Determinition of the Stoichiometry and Strength of Binding of Xanthophylls to the Photosystem II Light Harvesting Complexes*

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Xanthophylls have a crucial role in the structure and function of the light harvesting complexes of photosystem II (LHCII) in plants. The binding of xanthophylls to LHCII has been investigated, particularly with respect to the xanthophyll cycle carotenoids violaxanthin and zeaxanthin. It was found that most of the violaxanthin pool was loosely bound to the major complex and could be removed by mild detergent treatment. Gentle solubilization of photosystem II particles and thylakoids allowed the isolation of complexes, including a newly described oligomeric preparation, enriched in trimers, that retained all of the in vivo violaxanthin pool. It was estimated that each LHCII monomer can bind at least one violaxanthin. The extent to which different pigments can be removed from LHCII indicated that the relative strength of binding was chlorophyll b > neoxanthin > chlorophyll a > lutein > zeaxanthin > violaxanthin. The xanthophyll binding sites are of two types: internal sites binding lutein and peripheral sites binding neoxanthin and violaxanthin. In CP29, a minor LHCII, both a lutein site and the neoxanthin site can be occupied by violaxanthin. Upon activation of the violaxanthin de-epoxidase, the highest de-epoxidation state was found for the main LHCII component and the lowest for CP29, suggesting that only violaxanthin loosely bound to LHCII is available for de-epoxidation.

Xanthophylls are a class of carotenoids associated with the light harvesting complexes of plant chloroplast membranes (1). In most plants, there are three major xanthophylls, namely lutein, neoxanthin, and violaxanthin, the last of which can be reversibly de-epoxidized to antheraxanthin and zeaxanthin via the xanthophyll cycle (2). The reason for this diversity in xanthophyll composition is not entirely clear, although the conservation of xanthophyll composition across a range of plant species (3–5) indicates a specific role for each one. Although xanthophylls are bound to both LHCII* and LHCII, the nature of the binding has not been determined, and there are significant differences in the values reported for the numbers of pigments bound to particular complexes (6–11). In the structural model for the major LHCII component, LHCIIb, there are two carotenoid molecules that are presumed to be the two luteins that have been shown to be bound by this complex (12).

No other carotenoids were detected in this crystallographic study, despite the fact that there are either one or two other carotenoids present. For the other LHCII components, CP29, CP26, and CP24, there is even less certainty, with estimates of the number of bound carotenoids differing significantly (see reviews in Refs. 9 and 11).

In the case of the xanthophyll cycle carotenoids, establishing the stoichiometry of binding is of particular importance because this cycle plays a major role in controlling the efficiency of light harvesting (5, 13, 14): in light-limiting conditions, maximum efficiency of light harvesting is associated with the presence of violaxanthin, whereas de-epoxidation to zeaxanthin is correlated with down-regulation of light harvesting efficiency because of the nonradiative dissipation of excess energy. This latter process is important in the protection of the photosynthetic system from photodamage and is readily detected as the nonphotochemical quenching of chlorophyll fluorescence. For LHCIIb, the reported values for xanthophyll cycle carotenoid binding range from trace amounts (7) up to 1 per trimer (6). For CP29 and CP26, there are reports of between 0.65 (7, 9) and 1.5 (6) xanthophyll cycle carotenoids bound. This range of values gives rise to very different conclusions about the location of the xanthophyll cycle and its mode of action. With only trace amounts of xanthophyll cycle in LHCIIb, attention is focused on the minor complexes as the site of nonphotochemical quenching. The lowest estimates of the content of xanthophyll cycle carotenoids bound to these complexes lead to the suggestion that one of the internal luteins in the LHCIIb model is replaced by a violaxanthin (9). This internal location is consistent with a view that zeaxanthin, formed by de-epoxidation of this violaxanthin, directly quenches Chl excited states via Chl-car energy transfer (15, 16). Conversely, if one violaxanthin is bound per LHCIIb trimer, then at least 50% of the violaxanthin pool is associated with the main population of LHCII, bound at a site other than the lutein sites (8). Similarly, higher stoichiometries of binding to the minor complexes indicate that at least a proportion of the violaxanthin pool is not bound to the internal “lutein sites.” Such data are consistent with a peripheral location of violaxanthin and with the hypothesis that the xanthophylls cycle controls the structure of the LHCII system (13, 17, 18). Central to these arguments is also the question of which violaxanthin molecules can be de-epoxidized to zeaxanthin. In fact, the maximum de-epoxidation state of CP29 is rather low compared with LHCIIb (8), suggesting that peripheral violaxanthin is the preferred substrate for the violaxanthin de-epoxidase. A further question is whether violaxanthin and zeaxanthin are bound with equal strengths to LHCII; it has been suggested that only zeaxanthin (and antheraxanthin) can be associated with the complexes so as to initiate nonpho-
Xanthophyll Binding to LHCII

TABLE I

Pigment composition of LHCII, thylakoid membranes, and photosystem II BBY particles

| Sample       | %/MR Lut | Neo | Vio | Ant | β-Car | XC | Car/Chl | Chl a/b | Chl a | Chl b |
|--------------|----------|-----|-----|-----|------|----|---------|--------|-------|-------|
| LHCIIb       |          |     |     |     |      |    |         |        |       |       |
| MR           | 62.3 ± 0.7 | 32.2 ± 1.0 | 5.9 ± 0.3 | 0   | 0    | 5.9 ± 0.3 | 0.25 | 1.32 | 6.6 | 5.0 |
| CP26         | 39.2 ± 3.9 | 27.0 ± 3.1 | 31.0 ± 0.8 | 0   | 0    | 31.0 ± 0.8 | 0.27 | 2.50 | 7.5 | 3.0 |
| CP29         | 32.8 ± 2.1 | 20.8 ± 3.0 | 45.2 ± 1.6 | 0   | 0    | 45.2 ± 1.6 | 0.29 | 3.40 | 6.8 | 2.0 |
| PSHI (est)   | 34       | 17   | <7  | 10  | 0.9  | 1.2 | 0.29    | 3.10  | 4.0  | 6.0  | 2.0  |
| BBY          | 46.7 ± 1.8 | 21.1 ± 1.3 | 17.9 ± 1.8 | 1.7 ± 0.4 | 12.6 ± 2.7 | 19.6 ± 2.2 | 0.25 ± 0.02 | 2.05 ± 0.1 |
| Thylakoid    | 37.4 ± 2.6 | 14.7 ± 0.8 | 22.4 ± 1.4 | 1.2 ± 0.6 | 24.3 ± 1.9 | 22.4 ± 1.4 | 0.31 ± 0.01 | 3.60 ± 0.2 |
| A-band       | 49.7 ± 0.9 | 19.5 ± 2.1 | 24.1 ± 1.1 | 1.6 ± 1.1 | 5.1 ± 1.2 | 25.7 ± 1.1 | 0.33 ± 0.02 | 1.61 ± 0.15 |

In order to understand the way in which the xanthophyll cycle carotenoids, and the other xanthophylls, control the structure and function of LHCII, it is necessary to determine where and in what numbers these carotenoids are associated within the LHCII system. In this paper, we describe a systematic study of the binding stoichiometries of pigments to both the minor and major LHCII components and determine the relative binding strengths.

Experimental Procedures

Thylakoids and photosystem II BBY particles were prepared from spinach leaves as described previously (8). Light harvesting complexes of photosystem II were prepared from spinach by isoelectric focusing of either BBY particles or unstacked isolated thylakoids. The thylakoids were suspended in 0.33 M sorbitol, 1 mM EDTA, 50 mM HEPES, pH 7.6, and solubilized in 20 mM N-dodecyl β-D-maltoside (DM). To induce de-epoxidation of the xanthophyll cycle, spinach leaves were illuminated prior to isolation of BBY particles as described previously (8). Alternatively, in order to achieve the maximum de-epoxidation state, unstacked thylakoids were incubated in 0.33 M sorbitol, 1 mM EDTA, 30 mM HEPES, 20 mM MES, 40 mM ascorbate at pH 5.5 at 20 °C for 30 min (19). After incubation, thylakoids were diluted, centrifuged, and resuspended ready for solubilization and IEF, which was performed as described previously (8). Further purification of LHCII was carried out by sucrose density gradient centrifugation: sucrose gradients were seven step exponential gradients from 0.15 to 1.0 M sucrose dissolved in 20 mM HEPES buffer containing 20 μM DM. The run time was 18 h at 200,000 × g in a SW41 rotor at 4 °C. To remove pigments from LHCII, samples were incubated in different concentrations of detergent prior to centrifugation. Fractionation of thylakoids involved treatment of unstacked thylakoids with 10 mM DM with a ratio of DM/Chl of 8 on ice for 45 min, followed by centrifugation through a sucrose gradient. For fractionation of BBY particles the same procedure was used except that DM concentration was 8 mM (DM/Chl = 3).

The pigment composition of thylakoids, BBY particles, thylakoid subfractions, and LHCII samples was determined by reverse phase HPLC (4). SDS-polyacrylamide gel electrophoresis was used to determine the polypeptide composition of thylakoid membrane fractions. Absorption spectra were recorded using an Amino DW2000 spectrophotometer, and fluorescence emission spectra at 77 K were measured as described previously (20, 21).

RESULTS

Carotenoid Content of LHCII—Table I presents the carotenoid content of LHCIIb, CP29, and CP26 isolated by IEF of PSII membranes. Based on the known number of Chl b molecules bound to each complex (9) and the measured Chl a/b ratios, it was possible to determine the number of carotenoids bound. For LHCIIb, it was estimated that there were 2 molecules of lutein, 1 of neoxanthin, and a substoichiometric amount of violaxanthin (0.2 per monomer). CP26 bound over 1 molecule of lutein and 1 molecule each of neoxanthin and violaxanthin, whereas CP29 bound approximately 1 molecule of lutein, just over 1 of violaxanthin, and 0.6 of neoxanthin.

Using these values, and assuming that a PSII unit in the thylakoid membrane contains 1 molecule each of CP29, CP26, and CP24 and 5 trimers of LHCIIb, an estimate of the carotenoid composition of PSII can be made. These data predict that there is a maximum of 8 molecules of violaxanthin bound to PSII and that violaxanthin accounts for up to 10% of the total carotenoid pool. However, assay of the carotenoid content of the BBY PSII membranes used for the isolation of the complexes indicates that the content of violaxanthin was almost 20% of total carotenoid, a value consistent with measurements made for whole thylakoids. Thus, at least half of the xanthophyll cycle pool associated with PSII cannot be accounted for by the amounts bound to the purified complexes.

There are two explanations for this discrepancy: first, there could be a pool of violaxanthin that is not bound to LHCII but is either free in the lipid phase of the membrane or bound to another protein complex. Second, violaxanthin is only rather loosely bound to LHCII and is removed during solubilization of the BBY particles and complex purification. To distinguish between these hypotheses, the distribution of pigment in LHCII fractions and free pigment was determined using sucrose gradient centrifugation following solubilization of BBY PSII membranes with three different concentrations of detergent (Fig. 1). In all cases, the distribution of Chl was approximately the same: about 60% in the LHCIIb trimeric band, approximately 15% in the monomer band (mainly minor complexes CP26, CP29, and CP24), and the remaining 25% in PSII core complexes. Less than 5% of the Chl was found in the free pigment zone. However, in contrast to these results, the distribution of violaxanthin was very dependent on detergent concentration. At the lowest detergent concentration, nearly 80% of the violaxanthin was in the trimeric LHCIIb fraction, and approximately 5% was in the free pigment zone. At the highest detergent concentration, this distribution was reversed, with over 80% in the free pigment zone and less than 5% in the LHCIIb trimers. The distribution of violaxanthin in the monomer fraction was approximately 20% at each detergent concentration.

Carotenoid Composition of Oligomeric LHCII—These data showed that the xanthophyll cycle carotenoid not associated with purified LHCII was loosely associated with the complexes in the thylakoid membrane. Moreover, with low detergent concentrations, violaxanthin could be retained bound to solu-
Xanthophyll Binding to LHClI

Fig. 1. The distribution of pigment between PSII cores, LHClI trimers, LHClI monomers, and the free pigment zone following detergent solubilization of photosystem II BBY particles. Inset, diagrammatic representation of distribution of Chl in the centrifuge tube. Shown are Chl and violaxanthin (Viso) distribution following solubilization with 5 (8 mM), 8 (16 mM), and 14 (22 mM) molar ratio of DM to chlorophyll. Fp, free pigment zone; mon, monomeric LHClI; tr, trimeric LHClI; PSII, core complexes of PSII. Data are expressed as percentage of pigment loaded onto the gradient.

lized LHClI. Therefore, a different approach was taken to investigating the xanthophyll cycle content of the LHClI antenna: rather than isolating individual antenna complexes, gentle detergent treatment was used to try to remove an intact oligomeric LHClI light harvesting system containing the complete xanthophyll cycle pool. Treatment of either BBY particles or thylakoids with carefully established detergent concentrations was found to produce a series of Chl-containing bands on a sucrose gradient (Fig. 2). For BBY particles, there was less than 3% free Chl arising from this treatment, whereas there was none detectable with thylakoids. At 0.73 M sucrose, the band that contained 20–30% of the total Chl was referred to as the A-band.

The A-band had a Chl a/b ratio of 1.61 and a low content of β-carotene compared with BBY particles, suggesting a deficiency in the CP47/CP43/RC core (Table I). The carotenoid/Chl ratio was higher than either purified LHClI or PSII, and the violaxanthin content was 26% of total carotenoid. The absorption spectrum was very similar to a spectrum of LHClIb and the A-band. The fluorescence emission spectrum of the A-band showed the presence of the Lhcb1–2 polypeptides, and several bands in the 25–31-kDa region are present. The Lhcb1–3 polypeptides of LHClIb are shown, together with the polypeptides of CP26 and CP29. The trimer isolated from the A-band showed the presence of the Lhcb1–2 polypeptides, and these polypeptides were also detected in the monomer fraction. Densitometric estimates suggested that the Lhcb1–2 polypeptides accounted for more than 85% of the protein content in the A-band.

Binding Affinity of Carotenoid to LHClI—These data showed that a major fraction of violaxanthin, mostly bound to LHClIb, was easily lost upon detergent solubilization of either PSII membranes or oligomeric LHClI. Violaxanthin was clearly less tightly bound than Chl to this complex. Conversely, violaxanthin appeared to be more stably bound to monomeric LHClI. It was therefore decided to investigate in more depth the ease with which violaxanthin might be removed during detergent solubilization of LHClI, in comparison with the other bound pigments. Isolated LHClI fractions were incubated in different concentrations of DM and subjected to sucrose gradient centrifugation. The amounts of different pigments in the LHClI fractions and free pigment zones were then analyzed.

The results of this experiment using LHClIb are shown in Fig. 5. Treatment with a range of DM concentrations resulted in progressive loss of violaxanthin from this complex (Fig. 5A). At the highest concentration, less than 10% of the violaxanthin remained in the trimer band, with nearly 70% being released into the free pigment zone. DM treatment removed approximately 4.0% Chl, highly enriched in Chl a, but more of the carotenoid (the carotenoid/Chl ratio was 0.72 in the free pigment fraction). In comparison with the original IEF sample, the free pigment was depleted in neoxanthin and lutein but contained 20% violaxanthin (Fig. 5B). The carotenoids bound to the trimeric LHClIb band contained less than 1% violaxanthin. Ten percent of the Chl was found in a monomeric band, and it was of note that this complex had a violaxanthin content of approximately 20% and a lower content of neoxanthin compared with the trimer. It was estimated that the amounts of lutein and neoxanthin removed from LHClIb as free pigment by treatment with DM were only approximately 10 and 5%, respectively.
LHCBII trimers can be more efficiently broken into monomers by treatment with phospholipase (22). The carotenoid composition of monomers was again different from trimers (data not shown). In particular, again it was found that neoxanthin was 30% reduced, and the violaxanthin content was over 20 times higher than for trimeric LHCBII.

Broadly similar behavior was displayed by CP29 and CP26, although some important differences were found (Fig. 6). The free pigment zones again were enriched in carotenoid compared with the bands of purified LHCBII (in this case a monomeric band), and differed in carotenoid composition (Fig. 6A). The free pigment zone of CP26 was depleted in neoxanthin but...
enriched in both violaxanthin and lutein, compared with the initial IEF complex. Approximately 20% of Chl was removed by treatment with 0.2 mM DM, and this resulted in the loss of about 30% violaxanthin (Fig. 6C). There was negligible loss of either lutein or neoxanthin. Higher concentration of DM caused extensive loss of Chl from CP26, so that it was not possible to attempt to selectively remove any greater proportion of violaxanthin.

For CP29, the content of violaxanthin was almost the same in the IEF preparation, the free pigment zone, and the purified monomer, although there was some depletion in neoxanthin and enrichment in lutein in the free pigment zone (Fig. 6B). A 20% loss in violaxanthin was accompanied by an almost equivalent loss of Chl (Fig. 6D). As with CP26, higher concentrations of detergent removed Chl from the complex quite readily.

Using the data obtained from treatment of the complexes with DM, an index of relative binding strength was calculated for each of the pigment types: the pigment content in the initial IEF fraction (as a percentage of total pigment) divided by the pigment content in the free pigment zone. This ratio was normalized on the value for lutein, the one carotenoid for which a binding site within the complex has been structurally determined. The rationale is that if all pigments are bound equivalently, then the composition of the pigments released into the free pigment fraction would be identical to the initial composition of the complex. For the LHClIb trimer, neoxanthin was held as tightly as Chl b (Table II). This affinity was 5 times higher than lutein, which was in turn 5 times more strongly bound than violaxanthin, which was revealed to be the pigment most weakly associated with the trimeric complex. For LHClIb monomers, lutein, neoxanthin, and violaxanthin are bound with approximately equal efficiency, but less than Chl a and Chl b. For the minor complexes, neoxanthin was again the most tightly bound carotenoid, but the differences between the three carotenoids was much less. For CP26, violaxanthin and lutein were bound with equal strength, as for LHClIb monomers. Violaxanthin was bound more efficiently than lutein to CP29.

A similar analysis to that described above was carried out for LHClI in which violaxanthin had been partially de-epoxidized to zeaxanthin. As shown previously (8), the DEPS (de-epoxidation state of the xanthophyll cycle carotenoid pool (zeaxanthin + \(\frac{1}{2}\)antheroxanthin)/(violaxanthin + antheroxanthin + zeaxanthin)) varied among the different LHClI components (Table III). For LHClIb, the DEPS was around 40%, close to the overall value obtained for the PSII BYB particles in these experiments. After treatment with DM, the trimer band exhibited a DEPS of over 80%, but the monomer was less than 20%. These differences are caused by the depletion of violaxanthin from the trimer but the retention of zeaxanthin, and the converse for monomers. For CP26, the DEPS was almost the same as for LHClIb, whereas for CP29 it was the lowest, at only 15%. In order to increase DEPS to the maximum level, thylakoids were incubated at low pH in the presence of ascorbate (19). Here, an overall DEPS of 75–80% was observed. For CP29 isolated from these thylakoids by IEF, the DEPS was 52%.

Analysis of the A-band prepared from thylakoids with a high DEPS (70–80%) was carried out (Table III). The xanthophyll cycle carotenoid accounted for 25% of the total carotenoid, and the DEPS was 88%. The LHClIb trimer fraction prepared from the A-band was found to have a DEPS of 100% and the monomer fraction 79%. The difference between the monomer and the trimer appeared to result from the loss of violaxanthin from the latter, resulting in a lower xanthophyll cycle content (15%), compared with 21% in the monomer.

The relative binding strengths of zeaxanthin to different LHClI components is shown in Table II. For LHClIb trimers, it was found that zeaxanthin was significantly more tightly bound than violaxanthin. For LHClIb monomers, zeaxanthin and violaxanthin were both bound quite strongly, with zeaxanthin having somewhat less affinity. Zeaxanthin was also less tightly bound than violaxanthin to CP29. The violaxanthin remaining bound to CP29 after de-epoxidation was more
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The relative binding efficiency of pigments to each complex was determined as the ratio of the amount of carotenoid in the complex divided by the amount in the free pigment. DEP refers to whether the complex was prepared from de-epoxidised (+) or epoxidised (−) thylakoids. CP29 (thy) represents CP29 prepared from thylakoids treated to induce maximum de-epoxidation. Lut, lutein; neo, neoxanthin; vio, violaxanthin; zea, zeaxanthin.

| Sample         | DEP | Lut | Neo | Vio  | Zea  | Chl b | Chl a |
|----------------|-----|-----|-----|------|------|-------|-------|
| LHCIIb trimer  | −   | 1.0 | 5.4 ± 2.0 | 0.2 ± 0.01 | −   | 5.1 ± 0.3 | 2.2 ± 0.3 |
| +             | 1.0 | 4.3 ± 0.7 | 0.2 ± 0.02 | 0.7 ± 0.1 | 5.9 ± 0.5 | 2.4 ± 0.4 |
| LHCIIb monomer| −   | 1.0 | 1.0 ± 0.2 | 0.7 ± 0.1 | −   | 3.3 ± 1.1 | 2.2 ± 0.3 |
| +             | 1.0 | 0.7 ± 0.2 | 1.0 ± 0.2 | 0.7 ± 0.1 | 4.0 ± 1.2 | 2.7 ± 0.4 |
| CP26          | −   | 1.0 | 2.2 ± 0.1 | 1.1 ± 0.2 | −   | 4.5 ± 0.3 | 2.6 ± 0.3 |
| CP29          | −   | 1.0 | 2.6 ± 0.2 | 1.4 ± 0.1 | −   | 2.1 ± 0.2 | 2.1 ± 0.2 |
| +             | 1.0 | 2.7 ± 0.3 | 1.6 ± 0.1 | 1.1 ± 0.1 | 1.7 ± 0.4 | 1.8 ± 0.5 |
| CP29 (thy)    | −   | 1.0 | 1.9 ± 0.3 | 1.0 ± 0.2 | −   | 1.9 ± 0.3 | 2.4 ± 0.4 |
| +             | 1.0 | 2.4 ± 0.2 | 3.2 ± 0.4 | 0.5 ± 0.1 | 2.6 ± 0.3 | 1.9 ± 0.2 |

—, nondetectable.

Table II

Carotenoid composition of LHCII following induction of violaxanthin de-epoxidation

Complexes were prepared from de-epoxidised thylakoids prepared from light-treated leaves, except for CP29 (thy), for which the thylakoids were treated in vitro to induce maximum de-epoxidation state. DEPS is the de-epoxidation state \((zca^{1}ant)/(viol + ant + zea)\). For LHCIIb, data are from the IEF-prepared trimer, the trimer obtained by detergent treatment and subsequent sucrose gradient centrifugation as in Fig. 5, and the trimer derived from the A-band as in Fig. 2. Carotenoid values are the percentage of total carotenoid. Lut, lutein; neo, neoxanthin; vio, violaxanthin; ant, antheraxanthin; zea, zeaxanthin; XC, vio + ant + zea.

| Sample   | Lut   | Neo   | Vio  | Ant | Zea  | XC   | DEPS |
|----------|-------|-------|------|-----|------|------|------|
| LHCIIb IEF | 64.0 ± 2.2 | 30.1 ± 3.1 | 3.3 ± 1.1 | 0.2 ± 0.2 | 2.4 ± 0.8 | 6.5 ± 0.7 | 43.1 ± 6.1 |
| LHCIIb trimer | 54.3 ± 3.5 | 31.0 ± 3.3 | 1.5 ± 1.5 | 0   | 4.0 ± 0.6 | 5.9 ± 2.0 | 81.0 ± 15.0 |
| LHCIIb monomer | 65.0 ± 2.1 | 16.1 ± 3.0 | 15.5 ± 4.2 | 0   | 3.6 ± 0.3 | 29.3 ± 4.2 | 16.0 ± 3.0 |
| CP26 IEF | 38.3 ± 1.5 | 33.3 ± 3.5 | 17.7 ± 1.1 | 0   | 11.0 ± 1.2 | 28.7 ± 1.0 | 38.2 ± 4.3 |
| CP29 IEF | 39.3 ± 2.2 | 22.8 ± 2.8 | 32.0 ± 1.0 | 0   | 5.6 ± 0.6 | 37.6 ± 1.1 | 14.9 ± 2.4 |
| CP29 (thy) | 38.0 ± 0.6 | 25.0 ± 0.8 | 18.0 ± 1.2 | 0   | 17.6 ± 1.5 | 36.0 ± 1.8 | 52.0 ± 2.1 |
| A-band  | 52.2 ± 0.3 | 19.7 ± 0.4 | 4.3 ± 0.5 | 0.8 ± 0.4 | 19.8 ± 0.5 | 24.9 ± 0.6 | 81.1 ± 2.0 |
| LHCIIb (A) | 62.6 ± 2.0 | 20.9 | 0 | 0 | 15.8 | 15.5 | 100 |

* Single measurement.

Discussion

The Oligomeric A-Band—Most of the xanthophyll cycle pigment associated with PSII was found in an oligomeric LHCII fraction (the A-band). This fraction is larger than previously reported oligomeric LHCII preparations (23) and is different from PSII supercomplexes (24, 25), being deficient in PSII core proteins. The Chl a/b ratio indicates that 80% of the Chl is bound to LHCIIb, in agreement with the observations that 64% of the Chl was in trimeric LHCIIb, and approximately half of the monomeric band was also LHCIIb, the remainder being a mixture of CP29 and CP26. We estimate the A-band to be approximately 600 kDa, based on the estimates for other bands on the sucrose gradient (LHCII trimer, 100 kDa; PSII monomer, 236 kDa; PSII dimer, 430 kDa; PSI monomer, 500 kDa). This is consistent with its containing 5 LHCIIb trimers and 2–3 monomers of minor LHCII. The fluorescence yield was less than for LHCIIb trimers; the broadening of the fluorescence spectrum and increase in the relative contribution of the vibronic satellite band suggests a change in Chl environment within the oligomer that gives rise to fluorescence quenching (20, 26).

The Xanthophyll Cycle Content of LHCII—The xanthophyll cycle carotenoids were less tightly bound to LHCII than were Chl, lutein, and neoxanthin. The binding affinities of violaxanthin and zeaxanthin also differed. Differences in carotenoid binding strength are predicted because they have different end-group orientation and polarity (18). Gentle isolation procedures were needed to determine the amounts of these pigments bound to LHCII, and the weakness of binding means that it is impossible to exclude the possible distortion of results arising from the migration of pigments between binding sites during the solubilization procedure. The combination of the observed binding stoichiometries in different fractions and the efficiencies of binding strongly suggests that some previously published results have underestimated the number of xanthophyll cycle carotenoids bound to these complexes in vivo. In Fig. 7, the ratio of violaxanthin to neoxanthin is presented for the range of LHCIIb preparations. Because neoxanthin is so tightly bound to LHCIIb, with 1 molecule present per complex, this ratio provides a good measure of the binding stoichiometry. In purified LHCIIb, and samples obtained at higher DM concentrations, this ratio is less than 0.5. However, more gentle treatments lead to a higher ratio, with the maximum found for the A-band of just over 1; most significantly, the trimer purified from the A-band has a value between 0.9 and 1.0.

It is necessary to distinguish between tightly and loosely bound xanthophyll cycle carotenoid. For LHCIIb, there is no tightly bound violaxanthin, and almost all of the violaxanthin can be removed from this complex, explaining earlier results that there was no violaxanthin binding (7). The data in Fig. 7 suggest that there are 3 loosely bound violaxanthin molecules per trimer. For CP26, the binding strength of violaxanthin is rather similar to the value for monomeric LHCIIb; therefore, we suggest that CP26 does not contain a tightly bound violaxanthin but 1 molecule of loosely bound pigment. In contrast, CP29 has a violaxanthin content that is significantly higher than either lutein or neoxanthin. There is clearly a population of violaxanthin tightly bound to the complex, but in addition, there is an additional loosely bound violaxanthin. The much higher binding strength of the violaxanthin remaining after maximum de-epoxidation compared with the value obtained for the fully epoxidized sample is evidence for the existence of at least two binding sites, with only the violaxanthin at the loose
site available for de-epoxidation.

Therefore, CP26 and CP29 might together bind between 2 and 3 xanthophyll cycle carotenoids. If a similar stoichiometry is assumed for CP24 (9), then the minor LHCII will account for a maximum of 3–4 xanthophyll cycle carotenoids. The upper limit to that associated with LHCIIb would appear to be 3.0 per trimer. Assuming approximately 4–5 trimers per PSI unit, this would give another 12–15 xanthophyll cycle carotenoids. These data then predict that the total number associated with the PSII antenna would be 15–19 molecules per reaction center. The measured carotenoid composition of BBY particles suggested a value of approximately 15 molecules (Table I; see also Ref. 27), all of which are recovered in the A-band.

One factor that must be taken into account when considering the stoichiometry of xanthophyll binding to LHCII is that the carotenoid pool sizes are variable across different plant species and within the same species depending on growth conditions (4, 5). In spinach, the xanthophyll cycle pool size is approximately 22% of total carotenoid, whereas this may be as high as 30–35% in high light-grown plants and as low as 15% in shade plants. The data presented in this paper indicate that there may be an upper limit of 20 violaxanthin molecules associated with PSI. This suggests that under light-limited conditions, not all of the available xanthophyll cycle binding sites on LHCII are occupied. Furthermore, observations made on reconstitution of LHCII in vitro (28–31), as well as on mutants lacking particular carotenoids (32, 33), indicate that the carotenoid binding sites are not entirely specific and that variations in carotenoid composition may result from changes in the occupancy of these sites.

Multiple Carotenoid Binding Sites in LHCII—To explain the data presented here, we suggest that each LHCII monomer has four distinct carotenoid binding sites (Table IV). The structural model for LHCIIb identifies two carotenoids bound within the complex, and these are most likely two luteins (12). These sites are identified as L1 and L2. The location of the neoxanthin binding site (N) is not known, but the fact that it is a particularly tight binding site in LHCII trimers, but not in LHCIIb monomers, suggests it faces the interior of the trimeric structure. Although some caution should be applied in the application of the LHCIIb structural model to the minor complexes CP29 and CP26 (9), it is more than likely that the bound lutein occupies the same site in all three complexes. For both minor complexes, there is probably only one lutein site occupied. In CP29, the other site appears to be occupied by violaxanthin. This is essentially in agreement with the observations made on reconstituted CP29, although in this work, it was suggested that this site could be occupied by either violaxanthin or neoxanthin (9). Given that there is clearly an additional neoxanthin-specific site on LHCIIb, this seems unlikely. Therefore, we propose that CP29 also has an N site, which can be occupied by either neoxanthin or violaxanthin in order to explain the stoichiometric binding of neoxanthin. For CP26, it is less clear which, if any, carotenoid replaces the second lutein. We suggest that violaxanthin does not occupy this internal tight site, which may therefore remain vacant, and that CP26 has an N site that is occupied only by neoxanthin.

The loose binding site for violaxanthin (V) is most likely on the periphery of the complex, probably in equilibrium with the lipid phase. Violaxanthin is removed at low detergent concentration and appears to be lost during the previously published methods of LHCIIb purification. The empirically determined binding affinity of this site is 1 order of magnitude lower than Chl, lutein, or neoxanthin. When LHCIIb is monomerized, the affinity of this site increases significantly. Thus, the violaxanthin bound to CP26 is most likely bound to the V site, with an affinity equivalent to this site on LHCIIb monomers. For CP29, the binding stoichiometry of greater than 1 for violaxanthin suggests that there is a second site on this complex; because this binding is estimated to be 0.4 molecules or less, we suggest that this is bound to the N site.

Availability of Violaxanthin for De-epoxidation—Violaxanthin de-epoxidase is bound to the lumen surface of the thylakoid membrane (34). Recent studies suggest that violaxanthin has to be removed from the complex, inserted into the active site of the enzyme, de-epoxidized to antheraxanthin, released, and then de-epoxidized at the second end-group to form zeaxanthin (35). It is unlikely that this occurs with a carotenoid tightly bound to the L1/L2 site on the interior of the complex. Again, this indicates that the violaxanthin bound to the L2 site in CP29 is not available for de-epoxidation. In contrast to CP29, CP26 exhibits a high DEPS. It is therefore unlikely that violaxanthin in this complex is bound at the L1/L2 site. In studies of mutants lacking lutein, violaxanthin appears to replace the missing carotenoid, presumably becoming bound to the L1/L2 sites (32); this violaxanthin does not appear to be available for de-epoxidation. The conclusion that the violaxanthin involved in the xanthophyll cycle, and therefore in the control of nonphotochemical quenching, is peripheral is also consistent with the observed effects of endogenous xanthophyll cycle carotenoids on the structure and function of LHCIIb, CP29, and CP26 (17, 18, 21, 36).

The available violaxanthin appears to be associated with V sites on LHCII, and the majority of these are on LHCIIb. Under conditions leading to de-epoxidation, the higher affinity of the trimer V site for zeaxanthin relative to violaxanthin, not found for the monomeric complexes, predicts a higher relative concentration of de-epoxidized pigment in LHCIIb. It has been suggested that only a small fraction of the zeaxanthin pool may be needed for nonphotochemical quenching and that this is
associated only with the minor complexes (14). Although this idea cannot be ruled out by the present data, the association of the majority of the zeaxanthin pool with LHCIIb suggests that the site of action of the xanthophyll cycle in controlling non-photochemical quenching is not confined to the minor complexes but is a property of the whole antenna, including LHCIIb.

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