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ORIGINAL ARTICLE

Susanne Macko · Antje Wehner · Peter Jahns

Comparison of violaxanthin de-epoxidation from the stroma and lumen sides of isolated thylakoid membranes from *Arabidopsis*: implications for the mechanism of de-epoxidation

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Abstract The enzyme violaxanthin de-epoxidase (VxDE) is localized in the thylakoid lumen and catalyzes the de-epoxidation of membrane-bound violaxanthin (Vx) to zeaxanthin. De-epoxidation from the opposite, stroma side of the membrane has been investigated in the *npq1* mutant from *Arabidopsis thaliana* (L.) Heynh. – which lacks VxDE – by adding partially purified VxDE from spinach thylakoids. The accessibility of Vx to the exogenously added enzyme (exoVxDE) and the kinetics of Vx conversion by the exoVxDE in thylakoids from *npq1* plants were very similar to the characteristics of Vx conversion by the endogenous enzyme (endoVxDE) in thylakoids from wild-type plants. However, the conversion of Vx by exoVxDE was clearly retarded at lower temperatures when compared with the reaction catalyzed by endoVxDE. Since the exoVxDE – in contrast to the endoVxDE – has no access to the stacked regions of the membrane, where the xanthophylls bound to photosystem II are located, these results support the assumption of pronounced diffusion of xanthophylls within the thylakoid membrane.

Keywords *Arabidopsis* · Carotenoid · Energy dissipation · Violaxanthin de-epoxidase · Xanthophyll cycle

Abbreviations Ax: antheraxanthin · Chl: chlorophyll · endoVxDE: endogenous violaxanthin de-epoxidase · exoVxDE: exogenous violaxanthin de-epoxidase · PSI (II): photosystem I (II) · qE: pH-dependent quenching of chlorophyll fluorescence · Vx: violaxanthin · VxDE: violaxanthin de-epoxidase · WT: wild type · Zx: zeaxanthin

Dedicated to Prof. G. Heinrich Krause, Düsseldorf, on the occasion of his retirement.

S. Macko · A. Wehner · P. Jahns (✉)
Heinrich-Heine-Universität Düsseldorf,
Institut für Biochemie der Pflanzen,
Universitätsstrasse 1, 40225 Düsseldorf, Germany
E-mail: pjahns@uni-duesseldorf.de
Fax: +49-211-8113706

Introduction

The de-epoxidation of violaxanthin (Vx) to zeaxanthin (Zx) via the intermediate antheraxanthin (Ax) in the xanthophyll cycle (Yamamoto et al. 1962; Pfündel and Bilger 1994; Eskling et al. 1997) serves important functions in the protection of the photosynthetic apparatus against photo-oxidative stress (Demmig-Adams et al. 1987; Gilmore 1997; Niyogi 1999; Ort 2001). The central role of Zx in the energy- or pH-dependent mechanism, qE, of energy dissipation in the antenna of photosystem II (PSII) was underlined by the analysis of xanthophyll cycle mutants of *Chlamydomonas reinhardtii* and *Arabidopsis thaliana* (Niyogi et al. 1997, 1998). Algae and plants deficient in Vx de-epoxidase (VxDE), so-called *npq1* mutants, that were unable to generate Zx, showed little or no qE formation. On the other hand, when a permanently high Zx content was present in mutants deficient in Zx epoxidase, so-called *npq2* mutants, qE was generated more rapidly in comparison with wild-type (WT) plants/algae (Niyogi et al. 1997, 1998). Comparative analysis of different *npq* mutants demonstrated that the xanthophyll cycle pigments, in addition to their dissipative function in the antenna, may also play a crucial role in membrane fluidity, thermostability and in the protection of the membrane against lipid peroxidation (Havaux 1998; Havaux and Niyogi 1999; Havaux et al. 2000).

While the proposed function of Zx in energy dissipation is assumed to be related to xanthophylls bound to antenna proteins of PSII, the functions as membrane stabilizer and antioxidant should rather be achieved by xanthophylls that are not associated with proteins, thus indicating different pools of xanthophyll cycle pigments within the membrane. A heterogeneous organization of Vx has already been deduced in early studies from the observation that only a fraction (usually 50–80%) of the

total Vx can be converted to Zx (Siefermann and Yamamoto 1974; Siefermann-Harms 1984). Later studies with antenna-depleted plants corroborated the assumption that binding of xanthophylls to antenna proteins is limiting the substrate availability for the VxDE (Jahns 1995; Härtel et al. 1996). It remained unclear, however, to what extent the convertible fraction of Vx is bound to antenna proteins. Recent work with recombinant LHCII supports the view that both the extent and kinetics of Vx de-epoxidation are controlled by the carotenoid-binding site of a distinct antenna protein (Jahns et al. 2001), so that heterogeneous Vx pools could be explained by different binding affinities to antenna proteins. On the other hand, the large pool size of xanthophyll cycle pigments concomitant with a small antenna size in high-light-grown plants (Demmig-Adams 1998; Graßes et al. 2002) or young leaves of tropical forest trees (Krause et al. 1995) implies that under certain conditions definitely not all xanthophyll cycle pigments can be bound by antenna proteins.

Moreover, the heterogeneous organization of membranes in grana and stroma regions can be assumed to limit Vx de-epoxidation although it has been shown that Vx de-epoxidation occurs in both, grana and stroma lamellae to a similar extent (Siefermann and Yamamoto 1976; Arvidsson et al. 1997; Färber and Jahns 1998). Particularly, the rather low abundance of VxDE (about 1 VxDE per 20–100 electron transport chains; Arvidsson et al. 1996) can be expected to be critical for the enzyme–substrate interaction in the grana regions of the membrane. Since the mobility of both antenna proteins (to which the substrate is bound) and VxDE (which is bound to the membrane surface) should be limited, diffusion of xanthophylls might be proposed. This would require a rapid exchange of xanthophylls between proteins and the lipid phase, as has been assumed to be a general prerequisite for the de-epoxidation reaction (Rockholm and Yamamoto 1996).

In the present work the characteristics of Vx de-epoxidation from the stroma and the lumen side of the thylakoid membrane were compared. Vx conversion from the stroma side was studied with a VxDE-enriched extract prepared from spinach thylakoids added to thylakoids from *npq1* mutants that lack endogenous, lumen-localized VxDE (endoVxDE). As the exogenous VxDE (exoVxDE) should not have access to (i) the lumen side and (ii) the partitions of the grana region of the membrane, this approach should give information about the general accessibility of Vx from the stroma side and the possible requirement of xanthophyll diffusion for the de-epoxidation reactions.

Materials and methods

Plant material and growth conditions

Spinach (*Spinacia oleracea* L., cv. Polka) plants were grown in a greenhouse at 24 °C under short-day conditions (8.5 h light/15.5 h darkness) at a photon flux density of 150 μmol photons m^{-2} s^{-1} for 6 weeks. Plants of *Arabidopsis thaliana* (L.) Heynh. wild type (ecotype Columbia *gl1*, obtained from Prof. P. Westhoff, University of Düsseldorf, Germany) and *npq1* (obtained from the Nottingham *Arabidopsis* stock centre, UK) were cultured in soil under greenhouse conditions (80–100 μmol photons m^{-2} s^{-1}, 16 h light/8 h dark daily) for 4–6 weeks.

Thylakoid preparation

Thylakoids from *S. oleracea* were prepared according to standard procedures (Krause et al. 1985). For isolation of thylakoids from *A. thaliana*, leaves from 4-to 6-week-old plants were homogenised in a medium containing 330 mM sorbitol, 20 mM Tricine–NaOH (pH 7.6), 5 mM EGTA, 5 mM EDTA, 10 mM Na_2CO_3, 0.1% (w/v) BSA, and 330 mg/l ascorbate. After centrifugation for 5 min at 2,000 *g*, the pellet was resuspended in 300 mM sorbitol, 20 mM Hepes–KOH (pH 7.6), 5 mM $MgCl_2$, and 2.5 mM EDTA. With this procedure, high yields of broken chloroplasts were obtained, which retained high photosynthetic activity for several hours.

Electron transport measurements

Electron transport rates under saturating illumination with red light ($\lambda > 630$ nm) were derived from measurements of photosynthetic oxygen evolution using a Clark-type oxygen electrode. Thylakoids equivalent to 10 μg chlorophyll (Chl) ml^{-1} were used for all measurements. Linear electron transport ($H_2O \rightarrow K_3[Fe(CN)_6]$) was measured in a medium containing 330 mM sorbitol, 40 mM Hepes–NaOH (pH 7.6), 10 mM NaCl, 5 mM $MgCl_2$, using 0.5 mM $K_3[Fe(CN)_6]$ as terminal electron acceptor. 2 μM gramicidin D and 5 mM NH_4Cl were further added for determination of uncoupled electron transport rates.

Preparation of crude VxDE extracts

VxDE extracts were isolated from spinach essentially following the procedure described by Arvidsson et al. (1996). Briefly, isolated thylakoids were broken by sonification at pH 5.1 and VxDE was released from the resulting membrane fragments by increasing the pH to 7.2 for the final sonification step. After centrifugation, VxDE was precipitated from the supernatant by differential $(NH_4)_2SO_4$ fractionation and finally collected by ultracentrifugation (Arvidsson et al. 1996).

In vitro de-epoxidation

For pH-induced de-epoxidation (in the dark), thylakoids equivalent to 250 μg Chl were resuspended in 20 ml of a medium containing 5 mM $MgCl_2$, 10 mM NaCl, 100 mM Mes–NaOH (pH 5.1), 330 mM sorbitol, and the de-epoxidation reaction was started by addition of 30 mM ascorbate. For light-induced de-epoxidation, thylakoids equivalent to 37.5 μg Chl were resuspended in 3 ml of the same medium as before but using 40 mM Hepes–NaOH (pH 7.6) as buffer instead of Mes. 2 μM methyl viologen in the presence of 2 U ml^{-1} superoxide dismutase (1 U inhibits the auto-oxidation of pyrogallol red at 50%) served as electron acceptor. After addition of 30 mM ascorbate and 225 μl VxDE extracts, de-epoxidation was started by illuminating samples with saturating red light ($\lambda > 630$ nm).

Pigment analysis

Pigment analysis was carried out by reversed-phase HPLC as described elsewhere (Färber et al. 1997). For pigment extraction, aliquots of the de-epoxidation assays were added to a 4-fold volume of acetone, rapidly mixed and centrifuged for 2 min at 14,000 *g*.

Results

Light-induced electron transport and Vx de-epoxidation

Activation of VxDE requires a pH below 6. In intact isolated thylakoids, lumen acidification – which activates lumen-localized endoVxDE – can be induced by two different treatments: (i) by light-induced photosynthetic electron and proton transport or (ii) in the dark by acidification of the suspension medium. Activation of exoVxDE, which is localized on the stroma side of the membrane, on the other hand, can only be achieved by acidification of the medium, provided that the enzyme has no access to the thylakoid lumen space.

Electron transport rates and light-induced Vx conversion were determined for thylakoids from WT and *npq1* plants (Fig. 1). Photosynthetic electron transport was found to be fully functional in both types of thylakoid (Fig. 1a). The increase in electron transport rates in the presence of uncouplers by 80% and 180% in WT and mutant thylakoids, respectively, proves the integrity of the thylakoid membrane and implies that a transmembrane ΔpH (and thus a low lumen pH) is induced under our experimental conditions. The lower maximum electron transport rates in *npq1* thylakoids in comparison with WT thylakoids (see uncoupled rates in Fig. 1a) most likely reflect slight differences in the growth and thus of the Chl content of the two genotypes. Under the same experimental conditions, light-induced conversion of Vx to Ax and Zx was observed in WT thylakoids (although with rather slow kinetics), indicating that acidification of the lumen was sufficient to activate endoVxDE (Fig. 1b). In contrast, no de-epoxidation was found with *npq1* thylakoids, even in the presence of exoVxDE (Fig. 1c). This result corroborates not only the absence of endoVxDE in *npq1* thylakoids but underlines also that exoVxDE has no access to the thylakoid lumen. The general activity of VxDE at the given enzyme/substrate ratio has been confirmed in a separate experiment with isolated Vx (not shown).

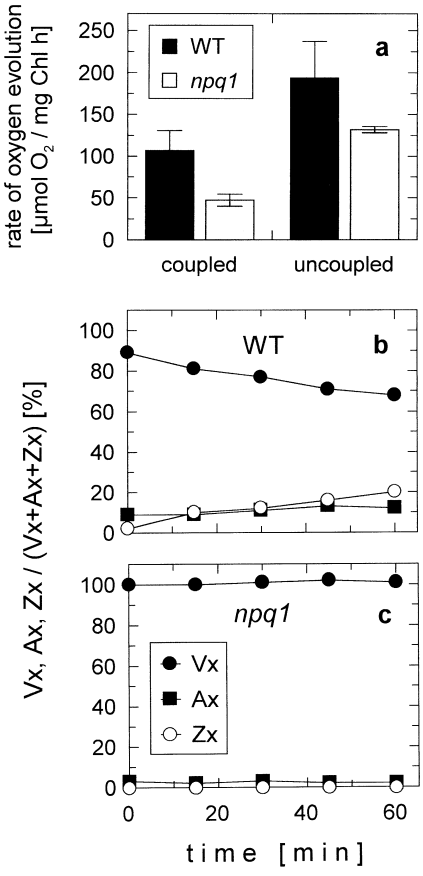

Fig. 1a–c Electron transport and light-induced Vx conversion by *Arabidopsis thaliana* thylakoids. **a** Electron transport rates were calculated from measurements of oxygen evolution under saturating illumination using a Clark-type electrode. $K_3Fe(CN)_6$ served as electron acceptor. Uncoupling of electron transport was achieved by addition of 2 µM gramicidin D and 5 mM NH_4Cl. Mean values ± SE of 3 independent measurements are shown. **b, c** Light-induced de-epoxidation was determined with thylakoids from WT (**b**) and *npq1* (**c**) plants. 2 µM methyl viologen in the presence of 2 U ml^{-1} superoxide dismutase served as electron acceptor. 30 mM ascorbate and VxDE extracts were added prior to illumination. At the given time, the reaction was stopped by mixing aliquots of the assay with 4-fold the volume of acetone. The pigment content of the acetone extract was analyzed by HPLC. Representative single experiments are shown

pH-induced Vx de-epoxidation

The pH-inducible conversion of Vx to Ax and Zx was determined in the presence and absence of exoVxDE for both types of thylakoid (Fig. 2). In the absence of exoVxDE, Vx de-epoxidation was found in WT thylakoids but not in *npq1* thylakoids, underlining again the absence of endoVxDE in *npq1* plants (Fig. 2a, b). In the presence of exoVxDE, however, Vx conversion was inducible to a similar extent and with similar (not identical) kinetics in both WT and *npq1* thylakoids (Fig. 2c, d). The significance of the slight retardation of the Vx to Ax conversion in *npq1* thylakoids in comparison with WT membranes cannot be evaluated from our data, particularly since the enzyme concentrations are unknown in both cases. The more pronounced retardation of the second step (Ax to Zx) of de-epoxidation in *npq1* thylakoids than in WT thylakoids, on the other hand, is most likely caused by the portion of Ax (roughly 10% of the total xanthophyll cycle pigment pool) present in WT thylakoids at $t = 0$ which accelerates the initial Ax-to-Zx conversion in WT membranes.

Obviously, the interaction between VxDE and Vx is not restricted by (i) the side at which the enzyme binds to the membrane or (ii) the exclusion of the exoVxDE from the partitions of the grana stacks. The latter conclusion was further corroborated in control experiments showing that unstacking of the membrane did not increase the convertibility of Vx in either type of thylakoid (data not shown). As VxDE has no access to the partitions of the grana stacks, where significant portions of

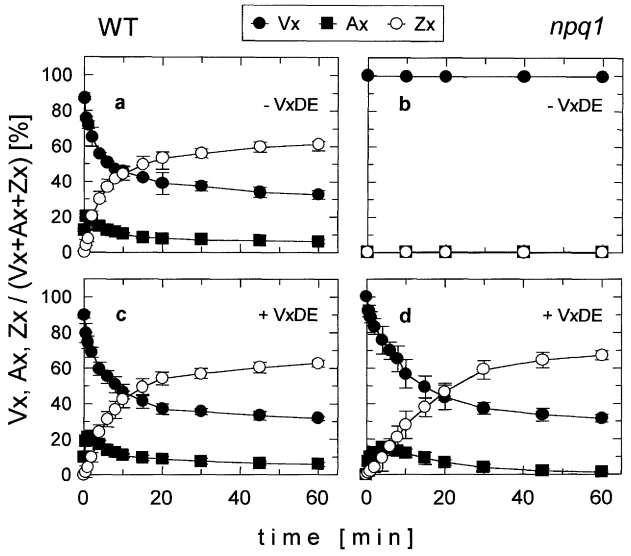

Fig. 2a–d Time course of pH-induced Vx de-epoxidation by *A. thaliana* thylakoids. Vx de-epoxidation at pH 5.1 was determined with thylakoids from WT (**a, c**) and *npq1* (**b, d**) plants. The reaction was started by adding ascorbate, yielding a final concentration of 30 mM. Measurements were performed in the absence (**a, b**) or presence (**c, d**) of an extract of partially purified VxDE from spinach thylakoids. At the given time, the reaction was stopped by mixing aliquots of the assay with 4-fold the volume of acetone. The pigment content of the acetone extract was analyzed by HPLC. Mean values ± SE of 3 independent measurements are shown

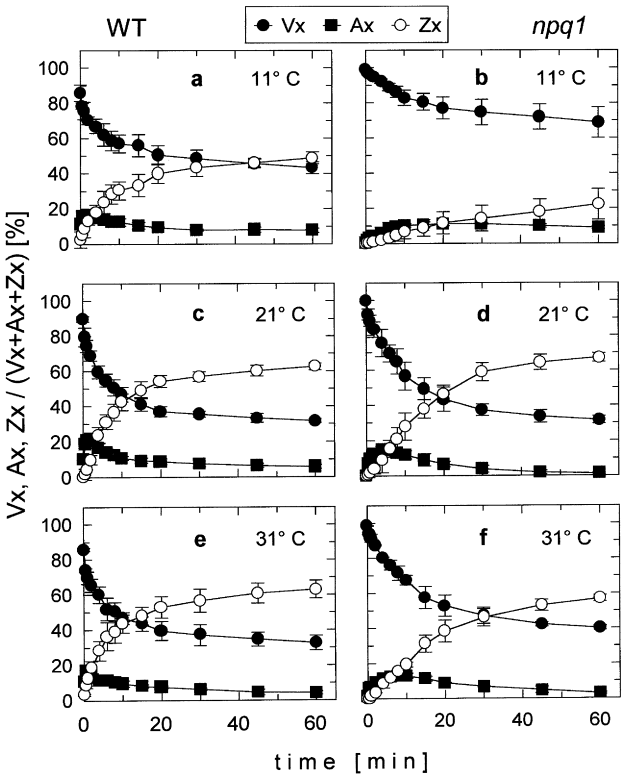

Fig. 3a–f Time course of pH-induced Vx de-epoxidation at different temperatures. Vx de-epoxidation was determined with thylakoids from WT (**a, c, e**) and *npq1* (**b, d, f**) *A. thaliana* plants at 11 °C (**a, b**), 21 °C (**c, d**) and 31 °C (**e, f**). All other conditions as in Fig. 2. Mean values ± SE of 3 independent measurements are shown

xanthophylls are located, these results indicate that diffusion of xanthophylls most likely plays an important role in the xanthophyll conversion. Moreover, the addition of VxDE to WT thylakoids did not accelerate Vx conversion (Fig. 2a, c), implying that the low abundance of the endoVxDE (estimated at 1 per 20–100 PSII; Arvidsson et al. 1996) is not limiting the de-epoxidation reactions.

Temperature dependence of de-epoxidation

The kinetics and extent of Vx de-epoxidation were determined at 11, 21 and 31 °C. While the characteristics of Vx conversion were very similar at 21 and 31 °C in both types of thylakoids, de-epoxidation was significantly retarded at 11 °C (Fig. 3). In *npq1* thylakoids, the retardation was more pronounced and particularly the portion of convertible Vx seemed to be strongly reduced in comparison with WT thylakoids. It cannot be ruled out that the remaining large portion of Vx in *npq1* thylakoids is convertible to a similar extent as in WT thylakoids, but with very slow kinetics. However, this seems rather unlikely for kinetic reasons. It is known from several studies with leaves and isolated thylakoids that Vx conversion is characterized by biphasic kinetics and that the major portion of Vx is converted with the fast kinetics (Siefermann and Yamamoto 1974; Pfündel and Dilley 1993; Jahns 1995; Härtel et al. 1996). This is also evident from the data obtained with WT thylakoids at all temperatures and with *npq1* thylakoids at the two higher temperatures (Fig. 3a, c–f) showing that about 80% of the convertible Vx is de-epoxidized with the fast kinetics (completed within the first 10–20 min.). Since the portion of this fast phase is strongly reduced at 11 °C in *npq1* thylakoids, we conclude that the overall convertibility is reduced to a similar extent. Therefore, this result could be understood as restriction of Vx availability in *npq1* thylakoids due to limited diffusion of Vx at lower temperatures.

Discussion

The results show that de-epoxidation of Vx from the stroma side of the membrane by exoVxDE is similar to the reaction catalyzed by endoVxDE from the lumen side. This conclusion can be drawn from our data, since our experimental conditions ensured that in the experiments with *npq1* thylakoids exclusively the activity from the stroma side was determined: (i) the absence of endoVxDE activity in *npq1* mutant plants (Niyogi et al. 1998; Figs. 1 and 2) excluded any residual endoVxDE activity and (ii) the control experiments shown in Fig. 1 demonstrated that added exoVxDE had no access to the lumenal space.

Similar characteristics of Vx conversion by endo-VxDE and exoVxDE have already been concluded in earlier work with spinach thylakoids (Arvidsson et al. 1997). In that case, however, endoVxDE was irreversibly inactivated by treatment with dithiothreitol/iodoacetamide and exoVxDE added after removal of the inhibitors. While only a little residual endoVxDE activity was found after the inhibitory treatment, a possible access of exoVxDE to the lumen side of the membrane was not ruled out in those experiments (Arvidsson et al. 1997).

The two photosystems (and thus Vx) are heterogeneously distributed in the thylakoid membrane. While PSII is mainly located in the grana stacks, PSI is found predominantly in the stroma lamellae (Andersson and Anderson 1980; Murphy 1986). On the other hand, it can be excluded that exoVxDE had direct access to the stromal membrane side in the appressed region of the membrane, since it has been shown in flash-spectrophotometric studies with pea thylakoids that the partitions of the grana region are not accessible even to small hydrophilic pH-indicating dyes (Polle and Junge 1986, 1989). This raises the question of how exoVxDE can interact with the substrate Vx as efficiently as endo-VxDE at the lumen side, where access to the central grana region should not be limited. Obviously, diffusion of the substrate Vx (whether or not bound to proteins) is required for enzyme–substrate interaction.

The distance between the central part of a granum and the membrane surface which is accessible to exo-VxDE can be estimated from electron micrographs of intact chloroplasts (e.g. Murphy 1986) as at least 250 nm. Due to the high protein density within the grana membrane (roughly 75% of the overall membrane area is assumed to be covered by membrane proteins; Murphy 1986) and the organization of PSII in large dimeric PSII–LHCII supercomplexes (Barber et al. 1999), the mobility of xanthophyll-binding antenna proteins should be strongly limited. Consequently, long-distance diffusion of non-protein-bound xanthophylls can be postulated as prerequisite of Vx de-epoxidation by exoVxDE. In agreement with earlier interpretations (Rockholm and Yamamoto 1996; Arvidsson et al. 1997) it is therefore very likely that xanthophyll conversion requires unbound Vx in the lipid phase of the membrane rather than a protein-bound substrate.

The low abundance of VxDE in spinach thylakoids (about 1 per 20–100 PSII; Arvidsson et al. 1996) and the similar characteristics of Vx conversion by exoVxDE and endoVxDE let us speculate that xanthophyll diffusion also occurs in intact chloroplasts, suggesting a rapid and permanent exchange of protein-bound xanthophylls with those in the lipid phase of the membrane under in vivo conditions, as well. It seems to be contradictory to this hypothesis that after mild solubilization of the thylakoid membrane most of the xanthophylls are found to be bound by antenna proteins (Thayer and Björkman 1992; Verhoeven et al. 1999). However, the high density of proteins within the membrane could ensure that the steady-state ratio of protein bound/non-protein bound xanthophyll is rather high.

The involvement of xanthophyll diffusion is further supported by the strong effect of low temperature on Vx conversion (Fig. 2). In a detailed study of the temperature dependence of de-epoxidation, the analysis of the activation energy for Vx de-epoxidation indicated a phase transition – most likely from the liquid-crystalline to the gel phase – of the thylakoid membrane between 12 and 16 °C, (data not shown). Consistent with our assumptions, the reduction of xanthophyll mobility due to this phase transition of the membrane could explain the observed temperature effect, in agreement with earlier observations in spinach thylakoids (Arvidsson et al. 1997).

Our model would not only consistently explain the results of the present study, but also a variety of other observations. It is well known that conversion of protein-bound and non-protein-bound Vx follows similar kinetics in vivo (Jahns 1995; Härtel et al. 1996) and in vitro (Jahns et al. 2001). This can easily be understood when a permanent rapid and reversible release of xanthophylls from their respective binding sites into the lipid phase of the membrane is postulated. The known multiphasic kinetics of Vx de-epoxidation will then be related to different binding affinities of the xanthophylls to the different binding sites. Moreover, differences in the convertibility of Vx among different species or sun and shade leaves (e.g. Demmig-Adams 1998) could be explained not only by differences in the pool size of non-protein-bound xanthophylls, but also by different membrane properties (fluidity, size of grana) that effect xanthophyll diffusion, as has been proposed in a study with two barley genotypes adapted to different climatic environments (Tardy et al. 1998). It is further worth noting that addition of exoVxDE to WT thylakoids did not accelerate Vx conversion although (i) exoVxDE itself was sufficient to catalyze Vx de-epoxidation at high rates and (ii) the amount of endoVxDE is assumed to be very low. Obviously, a factor other than the enzyme concentration is limiting the de-epoxidation reactions. In terms of our model this factor would be the release and diffusion of xanthophylls.

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