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The Effects of Illumination on the Xanthophyll Composition of the Photosystem II Light-Harvesting Complexes of Spinach Thylakoid Membranes[1]

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The xanthophyll composition of the light-harvesting chlorophyll *a/b* proteins of photosystem II (LHCII) has been determined for spinach (*Spinacia oleracea* L.) leaves after dark adaptation and following illumination under conditions optimized for conversion of violaxanthin into zeaxanthin. Each of the four LHCII components was found to have a unique xanthophyll composition. The major carotenoid was lutein, comprising 60% of carotenoid in the bulk LHCIIb and 35 to 50% in the minor LHCII components LHCIIa, LHCIIc, and LHCIId. The percent of carotenoid found in the xanthophyll cycle pigments was approximately 10 to 15% in LHCIIb and 30 to 40% in LHCIIa, LHCIIc, and LHCIId. The xanthophyll cycle was active for the pigments bound to all of the LHCII components. The extent of deepoxidation for complexes prepared from light-treated leaves was 27, 65, 69, and 43% for LHCIIa, -b, -c, and -d, respectively. These levels of conversion of violaxanthin to zeaxanthin were found in LHCII prepared by three different isolation procedures. It was estimated that approximately 50% of the zeaxanthin associated with photosystem II is in LHCIIb and 30% is associated with the minor LHCII components.

Leaves exposed to irradiance levels that are in excess of those capable of being utilized with maximum quantum yield induce nonphotochemical thermal dissipation of the excess-absorbed photons. Thermal dissipation is a mechanism for short-term adaptation to changes in irradiance, thereby protecting against photodamage to the photosynthetic membrane (Demmig-Adams and Adams, 1992a). This process is known as nonphotochemical quenching of Chl fluorescence, since increased heat evolution reduces fluorescence yield (Horton and Bowyer, 1990; Krause and Weis, 1991). There are three important features of nonphotochemical quenching of Chl fluorescence. First, the major part of it is induced as a result of the acidification of the thylakoid lumen that is associated with the formation of the proton motive force and has been referred to as qE (Briantais et al., 1979). Second, qE is a process by which energy is dissipated in the light-harvesting system of PSII, most probably in the LHCII protein complexes (Horton et al., 1991; Horton and Ruban, 1992; Ruban et al., 1991, 1992a, 1993b). Third, its formation is correlated with the activation of the xanthophyll cycle, an enzymic interconversion between violaxanthin and zeaxanthin (Demmig-Adams, 1990). Xanthophylls are found only in the light-harvesting complexes of the thylakoid membrane (Peter and Thornber, 1991; Thayer and Bjorkman 1992; Bassi et al., 1993), which is consistent with the role of LHCII in qE.

LHCII in higher plants consists of at least four different Chl *a/b*-binding proteins (Peter and Thornber, 1991). The bulk LHCII, which binds approximately 65% of PSII Chl, is referred to as LHCIIb, whereas the minor complexes, each accounting for only 5% of Chl, are called LHCIIa, LHCIIc, and LHCIId (Peter and Thornber, 1991). These minor complexes have also been named CP29, CP26, and CP24 (Jansson et al., 1992). Recent work has shown the importance of the minor LHCII complexes in qE; not only are these complexes enriched in violaxanthin (Peter and Thornber, 1991; Bassi et al., 1993), but they contain the sites that bind dicyclohexylcarbodiimide upon inhibition of qE (Ruban et al., 1992b; Horton et al., 1993). At least one of these is also a Ca^{2+}-binding protein (Irrgang et al., 1991), and these complexes may form the site of localized H^+/ion channels involved in the formation of qE (Horton and Ruban, 1992; Noctor et al., 1993).

Therefore, it is possible to suggest a simple model for qE in which zeaxanthin, formed in the minor LHCII components, acts as an energy trap. Recent work has suggested that the transition to the 2^1A_g excited state of zeaxanthin may have an energy level low enough to make energy transfer from the Chl-excited singlet state a possible mechanism for energy dissipation (Owens et al., 1992). However, a number of experimental observations suggest that qE is more complex. First, it has been shown that near maximum levels of qE can be formed in the complete absence of zeaxanthin (Noctor et al., 1991), despite the fact that no other xanthophyll would appear to have a 2^1A_g energy level capable of accepting energy from Chl. Second, a comparative study of a range of species reveals no evidence for a simple relationship between

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Abbreviations: LHCII: light-harvesting complex associated with photosystem II; pI, isoelectric point; qE, nonphotochemical quenching of chlorophyll fluorescence dependent on the presence of the thylakoid pH gradient.

zeaxanthin content and the capacity of qE (Johnson et al., 1993b). Third, Chl *b*-deficient mutants enriched in the minor LHCII species relative to the bulk LHCII have a reduced rather than an elevated capacity for qE (Genty et al., 1990). Fourth, the process of qE is specifically induced by protons; even if zeaxanthin is the quencher, changes in protein structure induced by protonation are required for quenching to occur. Distinct changes near A_{530} accompany this protonation (Bilger et al., 1989; Ruban et al., 1993b), the peak position of which depends on whether qE is formed in the presence or absence of zeaxanthin (Noctor et al., 1993). It has been suggested that these changes result from perturbations in the organization of the LHCII-bound xanthophylls (Horton and Ruban 1992; Ruban et al., 1993a), changes that are inhibited by antimycin A (Oxborough and Horton, 1987), stimulated by Mg ions (Noctor et al., 1993), and that can also be observed upon aggregation of LHCII in vitro (Ruban et al., 1992a). However, qE is associated with absorption changes in the Chl region, analogous to those accompanying quenching upon aggregation of LHCII in vitro, indicating that quenching may arise through a Chl/Chl interaction.

Clearly, the relationship among qE, the minor LHCII species, and the xanthophyll cycle requires further exploration. One particularly important feature, so far ignored, is that the LHCII system possesses five xanthophylls, each with different chemical structures that could confer specific energetic and structural properties to the complexes. Moreover, it seems that each of the four LHCII complexes has a unique xanthophyll composition (Peter and Thornber, 1991; Bassi et al., 1993). At present there is no explanation, either for this heterogeneity in the xanthophyll pool or for the specificity of the xanthophyll composition of each LHCII type, although it is tempting to suggest that both are related to the regulation of LHCII function in terms of switching between light harvesting and energy dissipation. Recently, it has been reported that the minor complexes bind most of the PSII-associated violaxanthin (Bassi et al., 1993), although other data have indicated that over half is bound to the bulk complex LHCIIb (Peter and Thornber, 1991). In neither case has it been shown if this violaxanthin was converted with uniform efficiency into zeaxanthin. In this paper we describe the results of an analysis of the xanthophyll composition of the LHCII complexes, prepared by a variety of methods from spinach (*Spinacia oleracea* L.) leaves before and after activation of the xanthophyll cycle. We provide strong evidence for an inhomogeneous distribution of xanthophylls in LHCII and show that the xanthophyll cycle is active on all of the LHCII complexes, including LHCIIb.

MATERIALS AND METHODS

Plant Material

Spinach (*Spinacia oleracia* L.) was grown hydroponically in a greenhouse with supplemental light as described by Walker (1987). Leaves of 6-week-old plants were used either dark adapted (dark) or after illumination (light) to induce conversion of violaxanthin to zeaxanthin using a previously published procedure (Noctor et al., 1991). Thylakoid membranes and PSII BBY particles (Crofts and Horton, 1991) were isolated from dark and light leaves as described previously. LHCII was prepared from Triton-solubilized thylakoids by the method of Burke et al. (1978), as modified by Ruban and Horton (1992).

IEF

To separate LHCII components by nondenaturing IEF, a procedure modified from that described by Bassi et al. (1991) was used with a Pharmacia Multiphor II Electrophoresis system and recipes. A slurry of volume 100 mL containing 4% Ultradex (LKB), 2% Ampholine carrier ampholites (pH 3.5–5.0), 1% Gly, and 0.06% dodecylmaltoside (Sigma) was prepared and poured into the 24.5 × 11.0 cm tray to form a homogeneous layer. After carefully removing air bubbles, the tray was placed 70 cm below a small fan on a balance to allow control of the weight of evaporated water; 15 g of water was evaporated in 2 h. Freshly prepared PSII particles with a total Chl concentration of 2.5 mg/mL were resuspended in 1 mL of deionized water, and 0.5 mL of 3% dodecylmaltoside was added on ice. Incubation with occasional stirring lasted for 30 min. Samples were centrifuged at 35,000*g* and the supernatants were applied 2 cm from the cathode to the precooled and prefocused gel (1 h of prefocusing at 8 W) using a sample applicator (10 × 2 cm). After the samples had been applied, the gel was allowed to equilibrate for 3 min before the start of focusing. The focusing procedure was carried out for 16 h at a constant power of 8 W at 4°C. The initial and final current values were normally about 13 and 5 mA, respectively. After measuring pH values, each green band was carefully collected using a spatula and eluted using columns with a minimum volume of a solution containing 100 mM Hepes (pH 7.6) and 0.06% dodecylmaltoside. Aliquots of samples were immediately taken for absorption and fluorescence spectral measurements as described previously (Ruban et al., 1992a).

Gel Electrophoresis

Nondenaturing PAGE followed the procedures of Thornber (Peter and Thornber, 1991) as described by Ruban et al. (1992a). Polypeptide analysis of samples was carried out by SDS-PAGE. Samples at 20 μg of Chl were denatured in a medium containing 1% SDS in 50 mM Tris-HCl buffer (pH 6.8) and run in a 15% acrylamide gel containing 6 M urea for 18 h at 7 mA constant current. For the staining procedure Coomassie brilliant blue R-250 was used. Apparent molecular masses were estimated using markers: egg albumin (45 kD), glyceraldehyde 3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), soybean trypsin inhibitor (24.1 kD), and α-lactalbumin (14.2 kD) (Sigma).

Pigment Analysis

Pigments were extracted from leaf samples by careful homogenization in redistilled ethanol and transferred to diethyl ether. Samples were dried under a steady stream of N_2 at −20°C. Pigment content and composition was determined by reversed-phase HPLC using a Spherosorb ODS2 column (25.0 × 0.46 cm) operating on a solvent gradient of 0 to 60%

ethyl acetate in acetonitrile:water (9:1, v/v) over 16 min, followed by a further 14 min at 60% ethyl acetate at a flow rate of 1.0 mL/min. This chromatographic system enabled resolution of all components and near baseline separation of lutein and zeaxanthin (Johnson et al., 1993a). Pigments were identified by their UV/visible absorption spectra in at least two different solvents, their retention times compared with standards and, where necessary, by MS. They were quantified using published extinction coefficients (Davies, 1976).

RESULTS

Identification of Fractions Obtained by IEF

Mild detergent solubilization of PSII BBY particles allows various pigment protein complexes to be separated by IEF (Fig. 1A). The majority of the Chl is located in eight bands between pI 4.05 and 4.66. The pattern shown in Figure 1 resembles that previously published for maize (Bassi et al., 1991; Bassi and Dainese, 1992) except that the separation of the bands labeled 5 to 8, pI 4.35 to 4.66, is much clearer here. The profile shown in Figure 1A was obtained from samples isolated from dark-adapted spinach leaves and from leaves illuminated so as to induce zeaxanthin formation; no significant differences were obtained in the IEF separation profile.

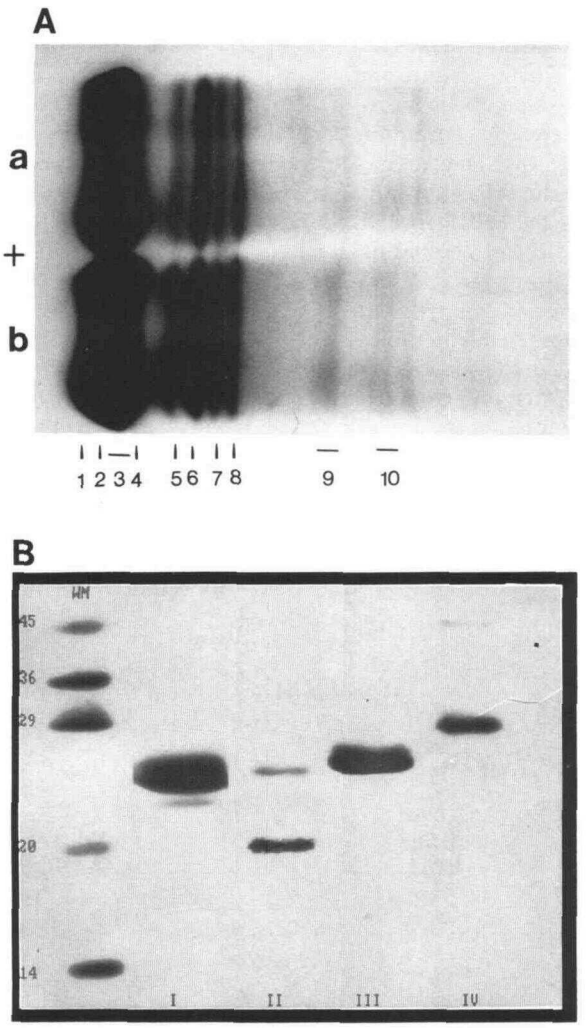

Figure 1. Separation of LHCII by IEF of spinach PSII particles. A, IEF profile of samples prepared from light-treated (a) and dark-adapted (b) leaves. The bands 1–4 and 5, 6, and 8, enriched in LHCII, are discussed in the text. Bands 7, 9, and 10 are enriched in PSII core polypeptides. B, SDS-polyacrylamide gel of LHCII fractions. I, Bands 1–4; II, band 5; III, band 6; IV, band 8; WM, mol wt markers.

The densely pigmented bands 1 to 4 contain approximately 65% of the PSII Chl and, by comparison with previous work, should contain the LHCIIb, whereas bands 5 to 8 are predicted to contain the minor LHCII components (Bassi et al. 1991; Bassi and Dainese, 1992). Denaturing PAGE in the presence of urea was carried out to confirm this prediction (Fig. 1B). Bands 1 to 4 (track I), pI 4.05 to 4.25, contained a strong band of the 28/27-kD doublet and a minor band at 25 kD, consistent with it containing LHCIIb. Band 5 (track II) was enriched in a polypeptide with apparent molecular mass of 21 kD, consistent with an identification as LHCIId. Band 6 (track III) showed only one polypeptide in the stained gel at 26 kD, as expected if it were LHCIIc. Finally, band 8 (track IV) contained a dominant polypeptide at 31 kD and a minor band at 43 kD, most likely LHCIIa and the PSII core protein CP43.

These identities were confirmed by a number of different assays, the results of which are shown in Table I. First, the absorption spectra were consistent with these assignments (Fig. 2); the LHCIIb fraction had strong peaks at 474 and 652 nm from Chl *b* (spectra I). The designated LHCIId fraction had only a shoulder at 652 nm (spectra II). The spectra for LHCIIa and LHCIId were similar (III and IV), both possessing the unusual minor peak with a λ_{max} at 641 nm. A fourth derivative analysis of these spectra shows the absence of the 664-nm band in LHCIIa and the addition of a 668-nm band, which explains the narrower bandwidth for this species. There are also differences in the Soret band between LHCIIa and LHCIIc. The ratios of Chl *a*/*b* for the four LHCII fractions also show the expected values for these identifications. The distribution of Chl between these fractions is also similar to that in previous work; the value of 65% for LHCIIb compares with 66% determined by Thornber by nondenaturing green gel separation, whereas the values of 5, 8, and 4% compare favorably with the 5% each for the minor LHCII components (Peter and Thornber, 1991). Also shown in Table I are the λ_{max} of Chl fluorescence of the complexes. There were small differences, with LHCIIa and LHCIIc showing a red shift and LHCIId showing a blue shift, relative to the 681- to 683-nm peak of LHCIIb. The fluorescence yields of each complex were approximately the same.

Finally, to confirm the identity of the 31- and 26-kD polypeptides in the LHCIIa and LHCIIc fractions, respectively, microsequencing of an N-terminal tryptic fragment was carried out. These were shown to be identical to that predicted from the published sequences of the *Lhcb4* and *Lhcb5* genes, respectively (R.G. Walters, personal communication).

Table I. *Characteristics of the LHCII bands obtained from IEF of PSII particles*

Data were derived from bands obtained from an IEF gel as shown in Figure 1. *a/b* is the Chl *a/b* ratio; absorption maxima were obtained from four derivatives of the spectra shown in Figure 3; percent Chl refers to the percent of the Chl loaded onto the gel that was recovered in each fraction; F_{max} is the wavelength maximum of the 77 K fluorescence emission.

IEF Band	pI	*a/b*	Percent Chl	Absorption Max	F_{max}
				nm	*nm*
LHCIIb (1–4)	4.05–4.25	1.35	65	436 444 463 474 488 641 652 664 673 681	681–683
LHCIId (5)	4.35	1.51	5	437 443 464 470 487 642 652 664 — 681	678–680
LHCIIc (6)	4.47	2.90	8	437 — 462 471 492 641 652 664 — 681	684–686
LHCIIa (8)	4.66	4.00	4	437 443 463 484 496 641 652 — 668 681	683–685

Carotenoid Analysis of the LHCII Fractions Obtained by IEF

Figure 3 shows the composition of carotenoids in the four LHCII fractions compared with the original thylakoids and BBY particles. Each of the five xanthophylls and carotene varied in their relative contents in the LHCII fractions. For example, β-carotene is completely absent in LHCIIb. Neoxanthin accounts for 30% of the carotenoid in LHCIIb but only 10 to 15% in LHCIId. Lutein is the main carotenoid in all the complexes, but varies from over 60% in LHCIIb to less than 40% in LHCIIa. Figure 3 also shows the composition after light treatment of the starting leaf material. In the thylakoids about 50 to 60% of the violaxanthin pool was converted to zeaxanthin. A slightly reduced conversion (40–50%) was retained in the solubilized BBY fraction loaded onto the IEF gel. It can be seen that zeaxanthin is present in all of the LHCII fractions, ranging from 20% in LHCIIc and LHCIId but accounting for less than 10% in LHCIIa and LHCIIb.

The xanthophyll contents of the complexes are presented in more detail in Table II. For LHCIIb the percent of carotenoid in the xanthophyll cycle pigments was found to be 8.8%, ranging from 4.9 to 15.6%; a deepoxidation state of 65% was obtained. For the minor complexes the content of

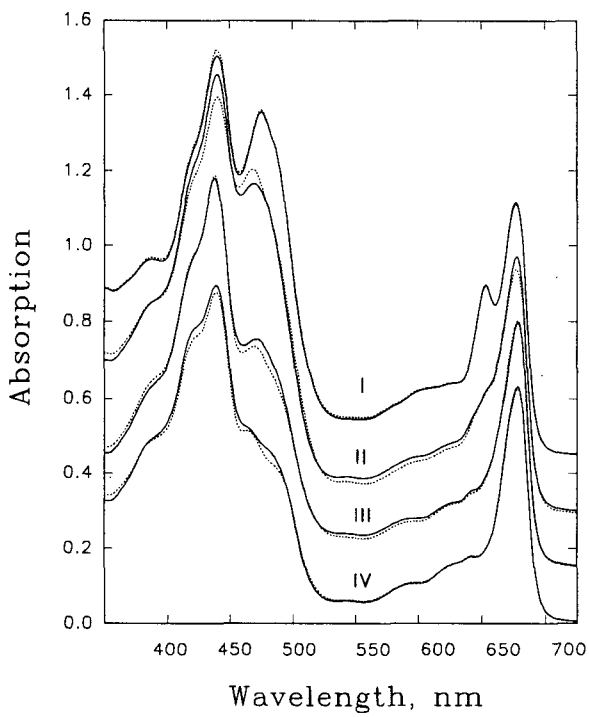

Figure 2. Absorption spectra of LHCII fractions. I, Bands 1–4 (LHCIIb); II, band 5 (LHCIId); III, band 6 (LHCIIc); IV, band 8 (LHCIIa). Dotted lines, Samples from light-treated leaves; solid lines, samples from dark-adapted leaves. Chl concentration, 10 μg/mL.

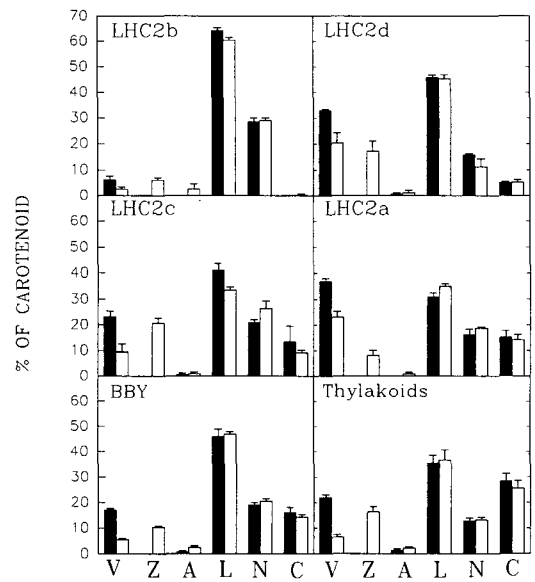

Figure 3. Carotenoid composition of LHCII fractions, PSII particles (BBY), and thylakoid membranes. Solid bars, Dark; open bars, light. V, Violaxanthin; Z, zeaxanthin; A, antheraxanthin; L, lutein; N, neoxanthin; C, β-carotene. Data are expressed as percent of total carotenoid in each sample.

Table II. *Xanthophyll composition of LHCII*

LHCII fractions were obtained from IEF bands as for Table I. Xanthophyll contents are expressed as the percent of the total xanthophyll found in each fraction. Values are the means of duplicate experiments from two leaf samples, ± indicating the range. Percent XC is the percent of the total xanthophyll pool found in the xanthophyll cycle carotenoids. DEPS, deexpoxidation state, is (zea + ½ant)/(zea + ant + viol)·100. X/Chl is the molar ratio of xanthophyll to Chl. D and L refer to samples isolated from dark-adapted and light-treated leaves, respectively. The DEPS value for the solubilized light PSII sample loaded onto the IEF gel was 45.3%.

Complex		Vio	Zea	Ant	Lut	Neo	Percent XC	DEPS	X/Chl
LHCIIb	D	6.2 ± 1.4		0.2 ± 0.1	64.3 ± 1.4	29.0 ± 1.0	6.4 ± 1.5	0.02	0.21 ± 0.01
	L	2.6 ± 0.9	6.0 ± 1.5	2.6 ± 2.0	60.0 ± 0.9	28.4 ± 0.6	11.2 ± 4.4	65.2	0.22 ± 0.02
LHCIId	D	34.5 ± 0.2		0.7 ± 0.2	48.5 ± 0.2	16.5 ± 0.2	35.2 ± 0.4	0.01	0.23 ± 0.02
	L	21.1 ± 3.8	16.0 ± 2.0	1.0 ± 1.0	47.8 ± 1.2	11.8 ± 2.5	38.1 ± 6.8	43.3	0.23 ± 0.02
LHCIIc	D	26.9 ± 1.2		0.7 ± 0.6	47.6 ± 0.4	24.8 ± 2.3	27.6 ± 1.8	0.01	0.19 ± 0.01
	L	10.0 ± 3.0	23.3 ± 3.0	1.1 ± 1.0	37.0 ± 3.4	26.4 ± 1.3	34.4 ± 7.0	69.3	0.19 ± 0.02
LHCIIa	D	44.3 ± 1.9			36.6 ± 0.4	19.0 ± 2.2	44.3 ± 1.9	0.00	0.16 ± 0.01
	L	26.5 ± 3.0	9.7 ± 2.0	1.4 ± 1.0	41.0 ± 1.1	21.5 ± 0.5	37.6 ± 6.0	27.7	0.15 ± 0.01

xanthophyll cycle carotenoids was 36.7% for LHCIId, 31.0% for LHCIIc, and 41.0% for LHCIIa. The extent of deepoxidation was highly variable between the three minor complexes: 28% for LHCIIa, 43% for LHCIId, and 69% for LHCIIc.

Table III presents the distribution of the five xanthophylls throughout the LHCII complexes. LHCIIb is the major complex and accounts for approximately 80% of the lutein and 85% of the neoxanthin. Violaxanthin was more evenly distributed, with LHCIIb binding only 30% in the dark-adapted thylakoids. Over 40% was recovered in the three minor LHCII fractions, with 20% found in LHCIId. In light-treated thylakoids, however, 52% of the zeaxanthin was found in the LHCIIb fraction. LHCIIc and LHCIId bound 17 and 11% of the zeaxanthin, respectively. The LHCIIa fraction contained only 4% of the PSII zeaxanthin. Although over 90% of the lutein and neoxanthin were recovered in these four LHCII fractions, the recovery of xanthophyll cycle carotenoids was less. In particular, 25% of the violaxanthin in samples from dark-adapted leaves was not found in the LHCII fractions; after light treatment, this proportion increased to 32%. The proportion of non-LHCII zeaxanthin was only 16%.

Carotenoid Analysis of LHCIIb

These data contrast with those recently published by Bassi and co-workers, who estimated that LHCIIb bound only 18% of the violaxanthin (Bassi et al., 1993). Data for the distribution of lutein and neoxanthin was comparable with that shown in Table III. Furthermore, it was stated that only the violaxanthin in the minor LHCII was convertible to zeaxanthin. In contrast, the data in Table II show that the second highest ratio of zeaxanthin to violaxanthin was found in LHCIIb. It was important, therefore, to prepare LHCIIb by alternative methods to confirm the availability of the LHCIIb-bound violaxanthin to deepoxidation and its 6 to 10% xanthophyll-cycle composition. First, the carotenoid composition of LHCII prepared by Triton solubilization of thylakoid membranes following the procedure of Burke et al. (1978) was determined (Table IV). A pigment analysis of LHCII prepared from dark-adapted leaves indicated that this preparation contained 19% xanthophyll-cycle carotenoid. The deepoxidation state was found to be 60%, in good agreement with the LHCIIb prepared by IEF. This LHCII preparation is highly enriched in LHCIIb but contains trace amounts of the minor LHCII species. Analysis by IEF reveals that LHCIIb accounts

Table III. *Distribution of xanthophylls between LHCII components*

Distribution of xanthophyll throughout the LHCII complexes was determined by expressing the carotenoid level in each of the four bands as percent of that in the material loaded onto the IEF gel. Data were obtained from a single experiment. Remainder refers to the difference between the total loaded and that recovered in the four LHCII fractions

Complex	Treatment	Vio	Zea	Ant	Lut	Neo
LHCIIb	Dark	31		52	84	86
	Light	24	52	31	80	82
LHCIId	Dark	22		22	6	5
	Light	18	11	12	5	9
LHCIIc	Dark	10		11	3	4
	Light	9	17	15	5	4
LHCIIa	Dark	12		15	2	2
	Light	17	4	7	3	3
Remainder	Dark	25			5	3
	Light	32	16	35	7	2

Table IV. *Xanthophyll content of LHCII components prepared by different procedures*

LHCIIb fractions were prepared from a Suc gradient following Triton solubilization of thylakoids (1), further purification of 1 by IEF (2), the trimeric LHCII band from a nondenaturing green gel separation of thylakoids (3); shown for comparison are the data for the LHCIIb from Table II (4). Combined minor LHCII (a + c + d) were obtained from the monomeric band on the nondenaturing green gel (5) and from the sum of the three IEF bands (6, data from Table II). Each xanthophyll is expressed as a percent total carotenoid. Percent XC is the percent of total xanthophyll in the xanthophyll cycle carotenoids. DEPS, deepoxidation state is (zea + ½ant)/(zea + ant + viol)·100. X/Chl is the molar ratio of xanthophyll to chlorophyll. D and L refer to samples prepared from dark-adapted and light-treated leaves, respectively.

Complex	Viol	Zea	Ant	Lut	Neo	Percent XC	DEPS	X/Chl
1 D	18.4			59.9	19.3	18.9	0	0.19
1 L	4.9	8.3	2.8	59.9	20.7	16.6	60.6	0.19
2 D	8.9		0.3	64.7	26.1	9.2	0.03	0.19
2 L	3.3	5.9	1.0	61.8	28.0	10.2	62.7	0.21
3 D	15.9		2.1	55.3	23.4	18.6	0.06	0.26
3 L	4.6	8.4	3.6	55.9	23.3	17.3	61.4	0.19
4 D	6.2		0.2	64.3	29.0	6.4	0.01	0.21
4 L	2.6	6.0	2.6	60.0	28.4	11.2	65.2	0.22
5 D	22.5	2.7	2.4	39.4	20.2	31.7	0.14	0.26
5 L	11.0	15.3	3.9	41.7	17.7	33.7	56.6	0.26
6 D	35.2		0.4	44.2	20.1	35.6	0.01	0.19
6 L	19.2	16.3	1.2	41.9	19.9	37.3	46.0	0.19

for 82% of the Chl, with LHCIId and LHCIIa containing 12 and 2.4%, respectively. Analysis of the purified LHCIIb shows that the xanthophyll-cycle carotenoids account for 10% of the bound xanthophyll (complex 2 in Table IV). A deepoxidation state of 63% was found in this fraction.

Both the IEF purification and the Suc gradient methods involved the use of Triton X-100 and long-term incubations; it could be argued that either of these could lead to artifactual reorganization of xanthophylls. Therefore, the rapid "green-gel" electrophoretic separation devised by Thornber was applied to the illuminated thylakoids. The main LHCIIb band was extracted and the carotenoid content was analyzed (Table IV). In this experiment the thylakoids showed a 51% conversion to zeaxanthin. The LHCIIb prepared by this method had a xanthophyll composition equivalent to that seen in LHCIIb prepared by the other methods. The percent carotenoid found in the xanthophyll cycle was 18.6%, with a deepoxidation state of 65% for the light-treated sample. The monomeric LHCII band obtained from this gel is enriched in the minor LHCII species (Peter and Thornber, 1991). A value of 32% was obtained for the content of xanthophyll cycle carotenoids in this band (complex 5 in Table IV) compared with a summed value of 36% for LHCIIa, LHCIIc, and LHCIId obtained from the data in Table II. A deepoxidation state of 57% was estimated for the monomeric LHCII fraction, compared with the value of 46% for the IEF fractions.

DISCUSSION

The light-harvesting system associated with PSII binds five different xanthophylls. These range from less than 5% of total carotenoid in the case of antheraxanthin to nearly 50% for lutein. In agreement with previous data (Peter and Thornber, 1991; Bassi et al., 1993), the four complexes that comprise the LHCII each appear to have a unique composition of these xanthophylls. The similarity between the data presented here and those of other studies suggests that they represent the composition of LHCII in situ. However, it is clear from Table IV that, particularly for LHCIIb, there is a reduction in xanthophyll-cycle content as the complex is subjected to purification. Thus, it is possible that some redistribution of xanthophylls may occur during thylakoid fractionation, and for this reason the exact binding stoichiometries should at present be interpreted with caution.

Lutein is the major xanthophyll in each complex but ranges from over 60% in LHCIIb to under 30% in LHCIIa. From the observed xanthophyll:Chl ratios and an estimate of 15 Chls per polypeptide, it is possible to calculate a stoichiometry of binding of pigments to the monomeric polypeptide. In each case it was estimated that there are three xanthophylls per polypeptide, slightly lower than the value of three to five estimated by Thornber et al. (1993). For LHCIIb there is 0.3 xanthophyll-cycle pigment per monomer; the functional unit of LHCIIb is the trimer, suggesting that each LHCIIb complex in vivo binds a single xanthophyll-cycle carotenoid. In contrast, each of the minor complexes binds one xanthophyll-cycle carotenoid/polypeptide; at present there is no indication of oligomerization of these complexes. In addition, LHCIIb binds two luteins and one neoxanthin per monomer; these values are one and one for LHCIIc and one and one-half for LHCIIa. LHCIId appears to bind one and one-half lutein and one-half neoxanthin but, given the presence of LHCIIc in this fraction, this may indicate the absence of neoxanthin from this complex and the binding of two molecules of lutein.

In this paper it has been shown that the violaxanthin pools associated with all the LHCII proteins can be enzymically deepoxidated when leaves are exposed to saturating light. In spinach leaves the maximum level of conversion of the

xanthophyll-cycle pool to zeaxanthin is approximately 60% (Noctor et al., 1991). The presence of a ceiling of the extent of deepoxidation of violaxanthin has been observed in previous studies (Siefermann and Yamamoto, 1974), although in some plant species as much as 90% conversion has been observed (Thayer and Bjorkman, 1990; Demmig-Adams and Adams, 1992b). There is at present no explanation for this restriction on the availability of violaxanthin to deepoxidation. One hypothesis, that the nonconvertible pool is bound to a particular LHCII complex, is not supported by the data shown here. However, the data in Table III suggest that a significant proportion of the violaxanthin may be present in fractions not containing LHCII; these are fractions containing PSII core components and also free pigment. It is significant that the value for this pool increases for the light-treated sample, perhaps indicating a low xanthophyll cycle activity for the non-LHCII carotenoid. Therefore, the presence of this pool of xanthophyll-cycle carotenoid could explain in part the restricted availability of violaxanthin, in addition to the physiological regulation of the xanthophyll cycle, as suggested previously (Siefermann and Yamamoto, 1975).

The percentage conversion (expressed as a deepoxidation state) of the xanthophyll cycle pool to zeaxanthin (and antheraxanthin) ranges from 28% for that bound to LHCIIa to 69% for that associated with LHCIIc. That bound to LHCIIb was found to be between 61 and 65% for LHCIIb prepared by three different methods. Similarly, estimates from two different procedures indicated that the deepoxidation state was less for the minor LHCII. It is interesting to note that there is no proportional relationship between the content of the xanthophyll-cycle pigments (as a percent total xanthophyll) and the deepoxidation state. In Figure 4 the percent zeaxanthin conversion in the light is plotted against the violaxanthin content in the dark; for the minor complexes it seems that a higher violaxanthin content is associated with less-efficient conversion to zeaxanthin.

The fact that the xanthophyll cycle is active on xanthophylls occurring throughout the LHCII system means that zeaxanthin will be distributed in the thylakoid membrane in a way that largely reflects Chl. Hence, over 50% of the PSII zeaxanthin was recovered in the LHCIIb fraction. However, the observed differences in the values for the efficiency of conversion and the relative content of xanthophyll-cycle carotenoids mean that the PSII-associated zeaxanthin will not follow Chl exactly. LHCIIc, which binds only 8% of PSII Chl, accounts for 17% of the zeaxanthin. Similarly, LHCIId binds 5% of the Chl and 11% of the zeaxanthin. In contrast, the LHCIIa fraction contains 4% of the Chl and 4% of the zeaxanthin, whereas 65% of the Chl is found in LHCIIb but only 52% of the zeaxanthin. Thus, although the majority of zeaxanthin is found in LHCIIb, there is considerable enrichment in the minor complexes, particularly in LHCIIc and LHCIId.

The enrichment of the xanthophyll-cycle pigments in the minor LHCII components means that if zeaxanthin is the quencher of singlet-excited Chl, quenching will be most powerful in these complexes. In particular, the enrichment in LHCIIc and the efficient conversion to zeaxanthin in this complex means that it will show the greatest change in zeaxanthin-related properties upon light activation of the xanthophyll cycle. These differences include not only the predicted value for the 2^1A_g energy level (Owens et al., 1992), but also the tendency for xanthophyll aggregation (Ruban et al., 1993a). However, it has yet to be demonstrated that any of these complexes has an obligatory role in the transfer of energy from the bulk LHCII to the PSII reaction center, a prerequisite for them being the location of unique quenching sites. Hence, it cannot yet be discounted that quenching also occurs in LHCIIb, even though the zeaxanthin to Chl ratio is lower than in LHCIIc and LHCIId, and the change in xanthophyll properties will be diluted by the dominating influence of lutein.

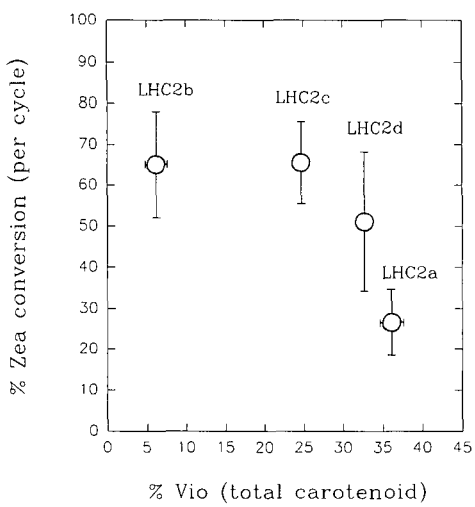

Figure 4. Relationship between the content of violaxanthin in LHCII fractions from dark-adapted leaves and the percent conversion of xanthophyll-cycle carotenoid to zeaxanthin.

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