Planta (2001) 213: 446–456
DOI 10.1007/s004250100589

ORIGINAL ARTICLE

Ruth Frommolt · Reimund Goss · Christian Wilhelm

The de-epoxidase and epoxidase reactions of *Mantoniella squamata* (Prasinophyceae) exhibit different substrate-specific reaction kinetics compared to spinach

Received: 24 November 2000 / Accepted: 22 March 2001 / Published online: 11 May 2001

Abstract In vivo the prasinophyceaen alga *Mantoniella squamata* Manton et Parke uses an incomplete violaxanthin (Vx) cycle, leading to a strong accumulation of antheraxanthin (Ax) under conditions of high light. Here, we show that this zeaxanthin (Zx)-depleted Vx/Ax cycle is caused by an extremely slow second de-epoxidation step from Ax to Zx, and a fast epoxidation from Ax back to Vx in the light. The rate constant of Ax epoxidation is 5 to 6 times higher than the rate constant of Zx formation, implying that Ax is efficiently converted back to Vx before it can be de-epoxidated to Zx. It is, however, only half the rate constant of the first de-epoxidation step from Vx to Ax, thus explaining the observed net accumulation of Ax during periods of strong illumination. When comparing the rate constant of the second de-epoxidation step in *M. squamata* with Zx formation in spinach (*Spinacia oleracea* L.) thylakoids, we find a 20-fold reduction in the reaction kinetics of the former. This extremely slow Ax de-epoxidation, which is also exhibited by the isolated *Mantoniella* violaxanthin de-epoxidase (VDE), is due to a reduced substrate affinity of *M. squamata* VDE for Ax compared with the VDE of higher plants. *Mantoniella* VDE, which has a similar K_m value for Vx, shows a substantially increased K_m for the substrate Ax in comparison with spinach VDE. Our results furthermore explain why Zx formation in *Mantoniella* cells can only be found at low pH values that represent the pH optimum of VDE. A pH of 5 blocks the epoxidation reaction and, consequently, leads to a slow but appreciable accumulation of Zx.

Keywords Antheraxanthin · *Mantoniella* (xanthophyll cycle) · Violaxanthin cycle · Violaxanthin de-epoxidase · Xanthophyll cycle · Zeaxanthin epoxidase

R. Frommolt · R. Goss (✉) · C. Wilhelm
Institut für Botanik – Pflanzenphysiologie,
Universität Leipzig, Johannisallee 21–23,
04103 Leipzig, Germany
E-mail: rgoss@rz.uni-leipzig.de
Fax: +49-341-9736899

Abbreviations Ax: antheraxanthin · Chl: chlorophyll · DTT: dithiothreitol · PFD: photon flux density · RM: reaction medium · Vx: violaxanthin · VDE: violaxanthin de-epoxidase · Zx: zeaxanthin

Introduction

Xanthophyll cycles play a major role in the short-term protection of higher plants and algae against an overexcitation of the photosynthetic apparatus under conditions where the light intensity exceeds photosynthetic capacity (for recent reviews, see Horton et al. 1996; Gilmore 1997). Another important function of xanthophyll cycles has been recently proposed by Lohr and Wilhelm (1999), who showed that xanthophyll cycling optimizes the biosynthesis of light-harvesting pigments under fluctuating light conditions. Higher plants and green algae possess a violaxanthin (Vx) cycle in which Vx is converted to zeaxanthin (Zx) in high light (Yamamoto et al. 1962). This two-step de-epoxidation sequence includes antheraxanthin (Ax) as an intermediate reaction product and is reversible in low light or darkness. A two-component xanthophyll cycle consisting of a one-step de-epoxidation from diadinoxanthin to diatoxanthin, and the respective epoxidation reaction, can be found in the algal classes Bacillariophyceae, Crysophyceae, Xanthophyceae, and Dinophyceae (Stransky and Hager 1970; Hager 1980). Very recently, Lohr and Wilhelm (1999) have been able to show that diadinoxanthin-containing algae possess both the diadinoxanthin and the violaxanthin cycle. Another interesting xanthophyll cycle has been reported in the prasinophyceaen alga *Mantoniella squamata* (Goss et al. 1998). Although this alga has the potential to convert Vx to Zx, illumination of intact cells with high light intensities induces only the first de-epoxidation step, from Vx to Ax.

The de-epoxidation reaction in the Vx cycle of higher plants is catalyzed by the enzyme violaxanthin de-epoxidase (VDE), which is localized in the lumen of the

thylakoid membranes (Hager 1969; Hager and Holocher 1994). The VDE with a pH optimum at pH 5.2 (Hager 1969), under in vivo conditions, is activated by the establishment of a light-driven proton gradient, and is supposed to bind to the lumenal side of the thylakoid membrane after activation (Hager and Holocher 1994). De-epoxidation of Vx does not necessarily rely on a light-driven pH gradient, but can also be induced by an incubation of thylakoid membranes or intact algal cells in reaction buffers adjusted to the pH optimum of VDE (Hager 1969; Goss et al. 1998). The acidic form of reduced ascorbate has been shown to be the co-substrate of the de-epoxidation reaction (Hager 1969; Bratt et al. 1995). From the studies of Bratt et al. (1995), it has also become clear that the pH optimum of VDE can be shifted to lower pH values when the ascorbate concentrations in the thylakoid lumen are low.

VDE has been purified from lettuce (Rockholm and Yamamoto 1996) and from spinach (Arvidsson et al. 1996; Havir et al. 1997; Kuwabara et al. 1999) by conventional protein purification methods. The amino acid sequence of the protein has been determined in tobacco, lettuce and *Arabidopsis* by molecular cloning (Bugos and Yamamoto 1996; Bugos et al. 1998), the sequence of spinach VDE is also available in the GenBank (accession number AJ250433). VDE has been identified as a member of the lipocalin family of proteins (Bugos et al. 1998). The protein shows three interesting domains, including a cysteine-rich domain, a lipocalin signature and a highly charged region (Bugos and Yamamoto 1996). The first domain contains 11 of the 13 total cysteines of the mature protein that probably form more than one disulfide bond. It is reasonable to believe that the cysteine-rich region is the site of action of the VDE inhibitor dithiothreitol (DTT; Yamamoto and Kamite 1972). The lipocalin signature is typical for a group of very diverse proteins that bind small hydrophobic molecules. The lipocalin domain typically contains a barrel-like structure that, in higher-plant VDE, is supposed to bind the hydrophobic substrates Vx and Ax. The third domain typical for VDE contains a high proportion of glutamic acid residues and is negatively charged. This domain may play a role in the activation of the enzyme and its binding to the thylakoid membrane when, under conditions of high proton concentrations, the negatively charged side chains of the amino acids become protonated.

The VDE of higher plants is specific for xanthophylls with a 3-hydroxy-5,6-epoxy group in a 3*S*, 5*R*, 6*S* configuration and with a polyene chain in the all-*trans* configuration (Yamamoto and Higashi 1978). The enzymes substrate affinity is twice as high for Ax as for Vx, a fact that leads to a much faster conversion of Ax compared with Vx (Yamamoto and Higashi 1978; Havir et al. 1997; Grotz et al. 1999).

The epoxidation reaction of the Vx cycle is catalyzed by the enzyme Zx epoxidase that is localized on the stromal side of the thylakoid membrane and has a pH optimum of pH 7.5 (Hager 1975; Siefermann and Yamamoto 1975). Zx epoxidase utilizes oxygen, FAD, and NAD(P)H as co-substrates (Büch et al. 1995). Zx epoxidase, originally thought to be identical to light-harvesting complex (LHC) II (Gruszecki and Krupa 1993), has recently been cloned and sequenced from tobacco (Marin et al. 1996), pepper (Bouvier et al. 1996), and tomato (Burbridge et al. 1997). A close comparison of these epoxidase protein sequences with already published sequences of lipocalins has revealed that Zx epoxidase also belongs to the lipocalin family of proteins (Bugos et al. 1998).

Epoxidation of Zx can best be observed under low light conditions or in darkness, but it has also been shown to occur in periods of high light illumination (Siefermann and Yamamoto 1975; Gilmore et al. 1994). Zx epoxidation under high light conditions is, however, less efficient than the contemporaneous Vx de-epoxidation, so that a net Zx accumulation can be observed in illuminated plants or thylakoid membranes.

It is the aim of our present study to demonstrate why, under high light conditions, the violaxanthin cycle of *M. squamata* is restricted to the first de-epoxidation step, from Vx to Ax. Furthermore, we point out the differences between the VDE of *M. squamata* and that of higher plants. The elucidation of the mechanism of this incomplete Vx cycle leads us to new insights into a functional and possible genetic heterogeneity of xanthophyll de-epoxidases of plants.

Materials and methods

Plant material

Batch cultures of *Mantoniella squamata* Manton et Parke (isolated by Desikachary; supplied by the Culture Collection Plymouth, Cambridge, UK; strain LB 1965/1) were cultivated at a photon flux density (PFD) of 40 μmol m^{-2} s^{-1} (photosynthetically active radiation) with a light:dark regime of 14:10 h in artificial seawater medium, according to Müller (1962). The temperature of the growth chamber was held constant at 20 °C. *Mantoniella* cells were harvested in the dark-adapted state at a chlorophyll (Chl) content of 2 mg Chl *a*/*b*/*c* l^{-1}. *Mantoniella* cells used for thylakoid isolation or preparation of VDE were grown at a PFD of 40 μmol m^{-2} s^{-1} in air-lift cultures and harvested at a chlorophyll content of 10 mg Chl *a*/*b*/*c* l^{-1}.

Fresh spinach (*Spinacia oleracea* L.) leaves were obtained from the local market.

Isolation of thylakoids

Dark-adapted *Mantoniella* cells (equivalent to 20 mg total Chl) were harvested by centrifugation with 2,500 *g* for 10 min at 4 °C (Varifuge, Heraeus). The cells were then resuspended in 50 ml isolation buffer [1 M sorbitol, 2 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes (pH 7.4), 0.2% BSA] and broken by three 15-s sonication pulses (Labsonic, Braun, Germany). After a second centrifugation step at 2,500 *g* for 4 min at 4 °C that removed remaining cell debris, the chloroplasts were osmotically shocked by a 2-min incubation in 30 ml shock medium [2 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes (pH 7.4)]. The broken chloroplasts were centrifuged again (2,500 *g*, 4 min, 4 °C), and the pellet containing the thylakoid membranes was resuspended in 1 ml VDE-preparation buffer (5 mM $MgCl_2$, 20 mM Tris, pH 7.5).

Dark-adapted spinach leaves (100 g) were homogenized in a blender (AKA Electric, Suhl, Germany) in 60 ml isolation buffer [400 mM sorbitol, 50 mM Mes (pH 6.5), 5 mM $MgCl_2$, 10 mM NaCl, 0.2% BSA]. The homogenate was filtered through a nylon net and centrifuged for 3 min at 1,500 *g* at 4 °C (Varifuge). The resulting pellet was washed with 20 ml of isolation buffer and centrifuged again. Chloroplasts were then osmotically shocked by a 2-min incubation in 20 ml shock medium [5 mM $MgCl_2$, 10 mM NaCl, 10 mM Mes (pH 6.5)], and after the last centrifugation step (2,500 *g*, 3 min, 4 °C), resuspended in 1 ml VDE-preparation buffer.

Dark incubation of *M. squamata* cells and spinach thylakoids at pH 5

Mantoniella cells were harvested by mild centrifugation at 1,000 *g* for 5 min at 15 °C (Varifuge), and then resuspended in reaction medium (RM) pH 5 consisting of 330 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 40 mM Mes and 30 mM ascorbate. After incubation times of 5, 9, 20, 60, and 120 min in the dark, 20 ml of incubated *Mantoniella* cells (equivalent to 40 µg total Chl) was collected on glass-fiber filters, immediately frozen in liquid nitrogen, and after freeze-drying, stored at –80 °C.

Spinach thylakoids were diluted in RM pH 5 (composition see above) to yield a final Chl concentration of 100 µg Chl *a*/*b* ml^{-1}. After 5, 9, 20, 60, and 120 min of incubation at pH 5 in the dark, 2 ml of the thylakoid suspension was collected on glass-fiber filters, frozen, freeze-dried, and stored at –80 °C.

Isolation of VDE (according to Hager and Holocher 1994)

The thylakoid suspensions of *M. squamata* and spinach (in VDE preparation buffer) were adjusted to a total Chl content of 2 mg ml^{-1}. The thylakoids were frozen in liquid nitrogen and then immediately thawed in a water bath adjusted to 29 °C. After the completion of seven freeze-thaw cycles, the suspensions were centrifuged for 20 min at 38,000 *g* and 2 °C (Allegra 64R; Beckman Instruments). The clear, colourless supernatant containing VDE was then used for the in vitro de-epoxidation assays.

The total protein content of the VDE extracts was determined according to Bradford (1976).

Isolation of Vx and Ax

Violaxanthin was extracted from dark-adapted spinach thylakoids. Spinach thylakoids with high contents of Ax were obtained by a short (15 s) incubation of thylakoid membranes in RM pH 5 at a reaction temperature of 29 °C. The thylakoid suspensions (3 mg total Chl) were centrifuged for 1 min at 2,500 *g* and 4 °C (Varifuge), and the photosynthetic pigments were extracted by the addition of 1 ml 100% acetone, and separated by HPLC (see below). Pure Vx and Ax were then dried under nitrogen and stored at −20 °C.

Enzyme assay at pH 5.2

The in vitro enzyme assay was performed according to Hager and Holocher (1994) with a modified reaction buffer consisting of 10 mM KCl, 5 mM $MgCl_2$, 40 mM Mes (pH 5.2), and 30 mM ascorbate. A 1-ml enzyme assay typically contained 25–100 µl of enzyme solution corresponding to 100 µg of total protein. In vitro de-epoxidation was started by the addition of purified Vx or Ax, and the reaction temperature was held constant at 29 °C.

To investigate the time-course of de-epoxidation, 1.2 μmol^{-1} Vx or 1 μmol^{-1} Ax was added to the assay, and samples were collected after de-epoxidation times of 0, 2.5, 5, 10, 30, 60, and 120 min.

To determine the apparent K_m values for Vx and Ax of both spinach and *M. squamata* VDE, we held the incubation time constant and added increasing pigment concentrations (in the range from 0.5 to 3.3 μmol^{-1}) to the in vitro assay. We found a reaction time of 10 min useful for the determination of the apparent *K*m values for Vx as substrate. The K_m determination for spinach VDE and Ax as substrate should be restricted to shorter incubation times (5 min).

The in vitro de-epoxidation reaction was terminated by the addition of 500 µl ethyl acetate, and 30 s of stirring transferred the pigments to the ethyl acetate phase. After a short centrifugation at 13,000 *g* for 2 min (Z 231 M; Hermle Gosheim, Germany), the pigments in the ethyl acetate phase were collected, dried under nitrogen, and stored at –20 °C for HPLC analysis.

The apparent K_m values were derived from the use of Lineweaver-Burk plots.

Pigment analysis by HPLC

Pigments were extracted in a medium consisting of 90% methanol/ 0.2 M ammonium acetate (90:10, v/v) and 10% ethyl acetate, centrifuged for 2 min at 13,000 *g* (Hermle Z 231 M), and injected into the HPLC column.

Reversed phase HPLC was performed on a Gynkotec HPLC system (Gynkotec, Germering, Germany) equipped with a photodiode array detector. The detection wavelength for integration was 440 nm. Quantification of pigments was done using calibration curves calculated from HPLC separations with purified pigment standards.

Pigments of *M. squamata* were separated on a Nucleosil column (ET 250/8/4, 120-3, C-18; Macherey & Nagel, Düren, Germany). The elution gradient was run with eluent A (0.1 ammonium acetate H_2O/methanol, 15:85, v/v) and eluent B (methanol/acetonitrile/ acetone, 44:43:13,v/v): 0 min 100% A, 32 min 75% A, 25% B, 47 min 100% B, 70 min 100% B. The flow rate was adjusted to 0.8 ml min^{-1}, the column temperature to 20 °C.

Pigment extracts of *S. oleracea* were analysed on a Nucleosil column (ET 250/8/4, 120-5, C18; Macherey & Nagel). The gradient was run with eluent A (acetonitrile/25 mM Tris, 97.5:2.5, v/v; pH 7.5) and eluent B (methanol/ethyl acetate, 75/25, v/v): 0 min 100% A, 18 min 100% A, 20 min 100% B, 35 min 100% B. The flow rate was 1 ml min^{-1}, the column temperature 20 °C.

Pigment extracts from the in vitro VDE assay containing only the xanthophyll cycle pigments Vx, Ax and Zx were analyzed, using a short-term HPLC separation on a Nucleosil 125/8/4, 120–5, C18 column (Chromatographie Service, Langerwehe, Germany). The gradient was run with eluent A (methanol/ 0.1 M ammonium acetate, 90/10, v/v) and eluent B (methanol/ ethyl acetate, 90/10, v/v): 0 min 100% A, 3 min 100% A, 7 min 100% B, 15 min 100% B. The flow rate was 1.5 ml min^{-1}, the column temperature 20 °C.

Illumination of spinach thylakoids and intact cells of *M. squamata*

Freshly prepared spinach thylakoids were diluted in RM pH 7.5 consisting of 330 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 40 mM Hepes (pH 7.5), and 30 mM ascorbate to yield a final Chl *a*/*b* concentration of 100 µg ml^{-1}. Ferricyanide (3 mM) was added as an artificial electron acceptor, and the thylakoids were illuminated for 20 min at a PFD of 400 µmol m^{-2} s^{-1}. The temperature was held constant at 20 °C. Directly after the end of the high light period, 2 ml of thylakoid membranes (equivalent to 200 µg total Chl) was harvested by filtration, freeze-dried, and stored at –20 °C. Intact *Mantoniella* cells were illuminated at a PFD of 250 µmol m^{-2} s^{-1} for 20 min or 6 h. The temperature of the algal cells was held constant at 20 °C. Carbon dioxide limitation was avoided by the addition of 10 mM $KHCO_3$ to the culture medium before the start of illumination. After the respective illumination times, 20 ml of *Mantoniella* cells (40 µg of total Chl) was filtered, freeze-dried, and stored for HPLC separation.

Epoxidase activity during illumination at high PFD

To investigate the kinetics of Ax epoxidation during actinic illumination, intact cells of *M. squamata* were exposed to a PFD of

200 μmol m^{-2} s^{-1}. Carbon dioxide limitation was avoided by the addition of 10 mM $KHCO_3$ to the culture medium before the start of illumination. After 10 min of the actinic light period, when appreciable amounts of Ax had been formed, VDE was blocked by the addition of 1 mM DTT. The epoxidation of Ax to Vx was then followed by taking samples 5, 10, 15, 20, 30 and 50 min after addition of DTT. A 20-ml sample of *Mantoniella* cells (equivalent to 40 μg of total Chl) was collected on glass fiber filters, immediately frozen in liquid nitrogen, freeze-dried, and stored at –20 °C for HPLC analysis.

Calculation of de-epoxidation and epoxidation rates

The rate constants for the first de-epoxidation step from Vx to Ax were calculated by fitting the Vx consumption to a mono-exponential decay function. The rate constants for the second de-epoxidation step from Ax to Zx were calculated assuming a stoichiometric conversion between the pigments of the xanthophyll cycle. Epoxidation from Ax to Vx in illuminated *Mantoniella* cells was calculated by a fit of the Ax decrease to a mono-exponential decay function.

Results

Xanthophyll cycling under in vivo conditions

Table 1 shows the differences in the light-induced conversions of the Vx cycle pigments in higher plants and in the prasinophyceaen alga *Mantoniella squamata*. Illumination of dark-adapted spinach thylakoids for 20 min at a PFD of 400 μmol m^{-2} s^{-1} led to a strong de-epoxidation of Vx to Zx. The Ax contents that were low in dark-adapted thylakoid membranes did not change during the high light exposure and remained low in the illuminated thylakoids. In *M. squamata*, illumination with saturating PFDs (20 min, 250 μmol m^{-2} s^{-1}) also resulted in a strong decrease in Vx. The product of the de-epoxidation sequence, however, was found to be exclusively Ax. Zx contents that were low in the dark-adapted intact *Mantoniella* cells did not increase during the 20-min high light treatment, and even prolonged exposures to saturating light intensities never resulted in an accumulation of Zx. This observation is in agreement with our earlier data on the Vx cycle of *M. squamata* (Goss et al. 1998). In this study, we also showed that *Mantoniella* cells incubated at pH 5 (the pH optimum of VDE) were able to form Zx. In the present study, we extend these findings and show that the Vx/Ax cycle of *Mantoniella* in intact cells during illumination is caused by an atypically slow de-epoxidation step from Ax to Zx and a fast epoxidation reaction. In cells incubated at pH 5, where the epoxidation is blocked by the low pH (pH optimum of the epoxidase is pH 7.5), we consequently expect a slow but appreciable Zx formation. This, on the other hand, means that incubation of intact cells at pH 5 is an essential prerequisite for acquiring information about the kinetics of the second de-epoxidation step from Ax to Zx in *M. squamata*.

Table 1 Pigment contents [mmol pigment (mol Chl *a*)$^{-1}$] of the Vx cycle pigments violaxanthin (Vx), antheraxanthin (Ax), and zeaxanthin (Zx) of dark-adapted or illuminated *Mantoniella squamata* cells and *Spinacia oleracea* thylakoids. Dark-adapted cells and thylakoids remained in darkness for at least 3 h prior to pigment determination. Cells of *M. squamata* were illuminated at a PFD of 250 μmol m^{-2} s^{-1} for 20 min or 6 h. Spinach thylakoids were illuminated at a PFD of 400 μmol m^{-2} s^{-1} for 20 min in the presence of 30 mM ascorbate and 3 mM ferricyanide. Mean values of three individual experiments with an SD of < 10% are shown

Conditions	Vx	Ax	Zx
M. squamata			
Dark-adapted	68.2	–	2.2
Light, 20 min	29.5	40.1	2.7
Light, 6 h	35.4	43.4	6.5
Spinach thylakoids			
Dark-adapted	160.2	10.6	5.4
Light, 20 min	75.6	9.0	97.2

Xanthophyll cycling at pH 5

Figure 1a shows the time-course of Vx de-epoxidation in spinach thylakoids incubated in RM pH 5 in the dark. Adjusting the pH of the reaction medium to the pH

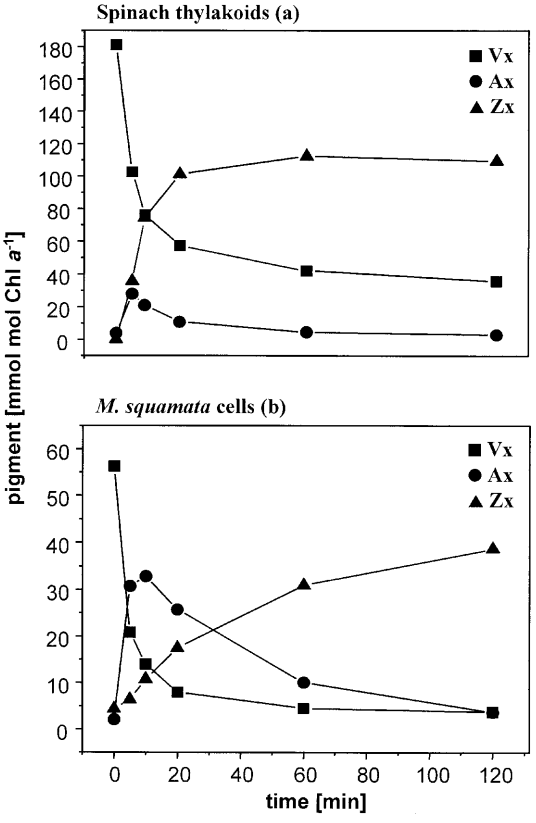

Fig. 1 The time course of Vx de-epoxidation (*squares*) and the synthesis of Ax (*circles*) and Zx (*triangles*) in isolated thylakoid membranes of *Spinacia oleracea* (**a**) and in intact *M. squamata* cells (**b**) in RM pH 5 in the dark. The chlorophyll concentration was adjusted to 100 μg Chl *a*/*b* ml^{-1} (**a**) and 2 μg Chl *a*/*b*/*c* ml^{-1} (**b**). The pigment concentrations are given as mmol pigment (mol Chl *a*)$^{-1}$. The figure shows the result of a typical experiment ($n = 3$, **a**; $n = 5$, **b**)

optimum of VDE led to a rapid decrease of Vx. Vx was directly converted to Zx, and increases in the Ax concentration could only be observed transiently during the first minutes of the de-epoxidation reaction. The largest part of the Zx formation was completed after 20 min of incubation at pH 5. Prolonged incubation times only led to further minor increases in the Zx concentration. Xanthophyll cycle pigment conversions proved to be substantially different in *Mantoniella* cells incubated at pH 5 (Fig. 1b). Vx that was de-epoxidized with kinetics similar to those in spinach thylakoids was not directly converted to Zx. De-epoxidation of Vx led to a strong increase in the Ax contents. Zx formation was not completely inhibited in *Mantoniella* cells, but Zx accumulated very slowly, and we observed high amounts of Zx only after long incubation times of 30–60 min. The kinetic differences in the formation of the de-epoxidation products between spinach and *Mantoniella* were most obvious after 10 min of pH-5 incubation. At that time we found high Zx and almost negligible Ax contents in spinach thylakoids, whereas *Mantoniella* cells showed high Ax and only low Zx concentrations. In contrast to the spinach thylakoids, and to what is known from other plant species (Siefermann and Yamamoto 1974; Pfündel and Dilley 1993), in *Mantoniella* the entire Vx pigment pool was convertible to Ax and Zx during the de-epoxidation at low pH.

The differences in the formation of the de-epoxidation products are also reflected by the rate constants calculated for both de-epoxidation steps in spinach and *Mantoniella* (Table 2). Similar rate constants were determined for the first de-epoxidation step, from Vx to Ax, for both spinach thylakoids and *Mantoniella* cells. Appreciable differences, however, became obvious for the second de-epoxidation reaction, from Ax to Zx. The rate constant for Zx formation was found to be very high for spinach thylakoids, whereas *Mantoniella* cells exhibited a drastically lowered rate constant for the second de-epoxidation step (17-times lower than in spinach). Taking into account the drastic differences in the rate constants of the Ax/Zx conversion, one can easily explain the differences in the formation of the de-epoxidation products in spinach and *Mantoniella*. Ax that is formed very efficiently in both spinach and *Mantoniella* is directly and very rapidly converted to Zx in spinach, whereas in *Mantoniella*, the strongly reduced ability to de-epoxidize Ax to Zx leads to the observed accumulation of Ax.

As will become evident from the in vitro experiments presented below, the differences in the de-epoxidation kinetics between *Mantoniella* and spinach were not due to the fact that intact cells (of *Mantoniella*) were compared with isolated thylakoid membranes (of spinach). The drastically different de-epoxidation rates could clearly be related to intrinsic differences between the VDEs of *M. squamata* and spinach.

Table 2 Rate constants of Vx/Ax de-epoxidation and Ax epoxidation in intact cells of *M. squamata*, and de-epoxidation of Vx/Ax by the isolated *M. squamata* VDE. De-epoxidation of Vx/Ax in spinach thylakoid membranes and by the isolated spinach VDE. For experimental conditions see Figs. 1, 2, 3, 4, and *Materials and methods* section. The calculated rate constants of a typical experiment are shown ($n = 3$)

Conditions	pH	De-epoxidation		Epoxidation
		Vx → Ax k (min^{-1})	Ax → Zx k (min^{-1})	Ax → Vx k (min^{-1})
Spinach thylakoids	5	0.14	0.5	
M. squamata cells	5	0.23	0.03	
Spinach VDE	5.2	0.163	0.691	
M. squamata VDE	5.2	0.176	0.021	
M. squamata cells, light	7.8			0.119

In vitro de-epoxidation of Vx by isolated VDE of spinach and *Mantoniella*

In in vitro assays employing isolated VDE of spinach or *Mantoniella* and purified Vx, we found a pattern of Vx de-epoxidation similar to that in spinach thylakoid membranes and intact *Mantoniella* cells. De-epoxidation of Vx started directly after Vx addition to the RM pH 5.2 containing spinach VDE (Fig. 2a). As in the de-epoxidation reaction in isolated thylakoids, Vx was directly converted to Zx, and Ax accumulation was never observed in in vitro assays with isolated spinach VDE. Conversion of Vx was slightly faster than in the intact thylakoid membranes, but the magnitudes of the rate constants of the first de-epoxidation step (Table 2) were found to be similar for both purified spinach VDE and thylakoids. *Mantoniella* VDE also showed a fast de-epoxidation of Vx (Fig. 2b). Zx, however, was formed very slowly, thus leading to the same de-epoxidation pattern as in intact *Mantoniella* cells with high Ax concentrations arising during the first minutes of the pH-5.2 incubation. In contrast to the de-epoxidation reaction in *M. squamata* cells, Ax was not completely converted to Zx during the later stages of the de-epoxidation. This observation can be explained if one considers that the epoxidized xanthophyll cycle pigments, Vx and Ax, are susceptible to pigment degradation, i.e. an isomerization to the furanoid-5,8-epoxides during the pH-5.2 incubation, whereas the de-epoxidized Zx remains stable under these mildly acidic conditions. This means that in the enzyme assay employing the *M. squamata* VDE, where especially Ax is converted to Zx with slow reaction kinetics, high Ax concentrations are present for relatively long time periods and can be converted to the respective furanoid, antherachrome. This isomerization product is then no longer accessible for de-epoxidation. Similar problems do not occur in enzyme assays with spinach VDE, where Vx and Ax are rapidly converted to the stable Zx. The complete conversion of Ax to Zx in the pH-5 treatment of intact *Mantoniella* cells can then be explained if one considers that the surrounding lipid

matrix, provided by the native thylakoid membrane, prevents the degradation of Vx and Ax.

The rate constants of the first de-epoxidation step from Vx to Ax (Table 2) were similar for both spinach and *Mantoniella* VDE, implying that Vx is a similarly well-suited substrate for both de-epoxidases, whereas Ax is not (see below for the estimation of the apparent K_m values of both enzymes). Spinach VDE exhibited a high rate constant for Zx formation, i.e. a very fast conversion of Ax to Zx, whereas *Mantoniella* VDE was characterized by a very low rate constant and slow kinetics of Ax de-epoxidation. It is noteworthy that, compared with the intact cells and thylakoid membranes, the rate constants for both de-epoxidation steps were only slightly enhanced in the in vitro assays with the isolated enzymes. This is a good indication that the kinetics of xanthophyll de-epoxidation in the thylakoid membrane reflect de-epoxidation kinetics of the respective enzyme, and are not substantially influenced by pigment accessibility or the existence of different Vx pigment pools in the thylakoid membrane (Siefermann and Yamamoto 1974, 1975; Pfündel and Dilley 1993).

In vitro de-epoxidation of Ax by isolated VDE of spinach and *Mantoniella*

The differences in the Ax de-epoxidation kinetics of the VDEs from spinach and *Mantoniella* were best seen when Ax was used as substrate for the isolated enzymes in the in vitro de-epoxidation assays (Fig. 3a, b). Spinach VDE showed a very fast de-epoxidation of Ax to Zx, with typical half-times of the de-epoxidation reaction of about 1 min, whereas for *Mantoniella* VDE, 30 min of reaction time was needed to convert half of the added Ax to Zx. This easily explains how the overall de-epoxidation process in thylakoid membranes is influenced by the drastically different de-epoxidation kinetics of the Ax-to-Zx conversion. For spinach thylakoids, this implies that as soon as Vx is converted to Ax, Ax is further de-epoxidized to Zx, and one will never observe an appreciable accumulation of Ax during the process of Vx de-epoxidation. The accumulation of Ax observed in *Mantoniella* cells or thylakoids is, on the other hand, caused by the interaction of a fast initial de-epoxidation step and the drastically lowered kinetics of the Ax-to-Zx conversion. Ax is formed very efficiently from Vx, but cannot be converted to Zx with the same efficiency. This,

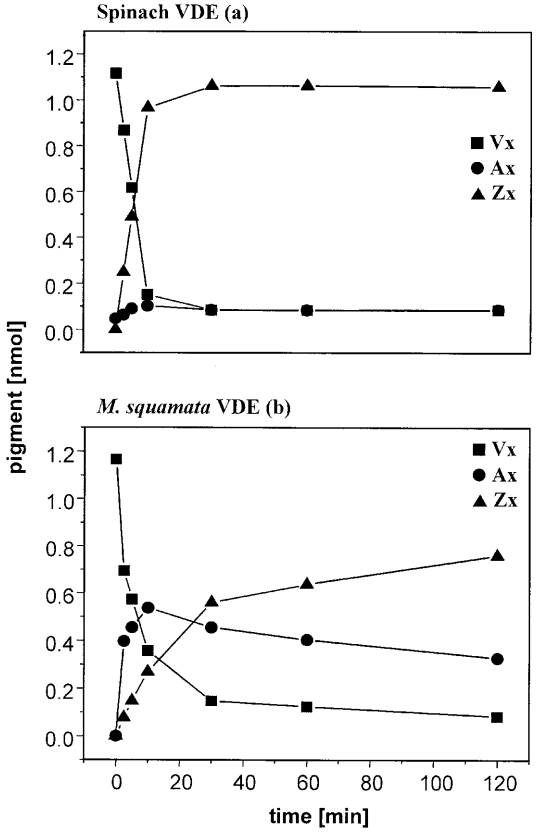

Fig. 2 Kinetics of the in vitro enzymic de-epoxidation of Vx (*squares*) to Ax (*circles*) and Zx (*triangles*) by spinach VDE (**a**) and *M. squamata* VDE (**b**). The reaction mixture (pH 5.2) contained isolated VDE (100 µg total protein), 30 mM ascorbate, and 1.17 µmol^{-1} Vx. The temperature of the in vitro assay was adjusted to 29 °C. Pigment concentrations are depicted as nmol pigment. The figure shows the result of a typical experiment ($n = 3$)

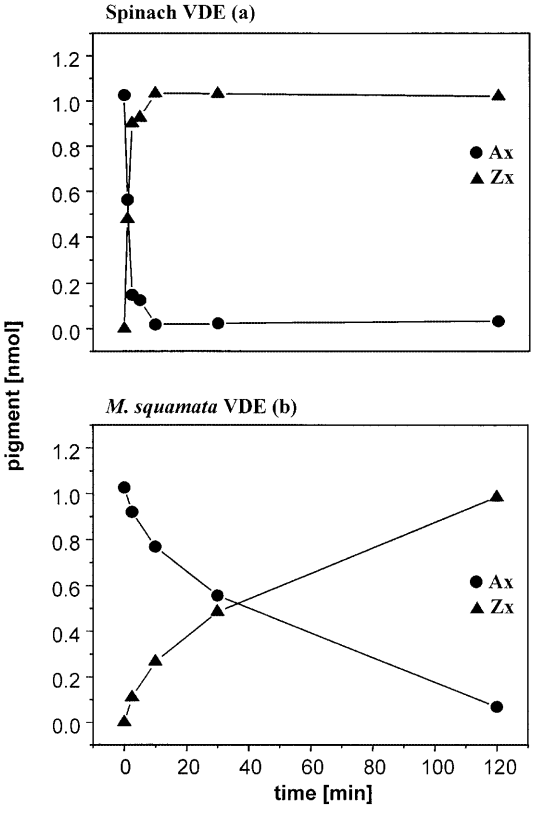

Fig. 3 Kinetics of the in vitro enzymic de-epoxidation of Ax (*circles*) to Zx (*triangles*) by spinach VDE (**a**) and *M. squamata* VDE (**b**). The reaction mixture (pH 5.2) contained isolated VDE (100 µg total protein), 30 mM ascorbate, and 1.03 µmol^{-1} Ax. The temperature of the in vitro assay was adjusted to 29 °C. Pigment concentrations are depicted as nmol pigment. The figure shows the result of a typical experiment ($n = 3$)

consequently, leads to the observed accumulation of the intermediate product of the violaxanthin cycle, Ax.

Apparent K_m values of *Mantoniella* and spinach VDEs for Vx and Ax as substrate

From our determination of the apparent K_m values of both spinach and *Mantoniella* VDE for Vx and Ax (Table 3), it becomes clear that the differences in the de-epoxidation kinetics were due to the different substrate affinities of spinach and *Mantoniella* VDEs for the substrate Ax. As expected from the similar rate constants of the first de-epoxidation step, from Vx to Ax, both enzymes exhibited similar K_m values for Vx. The fact that spinach VDE showed a lower apparent K_m, i.e. a higher substrate affinity for Ax than for Vx, is also in good agreement with the observed high rate constant of the second de-epoxidation step, from Ax to Zx in spinach thylakoid membranes. The apparent K_m values of spinach VDE, for both Vx and Ax, are in good agreement with those published by Hager (1980), Havir et al. (1997), and Grotz et al. (1999). The slow conversion rate of Ax to Zx in *Mantoniella* cells was reflected by a high apparent K_m value of *Mantoniella* VDE for the substrate Ax. Compared with spinach VDE, we observed a 5- to 6-fold higher K_m value for Ax, implying that Ax is a rather poor substrate for *Mantoniella* VDE and cannot be converted to Zx as efficiently as in spinach thylakoids. Although the high apparent K_m value of *Mantoniella* VDE for Ax was in good agreement with the low rate constant of the Ax-to-Zx conversion, it still seems to be underestimated (the apparent K_m value was found to be about 5 to 6 times higher in *Mantoniella* than in spinach, the rate constant of the second de-epoxidation step was estimated to be about 20 times lower). An explanation for this discrepancy could be that the relation between K_m and the rate constants of enzyme reactions only holds for substrate concentrations well below the K_m. The pigment concentrations (about 1 μmol^{-1} per assay) used in our enzyme assays were, on the one hand, necessary for an accurate quantification of the pigment conversions but may, on the other hand, have not been ideally suited to determine the exact correlation between K_m and rate constant.

Table 3 Apparent K_m values of the isolated VDEs from *M. squamata* and spinach for their substrates Vx and Ax. The apparent K_m values for Vx were determined after incubation at pH 5.2 for 10 min. For the determination of the apparent K_m of spinach VDE for Ax, the incubation time of the in vitro enzymic assay was restricted to 5 min. The apparent K_m of *M. squamata* VDE for Ax was determined after an incubation time of 10 min, and confirmed by independent measurements employing longer incubation times. The enzymic assay contained isolated VDE (100 µg total protein) and varying concentrations of Vx and Ax (0.3–3.3 μmol^{-1} pigment). The mean values of three independent K_m determinations with an SD of < 10% are shown

	Vx ($\mu mol\ l^{-1}$)	Ax ($\mu mol\ l^{-1}$)
Spinach VDE	3.3	1.6
M. squamata VDE	3.3	6

Epoxidation of Ax under high light conditions

To test our hypothesis that the Vx/Ax cycle of *M. squamata* is caused by the interaction of a slow de-epoxidation of Ax to Zx and a rather strong epoxidation reaction in the light, we illuminated *Mantoniella* cells at a PFD of 200 $\mu mol\ m^{-2}\ s^{-1}$ (Fig. 4). After high amounts of Ax had accumulated (corresponding to 10 min of high light), we added 1 mM DTT to block a further de-epoxidation from Vx to Ax. Pigment analysis of the xanthophyll cycle pigments during the 60 min after the addition of DTT clearly showed that the accumulated Ax was converted back to Vx, even under high light conditions. The rate constant of Ax epoxidation (Table 2) showed two important features. (i) It was lower than the rate constant of the first de-epoxidation step, from Vx to Ax. This means that de-epoxidation from Vx to Ax was favoured compared with the epoxidation from Ax to Vx and could induce the observed accumulation of Ax in the light. (ii) The rate constant of Ax epoxidation was higher than the rate constant of the second de-epoxidation step, from Ax to Zx. This means that under high light conditions two reactions with very different reaction kinetics were competing with each other for the substrate Ax, and that Ax was more efficiently converted back to Vx than in the forward reaction to Zx. Nevertheless, with high concentrations of Ax present, we would expect that a part of the Ax pool be converted to Zx. The total absence of Zx under

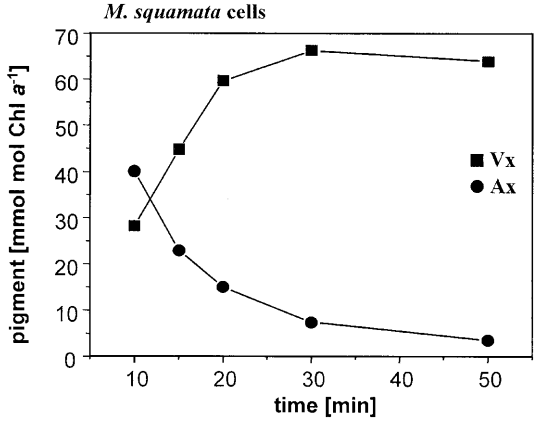

Fig. 4 The time course of Ax epoxidation (*circles*) and Vx formation (*squares*) in intact illuminated *M. squamata* cells. After 10 min of illumination at a PFD of 200 $\mu mol\ m^{-2}\ s^{-1}$ when substantial amounts of Ax had been formed (corresponding to the starting point of the figure), 1 mM DTT was added to block VDE. The PFD of 200 $\mu mol\ m^{-2}\ s^{-1}$ remained constant during the whole measuring period. The chlorophyll concentration of the cells was 2 µg Chl *a*/*b*/*c* ml^{-1}; 10 mM $KHCO_3$ was added to avoid carbon dioxide limitation. The pigment concentrations are depicted as mmol pigment (mol Chl *a*)$^{-1}$. The figure shows the result of a typical experiment ($n = 3$)

conditions of high light, however, implies that the first step of the epoxidation reaction, from Zx to Ax, also was taking place with a high rate constant and that any Zx formed was rapidly converted back to Ax. Table 2 lists the values of the different rate constants and shows that the rate constant of Ax epoxidation ($k = 0.119$ min^{-1}) was lower than the rate constant of the first de-epoxidation step from Vx to Ax ($k = 0.230$ min^{-1}), but, on the other hand, was substantially higher than that of the second de-epoxidation step, from Ax to Zx ($k = 0.030$ min^{-1}) in *Mantoniella* cells at pH 5.

Discussion

An explanation for the Vx/Ax cycle in *Mantoniella squamata*

In a previous paper (Goss et al. 1998), we were able to show that in vivo the prasinophyceaen alga *M. squamata* uses an incomplete Vx cycle. Illumination of intact *Mantoniella* cells with high light intensities led to a strong accumulation of Ax, whereas the product of the second de-epoxidation step Zx could never be observed, irrespective of the light intensity or the duration of the light period. We furthermore presented evidence that Ax was as efficient in photoprotection as Zx in higher plants. We also pointed out that VDE from *Mantoniella* possesses the ability to perform both de-epoxidation steps, and that incubation of *M. squamata* cells in a reaction buffer at pH 5 (the pH optimum of VDE, Hager 1969; Pfündel and Dilley 1993) induced appreciable concentrations of Zx. The results presented in our present study explain why illumination of intact cells leads to the observed accumulation of Ax, and also resolve the contradiction between the observed Zx formation at pH 5 and the missing Zx synthesis in the light. From our data, it is clear that the Vx/Ax cycle of *Mantoniella* is caused by the interaction of a slow, second de-epoxidation step from Ax to Zx and a strong epoxidation reaction in the light. For illuminated *M. squamata* cells this means that as soon as Vx is converted to Ax we find two reactions competing for the substrate Ax, namely the second de-epoxidation step, from Ax to Zx, and the epoxidation from Ax back to Vx. From the calculation of the rate constants of these two reactions it becomes clear that the epoxidation of Ax is more strongly favoured than the formation of Zx (rate constant is about 5 to 6 times higher). This implies that Ax is very efficiently converted back to Vx. Nevertheless, with high concentrations of Ax being present in high light, one should expect that a part of the Ax pool be converted to Zx. The total absence of Zx under conditions of high light illumination then implies that not only the epoxidation of Ax to Vx, but also the first epoxidation step from Zx to Ax exhibits very fast reaction kinetics and that any Zx formed will be rapidly converted back to Ax.

The rate constant of Ax epoxidation, however, is found to be lower than the rate constant of the first de-epoxidation step, from Vx to Ax, a fact that explains why we observe a net accumulation of Ax when illuminating the intact cells. Zx formation in intact cells at pH 5 can now easily be explained if one takes into account that the enzyme catalyzing the epoxidation reaction (Zx epoxidase) has a pH optimum at pH 7.5 (Hager 1975; Siefermann and Yamamoto 1975). Adjusting the pH of the incubation medium to pH 5 blocks the epoxidation reaction that normally competes with the second de-epoxidation step for their common substrate Ax and also inhibits the otherwise possible conversion of newly synthesized Zx back to Ax. Under conditions of low pH, Zx formation is the only process consuming Ax, and, consequently, we observe a slow but appreciable accumulation of Zx when *M. squamamta* cells are incubated at pH 5.

The slow de-epoxidation step from Ax to Zx in *Mantoniella*

It is a surprising result of our present study that, in *Mantoniella*, Ax is converted to Zx with extremely low reaction kinetics, especially if one takes into account the data on the second de-epoxidation step in higher plants. In spinach thylakoids, we find the Ax de-epoxidation to be almost 4 times faster than the Vx conversion, a result that is in accordance with data presented by Yamamoto and Higashi (1978), who used the 505-nm absorbance change to determine the velocity of the two de-epoxidation steps of the Vx cycle. This leads to the assumption that the Vx cycle of higher plants is optimized with regard to a complete conversion of Vx to Zx, and that an accumulation of the intermediate Ax is not a desirable condition for the higher-plant thylakoid membrane. While determining the apparent K_m values of spinach and *Mantoniella* VDEs for their substrates Vx and Ax does not explain why the *Mantoniella* xanthophyll cycle is not optimized in a similar way, it can, nevertheless, explain the different reaction kinetics for spinach thylakoids and *Mantoniella* cells. In spinach, we find the apparent K_m for Ax to be half of that for Vx. This is in good agreement with results published by Hager (1980) and Havir et al. (1997), and shows that Ax is a better substrate than Vx for spinach VDE. The higher substrate affinity of spinach VDE for the substrate Ax results in a faster second de-epoxidation step. *Mantoniella* VDE, on the other hand, shows a higher apparent K_m for Ax than for Vx. This means that Ax is not a good substrate for *Mantoniella* VDE and cannot be rapidly converted to Zx. Although the reduced substrate affinity of *Mantoniella* VDE for the substrate Ax can serve as an explanation for the low rate constant of the second de-epoxidation step and the observed Ax accumulation, the question of why Zx formation is strongly favoured in higher plants has to remain open.

The comparable rate constants of the de-epoxidation reactions in the in vitro assays and in the thylakoid

membranes or the intact cells show that the kinetics of the pigment conversions of the xanthophyll cycle pigments are not substantially influenced by xanthophyll availability in the thylakoid membrane (Siefermann and Yamamoto 1975; Pfündel et al. 1994) or by the existence of different Vx pools in the membrane (Siefermann and Yamamoto 1974; Demmig et al. 1987; Thayer and Björkman 1990; Pfündel and Dilley 1993). According to our results, it seems more likely that the overall de-epoxidation process in the thylakoid membrane can be attributed to the characteristic properties of the respective VDEs. We, nevertheless, find that a part of Vx is not convertible to Zx in the spinach thylakoid membranes, and might, therefore, represent Vx not available for de-epoxidation, as has been proposed by various authors (Siefermann and Yamamoto 1974, 1975; Pfündel and Dilley 1993). The fact that the Vx pool is completely convertible to Ax and Zx in *Mantoniella* can be related to the different structure of the *M. squamata* light-harvesting antenna, which contains only one light-harvesting complex (LHC) that serves as the antenna for both photosystems (Schmitt et al. 1993). This LHC is in some ways comparable to LHC IIb (Rhiel et al. 1993; Goss and Garab 2000), shown to exhibit the highest extent of de-epoxidation among the different light-harvesting proteins of higher plants (Ruban et al. 1994; Färber et al. 1997). Minor Chl *a*/*b*-binding proteins that show reduced Vx de-epoxidation (Ruban et al. 1994) are not present in the light-harvesting system of *Mantoniella*, and therefore cannot negatively influence the overall extent of de-epoxidation. The finding that Vx is bound to the LHC of *Mantoniella squamata* (Goss and Wilhelm 1998), but exhibits a weak pigment-protein binding, probably also contributes to the complete conversion of Vx. The weak pigment-protein coupling implies that during de-epoxidation, Vx is easily removed from the light-harvesting protein and may be completely accessible to VDE.

Possible molecular basis for the reduced substrate affinity of *Mantoniella* VDE for Ax

Our observation that the slow second de-epoxidation in *Mantoniella* is an intrinsic property of the *Mantoniella* VDE and is due to its decreased substrate affinity for Ax is an indication that *Mantoniella* VDE might show some modifications on the level of the protein structure compared with the VDE of higher plants. We propose that changes in region I, the cysteine-rich region of the protein, or in region II, the lipocalin signature (Bugos and Yamamoto 1996), could be responsible for the observed reduced ability of Ax conversion by *Mantoniella* VDE. Changes in the lipocalin region, which contains the hydrophobic binding pocket for the xanthophyll cycle pigments (Bugos et al. 1998), could reduce the binding efficiency of Ax to the enzyme VDE. This modification of the β-barrel structure has to be highly specific for Ax because from our data it can clearly be seen that the substrate affinity of *Mantoniella* VDE for Vx is not reduced compared with the VDE of higher plants. Therefore, we believe that a modification of the cysteine-rich region of the protein can serve as a more plausible explanation for the slow de-epoxidation of Ax to Zx. We were able to show (unpublished results) that low concentrations of DTT, which only partially inhibited spinach VDE, mainly affected the second de-epoxidation step and decreased the kinetics of the Ax/Zx conversion. This is in agreement with results published by Gilmore and Yamamoto (1993), who used low DTT concentrations to accumulate Ax in chloroplasts of *Pisum sativum* and *Lactuca sativa*. From the elucidation of the amino acid sequence of lettuce VDE (Bugos and Yamamoto 1996), it has become obvious that the cysteine-rich region of the protein contains 11 cysteine residues. As it is most likely that these cysteines form more than one disulfide bond, a partial inhibition of VDE by low DTT concentrations might be the result of the reduction of only a part of these linkages. Ax accumulation in partially inhibited higher-plant VDE gives rise to the speculation that *Mantoniella* VDE contains a cysteine-rich region with a lower amount of cysteine residues and disulfide linkages, resulting in the observed slow Ax-to-Zx conversion.

Xanthophyll epoxidation in periods of high light

Our present study clearly shows that the Vx/Ax cycle in *Mantoniella* is caused by the interaction of the slow second de-epoxidation step and the relatively fast epoxidation of Ax to Vx in high light. Epoxidation of Zx or Ax to Vx is normally described as taking place in low light or periods of darkness that follow illumination phases with high light intensities (Hager 1966, 1967). Epoxidation of Zx to Vx in high light is difficult to determine because the forward reaction of the xanthophyll cycle, i.e. Vx de-epoxidation is strongly favoured under these illumination conditions. By blocking Vx conversion with the VDE inhibitor DTT, various authors have been able to demonstrate Zx epoxidation under high light conditions in higher plants (Hager 1975; Siefermann and Yamamoto 1975; Gilmore et al. 1994) and in microalgae (Goss et al. 1998). It is another important characteristic of the epoxidation reaction that the reaction kinetics of Zx epoxidation depend on the light intensity used for Vx de-epoxidation and the length of the illumination period. High actinic light intensities and long periods of high light that lead to an over-excitation of PSII have been shown to reduce the epoxidation kinetics in higher plants (Demmig-Adams et al. 1998). Lohr and Wilhelm (1999) have also reported rate constants for diatoxanthin and Zx epoxidation in the diatom *Phaeodactylum tricornutum* that are lower than the rate constant for Ax epoxidation presented in our study. These differences might again be due to the differences in the illumination conditions used in these two studies, 6 h of 1,000 $\mu mol\ m^{-2}\ s^{-1}$ in the experiments

of Lohr and Wilhelm and 10 min of 250 μmol m^{-2} s^{-1} in our study, respectively. Although the length of the high light period seems to reduce the kinetics of xanthophyll epoxidation, we have to stress that even 6 h of high light never led to an accumulation of Zx in *Mantoniella*. This means that in *Mantoniella*, even after long high-light incubations, the rate constant of Ax epoxidation has to be higher than that of the second de-epoxidation step from Ax to Zx.

Functional heterogeneity of xanthophyll de-epoxidases

Mantoniella VDE is the second xanthophyll de-epoxidase from algal cells we have characterized and which differs functionally from higher-plant VDE. For *Phaeodactylum tricornutum* diadinoxanthin de-epoxidase (DDE), these differences are not related to a different affinity for the substrates Vx and Ax, as in *Mantoniella*, but to alterations in the pH optimum of the enzyme (Jakob et al. 2001). We were able to show that activation of *Phaeodactylum* DDE is significantly shifted to higher pH values compared with the VDE of higher plants. In *Phaeodactylum* thylakoids that contained the pigments of both the Vx cycle and the diadinoxanthin cycle (see also Lohr and Wilhelm 1999), we demonstrated that Vx and Ax are de-epoxidized in a way similar to that in higher plants, i.e. that conversion of Vx directly leads to the formation of Zx. On the other hand, we found that *Mantoniella* VDE, which exhibits a different substrate affinity for Vx and Ax, has a similar pattern of pH activation as the VDE of higher plants (data not shown). Our finding that different algal groups and higher plants contain functionally different xanthophyll de-epoxidases could also reflect a phylogenetic heterogeneity of these de-epoxidases. Future experiments are necessary to reveal whether or not these biochemical differences result from alterations of the protein and/or the gene structure. Determination of the amino acid sequence of the different xanthophyll de-epoxidases will provide new information about the protein domains responsible for pH activation and substrate binding and affinity.

Acknowledgements The authors express their thanks to Karen Böhme for providing the results of the 6-h light treatment of *M. squamata* cells, and to Dr. Martin Lohr for his help with the calculation of the rate constants. Financial support from the Deutsche Forschungsgemeinschaft (Go 818/1–2) is gratefully acknowledged.

References

Arvidsson P-O, Bratt CE, Carlsson M, Åkerlund H-E (1996) Purification and identification of the violaxanthin deepoxidase as a 43 kDa protein. Photosynth Res 49:119–129

Bouvier F, d'Harlingue A, Hugueney P, Marin E, Marion-Poll A, Camara B (1996) Xanthophyll biosynthesis: cloning, expression, functional reconstitution, and regulation of β-cyclohexenyl carotenoid epoxidase from pepper (*Capsicum anuum*). J Biol Chem 271:28861–28867

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bratt CE, Arvidsson P-O, Carlsson M, Akerlund H-E (1995) Regulation of violaxanthin deepoxidase activity by pH and ascorbate concentration. Photosynth Res 45:169–175

Büch K, Stransky H, Hager A (1995) FAD is a further essential cofactor of the NAD(P)H and O_2-dependent zeaxanthin-epoxidase. FEBS Lett 376:45–48

Bugos RC, Yamamoto HY (1996) Molecular cloning of violaxanthin de-epoxidase from romaine lettuce and expression in *Escherichia coli*. Proc Natl Acad Sci USA 93:6320–6325

Bugos RC, Hieber AD, Yamamoto HY (1998) Xanthophyll cycle enzymes are members of the lipocalin family, the first identified from plants. J Biol Chem 273:15321–15324

Burbridge A, Grieve T, Terry C, Corlett J, Thompson A, Taylor I (1997) Structure and expression of a cDNA encoding zeaxanthin epoxidase, isolated from wilt-related tomato (*Lycopersicon esculentum* Mill.) library. J Exp Bot 48:1749–1750

Demmig B, Winter K, Krüger A, Czygan F-C (1987) Photoinhibition and zeaxanthin formation in intact leaves. Plant Physiol 84:218–224

Demmig-Adams B, Moeller DL, Logan BA, Adams III WW (1998) Positive correlation between levels of retained zeaxanthin + antheraxanthin and degree of photoinhibition in shade leaves of *Schefflera arboricola* (Hayata) Merill. Planta 205:367–374

Färber A, Young AJ, Ruban AV, Horton P, Jahns P (1997) Dynamics of xanthophyll-cycle activity in different antenna subcomplexes in the photosynthetic membranes of higher plants. Plant Physiol 115:1609–1618

Gilmore AM (1997) Mechanistic aspects of xanthophyll cycle-dependent photoprotection in higher plant chloroplasts and leaves. Physiol Plant 99:197–209

Gilmore AM, Yamamoto HY (1993) Linear models relating xanthophylls and lumen acidity to non-photochemical fluorescence quenching. Evidence that antheraxanthin explains zeaxanthin-independent quenching. Photosynth Res 35:67–78

Gilmore AM, Mohanty N, Yamamoto HY (1994) Epoxidation of zeaxanthin and antheraxanthin reverses non-photochemical quenching of Photosystem II chlorophyll *a* fluorescence in the presence of trans-thylakoid ΔpH. FEBS Lett 350:271–274

Goss R, Garab G (2000) Organization of the pigment molecules in the chlorophyll *a*/*b*/*c* containing alga *Mantoniella squamata* (Prasinophyceae) studied by means of absorption, circular and linear dichroism spectroscopy. Biochim Biophys Acta 1457: 190–199

Goss R, Wilhelm C (1998) In *Mantoniella squamata* deepoxidized xanthophyll cycle pigments show a tighter pigment protein coupling than the respective epoxidized forms. In: Garab G (ed) Photosynthesis: mechanisms and effects, vol III. Kluwer, Dordrecht, pp 2317–2320

Goss R, Böhme K, Wilhelm C (1998) The xanthophyll cycle of *Mantoniella squamata* converts violaxanthin into antheraxanthin but not to zeaxanthin: consequences for the mechanism of enhanced non-photochemical energy dissipation. Planta 205: 613–621

Grotz B, Molnár P, Stransky H, Hager A (1999) Substrate specificity and functional aspects of violaxanthin de-epoxidase, an enzyme of the xanthophyll cycle. J Plant Physiol 154:437–446

Gruszecki WI, Krupa Z (1993) LHC II, the major light-harvesting pigment-protein complex is a zeaxanthin epoxidase. Biochim Biophys Acta 1144:97–101

Hager A (1966) Die Zusammenhänge zwischen lichtinduzierten Xanthophyll-Umwandlungen und Hill-Reaktion. Ber Dtsch Bot Ges 79:94–107

Hager A (1967) Untersuchungen über die Rückreaktion im Xanthophyll-Zyklus bei *Chlorella*, *Spinacia* und *Taxus*. Planta 76:138–148

Hager A (1969) Lichtbedingte pH-Erniedrigung in einem Chloroplasten-Kompartiment als Ursache der enzymatischen

Violaxanthin-Zeaxanthin-Umwandlung; Beziehungen zur Photophosphorylierung. Planta 89:224–243

Hager A (1975) Die reversiblen, lichtabhängigen Xanthophyllumwandlungen im Chloroplasten. Ber Dtsch Bot Ges 88:27–44

Hager A (1980) The reversible light-induced conversions of xanthophylls in the chloroplast. In: Czygan FC (ed) Pigments in plants. Fischer, Stuttgart, pp 57–79

Hager A, Holocher K (1994) Localization of the xanthophyll-cycle enzyme violaxanthin de-epoxidase within the thylakoid lumen and abolition of its mobility by a (light-dependent) pH decrease. Planta 192:581–589

Havir EA, Tausta SL, Peterson RB (1997) Purification and properties of violaxanthin de-epoxidase from spinach. Plant Sci 123:57–66

Horton P, Ruban AV, Waters RG (1996) Regulation of light harvesting in green plants. Annu Rev Plant Physiol Plant Mol Biol 47:655–684

Jakob T, Goss R, Wilhelm C (2001) Unusual pH-dependence of diadinoxanthin de-epoxidase activation causes chlororespiratory induced accumulation of diatoxanthin in the diatom *Phaeodactylum tricornutum*. J Plant Physiol 158:383–390

Kuwabara T, Hasegawa M, Kawano M, Takaichi S (1999) Characterization of violaxanthin de-epoxidase purified in the presence of Tween 20: effects of dithiothreitol and pepstatin A. Plant Cell Physiol 40:1119–1126

Lohr M, Wilhelm C (1999) Algae displaying the diadinoxanthin cycle also possess the violaxanthin cycle. Proc Natl Acad Sci USA 96:8784–8789

Marin E, Nussaume L, Quesada A, Gonneau M, Sotta B, Hugueney P, Frey A, Marion-Poll A (1996) Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*. EMBO J 15:2331–2341

Müller D (1962) Über jahres- und lunarperiodische Erscheinungen bei einigen Braunalgen. Bot Mar 4:140–155

Pfündel E, Dilley RA (1993) The pH dependence of violaxanthin deepoxidation in isolated pea chloroplasts. Plant Physiol 101:65–71

Pfündel E, Renganathan M, Gilmore AM, Yamamoto HY, Dilley RA (1994) Intrathylakoid pH in isolated pea chloroplast as probed by violaxanthin de-epoxidation. Plant Physiol 106:1647–1658

Rhiel E, Lange W, Mörschel E (1993) The unusual light-harvesting complex of *Mantoniella squamata*: supramolecular composition and assembly. Biochim Biophys Acta 1143:163–172

Rockholm DC, Yamamoto HY (1996) Purification of a 43-kilodalton lumenal protein from lettuce by lipid-affinity precipitation with monogalactosyldiacylglyceride. Plant Physiol 110:697–703

Ruban AV, Young AJ, Pascal AA, Horton P (1994) The effects of illumination on the xanthophyll composition of the photosystem II light-harvesting complexes of spinach thylakoid membranes. Plant Physiol 104:227–234

Schmitt A, Herold A, Welte C, Wild A, Wilhelm C (1993) The light-harvesting system of the unicellular alga *Mantoniella squamata* (Prasinophyceae): evidence for the lack of a photosystem I specific antenna complex. Photochem Photobiol 57:132–138

Siefermann D, Yamamoto HY (1974) 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 357:144–150

Siefermann D, Yamamoto HY (1975) Properties of NADPH and oxygen-dependent zeaxanthin epoxidation in isolated chloroplasts. Arch Biochem Biophys 171:70–77

Stransky H, Hager A (1970) Das Carotinoidmuster und die Verbreitung des lichtinduzierten Xanthophyllzyklus in verschiedenen Algenklassen. Arch Mikrobiol 73:315–323

Thayer SS, Björkman O (1990) Leaf xanthophyll content and composition in sun and shade determined by HPLC. Photosynth Res 23:331–343

Yamamoto HY, Higashi RM (1978) Violaxanthin de-epoxidase. Lipid composition and substrate specificity. Arch Biochem Biophys 190:514–522

Yamamoto HY, Kamite L (1972) The effects of dithiothreitol on violaxanthin de-epoxidation and absorbance changes in the 500 nm region. Biochim Biophys Acta 267:538–543

Yamamoto HY, Nakayama TOM, Chichester CO (1962) Studies on the light interconversions of the leaf xanthophylls. Arch Biochem Biophys 97:168–173