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Dynamics of Xanthophyll-Cycle Activity in Different Antenna Subcomplexes in the Photosynthetic Membranes of Higher Plants[1]

The Relationship between Zeaxanthin Conversion and Nonphotochemical Fluorescence Quenching

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The generation of nonphotochemical quenching of chlorophyll fluorescence (qN) in the antenna of photosystem II (PSII) is accompanied by the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin. The function of zeaxanthin in two mechanisms of qN, energy-dependent quenching (qE) and photoinhibitory quenching (qI), was investigated by measuring the de-epoxidation state in the antenna subcomplexes of PSII during the generation and relaxation of qN under varying conditions. Three different antenna subcomplexes were separated by isoelectric focusing: Lhcb1/2/3, Lhcb5/6, and the Lhcb4/PSII core. Under all conditions, the highest de-epoxidation state was detected in Lhcb1/2/3 and Lhcb5/6. The kinetics of de-epoxidation in these complexes were found to be similar to the formation of qE. The Lhcb4/PSII core showed the most pronounced differences in the de-epoxidation state when illumination with low and high light intensities was compared, correlating roughly with the differences in qI. Furthermore, the epoxidation kinetics in the Lhcb4/PSII core showed the most pronounced differences of all subcomplexes when comparing the epoxidation after either moderate or very strong photoinhibitory preillumination. Our data support the suggestion that zeaxanthin formation/epoxidation in Lhcb1–3 and Lhcb5/6 may be related to qE, and in Lhcb4 (and/or PSII core) to qI.

The induction of nonphotochemical, thermal dissipation of excitation energy is thought to provide protection against photooxidative damage of the photosynthetic apparatus under light-stress conditions, i.e. when more light energy is absorbed than can be used in photosynthetic electron transport. Two important mechanisms contribute to these processes: the energy- or pH-dependent mechanism (for review, see Horton et al., 1996) and photoinhibition (Aro et al., 1993; Osmond, 1994), giving rise to two qN components, qE, and qI, respectively. It has been suggested that the xanthophyll cycle, the reversible de-epoxidation of violaxanthin to Zea, is involved in both processes.

The qE component of qN is regulated by the lumen pH. The molecular mechanism by which the lumen pH controls the formation and relaxation of qE is still unclear. Most experimental evidence supports the view that qE originates from (pH-dependent) events in the PSII antenna (Horton et al., 1991, 1996; Gilmore and Yamamoto, 1993; Falk et al., 1994; Jahns and Krause, 1994; Härtel et al., 1996), rather than in the reaction center core (Weis and Berry, 1987; Krieger et al., 1992; Krieger and Weis, 1993). The generation of qE during acidification of the thylakoid lumen is accompanied by the formation of Zea in the de-epoxidation reactions of the xanthophyll cycle (Siefermann-Harms, 1977; Yamamoto, 1985; Pfündel and Bilger, 1994). The direct or indirect function of this carotenoid in the qE mechanism has been suggested by several authors (Demmig-Adams et al., 1990; Gilmore and Yamamoto, 1993; Gilmore et al., 1994; Jahns and Schweig, 1995; Horton et al., 1996). Binding of the xanthophyll-cycle pigments by antenna proteins (Bassi et al., 1993; Ruban et al., 1994; Lee and Thornber, 1995) supports the attribution of qE to events in the PSII antenna.

The photoinhibitory quenching component qI was assumed to originate from the reaction center core of PSII. Acceptor-side- and/or donor-side-related mechanisms of PSII inactivation that may lead to damage of the D1 protein have been proposed (Callahan et al., 1986; Barbato et al., 1991; de las Rivas et al., 1992; Vass et al., 1992; van Wijk and van Hasselt, 1993). Recovery from this inactivation state

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Abbreviations: Chl, chlorophyll; DEPS, de-epoxidation state; DM, *n*-dodecyl-β-D-maltoside; PFD, photon flux density; qE, pH-dependent quenching; qI, photoinhibitory quenching; qN, nonphotochemical quenching of Chl fluorescence; Zea, zeaxanthin.

was thought to be only reversible by replacement of the D1 protein (Aro et al., 1993; Leitsch et al., 1994). However, at least a part of qI has been suggested to occur in the antenna by a mechanism similar to qE (Ruban et al., 1993; Ruban and Horton, 1995) and to be symptomatic of the regulation of quenching capacity.

Similarly, the idea of a function of Zea in photoinhibitory processes (Demmig-Adams, 1990) has been supported by recent work showing that relaxation of qI is kinetically related to the epoxidation of Zea (Jahns and Miehe, 1996; Verhoeven et al., 1996). The concerted down-regulation of both the recovery of qI and Zea epoxidation with increasing degrees of photoinhibition implied a very close relation of both parameters (Jahns and Miehe, 1996). In vitro experiments with isolated thylakoids established that the reduction of the epoxidation rate (and of qI relaxation) was not attributable to a maintained transmembrane proton gradient under these conditions (Färber and Jahns, 1995), as might have been expected from other studies (Gilmore and Björkman, 1994a, 1994b). The close relation of qI relaxation and Zea epoxidation could then be based either on a co-regulation of epoxidase activity and recovery from photoinhibition or, as proposed for the qE mechanism of qN, on a direct or indirect function of Zea in qI.

Apart from a down-regulated epoxidase activity, decreasing epoxidation kinetics could in principle result from a reduced accessibility of the epoxidase to its substrate, e.g. from (light-dependent) alterations in the binding sites of Zea within the PSII antenna or conformational changes within xanthophyll-binding proteins. A possible association or a close contact of Zea with the PSII core antenna has been proposed to explain the correlation of qI relaxation and Zea epoxidation in intermittent light-grown plants that are devoid of most of the PSII antenna proteins (Jahns and Miehe, 1996). Based on these results, a possible redistribution of Zea within PSII under prolonged high-light stress has been hypothesized (Jahns and Miehe, 1996). So far, however, studies of the distribution of the xanthophyll-cycle pigments among the different pigment-binding proteins have brought no evidence for xanthophyll binding to the PSII core antenna in either the epoxidized or the de-epoxidized state (Bassi et al., 1993; Ruban et al., 1994; Lee and Thornber, 1995). However, it has been noticed that DEPS was variable between the light-harvesting complex II (LHCII) proteins, with the lowest found in the Lhcb4 protein complex (LHCIIa or CP29) (Ruban et al., 1994).

In this study, we have carried out a detailed investigation of the dynamics of Zea formation within different antenna subcomplexes under a wider range of illumination conditions. We focused on (a) possible differences between in vivo and in vitro experiments, (b) possible differences of Zea formation and distribution under illumination with different light intensities, and (c) possible differences of Zea epoxidation after preillumination under conditions that are known to induce different kinetics of qI relaxation (and Zea epoxidation). qI is defined in this study simply as the slowly relaxing (half time > 30 min) component of qN, irrespective of the underlying mechanism.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea* L.) was grown hydroponically in a greenhouse with supplemental light, as described by Walker (1987). Leaves of 6-week-old plants were used for the experiments. Pea (*Pisum sativum* L. cv Kleine Rheinländerin) plants were grown in a climate chamber in a 14-h light/10-h dark cycle at a PFD of 100 μmol m^{-2} s^{-1}. Leaves of 12- to 14-d-old plants were harvested at the end of the dark period.

De-Epoxidation Conditions

In vivo de-epoxidation was carried out under illumination of leaves with a 2 × 250-W halogen light source. During illumination leaves were floated on distilled water in a temperature-controlled cuvette. Unless otherwise indicated, all experiments were done at 20°C. For light-induced in vitro de-epoxidation, thylakoids (120 μg/mL Chl) were suspended in 0.33 M sorbitol, 1 mM EDTA, 1 mM $MgCl_2$, 40 mM ascorbate, 0.2 mM methyl viologen, and 50 mM Hepes/NaOH, pH 7.6, and illuminated (KL 1500 light source, Walz, Effeltrich, Germany). For pH-induced de-epoxidation in the dark, Mes buffer was used instead of Hepes and the pH was adjusted to 5.3. No electron acceptor was added in this case. All in vitro experiments were carried out at 20°C under gentle stirring of the samples.

IEF

Nondenaturing IEF was carried out following the protocol of Ruban et al. (1994), with the exception that unstacked thylakoid membranes were used instead of PSII particles. Thylakoids were prepared from pea and spinach leaves as described elsewhere (Polle and Junge, 1986). Unstacking of membranes was achieved by washing of thylakoids (equivalent to 1.5 mg/mL Chl) in 5 mM EDTA and resuspension in distilled water. The gel slurry used for IEF contained 1% (w/v) Gly, 0.06% (w/v) DM (Sigma), 2.5% (v/v) ampholine (Pharmacia), pH 3.5 to 5.0, and 4% (w/v) Ultrodex (Pharmacia).

Twenty-five grams of water was evaporated before starting prefocusing (60 min, 8 W, 4°C). Freshly prepared thylakoid membranes containing 1.5 mg of Chl were solubilized in 1% (w/v) DM and incubated on ice for 30 min, with thorough mixing every 5 min. After loading of the samples, focusing was started at a constant power of 8 W for 18.5 to 19 h at 4°C. The procedure resulted in formation of 13 distinct green bands. Each band was carefully collected using a spatula. The pigment protein complexes were eluted from the gel in an elution column (PEGG, Pharmacia) using a buffer containing 10 mM Hepes, pH 7.6, and 0.06% (w/v) DM, and stored at 4°C.

Pigment Analysis

Pigments were extracted by mixing 0.5 mL of sample, 0.5 mL of ethanol, 1 mL of diethyl ether, and 0.25 mL of water in a tube. The upper phase was collected, and the lower phase was washed again with 1 mL of diethyl ether. The

collected upper phases were dried under N_2 and stored at −20°C until resuspension in pure acetone for analysis.

Pigment separation was performed by reverse-phase HPLC (LiChrospher 100 RP-18 column with 5-μm particle size, Merck, Darmstadt, Germany). Solvent A was composed of acetonitrile, methanol, and 0.1 M Tris/NaOH, pH 8.0, in a ratio of 87:10:3; solvent B was a 4:1 mixture of methanol and hexane. The gradient from solvent A to solvent B was run from 9 to 12.5 min at a flow rate of 2 mL/min. Eluted pigments were monitored at 440 nm. Conversion factors allowing the calculation of pigment concentration from the integrated peak area were determined by calibration with pure pigments. Neoxanthin and violaxanthin were isolated by TLC. Chl *a* and *b* were purchased from Serva (Heidelberg, Germany), lutein from Sigma, β-carotene from Fluka (Neu-Ulm, Germany), and Zea was a kind gift from Hoffmann-LaRoche (Basel, Switzerland). The factors were 2772 (neoxanthin), 3211 (violaxanthin), 2192 (lutein), 2707 (Zea), 1609 (Chl *a*), 1298 (Chl *b*), and 2001 (β-carotene), expressed as peak area per picomole of the respective pigment. Antheraxanthin was estimated with the conversion factor for lutein.

SDS-PAGE and Western-Blot Analysis

SDS-PAGE and western-blot analysis were carried out as described previously (Jahns and Krause, 1994). Aliquots of the IEF samples were dried in a vacuum (Speed-Vac SC100, Savant, Farmingdale, NY) and resuspended in sample buffer. Proteins were separated in a 15% acrylamide gel containing 5.3 M urea and stained with Coomassie brilliant blue R-250. Apparent molecular masses of the proteins were estimated by molecular mass standards (SDS-7, Sigma). The following antibodies were used for identification of proteins in the western-blot analysis: anti-D1, raised against the D1 protein of the PSII reaction center (gift from Dr. D. Godde, Bochum, Germany); anti-16, raised against the extrinsic 16-kD subunit of the O_2-evolving complex (gift from Dr. B. Andersson, Stockholm, Sweden); anti-psaC and anti-psaD, raised against the C and D subunit of PSI (both gifts from Dr. H. Strotmann, Düsseldorf, Germany); and anti-Lhcb1–3 (gift from Dr. S. Berg, Winona, MN). Bound antibodies were visualized by using the enhanced chemiluminescence kit (Amersham).

Absorption and 77-K Fluorescence Measurements

Aliquots of the IEF samples were taken for absorption spectra measurements and 77-K fluorescence analysis as described by Ruban and Horton (1992).

Fluorescence Measurements and Quenching Analysis

Room-temperature chlorophyll fluorescence was measured using a pulse-amplitude-modulated fluorimeter (Walz). The kinetics of relaxation of qN were recorded according to established protocols (Quick and Stitt, 1989; Walters and Horton, 1991), except that illumination with actinic light was extended to 1 h for some of the experiments and light-saturation pulses were delivered at intervals of 120 s. The fast-relaxing (within the first 10 min of dark relaxation after light treatment) component of fluorescence quenching was assigned to the energy-dependent mechanism (qE) and the slowly relaxing (after 10 min of dark relaxation) component of fluorescence quenching was assigned to photoinhibitory processes (qI). Quenching parameters of the Stern-Volmer (SV) type were calculated according to Thiele et al. (1997) as follows:

$$qE(SV) = (F_M'/F_{MS} - 1) \times F_M/F_M' = F_M/F_{MS} - F_M/F_M' \text{ and}$$

$$qI(SV) = F_M/F_M' - 1$$

where F_M is the maximum fluorescence yield, F_{MS} is the quenched level of maximum fluorescence reached under illumination for different times with actinic light, and F_M' is the maximum fluorescence after 10 min of dark relaxation of the samples subsequent to illumination with actinic light.

RESULTS AND DISCUSSION

Identification of IEF Bands

In contrast to earlier experiments (Ruban et al., 1994), antenna proteins of whole thylakoids instead of PSII-enriched membrane fragments were separated by IEF. Thirteen green bands were obtained from the spinach thylakoids. The absorption spectra of bands 1 to 8 were found to be very similar to those of bands 1 to 8 obtained with PSII particles from spinach (Ruban et al., 1994). The spectra of the additional bands, particularly bands 11 to 13, indicated substantial amounts of Chl *a* absorbing at wavelengths > 700 nm, attributable to the presence of PSI components (data not shown). This was verified by the 77-K fluorescence emission spectra of each band (Fig. 1). Only bands 9 to 13 exhibited a significant portion of the long-wavelength fluorescence (λ_{max} about 730 nm), which most likely originates from the Lhca1 and Lhca4 proteins of the PSI antenna. The peak at about 680 nm of bands 9 to 13 can be attributed to the Lhca2 and Lhca3 proteins, because no PSII antenna proteins were detectable in these bands by western-blot analyses (see below).

Figure 2 shows a Coomassie blue-stained SDS gel of all IEF fractions. Samples 1 to 4 contained bands in the 25- to 27-kD range, identified by western-blot analysis as the major components of the PSII antenna, Lhcb1, Lhcb2, and Lhcb3. Samples 5 and 6 both contained a mixture of Lhcb5 and Lhcb6 and, in lower amounts, some other (not necessarily pigmented) proteins. A strong band at 29 kD was visible mainly in sample 8 (and to a lower extent in sample 7), containing the Lhcb4 protein. Additionally, significant amounts of other proteins were found in this fraction, identified by western-blot analysis as components of the PSII reaction center core (not shown). The protein content of samples 9 to 13 showed the typical pattern of the four LHCI proteins (Lhca1–4), in agreement with the fluorescence emission spectra shown in Figure 1. In addition to the antenna proteins, polypeptides of the reaction center core of PSI were found in these fractions, again identified

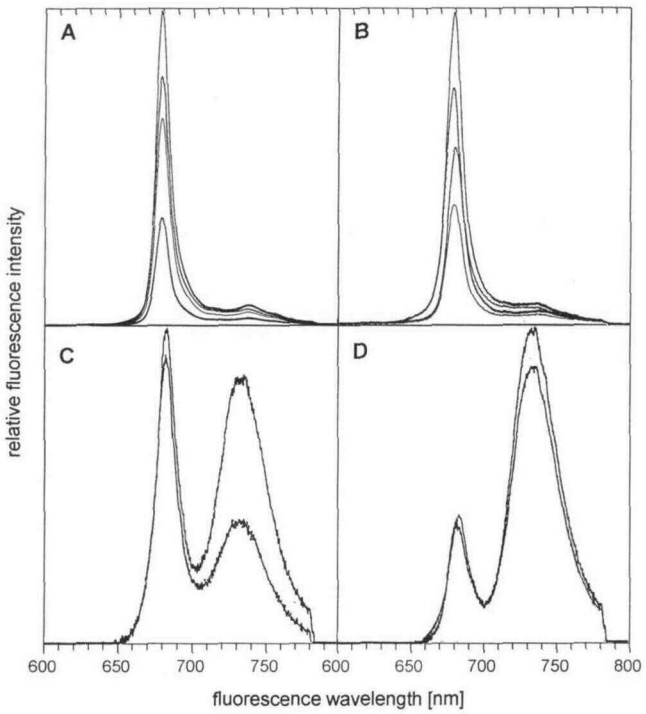

Figure 1. Emission spectra of Chl fluorescence at 77 K of all IEF fractions obtained with spinach thylakoids. A, Fractions 1 to 4; B, fractions 5 to 8; C, fractions 9 and 10; D, fractions 11 and 12. Spectra are arranged within each of the four parts (A–D) so that increasing peak heights correspond to increasing fraction numbers. The spectrum of fraction 13 was identical to that of fraction 12.

by western-blot analyses. A summary of these results is given in (Table I).

With pea thylakoids the pattern of IEF bands was very similar, with the exception that bands 5 and 6 (both containing Lhcb5 and Lhcb6 with spinach) ran as one single band and band 8 contained both PSII and PSI components. For the following analyses bands 1 to 4 were collected together as Lhcb1–3; bands 5 and 6 (only one band with pea

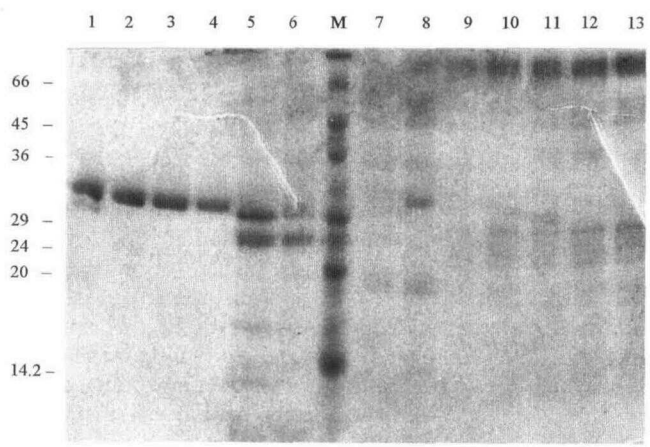

Figure 2. Coomassie blue-stained SDS gel of IEF samples. The numbers indicate the IEF fractions obtained with spinach thylakoids. M, Molecular mass standard (in kD).

Table I. *Identification of IEF band protein composition*

Data from absorption spectra, 77-K Chl fluorescence measurements (Fig. 1), and protein analyses of IEF bands (Fig. 2) are summarized.

Band No.	Absorption	77-K Fluorescence	SDS PAGE/Western-Blot Analysis
1	Lhcb1–3	PSII	Lhcb1–3
2	Lhcb1–3	PSII	Lhcb1–3
3	Lhcb1–3	PSII	Lhcb1–3
4	Lhcb1–3	PSII	Lhcb1–3
5	Lhcb5/Lhcb6	PSII	Lhcb5/Lhcb6
6	Lhcb5/Lhcb6	PSII	Lhcb5/Lhcb6
7	Lhcb4	PSII	Lhcb4 + D1/D2
8	Lhcb4	PSII	Lhcb4 + D1/D2
9	PSI	PSI	PSI
10	PSI	PSI	PSI
11	PSI	PSI	PSI
12	PSI	PSI	PSI
13	PSI	PSI	PSI

thylakoids) as Lhcb5/Lhcb6; bands 7 and 8 (only band 7 with pea thylakoids) as the Lhcb4/PSII reaction center core; and bands 9 to 13 as PSI.

Xanthophyll-Cycle Pigments of IEF Fractions

The xanthophyll composition of the four fractions from pea thylakoids is given in Table II. Compared with other data from the literature (Peter and Thornber, 1991; Bassi et al., 1993; Ruban et al., 1994), a somewhat higher content of the xanthophyll-cycle pigments was found in the three PSII fractions. This might originate from the use of whole thylakoids instead of PSII membrane fragments and could indicate that detergent treatment during the preparation of PSII particles in the other studies was accompanied by the loss of xanthophylls. Despite the contamination of the IEF fractions by reaction center core proteins, it proved possible to obtain reliable xanthophyll cycle contents by expressing the data on the basis of the content of Chl *b*.

Analysis of the pigment content supports the assumption that fraction I is composed of Lhcb1–3. The Chl *a/b* ratio of about 1.3 is in good agreement with other studies. Assuming that five Chl *b* are bound to one monomer (Kühlbrandt et al., 1994), we found about 0.5 xanthophyll-cycle pigments per monomer, somewhat higher than the value of 0.3 estimated by Ruban et al. (1994).

The Chl *a/b* ratio of about 3 in the Lhcb5/6 (fraction II) is not fully compatible with the values that have been reported for Lhcb5 (1.8–2.9) and Lhcb6 (about 1–1.5) in other studies (Peter and Thornber, 1991; Bassi et al., 1993; Ruban et al., 1994). This can simply be explained by a contamination of this fraction with proteins from the core antenna, since we also found unexpectedly high levels of β-carotene in this fraction (Table II). About 0.35 xanthophyll-cycle pigments per Chl *b* were detectable in Lhcb5/6. Under the assumption that three Chl *b* are bound to a monomer of Lhcb5 or Lhcb6, about one xanthophyll-cycle pigment per monomer is present in this fraction.

The third IEF fraction contained Lhcb4 and proteins of the PSII reaction center core, as was obvious from western-

Table II. *Pigment composition of IEF fractions*

Fractions I through IV were obtained from IEF experiments with pea thylakoids. The carotenoid content is expressed as percentage of total carotenoids. The amount of xanthophyll cycle pigments (violaxanthin + antheraxanthin + Zea [V + A + Z]) is also given on the basis of Chl *b*. Mean and SD values of 36 independent experiments performed under different illumination conditions are shown.

IEF Fraction	Complex	Chl *a*/*b*	Neoxanthin	Lutein	β-Carotene	V + A + Z	(V + A + Z)/Chl *b*
I (bands 1–4)	Lhcb 1–3	1.29 ± 0.05	20.6 ± 2.11	64.8 ± 1.7	3.7 ± 1.2	10.9 ± 1.8	0.11 ± 0.03
II (bands 5–6)	Lhcb5/6	2.95 ± 0.47	15.4 ± 1.8	50.6 ± 3.4	17.5 ± 6.1	16.5 ± 2.1	0.36 ± 0.08
III (band 7)	Lhcb4/PSII core	5.45 ± 1.27	9.1 ± 2.3	32.8 ± 5.5	43.1 ± 8.0	15.1 ± 2.6	0.39 ± 0.11
IV (bands 9–13)	Lhca1–4/PSI core	9.1 ± 0.9	1.8 ± 1.1	20.0 ± 3.1	64.6 ± 4.6	13.6 ± 1.8	0.49 ± 0.09

blot (not shown) and pigment analyses (Table II). Based on a stoichiometry of two Chl *b* per Lhcb4 (e.g. Giuffra et al., 1996), about 0.8 xanthophyll-cycle pigments per Lhcb4 can be estimated in this fraction, in agreement with other data (Peter and Thornber, 1991; Bassi et al., 1993; Ruban et al., 1994). Thus, all xanthophylls that are present in this band are probably associated with Lhcb4.

The pigment data of fraction IV confirm the assignment of this fraction to PSI. The high Chl *a*/*b* ratio and the very low neoxanthin content in particular are in good agreement with the pigment composition of PSI reported by Lee and Thornber (1995). Based on an estimated antenna size of roughly 200 Chl (*a* plus *b*) per PSI (Lee and Thornber, 1995; Färber and Jahns, 1997), about 10 xanthophyll-cycle pigments and 20 Chl *b* can be calculated for one PSI unit in pea thylakoids. With 6 to 8 LHCI proteins per PSI (Jansson, 1994; Lee and Thornber, 1995), a stoichiometry of 1 to 1.5 xanthophyll-cycle pigments per LHCI protein can be estimated.

In conclusion, the pigment data confirm the identification of the main proteins in the different IEF fractions based on fluorescence spectra, SDS-PAGE, and western-blot analyses. The stoichiometries of xanthophyll-cycle pigments per PSII antenna protein can be estimated with one xanthophyll per monomer for Lhcb4–6 and Lhca1–4, and with one per trimer for Lhcb1–3. With 5 Lhcb1–3 trimers per PSII (yielding a total PSII antenna size of about 250 Chl *a* plus *b*), and assuming a PSII/PSI stoichiometry of about 1, the xanthophyll-cycle pigments appear to be equally distributed between both photosystems. This is in contrast to the reported distribution of xanthophyll-cycle pigments (30% in PSI and 70% in PSII) in thylakoids isolated from cotton leaves (Thayer and Björkman, 1992). This discrepancy can be explained by different plant material and growth conditions or by an underestimation of the PSII/PSI stoichiometry in pea thylakoids (see below).

Comparison of Different De-Epoxidation Procedures

We determined the DEPS in whole thylakoids and the four IEF fractions after induction of the de-epoxidation reactions under in vivo and in vitro conditions. In vitro de-epoxidation was performed either by illumination at a PFD of 1 mmol m^{-2} s^{-1} or by low pH treatment of isolated spinach thylakoids, and in vivo de-epoxidation was performed by illumination of pea leaves with saturating PFDs of 2 mmol m^{-2} s^{-1} (Fig. 3). Comparing the three de-epoxidation procedures, we found similar degrees of de-epoxidation within each IEF fraction, with the pH treatment leading to a somewhat higher DEPS (Fig. 3). Very similar results were obtained with isolated pea thylakoids (data not shown).

However, marked differences were obtained when the different fractions were compared. The highest DEPS was always induced in Lhcb1–3 and Lhcb5/6, varying between about 70 and 80%. The lowest values were found in PSI (around 30%) and an intermediate DEPS of about 40 to 50% was detected in the Lhcb4/PSII core. Therefore, the earlier-reported differentiated xanthophyll conversion in the different PSII antenna subcomplexes (Ruban et al., 1994) was confirmed in these experiments. On the other hand, the DEPS in Lhcb4 was found to be clearly higher than in the former study. This is most likely because of the high light intensities and the long exposure times used here.

The most striking result, however, is the relatively low degree of de-epoxidation in PSI. These data suggest that the degree of de-epoxidation within distinct antenna sub-

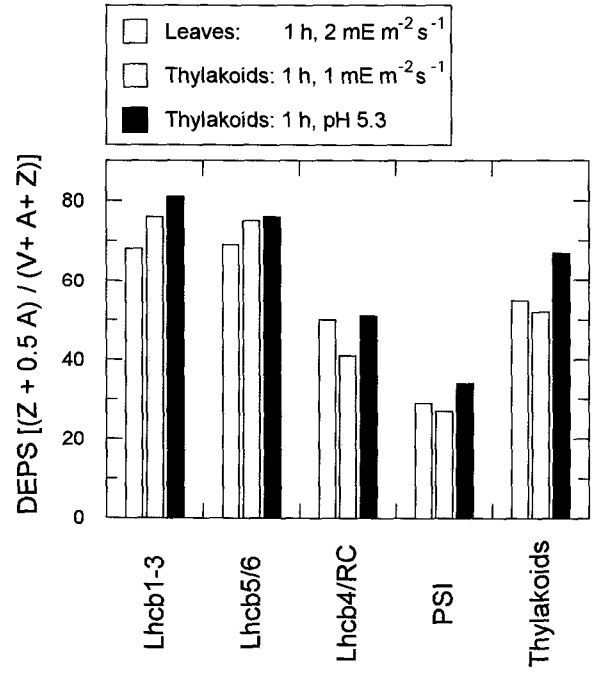

Figure 3. DEPS of the xanthophyll-cycle pigments in different antenna subcomplexes and thylakoids. De-epoxidation was induced under in vivo (with intact pea leaves) or in vitro (with isolated spinach thylakoids) conditions as indicated. V, Violaxanthin; Z, zeaxanthin; A, antheraxanthin. The data represent the mean value of one to four independent experiments.

complexes is determined by structural features that are specific for the different antenna subcomplexes. The differences between PSII and PSI antenna proteins might be explained by the proposed function of Zea formation in the PSII antenna in connection with thermal energy dissipation of excess light energy; no similar process of energy dissipation has been reported for PSI.

It is worth mentioning at this point that the above assumption of an equal distribution of the xanthophyll-cycle pigments between both photosystems is not consistent with the high DEPS obtained with whole thylakoids and an estimated PSII/PSI stoichiometry of 1. Thus, either the PSII/PSI stoichiometry must be higher than 1 (and thus more xanthophyll-cycle pigments are connected with PSII units), or there is a pool of nonprotein-bound xanthophylls, particularly in the de-epoxidized state. In any case, the xanthophyll-cycle pigments bound to PSI account for at least 30% (maximal 50%) of the total xanthophyll-cycle pool.

Dynamics of the De-Epoxidation in Different Antenna Proteins

Figure 4 shows the dynamics of the DEPS in the four IEF fractions during 1 h of illumination of pea leaves at PFDs of 500 and 2000 μmol m^{-2} s^{-1}. The lower light intensity has been shown to induce a high level of qE and a low level of qI, whereas the high intensity induces high levels of both qE and qI (Jahns and Krause, 1994; Jahns and Miehe, 1996; see also Table III).

At 500 μmol m^{-2} s^{-1} (Fig. 4), the time-dependent development of the DEPS was nearly identical in Lhcb1–3 and Lhcb5/6. It reached a maximum value after 20 min of about 40 to 45% and dropped to about 30% under prolonged illumination. In contrast, the DEPS in the two other fractions, the Lhcb4/PSII core and PSI, was much lower, with maximum values of about 25% under these conditions. In these two fractions no decrease of the DEPS was observable during prolonged illumination.

At 2000 μmol m^{-2} s^{-1} (Fig. 4), the highest DEPS was found in Lhcb5/6 and Lhcb1–3, about 65 to 70%, but no decrease of the DEPS was observable under prolonged illumination at the high light intensity in these two fractions. When comparing the two illumination conditions, the most pronounced increase in the maximum DEPS under strong illumination was detectable in the Lhcb4/PSII core. Moreover, a slow increase of the DEPS under prolonged illumination was obvious in this fraction. In PSI, however, the DEPS was only slightly increased and remained at a rather low level (30%) in comparison with the PSII fractions.

The increase of the DEPS in all subcomplexes at a PFD of 2000 μmol m^{-2} s^{-1} in comparison with the lower intensity can be explained by the increased de-epoxidase activity attributable to a lower lumenal pH at these saturating illumination conditions. The differences in the extent of the increase of the DEPS among the different antenna proteins most probably reflect different properties attributable to specific structural features of each antenna protein. These different properties could be based on either specific capacities of each protein in binding de-epoxidized xanthophylls or specific interactions of antenna proteins with the de-epoxidase. In either case, the differences might be closely related to the different sensitivities of the proteins to Zea-dependent and pH-induced energy dissipation.

From an earlier investigation of the dependence of xanthophyll-cycle activity on light intensity (Jahns, 1995), it can be concluded that the lower light intensity of 500 μmol m^{-2} s^{-1} does not fully saturate the photosynthetic electron transport in pea leaves. The decrease of the DEPS in Lhcb1–3 and Lhcb5/6 in the time range from 20 to 60 min of illumination can thus be attributed to an increase of the

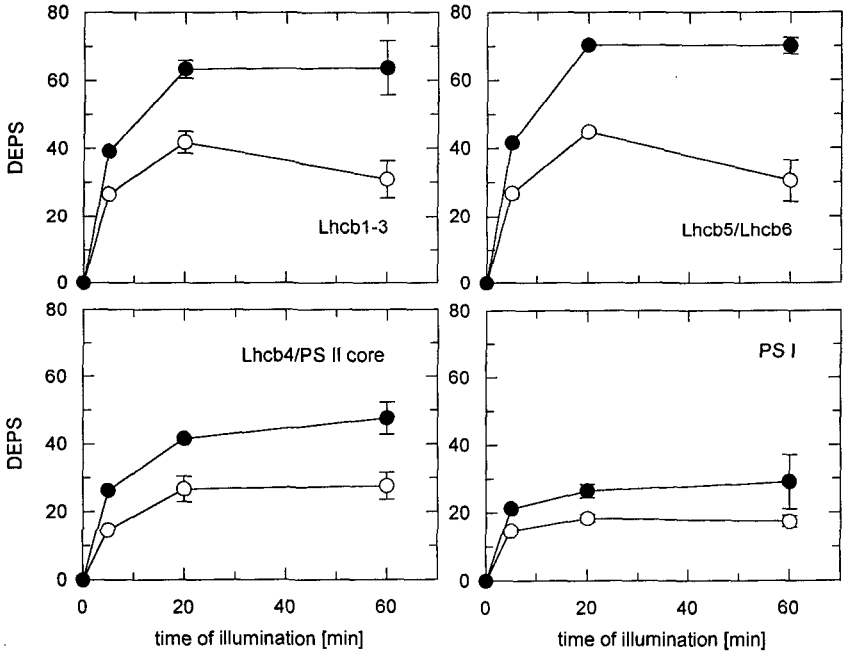

Figure 4. Time course of de-epoxidation in different antenna subcomplexes. De-epoxidation was induced by illumination of intact pea leaves at PFDs of 500 (●) or 2000 (○) μmol m^{-2} s^{-1}. Thylakoids were isolated at the different times and antenna proteins were separated by IEF. The data represent the mean value of two to four independent experiments. Error bars indicate either SE ($n \geq 3$) or the upper and lower limits of the respective data points ($n = 2$).

Table III. *Comparison of qN and the DEPS*

The extent of qE and qI was determined in intact leaves under the same conditions as the experiments shown in Figure 4. The data for the DEPS were taken from Figure 4. Values in parentheses show normalized data: within each column all data are given in relation to the value obtained after 20 min of illumination at a PFD of 500 μmol m^{-2} s^{-1}, which was set to 100 for all parameters. Mean values of two to four independent experiments are shown.

PFD	Time	qE	DEPS		qI	DEPS	
			Lhcb1–3	Lhcb5/6		Lhcb4	PSI
500 μmol m^{-2} s^{-1}	20 min	1.04 (100)	0.42 (100)	0.45 (100)	0.12 (100)	0.27 (100)	0.18 (100)
	60 min	0.68 (65)	0.31 (74)	0.31 (69)	0.22 (183)	0.28 (104)	0.17 (94)
2000 μmol m^{-2} s^{-1}	20 min	2.10 (202)	0.63 (150)	0.70 (156)	0.33 (275)	0.42 (155)	0.26 (144)
	60 min	2.12 (204)	0.64 (152)	0.70 (156)	0.48 (400)	0.48 (178)	0.29 (161)

lumenal pH after full activation of the Calvin cycle. However, it is not easy to understand why this effect can only be observed in these two fractions and not in Lhcb4 and PSI antenna proteins. A possible explanation might be that only complexes with higher DEPS are epoxidized under these conditions. This interpretation would be in agreement with the notion that under prolonged illumination (up to 3 h), the DEPS in Lhcb1–3 and Lhcb5/6 did not decrease below that in Lhcb4, which remained nearly constant within this period (not shown).

The similarity of the expected changes in lumenal pH and the kinetics of DEPS in Lhcb1–3 and Lhcb5/6 suggest that Zea formation in these complexes might be related functionally to the regulation of pH-dependent energy dissipation in PSII. In this case, a possible function of Lhcb4 in Zea-related thermal energy dissipation might then be in pH-independent processes, e.g. in connection with the qI component of qN. This would also explain the pronounced increase of the DEPS at the higher light intensity in this complex.

Determination of qN Parameters

We determined the extent of qE and qI (expressed as Stern-Volmer type) after 20 and 60 min of illumination under both light regimes. The result is summarized in Table III. The relative changes of qE very closely matched the relative changes of the DEPS in Lhcb1–3 and Lhcb5/6 with respect to the differences (in both the extent and the time course) between the two light intensities. This confirms our speculation that de-epoxidation in Lhcb1–3 and Lhcb5/6 may be involved in the generation of quenching centers that contribute to qE. The earlier suggestion that an interaction of Zea with Lhcb5 is involved in qE (Jahns and Schweig, 1995) is also supported by these data.

Our data are partly in contrast to the earlier reported direct correlation of the DEPS and the pH-dependent qN in whole thylakoids (Gilmore and Yamamoto, 1993; Gilmore et al., 1994), which suggested that the whole xanthophyll pool (and not only those xanthophylls that are bound to Lchb1–3 and Lhcb5/6) reacts with the same kinetics and is involved in qE. Here we show that the DEPS in Lhcb4 and PSI shows clearly different behavior in comparison with the other antenna complexes and also the level of qE. As stated above, the amount of Zea that is bound to Lhcb4 and PSI represents at least one-third of the total xanthophyll-cycle pool and should therefore significantly contribute to the overall DEPS in thylakoids.

The relative changes of qI, on the other hand, showed no clear correlation with the de-epoxidation in one of the subcomplexes (Table III). Only an increase in both qI and the DEPS in Lhcb4/PSII core at higher irradiance and longer illumination times, in contrast to the stationary qE and the DEPS in Lhcb1–3 and Lhcb5/6, might indicate that de-epoxidation in Lhcb4 is somehow related to the generation of quenching centers that are involved in a Zea-dependent qI. However, a further increase of qI under illumination of leaves at low temperature was not accompanied by a further increase of the DEPS in Lhcb4 (see below). This poor kinetic correlation of the generation of photoinhibitory fluorescence quenching and violaxanthin de-epoxidation must not necessarily argue against a postulated function of Zea in qI, but, rather, might reflect that (a) qI has different mechanistic bases, and (b) additional factors are required to establish qI.

Jahns and Miehe (1996) speculated that the generation of qI might originate from Zea-dependent energy quenching in the core antenna and thus might be accompanied by binding of Zea to the PSII core. Although we have not separated Lhcb4 and the PSII core in these experiments, the stoichiometry of xanthophyll-cycle pigments per Chl *b* (Table II) indicates that all xanthophylls in this fraction are more likely to be associated with Lhcb4 than with the PSII core. Therefore, any binding of Zea to the PSII core is not supported by our work. However, a possible function of CP47 and CP43 (which both are absent in this fraction) under in vivo conditions cannot be excluded by the present experiments.

Dynamics of the Epoxidation in Different Antenna Complexes

The possible involvement of Zea binding to distinct antenna proteins (particularly to Lhcb4) in the qI component of qN was further investigated by the following experimental approach. Leaves were illuminated at a PFD of 2 mmol m^{-2} s^{-1} for either 1 h at room temperature or 3 h at 4°C. These conditions can be expected to induce not only different degrees of qI, but also (after transfer of the leaves into low light) different kinetics of both qI relaxation and Zea epoxidation (Jahns and Miehe, 1996). Therefore, a specific function of Zea in one of the antenna complexes along

with qI might then be reflected by differences in the epoxidation kinetics between antenna subcomplexes.

The absolute values of the DEPS within each fraction were the same after both illumination procedures (not shown) and resembled the maximum DEPS in each fraction determined in the earlier experiment at the higher light intensity after 1 h shown in Figure 4. On the other hand, qI increased (from 0.48 to 0.70) during prolonged illumination at low temperature. Thus, the increase of qI under the more extreme light-stress treatment was not accompanied by an increase of the DEPS in any of the antenna proteins. Moreover, analyses of the absolute stoichiometries of xanthophyll-cycle pigments per antenna protein in each fraction (based on the estimations given above) indicated that neither an additional binding of xanthophylls in distinct complexes nor a shift of xanthophylls from one complex to the other occurred in parallel to the increase of qI (not shown). Therefore, as discussed previously (Krause and Weis, 1991; Horton, 1996), it is important to emphasize the extreme heterogeneity of qI, which undoubtedly results from several effects of light stress on the thylakoid membrane.

The time course of epoxidation in the different antenna subcomplexes (induced in intact leaves under low light subsequent to the photoinhibitory illumination) is shown in Figure 5. For a better comparison of the kinetics, the DEPS at the beginning of the epoxidation experiment was normalized to 100 in each fraction. It is clear that prolonged illumination at low temperatures led to a decrease of the epoxidation kinetics in all complexes. However, the four fractions differed markedly from each other in the extent of this decrease. Only small effects of prolonged illumination at low temperatures on the epoxidation kinetics were found in Lhcb1–3 and Lhcb5/6. In contrast, large differences in the epoxidation kinetics were detected in the Lhcb4/PSII core fraction, and intermediate values were obtained for PSI (see Fig. 5).

The high sensitivity of the epoxidation kinetics in Lhcb4 with respect to the preillumination conditions corroborates the above-suggested involvement in qI of xanthophylls bound to this complex. The lower sensitivity of the other complexes, particularly of Lhcb1–3, is also in agreement with the proposed function of Zea in these complexes in qE rather than in qI. On the other hand, the large effect of the different illumination conditions on the epoxidation kinetics in the PSI fraction indicates that the regulation of the epoxidation rate might not simply be related to the function of the xanthophyll-binding proteins in qN, but, rather, might reflect that the epoxidase activity itself is downregulated after stronger photoinhibitory illumination. This interpretation, however, could not explain the varying epoxidation kinetics in the different antenna subcomplexes. It seems to be more likely that the DEPS (and thus the epoxidation rate) in each antenna complex is largely determined by factors that are specific for the different antenna proteins and related light-stress phenomena. The effect of strong light on PSI antenna proteins might then be attributable to an unrecognized function of Zea in these complexes.

The molecular basis for the reduction of the epoxidation rate might be a reduced accessibility of the epoxidase to its substrates Zea and antheraxanthin. This could originate from conformational changes possibly induced by phosphorylation of antenna proteins, as has been shown for Lhcb4 under photoinhibitory illumination (Bergantino et al., 1995; Croce et al., 1996). Phosphorylation of antenna proteins could therefore not only explain the reduction of the epoxidation rate but also the mechanistic differences between qE and the Zea-related component of qI. The dynamic interaction between protonation of Lhcb proteins and the DEPS state in the regulation of qE has already been established (Noctor et al., 1991; Rees et al., 1992); such interaction is best explained by the existence of different

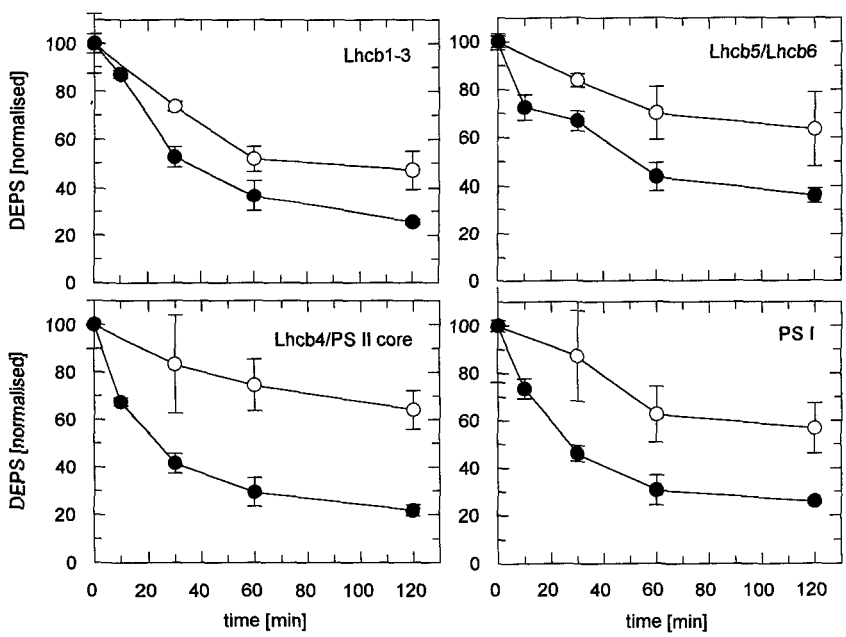

Figure 5. Time course of epoxidation in different antenna subcomplexes. Epoxidation was induced in intact pea leaves under low light (PFD = 15 μmol m^{-2} s^{-1}) after preillumination at a PFD of 2 mmol m^{-2} s^{-1} for either 1 h at 20°C (●) or 3 h at 4°C (○). Thylakoids were isolated at the different times and antenna complexes were separated by IEF. Data are normalized to the DEPS at the beginning of the experiment. Absolute values of the DEPS in each fraction were: Lhcb1–3: 68% (○) and 65% (●); Lhcb5/6: 71% (○) and 70% (●); Lhcb4/PSII core: 46% (○) and 48% (●); PSI: 29% (○) and 33% (●). The data represent the mean value of two to three independent experiments. Error bars indicate either SE (n = 3) or the upper and lower limits of the respective data points (n = 2).

defined conformational states of the Lhcb proteins (Horton et al., 1991).

The data shown here suggest further states formed under more extreme conditions that may respond differently to epoxidation, and also suggest that there are subtle but important differences in the dynamic behavior between different Lhcb proteins. Indeed, it has been found that there are differences in responsiveness of the fluorescence yield of different isolated Lhcb proteins to acidification and xanthophyll addition (Ruban et al., 1996). Further work is now urgently needed to explore the relationships and interactions between all of the modifications to these proteins that occur under different light conditions: the binding of protons, the de-epoxidation of xanthophyll-cycle carotenoids, and phosphorylation. As stated previously, these are likely to be central to the regulation of light harvesting (Horton, 1989).

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