Carotenoid-Dependent Oligomerization of the Major Chlorophyll a/b Light Harvesting Complex of Photosystem II of Plants†

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ABSTRACT: Under many environmental conditions, plants are exposed to levels of sunlight in excess of those required for photosynthesis. Then, a regulated increase in the rate of nonradiative dissipation of excess excitation energy in the thylakoid membrane correlates with the conversion of the carotenoid violaxanthin into zeaxanthin and provides protection from the damaging effects of excessive irradiation. The hypothesis that these carotenoids specifically control the oligomerization of the light harvesting complexes of photosystem II was tested by investigating the effects of violaxanthin and zeaxanthin on the behavior of the major complex, LHCIIb, on sucrose gradients; it was found that zeaxanthin stimulated the formation of LHCIIb aggregates with reduced chlorophyll fluorescence yield whereas violaxanthin caused the inhibition of such aggregation and an elevation of fluorescence. Measurements of 77 K fluorescence indicated that zeaxanthin was not exerting an additional direct quenching of chlorophyll fluorescence. These effects can explain the physiological control of the light harvesting system by the xanthophyll cycle.

Light harvesting in plant photosystem II is carried out by an array of chlorophyll a/b−carotenoid protein light harvesting complexes (LHCII)[1] which form the photosynthetic antenna in the chloroplast membrane. LHCII comprises six different homologous proteins encoded by the *Lhcb* gene family (Jansson, 1994). Three of these (encoded by the *Lhcb1-3* genes) comprise the bulk LHCII protein known as LHCIIb, whereas the other three, LHCIIa, LHCIIc, and LHCIId are referred to as the minor LHCII. All LHCII proteins show dynamic behavior, which is thought to contribute to the control over the efficiency of light harvesting *in vivo* (Horton, 1989; Horton & Ruban, 1992; Horton et al., 1996): the state of photosystem II giving highly efficient energy utilization in low light is replaced by a state in high light in which there is effective nonradiative dissipation of excess excitation energy, a molecular switch which controls the flow of excitation energy to the reaction center. The switch between these two conditions is essential if there is to be both efficient photosynthesis in low light and protection from the photoinhibitory effects of excessive irradiation in high light.

The process of energy dissipation is observed by its effect on chlorophyll fluorescence; a decrease in yield is a consequence of the increase in rate of dissipation. The principal component of such nonphotochemical quenching of fluorescence is referred to as qE (Krause & Weis, 1991). It has been established that two signals cooperatively control qE (Horton et al., 1991, 1996; Bilger & Björkman, 1994; Gilmore & Yamamato, 1992a): firstly, there is the pH gradient built up across the thylakoid membrane in excess light (Briantais et al., 1979), the associated acidification of the thylakoid lumen being thought to cause protonation of one or more of the proteins of LHCII (Horton & Ruban, 1992; Walters et al., 1996; Pesaresi et al., 1997). Secondly, the reversible enzymatic de-epoxidation of the carotenoid violaxanthin into zeaxanthin (the xanthophyll cycle) has been shown to correlate with the formation of qE in a large number of experiments *in vitro* and *in vivo* (Demmig-Adams & Adams, 1992; Gilmore & Yamamoto, 1992b). The xanthophyll cycle carotenoids have been found to be bound by LHCII (Peter & Thornber, 1990; Thayer & Björkman, 1992; Bassi et al., 1993; Ruban et al., 1994b). Although these carotenoids are enriched in the minor LHCII proteins, binding ca. 1 per monomer, ca. 50% of the total pool is associated with LHCIIb, which binds ca. 1 mol per trimer. At least in the case of LHCIIb, these carotenoids appear to be rather loosely bound to the protein periphery, and purification of the complex in detergents progressively lowers the amount of bound xanthophyll cycle carotenoid.

It is important to discover how the conversion of violaxanthin to zeaxanthin may exert control over the function of LHCII. It has been suggested that zeaxanthin *directly* quenches chlorophyll excited states (Demmig, 1990; Owens et al., 1992; Frank et al., 1994; Gilmore et al.,1996a), although there is no evidence of chlorophyll to carotenoid energy transfer in LHCII either *in vitro* or *in vivo*. A correlation between the quenching of fluorescence in isolated LHCIIb and the S_1 energy level of exogenous carotenoid has been found, although a direct role of chlorophyll to

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[1] Abbreviations: DM, *n*-dodecyl *β*-maltoside; LHCII, light harvesting complexes of photosystem II; qE, nonphotochemical quenching of chlorophyll fluorescence dependent of the thylakoid pH gradient.

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carotenoid energy transfer was suggested to be secondary to an indirect structural effect (Phillip et al., 1996). In fact, there is experimental evidence indicating that the xanthophyll cycle *indirectly* controls the efficiency of quenching (Noctor et al., 1991). It was proposed that violaxanthin and zeaxanthin perform this function by control of protein−protein interaction between the LHCII proteins (Horton et al., 1991); violaxanthin was proposed to stabilize the unquenched state of LHCII that is efficient in light harvesting whereas zeaxanthin promotes a quenched state of the complexes displaying effective energy dissipation. This hypothesis is consistent with observations suggesting that the PSII light harvesting system *in vivo* existed in a partially aggregated state (Bassi & Dianese, 1992; Garab et al., 1991; Kolubayev et al., 1986), which may allow efficient long-range energy migration (Barzda et al., 1995) and that formation of qE was associated with changes in the macrostructure of the thylakoid (Ruban et al., 1993b; Bilger & Björkman, 1994, Garab et al., 1988)

Aggregation of isolated LHCIIb protein is associated with a strong quenching of chlorophyll fluorescence (Mullet & Arntzen, 1980; Ruban & Horton, 1992). The main features of the quenching of fluorescence in LHCII aggregates are the same as for quenchnig *in vivo* (Ruban et al., 1992, 1994a). These observations were initially made using LHCIIb, but it was later found that the minor LHCII behaved in the same way (Ruban et al., 1996). The exact nature of the quenching process is not known, but changes in the absorption spectrum (Ruban et al., 1994a), fluorescence excitation spectrum (Ruban & Horton, 1992), Raman spectrum (Ruban et al., 1995), and yield of Z band thermoluminescence (Hagen et al., 1996) provided evidence for alteration in pigment conformation and/or environment in the aggregated state of isolated LHCII.

In this paper the results of experiments are described which were designed to directly test the central feature of this hypothesis, i.e., do the xanthophyll cycle carotenoids modulate the state of oligomerization of the LHCII proteins? It was found, using LHCIIb, that violaxanthin and zeaxanthin induce disaggregation and aggregation, respectively.

MATERIALS AND METHODS

The LHCII protein used in this study is LHCIIb. It was isolated from dark adapted spinach leaves by isoelectric focusing of detergent solubilized photosystem II BBY particles as described previously (Ruban et al., 1994b). No zeaxanthin was present in the complex. LHCIIb was dissolved in 200 μM dodecylmaltoside (DM) and 20 mM HEPES, pH 7.6, at a chlorophyll concentration of 200 μM. Before centrifugation, the sample was diluted to 25 μM chlorophyll and incubated for 5 min as described in the figure legends. Where indicated, violaxanthin or zeaxanthin, purified as described previously (Ruban et al., 1993) and dissolved in ethanol, were included at 25 μM in the incubation medium. The final ethanol concentration was less than 1.5%. Sucrose gradients were seven step exponential gradients from 0.16 to 0.87 M sucrose dissolved in HEPES buffer containing either 200 μM DM (Figure 1, tubes 1−3) or 250 μM dibucaine (Figure 1, tubes 4 and 5). The run time was 17 h at 200000*g* using a SW41 Rotor at 4 °C. Sucrose density was determined by refractometry. From the gradients, 7−10 fractions were collected and assayed for carotenoid composition (Ruban et al., 1994b), fluorescence yield at 77 K and room temperature (Ruban et al., 1996), and chlorophyll content.

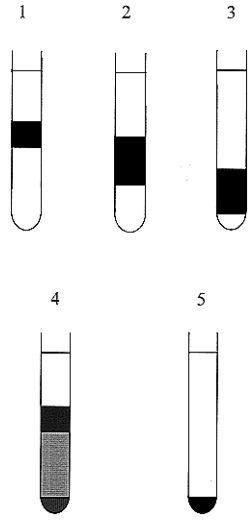

FIGURE 1: The effect of zeaxanthin on the sucrose density profile of different aggregation states of LHCII. Diagramatic representation of sucrose gradients of LHCII preincubated in 200 μM DM (1); 25 μM DM (2); 25 μM DM in the presence of 25 μM zeaxanthin (3); 25 μM DM + 250 μM dibucaine (5), and 25 μM DM + dibucaine in the presence of 25 μM violaxanthin (4). For conditions of centrifugation, see Materials and Methods.

RESULTS

To study the effects of violaxanthin and zeaxanthin on LHCII organization required careful choice of conditions because it was necessary to simulate the aggregation/disaggregation forces imposed on LHCII in the thylakoid membrane environment where the complex is partially aggregated. Carefully designed conditions for sucrose gradient centrifugation of detergent solubilized LHCII were devised, which allowed the dynamics of oligomerization to be investigated. The experimental strategy was to preincubate LHCII with added xanthophyll cycle carotenoid so as to bring about complex formation. These conditions had been established in earlier fluorescence quenching experiments (Ruban et al, 1994a), which showed that a molar ratio of 1 car:1 Chl gave near maximum effect.

Figure 1 shows the results of sucrose gradient centrifugation of LHCII in different states of oligomerization. Tubes 1−3 show three different banding patterns produced in the presence of 200 μM DM, after preincubation at a reduced detergent concentration in the absence and in the presence of zeaxanthin, respectively. Measurement of the chlorophyll content of fractions collected from the gradient revealed the profiles for these three states (Figure 2A): the triangles show the profile of LHCII in its trimeric state; this is the state of LHCII found *in vitro* and most probably *in vivo*. After incubation at 6 μM DM, some aggregation occurred; on the gradient this profile is shown as filled circles and is found at ca. 0.6 M sucrose. In the presence of zeaxanthin, aggregation of LHCIIb occurred (Figure 2A, squares). Carotenoid distributes in the gradient in a characteristic pattern (Figure 2B). Most is washed from the complex but in the LHCII aggregate there is 1.1 zeaxanthin per 42 chlorophyll; this is ca. 1 zeaxanthin per LHCII trimer, the same as the content of xanthophyll cycle in LHCII gently

Table 1: Chlorophyll Fluorescence Parameters for LHCII[a]

LHCII	fluorescence yield	heat dissipation constant	F700/F680
trimer	1.00	0.00	0.10 ± 0.01
control (+DM)	0.81 ± 0.02	0.23 ± 0.03	0.13 ± 0.01
+ zeaxanthin	0.29 ± 0.02	2.45 ± 0.23	0.41 ± 0.02
+ violaxanthin	0.46 ± 0.03	1.17 ± 0.15	0.30 ± 0.02
control (+ dibucaine)	0.22 ± 0.02	3.55 ± 0.45	0.47 ± 0.02

[a] Data were taken from samples prepared as in Figures 1 and 2. Fluorescence yield was measured at 20 °C and is expressed relative to the LHCII trimer recorded at the same chlorophyll concentration. Heat dissipation constant was calculated from $(F_{trimer} - F_{sample})/F_{sample}$. F700/F680 was calculated from the fluorescence emission spectra at 77 K using the signal amplitudes at 680 and 700 nm. Data are means of 3−4 replicates ± sem.

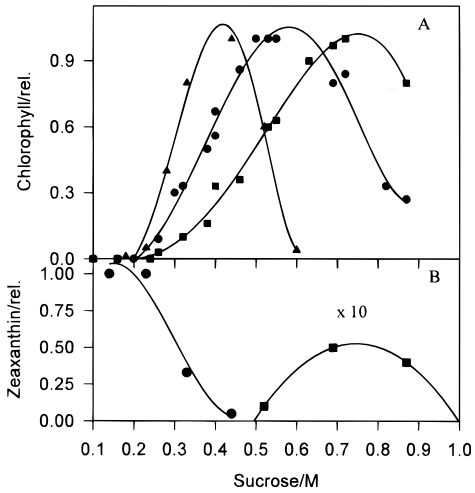

FIGURE 2: The distribution of pigments in fractions taken from sucrose gradients of the type shown in Figure 1. (A) Distribution of chlorophyll; (B) distribution of zeaxanthin, in relative units. LHCII preincubated in 200 μM DM (▲); 25 μM DM (●); 25 μM DM in the presence of 25 μM zeaxanthin (■). For panel B, the zeaxanthin content for fractions marked as ■ are multiplied by 10.

isolated from thylakoid membranes (Ruban et al., 1994b). Although we can not accurately determine the size of the aggregates from the sucrose gradient, we estimate that the band at 0.6 M sucrose corresponds to 4−6 LHCII trimers.

The fluorescence characteristics of these fractions are shown in Table 1. In the control aggregate, the fluorescence was quenched by ca. 19% compared to the trimeric state. In the fraction with added zeaxanthin LHCII fluorescence was highly quenched (yield = 0.29). A heat dissipation constant was also calculated from the extent of quenching; this increased over 10-fold due to the presence of zeaxanthin. Aggregation-dependent quenching in LHCII is associated with an increase in the relative intensity of emission of fluorescence at 700 nm (Ruban & Horton, 1992; Ruban et al., 1996) observed at 77 K. Values of F700/F680 are shown for each LHCII aggregation state; the sample plus zeaxanthin had a high value compared to the trimeric state and the control oligomer.

Figures 1 and 2 clearly show the induction of LHCII aggregate formation by zeaxanthin. If violaxanthin was used instead of zeaxanthin, a profile identical to the control was observed (data not shown). Figure 1 (tubes 4 and 5) shows the results of an experiment aimed at examining the role of violaxanthin in controlling LHCII organization. For this experiment, LHCII aggregation was induced by the inclusion of dibucaine in the sucrose gradient medium. This reagent,

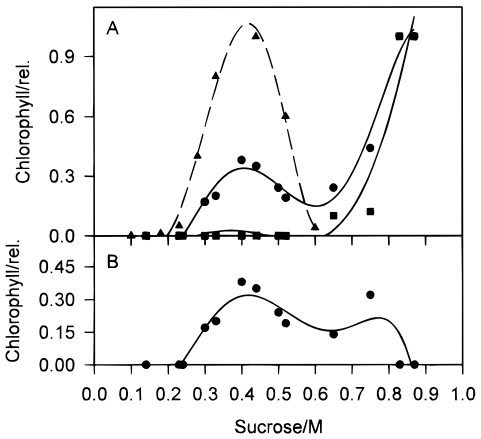

FIGURE 3: Distribution of chlorophyll in fractions taken from the gradients (A), control (■) and + violaxanthin (●). Also shown is the profile of LHCII trimers taken from Figure 2 (▲). (B) difference in chlorophyll distribution due to violaxanthin, all in relative units. Other details as for Figure 1.

first characterized as a local anaesthetic, is a promoter of qE in isolated chloroplasts (Laasch & Weis, 1989) and, most importantly, it has been shown to induce quenching and aggregation in isolated LHCII (Ruban et al., 1994a). It mimics the effect of low pH on LHCII but has the advantage of not inducing irreversible effects on protein structure and more importantly does not induce formation of violaxanthin isomers which can occur at low pH under the long experimentation times used for centrifugation. In the presence of dibucaine, the possible role of violaxanthin as an inhibitor of aggregation could be investigated. The control sample (tube 5) formed a pellet at the bottom of the tube indicating a more extensive aggregation than found in tube 3, owing to the absence of detergent in this gradient. The presence of violaxanthin (tube 4) caused the appearance of a band in the trimer region and a diffuse area below, whereas zeaxanthin was without effect (data not shown), clearly violaxanthin inhibited the process of oligomerisation.

The distribution of chlorophyll in this experiment is shown in Figure 3A. The difference in chlorophyll distribution (Figure 3B) shows that the presence of violaxanthin mainly increases two bands; one, at 0.4 M sucrose, is most likely a trimeric state whereas the other, at 0.8 M, is coincident with the aggregate band found in the zeaxanthin sample in Figure 2. In between these there is probably a gradation of aggregation states.

The average fluorescence yield of the complex in the presence of violaxanthin was 0.46 compared to 0.22 in the control with dibucaine (Table 1). The low yield in the latter sample was consistent with its more extensive aggregation as revealed by behavior on the sucrose gradient. The 700:680 emission ratio of the dibucaine sample was also the highest, and a value intermediate between the trimer and aggregate was found for the sample with added violaxanthin.

Figure 4 shows the relationship between fluorescence yield and the 700:680 emission ratio for the various samples produced in these experiments. Also included on this graph are data taken from a number of different experiments in which the fluorescence yield had been manipulated as described by Ruban et al. (1996). All the data fit a single line, including that with added zeaxanthin. This suggests that the quenching of fluorescence in all these samples was due to the aggregation and not to a direct quenching by

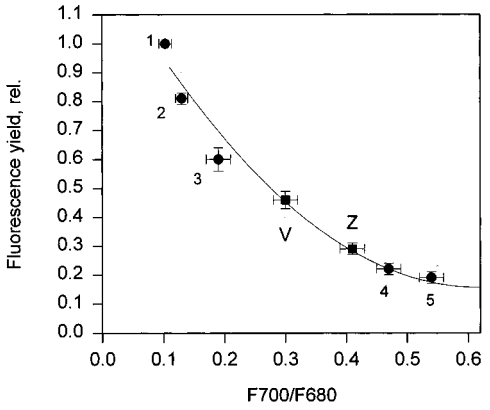

FIGURE 4: The relationship between the yield of chlorophyll fluorescence at 20 °C and the ratio of emission at 700−680 nm (F700/F680) recorded at 77 K. Data points 1, 2, and 4 refer to the samples shown in Figure 1 tubes 1, 2, and 5. Points V and Z refer to samples in the presence of violaxanthin and zeaxanthin. Point 3 and 5 are samples prepared after incubation of LHCII into 6 μM DM prior to centrifugation and after decrease in pH to 5.5 as described in Ruban et al. (1996). Each point is the mean of 3−4 replicates, error bars are sem.

energy transfer to zeaxanthin; any extra direct quenching would be observed by the displacement of the "Z" data point below the line shown in Figure 4.

DISCUSSION

These data show that the macro-organization of LHCIIb can be manipulated in an opposing manner by violaxanthin and zeaxanthin. In the absence of added carotenoid, a partially aggregated state was found; this is probably the state of LHCIIb found in dark-adapted thylakoids which gives rise to an efficient light harvesting function. Violaxanthin was found to stabilize this state, opposing the tendency for aggregation in the presence of dibucaine. In contrast, zeaxanthin induces aggregate formation and a state of high thermal energy dissipation. There is evidence that the xanthophyll cycle carotenoids are peripherally bound to the complexes (Kühlbrandt et al., 1994), explaining why exogenous pigment can so readily modify the properties of LHCIIb. Thus it is peripheral, not intrinsic, xanthophyll that controls energy quenching in LHCII. It has been found that the presence of zeaxanthin within LHCIIb, where it seems to replaces one of the two internal lutein molecules in the *Arabodopsis aba* mutant does not lead to an increase in quenching (Hurry et al., 1997).

We suggest that the key molecular feature in the effects on LHCIIb oligomerization is the structure of the carotenoid molecule. The extension of the conjugated bond system into the β-end-groups of zeaxanthin causes the molecule to adopt an energetically favored near planar ring-chain conformation. In contrast, in violaxanthin, the presence of epoxide groups in the C5,6 position removes conjugation and the end-group occupies a perpendicular position relative to the main chain. These differences were used to explain the significant differences in the solubility of the molecules in ethanol-water mixtures (Ruban et al., 1993a) and could alter the association between LHCIIb and the carotenoid. In turn, this could control the interaction between LHCIIb proteins. Violaxanthin would, in effect, act as an antiquencher, stabilizing the complex in a state capable of efficient light harvesting. In contrast, the removal of violaxanthin and/or a weaker interaction between zeaxanthin and LHCIIb may give rise to a protein structural change that leads to oligomerization. *In vivo*, these effects observed in isolated LHCIIb may occur in addition to, instead of, or be replaced by, the direct quenching of Chl excited states by energy transfer to zeaxanthin (Frank et al., 1994). However, it should be added that in the experiments described here, the relationship between quenching and the alteration in the ratio of emission at 700 nm relative to 680 nm shown in Figure 4 indicates that the direct mechanism is not playing a significant role.

It should also be emphased that, *in vivo*, changes in xanthophyll cycle de-epoxidation state alone are not sufficient to bring about quenching since there is an obligatory requirement for the thylakoid pH gradient (Noctor et al., 1991). This has been clearly demonstrated in *Arabidopsis* mutants which, despite a constitutively high zeaxanthin level, do not show any enhancement of qE (Hurry et al., 1997). It is established that de-epoxidation and protonation act synergistically together to induce qE (Horton et al., 1991; 1996, Bilger & Björkman, 1994; Gilmore & Yamamato, 1992), and we have proposed that both provide the stimulus for LHCII oligomerization (Horton et al., 1991). In the experiments described here, we have shown the effect of zeaxanthin. Other *in vitro* experiments have shown the effect of low pH on LHCII fluorescence and oligomerization (Ruban et al., 1994a, 1996).

Upon the basis of the location of DCCD-binding sites on the minor LHCII, it has been concluded that these LHCII proteins have an important role in qE (Horton & Ruban, 1991; Walters et al., 1994, 1996). In fact, it has been suggested that these proteins exclusively contain the sites of quenching (Bassi et al., 1994; Crofts & Yerkes, 1994; Pesaresi et al., 1997; Gilmore et al., 1996a,b). Studies of mutants and/or developing chloroplasts which lack LHCIIb but which still show qE are consistent with this suggestion (Härtel et al., 1996; Jahns & Schweig, 1995; Gilmore et al., 1996b), although it is perhaps very important that in these plants quenching is less efficient (Briantais, 1994); this has been assessed quantitatively in terms of quenching efficiency with respect to the de-epoxidation state of the xanthophyll pool (Gilmore et al., 1996b) and ΔpH (Schonknecht et al., 1996). Efficient quenching, and its physiological regulation, may therefore require a complete system containing all the LHCII proteins, implying that the xanthophyll cycle exerts overall control over the organization of whole PSII antenna. The effects of violaxanthin and zeaxanthin on LHCIIb observed here are consistent with this view. It should be emphasized that ca. 50% of the xanthophyll cycle pool are bound by LHCIIb (Ruban et al., 1994b) and that this is active in the control of quenching because it is peripheral. The more tightly bound, perhaps internal, xanthophylls of the minor complexes may not be active and may not necessarily exert structural effects. However, it should also be emphasized that *all* LHCII proteins show qualitatively similar quenching behavior *in vitro* (Ruban et al., 1996), this is not unexpected given the high degree of structural homology among these proteins (Green & Durnford, 1996; Jansson, 1994). We predict that the xanthophyll cycle carotenoids similarly control the interactions between all LHCII proteins; further experiments are in progress to investigate this.

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