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Membrane curvature stress controls the maximal conversion of violaxanthin to zeaxanthin in the violaxanthin cycle—influence of α-tocopherol, cetylethers, linolenic acid, and temperature

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Abstract

Zeaxanthin, an important component in protection against overexcitation in higher plants, is formed from violaxanthin by the enzyme violaxanthin de-epoxidase. We have investigated factors that may control the maximal degree of conversion in the violaxanthin cycle. The conversion of violaxanthin to zeaxanthin in isolated spinach thylakoids was followed at different temperatures and in the presence of lipid packing modifiers. The maximum degree of conversion was found to be 35%, 70% and 80% at 4 °C, 25 °C and 37 °C respectively. In the presence of membrane modifying agents, known to promote non-lamellar structures (H_{II}), such as linolenic acid the conversion increased, and the maximal level of violaxanthin de-epoxidation obtained was close to 100%. In contrast, substances promoting lamellar phases (L_α), such as α-tocopherol and 8-cetylether ($C_{16}EO_8$), only 55% and 35% of the violaxanthin was converted at 25 °C, respectively. The results are interpreted in light of the lipid composition of the thylakoid membrane, and we propose a model where a negative curvature elastic stress in the thylakoid lipid bilayer is required for violaxanthin de-epoxidase activity. In this model zeaxanthin with its longer hydrophobic stretch is proposed to promote lamellar arrangements of the membrane. As a result, zeaxanthin relieves the curvature elastic stress, which in turn leads to inactivation of violaxanthin de-epoxidase.

Keywords: Membrane curvature stress; Inverted hexagonal phase (H_{II}); Thylakoid membrane; Violaxanthin de-epoxidase (VDE); Zeaxanthin; Xanthophyll cycle

1. Introduction

The xanthophyll cycle (XC)/violaxanthin cycle in higher plants is a thylakoid membrane associated photoprotective mechanism. Under normal, unstressed conditions, the predominant carotenoid in the cycle is violaxanthin. When overexcited, the lumenal pH of thylakoids drops to values below 6. This causes a conformational change of the enzyme, violaxanthin de-epoxidase (VDE) presumably through protonation of conserved histidines [1]. Subsequently, VDE docks to the lumenal side of the membrane and starts to convert violaxanthin to zeaxanthin through the intermediate antheraxanthin using ascorbic acid as a co-substrate. Zeaxanthin plays a key role in harmless dissipation of excess energy, measured as a component of non-photochemical quenching (NPQ) [2]. The energy dissipation also requires ΔpH across the thylakoid membrane and PsbS, a PSII subunit [3,4]. For further information on the XC see reviews by Demmig-Adams and Adams [5], Eskling et al. [6] and Grzyb et al. [7].

As expected the degree of violaxanthin to zeaxanthin conversion varies in response to the stress level, and the level of zeaxanthin (+antheraxanthin) directly correlates to the degree of NPQ under restricted conditions [8]. However, the extent of violaxanthin conversion has in most cases been far from complete, even if the stress level has been increased and prolonged.

Abbreviations: $C_{16}EO_x$, Poly(oxyethylene) hexadecyl ethers and x is the number of oxyethylene units in the polar head groups; $C_{16}EO_8$, octaethylene glycol monohexadecyl ether; DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; H_{II}, inverted hexagonal phase; HPLC, high performance liquid chromatography; L_α, lamellar liquid crystal phase; L_β, lamellar gel phase; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; NPQ, non-photochemical quenching; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PSII, photosystem II; VDE, violaxanthin de-epoxidase; XC, xanthophyll cycle

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The amount of violaxanthin remaining unconverted normally ranges between 20 and 50%. The reason to the limitation has not been clarified, but it has often been assumed that this part of the violaxanthin pool is 'inaccessible' [9,10]. The specific location of the XC-pigments in the membrane is controversial. Many studies suggest that XC-pigments are protein-bound as they can be found in pigment–protein complexes after fractionation or purification [11–13], and have even been found in the crystal structure of the major light-harvesting complex [14,15]. It has also been shown that XC-pigments can occupy the binding site for lutein and neoxanthin in recombinant light harvesting complexes [16]. Thus the light-harvesting complexes have the ability to bind violaxanthin. One view is therefore that the 'inaccessible' violaxanthin would be strongly bound to proteins and not available for conversion by VDE. However, it is well known that the binding of xanthophylls to light-harvesting proteins is much weaker than that of other carotenoids and mild conditions are needed to retain violaxanthin during isolation of complexes [17].

The other main view regarding the location of 'inaccessible' violaxanthin is that at least a substantial part of XC-pigments are free in the lipid matrix of the thylakoid membrane. This assumption is based on the following findings, that (i) higher amount of XC-pigments were found in plants grown at high light although the amount of pigment–protein complexes remained constant or even reduced [18], (ii) violaxanthin can be converted to the same extent from both sides of the thylakoid membrane [19], (iii) fractionation of thylakoid membranes in the absence of detergents revealed that XC-pigments were almost equally distributed between different regions of the membrane, in strong contrast to other pigments and proteins [19], and (iv) restricted conversion of violaxanthin by VDE was obtained in pure lipid systems in the absence of any pigment–protein complexes [20].

Less likely explanations to the restricted conversion of violaxanthin have been, direct product feed-back inhibition of VDE by zeaxanthin [20,21], and that some of the violaxanthin is in the *cis*-form [20] rather than the all-*trans* form required by VDE for active de-epoxidation reaction.

An interesting finding is that VDE has a specific lipid requirement and prefer monogalactosyldiacylglycerol (MGDG) over digalactosyldiacylglycerol (DGDG) for activity [22,23], although recent data [24] indicate that even DGDG may support activity under some conditions. In a comparative study, Latowski et al. [23] found that phosphatidylethanolamine (PE) was as efficient as MGDG to support the de-epoxidation of violaxanthin while no conversion was observed with either DGDG or phosphatidylcholine. Notably, neither pure MGDG nor pure PE can form lamellar structures when dispersed in aqueous media but instead forms inverted hexagonal phases (H_{II}) [25]. The authors concluded that the structure formed by the lipids determines the activity of VDE, rather than interactions with the lipid head groups [23]. The head group of MGDG and DGDG is polar but uncharged while PE and PC have zwitter-ionic head groups. Since both MGDG and PE have been shown to be sufficient for VDE [23], it is reasonable to assume that VDE docks to the membrane through hydrophobic interactions between lipid head groups rather than through ionic interactions.

MGDG is the dominant lipid of the thylakoid membrane accounting for 50 mol% of the total polar lipids. The other major component is DGDG comprising about 30 mol% [26]. As mentioned above, pure MGDG cannot form lamellar membranes. This is because the ratio between the areas occupied by the head group of MGDG relative to the area occupied by the polyunsaturated fatty acid side chains is below unity. In the presence of DGDG and proteins the overall lamellar structure of thylakoids is formed even at such high non-lamellar lipid content. However, the high amount of MGDG in the membrane is expected to make the membrane less tightly packed at the water–membrane interface and to impose negative intrinsic curvature stress to the membrane.

The group of Brown found that non-lamellar prone lipids affect the photochemical activity of rhodopsin and proposed a flexible surface model involving intrinsic membrane curvature [27,28]. More recently, intrinsic curvature stress of biological membranes has been shown to affect the activity of a number of enzymes [29–31]. It was therefore of interest to investigate specifically what effect intrinsic curvature stress has on the conversion of violaxanthin to zeaxanthin.

In this study, we show that the degree of conversion from violaxanthin to zeaxanthin can be altered by change of temperature and by addition of lipid packing modifiers such as linolenic acid, α-tocopherol and specific cetylethers, which all are expected to affect the intrinsic curvature stress. Nearly 100% violaxanthin de-epoxidation was observed in the presence of linolenic acid, while α-tocopherol and specific cethylethers restricted the conversion of violaxanthin. A model is proposed in which the intrinsic membrane curvature stress controls the activity of VDE and thus the conversion of violaxanthin to zeaxanthin in the violaxanthin cycle.

2. Materials and methods

2.1. Isolation of thylakoids

Thylakoids were isolated from spinach (Spinacia oleracea) mainly according to Yamamoto and Higashi [22]. Prior to isolation, leaves were dark-adapted for 12 h so that all zeaxanthin was converted to violaxanthin. Plant material was homogenized in 100-g portions in a blender with 200 mL medium A (400 mM sorbitol, 50 mM MOPS–NaOH, pH 7.0, and 10 mM NaCl). The homogenate was filtered through four layers of 20-μm nylon cloth and the filtrate was centrifuged at 2500×*g* for 7 min. The pellet was re-suspended in the same medium, centrifuged at 5000×*g* for 5 min, resuspended in 50 mL distilled water per 100 g plant material, followed by centrifugation at 37,000×*g* for 10 min. Finally, the thylakoid pellet was resuspended in medium B (400 mM sucrose, 50 mM MES–NaOH, pH 6.5, 15 mM NaCl). The chlorophyll content was determined according to Porra [32]. Dimethyl sulfoxide (DMSO) was added to a final concentration of 5% (v/v), and samples were stored at −80 °C until further use.

2.2. Violaxanthin to zeaxanthin conversion at different temperatures (including temperature-shift and jump experiments)

The de-epoxidation reaction was monitored at 4, 25 and 37 °C, including the shift from low to high temperatures, at 0.08 mg chlorophyll mL^{-1} in 50 mM MES–NaOH, pH 5.1, in darkness with gentle stirring. A time-range of 0–180 min was used. The reaction was initiated by the addition of ascorbate to a final concentration of 30 mM. In the shift experiments (Fig. 1), after 50 min reaction at 4 °C, the reaction mixtures were transferred to 25 °C or 37 °C for another 130 min reaction. In the case of temperature-jump experiment (Fig. 7),

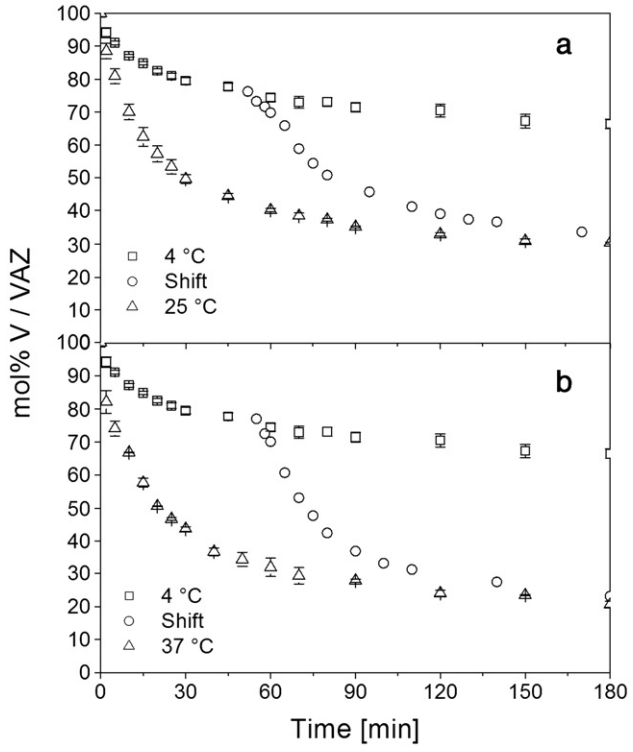

Fig. 1. Time-course of violaxanthin conversion at different temperatures. (a) Isolated spinach thylakoids were incubated at 4 °C (open square), at 25 °C (open triangle) and a shift (open circle) from the low temperature to 25°C. (b) Incubation at 4 °C (open square) and at 37 °C (open triangle), as well as a shift (open circle) from the low temperature to 37 °C. The reactions were performed as described in Materials and methods. The amount of violaxanthin (V) was measured as mol% of the total XC-pool pigments (VAZ). Data are shown as average ± standard error for 3 independent sets of experiments, shift experiments were run once.

the 2-h reaction at 4 °C with active VDE was followed by 20 min incubation at 37 °C either (a) at high pH (VDE inactive) or (b) after removal of ascorbate by two consecutive sedimentations and resuspensions in ascorbate free medium at pH 5.1. Finally, the conditions were changed back to the low temperature combined with (a) low pH (5.1) or (b) addition of ascorbate. Then, the conversion was continued at 4 °C for 2 h. For pigment analysis samples of 1 mL were withdrawn after each time point of the curves.

2.3. Treatment with lipid packing modifiers

Thylakoids at a concentration of 0.08 mg chlorophyll mL^{-1}, in 50 mM MES–NaOH, pH 5.1, were treated with either fatty acids such as oleic acid, linoleic acid and linolenic acid; or α-tocopherol, or cethylether derivatives (poly (oxyethylene) hexadecyl ethers, $C_{16}EO_x$). The number of oxyethylene units (x) was: 2, 6, 8 and 10. All chemicals were obtained from Sigma. Freshly prepared 0.05 g mL^{-1} stock solutions in ethanol were used in all experiments. The effect of ethanol (0.5%, v/v) in the system was negligible and had no effect on the violaxanthin to zeaxanthin conversion. The de-epoxidation reaction was initiated by the addition of ascorbate (30 mM). Violaxanthin conversion was monitored at 25 °C for 180 min in the kinetic experiments. At each time point, 1 mL mixture was withdrawn for pigment analysis and the reaction was terminated by pH adjustment to above 7.0. In concentration dependency studies, samples of 1 mL were run for 90 min. Prior to HPLC analysis carotenoids and chlorophylls were extracted as described below.

2.4. Pigment extraction

In all cases, the de-epoxidation reaction in the 1-mL samples were terminated by addition of 50 μL 1 M NaOH to give a pH above 7.0 where VDE is inactive. Thylakoids were then sedimented by full-speed centrifugation in an Eppendorf bench top centrifuge for 4 min, resuspended in 130 μL 100 mM phosphate buffer, pH 7.0, ice cold 100% acetone (430 μL) was added to extract XC-pigments; the mixture was incubated on ice for 4 min in darkness and centrifuged as above. The obtained supernatant was withdrawn and further diluted with water to get 50% (v/v) acetone and directly subjected to HPLC analysis as described below.

2.5. HPLC determination of the XC-pigments

XC-pigments were analyzed by reversed-phase HPLC (Waters 600E) essentially according to Thayer and Björkman [33]. Pigment extracts (150 μL) were subjected to a Zorbax ODS 4.6 × 250 mm non-end capped cartridge column, where xanthophylls were separated in a methanol/acetonitrile mixture (15%/85%, v/v) run isocratically for 6 min, followed by a rapid elution of other pigments in methanol/ethyl acetate mixture (50%/50%, v/v) for 6 min. All solvents were HPLC grade. The flow rate was constant at 1 mL min^{-1}. Pigments were detected at 445 nm and quantified by peak area integration using data acquisition software.

3. Results

When isolated thylakoids were provided with ascorbic acid at low pH a typical conversion of violaxanthin to zeaxanthin was seen (Fig. 1, cf. [19]). Within 10–30 min more than half of the convertible violaxanthin, at the respective temperatures, was de-epoxidised. However, even after 3 h of enzyme reaction a large proportion of violaxanthin (65%, 30% and 20% at 4 °C, 25 °C and 37 °C, respectively) remained in the samples. The initial rate of conversion was much higher at 25 and 37 °C compared to at 4 °C. The crucial observation is, however, that the final level of conversion obtained was strongly temperature-dependent. In order to exclude the possibility that the enzyme, VDE had become irreversibly inactivated during the long incubation time, temperature-shift experiments were carried out. Samples run at 4 °C for 50 min, where the reaction had almost stopped, was shifted to 25 °C (Fig. 1a) or 37 °C (Fig. 1b). Active conversion was rapidly resumed and the final level of violaxanthin conversion was the same as for the samples only exposed to the higher temperatures. Thus, the amount of 'inaccessible' violaxanthin was strongly affected by the temperature, in good agreement with earlier findings [19].

To test the possibility that lipid packing may modulate VDE activity, experiments with addition of membrane modifying agents to thylakoid membranes were carried out. Prades and co-workers [34] demonstrated that membrane lipid composition in model DEPE (1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine) membranes could be modulated by the addition of free fatty acids. Incorporation of C-18 unsaturated fatty acids was shown to affect the packing parameters and facilitate L_α–H_{II} phase transition. Linolenic acid was the most efficient and was therefore used here to increase intrinsic membrane curvature stress.

As seen in Fig. 2, addition of linolenic acid to thylakoids had a strong stimulatory effect on the final degree of violaxanthin to zeaxanthin conversion at all temperatures, despite a slightly reduced initial rate of conversion. The effect was more pronounced at elevated temperatures (Fig. 2 bV, bA and bZ). Almost the entire violaxanthin pool was converted to zeaxanthin at 37 °C. Even at the lowest temperature used (4 °C) a significant

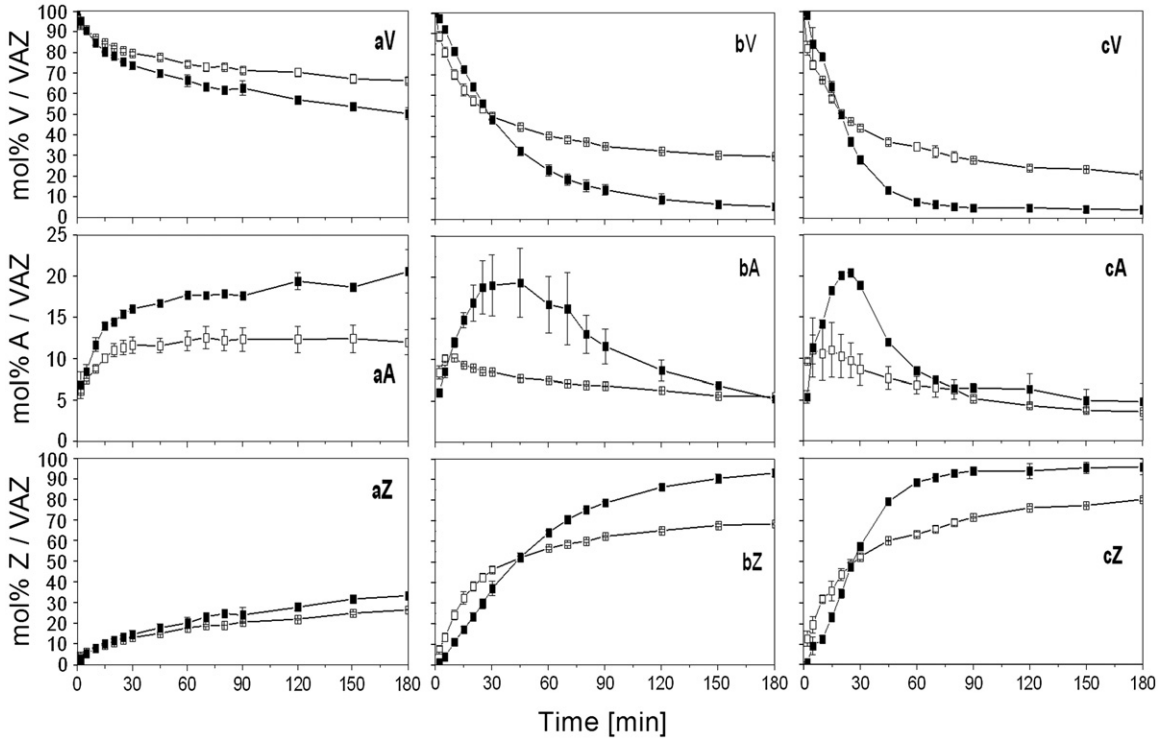

Fig. 2. Time-course of violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z) conversion in the absence (open squares) and presence of 1.2 mM linolenic acid (filled squares). The reaction was monitored at (a) 4 °C (aV, aA, aZ), (b) 25 °C (bV, bA, bZ) and (c) 37 °C (cV, cA, cZ). Each point represents an individual sample. The amount of violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) were measured as mol% of the total XC-pool pigments (VAZ). Error bars denote standard error for 2–3 independent sets of experiments.

stimulatory effect of linolenic acid was observed (Fig. 2 aV, aA and aZ). Antheraxanthin accumulated to somewhat higher extent at 25 and 37 °C, as compared to the control, but it was finally converted to zeaxanthin. The increase in antheraxanthin could indicate that linolenic acid affect the required flip-flop of antheraxanthin [35].

Linoleic acid (*18:2cc*Δ*9,12*) also increased the final degree of conversion of violaxanthin (Fig. 3) although to a less extent than linolenic acid. Oleic acid (*18:1c*Δ*9*), with only one double bond in the acyl chain, and stearic acid showed no significant effect on the violaxanthin conversion. The stimulatory effect was dependent on the fatty acid concentration and was detected already at 0.4 mM for linolenic acid (Fig. 3). It is worth noting that even at the two highest fatty acid concentrations used, thylakoids could be sedimented by low speed centrifugation. This means that the membrane was not solubilised. The amount of linolenic acid compared to total membrane lipids would be 1:1 on a weight basis at 1.2 mM linolenic acid. This was calculated with the assumptions that linolenic acid partitions completely to the thylakoid membrane, that the pigments constitute about 26% of the total lipids [36] and that chlorophyll constitute about 90% of the pigment weight [19]. Thus, the amount of linolenic acid in our experiments would be sufficiently high to affect the structure of the lipid bilayer. Taken together, the results show that the 'accessibility' of violaxanthin can be increased by linolenic and linoleic acids but not with the more saturated oleic and stearic acids.

Another substance known to promote lipid packing is 8-cetylether ($C_{16}EO_8$), a poly(oxyethylene) alkyl ether with a hexadecyl chain and eight oxyethylene units in the head group region. This has been shown nicely in experiments on growing mycoplasma [37]. The mycoplasma easily incorporates hydrophobic substances into the membrane and adjusts lipid head

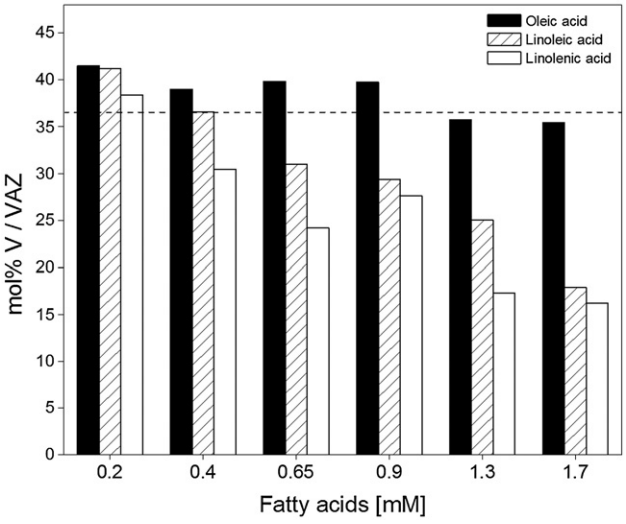

Fig. 3. Effect of fatty acids on the amount of violaxanthin in the thylakoid membrane. C-18 fatty acids such as oleic (filled bars), linoleic (hatched bars) and linolenic acid (open bars) were added at various concentrations as indicated in the figure. The thylakoid samples were incubated at 25 °C for 90 min in the presence of fatty acids and ascorbate (30 mM). The amount of violaxanthin (V) remaining after the conversion was measured as mol% of the total XC-pool pigments (VAZ). The dashed line indicates the value of the control sample (36.5 mol%).

groups to restore membrane integrity and function. Addition of 8-cetylether leads to a compensatory reaction by the bacteria leading to an increased monoglucosyldiglyceride/diglucosyldiglycerade ratio [37].

As shown in Fig. 4 addition of $C_{16}EO_8$ to thylakoids caused a severe inhibition of violaxanthin conversion, both with respect to rate of conversion and in the degree of conversion reached after 3 h. Thus, the effect of $C_{16}EO_8$ was opposite to that of the fatty acids. The degree of inhibition, seen as the amount of violaxanthin remaining after 90 min of reaction, was dependent on the number of oxyethylene units in the head group of the cetylether added (Fig. 5). The inhibition was more pronounced with larger head group area in the molecules. These results are in agreement with the ability of cetylethers to change the intrinsic membrane curvature stress and the equilibrium between lamellar and non-lamellar phases in the membrane [37]. Thus, cetylethers with larger head groups are able to increase the proportion of 'inaccessible' violaxanthin.

α-Tocopherol is a naturally occurring lipid-soluble component of the thylakoid membrane that has been suggested to modulate lipid packing, analogous to cholesterol in eukaryotic membranes [38]. α-Tocopherol is expected to orient in the membrane with the bulky hydrophobic group close to the lipid–water interface in such a way that the negative curvature stress is relieved. In this context, it is interesting that high levels of α- and γ-tocopherol accumulation was observed in the Arabidopsis mutant (*npq1*) lacking functional VDE, and thus unable to convert violaxanthin to zeaxanthin [39]. It was therefore of interest to see if addition of α-tocopherol could affect the degree of violaxanthin conversion.

Indeed, addition of α-tocopherol to the thylakoids caused a decrease both in the rate and in the final amount of violaxanthin that could be converted (Fig. 4). The effect of α-tocopherol was also concentration-dependent and already at 0.23 mM, the lowest concentration used, a reduction in violaxanthin conversion was seen (Fig. 6). Thus, also α-tocopherol increased the proportion of 'inaccessible' violaxanthin.

Taken together, the effect of temperature and the addition of membrane modifiers point to the role of the lipid matrix in the restriction of violaxanthin conversion. One possibility is that the membrane is divided into fluid, liquid-disordered (Lα) and less

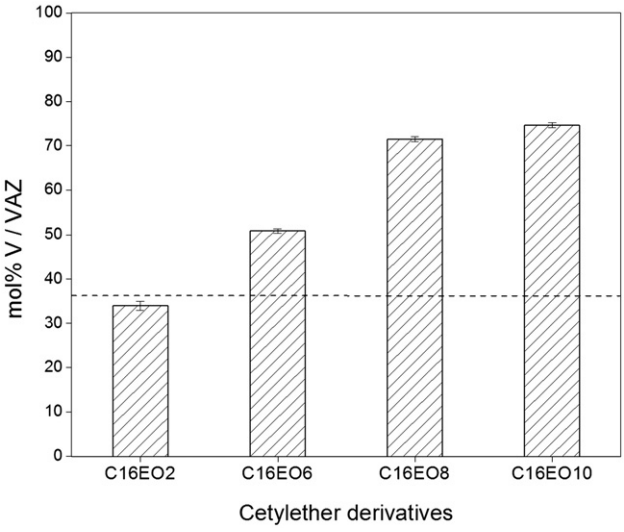

Fig. 5. The effect of different cethylethers ($C_{16}EO_x$) with increasing head group size (x=2, 6, 8 and 10) on violaxanthin de-epoxidation. Thylakoid membranes were treated with the same amount of additions (on a weight-base). The amount of violaxanthin (V) remaining after the 90-min conversion was measured as mol % of the total XC-pool pigments (VAZ). The value of the control sample (36.5 mol% V) is indicated as a dashed line. Data are shown as average± standard error for 3 independent sets of experiments.

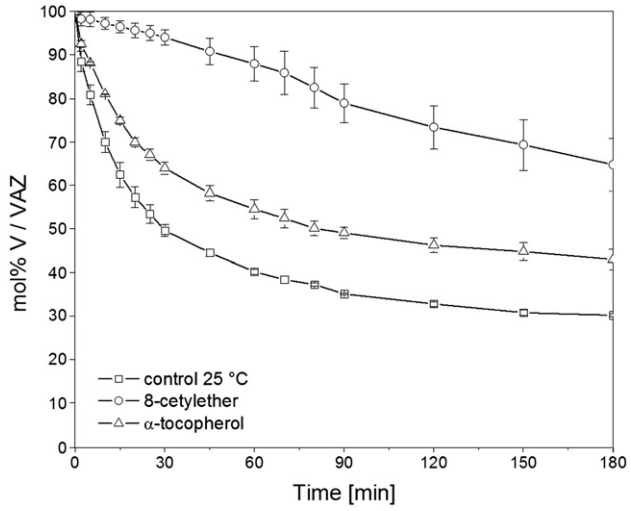

Fig. 4. Time-course of violaxanthin de-epoxidation at 25 °C (open square), the effect of 0.2 mM 8-cethylether (octaethylene glycol monohexadecyl ether, $C_{16}EO_8$) (open circle) and 2.2 mM α-tocopherol (open triangle). The amount of violaxanthin (V) was measured as mol% of the total XC-pool pigments (VAZ). Data are shown as average±standard error for 2 (in the case of 8-cethylether and α-tocopherol) and 3 (control) independent sets of experiments.

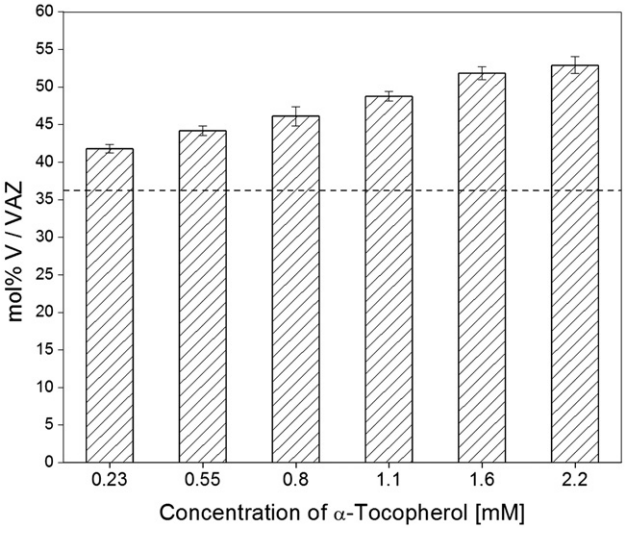

Fig. 6. The effect of α-tocopherol on the amount of violaxanthin in the XC. The concentration of α-tocopherol ranged between 0.23 and 2.2 mM in the course of violaxanthin conversion. The amount of violaxanthin (V) was measured as mol % of the total XC-pool pigments (VAZ).The dashed line indicates the value of the control sample (36.5 mol%) after 90 min de-epoxidation reaction at 25 °C. Data are shown as average±standard error for 3 independent sets of experiments.

fluid, liquid-ordered (L_β) regions (with or without light harvesting proteins involved). In this model, violaxanthin would be converted in the fluid region [40], but violaxanthin present in the liquid-ordered regions or bound to light-harvesting proteins would be 'inaccessible'. It is therefore reasonable that temperature and modifiers affect the proportion between fluid and liquid crystalline regions, and hence the maximum degree of conversion. To test this possibility, thylakoids were subjected to a temperature-jump experiment (Fig. 7).

Violaxanthin de-epoxidation was first run at 4 °C for 120 min, to reach essentially full conversion at that temperature. The enzyme reaction was reversibly stopped by either increasing the pH in the reaction medium (Fig. 7a) or by removing ascorbate (Fig. 7b). The temperature was then raised to 37 °C where more violaxanthin should be available. Here violaxanthin and zeaxanthin should have the possibility to exchange with 'inaccessible' violaxanthin whether it was present in the liquid crystalline regions of the membrane or bound to proteins (cf. Fig. 1). Finally, the temperature was again lowered and the conditions were changed back to low pH (Fig. 7a) or by addition of ascorbate (Fig. 7b) to resume VDE activity. Remarkably, no further conversion was observed. If it were just a question of availability a significant increase in the amount of zeaxanthin would be expected. In this respect, the thylakoids appear to behave like a state function. Our interpretation is that the intrinsic parameters of the membrane at the specified temperature are the determining factor for VDE activity and not the history of the system.

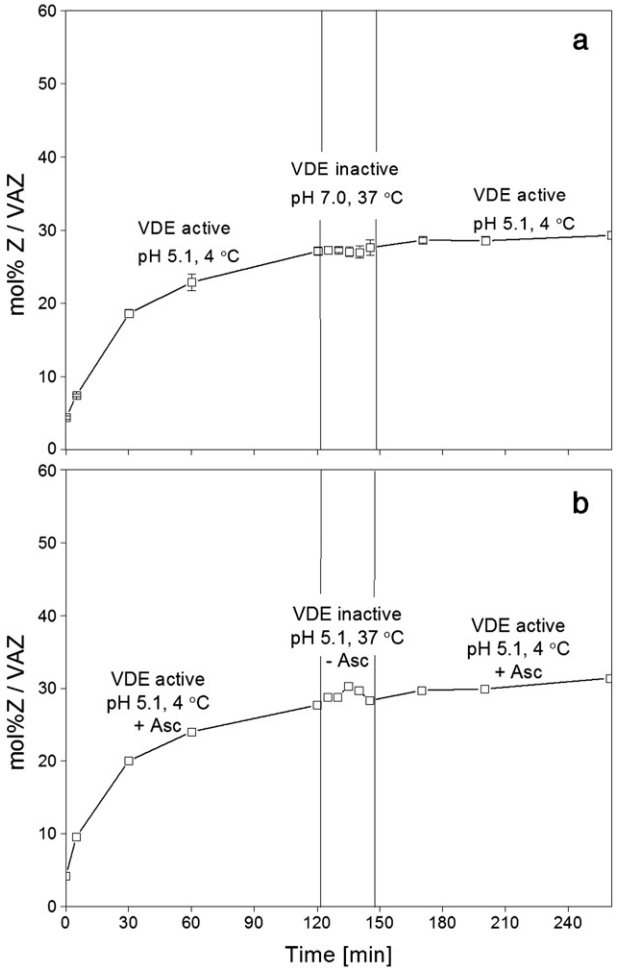

Fig. 7. Effect of temperature-jump on the amount of zeaxanthin formed in the XC. VDE was active at pH 5.1 (4 °C) for 120 min then the formation of zeaxanthin (Z) was stopped for 20 min either (a) by adjusting the pH in the medium to 7.0 or (b) removal of ascorbate. At the same time the temperature was raised to 37 °C to allow pigment-exchange between the liquid-ordered (Lα) and the liquid-disordered (L_β) regions. Afterwards, violaxanthin conversion was resumed at 4 °C by lowering the pH to 5.1. The amount of zeaxanthin was measured as mol% of the total XC-pool pigments (VAZ). Error bars denote standard error for two individual sample sets.

4. Discussion

The results presented in this paper show that in the isolated spinach thylakoid system lipid packing modifiers and temperature had a strong effect on the degree of violaxanthin conversion. The question, however, remains why conversion became restricted at a specific level. The temperature-jump experiments (Fig. 7) show that the restricted conversion of violaxanthin was not due to accessibility limitations. Instead, we suggest that the activity of VDE is controlled by the intrinsic curvature stress of the membrane and that zeaxanthin affects membrane packing and thus indirectly inhibit VDE (Fig. 8).

It is known that violaxanthin de-epoxidase requires lipids that form inverted hexagonal phase [22] regardless of their chemical nature [23]. Physical properties of membrane lipids are greatly affected by temperature. At low temperatures hydrocarbon chains in the lipid molecules are highly ordered and the overall structure is lamellar. By increasing the temperature, the motional freedom of hydrocarbon tails is facilitated; the membrane becomes more fluid, liquid-like lamellar phase and less tightly packed. At even higher temperatures lipids can give rise to non-lamellar structures such as the H_{II} phase [26]. In the thylakoid membrane both lamellar and non-lamellar structure-forming lipids exist, mainly DGDG (30%) and MGDG (50%) [26]. Fatty acyl chains in both galactolipids are mostly unsaturated but the lipids are heterogeneous and consist of 10% with a high melting temperature [26]. Therefore, the thylakoid membrane does not give a specific melting point. MGDG, with a small polar head group and a bulky tail, is a conically shaped molecule. It forms inverted hexagonal phase (H_{II}) upon hydration at physiological temperatures. DGDG with a larger head group and a tail area equal to the head group is more cylindrical in shape and therefore form lamellar phase (L_α) structure under the same conditions. As mentioned in Introduction, the high amount of MGDG in the thylakoid membrane is expected to impose negative intrinsic curvature stress to the membrane and the existence of inverted hexagonal phases in the thylakoid membranes has even been indicated by various methods [41,42].

Goss et al. [43] suggested that the presence of inverted hexagonal phase forming lipids is crucial for both pigment solubilisation and VDE activity [43]. Moreover, they hypothesised that lateral segregation of MGDG into proper H_{II} structures within the plane of the membrane occurs upon high light providing a preferred docking site for the pH-activated VDE. Also, violaxanthin would migrate preferentially into these

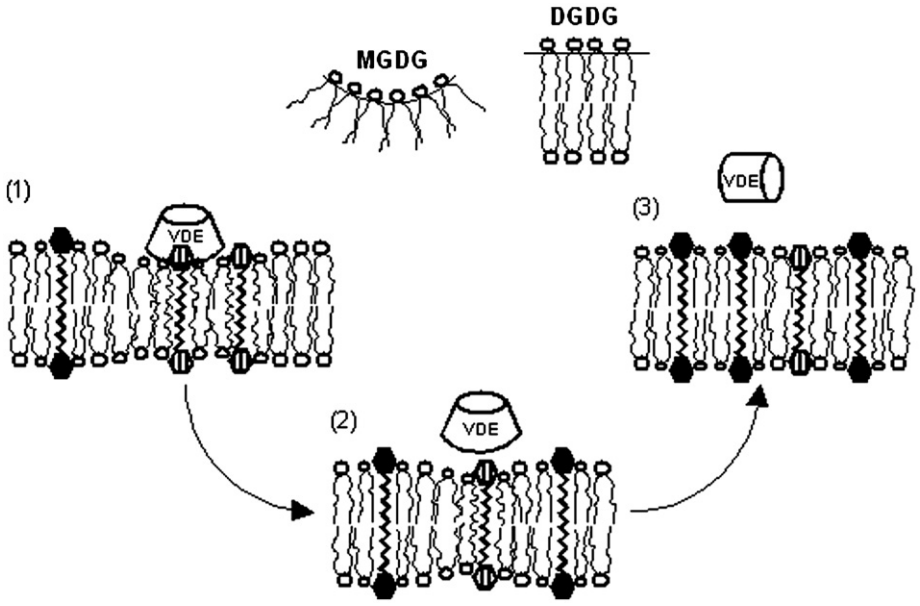

Fig. 8. Schematic model of the effect of curvature stress on XC. The thylakoid membrane is composed of 50 mol% MGDG (H_{II} lipid) and 30 mol% DGDG (Lα). MGDG has a high propensity for interfacial curvature. Upon high light, the formation of the MGDG-rich regions gives rise to curvature stress in the bilayer. MGDG serves as an efficient host for violaxanthin (hatched hexagon) and is also required by the enzyme, VDE (truncated cone). (1) The enzyme docks to the membrane and converts violaxanthin to zeaxanthin (filled hexagon). (2) As the conversion proceeds more hydrophobic and stretched zeaxanthin is formed, (3) the membrane expands and brings about a release of curvature stress leading to a less favoured lipid environment for VDE (cylinder). Elevated temperature and the presence of linolenic acid facilitate zeaxanthin formation, whereas low temperature, the presence of other membrane modifiers such as 8-cethylether and α-tocopherol during the conversion retarded the formation of zeaxanthin.

MGDG-enriched regions. Our results corroborate the model of Goss et al. [43]. However, we prefer to interpret our findings in terms of intrinsic curvature stress in the membrane, as this is more compatible with the rapid response of violaxanthin conversion to temperature-shift and membrane modifiers.

Zeaxanthin has a crucial role in our model. It has a longer hydrophobic stretch (11 double bonds instead of 9 in violaxanthin), and should therefore govern a stretching of the structure of the membrane lipids. This would lead to a more densely packed and a slightly thicker membrane relieving the intrinsic curvature stress caused by MGDG. The reduction in curvature stress is then thought to inactivate VDE. This could explain why the level of zeaxanthin remained unchanged even when VDE was active again at 4 °C. This resembles the flexible surface model on rhodopsin function [27,28] including the effects of lipid polymorphism and membrane curvature stress.

Our results are most easily explained if XC-pigments are located in the lipid part of the membrane. However, the localization of these pigments is controversial. In one view, XC-pigments are free in the membrane. The most important observation supporting this view is the lack of correlation between the XC-pigments and pigment-protein complexes. Numerous studies have shown that light stress within days lead to a drastic increase in the XC-pigments (e.g. [18], and references therein). At the same time the amount of light harvesting complexes decrease or stay constant. Also, fractionation of mechanically fragmented thylakoids in the absence of detergents shows an almost random distribution of XC-pigments despite drastic differences in the type of light-harvesting complexes found in the different fractions [19]. All other pigments showed clear preferences for different membrane regions.

The other view is that the XC-pigments are bound to the light-harvesting complexes, although the binding is generally thought to be weak. The strongest argument for proteins binding XC-pigments is the presence of violaxanthin in the crystal structure of LHCII [14,15]. Another argument is that the process of NPQ occurs in the antenna system and requires zeaxanthin. If all XC-pigments, or just the strongly bound pigments are bound to proteins we have to explain how the treatments made in our work could affect the binding.

Changes in temperature could change the equilibrium between free and bound violaxanthin. However, the change by temperature should be gradual and still the bound violaxanthin should be so strongly bound that it does not exchange within hours with free xanthophylls. Another possibility would be that there are many different binding sites with different temperature thresholds for release of pigments, but we are not aware of any results that point in that direction.

The effect of additives (fatty acids, cetylethers and α-tocopherol) might be argued to directly affect the binding site of violaxanthin in the light-harvesting complex. For linolenic acid and linoleic acid the increase in violaxanthin conversion could be explained by a direct competition for the binding site. However, oleic acid and stearic acid, although similar in structure, could not increase the conversion of violaxanthin (Fig. 3). It is even more difficult to explain how cetylethers and α-tocopherol could increase binding of violaxanthin through a direct effect on the binding site. We suggest a compromise where most observations can be accommodated. In this model, a

significant part of the XC-pigments are free in the membrane at any particular moment but at the same time in rapid equilibration with all XC-pigments bound to the light-harvesting complexes. The final level of conversion would be reached because VDE becomes inactivated when the intrinsic curvature stress of the membrane is relieved through zeaxanthin formation. Additives would just change the amount of zeaxanthin required to relieve the curvature stress.

Interesting questions for future research would be to determine the membrane thickness of thylakoids in the violaxanthin and zeaxanthin form, respectively, and to study how the binding properties of VDE to thylakoid membranes are controlled by different levels of curvature stress.

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References

[1] A. Gisselsson, A. Szilágyi, H.-E. Åkerlund, Role of histidines in the binding of violaxanthin de-epoxidase to thylakoid membrane as studied by site-directed mutagenesis, Physiol. Plant. 122 (2004) 337–343.
[2] B. Demmig, K. Winter, A. Krüger, F.-C. Czygan, Photoinhibition and zeaxanthin formation in intact leaves, Plant Physiol. 84 (1987) 218–224.
[3] R.B. Peterson, E.A. Havir, Photosynthetic properties of an *Arabidopsis thaliana* mutant possessing a defective PsbS gene, Planta 214 (2001) 142–152.
[4] X.P. Li, A. Phippard, J. Pasari, K.K. Niyogi, Structure–function analysis of photosystem II subunit S (PsbS) in vivo, Funct. Plant Biol. 29 (2002) 1131–1139.
[5] B. Demmig-Adams, W.W. Adams, The role of xanthophyll cycle carotenoids in the protection of photosynthesis, Trends Plant Sci. 1 (1996) 21–26.
[6] M. Eskling, P.O. Arvidsson, H.-E. Åkerlund, The xanthophyll cycle, its regulation and components, Physiol. Plant. 100 (1997) 806–816.
[7] J. Grzyb, D. Latowski, K. Strzalka, Lipocalins — a family portrait, J. Plant Phyiol. 163 (2006) 895–915.
[8] A.M. Gilmore, H.Y. Yamamoto, Linear models relating xanthophylls and lumen acidity to non-photochemical fluorescence quenching. Evidence that antheraxanthin explains zeaxanthin-independent quenching, Photosynth. Res. 35 (1993) 67–78.
[9] H. Siefermann, H.Y. Yamamoto, Light-induced de-epoxidation of violaxanthin in lettuce chloroplasts. III. Reaction kinetics and effect of light intensity on de-epoxidase activity and substrate availability, Biochim. Biophys. Acta 357 (1974) 144–150.
[10] H. Siefermann, H.Y. Yamamoto, Light-induced de-epoxidation of violaxanthin in lettuce chloroplasts. IV. The effect of electron-transport conditions on violaxanthin availability, Biochim. Biophys. Acta 387 (1975) 149–158.
[11] A.V. Ruban, A. Pascal, P.J. Lee, B. Robert, P. Horton, Molecular configuration of xanthophyll cycle carotenoids in photosystem II antenna complexes, J. Biol. Chem. 277 (2002) 42937–42942.
[12] P. Jahns, A. Wehner, H. Paulsen, S. Hobe, De-epoxidation of violaxanthin after reconstitution into different carotenoid binding sites of light-harvesting complex II, J. Biol. Chem. 276 (2001) 22154–22159.
[13] A.S. Verhoeven, W.W. Adams, B. Demmig-Adams, R. Croce, R. Bassi, Xanthophyll cycle pigment localization and dynamics during exposure to low temperatures and light stress in Vinca major, Plant Physiol. 120 (1999) 727–738.
[14] Z. Liu, H. Yan, T. Kuang, J. Zhang, L. Gui, X. An, W. Chang, Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution, Nature 428 (2004) 287–292.
[15] R. Standfuss, A.C.T. van Scheltinga, M. Lamborghini, W. Kuhlbrandt, Mechanisms of photoprotection and nonphotochemical quenching in pea light-harvesting complex at 2.5 Å resolution, EMBO J. 24 (2005) 919–928.
[16] R. Croce, S. Weiss, R. Bassi, Carotenoid-binding sites of the major light-harvesting complex II of higher plants, J. Biol. Chem. 274 (1999) 29613–29623.
[17] A.V. Ruban, A.J. Young, A.A. Pascal, P. Horton, The effects of illumination on the xanthophyll composition of the photosystem-II light harvesting complexes of spinach thylakoid membranes, Plant Physiol. 104 (1994) 227–234.
[18] M. Eskling, H.-E. Åkerlund, Time course of changes in the quantities of violaxanthin de-epoxidase, xanthophylls and ascorbate in spinach upon shift from low to high light, Photosynth. Res. 57 (1998) 41–50.
[19] P.O. Arvidsson, M. Carlsson, H. Steffánsson, P.Å. Albertsson, H.-E. Åkerlund, Violaxanthin accessibility and temperature dependency for de-epoxidation in spinach thylakoid membranes, Photosynth. Res. 52 (1997) 39–48.
[20] D. Latowski, J. Kruk, K. Burda, M. Skrzynecka-Jaskier, A. Kostecka-Gugala, K. Strzalka, 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 (2002) 4656–4665.
[21] E.A. Havir, S.L. Tausta, R.B. Peterson, Purification and properties of violaxanthin de-epoxidase from spinach, Plant Sci. 123 (1997) 57–66.
[22] H.Y. Yamamoto, R.M. Higashi, Violaxanthin de-epoxidase—Lipid composition and substrate specificity, Arch. Biochem. Biophys. 190 (1978) 514–522.
[23] D. Latowski, H.-E. Åkerlund, K. Strzalka, Violaxanthin de-epoxidase, the xanthophyll cycle enzyme, requires lipid inverted hexagonal structures for its activity, Biochemistry 43 (2004) 4417–4420.
[24] H.Y. Yamamoto, Functional roles of the major chloroplast lipids in the violaxanthin cycle, Planta 224 (2006) 719–724.
[25] P.J. Quinn, W.P. Williams, Plant lipids and their role in membrane-function, Prog. Biophys. Mol. Biol. 34 (1978) 109–173.
[26] E. Selstam, A.W. Wigge, Chloroplast lipids and the assembly of membranes, in: C. Sudnqvist, M. Rydberg (Eds.), Pigment–Protein Complexes in Plastids — Synthesis and Assembly, Academic Press, ISBN: 0-12-676960-5, 1993, pp. 241–277.
[27] N.J. Gibson, M.F. Brown, Lipid headgroup and acyl chain composition modulate the MI–MII equilibrium of rhodopsin in recombinant membranes, Biochemistry 32 (1993) 2438–2454.
[28] A.V. Botelho, N.J. Gibson, R.L. Thurmond, Y. Wang, M.F. Brown, Conformational energetics of rhodopsin modulated by non-lamellar-forming lipids, Biochemistry 41 (2002) 6354–6368.
[29] G.S. Attard, R.H. Templer, W.S. Smith, A.N. Hunt, S. Jackowski, Modulation of CTP:phosphocholine cytidyltransferase by membrane curvature elastic stress, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 9032–9036.
[30] S. Hubner, A.D. Couvillon, J.A. Kas, V.A. Bankaitis, R. Vegners, C.L. Carpenter, P.A. Janmey, Enhancement of phosphoinositide 3-kinase (PI 3-kinase) activity by membrane curvature and inositol-phospholipid-binding peptides, Eur. J. Biochem. 258 (1998) 846–853.
[31] A.E. Drobnies, S.M.A. Davies, R. Kraayenhof, R.F. Epand, R.M. Epand, R.B. Cornall, CTP:phosphocholine cytidyltransferase and protein kinase C recognize different physical features of membranes: differential responses to an oxidized phosphatidylcholine, Biochim. Biophys. Acta-Biomemb. 1564 (2002) 82–90.
[32] R.J. Porra, The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b, Photosynth. Res. 73 (2002) 149–156.
[33] S.S. Thayer, O. Björkman, Leaf xanthophyll content and composition in sun and shade determined by HPLC, Photosynth. Res. 23 (1990) 331–343.
[34] J. Prades, S.S. Funari, P.V. Escribá, F. Barceló, Effects of unsaturated fatty acids and triacylglycerols on phosphatidylethanolamine membrane structure, J. Lipid Res. 44 (2003) 1720–1727.
[35] W.I. Gruszecki, Z. Krupa, LHCII, the major light-harvesting pigment–protein complex is a zeaxanthin-epoxidase, Biochim. Biophys. Acta 1144 (1993) 97–101.
[36] R.G. Hiller, J.K. Raison, The fluidity of chloroplast thylakoid membranes

and their constituent lipids—A comparative-study by electron-spin-resonance, Biochim. Biophys. Acta 599 (1980) 63–72.
[37] Å. Wieslander, L. Rilfors, G. Lindblom, Metabolic changes of membrane lipid composition in *Acholeplasma laidlawii* by hydrocarbons, alcohols and detergents: arguments for effects on lipid packing, Biochemistry 25 (1986) 7511–7517.
[38] S. Munne-Bosch, L. Alegre, The function of tocopherols and tocotrienols in plants, Crit. Rev. Plant Sci. 21 (2002) 31–57.
[39] M. Havaux, J.-P. Bonfils, C. Lütz, K.K. Niyogi, Photodamage of the photosynthetic apparatus and its dependence on the leaf development as stage in the *npq1 Arabidopsis* mutant deficient in the xanthophyll cycle enzyme violaxanthin de-epoxidase, Plant Physiol. 124 (2000) 273–284.
[40] A. Wisniewska, W.K. Subczynski, Effects of polar carotenoids on the shape of the hydrophobic barrier of phospholipid bilayers, Biochim. Biophys. Acta 1368 (2006) 235–246.
[41] A. Sen, W.P. Williams, A.P.R. Brain, M.J. Dickens, P.J. Quinn, Formation of inverted micelles in dispersions of mixed galactolipids, Nature 293 (1981) 488–490.
[42] K. Haraczyk, K. Strzalka, W. Dietrich, J.S. Blicharski, ^{32}P-NMR observation of the temperature and glycerol induced non-lamellar phase formation in wheat thylakoid membranes, J. Biol. Phys. 21 (1995) 125–139.
[43] R. Goss, D. Latowski, J. Grzyb, A. Vieler, M. Lohr, C. Wilhelm, K. Strzalka, Lipid dependence of diadinoxanthin solubilization and de-epoxidation in artificial membrane systems resembling the lipid composition of the natural thylakoid membrane, Biochim. Biophys. Acta 1768 (2007) 67–75.