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Analysis of the Pigment Stoichiometry of Pigment-Protein Complexes from Barley (*Hordeum vulgare*)[1]

The Xanthophyll Cycle Intermediates Occur Mainly in the Light-Harvesting Complexes of Photosystem I and Photosystem II

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The carotenoid zeaxanthin has been implicated in a nonradiative dissipation of excess excitation energy. To determine its site of action, we have examined the location of zeaxanthin within the thylakoid membrane components. Five pigment-protein complexes were isolated with little loss of pigments: photosystem I (PSI); core complex (CC) I, the core of PSI; CC II, the core of photosystem II (PSII); light-harvesting complex (LHC) IIb, a trimer of the major light-harvesting protein of PSII; and LHC IIa, c, and d, a complex of the monomeric minor light-harvesting proteins of PSII. Zeaxanthin was found predominantly in the LHC complexes. Lesser amounts were present in the CCs possibly because these contained some extraneous LHC polypeptides. The LHC IIb trimer and the monomeric LHC II a, c, and d pigment-proteins from dark-adapted plants each contained, in addition to lutein and neoxanthin, one violaxanthin molecule but little antheraxanthin and no zeaxanthin. Following illumination, each complex had a reduced violaxanthin content, but now more antheraxanthin and zeaxanthin were present. PSI had little or no neoxanthin. The pigment content of LHC I was deduced by subtracting the pigment content of CC I from that of PSI. Our best estimate for the carotenoid content of a LHC IIb trimer from dark-adapted plants is one violaxanthin, two neoxanthins, six luteins, and 0.03 mol of antheraxanthin per mol trimer. The xanthophyll cycle occurs mainly or exclusively within the light-harvesting antennae of both photosystems.

Excess excitation energy can be lethal to a plant. The triplet Chl or singlet oxygen species that result from excess excitation of a photosystem can rapidly react with membrane lipids, aromatic amino acids, and purines, causing irreparable damage (Siefermann-Harms, 1987). Thus, plants have evolved various photoprotective mechanisms to minimize the destructive effects of too much light energy. It has already been demonstrated that carotenoids, in addition to a light-harvesting function, also play a role in photoprotection. Carotenoids can bring about the nonradiative dissipation of excess excitation energy by reacting with triplet Chl or singlet oxygen (Siefermann-Harms, 1987). Many recent experiments indicate that the carotenoids of the xanthophyll cycle are involved in photoprotection. Under conditions of excess excitation energy, violaxanthin is deepoxidated to zeaxanthin via antheraxanthin as an intermediate (Yamamoto, 1979; Demmig-Adams, 1990). The presence of zeaxanthin has been correlated with a rapid quenching of Chl fluorescence (Demmig et al., 1987), and inhibition of zeaxanthin formation abolishes this quenching (Demmig-Adams et al., 1990).

The biochemistry of the xanthophyll cycle has been known for some time (Yamamoto, 1979), and other papers have addressed the physiological role of the xanthophyll cycle under various environmental conditions such as water and temperature stresses (Demmig et al., 1988; Demmig-Adams et al., 1989). However, the precise molecular mechanism and the site of quenching of excited Chls by zeaxanthin in the plant has not been fully elucidated. Being able to determine the location of zeaxanthin within the thylakoid membranes would be a first step in determining the site and mechanism of zeaxanthin activity. So far, two papers (Thayer and Björkman, 1992; Ruban et al., 1994) have addressed this problem. Thayer and Björkman (1992) determined that zeaxanthin was associated with both PSI and PSII. However, some of their LHCs contained core components, and therefore, they could not determine whether zeaxanthin was localized in the core, antenna, or both. Ruban et al. (1994) presented a more detailed analysis than is presented here, but restricted their analysis to the polypeptides of the PSII antenna. They concluded that the major as well as the minor LHC II polypeptides all bound zeaxanthin to varying degrees.

In addition to determining the localization of zeaxanthin within the multiple pigment-protein complexes, we are also interested in quantitating the other carotenoids that are also associated with these complexes. The carotenoid stoichiometry of PSI is not as well studied as that of PSII. Previous reports have focused either on the carotenoids of

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Abbreviations: CC, core complex; Deriphat-160, disodium *N*-lauryl iminodipropionate; LHC, light-harvesting complex; OEE, oxygen-evolving enhancer.

PSII (Peter and Thornber, 1991a; Bassi et al., 1993; Ruban et al., 1994) or primarily (although not exclusively) on the xanthophyll cycle carotenoids (Thayer and Björkman, 1992). Also, harsher detergent conditions than those used here were used in the latter study. In this paper, we have characterized the pigment content, from dark- and high-light-treated barley (*Hordeum vulgare*) seedlings, of both the PSI holocomplex and subcomplexes of PSI and PSII without any significant removal of Chl and carotenoid from their in situ locations. Using a gentle glycosidic detergent solubilization and the nondenaturing Deriphat-PAGE system developed in this laboratory, we have been able to solubilize photosynthetic membranes and to resolve the photosynthetic components into five pigment-protein complexes: PSI; CC I, the core of PSI; CC II, the core of PSII; LHC IIb, an oligomeric complex of the major light-harvesting Chl *a/b* binding protein; and LHC IIa, c, and d, a monomeric complex of the minor light-harvesting proteins of PSII. LHC IIa, c, and d are also known as CP 29, 26, and 24, respectively (Jansson, 1994). Since our PSI contained contaminating CC II polypeptides, we have also analyzed the pigment content of PSI derived from PSI-enriched stromal lamellae membranes. These latter PSI complexes contained only PSI polypeptides. Both our CC I and CC II fractions contained small amounts of LHC polypeptides.

Our results indicate that the majority if not all of the xanthophyll cycle activity occurs within the light-harvesting components of both PSI and PSII and that zeaxanthin occurred in both the LHC I and LHC II fractions. Although the pigment content of LHC I was not measured directly, it was estimated by subtracting the pigments of CC I from those of PSI. We also present an estimate of the carotenoid content of the LHC IIb oligomer and the monomeric complex consisting of LHC IIa, c, and d. It is interesting to note that our analysis of trimeric LHC IIb leads us to assign one more neoxanthin molecule per trimer than was reported by Ruban et al. (1994) and two more neoxanthins and one more violaxanthin than was reported by Kühlbrandt et al. (1994).

MATERIALS AND METHODS

Growth of Barley Plants and Light Treatment

Barley (*Hordeum vulgare* var Prato) seedlings were grown at 21°C in a growth chamber under continuous light of 100 μE m^{-2} s^{-1}. Seven-day-old seedlings were either treated with high light for 20 min or put into total darkness for 14 to 18 h before harvesting on the 7th d. High-intensity light was provided by four 500-W halogen lamps arranged so that a filter of chilled circulating water was placed between the plants and the lamps to absorb the heat. The vertical blades of the barley seedlings were bent 30° to 90° to maximize the surface area exposed to the lamps placed directly overhead. The lamp distance was adjusted so that the top one-third of the seedlings was exposed to 1600 to 2000 μE m^{-2} s^{-1}. Only the top one-third of both the light-treated and dark-adapted seedlings was harvested for membrane isolation.

Membrane Isolation

Thylakoid membranes were isolated according to Peter and Thornber (1991a). Stromal lamellae membranes were isolated following the procedure of Preiss et al. (1993). To minimize losses during membrane isolation, the NaBr wash was omitted in both of the above procedures. All membranes were adjusted to a final concentration of 1.1 mg Chl/mL using the equations of Arnon (1949).

Solubilization of Membranes and Fractionation of Pigment-Protein Complexes

Pigment-protein complexes were extracted from thylakoid or stromal lamellae membranes by mixing membranes with a 10% (w/v) stock of decyl-β-D-maltoside (Calbiochem, La Jolla, CA) to a final weight ratio of Chl:surfactant of 10:1. The membrane/surfactant mix was incubated at 4°C for 5 min and then microfuged for 5 min before the supernatant was loaded onto a Deriphat-160 (Henkel Corp., Hoboken, NJ) polyacrylamide gel. Deriphat-PAGE was performed following the methods of Peter and Thornber (1991a) but with the following modifications: an 8% gel was used at 100 V for 1 h using only Ultrapure reagents (Sigma). To obtain sufficient material for each pigment-protein, two or three 2-mm-thick preparatory gels were usually run simultaneously with 495 mg of Chl loaded per gel. The different pigment-protein complexes occur as green bands on the gel.

To determine the polypeptide composition of the various pigment-protein complexes fractionated on Deriphat-PAGE, each band was excised, eluted from the gel strips (see below), and analyzed by SDS-PAGE following the protocol of Peter and Thornber (1991a).

Pigment Analysis of Pigment-Protein Complexes Separated by Deriphat-PAGE

To elute the pigment-protein complexes, the protocol of Thornber et al. (1967) was used with some modifications. The excised green band of interest was chopped into small pieces and forced once through a coarse nylon mesh (170 μm, Fisher Scientific) and then twice through a finer mesh (90 μm, Fisher Scientific). The gel slush was extracted three times by a 5-min incubation at 4°C in elution buffer (Deriphat-PAGE reservoir buffer, pH 7.5, 10 mM ascorbate, 0.1% decyl-β-D-maltoside) and centrifugation at 13,600*g* for 5 min. The supernatants from the three extractions (containing the eluted pigment-protein complexes) were pooled, filtered through 0.4-μm Nylon-66 filters (Rainin, Woburn, MA) to remove any fine gel pieces, and concentrated on 30-kD Centricon microconcentrators (Amicon, Beverly, MA) by centrifuging at 3,000*g* for 1.5 h. An aliquot of the pigment-protein complexes was removed for polypeptide analysis by SDS-PAGE. Pigments were extracted by the addition of 100% HPLC-grade acetone (Fisher Scientific) to give a final concentration of 80% (v/v) aqueous acetone. The free-pigment band was also eluted as described above, but since the free pigments are smaller than the pore size of the microconcentrators, the supernatant containing the pigments was concentrated by passage

through a C-18 Plus column (Millipore, Bedford, MA) that had been previously washed with 10 mL of HPLC-grade acetonitrile (Fisher Scientific) followed by 10 mL of water. Pigments were eluted from the column using mobile phase B from the HPLC protocol (see below) and further concentrated in a Savant (Farmingdale, NY) Speed-Vac for 1 h. Before analysis by HPLC, all pigment extracts were microfuged for 5 min and passed through a 0.2-μm Nylon-66 filter (Micron Separations, Inc., Westboro, MA). All isolation procedures were carried out in darkness or in dim light and, whenever possible, at 4°C. HPLC analysis used the mobile phases A1 and B of Gilmore and Yamamoto (1991). However, with our column it was necessary to lengthen the isocratic separation to 14 min of A1 at 1 mL/min in order to obtain the optimal separation of lutein and zeaxanthin.

Quantitation of Pigments

Pigments for calibration were collected from leaf tissue ground in liquid nitrogen and extracted twice in 80% acetone. These pigments were separated on Baker-flex silica gel 1B TLC plates (J.T. Baker, Phillipsburg, NJ) using hexane:acetone (60:40, v/v) as the mobile phase. The bands on the TLC plates were scraped off and the pigments were eluted by 95% ethanol. Zeaxanthin and lutein were gifts from H. Yamamoto (University of Hawaii, Honolulu), and β-carotene was purchased from Sigma. Pigment concentrations were calculated spectrophotometrically using known extinction coefficients for pigments in ethanol (Davies, 1976), and the equations of Lichtenthaler (1987) for pigments in 95% ethanol were used for Chl *a* and *b* estimations. Known quantities of each pigment were injected into the HPLC system, and an equation correlating peak area to pigment concentration was determined.

RESULTS

Solubilization of Thylakoid Membranes

Thylakoid membranes were solubilized in decyl-β-D-maltoside and fractionated on a nondenaturing Deriphat-PAGE system (Fig. 1, lane 1). Each band (except the free pigment) is a pigmented multiple-protein complex. The complexes fractionated on this gel system are in order of decreasing size: the PSI holocomplex (CC I plus LHC I), the core of PSI (CC I), the core of PSII (CC II), an oligomeric form of the major light-harvesting Chl *a*/*b* binding protein (LHC IIb), and a monomeric complex containing the minor PSII Chl *a*/*b* binding light-harvesting polypeptides (LHC IIa, c, and d). The relatively faint band at the bottom is the free-pigment fraction. Decyl-β-D-maltoside is a gentle solubilizing detergent that minimizes the amount of free pigment and the dissociation of the LHC IIb oligomer into its monomeric units (Peter and Thornber, 1991a). There was no difference in the banding pattern between solubilizations of thylakoids isolated from dark- or high-light-treated barley (data not shown).

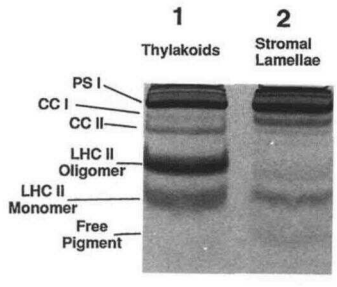

Figure 1. Nondenaturing Deriphat-PAGE of barley thylakoid (lane 1) or stromal lamellae (lane 2) membranes solubilized with decyl-β-D-maltoside.

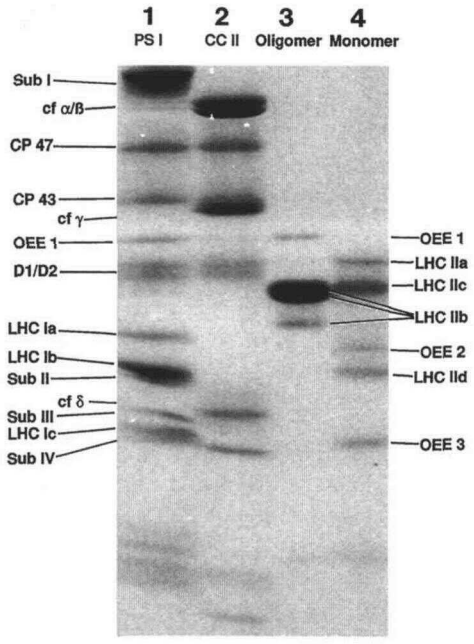

Figure 2. Fully denaturing urea-SDS-PAGE of the complexes obtained from the gel shown in Figure 1, lane 1. Sub I to Sub IV, The nonpigmented subunits of PSI; cf, the various subunits of ATPase; CP43/47 and D1/D2, the proteins of the PSII core; LHC Ia, b, and c, the light-harvesting polypeptides of PSI; LHC IIa, b, c, d, the light-harvesting polypeptides of PSII.

To determine the polypeptide composition of these complexes, the pigment protein complexes eluted from each green band were subjected to SDS-PAGE (Fig. 2). Lane 2 shows the polypeptides of CC II: CP 43/47 and D1/D2. Lane 3 shows the polypeptide composition of the trimeric form of the major LHC, LHC IIb. LHC IIb in barley consists of three polypeptides with sizes of 28, 27, and 25 kD. The 33-kD band is due to co-migration of the dimer of OEE1 polypeptide (Peter and Thornber, 1991b). Lane 4 shows the monomeric PSII light-harvesting polypeptides derived from LHC II a, c, and d, and running at 31, 29, and 26 kD, respectively, again with OEE polypeptides present. Lane 1 (PSI) contains the core and light-harvesting subunits of PSI. However, this fraction also contains significant amounts of CP 43/47 and D1/D2, which are components of CC II. All fractions have detectable amounts of the OEE proteins and/or the various subunits of ATPase. Because we wanted to minimize pigment loss from thylakoids, the

membranes were washed with neither NaBr nor Tris buffer and, therefore, abundant amounts of the OEE proteins and the subunits of ATPases are present in the detergent extracts. However, these proteins are not pigmented, and their presence does not affect the pigment analysis of the green bands. Thus, with the exception of the PSI fraction, all of these bands are homogeneous pigmented complexes.

Solubilization of Stromal Lamellae

Because the isolated PSI fraction contained CC II polypeptides, we used PSI-enriched stromal lamellae membranes to obtain a more purified PSI preparation. Figure 1, lane 2, shows a nondenaturing gel of solubilized stromal lamellae membranes. Compared to solubilized thylakoid membranes (Fig. 1, lane 1), there is a greatly reduced amount of CC II and the major and minor LHC II components. The PSI and CC I bands from solubilized stromal lamellae were excised, eluted, and analyzed on SDS-PAGE (Fig. 3). This PSI fraction does not contain the CC II polypeptides (compare Fig. 2, lane 1, to Fig. 3, lane 2). Our CC I fraction was essentially pure except for a band at approximately 36 kD (Fig. 3, lane 1, band indicated by "?"). Two polypeptides that run in this region are the γ subunit of ATPase (cf γ) or the 43-kD polypeptide of CC II (CP 43).

Pigment Analysis of Green Bands

The pigment content was determined for all the pigmented complexes as well as the free-pigment fraction isolated from thylakoid membranes and for PSI complexes isolated from stromal lamellae. Figure 4 shows the pigment composition of the three PSII complexes identified in the nondenaturing PAGE gel shown in Figure 1, lane 1. To varying degrees, all three PSII complexes demonstrated a decrease in violaxanthin with an increase in antheraxanthin and zeaxanthin when thylakoids from high-light-treated plants were compared to those from dark-adapted plants. Antheraxanthin was associated with all three complexes even in dark-treated plants, consistent with other reports of low antheraxanthin levels in dark-treated plants (Thayer and Björkman, 1992; Ruban et al., 1994). The pigments not involved in the xanthophyll cycle (neoxanthin, β-carotene, Chl *a*, and Chl *b*) showed little or no change in dark- versus high-light-treated plants for all three PSII complexes examined (Fig. 4).

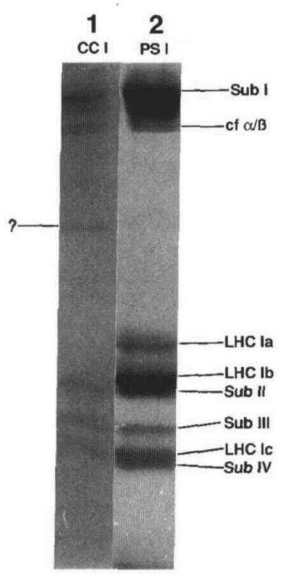

Figure 3. Fully denaturing urea-SDS-PAGE of the complexes obtained from the gel shown in Figure 1, lane 2. For further explanation of abbreviations, see the legend of Figure 2.

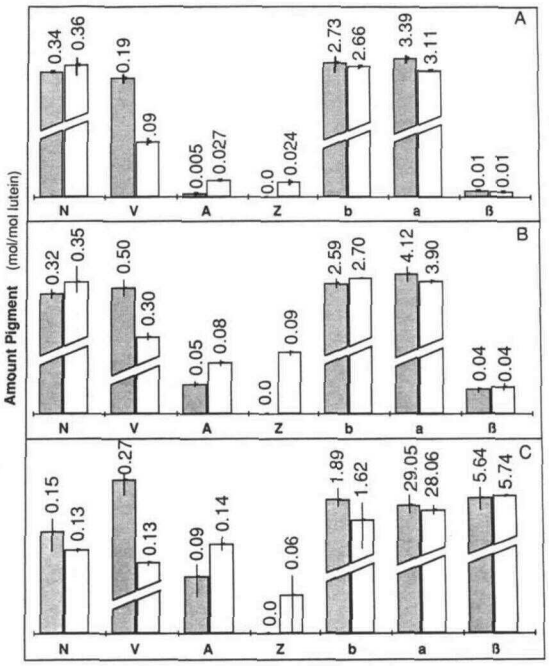

Figure 4. *Pigment content of the PSII complexes isolated from nondenaturing PAGE (Fig. 1) of solubilized thylakoid membranes derived from dark-adapted and high-light-treated barley seedlings. A, LHC II oligomer; B, LHC II monomer; C, CC II. Stippled bars, Dark-treated barley; open bars, high-light-treated barley. N, Neoxanthin; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; a, Chl a; b, Chl b; β, β-carotene. All numbers are in mol and are reported relative to 1 mol of lutein. Each column represents the mean of three experiments, with the error bars indicating the minimum and maximum values.*

The oligomeric LHC IIb complex decreased in violaxanthin content from 0.19 mol/mol lutein to 0.09 mol/mol lutein (a 53% decrease) upon light treatment, and its antheraxanthin content increased from 0.005 to 0.027 mol/mol lutein and its zeaxanthin from 0.0 to 0.024 mol/mol lutein. This represents a shift in the deepoxidation state from 1.3% in dark-treated seedlings to 26.6% in high-light-treated seedlings. Table I summarizes the percent change in violaxanthin content and the changes in deepoxidation state for all pigment-protein complexes. Small amounts of β-carotene were detected in the LHC IIb band.

In the monomeric LHC II complex, the violaxanthin level decreased by 40% from 0.50 to 0.30 mol violaxanthin/mol lutein. Antheraxanthin content increased from 0.05 to 0.08 mol/mol lutein, and zeaxanthin levels increased from 0.0 to 0.09 mol/mol lutein. These changes represent a dee-

Table I. *Percent violaxanthin decrease and changes in deepoxidation state for various pigment-protein complexes*

Percent violaxanthin decrease is calculated as amount of violaxanthin decrease over violaxanthin levels in dark-adapted plants × 100. Deepoxidation state is calculated as $[(Z + 0.5\ A)/(V + A + Z)] \times 100$. PSI tk, PSI from thylakoid membranes. PSI str lam, PSI from stromal lamellae. For further explanations of abbreviations, see legend of Figure 4.

Complex	Percent Violaxanthin Decrease	Deepoxidation State	
		Dark	Light
LHC II oligomer	53	1.3	26.6
LHC II monomer	40	4.2	28.2
CC II	52	12	39
PSI tk	21	1.3	22.4
PSI str lam	28	0.8	26.2
CCI	60	0.0	35.2

poxidation state shift from 4.2 to 28.2%. The β-carotene content of this band was 0.04 mol/mol lutein.

A similar pattern of carotenoid changes was observed for the CC II complex. Comparing dark- to high-light-treated plants, the violaxanthin content of CC II decreased 52%, from 0.27 to 0.13 mol/mol lutein. Antheraxanthin increased from 0.09 to 0.14 mol/mol lutein and zeaxanthin levels increased from 0.0 to 0.06 mol/mol lutein. These changes represent an increase in deepoxidation state for CC II from 12 to 39%. The presence of small amounts of Chl *b* in our CC II fraction indicates that there are LHC II polypeptides within this fraction, since CC II has been shown to be devoid of Chl *b* (Siefermann-Harms, 1985). Most likely there is some co-migration of LHC IIa, the 31-kD apoprotein that migrates in the same region of the gel as the D1/D2 proteins. LHC IIa has been reported to be the most tightly bound to CC II of all non-CC II components (Camm and Green, 1989; Peter and Thornber, 1991a) and has been demonstrated to bind zeaxanthin upon illumination with high light (Ruban et al., 1994). Thus, it is possible that all the zeaxanthin detected in our CC II fraction is actually associated with the co-migrating LHC IIa protein. The predominant carotenoid in CC II is β-carotene, at 5.6 mol/mol lutein.

We next analyzed the pigment content of the PSI holocomplex. Since the PSI complex extracted from thylakoids was contaminated with CC II components, we also examined the pigment content of the much purer PSI extracted from stromal lamellae membranes, which is essentially free of any contaminating CC II polypeptides (Fig. 3). Figure 5, A and B, show the pigment analysis of both PSI preparations. Although there are some differences, both preparations give very similar results, indicating that CC II did not contribute a significant amount of pigment to the PSI fraction obtained from thylakoids. Both PSI preparations have an extremely low content of neoxanthin; small amounts of antheraxanthin and no zeaxanthin are detected in dark-adapted plants. Upon high-light treatment, PSI preparations demonstrate a decrease in violaxanthin and an increase in antheraxanthin and zeaxanthin. In PSI from stromal lamellae, violaxanthin decreased 28%, from 0.65 to 0.47 mol/mol lutein, whereas antheraxanthin increased from 0.01 to 0.08 mol/mol lutein and zeaxanthin increased from 0.0 to 0.14 mol/mol lutein. This represents a change in deepoxidation state from 0.8 to 26.2%. The similarity of results between the two preparations is surprising considering that a much more involved treatment with surfactants is needed to isolate stromal lamellae. PSI seems to be a particularly stable complex that can withstand fairly harsh isolation procedures. The only difference between the two preparations is that β-carotene is sharply reduced in high light in PSI from thylakoid membranes but remains at the same level in stromal lamellae PSI. The significance of this observation is unknown.

Our CC I fraction from stromal lamellae membranes contained either cf γ or CP43 (Fig. 3, lane 1). Since the contamination was minor, we proceeded to determine the pigment content of our CC I fraction from dark- and high-light-treated plants (Fig. 6). This CC I fraction contained zeaxanthin in high-light-treated plants with a concomitant 60% decrease in violaxanthin content. The level of deepoxidation increased from 0 to 35%. At present we cannot explain the presence of very minor amounts of Chl *b* in this fraction, since CC I does not contain Chl *b* (Siefermann-Harms, 1985) and there seem to be no LHC I polypeptides, which could bind Chl *b*, co-migrating with this fraction. In addition, we were surprised to observe that neoxanthin levels increased, essentially from zero, in high-light-treated plants, since neoxanthin content usually does not vary with

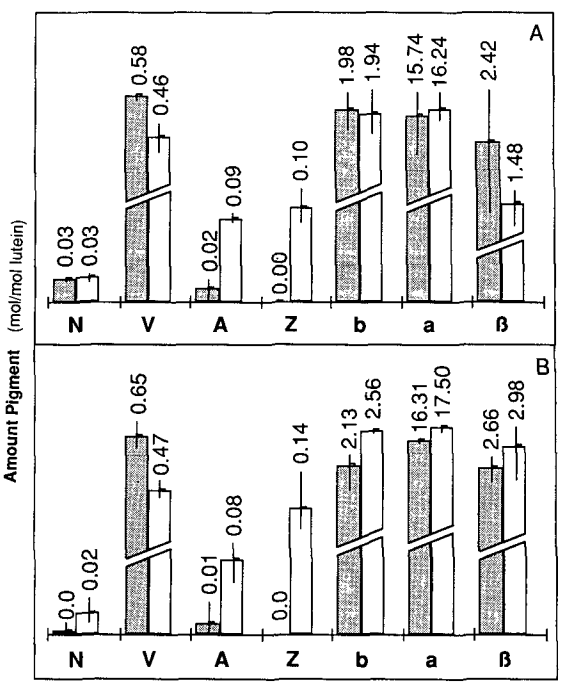

Figure 5. Pigment content of PSI isolated from nondenaturing PAGE (Fig. 1) of solubilized thylakoid or stromal lamellae membranes derived from dark- or high-light-treated barley seedlings. A, PSI from thylakoid membranes; B, PSI from stromal lamellae. Stippled bars, Dark-treated barley; open bars, high-light-treated barley. For further explanation of abbreviations, see legend to Figure 4.

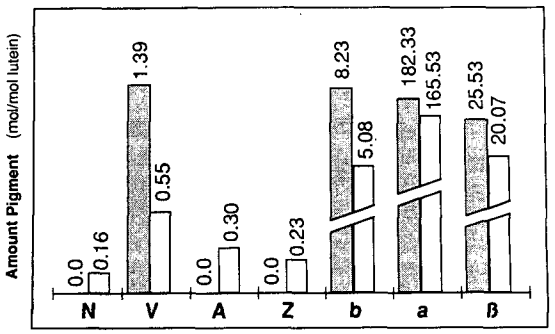

Figure 6. Pigment content of CC I isolated from stromal lamellae and fractionated by nondenaturing PAGE (Fig. 1). Stippled bars, Dark-treated barley; open bars, high-light-treated barley. Each bar represents one experiment. All numbers are in mol and are reported relative to 1 mol of lutein. For further explanation of abbreviations, see legend to Figure 4.

light treatment (Fig. 4; Ruban et al., 1994). In this fraction, there may be a small decrease in β-carotene upon high-light treatment, from 25.5 to 20.1 mol/mol lutein.

The free-pigment fraction on our nondenaturing gel system was excised and analyzed as well (Fig. 7). There is a 68% decrease in the amount of violaxanthin along with an increase in antheraxanthin (57%) and zeaxanthin (0 to 0.12 mol/mol lutein) in this fraction under high-light treatment. The other pigments decrease between dark and light treatment, although there is a broad margin of error and it is difficult to determine if these changes are significant. These pigments are expressed relative to the amount of lutein in the free-pigment fraction. We chose lutein to be consistent with the rest of our data but it is not known whether the levels of lutein vary within this fraction. Thus, the shifts in pigment content within the free-pigment fraction should be interpreted with caution. However, when expressed relative to the other pigments (Chl *a*, Chl *b*, or neoxanthin), violaxanthin still decreases and antheraxanthin and zeaxanthin still increase upon high-light exposure. Thus, the changes in the xanthophyll-cycle carotenoids within the free-pigment pool are not the result of shifts in lutein levels.

Our deepoxidation level of 22 to 28% is much lower than the 40 to 65% reported elsewhere (Thayer and Björkman, 1992; Ruban et al., 1994). Nevertheless, the levels of zeaxanthin observed are well within the 20 pmol detection limit for this HPLC method (Gilmore and Yamamoto, 1991). It is advantageous for us to report our data relative to the lutein level within each complex because, unlike neoxanthin or β-carotene, lutein is detected in all our complexes but at much lower levels than Chl *a* or Chl *b*. Lutein was selected as an internal standard because several lines of evidence indicated that the amount of lutein within each complex does not change between light and dark treatments. Although it has been reported that with high-light treatment the total leaf lutein content decreases slightly (Demmig et al., 1987), other analyses have indicated that lutein remains at essentially the same level under conditions of excess excitation energy at 0% CO_2 (Demmig et al., 1987) and within pigment-protein complexes from plants treated with high-light intensities (Thayer and Björkman, 1992; Ruban et al., 1994). In agreement with these reports, the fact that the level of most nonxanthophyll-cycle pigments in our experiments do not vary between dark and light treatment when expressed relative to lutein is a good indication that the lutein content of these complexes also does not vary between dark and light treatments.

DISCUSSION

Our nondenaturing Deriphat gel system fractionates intact photosynthetic pigment-protein complexes with little loss of pigment (Fig. 1). Polypeptide analysis (Figs. 2 and 3) of these complexes demonstrates that we obtained highly purified fractions. We were interested in characterizing their pigment composition and whether there were any shifts in pigment content upon high-light treatment. In particular, we hoped to determine if changes characteristic of the xanthophyll cycle (i.e. decrease in violaxanthin and increase in antheraxanthin and zeaxanthin) occurred in any of our pigmented proteins. To varying degrees we detected such a pattern of carotenoid changes upon high-light treatment in all the complexes studied as well as in the free-pigment fraction. In addition, by characterizing the pigment stoichiometry, we hoped to glean additional information concerning the arrangement of pigments within their respective proteins and possibly the organization of these pigmented complexes within the photosynthetic membranes.

In dark-adapted plants, low levels of antheraxanthin and no zeaxanthin were detected in the oligomeric LHC IIb complex. The ratio of the other xanthophylls, neoxanthin and violaxanthin, relative to one lutein were 0.19 and 0.34, respectively (Fig. 4A). The LHC IIb complex occurs as a trimer with two luteins per monomer (Kühlbrandt et al., 1994). Therefore, per trimeric complex, our data indicate a carotenoid molar content of 1.14 violaxanthin, 2.04 neoxanthin, 0.03 antheraxanthin, and no zeaxanthin from dark-adapted plants. Rounding off the above numbers, we postulate that per LHC IIb trimer, there are 1 violaxanthin, 2 neoxanthins, 6 luteins, and on average 0.03 antheraxanthin molecules. This integral stoichiometry of only 1 violaxanthin and 2 neoxanthins per trimer suggests that the LHC IIb

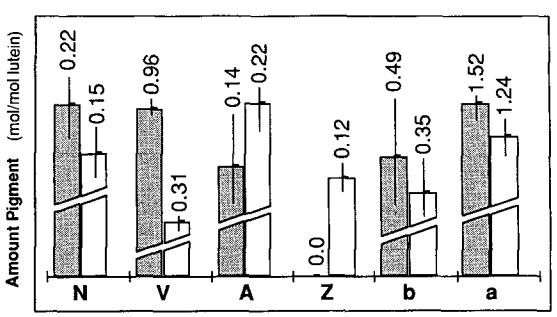

Figure 7. Pigment content of the free-pigment fraction from nondenaturing Deriphat-PAGE (see Fig. 1) of solubilized thylakoid membranes from dark-adapted and high-light-treated barley seedlings. Stippled bars, Dark-treated barley; open bars, high-light-treated barley. For further explanation of abbreviations, see legend to Figure 4.

trimer is the functional entity rather than the monomeric LHC IIb pigment-proteins. With exposure to high light, the violaxanthin decreases to 0.54 mol/mol LHC IIb trimer and antheraxanthin increases to 0.16 and zeaxanthin to 0.14 mol per trimer. Neoxanthin levels remain the same at 2 per trimer. Our data differ from those of Ruban et al. (1994) and Kühlbrandt et al. (1994). The former group concluded that the trimer binds 1 violaxanthin and only 1 rather than 2 neoxanthin molecules, whereas the latter group observed 2 luteins as the only xanthophyll in their monomeric LHC IIb preparation. The difference may reflect different growth conditions, different plants (barley versus spinach), or different isolation and solubilization procedures for LHC IIb.

The Chl *a*/*b* ratio of the LHC IIb oligomer was calculated to be 1.24 (Fig. 4A), and, again assuming 6 luteins per trimer, we estimate that there are 16 Chl *b* and 20 Chl *a* per trimer. Other laboratories (Bassi et al., 1993; Ruban et al., 1994), including ours (Peter and Thornber, 1991a), have reported a Chl *a*/*b* ratio of 1.33, whereas recent crystallographic work has reported a ratio of 1.17 (Kühlbrandt et al., 1994). The difference may be due to decyl-β-D-maltoside solubilization retaining more pigment (viz. Chl *b*) with the trimer than dodecylmaltoside or other detergents. A less likely explanation is that the difference may be due to different extinction coefficients (some more precise than others) for Chl determination being utilized by the various groups.

The LHC II monomeric complex (Fig. 1, lane 1, and Fig. 2, lane 4), a heterogeneous mix of LHC IIa, c, and d, displayed a pattern of carotenoid changes in high light similar to those of the LHC IIb trimeric complex. Because the LHC II polypeptides all show sequence similarity (Green et al., 1991; Thornber et al., 1993) and all have light-harvesting functions, they must be essentially structurally similar to the Kühlbrandt et al. (1994) structure of the LHC IIb monomer. If we assume that each of the minor LHC II polypeptides is stabilized by a pair of lutein molecules, as occurs in LHC IIb, then on average, from dark-adapted plants there would be 1 violaxanthin, 0.64 neoxanthin, 0.1 antheraxanthin, no zeaxanthin, 0.08 β-carotene, and 13 to 14 Chl molecules per minor LHC II polypeptide. A value of 14 Chls per polypeptide is quite probable (cf. LHC IIb). After high-light treatment, the xanthophyll cycle carotenoids change to 0.6 violaxanthin, 0.16 antheraxanthin, and 0.18 zeaxanthin per polypeptide, and the levels of the remaining pigments are unchanged. Note that these numbers are the average pigment content per LHC II polypeptide within a mixture of LHC IIa, c, and d. LHC IId contains little (Peter and Thornber, 1991a) or no (Bassi et al., 1993) neoxanthin, whereas LHC IIa and c each have 1 neoxanthin per polypeptide (Peter and Thornber, 1991a). Averaging the pigment content of the three probably explains the nonstoichiometric amount of neoxanthin in our data. In the study by Ruban et al. (1994), the pigment content per individual LHC II polypeptides from dark-adapted plants was calculated assuming a not unreasonable value of 15 Chl molecules per polypeptide. Each minor LHC II polypeptide contained 1 violaxanthin molecule, with LHC IIa having 1 lutein and 0.5 neoxanthin, LHC IIc with 1 each of lutein and neoxanthin, and LHC IId with 1.5 lutein and 0.5 neoxanthin, although this latter fraction may have had a small amount of LHC IIc. We prefer to base our calculations on 2 lutein molecules per polypeptide because, although the Chl content may vary between LHC IIa, c, and d, each minor LHC II polypeptide very likely has 2 lutein molecules.

To our surprise, we detected changes characteristic of the xanthophyll cycle within the CC II complex (Fig. 4C). Our results and the results of Bassi et al. (1993) indicate that CC II apparently contains some xanthophyll carotenoids. Bassi et al. (1993) detected β-carotene in both the PSII reaction center and its core antenna but lutein only in the core antenna. They did not report any neoxanthin, violaxanthin, or antheraxanthin associated with this complex. As described in "Results," we detected all the xanthophyll carotenoids, including zeaxanthin, in the CC II complex, albeit in greatly reduced amounts compared to the LHC fractions. The most likely source is LHC IIa, which is difficult to remove completely from the core (see "Results"; Camm and Green, 1989; Peter and Thornber, 1991a). However, we cannot rule out the possibility that the core's antenna has minor amounts of xanthophylls.

In addition to PSII, the xanthophyll cycle also operates within PSI. This is in agreement with a previous report of the presence of zeaxanthin within PSI (Thayer and Björkman, 1992). Both the PSI complexes isolated from thylakoids and those isolated from stromal lamellae exhibit decreases in violaxanthin content (21–28%, Table I) that are lower than in the PSII complexes (40–53%). It may be that less of the PSI xanthophyll cycle pool is available for conversion into antheraxanthin and zeaxanthin. An alternative but less likely possibility is that the PSII violaxanthin is more easily dissociated during fractionation, resulting in the greater percentage of decrease in violaxanthin content.

We followed pigment analysis of PSI (i.e. CC I + LHC I) with one of CC I to determine whether the xanthophyll cycle activity occurred within LHC I or CC I alone or within both complexes. CC I from dark-adapted seedlings contained no neoxanthin or antheraxanthin but did contain violaxanthin, lutein, β-carotene, Chl *a*, and Chl *b* (Fig. 6). We are puzzled by the small amount of Chl *b* detected within this fraction as well as by the appearance of neoxanthin in CC I in high-light-treated plants. CC I should not contain Chl *b* (Siefermann-Harms, 1985), yet the only non-CC I polypeptides we are able to detect on our denaturing gels are possibly CP 43 of the PSII core or cf γ, which also do not contain Chl *b*. We cannot explain the appearance of a small amount of neoxanthin after high-light treatment. Thus far, neoxanthin has not been implicated in photoprotection. Small amounts of zeaxanthin were found within the CC I fraction upon high-light treatment. However, it is not clear whether this zeaxanthin is associated with the CC I complex or with possible LHC contaminants, which might also explain the presence of Chl *b* in CC I.

Since LHC I has not been isolated as an intact multiple pigment-protein complex by our nondenaturing gel system, we can only deduce the pigment content of LHC I by subtracting the pigment content of CC I from that of PSI in

dark-treated plants. Assigning 200 Chl molecules to PSI (Malkin, 1987; Zipfel and Owens, 1991) and using the Chl *a*/*b* ratio of 7.7 as derived from Figure 6, there would be approximately 7 violaxanthin, 0.11 antheraxanthin, 11 lutein, 23 Chl *b*, 177 Chl *a*, and 29 β-carotene molecules per PSI particle. There have been various determinations of the Chl and/or P700 content of CC I and, depending on the preparation, the Chl content of CC I has ranged from 90 to 120 Chl *a* molecules per unit or per P700 molecule (Siefermann-Harms, 1985; Malkin, 1987; Zipfel and Owens, 1991). If we assign 120 Chl *a* molecules to CC I, then our CC I analysis yields approximately 0.9 violaxanthin, 0.65 lutein, and 16.8 β-carotene molecules per CC I particle. Subtracting the putative pigment content of CC I from that of PSI results in approximately 6.2 violaxanthin, 0.11 antheraxanthin, 10.1 lutein, 23 Chl *b*, 57 Chl *a*, and 12.0 β-carotene molecules per LHC I complex. Although the LHC I complex consists of four distinct pigment-proteins (LHC Ia, b, c, and d), LHC Ia and b form the bulk of the LHC I complex (Thornber et al., 1993). There is evidence to suggest that these two polypeptides are organized as trimers (Preiss et al., 1993; Dreyfuss and Thornber, 1994). Since LHC Ia and b show a high degree of homology to LHC IIb, we think it likely that each monomeric LHC I polypeptide will contain two luteins. Thus, we propose that per CC I entity, the LHC I complex (consisting mostly of LHC Ia and b) is composed of six polypeptides possibly organized as two trimers analogous to LHC IIb. This arrangement gives us a total of 12 luteins per LHC I complex, which is close to our estimated value of 10 luteins. Each LHC I monomer, in addition to the two luteins, will also have 1 violaxanthin, 0.02 antheraxanthin, 4 Chl *b*, 10 Chl *a*, and 2 β-carotene. The xanthophyll stoichiometry in this case, unlike the situation with LHC IIb, does not necessarily indicate a trimer (see above). However, if, as seems probable, LHC I polypeptides form a trimer, there will be 3 violaxanthin molecules per LHC I trimer in contrast to 1 violaxanthin per LHC IIb trimer. A surprising result of these calculations is that there are two β-carotene molecules per monomer, which is unlike the situation with the LHC II polypeptides. Because of the nature of the calculation, the unexpectedly large amount of β-carotene may be explained by some of the β-carotene in CC I in the PSI fraction being lost upon treatment of PSI to CC I.

It is certainly true that the xanthophyll cycle intermediates are present in LHC I. Using the data from Figures 5B and 6 and the same reasoning as above, we estimate that under high-light conditions LHC I monomer has approximately 0.7 violaxanthin, 0.10 antheraxanthin, and 0.21 zeaxanthin, with the other nonxanthophyll pigments remaining at about the same levels.

At this time, whether or not the xanthophyll cycle operates in the CCs of PSI and/or PSII is equivocal. The light-harvesting antennae contain the bulk of the xanthophylls; therefore, the xanthophyll cycle must be operating predominantly if not exclusively within the antenna complexes of both photosystems. In contrast to Bassi et al. (1993) and in agreement with Ruban et al. (1994), we found both the LHC IIb complex and the minor LHC II pigment-proteins to be potential sites of xanthophyll-cycle-associated fluorescence quenching. If the LHC IIb trimer, which is probably the functional entity rather than the monomer, is compared to the minor LHC II monomeric polypeptides, then the carotenoid content per two lutein molecules is very similar in both fractions. Our results indicate that in dark-adapted plants, both are associated with 1 violaxanthin, and upon high-light treatment, both type of complexes are associated with 0.16 antheraxanthin and 0.14 (LHC IIb) or 0.18 (minor LHC IIs) zeaxanthin. Ruban et al. (1994) have demonstrated varying degrees of deepoxidation within the PSII antenna (from 65% for LHC IIb to 27% for LHC IIa) upon high-light treatment. In our system, the LHC IIb oligomer and the minor LHC II polypeptides both reached a similar deepoxidation state of 26.6 and 28%, respectively. Again, the difference may be due to different plant species or to differences in growth light regime. Perhaps in a plant that exhibits a higher degree of zeaxanthin synthesis different LHC II complexes can be deepoxidated to greater or lesser extents.

Analysis of the free-pigment zone reveals that all pigments except β-carotene are within this fraction, with violaxanthin being particularly high relative to the other carotenoids. Thus, our present pigment analysis may indicate the minimum amount of pigment that is associated with each complex. However, the amount of the total thylakoid pigment in the free-pigment zone appears to be very small (compare LHC II monomer, which represents approximately 9% of the total Chl [Thornber et al., 1993] with the free pigment in Fig. 1, lane 1). It is possible that a population of xanthophyll-cycle carotenoids are more loosely or not directly attached to LHC pigment-proteins or to other proteins and therefore will be found in the free-pigment fraction (see below). Further, a new, xanthophyll-rich minor LHC II, LHC IIe, migrates close to the free-pigment zone (Peter and Thornber, 1991a), and it may contribute some of the pigments in the free-pigment zone analysis. We are currently determining conditions that will consistently separate LHC IIe from the free-pigment zone. No β-carotene was detected in the free-pigment zone. Therefore, the β-carotene content of our complexes represents the situation in the thylakoid membranes prior to solubilization with detergents.

At present little is known concerning the mechanism of the xanthophyll cycle in fluorescence quenching. Among the many puzzling aspects is the nonstoichiometric amounts of carotenoids per polypeptide within each pigment-protein complex. In some instances, the mol pigment/mol polypeptide ratio is quite small (e.g. 0.02). This is explained either by contamination (e.g. β-carotene in LHC IIb) or by a small fraction of the 1 mol violaxanthin per mol LHC monomer or trimer having been converted into antheraxanthin with or without zeaxanthin. Two suggestions for LHC IIb, which can also apply to other LHC protein complexes, are that the nonstoichiometric carotenoids are placed at the periphery of each complex or between complexes (Kühlbrandt et al., 1994) or that the carotenoids bind preferentially to one of the three types of polypeptides that make up the LHC IIb complex (Bassi et

al., 1993). Another possibility is that a fraction of the xanthophyll-cycle pool is always present as carotenoids loosely associated with or not associated with protein complexes, thus leading to the substoichiometric amounts. This scenario may explain the presence of the xanthophyll-cycle carotenoids in the free-pigment zone. Carotenoids are about 30 Å in length (Kühlbrandt et al., 1994), suggesting that they span the thylakoid membranes. Yet, the deepoxidase, which converts violaxanthin to zeaxanthin, is likely to be on the lumenal side of the thylakoid membranes (Yamamoto, 1979; Hager and Holocher, 1994), whereas it is postulated that the epoxidase is located within the membrane (Gruszecki and Krupa, 1993) or on the stromal side (Yamamoto, 1979). Therefore, the carotenoids of the xanthophyll cycle may have to be loosely associated with their respective complexes in order to be deepoxidated and epoxidated in different locations.

Upon high-light treatment, violaxanthin peripherally associated with protein or already within the free-pigment pool may be deepoxidated on the lumenal side of the thylakoid membranes, forming zeaxanthin. The newly formed zeaxanthin might then move into the membrane to be attached to the LHC polypeptides. This loose association of xanthophyll to proteins may be reflected in the carotenoids of the free-pigment fraction. Since lutein has been reported to be in the center of LHC IIb, forming the scaffolding for the protein, the presence of lutein in the free-pigment fraction could indicate some disruption of intact pigment-protein complexes during fractionation or that there is a special function for the free-pigment carotenoids. Therefore, the free-pigment fraction may indicate artifactual loss of pigment and/or perhaps the existence of biochemical processes that occur under environmental stress conditions.

Our results concur with many of the conclusions from other laboratories (Thayer and Björkman, 1992; Ruban et al., 1994). The differences in results illustrate the difficulty of quantitating the pigment content of these protein complexes. There is always the possibility of artifactual loss of pigments during membrane isolation and solubilization or fractionation of pigmented complexes. It can be difficult to prepare highly purified complexes without using unduly harsh detergents, which can lead to pigment loss. As Ruban et al. (1994) demonstrate, the carotenoid content of LHC IIb varies slightly with the isolation and solubilization procedure. In many, although not all, of our complexes, we were able to obtain stoichiometric amounts of pigments, particularly neoxanthin and violaxanthin. The pigments that have been shown to remain unchanged in the whole leaf during high-light treatment did not fluctuate between dark and light treatments in our experiments. However, the presence of Chl *b* within both of our CC fractions indicates the possibility of LHC contaminants and, therefore, we cannot be certain whether the xanthophyll cycle is also active in the CCs. Neither can we explain at this time the small increase in neoxanthin within the CC I fraction upon high-light treatment. The small amounts of β-carotene within our LHC II fractions (Fig. 4, A and B) may have come from extraction of CC II components, although we cannot say for certain. However, the amount is small and, more importantly, does not vary between light and dark treatments, whereas antheraxanthin and zeaxanthin do. Therefore, rather than relying on one method, the results of several experimental approaches may be needed to reach a consensus on the exact pigment stoichiometry of these complexes.

Because zeaxanthin is associated with both PSI and PSII complexes, all of these complexes are candidates for the site of the high-energy, DTT-sensitive fluorescence quenching that has been associated with the presence of zeaxanthin (Demmig-Adams, 1990). It is not known whether all complexes that contain zeaxanthin are capable of fluorescence quenching. The Chl *b*-less *chlorina* f2 mutant of barley lacks some of the LHC II polypeptides (Peter and Thornber, 1991a), yet this mutant exhibits the ability to quench Chl fluorescence, suggesting that the remaining complexes (CC II, PSI, and/or the remaining LHC II) are capable of fluorescence quenching (Leverenz et al., 1992). Fluorescence quenching studies in mutants that lack a portion of the photosynthetic apparatus, along with electron diffraction analysis to determine the orientation of the carotenoids within their protein complexes, will be needed to elucidate the mechanism of the xanthophyll cycle and its role in photoprotection.

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