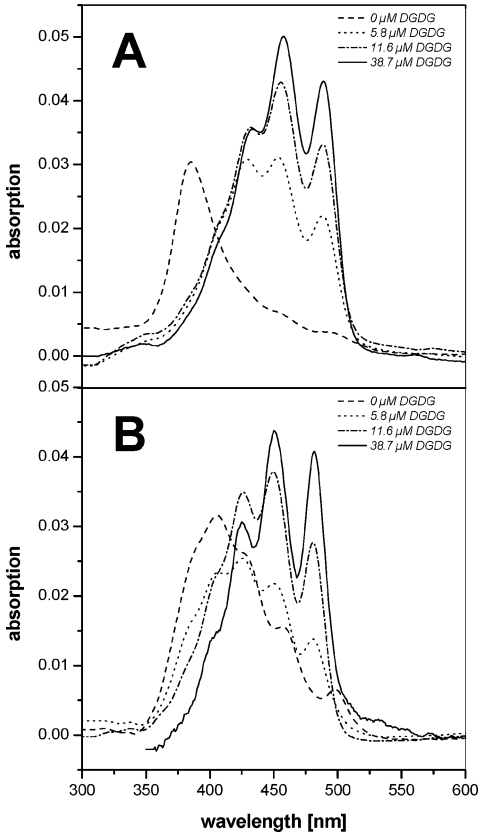

FIGURE 6: (A) Absorption spectra of Ddx in reaction medium at pH 5.2 in the presence of different concentrations of bilayer-forming galactolipid DGDG. The Ddx concentration is fixed at 0.4 μM. (B) Absorption spectra of Vx in reaction medium at pH 5.2 in the presence of different concentrations of DGDG. The Vx concentration is fixed at 0.4 μM.

PC concentrations (Figure 5B). At PC concentrations below 38.7 μM, the Vx spectra are substantially distorted, showing the most prominent absorption maxima in the blue region of the spectrum. The characteristic Vx absorption spectrum with an absorption maximum at 452 nm and a pronounced long-wave peak around 480 nm can only be seen at the highest PC concentration that was employed. In the case of Vx, the PC concentrations needed for a full solubilization are ~4 times higher than the respective concentrations of the hexagonal structure-forming lipids.

The decreased efficiency of PC in the process of xanthophyll solubilization is not restricted to this special lipid but occurs in a very similar way when another bilayer-forming lipid, DGDG, is utilized (Figure 6).

The dependence of both the Ddx (Figure 6A) and Vx spectra (Figure 6B) on the DGDG concentrations is comparable to the results obtained with PC. In either case, 38.7 μM lipid is needed to completely solubilize Ddx and Vx, and the shapes of the absorption spectra at the different lower lipid concentrations are also very similar for both lipids. However, a direct comparison of the Vx absorption spectra at 5.8 μM DGDG and PC (compare Figures 5B and 6B) indicates a slightly higher solubilization efficiency of Vx in PC. In general, one can conclude that the bilayer-forming lipids are significantly less efficient in the solubilization of the xanthophyll cycle pigments than the hexagonal structure-forming lipids. The use of lipids that are structurally related (MGDG vs DGDG and PE vs PC) furthermore indicates that it is not the molecular structure of the lipid itself but the higher-order structure, which is formed by the respective lipid, that determines xanthophyll solubility.

Xanthophyll De-Epoxidation Is Not Only Determined by Xanthophyll Solubility. The experiments with the hexagonal structure-forming lipids show that the solubilization of the xanthophyll cycle pigments at lower lipid concentrations is one of the key factors determining the de-epoxidation activity, i.e., that enhanced solubilization of Vx and Ddx leads to increased rates of de-epoxidation. Ddx and Vx de-epoxidation in the presence of bilayer-forming lipids PC and DGDG (Figure 7) indicates that solubilization is not the only factor which controls de-epoxidation. Even in the presence of high concentrations of PC and DGDG, where both xanthophyll cycle pigments are completely solubilized, Ddx de-epoxidation and Vx de-epoxidation are significantly restricted. Ddx de-epoxidation proceeds slowly in the presence of both PC and DGDG (Figure 7A), and after 10 min of the in vitro de-epoxidation assay, only ~25% of Ddx has been converted to Dtx (compared with the almost complete Ddx de-epoxidation after 2 min in the presence of 2 μM MGDG). Notably, the Ddx de-epoxidation rate at DGDG or PC concentrations of 38.7 μM is ~10 times lower than the de-epoxidation rate in the presence of 38.7 μM MGDG (data not shown).

Vx de-epoxidation in the presence of PC is even more inefficient, and the reaction is almost completely suppressed in the presence of DGDG (Figure 7B). It has to be noted

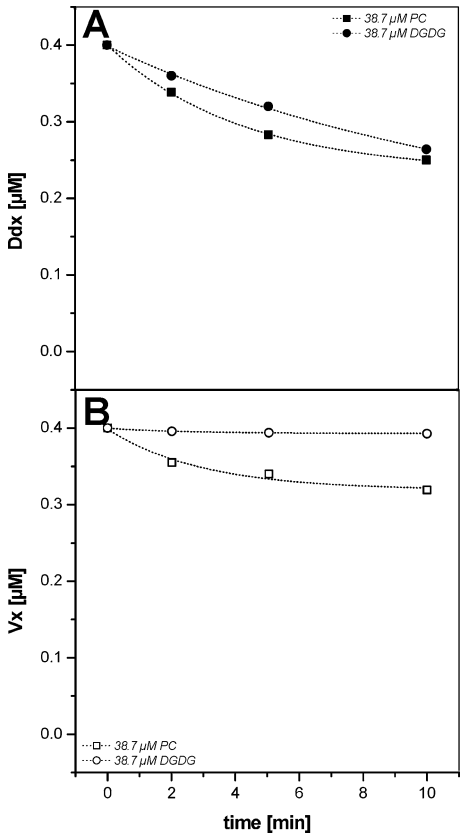

FIGURE 7: (A) Time course of Ddx de-epoxidation by the *C. meneghiniana* DDE in reaction medium at pH 5.2 in the presence of DGDG and PC. The Ddx concentration is 0.4 μM, and ascorbate (final concentration of 30 mM) is added to the reaction medium to induce Ddx de-epoxidation. (B) Time course of Vx de-epoxidation by wheat VDE in reaction medium at pH 5.2 in the presence of DGDG and PC. The Vx concentration is 0.4 μM, and ascorbate (final concentration of 30 mM) is added to the reaction medium to induce Vx de-epoxidation. Mean values of three (A) and five (B) independent experiments with standard deviations of $<5\%$ for both panels A and B.

that both Ddx de-epoxidation and Vx de-epoxidation at lower concentrations of PC and DGDG, where complete solubilization has not been achieved, proceed even more slowly than the de-epoxidation at a lipid concentration of 38.7 μM (data not shown). Further increases in the lipid concentrations are also not able to enhance the Ddx and Vx de-epoxidation rates above the rate shown here. These results indicate that, besides the solubilization of the xanthophyll cycle pigments, the provision of the inverted hexagonal structures is the second key feature which determines efficient xanthophyll de-epoxidation. Although PC and DGDG are able to solubilize Ddx and Vx, albeit at higher lipid concentrations compared with MGDG and PE, they do not provide the hexagonal structures needed for a high xanthophyll de-epoxidase activity.

DISCUSSION

Importance of Substrate Solubilization for Xanthophyll De-Epoxidation. Our data show that one of the key functions of the lipids in xanthophyll de-epoxidation is to solubilize the xanthophyll cycle pigments, i.e., to provide the substrates Ddx and Vx in a form that is accessible for VDE or DDE. Injection of xanthophyll cycle pigments into an aqueous reaction medium buffered to pH 5.2, which simulates the properties of the thylakoid lumen, leads to an uncharacteristic carotenoid absorption spectrum dominated by a broad absorption maximum in the UV or blue region of the spectrum. The formation of this maximum is caused by an aggregation of the lipophilic pigment molecules in the aqueous environment (*25*). The aggregated xanthophyll cycle pigments are characterized by an altered distribution of the electrons in the chromophore system, which leads to the observed changes in the light absorption of the molecules. Ddx and Vx in the aggregated form cannot be used as substrates by enzymes VDE and DDE, as can be seen by the complete suppression of Vx de-epoxidation by VDE in the absence of lipids. Ddx de-epoxidation by DDE without addition of supplementary lipids is also strongly inhibited; the minor Ddx de-epoxidation we observed is most probably caused by very low concentrations of residual MGDG still present in the DDE enzyme preparation. Aggregation of the xanthophyll cycle pigments in the aqueous environment is prevented by the presence of solubilizing lipids. Solubilization of Ddx and Vx, which is indicated by a complete restoration of the characteristic three-peak absorption spectrum, is a prerequisite for their efficient de-epoxidation and depends on the nature and the concentration of the lipids employed. MGDG, the most abundant thylakoid membrane lipid, is very effective in substrate solubilization. Only low concentrations of the hexagonal structure-forming lipid are needed to fully dissolve the pigments and saturate xanthophyll de-epoxidation. This observation is in agreement with results published in ref *16*, where the first evidence that MGDG activates Vx de-epoxidation through substrate solubilization was presented. Our data extend this finding and show that other lipids are also able to solubilize the xanthophyll cycle pigments. However, we observed a substantial difference in the efficiency of Ddx and Vx solubilization exerted by different lipids. PE, another hexagonal structure-forming lipid, exhibited an efficiency in pigment solubilization similar to that of MGDG. Low lipid concentrations led to the complete solubilization of Ddx and Vx, thereby saturating the de-epoxidase reaction. Bilayer-forming lipids such as DGDG and PC were also able to solubilize Ddx and Vx, albeit at significantly higher concentrations. The differences in substrate solubilization are not related to the intrinsic structures of the different lipids, as MGDG and DGDG, on one hand, and PE and PC, on the other hand, possess virtually the same molecular structure. Solubilization, therefore, seems to depend on the higher-order structure that the different lipids provide, i.e., the hexagonal structure in the case of MGDG and PE and the bilayer structure in the case of DGDG and PC. Bilayer-forming lipids, although capable of solubilizing the xanthophyll cycle pigments, were, however, not able to activate efficient xanthophyll de-epoxidation (see below). The significant differences in the solubility of the xanthophyll cycle pigments in the hexagonal phases or bilayer-forming lipids may have consequences for the distribution of the xanthophyll cycle pigments in the natural thylakoid membrane, which consists to a large extent of galactolipids MGDG and DGDG. On the basis of our results, we can conclude that the xanthophyll cycle pigments, after their release from the light-harvesting proteins, are preferentially located in the MGDG-rich domains of the membrane, where the actual de-epoxidation reaction is taking place (refs *18* and *26*; see also discussion below).

Significant Differences in the Lipid Solubility of Ddx and Vx. Another important result of our study is the observation that Ddx and Vx exhibit prominent differences with respect to their lipid solubility. For a complete solubilization of Ddx, concentrations of MGDG and PE significantly lower than for the solubilization of Vx are needed. If the importance of substrate solubilization for efficient de-epoxidation outlined above is taken into account, it becomes clear why we observe much higher rates of Ddx de-epoxidation at lower concentrations of MGDG and PE compared with Vx de-epoxidation. At present, it is not clear why Ddx shows a better lipid solubility than Vx. One can only speculate that either the asymmetry of the Ddx molecule or the difference in polarity of the two xanthophylls is responsible for this, but further experiments are needed to clarify this question. However, the saturation of Ddx de-epoxidation at a significantly lower lipid:pigment ratio may be related to the natural situation in the diatom photosynthetic apparatus. Various studies (*12*, *27*, *28*) have shown that the Ddx cycle pool size can be significantly larger than the respective Vx cycle pool size in vascular plants and green algae. This means, that in diatoms, which are illuminated with actinic high light, a larger amount of Ddx molecules can be released from the light-harvesting proteins to the free lipid phase of the membrane. Complete solubilization of a large number of Ddx molecules can only be achieved under the assumption that a low lipid:pigment ratio is sufficient for solubilization. The increased solubility of the Ddx molecules compared with Vx would then enable an efficient de-epoxidation of a large number of Ddx molecules within a short time period, thereby providing rapid photoprotection to the photosynthetic apparatus. The higher solubility of Ddx compared with Vx is in line with other results on the Ddx cyle of diatoms, which show that the Ddx cycle is optimized in several ways compared with the Vx cycle of higher plants. Among these are the important observations that DDE activity starts at higher pH values than the respective VDE activity in higher plants (*19*), that Ddx in vivo is de-epoxidized by DDE much faster than Vx (*29*), and that Dtx epoxidation is completely suppressed by the transmembrane pH gradient under high light illumination (*28*, *30*).

Importance of Hexagonal Structures for Xanthophyll De-Epoxidation. The second key function of the lipids in xanthophyll de-epoxidation is the provision of a structural environment suitable for efficient conversion of Ddx and Vx. Our data corroborate the findings presented in refs *18* and *26*, which showed that the presence of inverted hexagonal phase domains is necessary for Vx de-epoxidation and that xanthophyll de-epoxidase activity cannot be observed in lipid bilayers, which do not contain inverted hexagonal phase-forming lipids. We extend these findings and show that the absence of efficient xanthophyll de-epoxidation in a membrane solely composed of bilayer-forming lipids, i.e., DGDG or PC, cannot be explained by an insufficient solubilization of the xanthophyll cycle pigments in these lipids. Although both lipids are significantly less efficient in Ddx and Vx solubilization compared with hexagonal phase-forming lipids MGDG and PE, complete substrate solubilization can be achieved at higher lipid concentrations. However, complete Ddx and Vx solubilization at these concentrations does not induce efficient xanthophyll de-epoxidation. High rates of Ddx and Vx conversion are only possible when, apart from complete substrate solubilization, inverted hexagonal structures are provided by MGDG or PE. The presence of such H_{II} phases associated with natural thylakoid membranes has already been reported (*33*, *34*). The question of whether these hexagonal phases reside within the natural membrane bilayer (*18*) or are associated with, for example, the lumenal side of the thylakoid membrane, however, is still open (for a discussion of the possible ways of bilayer and nonbilayer coexistence, see ref *31*).

Besides the formation of inverted hexagonal structures, nonbilayer lipids such as MGDG and PE exert other characteristics which are in favor of an efficient xanthophyll de-epoxidation. According to the data published in ref *32*, where the effect of nonbilayer lipids on membrane binding and insertion of the catalytic domain of leader peptidase was studied, the smaller headgroup sizes of the non-bilayer-forming lipids reduce the packing density at the membrane−water interface. The nonbilayer lipids thereby create hydrophobic insertion sites, which foster the insertion of membrane-associated proteins. With regard to VDE and DDE, this would mean that insertion of the catalytic site of the enzyme is strongly facilitated in membrane domains enriched in MGDG. This would allow the enzyme to reach the epoxyionon ring of Ddx and Vx, which is located at the lumenal side of the thylakoid membrane.

We conclude that the role of the naturally occurring thylakoid lipid MGDG is twofold. MGDG is, on one hand, perfectly suited to solubilize the xanthophyll cycle pigments, even at low concentrations. On the other hand, it forms inverted hexagonal structures associated with the membrane bilayer, thereby providing the higher-order structure essential for efficient xanthophyll de-epoxidase membrane insertion and activity. It is interesting to note that the minor Chl *a*/*b* binding antenna proteins of higher plants, which have been shown to contain the majority of the Vx cycle pigments (*35*, *36*), are also enriched in MGDG (*37*). We propose that MGDG, located in the vicinity of these proteins, solubilizes Vx after its detachment from the LHC protein and provides the suitable structure for Vx de-epoxidation. MGDG also enables the binding of the formerly inactive and water-soluble VDE to the thylakoid membrane (*13*, *38*), thereby targeting the enzyme to the site now enriched in free Vx. We believe that such a localized Vx de-epoxidation would be advantageous for a fast and efficient rebinding of Zx to the LHC protein, once Zx has been formed from Vx.

REFERENCES

1. Yamamoto, H. Y., Nakayama, T. O. M., and Chichester, C. O. (1962) Studies on the light interconversions of the leaf xanthophylls, *Arch. Biochem. Biophys. 97*, 168−173.
2. Hager, A. (1966) Die Zusammenhänge zwischen lichtinduzierten Xanthophyll-Umwandlungen und Hill-Reaktion, *Ber. Dtsch. Bot. Ges. 79*, 94−107.
3. Latowski, D., Burda, K., and Strzalka, K. (2000) A mathematical model describing kinetics of conversion of violaxanthin to zeaxanthin via intermediate antheraxanthin by the xanthophyll cycle enzyme violaxanthin de-epoxidase, *J. Theor. Biol. 206*, 507−514.
4. Stransky, H., and Hager, A. (1970) Das Carotinoidmuster und die Verbreitung des lichtinduzierten Xanthophyllzyklus in verschiedenen Algenklassen, *Arch. Mikrobiol. 73*, 315−323.
5. Hager, A. (1980) The reversible light-induced conversions of xanthophylls in the chloroplast, in *Pigments in Plants* (Czygan, F. C., Ed.) pp 57−79, Fischer, Stuttgart, Germany.

6. Latowski, D., Grzyb, J., and Strzalka, K. (2004) The xanthophyll cycle: Molecular mechanism and physiological significance, *Acta Physiol. Plant. 26*, 197−212.
7. Demmig-Adams, B., Adams, W. W., III, Heber, U., Neimanis, S., Winter, K., Krüger, A., Czygan, F. C., Bilger, W., and Björkman, O. (1990) Inhibition of zeaxanthin formation and of rapid changes in radiationless energy dissipation by dithiothreitol in spinach leaves and chloroplasts, *Plant Physiol. 92*, 293−301.
8. Horton, P., and Ruban, A. V. (1992) Regulation of photosystem II, *Photosynth. Res. 34*, 375−385.
9. Gilmore, A. M., and Yamamoto, H. Y. (1993) Linear models relating xanthophylls and lumen acidity to non-photochemical fluorescence quenching: Evidence that antheraxanthin explains zexanthin-independent quenching, *Photosynth. Res. 35*, 67−78.
10. Olaizola, M., La Roche, J., Kolber, Z., and Falkowski, P. G. (1994) Non-photochemical fluorescence quenching and the diadinoxanthin cycle in a marine diatom, *Photosynth. Res. 41*, 357−370.
11. Lavaud, J., Rousseau, B., and Etienne, A.-L. (2002) In diatoms, a transthylakoid proton gradient alone is not sufficient to induce a non-photochemical fluorescence quenching, *FEBS Lett. 523*, 163−166.
12. Goss, R., Pinto, E. A., Drumm, T., and Richter, M. (2004) Different regulation of the PS II antenna function in Vx and Ddx cycle containing plants. I. The de-epoxidized xanthophyll cycle pigments Zx and Dtx have different quenching capabilities, *Planta* (submitted for publication).
13. Hager, A., and Holocher, K. (1994) Localization of the xanthophyll cycle enzyme violaxanthin de-epoxidase within the thylakoid lumen and abolition of its mobility by a (light-dependent) pH decrease, *Planta 192*, 581−589.
14. Hager, A. (1969) Lichtbedingte pH-Erniedrigung in einem Chloroplasten-Kompartiment als Ursache der enzymatischen Violaxanthin-Zeaxanthin-Umwandlung: Beziehungen zur Photophosphorylierung, *Planta 89*, 224−243.
15. Pfündel, E., Renganathan, M., Gilmore, A. M., Yamamoto, H. Y., and Dilley, R. A. (1994) Intrathylakoid pH in isolated pea chloroplasts as probed by violaxanthin de-epoxidation, *Plant Physiol. 106*, 1647−1658.
16. Yamamoto, H. Y., and Higashi, R. M. (1978) Violaxanthin de-epoxidase. Lipid composition and substrate specifity, *Arch. Biochem. Biophys. 190*, 514−522.
17. Latowski, D., Kostecka, A., and Strzalka, K. (2000) Effect of monogalactosyldiacylglycerol and other thylakoid lipids on violaxanthin de-epoxidation in liposomes, *Biochem. Soc. Trans. 28*, 810−812.
18. Latowski, D., Kruk, J., Burda, K., Skrzynecka-Jaskier, M., Kostecka-Gugala, A., and Strzalka, K. (2002) Kinetics of violaxanthin de-epoxidation by violaxanthin de-epoxidase, a xanthophyll cycle enzyme, is regulated by membrane fluidity in model lipid bilayers, *Eur. J. Biochem. 269*, 4656−4665.
19. Jakob, T., Goss, R., and Wilhelm, C. (2001) Unusual pH-dependence of diadinoxanthin de-epoxidase activation causes chlororespiratory induced accumulation of diatoxanthin in the diatom *Phaeodactylum tricornutum*, *J. Plant Physiol. 158*, 383−390.
20. Provasoli, L., McLaughlin, J. J. A., and Droop, M. R. (1957) The development of artificial media for marine algae, *Arch. Mikrobiol. 25*, 392−428.
21. Lohr, M., and Wilhelm, C. (2001) Xanthophyll synthesis in diatoms: Quantification of putative intermediates and comparison of pigment conversion kinetics with rate constants derived from a model, *Planta 212*, 382−391.
22. Havir, E. A., Tausta, L. S., and Peterson, R. B. (1997) Purification and properties of violaxanthin de-epoxidase from spinach, *Plant Sci. 123*, 57−66.
23. Kraay, G. W., Zapata, M., and Veldhuis, M. J. W. (1992) Separation of chlorophylls c_1, c_2 and c_3 of marine phytoplankton by reversed-phase C_{18}-high-performance liquid chromatography, *J. Phycol. 28*, 708−712.
24. Wilhelm, C., Volkmar, P., Lohmann, C., Becker, A., and Meyer, M. (1995) The HPLC-aided pigment analysis of phytoplankton cells as a powerful tool in water quality control, *Aqua 44*, 132−141.
25. Hager, A. (1970) Ausbildung von Maxima im Absorptionsspektrum von Carotinoiden im Bereich um 370 nm; Folgen für die Interpretation bestimmter Wirkungsspektren, *Planta 91*, 38−53.
26. Latowski, D., Åkerlund, H.-E., and Strzalka, K. (2004) Violaxanthin de-epoxidase, the xanthophyll cycle enzyme, requires lipid inverted hexagonal structures for its activity, *Biochemistry 43*, 4417−4420.
27. Lavaud, J., Rousseau, B., and Etienne, A.-L. (2003) Enrichment of the light-harvesting complex in diadinoxanthin and implications for the nonphotochemical fluorescence quenching in diatoms, *Biochemistry 42*, 5802−5808.
28. Pinto, E. A., Goss, R., Wilhelm, C., and Richter, M. (2004) Different regulation of the PS II antenna function in Vx and Ddx cycle containing plants. II. The importance of a highly active and ΔpH-regulated diatoxanthin epoxidase, *Planta* (submitted for publication).
29. Lohr, M., and Wilhelm, C. (1999) Algae displaying the diadinoxanthin cycle also possess the violaxanthin cycle, *Proc. Natl. Acad. Sci. U.S.A. 96*, 8784−8789.
30. Mewes, H., and Richter, M. (2002) Supplementary ultraviolet-B radiation induces a rapid reversal of the diadinoxanthin cycle in the strong light-exposed diatom *Phaeodactylum tricornutum*, *Plant Physiol. 130*, 1527−1535.
31. Garab, G., Lohner, K., Laggner, P., and Farkas, T. (2000) Self-regulation of the lipid content of membranes by non-bilayer lipids, *Trends Plant Sci. 5*, 489−494.
32. Van den Brink-van der Laan, E., Dalbey, R. E., Demel, R. A., Killian, J. A., and de Kruijff, B. (2001) Effect of nonbilayer lipids on membrane binding and insertion of the catalytic domain of leader peptidase, *Biochemistry 40*, 9677−9684.
33. Gounaris, K., Sen, A., Brain, A. P. R., Quinn, P., and Williams, W. P. (1983) The formation of non-bilayer structures in total polar lipid extracts of chloroplast membranes, *Biochim. Biophys. Acta 728*, 129−139.
34. Haranczyk, H., Strzalka, K., Dietrich, W., and Blicharski, J. S. (1995) ^{31}P NMR observation of the temperature and glycerol induced non-lamellar phase formation in wheat thylakoid membranes, *J. Biol. Phys. 21*, 125−139.
35. Bassi, R., Pineau, B., Dainese, P., and Marquardt, J. (1993) Carotenoid-binding proteins of photosystem II, *Eur. J. Biochem. 212*, 297−303.
36. Goss, R., Richter, M., and Wild, A. (1997) Pigment composition of PS II pigment protein complexes purified by anion exchange chromatography. Identification of xanthophyll cycle pigment binding proteins, *J. Plant Physiol. 151*, 115−119.
37. Tremolieres, A., Dainese, P., and Bassi, R. (1994) Heterogeneous lipid distribution among chlorophyll-binding proteins of photosystem II in maize mesophyll chloroplasts, *Eur. J. Biochem. 221*, 721−730.
38. Bugos, R. C., and Yamamoto, H. Y. (1996) Molecular cloning of violaxanthin de-epoxidase from romain lettuce and expression in *Escherichia coli*, *Proc. Natl. Acad. Sci. U.S.A. 93*, 6320−6325.

BI047464K