The Binding of Xanthophylls to the Bulk Light-harvesting Complex of Photosystem II of Higher Plants

A SPECIFIC REQUIREMENT FOR CAROTENOIDS WITH A 3-HYDROXY-β-END GROUP*

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The pigment composition of the light-harvesting complexes (LHCs) of higher plants is highly conserved. The bulk complex (LHCIIb) binds three xanthophyll molecules in combination with chlorophyll (Chl) α and β. The structural requirements for binding xanthophylls to LHCIIB have been examined using an in vitro reconstitution procedure. Reassembly of the monomeric recombinant LHCIIB was performed using a wide range of native and nonnative xanthophylls, and a specific requirement for the presence of a hydroxy group on C-3 on a single β-end group was identified. The presence of additional substituents (e.g. at C-4) did not interfere with xanthophyll binding, but they could not, on their own, support reassembly. cis isomers of zeaxanthin, violaxanthin, and lutein were not bound, whereas all-trans-zeaxanthin and different chiral forms of lutein and zeaxanthin were incorporated into the complex. The C-3 and C-3′ diols lactucaxanthin (a carotenoid native to many plant LHCs) and eschscholtzaxanthin (a retro-carotenoid) both behaved very differently from lutein and zeaxanthin in that they would not support complex reassembly when used alone. Lactucaxanthin could, however, be bound when lutein was also present, and it showed a high affinity for xanthophyll binding site N1. In the presence of lutein, lactucaxanthin was readily bound to at least one lutein-binding site, suggesting that the ability to bind to the complex and initiate protein folding may be dependent on different structural features of the carotenoid molecule. The importance of carotenoid end group structure and ring-to-chain conformation around the C-6–C-7 torsion angle of the carotenoid molecule in binding and complex reassembly is discussed.

In contrast to the algae and photosynthetic bacteria, the carotenoid composition of higher plant photosynthetic tissues is highly conserved (1). Under conditions of low irradiance, three xanthophylls, namely lutein ((3R,3′R)-β,ε-carotene-3,3′-diol), violaxanthin ((3S,5R,6S,3′S,5′R,6′S)-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol), and 9′-cis neoxanthin ((3S,5R,6R,3′S,5′R,6′S)-6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-triol) together with the carotene, β-carotene (β,β-carotene), are typically found. In addition, the leaves of some species may contain small amounts of one or more of the following carotenoids: lutein-5,6-epoxide (5,6-epoxy-5,6-dihydro-β,ε-carotene-3,3′-diol), lactucaxanthin ((3S,3′S,6S,6′S)-ε,ε-carotene-3,3′-diol), and α-carotene (β,ε-carotene). The xanthophyll composition may alter in the short term when tissues are exposed to high irradiances by the de-epoxidation of violaxanthin into zeaxanthin ((3R,3′R)-β,β-carotene-3,3′-diol) and antheraxanthin (5,6-epoxy-5,6-dihydro-β,β-carotene-3,3′-diol) (the xanthophyll cycle; see Ref. 2 for a recent review). Changes in the carotenoid composition may also be observed over the long term, again in response to growth irradiance (sun/shade response). This may alter the xanthophyll cycle pool size but may also bring about alterations to the relative amounts of some carotenoids (e.g. the ratios of α-carotene/β-carotene or lactucaxanthin/lutein), perhaps simply reflecting changes to the pigment antenna bed and reaction center (so-called “regulation of light harvesting”) (3) and/or changes to the biosynthetic flux to form the β,β- or ε,ε-carotenoids.

The precise location of these pigments within the photosynthetic apparatus of higher plants has been determined by a number of studies in recent years (e.g. Refs. 4–7). The xanthophylls are associated with the various chlorophyll (Chl) α/β light-harvesting complexes of photosystems I and II (LHCl and LHCI, respectively). The major light-harvesting complex of photosystem II, LHCIIB, contains the bulk of chlorophyll and xanthophylls. The three-dimensional structure of this complex, determined at a resolution of 3.4 Å, revealed the presence of two xanthophylls occupying a central position in the complex (8). These two molecules were both tentatively identified as lutein (on the basis of the pigment stoichiometry of the complex) and have been assigned to binding sites L1 and L2 in the complex. It is, however, generally accepted that this particular pigment-protein complex has four binding sites (L1, L2, N1, and V1), one of which (V1) apparently binds its pigment rather loosely in vitro (7) and may in fact only be occupied in vivo in plants maintained at high irradiances (9). The precise location of N1 is not known but is thought to be in a domain located between helix C and the A and B cross-helix (10). In addition,

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1The abbreviations used are: Chl, chlorophyll; HPLC, high-performance liquid chromatography; LHC, light-harvesting complex; LHCIIB, major light-harvesting complex of photosystem II of higher plants.
the fourth (xanthophyll cycle) binding site may not always be fully occupied, especially in conditions of low irradiance (9). The apparent strength of binding of these xanthophylls to native LHC has been determined (7). The pigments show affinity to the native (trimeric) complex in the order Chl b > neoxanthin > Chl a > lutein > zeaxanthin > violaxanthin. This affinity can alter upon monomerization of the complex (e.g. neoxanthin is tightly bound to trimeric LHCIIb but not to monomers of the complex, suggesting that it occupies a site in the interior of the trimeric array). In contrast, violaxanthin appears to be loosely bound to LHCIIb, suggesting a location (V1) on the periphery of the complex. The relative affinity of violaxanthin is increased upon monomerization of the complex.

In vitro reconstitution has proved to be a powerful tool in the study of pigment-protein complexes in higher plants (11, 12), algae (13), bacteria (14), and crustaceans (15). The technique was first employed in the study of pigment binding in LHCIIb by Plumley and Schmidt (11). Reconstitution was achieved using denatured thylakoid polypeptides from dark-adapted spinach and a pigment mixture (also extracted from spinach) containing Chls a and b and native xanthophylls (i.e. lutein, neoxanthin, and violaxanthin). Paulsen et al. (12) overexpressed the 28-kDa polypeptide from pea in Escherichia coli, enabling the production of large amounts of homogenous protein. Initial experiments demonstrated that maximum yields of reconstituted complex were obtained using a mixture containing native xanthophylls and Chls a and b. Complexes were also successfully produced using Chls a and b in combination with only two of the three native xanthophylls and also using lutein as the only xanthophyll molecule. Yields of these complexes, however, were poor, and the complexes were less stable than the complex assembled using the full native complement of pigments. These studies indicated that lutein was an essential component for the assembly of LHCIIb. More recent evidence suggests that, in vivo, there is some plasticity with regard to the specificity of xanthophyll binding and that some plants deficient in lutein are indeed viable (16).

In vitro, some specificity for the preferential binding of lutein to reconstituted LHCIIb has been shown (10, 17).

The aim of the present investigation was to determine which structural features of the xanthophyll molecule facilitated reassembly of LHCIIb by Plumley and Schmidt (11). Reconstitution was achieved using denatured thylakoid polypeptides from dark-adapted spinach and a pigment mixture (also extracted from spinach) containing Chls a and b and native xanthophylls (i.e. lutein, neoxanthin, and violaxanthin). Paulsen et al. (12) overexpressed the 28-kDa polypeptide from pea in Escherichia coli, enabling the production of large amounts of homogenous protein. Initial experiments demonstrated that maximum yields of reconstituted complex were obtained using a mixture containing native xanthophylls and Chls a and b. Complexes were also successfully produced using Chls a and b in combination with only two of the three native xanthophylls and also using lutein as the only xanthophyll molecule. Yields of these complexes, however, were poor, and the complexes were less stable than the complex assembled using the full native complement of pigments. These studies indicated that lutein was an essential component for the assembly of LHCIIb. More recent evidence suggests that, in vivo, there is some plasticity with regard to the specificity of xanthophyll binding and that some plants deficient in lutein are indeed viable (16). In vitro, some specificity for the preferential binding of lutein to reconstituted LHCIIb has been shown (10, 17).

EXPERIMENTAL PROCEDURES

Overexpression of pLHCP in E. coli—Plasmids were constructed using standard techniques as previously described (12). The bacterial host was E. coli strain JM101 (Stratagene, Heidelberg, Germany).

Isolation of Overexpressed LHCIIb from Bacteria—The method used was that of Paulsen et al. (12). Routinely, bacteria that had been transformed to accumulate LHCIIb from pea were grown at 37 °C in 100-ml flasks until the culture density gave an A665 of 0.4–0.5. Bacteria were cultured in Luria-Bertani medium: 1% (w/v) Bactotryptone, 0.5% (w/v) NaCl (Merck), and 1% (w/v) Bactopeptone (Oxoid, Basingstoke, UK), 0.1% (w/v) Bactoyeast (Oxoid, Basingstoke, UK), and 1% (w/v) NaCl (Merck) to which ampicillin (50 μg/ml) was added. Expression of LHCP IIb was induced by the addition of isopropyl-β-D-thiogalactopyranoside (1 mM). After a further 4 h at 37 °C, the cell mass was cooled to 0 °C and centrifuged at 5000 × g for 5 min. Isolation of LHCIIb was carried out as detailed by Paulsen et al. (12), except that Igepal CA-630 (ICN Biomedicals) was substituted for Nonidet P-40. Unless stated otherwise, all chemicals were purchased from Sigma.

Isolation of Pigments—Xanthophylls were isolated from a range of natural sources. Prior to use, they were purified using a combination of TLC (silica and MgO), column chromatography, and HPLC. Final concentrations were quantified by UV-visible spectroscopy (HP8453; Agilent Technologies, Stockport, UK) using the appropriate extinction coefficients. Chls a and b were purchased from Sigma. cis isomers of lutein violaxanthin and zeaxanthin and chiral-isomers of lutein and zeaxanthin were isolated using a combination of TLC, column chromatography, and HPLC. Identification was made on the basis of Rf values together with UV-visible spectral characteristics in comparison with authentic standards. Unless otherwise stated, all carotenoids are all-trans. Structures of the compounds used in this study are given in Figs. 1 (carotenoids native to plant LHC) and 2 (nonnative carotenoids).

I. Binding of Xanthophylls to LHC

II. In Vitro Reconstitution of Pigment-Protein Complexes

—The low stringency method of Paulsen et al. (18) was used unless otherwise stated. LHCIIb (4 μg) was solubilized in buffer composed of 100 mM Tris-HCl (pH 11), 5 mM 6-aminoacaproic acid, 1 mM benzamidine, 12.5% (w/v) sucrose, and 2% (w/v) lithium dodecyl sulfate. The mixture was heated to 100 °C for 5 min. After cooling, diiothreitol was added to a final concentration of 1% (w/v) with vigorous mixing. The solution was allowed to stand for a minimum of 5 min. The majority of the dodecyl sulfate was precipitated by centrifugation (10,000 × g, 3 min), and the supernatant was retained. The reconstituted complex was isolated and purified by carrying out nondenaturing PAGE on the supernatant (see below).

The high stringency protocol of Plumley and Schmidt (11) was also performed as follows. Isolated LHCIIb polypeptides or delipidated thylakoid polypeptides were resuspended in solubilization buffer (polyethylene glycol concentration and solubilization composition was the same as used in the low stringency protocol detailed above). Following heating at 100 °C for 5 min and subsequent cooling, pigments (in ethanol) were introduced to the solubilized protein (see above for concentrations). Reconstitution was achieved by freezing the mixture at −20 °C. A series of three freeze-thaw cycles (6–12 h of freezing followed by 15 min of thawing) was employed to complete the reconstitution process. Reconstituted complexes were isolated as detailed below.

Partially Denaturing PAGE and SDS-PAGE—Partially denaturing PAGE was carried out at 4 °C using discontinuous gels and the buffer system of Laemmli (in which sodium dodecyl sulfate replaced SDS in the electrode buffer) as previously described (19). Electrophoresis was carried out using a Sigma midget vertical gel system with water cooling. Gels were run at 100 V until the free pigment band had migrated ~7 cm into the resolving gel.

Following partially denaturing PAGE, pigmented complexes were cut from the gel using a scalpel. The complex was electrodialuted from the gel using the Blue Tank (Isco). The electrophoresis tank contained buffer with the same composition as that used during partially denaturing PAGE. The nanotraps contained the same buffer that had undergone a 10-fold dilution. Electroelution was carried out for 20–30 min with a current of 2 mA/trap. When electrophoresis was complete, room temperature absorption spectra were obtained using an Agilent Technologies 8453 spectrophotometer (Stockport, UK).

Fully denaturing PAGE was carried out as previously described (12). Green bands were excised from partially denaturing gels and heated to 100 °C for 2 min. The band was then placed in the gel slot of an SDS-polyacrylamide gel. SDS replaced lithium dodecyl sulfate in the electrode buffer.

Preparation of Delipidated Thylakoids—Thylakoids were prepared from spinach by the method of Berthold et al. (20). Acetone delipidation of the thylakoids was carried out as described by Plumley and Schmidt (11). Reconstitution of solubilized delipidated thylakoids was carried out using the detergent exchange technique described above.

HPLC of Pigments in Complexes—Where more than one xanthophyll was used in the reconstitution mixture, HPLC analysis of the pigment content of the complex was carried out to determine the ratio of bound xanthophylls. Pigments were extracted from gel slices using the method of Martinson and Plumley (21). Pigments in the complex were separated using an HPLC system (Spherisorb ODS2 column; 250 × 4.6 mm, Phenomenex, Macclesfield, UK) with an Agilent Technologies HP1040 diode array detector and Gynkotek 480 pump (Dionex Ltd., Leeds, UK). The solvent system used was as described in Ref. 6.

Estimation of Chls a and b and Xanthophylls—Pigment-protein complexes were excised from gel slices following partially denaturing PAGE. Pigments were carefully extracted using acetone/ethanol (1:1, v/v), partitioned into diethyl ether, and washed several times with
water. Following extraction the solvent was evaporated using oxygen-free nitrogen. Samples were resuspended in acetone and quantitative analysis of Chls \( a \) and \( b \) and xanthophylls was carried out using the method of Lichtenthaler and Wellburn (22). The yield of reassembled complex was calculated as a percentage of the recovery of total bound chlorophyll in the reassembled complex as a function of the level obtained in the complex reassembled with native xanthophylls.

RESULTS

Binding of Native Xanthophylls—A comparison of the procedures for the in vitro reconstitution of the 28-kDa polypeptide from pea overexpressed in \( E. \ coli \) and the high stringency procedure utilizing solubilized spinach thylakoid polypeptides was initially performed. A 1:1 ratio of Chls \( a \) and \( b \) (50 \( \mu \)g) and a mixture of native xanthophylls (1.7 \( \mu \)g of lutein, 0.9 \( \mu \)g of 9'-cis neoxanthin, 1.4 \( \mu \)g of violaxanthin) were used, giving a Chl/xanthophyll ratio of 12.5:1. In the present study, the resulting stoichiometry of the reassembled complex using all three procedures was comparable with ratios of Chl \( a/b \) in the range 1.11–1.18:1 and a ratio of Chl/xanthophyll of 4:1 (Table I). These are very close to that of native spinach LHCIIb (pu-
rified by isoelectric focusing) in which the ratio of Chl a/b was 1.22:1 and the ratio of Chl/xanthophyll was 4.1:1. The room temperature absorption spectra of the complex reconstituted using a mixture of xanthophylls by the low stringency technique is almost identical to that seen for native LHCIIb (Fig. 3). Hobe et al. (17) have previously demonstrated the use of the low stringency procedure to reassemble LHCIIb with single xanthophylls, including relatively unstable Chl a/b complexes containing lutein, zeaxanthin, violaxanthin, or 9'-cis-neoxanthin. This particular method was chosen for the remainder of this study, since it permitted a systematic analysis of the structural requirements for xanthophyll binding employing a range of carotenoids, many of which resulted in the formation of relatively unstable pigment-protein complexes.

With the exception of lactucaxanthin (see below), all of the xanthophylls native to the photosynthetic tissues of higher plants were able to support reassembly of LHCIIb when used alone with Chls a and b, using the low stringency procedure. Yields of the reassembled complex were, however, always lower than that achieved for the mixture of native xanthophylls (see above) at ~60% for lutein, zeaxanthin, and antheraxanthin and dropping to only 40–45% for violaxanthin and 9'-cis-neoxanthin (Table II). The relatively poor efficacy demonstrated by violaxanthin and 9'-cis-neoxanthin was reflected in a reduction in the ratio of Chl a/b, especially with 9'-cis-neoxanthin, as previously reported (17). However, other low yielding complexes (e.g. with capsanthin; Table III) had a normal ratio for Chl a/b, so that efficiencies of complex reassembly and Chl stoichiometry are not linked. For carotenoids other than lutein and zeaxanthin, the xanthophyll stoichiometry was generally in the range of 2.3–2.7 per monomer, possibly reflecting partial filling of one binding site (probably N1 (21)).

The room temperature difference absorption spectra for selected single xanthophyll Chl a/b complexes are shown in Fig. 4, A and B. The spectra for the lutein complex in particular is almost identical to that of the complex reassembled with a mixture of native xanthophylls. The difference spectra (native LHC minus reassembled complex) reveals only very small changes in intensity of absorption in the Soret region (Fig. 4A). In contrast, the difference spectra for complexes reassembled with other native

Fig. 2. Structures of nonnative all-trans-xanthophylls used in the reconstitution of LHCIIb. All xanthophylls supported reassembly of LHCIIb unless otherwise indicated. *, failed to support complex reassembly.

| Procedure (Ref.) | Ratio of Chl a/b | Xanthophyll stoichiometry |
|------------------|-----------------|---------------------------|
|                  |                 | Lutein | Neoxanthin | Violaxanthin |
| LHCIIb (10)      | 1.11:1 ± 0.05   | 1.9    | 0.9        | 0.2          |
| Low stringency LHCIIb (12) | 1.18:1 ± 0.06 | 1.8    | 1.0        | 0.2          |
| High stringency thylakoids (11) | 1.16:1 ± 0.05 | 1.8    | 1.0        | 0.2          |
xanthophylls reveal more marked changes in their UV-visible spectra (Fig. 4, A and B). The spectrum of the complex reconstituted with violaxanthin (Fig. 4A) shows marked changes in the Qy transition region at 650 (decrease), 661 (increase), and 678 nm (decrease). The spectra from the zeaxanthin, 9-cis neoxanthin (both Fig. 4B), and antheraxanthin (Fig. 4A) complexes are similar, with marked changes at 648 (decrease), 662 (increase), and 682 nm (decrease). These spectra also reveal changes in the Soret region of the spectrum that are more profound than that seen in either the native or the lutein-Chl a/b complex, with decreases in absorption at 470 nm (violaxanthin) and 487–488 nm (zeaxanthin, antheraxanthin, and 9′-cis-neoxanthin).

The epoxide group at C-5–C-6 appears to be an important structural feature of the carotenoid molecule, since the furanoid derivatives (produced by acid treatment of isolated xanthophylls) of violaxanthin and antheraxanthin (namely auroraxanthin (3,3′-dihydroxy-5,8,5′,8′-diepoxo-β-carotene) and mutatoxanthin (3,3′-dihydroxy-5,8-epoxy-β-carotene), respectively), in which the epoxide group is rearranged to C-5–6-dihydroxy-5,8,5′-dioxanthin (3,3′-R,S-diols), and cucurbitaxanthin B (3,3′,S,R-diols) supported a low yield of reassembly of the complex (see below).

The diol lactucaxanthin, which is common in the LHC of a number of plants (e.g. lettuce (6)) but not pea from which the apoprotein was cloned, would not support complex assembly when used as the only xanthophyll. In addition, it would not support reassembly of the complex (albeit with low efficiency, ~40%). The all-trans form of neoxanthin (isolated from non-photosynthetic tissues of plants) supported reconstitution with a similar yield to the cis form, suggesting that both isomeric forms of this carotenoid can bind to LHC, although it is not clear whether they can occupy the same binding site.

Native LHCIIb binds the 3R,3′R form of zeaxanthin and the 3R,3′,6R,6′R form of lutein. In vitro, all three chiral forms of zeaxanthin (namely the 3R,3′S, 3S,3′S, and 3R,3′R forms) all supported reconstitution with identical yields (~60% of the native xanthophyll mixture). In addition, the 3R,3′S,6′S diastereoisomer of lutein and 3′-epilutein (3R,3′S,6′R) were as effective as the native form of this carotenoid in supporting reassembly of the complex.

Binding of Nonnative Xanthophylls—The first series of xanthophylls studied were a range of compounds possessing a C-3-hydroxy β-end group. The monohydroxycarotenoids, β-cryptoxanthin (3R,R)-β-carotene-3-ol) and α-cryptoxanthin (3R,6′R)-β-carotene-3-ol), both supported reassembly of LH-CIIb, with a ratio of Chl a/b similar to other reassembled complexes (in the range of 1.18–1.20:1) but with a relatively low yield at 32–34% (Table III). The difference spectra are shown in Fig. 5A and reveal marked changes from the native complex and that reassembled with a mixture of native xanthophylls (cf. Fig. 4). Of more interest is a comparison of their difference spectra with those obtained for their dihydroxy equivalents, namely lutein and zeaxanthin, which reveal profound changes in the intensity of absorbance primarily in the Soret region (~456 nm). The difference spectra for a number of other carotenoids including capsanthin (3R,3′,S,5′R)-3,3′,6-dihydroxy-β-carotene-4′,6′-one) are almost identical to these (data not shown). For capsanthin, the predicted shift of the absorbance in the Soret region (due to the red-shifted absorbance of the carotenoid at ~470 nm compared with native xanthophylls) was not observed. In contrast, the spectrum (Fig. 5B) obtained for the ketocarotenoid astaxanthin (3R,3′S,5′-3,3′,6-dihydroxy-β-carotene-4′,4′-dione) reveals an additional significant increase in absorption at 470 nm, reflecting its inherent spectral properties. The two monocyclic 3-hydroxyxcarotenoids tested (namely gellidoxanthin (3-hydroxy-4-oxo-3′,4′-didehydro-2′-apo-β-caroten-2′-al) and rubixinthin ((3R,R)-β,R-carotene-3-ol)) both supported reassembly of the complex.

The diols isozeaxanthin (β,β-carotene-4′,4′-diol), with hydroxy groups at C-4 and C-4′, and the retro-carotenoid diol eschscholtzaxanthin (4′,5′-didehydro-4′,5′-reto-β,β-carotene-3,3′-diol) failed to support reconstitution. A number of other epoxides failed to support reassembly of the complex (see below).

The diol lactucaxanthin, which is common in the LHC of a number of plants (e.g. lettuce (6)) but not pea from which the apoprotein was cloned, would not support complex assembly when used as the only xanthophyll. In addition, it would not support reassembly of the complex using thylakoids isolated from either spinach or lettuce. Lactucaxanthin could, however, be incorporated into LH-CIIib provided that lutein was also present (see below). Neither β-carotene nor the acyclic carotene lycopene supported reassembly of the complex.

Binding of Geometric Isomers and Stereoisomers of Xanthophylls—The pigment-protein complexes of photosynthetic organisms show a high degree of selectivity toward certain geometric forms of carotenoids. In the present study, the ability of selected geometric isomers of lutein, zeaxanthin, and violaxanthin to support reassembly of LH-CIIib was examined (see Fig. 1 for structures). In contrast to the all-trans configurations, the 9-cis and 13-cis forms of violaxanthin and zeaxanthin and 13-cis lutein all failed to support reassembly. A mixture of di-cis isomers (unidentified) of zeaxanthin also failed in this respect. Neoxanthin is naturally present as the 9-cis form in the photosynthetic tissues of higher plants (23), and this can support reassembly of the complex (albeit with low efficiency, ~40%). The all-trans form of neoxanthin (isolated from non-photosynthetic tissues of plants) supported reconstitution with a similar yield to the cis form, suggesting that both isomeric forms of this carotenoid can bind to LHC, although it is not clear whether they can occupy the same binding site.

Native LHCIIb binds the 3R,3′R form of zeaxanthin and the 3R,3′,6R,6′R form of lutein. In vitro, all three chiral forms of zeaxanthin (namely the 3R,3′S, 3S,3′S, and 3R,3′R forms) all supported reconstitution with identical yields (~60% of the native xanthophyll mixture). In addition, the 3R,3′S,6′S diastereoisomer of lutein and 3′-epilutein (3R,3′S,6′R) were as effective as the native form of this carotenoid in supporting reassembly of the complex.
diepoxo-5,6,5',6'-tetrahydro-β,β-carotene-3,5'-dil). Both lorio-
exanthin ((3R,3'R,5',5'R,6'R,6''R)-3,19,3',tri-hydroxy-β,β-carotene) and siphonaxanthin ((3R,3'R,5',5'R,6'R,6''R)-3,19,3',tri-hydroxy-7,8-dihydro-β,β-
carotene), which possess in-chain methoxy groups at C-19, also
supported complex reassembly.

In contrast to these compounds, carotenoids that lack hy-
droxy groups at C-3 and C-3' such as the ketocarotenoids
canthaxanthin (β,β-carotene-4,4'-dione) and echinenone (β,β-
caroten-4-one) and the retro-carotenoid rhodoxanthin (4',5',
didehydro-4',5'-retro-β,β-carotene-3,3'-dione) do not support
complex reassembly when used alone with Chls a and b. The
di-C-3-C-6 epoxydicycloviolaxanthin ((3S,5'R,6'R,3'S,5'S,6'R,6''R)-
3,6,3',6'-diepoxo-5,5',6'-tetrahydro-β,β-carotene-5,5'-dil), which has
hydroxy groups at C-5 and C-5', also failed to support
reconstitution.

**Competition between Xanthophylls for Binding Sites**—The
possible interaction between different xanthophylls in the re-
constitution procedure was examined. A general observation
was that the presence of two xanthophylls in the reconsti-
tution mix resulted in an increase in yield (compared with that
achieved with a single xanthophyll) until the ratio of lutein/
nonnative xanthophyll was <1:3. For nonnative xanthophylls
such as crustaxanthin, β-cryptoxanthin, and astaxanthin the
ratio of lutein/nonnative xanthophyll in the resulting complex
was 2:1, regardless of the ratio of xanthophylls employed in
the reconstitution mixture. When this ratio was significantly
increased, the yield of reconstituted complex decreased. The
affinity of the complex to astaxanthin, crustaxanthin, and
β-cryptoxanthin shown in Fig. 6 is almost identical to that seen
for 9'-cis neoxanthin (data not shown, but see Ref. 17), suggest-
ing that they can also occupy the third binding site (N1).

As seen above, luteoyxanthin does not, when presented as
the only xanthophyll, support the in vitro reassembly of LH-
CIIb. However, when a mixture consisting of lutein and lactu-
caxanthin was used, lactucaxanthin was readily incorporated

### Table II

**Stoichiometry of LHCIIb reconstituted using single native xanthophylls and Chls a and b using the low stringency (12) procedure (n = 6 ± S.E.)**

| Reconstituted xanthophyll-Chl a/b pigment-protein complex | Ratio of Chl a/b | Xanthophyll stoichiometry | Yield |
|-----------------------------------------------------------|-----------------|---------------------------|-------|
| Native xanthophyll mixture                                | 1.18:1 ± 0.06   | 3.0 ± 0.1                 | 100   |
| Lutein                                                   | 1.22:1 ± 0.05   | 1.9 ± 0.1                 | 63 ± 2.4 |
| 9'-cis-Neoxanthin                                         | 0.99:1 ± 0.03   | 2.3 ± 0.1                 | 40 ± 1.5 |
| Violaxanthin                                             | 1.10:1 ± 0.03   | 2.7 ± 0.1                 | 45 ± 1.8 |
| Antheraxanthin                                           | 1.15:1 ± 0.03   | 2.6 ± 0.1                 | 59 ± 2.1 |
| Zeaxanthin                                               | 1.18:1 ± 0.04   | 1.9 ± 0.1                 | 60 ± 1.9 |

### Table III

**Stoichiometry of LHCIIb reconstituted using single nonnative xanthophylls and Chls a and b using the low stringency (12) procedure**

The xanthophyll stoichiometry is reported per 12 Chl molecules (n = 6 ± S.E.). The yield of reassembled complex is calculated as a percentage of the native xanthophyll mixture.

| Reconstituted xanthophyll-Chl a/b pigment-protein complex | Ratio of Chl a/b | Xanthophyll stoichiometry | Yield |
|-----------------------------------------------------------|-----------------|---------------------------|-------|
| Native xanthophyll mixture                                | 1.18:1 ± 0.06   | 3.0 ± 0.1                 | 100   |
| Adonirubin                                               | 0.97:1 ± 0.05   | 2.1 ± 0.1                 | 33 ± 1.4 |
| Astaxanthin                                              | 1.16:1 ± 0.04   | 2.5 ± 0.1                 | 46 ± 1.8 |
| Capsanthin                                               | 1.20:1 ± 0.06   | 2.6 ± 0.1                 | 35 ± 1.2 |
| Crustaxanthin                                            | 0.97:1 ± 0.02   | 2.2 ± 0.1                 | 38 ± 1.7 |
| α-Cryptoxanthin                                          | 1.22:1 ± 0.05   | 2.5 ± 0.1                 | 34 ± 1.4 |
| β-Cryptoxanthin                                          | 1.18:1 ± 0.03   | 2.4 ± 0.1                 | 32 ± 1.5 |
| Fucoxanthin                                              | 1.20:1 ± 0.04   | 2.6 ± 0.1                 | 40 ± 1.8 |
| All-trans-neoxanthin                                     | 0.97:1 ± 0.03   | 2.2 ± 0.1                 | 38 ± 1.2 |
| Astaxanthin/lutein (1:3)                                  | 1.19:1 ± 0.04   | 2.7 ± 0.2                 | 68 ± 2.6 |
| Lactucaxanthin/lutein (1:3)                               | 1.21:1 ± 0.05   | 2.7 ± 0.2                 | 73 ± 3.0 |

Fig. 4. Room temperature difference spectra of native LHCIIb minus LHCIIb reconstituted with single native xanthophylls. A, lutein (thin solid line), violaxanthin (thick solid line), and antheraxan-
thin (dashed line). B, zeaxanthin (solid line) and 9'-cis neoxanthin
dashed line).
the ratio of Chl \(a/b\) dropped markedly so that the presence of lactucaxanthin or absence of lutein apparently inhibited protein folding. This is consistent with the failure of lactucaxanthin to support complex reassembly as the sole carotenoid (see above). The ratio of lutein/lactucaxanthin in the complex reflected the ratio of the two xanthophylls provided in the reconstitution mix (\(r^2 = 0.979\)) but saturated at a ratio of \(2.5:1.0\) when lutein was provided in a 3-fold excess (Fig. 6). The stoichiometry of the complexes produced (consistent at \(2.7\) per LHC monomer) reveals that two lutein and one lactucaxanthin or two lactucaxanthin and one lutein can be readily incorporated into a stable complex. This is consistent with the behavior of lutein and zeaxanthin (see above (17)). Furthermore, the data suggest that lactucaxanthin is not discriminated against in two binding sites, whereas the other site has a much higher preference for lutein. However, the shape of the curve shown in Fig. 6 reveals a complex pattern of behavior for lactucaxanthin, and it is difficult to interpret further. Whereas it is clear that lactucaxanthin can be bound to the specific carotenoid binding sites in LHCBII, its presence on its own does not permit complex assembly.

The behavior of lactucaxanthin was in marked contrast to the binding of several other (nonnative) carotenoids (e.g., astaxanthin, crustaxanthin, and \(\beta\)-cryptoxanthin) when used in combination with lutein (Fig. 6). These showed similar behavior to that previously reported for neoxanthin (17), clearly showing binding to one carotenoid-binding site, namely N1. Notably, canthaxanthin (and some other carotenoids) failed to bind to the complex even in the presence of lutein, indicating a degree of structural specificity (probably related to the lack of a substituent at C-3) of binding in each of the three carotenoid binding sites. Their presence did, however, reduce the yield of complex compared with lutein alone (Table III). In all complexes prepared using combinations of carotenoids, a strong preference for lutein was exhibited at all times, suggesting that sites L1 and L2 show a strong preference for this particular carotenoid (and zeaxanthin (17)).

**DISCUSSION**

The data obtained in the current study reveal that for all three procedures available for reconstitution of LHCBII from higher plants, a maximum of three molecules of xanthophyll are bound per monomer of reassembled LHCBII. The pigment composition of the complex reassembled using a mixture of native xanthophylls resulted in a pigment stoichiometry almost identical to that of native LHCBII (Table I). The resulting room temperature UV-visible absorption spectra were identical for complexes produced from all procedures and nearly identical to that of native spinach LHCBII. The use of the low stringency procedure for the reassembly of the complexes permitted a study of single-xanthophyll Chl \(a/b\) complexes. A similar observation was made by Hobe and colleagues (17), although the ratio of Chl \(a/b\) is consistently higher in the present study. Variations in the stoichiometry for complexes reassembled with single xanthophylls were seen (Tables I–III) and have been previously reported (10, 17, 24). Such variation may simply reflect a low affinity of xanthophylls for one or more of the binding sites (especially N1 (24)). The data support the hypothesis that monomeric LHCBII possesses three carotenoid binding sites (see Refs. 7 and 10). The proposed fourth binding site (V1; Refs. 7 and 9) was not occupied by any carotenoids using this in vitro reconstitution procedure, suggesting that its occupancy is indeed dependent upon the trimeric, in vivo, state, and is perhaps inter- rather than intracomplex in nature (7). Indeed, in vivo, under light-limiting conditions, it is possible that not all of the xanthophyll-cycle binding sites are fully occupied (7).

**Fig. 6.** Binding affinity for selected single xanthophylls in combination with lutein to LHCP: lactucaxanthin (.), crustaxanthin (.), \(\beta\)-cryptoxanthin (.), and astaxanthin (.)). Data are mean values from at least six separate determinations ± S.E.
An important observation from the current study was that the use of lutein as the sole xanthophyll resulted in a pigment-protein complex that was almost identical to the native complex both in terms of pigment stoichiometry and absorption spectrum. The use of the low stringency procedure also permitted the assembly of a stable Chl a/b complex using 9'-cis neo-oxanthin as the single xanthophyll, which, in an earlier study, could only be bound to the complex in the presence of lutein (10). 9'-cis-neoxanthin displays a high affinity to native trimeric but not monomeric LHCIIb, suggesting that it occupies a site on the inner face of the trimeric complex (7). The data here suggest that its structure does not preclude it from occupying L1 and L2, although there is a clear preference for lutein when present (17). The absorption spectra of complexes reassembled with 9'-cis neoxanthin and with zeaxanthin are almost identical (Fig. 1).

Croce and colleagues (10) observed that complexes reassembled with zeaxanthin had a substantially increased ratio of Chl a/b compared with both the native and lutein complexes at 2:3:1. This was interpreted as evidence of zeaxanthin-mediated interference with the binding sites for Chl b in LHCIIb due to differences in the ring-to-chain conformation between lutein and zeaxanthin. However, this was not seen in the present study, possibly as the result of the use of the particular reconstruction procedure employed, and the zeaxanthin complex had a ratio of Chl a/b of 1.18:1, typical of that seen for native LHCIIb (see Table I).

Both lutein and zeaxanthin were incorporated at ~1.9 molecules per monomer in these single xanthophyll complexes. In contrast, the stoichiometry of the complexes reassembled with antheraxanthin, violaxanthin, and 9'-cis-neoxanthin was consistently higher at ~2.3–2.7 per monomer, suggesting that a third binding site (N1) is at least partly occupied. Unlike the mixture of native xanthophylls, no single carotenoid could be bound in a ratio of three molecules per monomer (Tables I and II). This supports the observation that lutein and zeaxanthin are discriminated against for in N1 because of their structures. Occupancy of N1 is not required for complex reassembly (or subsequent stability (26, 27)), but binding of neoxanthin greatly improves the complex yield. Carotenoids with a C-5–C-6 epoxide, but not the C-3–C-6, on at least one end group can occupy N1, as can the diol lactucaxanthin (see below). Thus, carotenoids that only possess hydroxy groups on a β-end group at C-3 and/or C-3' apparently cannot bind to this third xanthophyll binding site on LHCIIb. This suggests that zeaxanthin may, in fact, be excluded from a binding site that may be readily occupied by violaxanthin or antheraxanthin, although Jahns et al. (24) demonstrated that violaxanthin can be converted into zeaxanthin by violaxanthin diepoxidase at both L2 and N1 (but not L1).

Assuming that one end group is a 3-hydroxy β-type, the structure of the second end group of the carotenoid molecule appears to be much less important. Thus, capsanthin, which has a γ-end group at one end of the molecule, supports reassembly of LHCIIb. In addition, monocyclic xanthophylls (e.g. rubixanthin) and the monohydroxycarotenoids (e.g. α-cryptoxanthin, a major constituent of the lat5 mutant of Arabidopsis (16)) can occupy L1 (and either L2 or N1) and initiate protein folding, albeit with a much reduced yield of complex.

Monoepoxides (e.g. lutein-5,6-epoxide) support reconstitution with a similar yield and stoichiometry to that seen with the diepoxide violaxanthin. Lutein-5,6-epoxide is found in the photosynthetic tissues of many plants (28), including Cuscuta reflexa (29), in which it appears to replace 9'-cis neoxanthin in situ. The data presented here indicate that this epoxide can occupy N1.

The inability of carotenoid furanoids to bind to LHCII was first observed by Plumley and Schmidt (11). The structure of auroxanthin is such that the ring-to-chain conformation is fixed in a near-planar position with little opportunity for rotation of the end group. This implies that the conformation of a carotenoid molecule may be a key factor in facilitating binding to the complex, or it may indicate that the carotenoid molecule must be able to rotate its end group relative to the chromophore (either during binding or protein folding; see below for further discussion). The x-ray structure of LHCIIb shows a clear twisting of the of lutein molecule (8), and carotenoids reassembled into bacterial LHC are known to adopt a conformation appropriate to their function in terms of energy transfer (30). The data presented here suggest that the difuranoid auroxanthin (implicated in fluorescence quenching (31)), if formed in vivo, may not bind to (monomeric) LHCIIb, but this does not preclude its binding or association with trimeric complexes.

End Group Structure and Ring-to-chain Conformation—The diol lactucaxanthin is a major component of the LHC of some plant species (notably lettuce (1, 6)), levels of which (under conditions of low irradiance) may readily exceed that of lutein.2 The LHC pool size of lutein plus lactucaxanthin is relatively constant regardless of the growth conditions (6). Since the xanthophyll binding sites L1 and L2 are conserved in all LHC proteins, the failure of lactucaxanthin to support reassembly of the complex when used on its own was unexpected. We have demonstrated that lactucaxanthin clearly cannot support complex reassembly on its own but can successfully compete with lutein in competition studies (Fig. 6). This demonstrates that lactucaxanthin can in fact readily bind to at least two xanthophyll binding sites, namely N1 and at least one of the lutemin-binding sites. The inability of lactucaxanthin to permit complex assembly suggests that it is omitted from L1, occupancy of which is obligatory for protein folding (26, 27), and instead occupies L2. Binding saturates at approximately two molecules of lutein per molecule of lactucaxanthin for each complex (assuming 12 Chl per LHC), indicating that lactucaxanthin readily occupies N1, whereas lutein is excluded. Overall, the behavior of lactucaxanthin is unlike that of any other xanthophyll examined in either this or an earlier study (17) and is not readily explained. Identical behavior is seen using the recombinant protein and thylakoids prepared from lettuce (which naturally possess lactucaxanthin) or spinach.

The ability of lactucaxanthin to partially substitute for (but not replace) lutein in LHC is also seen in whole plants. Treatment of lettuce with the substituted triethylamine 2-(4-methylpenoxytriethylamine) (a selective inhibitor of ε-cyclase) significantly alters the relative levels of lactucaxanthin and lutein, and the LHCIIb reveals that an absolute maximum of about two lactucaxanthin molecules are bound for every lutein molecule (very similar to that seen in the reconstitution studies described here). Again, this indicates that N1 and L2 can be fully occupied by lactucaxanthin but that replacement of lutein by lactucaxanthin in L1 is not permitted (neoxanthin was present in substoichiometric amounts in these plants).

The failure of lactucaxanthin to permit LHC assembly is an important observation, and as a result, it is interesting to consider the structures of these xanthophylls very carefully. The structural differences between the diols zeaxanthin, lutein, and lactucaxanthin at first appear to be relatively minor, since all three possess hydroxy groups at C-3 and C-3’. Lactucaxanthin possesses ε-end groups at both ends of the molecule, whereas zeaxanthin has two β-end groups, and lutein has one β- and one ε-end group. This is reflected in the conjugated chain

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2 D. Phillip, unpublished data.
lengths of the molecules and the overall shape of the carotenoid, in particular the ring-to-chain conformation around C-6–C-7. The structures of the end groups found in zeaxanthin and luteoxanthin (from MNDO-AM1 optimization (32)) revealed marked differences in the bond angles and dihedral (torsion) angles, especially in the cyclohexene ring region of the molecule (Fig. 7). The most stable structure of the β-end groups found in zeaxanthin (and lutein) had a C-5–C-6–C-7–C-8 dihedral angle of −48.7°, compared with +124.5° for the ε-end groups of luteoxanthin. The predicted stable end group structure of luteoxanthin (depicted as type B in Fig. 7) is therefore very different from that seen in other native xanthophylls that can bind to L1 (which possess type A structures on at least one end group). Note that the structures of the ε-end groups found in lutein (3′R,6′R) and luteoxanthin (3S,6S,3′S,6′S) are very different. The torsion angle that describes the ring-to-chain conformation around C-6–C-7 in luteoxanthin is unique. The most striking difference in the adiabatic potential energy curves for this torsion angle is that the end groups found in lutein, zeaxanthin, and violanoxanthin all possess relatively symmetrical potential energy curves, but that calculated for luteoxanthin is asymmetrical.

Furthermore, lactuxanthin is only stable over a very narrow range of the C-6–C-7 torsion angle and is not able to twist around its end groups (e.g., as might occur during protein folding). Such differences in structure may account for the quite different behavior exhibited by these carotenoids in their ability to promote complex assembly in vitro.

The behavior of the retro-dihydroxyxcarotenoid eschscholtzxinanthin in failing to facilitate reassembly of LHClIb further highlights the issue of carotenoid shape (especially the ring-to-chain conformation around C-6–C-7) as being an important factor in binding (33). Whereas its stable structure has not been determined, the conjugated double bond system is shifted so that the C-4–C-5 and C-6–C-7 double bonds are effectively co-planar, so that twisting of the molecule between ring and chain is thought not to occur (34).

The observations made in this study suggest that the structural requirements in order to facilitate protein folding in complex reassembly may be different from those required for binding to the protein per se. Thus, the carotenoid molecule should possess a 3-hydroxy β-end group (type A; Fig. 7) in order to permit reassembly (and subsequent stabilization) of the complex. The observation that 9′-hydroxyxanthophylls, which have a β-end group structure identical to that found in zeaxanthin, can support reassembly of LHClIb supports this view. Once this requirement is satisfied, other carotenoids can be bound, presumably on a site peripheral to the complex, but even then certain carotenoids may still not be bound (e.g., canthaxanthin).

Native plant LHC exhibits (with the exception of 9′-cis neoxanthin) a strong preference for binding all-trans xanthophylls. Native LHClIb (spinach) contains only trace amounts of cis isomers of lutein, principally the 13,13' and 15-cis forms (35). This stereoselectivity is probably related to the different photochemistry exhibited in situ by all-trans and cis forms of these pigments (36). Attempts to bind central cis isomers of lutein and di-cis isomers of zeaxanthin to LHClIb in the current study were, however, unsuccessful, although both all-trans- and 9′-cis-neoxanthin did produce stable Chl a/b complexes. trans → cis isomerization of xanthophylls may occur in situ in the light-harvesting complexes of plants as a result of triplet-excit state photoisomerization, with Chls acting as the sensitizers (37). Indeed, the reversible formation of 13-cis-violaxanthin from the all-trans form in parallel to deepoxidation upon illumination of leaves has been reported (37, 38). This particular xanthophyll isomer is not bound to LHClIb in vitro, while the all-trans form is readily bound (see also Ref. 10), suggesting that the cis form may be excluded from the (monomeric) complex on formation.

The ability of LHClIb to bind nonnative carotenoids has been previously demonstrated, and this study further reveals a plasticity inherent in other pigment-protein complexes. For example, total pigment extracts from the algae of Euglena gracilis (containing neoxanthin and diadinoxanthin (5,6-epoxy-7′,8′-didehydro-5,6-dihydro-β-carotene-3,3′-dial) and Chiamydomonas reinhardtii (containing loroxanthin) have been used in heterologous reconstitution of spinach light-harvesting complexes (39). More recently, capsanthin (which supports in vitro reassembly of LHClIb; see above) has been shown to be a functional component (at ~36% of total carotenoid) of transformed plants of Nicotiana benthamiana (25).

Whereas the carotenoid composition of plant light-harvesting complexes is highly conserved, the complexes themselves

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Fig. 7. Stable end group structures predicted by MNDO-AM1 molecular orbital calculations (32). 1, type A for the 3-hydroxy β end group found in zeaxanthin, antheraxanthin, and lutein; 2, type A for the C-5–C-6 epoxide 3-hydroxy end group of antheraxanthin and violaxanthin; 3, type B for the 3-hydroxy ε end group of lactoxanthin. C-5, C-6, C-7, and C-8 carbon atoms and oxygen atoms are omitted for simplicity.
exhibit a high degree of plasticity in terms of their ability to bind carotenoids, provided that these molecules possess a 3-hydroxy-β-end group. Both the presence of key functional groups and the overall shape of the carotenoid molecule, especially ring-to-chain conformation, are important factors governing binding to LHCCIb and subsequent complex reassembly.

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The Binding of Xanthophylls to the Bulk Light-harvesting Complex of Photosystem II of Higher Plants: A SPECIFIC REQUIREMENT FOR CAROTENOIDS WITH A 3-HYDROXY- β-END GROUP

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