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Review

Mechanism and regulation of the violaxanthin cycle: The role of antenna proteins and membrane lipids

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ABSTRACT

The violaxanthin cycle describes the reversible conversion of violaxanthin to zeaxanthin via the intermediate antheraxanthin. This light-dependent xanthophyll conversion is essential for the adaptation of plants and algae to different light conditions and allows a reversible switch of photosynthetic light-harvesting complexes between a light-harvesting state under low light and a dissipative state under high light. The photoprotective functions of zeaxanthin have been intensively studied during the last decade, but much less attention has been directed to the mechanism and regulation of xanthophyll conversion. In this review, an overview is given on recent progress in the understanding of the role of (i) xanthophyll binding by antenna proteins and of (ii) the lipid properties of the thylakoid membrane in the regulation of xanthophyll conversion. The consequences of these findings for the mechanism and regulation of xanthophyll conversion in the thylakoid membrane will be discussed.

1. Introduction

Aerobic photosynthetic organisms have developed a number of strategies to avoid or minimize damage related to the light-induced formation of reactive oxygen species (=photo-oxidative stress). Among those, the dissipation of heat by carotenoids represents the most important photoprotective mechanism active in thylakoid membranes of plants and algae. The deactivation of excited triplet chlorophyll (^{3}Chl) and singlet oxygen ($^1O_2$) in the reaction centre and antenna proteins is constitutively provided by tightly bound carotenoids (e.g. β-carotene or lutein). In addition to this constitutive protection mechanism, the light-regulated and reversible induction of a dissipative state in the antenna of photosystem II (PSII) is facilitated by the action of so-called xanthophyll cycles in plants and algae, and allows a reversible switch of photosynthetic light-harvesting complexes between a light-harvesting state under low light and a dissipative state under high light. Three different xanthophyll cycles have been described in the literature: (i) the violaxanthin cycle (Vx-cycle; in all plants and green algae) [1], (ii) the diadinoxanthin cycle (Ddx-cycle; in some algae) [2] and (iii) the lutein-epoxide cycle (Lx-cycle; found in some plant species) [3,4] (Table 1). In all three cycles, high light induces the de-epoxidation of the respective xanthophylls (violaxanthin, diadinoxanthin and lutein-epoxide) and by that the reduction of the light-harvesting efficiency in the antenna. By contrast, the reconversion (=epoxidation) of the formed xanthophylls (zeaxanthin, diatoxanthin and lutein, respectively) in low light (or darkness) reverses these changes. In this review, we will focus on the current understanding of the mechanism and regulation of the Vx-cycle and its relation to photoprotective functions.

2. The basic reactions of the Vx-cycle

The Vx-cycle (Fig. 1) is distributed among all higher plants and some green algae [1] and was the first xanthophyll cycle which has been described in the literature [5]. Pioneering work by the groups of Yamamoto and Hager in the 1960th and 1970th established the basic biochemical characteristics of the Vx-cycle [6] in which violaxanthin (Vx) is reversibly converted to zeaxanthin (Zx) via the intermediate

Abbreviations: ASC, ascorbate; ASCH, acid form of ascorbate; Ax, antheraxanthin; Chl, chlorophyll; Ddx, diadinoxanthin; DHA, dehydroascorbate; DGDG, digalactosyldiacylglycerol; Dtx, diatoxanthin; LHCII, light-harvesting complex of photosystem II; MGDG, monogalactosyldiacylglycerol; NPQ, non-photochemical quenching; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PSII, photosystem II; VAZ, sum of violaxanthin, antheraxanthin and zeaxanthin; Vx, violaxanthin; VDE, violaxanthin de-epoxidase; Zx, zeaxanthin; ZE, zeaxanthin epoxidase

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antheraxanthin (Ax) [7]. These two-stepped reactions, during which two epoxy groups are stepwise removed (in the de-epoxidation reactions) or inserted (in the epoxidation reactions), are catalyzed by two different enzymes which are located on the opposite sites of the thylakoid membrane: the Vx de-epoxidase (VDE) in the thylakoid lumen and the Zx epoxidase (ZE) in the chloroplast stroma (Fig. 1). The VDE activity – and thus Zx formation – is strictly regulated by the pH of the thylakoid lumen [6]. The enzyme becomes activated at pH values below 6.2 [8,9] and requires ascorbate as cofactor [10]. The conversion of Vx to Zx proceeds in about 10–30 min under both *in vivo* and *in vitro* conditions [11]. The regulation of the VDE activity by the lumen pH ensures that Zx accumulates only at saturating light intensities under *in vivo* conditions. In low light (or darkness) Zx is reconverted to Vx by the ZE [12]. These epoxidation reactions are about 5 to 10 times slower than the de-epoxidation reactions [13,14] The ZE is most likely localized at the stroma side of the thylakoid membrane. Up to now, the regulation of the ZE activity is not well understood. However, down-regulation of the ZE activity under extreme photo-oxidative stress conditions in the long-term [15] and short-term [16,17] has been reported.

3. The functions of the Vx-cycle

The possible function of the Vx-cycle remained unclear until a role of Zx in the dissipation of excess light energy in PSII was proposed in 1987 [18]. Since then numerous studies on the xanthophyll cycle, the possible function of Zx and the molecular mechanism of energy dissipation have been performed. The discovery and characterization of xanthophyll cycle mutants in *Chlamydomonas* [19] and *Arabidopsis* [20] represented a further breakthrough in the understanding of the function of the xanthophyll cycle and underlined the essential role of Zx in photoprotection. According to present understanding, Zx plays a central role in different photoprotective mechanisms in chloroplasts: It contributes essentially to the dissipation of excess excitation energy (=non-photochemical quenching, NPQ) in the antenna of PSII (so-called qE-mechanism) [18,20] and additionally acts as antioxidant in the lipid phase of the thylakoid membrane or the protein/lipid interface [21–23]. Furthermore, Zx is supposed to be involved in more slowly developing NPQ processes (=qI-component of NPQ) which may et least partially be related to photoinhibitory processes in the reaction centre of PSII [24,25]. The exact role of Zx in the qE-mechanism of NPQ is still under debate. It is particularly unclear whether Zx has a direct [26,27] or indirect [28,29] function in these processes. Although it is generally assumed that most (if not all) of the Vx-cycle pigments (VAZ-pool) are bound to the antenna proteins of both photosystems [30–32], the different photoprotective functions of Zx are likely to require different specific sites of action.

Another postulated function of carotenoids is their involvement in the modulation of the molecular dynamics of membranes (for review see [33]). Vx and Zx are among those xanthophylls which strongly affect the thermodynamic parameters of membranes [34].

Table 1
Occurrence of different forms of the xanthophyll cycle in photoautotrophic organisms [1–4]

Violaxanthin cycle	Diadinoxanthin cycle	Lutein-epoxide cycle	No xanthophyll cycles
Higher plants	Bacillariophyceae	*Cuscuta reflexa*	Cyanobacteria
Ferns	Chrysophyceae	*Amyema miquelli*	Photosynthetic bacteria
Mosses	Xanthophyceae	*Quercus* sp.	Most Rhodophyta
Phaeophyta	Rhaphidophyta	*Inga* sp.	Cryptophyta
Chlorophyta	Dinophyceae		
Some Rhodophyta	Euglenophyta		

4. The two enzymes involved in the Vx-cycle: structural aspects and cofactors

4.1. Violaxanthin de-epoxidase

VDE and ZE are two of the six known plant lipocalin proteins. So far, a biological function of plant lipocalins was described only for VDE and ZE [35,36]. Lipocalins have been widely described for animals [37] and *Procaryota* [38] and are characterized by a similar tertiary structure and similar functions [39]. Lipocalins are supposed to contain eight antiparallel β-sheets and three highly conservative motifs, known as SCR [40]: Motif I is composed of the first of the eight β-sheets and a short fragment of the preceding α-helix. Motif II is composed of the loop between β-sheets six and seven and parts of the end of β-sheet six and the beginning of β-sheet seven. Motif III is composed of the end of β-sheet eight and part of the C-terminal α-helix including the loop between both fragments. VDE and ZE are conserved in two or only one SCR, respectively [41].

Crystallographic research of known lipocalins showed that the characteristic structure of β-sheets is responsible for creation of a deep, conical hollow, necessary for substrate binding. The depth of the hollow in examined proteins is about 40 Å [42,43]. The structure is typical for lipocalins and is associated with their function. All proteins belonging to this class are able to bind and carry small hydrophobic molecules [44]. The presence of the hollow in VDE molecules was already predicted [7]. The depth of this hollow fits the length of Vx molecule and this is why VDE is strictly specific to 3 OH, 5,6-epoxycarotenoids in configuration 3R, 5S, 6R [7,45]. Aside from VDE and ZE there is only one other known lipocalin with enzymatic activity — prostaglandin D synthase [46].

VDE is encoded in the nucleus. In 1996, the cDNA of VDE was cloned for the first time and expressed in *Escherichia coli* [47]. These experiments allowed to determine the number of amino acids (348 residues), and to calculate the molecular mass of VDE with 39.9 kDa, which is close to the molecular mass of 43 kDa determined by SDS-PAGE [48–50]. The calculated isoelectric point is 4.57, as compared with the 5.4 found experimentally [49].

Besides the lipocalin domain, two other characteristic domains exist in VDE: First, the N-terminal region enriched in cystidyl moieties (11 out of 13 present in VDE). This region is most probably composed of α-helices. Second, the C-terminal domain which is charged and enriched in glutamyl residues, and probably composed of long α-helices. Comparative analysis of the amino acid sequences (deduced from cDNA) indicates a high degree of homology among VDE proteins from different plants. VDEs from *Arabidopsis thaliana*, *Nicotiana tabacum* and *Lactuca sativa* differ just in nine amino acid residues [36]. Knowledge of the amino acid sequence allowed the prediction of some specific properties of VDE in relation to the primary structure. The cysteine enriched domain is likely to be responsible for inhibitory effect of dithiothreitol, which reduces the disulphide bonds in the enzyme molecule [47,51]. The inhibition of VDE by dithiothreitol (at pH 5.2 and 5.7) is reversible, while inhibition by iodoacetamide treatment is not [48]. When the treatment by inhibitors is performed at pH 7.2, the chemicals do not influence the enzyme activity, which indicates that the disulphide bonds may be not exposed to the surrounding bulk phase at neutral pH values. Thus pH-dependent conformational changes in the VDE molecule are required for enzymatic activity. In general, the activity of isolated VDE can easily be measured under *in vitro* conditions at a pH<5.8 in presence of Vx, monogalactosyldiacylglycerol (MGDG) and ascorbic acid [7,45,47–51].

4.2. Zeaxanthin epoxidase

The ZE is the second identified plant lipocalin. Its cDNA was first characterized for *Nicotiana plumbaginifolia* [14] and later for *Capsicum annum* [12], *Lycopersicon esculentum* [52], *Arabidopsis thaliana* and

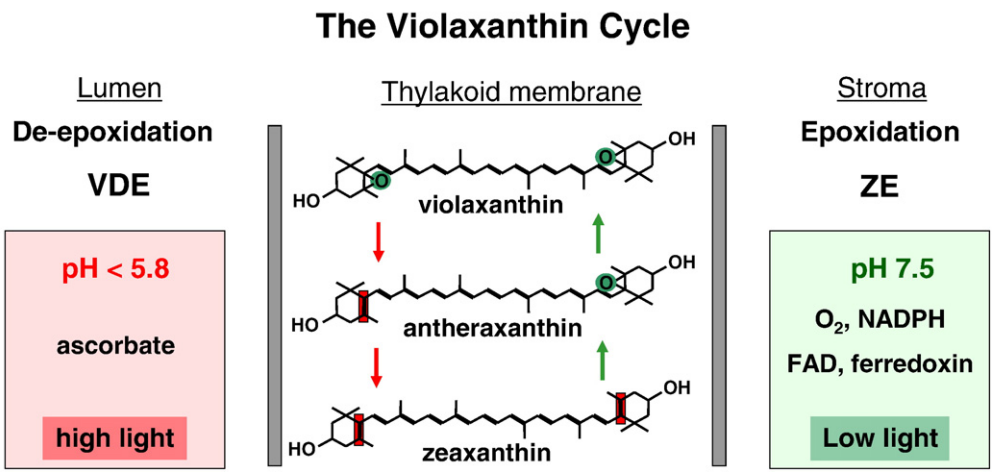

Fig. 1. Scheme of the reactions of the xanthophyll cycle.

Prunus armeniaca [36]. Like the VDE, also the ZE sequence reveals characteristic lipocalin features, but differs from the VDE with respect to the number of amino acids between motifs I and II. In case of ZE from *Nicotiana* 103 residues exist in this region, but 65 to 73 residues were found in ZEs from other organisms. The increase in amino acid number provides evidence for a longer loop or the existence of an additional β-sheet in that region of the protein [35]. The degree of homology between motif II and III of ZE and the respective motifs of other lipocalins seems to be rather low [37,53]. The homology to VDE is also low [36]. However, it should be noticed that motif I of different ZEs (except for ZE of *Prunus*) aligns well with other lipocalins. In this region, an unusual cysteine moiety (subsequent to tryptophan and tyrosine residues) was found to be conserved in every known lipocalin [36]. Besides the similarity in the tertiary structure, no similarities among the amino acid sequences of ZE and VDE are given. ZE is thought to be a monooxygenase, catalysing the epoxidation in positions 5 and 6 of xanthophyll rings [36]. In plants, this process occurs in the dark or in weak light. Some data indicate, that epoxidation can be observed also in high light [54–56]. The optimum pH for ZE activity is 7.5 [12,54]. Until now, in spite of many attempts, an active form of this enzyme has not been isolated. Only in one case over-expression of an active ZE in *Escherichia coli* has been reported [14].

4.3. Cofactors of the Vx-cycle enzymes

4.3.1. Ascorbate

The VDE requires ascorbate (ASC) as reductant to convert Vx to Ax and further to Zx [6,7]. Bratt et al. [57] showed that the optimum ASC concentration for VDE activity is strongly pH-dependent. At pH 4.5–5.5 the enzyme becomes saturated at 10–20 mM ASC, whereas at pH 6.0 it is not saturated below 100 mM (Table 2). The ASC concentration in chloroplasts is supposed to be in the range of 10–50 mM [58–60] although it may increase in response to stress factors such as high light irradiances and chilling [60,61].

The ASC-dependent pH optimum is similar for both VDE located in the thylakoids and partially purified enzyme VDE [7], although the partially purified enzyme shows a broader pH optimum and a shift to higher pH in comparison with VDE in thylakoids. These differences may be caused by the limitation of ASC passage across the thylakoid membrane at higher pH, due to the negative charge of the base form of ASC. These results suggested that VDE has a pH-dependent K_M for ASC (Table 3) and that ASC is not simply a cofactor but a co-substrate for VDE [57,62].

Interestingly, all determined values of the K_M for ASC fell within the range of 0.10±0.02 mM when ASC concentrations are expressed for the acid form of ASC (ASCH) and assuming a pK_a of 4.1. This suggests that not the negatively charged ASC but rather the acidic form ASCH is the substrate for VDE.

ASCH as a protonated form of ASC is an endogenous electron and proton donor for de-epoxidation and activates VDE [7,57,62–64]. VDE catalyzes electron and proton transfer from ASCH to one epoxide group of Vx in the first step and to the epoxide group of Ax in the second step, by that creating Zx, water and oxidized dehydroascorbate (DHA) [6]. Since no mechanism engaged in rereduction of DHA into ASC in thylakoid lumen is known, the existence of a DHA transporter in the thylakoid membrane has been postulated [57] which may allow the rereduction of DHA to ASC in the stroma via the glutathione cycle consuming NADPH and glutathione. Because the negatively charged form of ASC may not penetrate the membrane by simple diffusion, the transport of ASC into thylakoid lumen might be facilitated by a postulated DHA transporter in exchange for DHA [62]. Protonation of ASC to create the required ASCH for de-epoxidation would finally be possible at a low lumen pH as given in the light. The requirement of an ASC transporter in the thylakoid membrane and the possible limitation of VDE activity by ASC consuming reactions in the stroma have further been supported by studies with isolated chloroplasts [64].

Grouneva et al. [65] found that partially purified diadinoxanthin de-epoxidase (DDE) has a 3–4 times higher affinity for the ASCH than VDE. The K_M value of DDE for ASC at pH 5 was determined to be 0.7 mM while the K_M for VDE was 2.3 mM. At high ASC concentrations a strong shift of the pH optimum towards higher pH values was observed and DDE was found to be still active at almost neutral pH values, allowing a very fast and strong response of the DDE activity to small pH changes in the thylakoid lumen [65].

4.3.2. Other cofactors

Other cofactors required for the Vx-cycle are related to the back reaction of the cycle catalyzed by ZE and include NADPH, FAD, ferredoxin and molecular oxygen [12,66–68]. Other epoxidases are known to require also additional proteins for their activity: For example, squalene epoxidase requires flavoprotein oxidase and NADPH-cytochrome P450 oxidase [12,69]. A similar requirement has been proposed for the ZE. Bouvier et al. [12] suggested that electrons from NADPH may be transferred through ferredoxin:$NADP^+$

Table 2
The pH-optimum of VDE activity in dependence on the ASC concentration [57]

ASC concentration [mM]	Optimum pH of VDE activity
0.5	4.7
3	4.8–4.9
30	5.0

Table 3
K_M-values for ASC of partially purified VDE in dependence of the pH [57,62]

pH	K_M for ASC [mM]
4.5	0.3
5.0	1.0
5.5	2.5
6.0	10

oxidoreductase to ferredoxin and further to FAD. Reduced FAD is able to bind an oxygen molecule and to form a hydroperoxiflavin intermediate. Cleavage of this intermediate may allow the incorporation of one oxygen atom into Zx and the reduction of the other to water [12]. In an analogous reaction, the formed Ax may then be converted to Vx in a second reaction [12].

5. The regulation of the Vx cycle

The light-dependent regulation of xanthophyll conversion is essentially required in plants to avoid photodamage of the photosynthetic apparatus in high light and to allow efficient light harvesting in low light. The functional importance of the Vx-cycle was established by studies with the xanthophyll cycle mutants *npq1* (defective VDE) and *npq2* (defective ZE) [20]. The suppression of Zx formation in *npq1* plants has been shown to result in an increased damage of PSII in the short- and long-term [20,21,70] which is accompanied by an increased rate of lipid peroxidation [21,71]. These data proved the general antioxidative function of Zx and underlined the role of Zx located in the lipid phase or at the protein/lipid interface. By contrast, the constitutive accumulation of high levels of Zx in *npq2* mutants leads to a higher NPQ level at low and moderate light intensities [20,70,72] and a more rapid generation of NPQ than in wild-type plants [20,72,73]. Furthermore NPQ relaxation was found to be slower in *npq2* plants than in the wild type [20,73]. These particular features of the NPQ dynamics in presence of permanent high levels of Zx are likely to be the cause for the observed reduction of growth in *npq2* under limiting light conditions when compared to the wild type [70,74]. In fact, it has been shown that *npq2* plants exhibit sustained energy quenching in the dark-adapted state in contrast to wild-type and *npq1* plants [74]. In addition to these changes in light utilization, *npq2* plants further show a reduced stability of LHCII trimers [70,74–77]. These observations underline the requirement for an efficient regulation of the Zx content in response to different light conditions.

5.1. The regulation of Vx de-epoxidation

The formation of Zx is essentially regulated by the lumen pH which controls the Vx conversion at different levels:

1. Prior to the activation of the VDE, the enzyme has to become tightly bound to the thylakoid membrane. VDE binding to the thylakoid membrane is induced at pH values below 6.5 [9,57] and the binding/release of the enzyme occurs with a cooperativity of about 4 (with respect to protons) and in a very narrow pH range of about 1 pH unit, with an inflection point of about 6.6 [57] (cf. also Section 5.4.1). At higher pH values (lumen pH > 7, i.e. at low light intensities or in the dark) the enzyme is supposed to be mobile within the lumen [9].
2. The VDE activity itself is regulated by the lumen pH. Activation of the VDE is induced upon binding of the enzyme to the membrane and highest activities are achieved at pH values ≤ 5.8. Also this process occurs in a highly cooperative manner with a cooperativity for protons of about 5.5 and an inflexion point around pH 6 [8,78]. These determinations from *in vitro* studies with spinach and pea chloroplasts have been supported recently by the characterization of the Arabidopsis *pgr1* mutant [79] which is unable to acidify the thylakoid lumen below pH 6.0 [80] and thus shows reduced rates of Vx de-epoxidation in comparison with wild-type plants.
3. The lumen pH further controls the pK of the VDE for the acid form of ascorbate, which is supposed to be the substrate for the VDE [57,62]. As a consequence, the pH-regulation of Vx de-epoxidation is also dependent on the ascorbate concentration in the chloroplast or, more precisely, in the thylakoid lumen. The importance of the ascorbate concentration under *in vivo* conditions has been underlined by studies of the ascorbate deficient Arabidopsis *vtc2* mutant which accumulates only about 25% of ascorbate wild-type levels [81]. In this mutant, the accumulation of Zx under high light was found to be reduced concomitant with the reduction of NPQ [82].
4. Finally, the lumen pH can be expected to control the release of Vx from the Vx binding sites (V1 or L2) in the different antenna proteins [32,83]. This aspect will be considered in more detail below (Section 5.3.).

In addition to the pH-regulation of the VDE activity, the conversion of Vx might be expected to be controlled by the expression level of the VDE, particularly since a very low amount of VDE with about 1 VDE per 20–100 electron transport chains has been estimated to be present in spinach chloroplasts [48]. However, only a slight increase in the rate of Vx de-epoxidation has been reported for tobacco plants over-expressing the *Arabidopsis* VDE, although a nearly 20fold increase of the specific VDE activity was found in such plants [84] and similar results were obtained upon homologous over-expression of the tobacco VDE [85]. These observations argue against a limitation of Vx de-epoxidation by the amount of VDE as has also been derived from studies with isolated *Arabidopsis* thylakoids showing that addition of isolated VDE does not lead to an increased rate of Vx conversion [86]. Nonetheless, the expression levels of VDE have been shown to vary in response to growth light intensities and concomitant changes in the VAZ pool size [87]. However, analysis of the expression of the VDE gene during adaptation of *Arabidopsis* plants to excess light provided evidence that the activity of VDE is regulated predominantly on the post-transcriptional level although expression of the VDE gene changed diurnally [88].

It has further been suggested by Yamamoto and co-workers that Vx exchange between thylakoid membranes and the chloroplast envelope as well as feedback inhibition of VDE activity by Zx could be involved in the regulation of the Vx-cycle activity [89]. Although the exchange of Vx (but not Zx) between the envelope and thylakoid membrane has already been postulated earlier [90], direct experimental evidence for such a mechanism is still missing. To what extent feedback inhibition of VDE activity by Zx may contribute to the regulation of the Vx de-epoxidation under *in vivo* conditions is unclear.

A very specific regulation of Vx de-epoxidation has been reported for the Vx-cycle in the prasinophyceaen alga *Mantoniella squamata* [56,91]. Under *in vivo* conditions, i.e. in high light, Vx can only be converted to Ax but not to Zx in this alga, although Zx formation can artificially be induced by low pH treatment [91]. This limited VDE activity has been related to a lower substrate specificity of the VDE to Ax and a much faster epoxidation rate in *Mantoniella squamata* in comparison with higher plants [56].

Another important factor that is supposed to control the formation of Zx in the de-epoxidation reactions is the lipid environment given in thylakoid membranes. In fact, it was discovered during the last years in studies with model lipid bilayers and artificial membrane systems that the lipid properties of the thylakoid membrane have a strong impact on xanthophyll conversion [92–96]. Closely related to this feature is the notion that the kinetics of Vx de-epoxidation is likely to be limited by xanthophyll diffusion within the membrane [86]. The latter assumption is supported by the fact that the first step of de-epoxidation (the Vx

to Ax conversion) exhibits a 4–6 times higher rate constant than the second step (the Ax to Zx conversion) [13,97,98] and thus represents the rate-limiting step of de-epoxidation. These topics will be considered in more detail below (see Sections 5.3 and 5.4.).

5.2. The regulation of Zx epoxidation

In contrast to the case of the VDE the knowledge for the regulation of the ZE is rather poor. In general the ZE activity seems to be constitutive. The ZE is not inhibited by the transmembrane pH-gradient [67,99] and under *in vivo* conditions also not regulated by the stromal pH [54]. Since ZE requires molecular oxygen as second substrate and NADPH as cofactor, both factors might principally control or limit the ZE activity. However, no reports are available so far, whether limiting O_2 might reduce the ZE activity under *in vivo* conditions. By contrast, a role of the NADPH concentration as a critical parameter for the ZE activity has been proposed in a study with the *nadk2* mutant of Arabidopsis [100]. This mutant, which is defective in a chloroplast NAD kinase and therefore contains only about 50% of the amount of NADPH found in wild-type plants, accumulates high levels of Zx even in low light and in the dark [100]. But NADPH limitation of Zx epoxidation under *in vivo* conditions in wild-type plants has not been reported in the literature so far.

It has been shown, however, that Zx epoxidation can be down-regulated after exposure of plants to high light conditions [17,24,25]. In extreme cases of severe stress, like in over-wintering evergreen plants, high levels of Zx can be permanently retained during winter along with sustained down-regulation of photosystem II (PSII) activity [101]. The molecular basis of this down-regulation is unclear but there is some experimental evidence that phosphorylation reactions might be involved in these processes: A direct inactivation of the ZE activity by phosphatase inhibitors has been found in rice [102] and the phosphorylation of the D1 protein of PSII and the activation of TLP40 – a phosphatase inhibitor located in the thylakoid lumen – has been correlated with the inhibition of Zx epoxidation under severe light stress conditions [103,104]. Recent work on *Arabidopsis* mutants *stn7* and *stn8*, which are defective in thylakoid protein phosphorylation provided evidence that the phosphorylation of PSII proteins is not directly involved in the down-regulation of ZE activity [17]. It was further shown in the same work that ZE activity is stepwise down-regulated in the short-term concomitant with increasing photo-oxidative stress [17]. The molecular mechanism giving rise to the inactivation, however, remained unclear. By contrast, a recent study with duckweed (*Lemna trisulca*) showed that amino sugars are efficient inhibitors of Zx epoxidation and it was speculated that this class of substances may be involved in the sustained down-regulation of Zx epoxidation under *in vivo* conditions [93]. The inhibition of ZE activity by cadmium has been related to the interaction of heavy metals with cysteine residue located in motif I of ZE [105]. Thus clearly more work is required to identify the molecular processes that are responsible for the inhibition of Zx epoxidation under photo-oxidative stress conditions. Nonetheless, it is clear that the ZE activity, similar to the case of the VDE, is regulated on the post-transcriptional level. It has been shown that expression of the ZE gene is indeed regulated diurnally in *Nicotiana plumbaginifolia* [106], *Arabidopsis thaliana* [88] and *Lycopersicon esculentum* [107] but the expression level was found to be constant during the day/night-cycle in all cases. Moreover, over-expression of the ZE in tomato showed that the extent of Zx formation in high light was only partially reduced concomitant with a similar reduction in NPQ formation and an increased rate of Zx epoxidation [107]. These data indicate that the activity of both Vx-cycle enzymes – the VDE and the ZE – is not limited by the amount of the enzyme present in the chloroplast. Consequently, the interconversion of Vx and Zx, and thus the steady state level of Zx present in the membrane, must be controlled by other factors.

5.3. The role of antenna proteins

The binding of xanthophylls by antenna proteins of both photosystems is central to the regulation and function of the Vx-cycle for two reasons: (1) antenna proteins bind the substrates, Vx and Zx, of the two Vx-cycle enzymes, VDE and ZE, respectively and (2) antenna proteins are supposed to be the site for the Zx-dependent dissipation of excess light energy. The antenna of PSII is composed of trimeric LHCII (consisting of Lhcb1–3 proteins) and the three monomeric proteins Lhcb4 (CP29), Lhcb5 (CP26) and Lhcb6 (CP24), while the PSI antenna is composed of two heterodimers, LHCI-730 and LHCI-680, built-up by Lhca1/Lhca4 and Lhca2/Lhca3, respectively [108]. In the dark-adapted state, each antenna protein binds at least 1 Vx per monomer and the Vx binding site is supposed be located at either the peripheral V1 site (in LHCII, Lhca3 and possibly also Lhcb5) or the L2 site (in all other antenna complexes) [30,32] (see Table 4). Only for Lhca1 and Lhcb5 additional binding of Vx to the N1 site has been proposed [30,109].

It should be noted that the exact stoichiometry of xanthophyll binding to antenna proteins is not easy to determine. In fact, strongly different stoichiometries of Vx per monomer have been reported for each antenna protein, varying e.g. for each monomer of trimeric LHC II from values <0.2 [110] to values of about 1 [30]. Moreover stoichiometries of 1–1.5 Vx per monomer have been reported for Lhcb4 and Lhcb5 [30] and LHCI proteins [111]. In general, lowest stoichiometries are usually determined for reconstituted complexes, while the stoichiometries found in isolated native complexes vary in dependence of the solubilization conditions (kind and concentration of the detergent). Particularly those xanthophylls which are located at the protein/lipid interface or the interaction sites between antenna proteins (and thus represent a fraction of loosely bound xanthophylls) may easily be lost during isolation or not bound by reconstituted complexes.

5.3.1. Antenna proteins and Vx de-epoxidation

It is now generally accepted that the conversion of Vx to Zx occurs in the lipid matrix of the thylakoid membrane [9,32,62,112]. Furthermore, strong evidence exists that the de-epoxidation of Vx takes place in inverted hexagonal phase domains [92,94,95] which are likely to be formed by the galactolipid monogalatosyldiacylglycerol (MGDG) in the thylakoid membrane. Hence, the release of Vx from the respective xanthophyll binding site of a distinct antenna protein is a prerequisite for its conversion to Zx. The driving force for the release of xanthophylls from the respective binding sites is not clear. It could be assumed that simply the low lumen pH induces the release of Vx. In fact, pH-inducible release of Vx has been reported in a study with recombinant antenna proteins [113]. However, antenna proteins reconstituted with Vx as the only xanthophyll have been used in that work and the reduction of the Vx content was rather low in comparison with experiments in presence of active VDE [113]. By contrast, low pH incubation of intact thylakoids did not result in the release of significant amounts of Vx from antenna proteins (Jahns, unpublished results). Hence, it seems unlikely that a low pH alone is the driving force for Vx release under *in vivo* conditions. However, Bassi and co-workers further reported that the pH-dependent release of Vx from recombinant Lhcb5 and Lhca4 antenna proteins (again reconstituted with Vx as only xanthophyll) was increased when Zx was present, implying that Vx can be substituted by Zx at low pH in absence of active VDE [113]. Although these results remain to be verified also for native antenna complexes, such a scenario is likely to reflect the *in vivo* situation (see below) and can consistently explain the known characteristics of Vx to Zx conversion in intact leaves.

Consequently, Vx de-epoxidation in intact leaves can be expected to depend strongly on the binding affinity of Vx to its specific binding site in each of the antenna proteins. Indeed, the limitation of Vx de-epoxidation by xanthophyll binding to antenna proteins has been

Table 4
Vx binding sites and Vx conversion rates in different antenna complexes

Protein	Vx binding site[a]	Vx conversion rate	
LHCII	V1	Rapid	10 min
Lhcb4	L2 (N1)	Very slow	>90 min
Lhcb5	L2 (V1)	Slow	90–120 min
Lhcb6	L2	Slow	60 min
Lhca1	L2 (N1)	Very slow	>90 min
Lhca2	L2	Very slow	>90 min
Lhca3	V1	Rapid	10–20 min
Lhca4	L2	Slow	90–120

[a] The assignment of the putative binding sites were adapted from [30,32], the conversion rates were taken from [83,117,118].

derived earlier from studies with antenna depleted thylakoids from pea [16] and barley [13]. In absence of most of the antenna proteins both the extent and the rate of Vx de-epoxidation was found to be increased [13,16]. The faster kinetics of Vx conversion in absence of antenna proteins supported the assumption that non protein-bound Vx is the substrate for the VDE and thus that Vx de-epoxidation occurs in the lipid matrix of the membrane. On the other hand, the increased convertibility of the VAZ-pool in absence of antenna proteins indicated that xanthophyll binding by antenna proteins is responsible for the (well-known) restriction of Vx conversion observable in nearly all plants. Consequently, an increase of the VAZ-pool size in mutants over-expressing β-carotene hydroxylase by a factor of 2–3 did not result in a significant increase of the amount of non-convertible Vx [114,115]. In fact, more detailed *in vivo* analysis of the dynamics of xanthophyll conversion in spinach demonstrated that the extent and kinetics of Zx formation differ in single antenna subcomplexes [116], most likely reflecting different affinities of Vx binding sites in different antenna proteins. This interpretation was verified by detailed *in vitro* studies of the de-epoxidation kinetics in recombinant and native antenna proteins of both photosystems [83,117,118]. Using recombinant Lhcb1 proteins as a model system for LHCII it was possible to show that Vx bound to the V1 site (which represents the Vx binding site in the native Lhcb1 protein [119]) is rapidly convertible to Zx with similar kinetics as determined with non protein-bound Vx [118]. From experiments with Lhcb1 reconstituted with varying xanthophylls (in order to replace either Nx or lutein by Vx) it was further derived that Vx bound to the L2 site (which is occupied by lutein in the native Lhcb1 complex) is convertible to Zx with clearly slower (factor of 4) kinetics and that Vx bound to the L1 site (although not occurring at all in native antenna proteins) is generally not convertible to Zx [118]. Extending these experiments to the other antenna proteins of PSII [117] and PSI [83] it was found that Vx bound to homologous binding sites (in particular L2 and V1) has similar (but not identical) conversion characteristics (and thus binding affinities) in the different antenna proteins.

The following general rules were derived from these experiments: Vx bound to V1 (given in LHCII and Lhca3) is rapidly (within about 10 min) convertible to Zx, and Vx bound to L2 (given in all other antenna proteins) is either slowly (within about 60 min in Lhcb5, Lhcb6, Lhca4) or not (in Lhcb4, Lhca1, Lhca2) convertible to Zx (Table 4). Obviously, each antenna protein provides a specific binding affinity for Vx. Based on these characteristics determined in recombinant antenna proteins, the multiphasic and incomplete conversion of Vx to Zx found under *in vivo* conditions in intact leaves or under *in vitro* conditions in isolated thylakoid membranes can at least partly be explained by the different binding affinities to Vx of antenna proteins, as summarized in Fig. 2. While this simplified model might in general explain the basic Vx conversion characteristics with respect to the multiphasic behaviour and the limited Vx convertibility, the situation under *in vivo* conditions is most likely much more complex. Particularly under environmental stress conditions, redistribution of VAZ pigments among different antenna proteins may occur [120] and also an altered macromolecular organization of the photosystems and/or antenna proteins may further modulate the Vx convertibility under *in vivo* conditions.

The *in vivo* situation is further complicated by the fact that the de-epoxidation of Vx takes place in the lipid phase of the membrane and that diffusion of Vx (from the respective antenna protein, where it is released, to the conversion site, where it interacts with the VDE) is required to allow de-epoxidation. From studies with partially purified VDE that has been added to the stromal side of intact thylakoid membranes without intrinsic VDE activity (either by chemical inhibition of endogenous VDE [112] or by using the VDE deficient *npq1* mutant [86]) it is known that the VDE shows similar activities from both sides (stroma and lumen) of the membrane. This interesting feature not only excludes any requirement of a specific interaction of VDE with antenna proteins for de-epoxidation, but further implies that rather diffusion of Vx is required for the interaction of the VDE with its substrate [86]. The latter conclusion can be drawn from the fact that exogeneously added VDE has no access to the partitions of the grana stacks and thus can interact with Vx only at the stroma lamellae or at the margins of the grana stacks, so that it should be impossible to convert Vx in the majority of PSII antenna proteins [86]. Since it is further very unlikely to assume that a high mobility of xanthophyll-binding antenna proteins facilitates the transport of xanthophylls to these regions of the membrane, long-distance diffusion of xanthophylls can be postulated as prerequisite of Vx de-epoxidation [86].

In conclusion, these data support the model that the release of Vx from its binding site at a distinct antenna protein and the subsequent diffusion of Vx represent the rate-limiting steps for de-epoxidation. This scenario sufficiently explains the notion that the first step of de-epoxidation (i.e. the Vx to Ax conversion) is much slower than the second one (Ax to Zx conversion) [13,97,98]. The loosely bound and most rapidly convertible Vx-pool bound to V1 in LHCII (and possibly also in Lhca3) is thus likely to provide the pool of rapidly formed Zx which in turn can then substitute for Vx bound to other antenna proteins and by that allowing further de-epoxidation of Vx (see also Fig. 2).

5.3.2. Antenna proteins and Zx epoxidation

Only very few studies have been done that allow reliable predictions for the possible role of antenna proteins for the reconversion of Zx to Vx. Since no *in vitro* studies with recombinant antenna proteins and using isolated ZE protein have been performed so far, the present knowledge is based on *in vivo* studies only. In

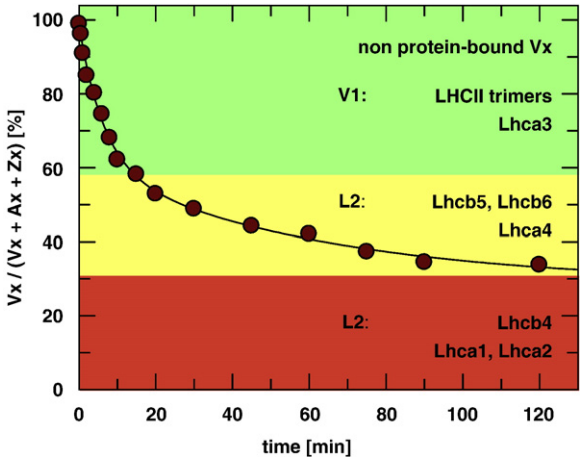

Fig. 2. Time course of Vx de-epoxidation in isolated thylakoid membranes. The data show a typical time course of Vx de-epoxidation in isolated thylakoid membranes from spinach measured at 20 °C. The coloured areas indicate three main pools of Vx as distinguished by the characteristics of the convertibility to Zx. For further details see text and Table 4. Adapted from [83] with kind permission of the 'American Society for Biochemistry and Molecular Biology (ASBMB)'.

comparison with the de-epoxidation reactions, the role of antenna proteins seems to be different for the epoxidation reactions. Two studies with antenna depleted plants from pea [16] and barley [13] showed that the rate of epoxidation is reduced by a factor of 2–10 in comparison with wild-type plants. This finding implies that the ZE requires protein-bound Zx as substrate rather than Zx in the lipid phase of the membrane in marked contrast to the VDE. It is further worth to note that in the case of the ZE the second step of the reaction (i.e. the Ax to Vx conversion) is slower (by a factor of at least 2) than the first step (Zx to Ax) [13,16] again in contrast to the VDE. Obviously both enzymes of the Vx-cycle follow different mechanisms of xanthophyll conversion. The molecular reason for this difference is unclear.

However, some information can be derived from a comparison of the Vx-cycle with the diadinoxanthin cycle (Ddx-cycle) of diatoms [2] in which Ddx is converted to diatoxanthin (Dtx) in a one-step reaction. The Ddx-cycle is not active in higher plants and has been described for the algae classes Bacillariophyceae, Crysophyceae, Xanthophyceae, Haptophyceae and Dinophyceae (Table 1) [2]. In general, these algae possess only an active Ddx-cycle, but the additional induction of a functional Vx-cycle after long-lasting high light treatment has been found in the diatom *Phaeodactylum tricornutum* [121]. Like de-epoxidation of Vx in the Vx-cycle, the de-epoxidation of Ddx to diatoxanthin (Dtx) is also induced by high light (and thus by a low lumen pH) in the Ddx-cycle. This reaction occurs with similar kinetics as known for the Vx-cycle, i.e. in about 10–30 min (e.g. [122]). By contrast, the back reaction, i.e. the epoxidation of Dtx to Ddx, is regulated by the transthylakoid pH-gradient and is, unlike the de-epoxidation, inhibited by a high pH gradient in the light [122]. Under low light, epoxidation of Dtx is completed within 15–30 min [122] and thus much faster than the epoxidation of Zx in the Vx-cycle.

The parallel existence of the Vx-cycle and the Ddx-cycle allowed a direct comparison of both cycles in *P. tricornutum*. [121]. In comparison to the Vx de-epoxidation the de-epoxidation of Ddx was accelerated by a factor of about 4, while the epoxidation of Dtx and Zx exhibited similar (and thus rapid) kinetics. Since it is assumed that, in *P. tricornutum*, the enzymes which are active in the Ddx-cycle are identical with those active in the Vx-cycle [121], the reduced rate of Zx epoxidation in plants is likely to be related to specific alterations of either the properties of (i) the ZE, (ii) the substrate availability or (iii) the lipid environment. To clarify these points it will be required to study the activity of the Dtx epoxidase from *P. tricornutum* in plant chloroplasts and *vice versa*.

5.4. The role of membrane lipids

5.4.1. Lipid–protein-interactions

The VDE has been characterized as a water soluble protein localized in the thylakoid lumen [6] and the pH-dependent binding to (at low pH) and release from (at high pH) the thylakoid membrane is thought to be central to the regulation of VDE activity [9]. Binding of the VDE to the thylakoid membrane occurs most likely through the charged C-terminal domain of the enzyme and an important role of histidine residues in the binding process has been proposed [123]. Since the co-operativity for binding of VDE, with respect to protons, and the pH value of the respective inflexion point was found to be strongly dependent on the numbers of histidine residues, it was proposed that protonation of the histidine residues at low pH induces a conformational change of the VDE, and by that contributes to the regulation of binding of VDE to the thylakoid membrane [123]. A similar role has earlier been postulated for glutamic acid residues, which are located in the C-terminal domain of the VDE [36,39]. Protonation of these glutamic acid residues at low pH might facilitate the binding of the VDE to the membrane [36,47].

All xanthophyll de-epoxidases characterized so far require for their *in vitro* activity the major thylakoid lipid monogalactosyldiacylglycerol (MGDG) [54,98]. Indeed, a specific interaction of VDE with MGDG was derived from the characteristics of lipid-affinity precipitation of the VDE [49]. In comparison with other thylakoid membrane lipids, MGDG was found to be 4 to 38 times more efficient in precipitating VDE [49]. The essential role of MGDG for VDE and DDE activity was further confirmed in recent studies on the de-epoxidation in liposomes [92,94,95,124].

It has been postulated that xanthophyll de-epoxidases do not bind randomly to the thylakoid membrane but rather attach only to certain membrane domains which are enriched in MGDG, and that the de-epoxidation reactions take place in these domains [95]. The existence of such domains in model and natural biological membranes has been reported in numerous studies [125–130].

The lipid requirement of the ZE is not clear, although mixtures of MGDG and DGDG have been used successively for measurements of ZE activity under *in vitro* conditions [12]. Possibly, these lipids generally facilitate substrate accessibility for the Vx-cycle enzymes. However, the influence of other lipids on ZE activity under *in vitro* conditions has not been studied so far in more detail.

5.4.2. Lipids and membrane properties

Yamamoto was the first who provided a possible explanation for the role of MGDG in Vx de-epoxidation under *in vitro* conditions and proposed that Vx may be located in micelles formed by this galactolipid [98]. It was estimated that MGDG micelles consist of about 28 MGDG molecules and that this structure allows the oscillation of Vx and by that the accessibility of Vx to the substrate binding site of the VDE and hence the formation of Ax [98]. The required flip-flop of Ax (which is inevitably necessary to allow enzymatic conversion of the second epoxy group) could then be facilitated by the mobility of Ax within the MGDG micelles. Based on the physicochemical properties of MGDG – hydratation degree of about 5 water molecules per one molecule of MGDG [131–134] and the value of critical packing parameter [135] – however, it has been derived that MGDG does not form micelles in water, but rather inverted hexagonal structures (H_{II}) [136,137].

Based on these features a more refined model for the molecular mechanism of de-epoxidation was derived from *in vitro* experiments with phosphatidylcholine (PC) liposomes enriched in MGDG and Vx or Ddx. This system resembles more the thylakoid membrane than the commonly used system composed of MGDG aggregates only. In fact, the presence of the H_{II} domains inside native thylakoid membranes and the liposomes composed of PC and MGDG has been described by several authors [125–130,138,139].

In liposomes composed of PC and MGDG, the de-epoxidation rate of Vx or Ddx depends on the MGDG/Vx or MGDG/Ddx ratio. In order to be converted to Ax, Vx molecules present in the bilayer part of the liposome have to get access to the MGDG domain by lateral diffusion. Thus the rate of de-epoxidation will depend on the rate of the lateral diffusion of Vx to these structures [92,95,140]. Due to the presence of HII phases in the MGDG domain, Ax can easily turn in the membrane and hence performing the required flip-flop type of movement. This will allow the VDE to get access to the second epoxy group and thus to convert Ax further to Zx. The proposed key role of H_{II} structures in Vx and Ax de-epoxidation has been confirmed in experiments with another non bilayer-forming lipid, phosphatidylethanolamine (PE). Efficient Vx or Ddx de-epoxidation was observed when MGDG was replaced by this lipid [94,141]. Despite the difference in the head group structure and chemical characteristics, PE provides similar structural features as MGDG (hydratation degree, critical packing parameter) and is supposed to form inverted hexagonal structures as well [131,139,142]. The requirement of H_{II} structures was also predicted for the conversion of Ddx to Dtx by DDE in the Ddx-cycle [94]. Based on the chemical characteristics of PE, it was concluded that rather the structure formed by the lipids determines the activity of VDE (DDE) than the interaction with the lipid head groups [141]. The head group of MGDG and DGDG is polar but uncharged, while PE and

PC contain zwitter-ionic head groups. Since both MGDG and PE have been shown to be effective for VDE [141] and DDE [94] activity, it is reasonable to assume that VDE docks to the membrane through the interaction with lipid head groups by forming hydrogen bonds rather than through ionic interactions.

H_{II} phases have also been shown to allow a better solubilization of xanthophyll cycle pigments. For complete solubilization of Vx, only 20 MGDG (or PE) molecules per 1 Vx molecule are required, while for DGDG (or PC) the same solubility could be obtained only at significantly higher ratios of about 100 molecules of lipid per 1 Vx. In case of Ddx, both ratios were lower, but the tendency was the same. Most probably, the asymmetry of the Ddx molecule is responsible for these solubilization characteristics [124]. However, solubilization of xanthophylls per se is not sufficient to allow de-epoxidation. In fact, Ddx and Vx were found to be not convertible to Dtx and Zx, respectively, when solubilized in PC and DGDG. In terms of the former considerations, this can easily be explained by the fact that PC and DGDG do not provide the hexagonal structures needed for xanthophyll de-epoxidase activity. It can thus be concluded that in thylakoid membrane both Vx and Ddx de-epoxidation takes place in domains rich in H_{II} forming lipids [94,95,124,141,143].

The products of Vx or Ddx de-epoxidation, Zx and Dtx, respectively, are supposed to play an additional important role as regulators of the membrane fluidity. Like cholesterol, Zx may regulate physical properties of thylakoid membrane. Accumulation of Zx affects not only the membrane fluidity [144] but influences also the value of the thylakoid membrane order parameter [145]. The rigidifying effect of this xanthophyll was also determined upon incorporation of exogenous Zx into isolated thylakoid membranes [146] and it was further shown most recently by laurdan fluorescence spectroscopy, that the conversion of Vx to Zx increases the order of the thylakoid membrane [143]. The rigidifying effect of epoxidized xanthophylls may also play an important role as self-regulation system in the molecular mechanism of xanthophyll conversion. When the level of Zx or Dtx is high (e.g. under high light conditions), the rate of lateral diffusion of xanthophyll cycle substrates, like Vx or Ddx is slower and by that also the rate of Vx or Ddx de-epoxidation [94,95,124,140].

5.4.3. Lipids, xanthophyll location and LHCII aggregation

As already pointed out in Section 5.3, binding of xanthophylls to antenna proteins is likely to affect the rate of xanthophyll conversion in thylakoid membranes by controlling the release of xanthophylls into the lipid phase of the membrane. While it is generally assumed that most of the VAZ pigments are bound to antenna proteins, at least a portion of the VAZ pigments may be expected to be present as free pigments in the lipid matrix of the thylakoid membrane. In thylakoid membranes isolated from Arabidopsis plants grown at a light intensity of 100 μmol photons m^{-2} s^{-1}, about 35–40 mmol VAZ pigments per mol Chl (a+b) are present [147,148]. Assuming that (i) each antenna protein may bind up to 1 VAZ pigment, (ii) a PSII/PSI ratio of about 1.5 [77] and (iii) an antenna size of about 200 Chl (a+b) per PSII (corresponding to the presence of one copy of each of the minor monomeric Lhcb protein (Lhcb4, 5 and 6) and three copies of LHC II trimers (i.e. in total 9 monomers)) and of about 150 Chl (a+b) per PSI (corresponding to one copy of each of the Lhca proteins (Lhca1–4)), the amount of roughly 40 mmol VAZ pigments per mol Chl (a+b) can easily be completely bound by antenna proteins. However, the majority of the VAZ pigments should be bound to trimeric LHCII proteins and the binding strength of xanthophylls to LHCII is supposed to be rather weak [30]. Hence, a reasonable fraction of the VAZ pigments may easily and rapidly be released into the lipid matrix of the membrane. The situation will certainly be different when plants are acclimated to high light intensities. In this case, the amount of VAZ pigments per Chl can increase 2 to 3 fold concomitant with the reduction of the amount of LHC proteins, so that a reasonable fraction of VAZ pigments may be present in the lipid matrix of the membrane, provided that binding VAZ pigments by other proteins like early light-inducible proteins (ELIPs, [149]) or high light-inducible proteins (HLIPs, [150]) is excluded. Independent of these considerations on the general location of VAZ pigments in dark-adapted plants it is clear that non protein-bound Vx is required for the de-epoxidation by VDE upon illumination. The release of Vx from xanthophyll binding sites in LHCII has indeed been derived from studies of the pigment composition of antenna proteins isolated before and after illumination of leaves [145].

It is likely that these events are directly related to conformational changes of LHCII proteins that have been shown to take place upon acidification of the thylakoid lumen concomitant with the generation of NPQ processes in PSII [28,151,152]. LHCII aggregation can further be supposed to be accompanied by changes in the organization of lipids. In the thylakoids of vascular plants and diatoms, light-harvesting complexes (LHC, FCP) enriched in xanthophyll cycle pigments [110,153,154] are located in lipid bilayers. In vascular plants, LHC proteins were found to be surrounded by a high concentration of the non-bilayer lipid MGDG [155], which is forced into the bilayer structure by the respective antenna proteins and thus prevents the formation of H_{II} phases [156]. In dark-adapted plants or algae, the LHC/FCP is in a non-aggregated state and Ddx or Vx are bound to the antenna complexes. Aggregation of LHCII proteins upon high light illumination (and thus NPQ induction) may lead to the displacement of MGDG from the bilayer and by that may induce the assembly of parts of the MGDG in H_{II} phases. Such a scenario is supported by the fact that the amount of MGDG which can be incorporated into a bilayer structure depends on the conformation of the LHC proteins and that additional MGDG molecules may form H_{II} phases which remain closely associated with the LHC/MGDG bilayer [156,157]. Once these H_{II} phases are formed, Ddx or Vx might easily be disconnected from the LHC and migrate preferentially into these MGDG enriched regions which in both cases still form a continuous space with the membrane bilayer. Decreasing the amount of LHCII in the membrane (e.g. during acclimation to high light or in plants with reduced Chl b synthesis) will consequently generally increase the probability of H_{II} phase formation and thus allow rapid and efficient xanthophyll de-epoxidation.

The aggregation of LHCII may further be influenced by the isomerization state of xanthophylls. It has been shown that the isomerization of the all-*trans* form of Vx to 13-*cis* and 9-*cis* conformers affects the LHCII aggregation state so that the accumulation of *cis* forms of Vx upon high light illumination may induce the disassembly of large LHCII aggregates [158,159] and by that also control the availability of all-*trans* Vx for de-epoxidation. On the other hand, the accumulation of all-*trans* zeaxanthin in the thylakoid membranes facilitates the formation of large LHCII aggregates [158], which are supposed to contribute to NPQ processes [28,151,152].

6. Model for the mechanism of xanthophyll conversion

Based on the recent data summarized above, we derived a new model for the mechanism of xanthophyll conversion (Fig. 3). In our hands, the de-epoxidation of Vx/Ddx to Zx/Dtx essentially requires (i) the presence of H_{II} phases and (ii) non protein-bound Vx. While it is unclear, whether both requirements are already given in thylakoids of dark-adapted plants, it is reasonable to assume that both features are at least established under illumination conditions suitable to induce NPQ processes. The model further proposes that the transition to the light-adapted state is accompanied by conformational changes in the thylakoid membrane, in particular by the aggregation of LHCII complexes, which strongly facilitates the stability of H_{II} domains and the release of Vx bound to the loose V1 binding site of LHCII. The MGDG phases are assumed to be the preferential location site of non protein-bound Vx in the membrane and further represent the docking site for the VDE (or DDE). After binding of the VDE (or DDE) to the segregated MGDG phases, Vx (or Ddx) are de-epoxidized to Zx (or

Dtx). Once substantial amounts of Zx (or Dtx) have been formed, these de-epoxidized xanthophylls can rebind to LHCII or diffuse to other parts of the lipid bilayer and exchange for Vx bound to more strongly binding sites (mainly L2) in other antenna proteins (Fig. 3). The rebinding of Zx (or Dtx) to LHC proteins may be facilitated by the different chemical properties of the de-epoxidized xanthophyll cycle pigments (most probably due to a decreased solubility in MGDG) compared with epoxidized xanthophyll [124]. Although the solubility of Ddx and Dtx in the bilayer forming lipid PC is comparable, the difference in Dtx solubility between the MGDG and the PC phase is much less pronounced than that of Ddx solubility, thereby supporting the re-entrance of Dtx or Zx into the bilayer phase of the membrane where the LHC proteins are located [124]. The differences in solubility between epoxidized and de-epoxidized xanthophyll cyle pigments may also be responsible for the increase in membrane rigidity observed upon the light-induced conversion of Vx to Zx in higher plants [144]. It should be emphasized that the hydrophobic area of the segregated MGDG phases should form a continuity with the

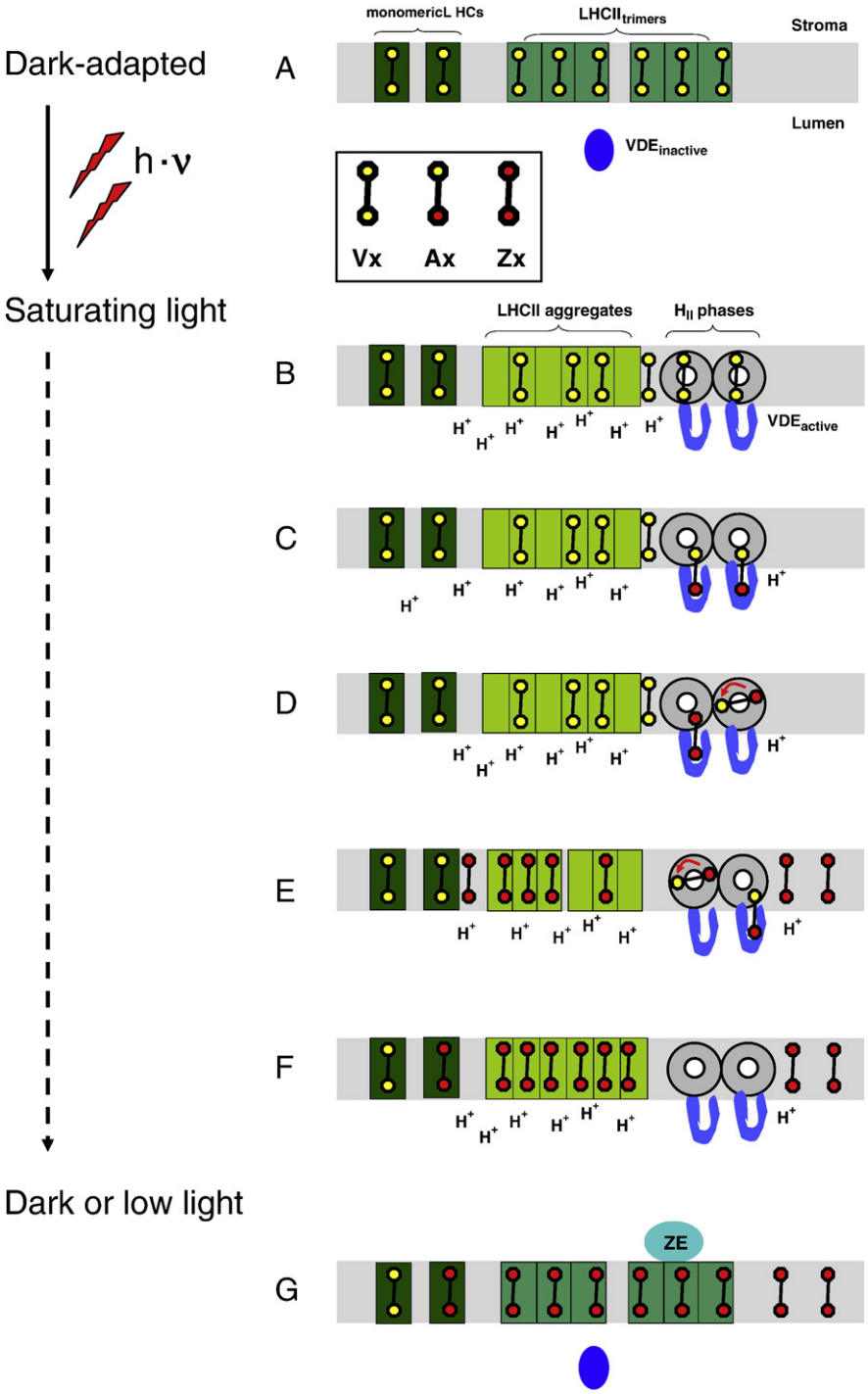

Fig. 3. Model for the mechanism of xanthophyll conversion. In the dark-adapted state (A), only Vx is bound to the antenna proteins. Upon the transition from dark to light (B), the lumen pH drops below pH 6. Due to the pH-shift, LHCII aggregates, H_{II}-phases are formed and VDE is bound to the membrane. Concomitantly with LHCII aggregation a fraction of Vx is released into the lipid phase of the membrane. Ongoing illumination (C–F) leads to the formation of Zx, which is released from the H_{II}-phase and binds either to empty xanthophyll binding sites or exchanges for Vx bound to more tightly xanthophyll binding sites in monomeric antenna proteins. Upon the transition from light to dark (G), the lumen pH increases, VDE is inactivated and the ZE can reconvert Zx back to Vx. For further details see text.

hydrophobic area of the membrane bilayer and the integral membrane proteins. Such an arrangement would minimize the diffusion times of the xanthophyll cycle pigments between their protein binding sites and the sites of de-epoxidation, leading to the fast rebinding of de-epoxidized xanthophylls necessary for efficient photoprotection. The formation of LHCII aggregates in the light-adapted state will allow rapid dissipation of excess light energy in these complexes [28,160] and binding of Zx to the minor antenna proteins of PSII (Lhcb4–6) could facilitate the proposed dissipation of excitation energy via a Zx cation radical in these complexes [26,27,161]. During transition from the light-adapted state back to the dark-adapted state (Fig. 3), the break-down of the transmembrane proton gradient will reverse LHCII aggregation and further allow the reconversion of Zx (or Dtx) to Vx (or DDx). So far it is unclear whether the ZE also requires H_{II} domains for activity.

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