

Decreasing the Chlorophyll *a/b* Ratio in Reconstituted LHCII: Structural and Functional Consequences[†]

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ABSTRACT: Trimeric (*bT*) and monomeric (*bM*) light-harvesting complex II (LHCII) with a chlorophyll *a/b* ratio of 0.03 were reconstituted from the apoprotein overexpressed in *Escherichia coli*. Chlorophyll/xanthophyll and chlorophyll/protein ratios of *bT* complexes and 'native' LHCII are rather similar, namely, 0.28 vs 0.27 and 10.5 ± 1.5 vs 12, respectively, indicating the replacement of most chlorophyll *a* molecules with chlorophyll *b*, leaving one chlorophyll *a* per trimeric complex. The LD spectrum of the *bT* complexes strongly suggests that the chlorophyll *b* molecules adopt orientations similar to those of the chlorophylls *a* that they replace. The circular dichroism (CD) spectra of *bM* and *bT* complexes indicate structural arrangements resembling those of 'native' LHCII. Thermolysin digestion patterns demonstrate that *bT* complexes are folded and organized like 'native' trimeric LHCII. Surprisingly, in the *bT* complexes at 77 K, half of the excitations that are created on either chlorophyll *b* or xanthophyll are transferred to chlorophyll *a*. No or very limited triplet transfer from chlorophyll *b* to xanthophyll appears to take place. However, the efficiency of triplet transfer from chlorophyll *a* to xanthophyll is close to 100%, even higher than in 'native' LHCII at 77 K. It is concluded from the triplet-minus-singlet and CD results that the single chlorophyll *a* molecule that on the average is present in each *bT* complex binds preferably next to a xanthophyll molecule at the interface between the monomers.

Light-harvesting complex II (LHCII)¹ is the most abundant pigment–protein complex in higher plants. Its main task is the absorption of sunlight and the subsequent transportation of excitation energy toward the reaction center of mainly photosystem II (1). The complex exists in a trimeric form, and each monomeric unit contains besides at least 3 xanthophylls (Xan) approximately 7–8 chlorophyll *a* (Chl *a*) and 5–6 chlorophyll *b* (Chl *b*) molecules (2, 3). Together with 2 xanthophylls, 12 chlorophylls were resolved per

monomer in the 3.4 Å crystal structure. The seven chlorophylls located closest to the resolved central pair of xanthophylls were assigned as Chl *a* (4). This assignment is mainly based on the idea that due to fast Chl *b* to Chl *a* singlet energy transfer, triplet states will predominantly be formed on Chl *a* and in order to achieve effective triplet quenching all Chl *a* pigments will have to be in close contact with the xanthophylls. However, it is not clear whether this assignment is correct and this hampers a good understanding of the structure–function relationship.

The possibility to reconstitute LHCII from the apoprotein overexpressed in *Escherichia coli* and pigments extracted from chloroplast membranes (5, 6) offers several ways to characterize the binding preference of various Chl binding sites: (i) using site-specific mutagenesis, binding sites can selectively be mutated and the effect on the pigment stoichiometry of reconstituted complexes can be studied; (ii) the pigment stoichiometry of the reconstitution mixture can be varied in order to obtain reconstituted complexes with various pigment stoichiometries. Both approaches were used for CP29 (7, 8), which is a minor light-harvesting complex that shows a strong sequence homology with LHCII (7, 9).

Besides xanthophylls, 'native' CP29 binds 6 Chls *a* and 2 Chls *b*. It was reported that stable reconstituted CP29 complexes could also be formed with a Chl *a/b* ratio of ~0.4, whereas the Chl/protein ratio did not change, thereby showing that at least part of the Chl binding sites can be occupied by either Chl *a* or Chl *b* (8). In (7) it is shown that

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¹ Abbreviations: *abT* complexes, trimeric reconstituted LHCII complexes containing Xan and Chl *a/b* in a ratio of 0.7; *bT* complexes, trimeric reconstituted LHCII complexes containing Xan, Chl *b*, and traces of Chl *a*; *bM* complexes, monomeric reconstituted LHCII complexes containing Xan, Chl *b*, and traces of Chl *a*; DADS, decay-associated difference spectrum; CD, circular dichroism; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; DM, *n*-dodecyl- β , β -D-maltoside; DP, deletion product; EDTA, ethylenediaminetetraacetate; FWHM, full width at half-maximum; LD, linear dichroism; LDS, lithium dodecyl sulfate; LHCII, light-harvesting complex of photosystem II of higher plants; LHCP, light-harvesting complex protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 1–T, 1–transmission; T–S, triplet-minus-singlet; v/v, volume per volume; w/v, weight per volume; WT, wild type; Xan, xanthophyll.

when Chl *a/b* ratios ranging from 8 to 18 are applied in the reconstitution mixture, reconstituted CP29 complexes are formed with the 'native' pigment composition. Therefore, it is suggested that the 'native' conformation corresponds to a high stability state for the proteins (7). The construction of point mutants on putative Chl binding sites has in the case of CP29 led to the identification of the nature of at least one binding site (7). However, in general the assignment is not very straightforward, since some binding sites might not exhibit a strong preference for binding either Chl *a* or Chl *b*, at least during the reconstitution process (8).

The dependence of the Chl *a/b* ratio in the reconstituted complex on the Chl *a/b* ratio during LHCII refolding is not fully understood. In order to investigate within what ranges of pigment composition "LHCII-like" complexes could be formed, both pigment stoichiometry and pigment to protein ratio of the reconstituted algal LHCII complexes of *Chlorella fusca* were varied in (10). It was demonstrated that complexes containing Chl *a/b* ratios from 0.9 to 1.5 share a very similar CD spectrum in the visible region. This was interpreted in terms of a central cluster of pigment molecules being responsible for the characteristic circular dichroism and being required for the folding of a large portion of the protein into the correct secondary structure. This cluster seemed to be complemented by more peripheral pigment binding sites which are rather tolerant to either Chl *a* or Chl *b*, but do not lead to a more complete restoration of secondary structure (10).

Reconstitution of LHCII with LHCP overexpressed in *E. coli* and various Chl *a/b* ratios in LDS micelles has shown that deviation from an optimal ratio of Chl *a/b* = 1 in the reconstitution mixture leads to a decrease in complex formation rather than a variation in the Chl *a/b* ratio in the reconstituted complexes. However, when complex formation is achieved by a detergent exchange to octyl glucopyranoside, less stable complexes with largely varying Chl *a/b* ratios can be obtained (Paulsen and Hobe, unpublished).

Here we have investigated such reconstituted complexes. We present a detailed spectroscopic study concerning reconstituted complexes containing, besides xanthophylls, Chl *a/b* ratios of respectively 0.7 and 0.03 (1 Chl *a* per trimer). The latter complex allows us to investigate to what extent Chl *a* can be replaced by Chl *b* and how this affects structure and function of the complex. Circular dichroism (CD), linear dichroism (LD), absorption (OD), fluorescence emission, fluorescence excitation, and triplet-minus-singlet (T-S) measurements on these reconstituted monomeric and trimeric complexes have been performed. Spectra are compared to spectra of 'native' and 'native' reconstituted LHCII. In (11) it was shown that the spectroscopic properties of LHCII reconstituted with all pigments in 'native' ratios were similar (though not identical) to those of 'native' LHCII. Furthermore, the efficiencies of singlet and triplet excitation energy transfer are estimated.

MATERIALS AND METHODS

Preparation of Reconstituted Complexes. Monomeric and trimeric reconstituted LHCII complexes were prepared as in (12, 13) with the following modifications. Pigments for reconstitution were composed of either a Chl-free Xan extract of pea thylakoids to which Chl *a* and Chl *b* were added in

a ratio of 0.01/1 to obtain the complexes containing only trace amounts of Chl *a*, or a Xan extract to which Chl *a* and Chl *b* were added in a ratio of 1/1, leading to the formation of complexes with a final Chl *a/b* ratio of 0.7. Pigment/protein ratios in the reconstitution mixtures were 70 for Chl *a* + Chl *b* and 17 for the xanthophylls. The molar ratio of neoxanthin/violaxanthin/lutein was 1/1/3. Monomeric and trimeric complexes were separated on sucrose gradients as in (12, 13).

Some of the complexes having a Chl *a* to Chl *b* ratio of 0.7 were prepared by a modified method which raised the yield of trimers as compared to the original trimerization assay (12, 13) but changed neither the Chl *a/b* ratio nor the pigment/protein stoichiometry. After dialysis and ultrafiltration (12, 13), the reconstituted LHCII suspension was solubilized in 0.5% Triton X-100 at a Chl concentration of 0.5 mg/mL and precipitated on ice for 30 min with 300 mM KCl. The spun-down pellet (4 °C, 15000g) was washed once in ice-cold water and centrifuged again. The pellet was then solubilized in 0.1% *n*-dodecyl β , β -maltoside (DM) for 30 min and applied to sucrose gradients as before (12, 13).

In the present work, trimeric complexes with a Chl *a/b* ratio of 0.7 are referred to as *abT* complexes. Monomeric and trimeric complexes containing mainly Chl *b* and only trace amounts of Chl *a* will be referred to as *bM* and *bT* complexes, respectively.

Determination of Pigment Composition and Pigment to Protein Ratio. Pigment stoichiometries of the complexes isolated from sucrose gradients were determined after extraction of the pigments following the method described in (14). Aliquots of 100 μ L of the removed sucrose gradient bands were mixed with 100 μ L of *sec*-butanol and 25 μ L of 5 M NaCl. After 30 min on ice, phase separation was enhanced by spinning for 10 min at 4 °C and 15000g. The upper organic phase was carefully removed, and the water phase was extracted a second time with 40 μ L of butanol. Butanol phases were pooled and incubated at -20 °C overnight in order to exclude the possibility of water remaining. When there was no phase separation (frozen water at the bottom), the volume of the butanol phase was determined gravimetrically ($d = 0.81$ g/mL).

Pigment composition was determined by directly applying the butanol phase to an analytical HPLC. Pigments were separated on a reversed-phase column (Symmetry C8, 5 μ m, 4.6 \times 150 mm; Waters, Eschborn, Germany) using a linear gradient of 70–100% acetone at a flow rate of 1 mL/min. Eluting bands were detected by their absorption at 440 nm and quantified using calibration curves established for purified pigments. Samples for calibration points were quantified using the algorithm of (15) for Chls and the extinction coefficients for Xans as determined in (16).

Protein quantitation was done by densitometric analysis of stained bands after denaturing SDS-PAGE. Complexes were heat-denatured for 2 min at 100 °C after mixing 40 μ L of the sucrose band with 4.5 μ L of 10% SDS and directly loaded. As an internal standard, aliquots of purified overexpressed and denatured mature LHCP were loaded on the same gel. Standard samples were calibrated using a gravimetrically determined extinction coefficient, $E_{1\%,1\text{cm},280\text{nm}} = 19$, in 10 mM Tris, pH 6.8, 2% SDS, 1 mM β -mercaptoethanol. Gels were run at 60 V and at 20 °C for about 3 h using a running buffer with reduced (0.05%) SDS concentra-

tion. Gel staining with Coomassie Brilliant Blue followed the standard protocol. Destained gels were scanned on a flat-bed scanner. Two-dimensional densitometric analysis of the resulting Tiff-files was performed using AIDA (Raytest Isotopenmessgeräte GmbH, Germany). The determined pigment/protein ratios had an accuracy limit of $\pm 15\%$.

Thermolysin Digestion. Complexes were removed from sucrose gradients and incubated at 25 °C for 30 min after the addition of 10 mM Hepes–KOH (pH 8.0), 0.5 mM CaCl₂, and 0.1 mg/mL thermolysin. Digestion was terminated by adding 10 mM EDTA and subsequent heat-denaturing.

Fully Denaturing Electrophoresis and Immunoblot. Samples were heat-denatured (2 min, 100 °C) after adding 1% SDS and applied to fully denaturing SDS–PAGE (12% polyacrylamide). Immunodetection was done as described earlier (6).

Spectroscopy. For spectroscopic measurements at 77 K, the samples were diluted in the following buffer: 20 mM HEPES (pH 7.5), 0.1% *n*-dodecyl β ,D-maltoside, and 80% (v/v) glycerol. For these measurements at cryogenic temperatures, an Oxford Instruments DN1704 liquid nitrogen cryostat was used. Absorption spectra were measured on a double-beam Cary 219 spectrophotometer with a bandwidth of 1 nm. Circular dichroism and linear dichroism measurements were performed on a home-built spectropolarimeter (17). Fluorescence emission spectra were recorded with a CCD camera (Chromex 500IS) via a 0.5 m spectrograph (Chromex 1) with a bandwidth of 1 nm and were corrected for the wavelength sensitivity of the detection system. Excitation light was provided by a 250 W tungsten–halogen lamp via a band-pass filter at 475 nm with a FWHM of 20 nm. Fluorescence excitation spectra (1 nm resolution) were measured on a home-built setup. Modulated excitation light was provided by a tungsten–halogen lamp via a 0.5 m monochromator (Chromex 500SM, spectral bandwidth 1 nm) and a mechanical chopper. Fluorescence light was detected in a 90° geometry via a 690 nm high-pass filter by an S-20 photomultiplier connected to a lock-in amplifier. The setup was calibrated for the wavelength dependence of the intensity of the excitation light using three different dyes (Sternyl 9M, DCM, and LD722, all in methanol).

T–S absorption difference measurements were performed using a pulsed dye laser (FWHM 8 ns, 10 mJ per pulse) for excitation at 590 nm and a pulsed (20 ms) xenon lamp (450 W) for detection (18). Transmitted light was detected via a 1/4 m monochromator with a photomultiplier (Hamamatsu R928). The instrument response time was less than 0.5 μ s. Kinetic traces were averaged 128 or 256 times at many wavelengths. The data were analyzed using a global analysis routine (19).

The Chl to Xan triplet transfer was estimated by dividing the amount of Chl triplets by the total amount of triplets (Chl + Xan). The Chl *b* bleaching at 472 nm, the Chl *a* bleaching at ~ 675 nm, and the ³Xan peak at 507–515 nm were used to calculate relative concentrations using the following extinction coefficients: Chl *b*, 1.56×10^5 cm⁻¹ M⁻¹ at 472 nm; Chl *a*, 1×10^5 cm⁻¹ M⁻¹ at 675 nm; and Xan, 2.4×10^5 cm⁻¹ M⁻¹ at 507–515 nm (20, 21).

RESULTS

Pigmentation and Folding of Reconstituted LHCII with Reduced Chl *a/b* Ratio. Recombinant LHCII with Chl *a/b*

Table 1: Pigment Composition of the Reconstituted *bT* and *abT* Complexes^a

pigments	<i>bT</i>	<i>abT</i>
neoxanthin	1.19 \pm 0.12	1.10 \pm 0.16
violaxanthin	0.14 \pm 0.02	0.07 \pm 0.01
lutein	2.00 \pm 0.26	2.12 \pm 0.47
Chl <i>a</i>	0.37 \pm 0.04	4.93 \pm 0.10
Chl <i>b</i>	11.63 \pm 0.04	7.07 \pm 0.10

^a Numbers are mean values of 3 determinations and were normalized to a total of 12 chlorophylls.

ratios of 0.7 and 0.03 were obtained when reconstitution mixtures contained Chl *a* and Chl *b* at ratios of 1 and 0.01, respectively. Complexes with a Chl *a/b* ratio of 0.7 contain the same overall number of 10.5 ± 1.5 Chls per protein as the ones with a Chl *a/b* ratio of 0.03. The xanthophyll composition is not affected by the altered Chl *a/b* ratio, since we observe, like in ‘native’ LHCII isolated from pea, ratios of about 2 luteins, 1.1 neoxanthins, and substoichiometric amounts (~ 0.1) of violaxanthin per 12 Chls (see Table 1). Because pigment analysis was done on green bands from sucrose gradients comigrating with ‘native’ LHCII trimers isolated from pea (see below), complexes are termed *bT* (Chl *a/b* ratio = 0.03) and *abT* (Chl *a/b* ratio = 0.7) in Table 1. Some spectroscopic data were also collected from monomeric complexes with a Chl *a/b* ratio of 0.03 (*bM*). Due to minor contaminations with unbound carotenoids, these samples show a 1.5-fold higher Xan/Chl ratio as compared with *bT* or ‘native’ LHCII.

Reconstituted LHCII with a lowered Chl *a/b* ratio forms trimers in vitro under the experimental conditions used for the trimerization of recombinant LHCII with a more ‘native’ pigment composition (13). The trimers containing almost exclusively Chl *b* (*bT*) behave very similarly to ‘native’ LHCII isolated from pea with regard to their sedimentation in sucrose gradients (Figure 1A) and migration in partially denaturing polyacrylamide gels (not shown).

In order to test whether the isolated complexes are correctly folded and organized like ‘native’ LHCII trimers, we assayed their resistance to proteolytic attack after isolation and spectroscopic measurements. It has been shown before that ‘native’ LHCII as well as reconstituted LHCII trimers with a ‘native’-like Chl *a/b* ratio are largely protected against protease, giving rise to an N-terminally shortened digestion product (DP) of 24 kDa (22). Upon dissociation of ‘native’ LHCII into monomeric complexes, a larger part of the N-terminal domain becomes accessible to protease, yielding a 20 kDa digestion product (DP*) (22). Figure 1B shows immunoblots of the thermolysin digestion products of ‘native’ LHCII trimers and monomers and the corresponding assays on complexes, reconstituted at low Chl *a/b* ratio (*bT*, *bM*). Both monomeric samples result in two deletion products of 24 kDa (DP) and 20 kDa (DP*). For *bM* complexes, DP* is the most prominent band while ‘native’ monomers are only partially degraded all the way to DP*. On the other hand, ‘native’ trimeric LHCII yields the larger DP exclusively, and in *bT* this is the predominant product of the protease reaction, with only trace amounts of DP* appearing.

Absorption. The 77 K absorption spectra of the *bT*, *abT*, and *bM* complexes are shown in Figure 2A. The absorption spectrum of the *abT* complex has peaks at 435, 473, 600,

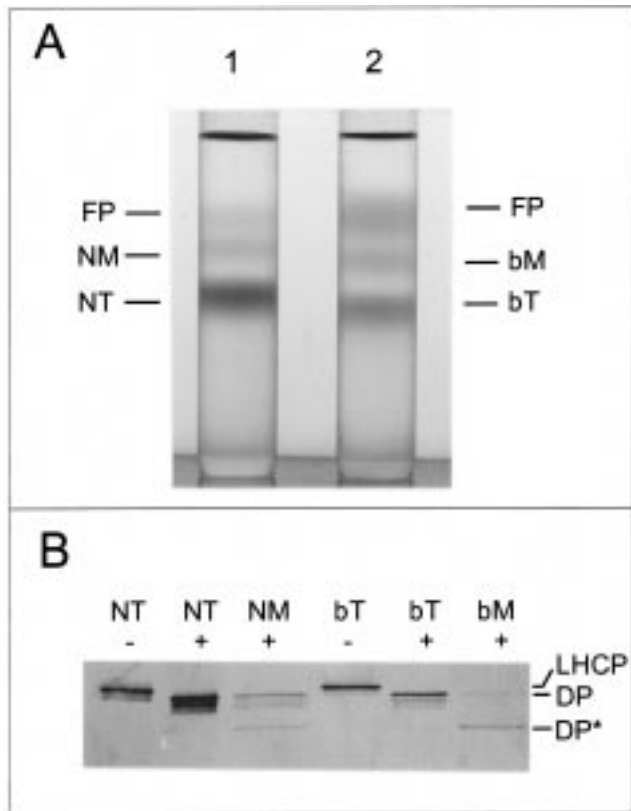


FIGURE 1: Sucrose density centrifugation (panel A) of 'native' LHCII and products of reconstitution and trimerization at low Chl *a/b* ratio and immunoblot (panel B) of thermolysin digestion products. (A) 'Native' LHC II (1) and the trimerization product of low Chl *a/b* ($=0.01$) reconstitution (2) were applied to sucrose density gradients, containing 0.1% LM. NT = 'native' trimeric LHCII; NM = 'native' monomeric LHCII; FP = unbound pigment; bM = monomeric reconstitution product; bT = trimeric reconstitution product. (B) Bands from gradients in panel A were digested with 0.1 mg/mL thermolysin, electrophoresed, blotted, and immunostained. -/+ indicates absence/presence of thermolysin. LHCP = mature size of LHCP; DP = deletion product at 24 kDa; DP* = deletion product at 20 kDa.

649, 661, 670, and 675 nm and is to a large extent similar to the absorption spectrum of the 'native' reconstituted complex presented in (11). The spectra of bT and bM complexes show peaks at 464 nm (Soret band of Chl *b*), at 600 nm (Q_x band of Chl *b*), and at 649 nm (Q_y band of Chl *b*). The difference in the Soret region between monomers and trimers is partly due to variations in the scattering background. However, the difference spectrum ($bM - bT$) shows features (data not shown) which are due to the fact that relatively more xanthophyll is present in the bM preparations.

In Figure 2B, blowups of the spectra in the Q_y region of bT (solid) and abT (dashed) complexes and their second derivatives are shown. Both preparations show a Chl *b* peak at 648–649 nm. The peak at 661 nm is most probably Chl *b* in the bT complexes whereas in 'native' LHCII this peak was assigned to Chl *a* (24). In the spectrum of the bT complexes, Chl *b* peaks have appeared at 641 and 654 nm. This is similar to the band positions assigned to Chl *b* in CP29 reconstituted with a Chl *a/b* ratio of ~ 0.4 (8). The second-derivative spectrum of the bT complexes suggests that two small Chl *a* peaks (670 and 676 nm) are hidden in the tail of the bT absorption spectrum. Similar features were

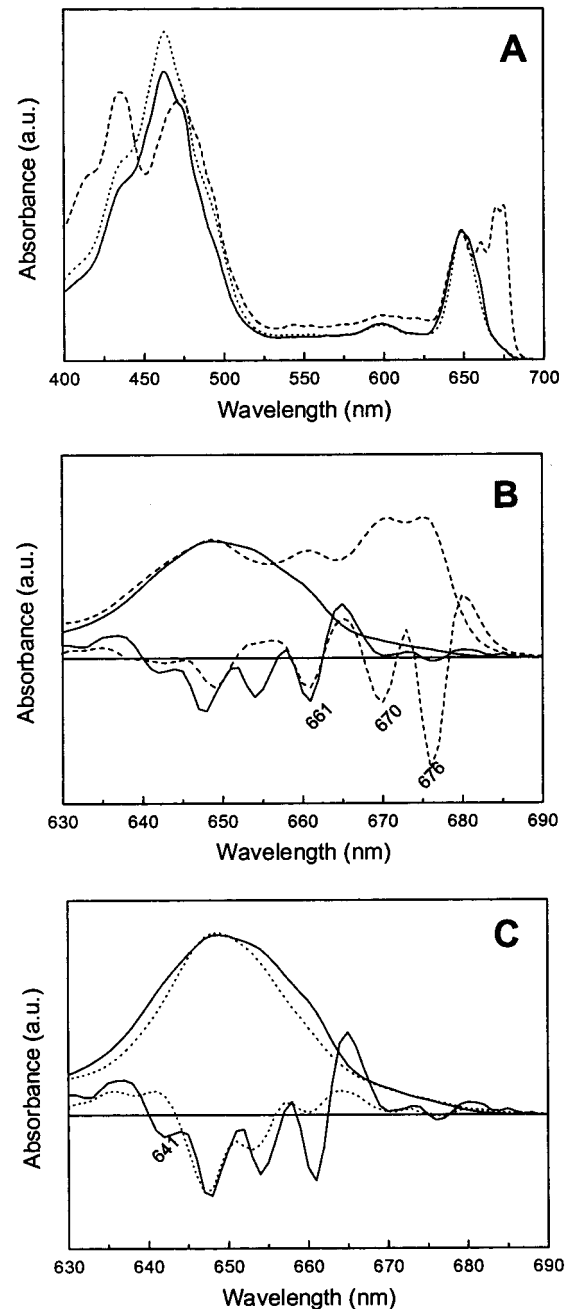


FIGURE 2: (A) Absorption spectra at 77 K of bT (solid), abT (dashed), and bM (dotted) complexes. (B) Absorption spectra of the Q_y region of bT (solid) and abT (dashed) complexes. Also shown are the second derivatives of both spectra. (C) Same as (B) but of bT (solid) and bM (dotted) complexes.

observed for preparations containing three Chls *a* per trimer (data not shown). In Figure 2C, the spectra in the Q_y region of bT and bM complexes and their second derivatives are shown. The same peaks are present, but the peaks at 661 and 654 nm are less pronounced for monomers. The bM complexes lack a peak at 641 nm that is present in the bT complexes. Similarly, trimeric 'native' LHCII shows a peak at 639.5 nm while monomers prepared with phospholipase or chymotrypsin do not show that band in the absorption spectrum (17).

Circular Dichroism. The room temperature CD spectra of bT (solid) and bM (dotted) complexes are given in Figure 3. In the Soret region, a negative peak at 478 nm is present in the spectrum of the bT complexes, but it is missing in the

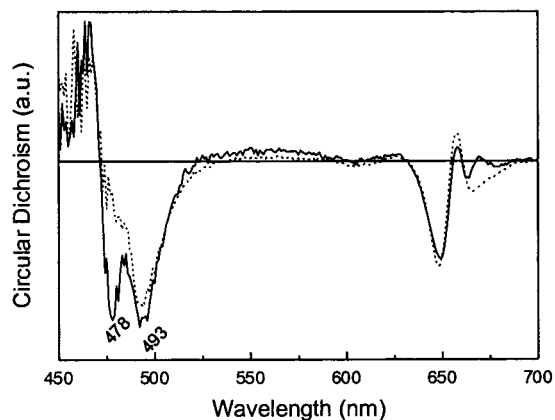


FIGURE 3: CD spectra of *bT* (solid) and *bM* (dotted) complexes at room temperature.

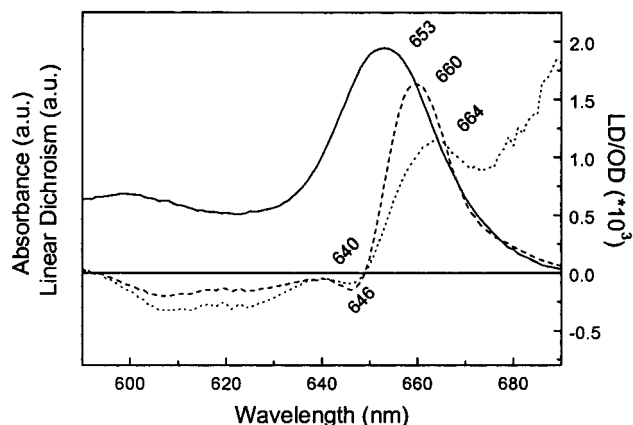


FIGURE 4: LD (dashed), OD (solid), and reduced LD (dotted) spectra of *bT* complexes at room temperature.

bM complexes. This characteristic difference in the Soret region was reported previously for ‘native’ reconstituted LHCII and ‘native’ LHCII (11, 13, 25), confirming that indeed trimeric *b* complexes are formed.

The Chl *b* Q_y region of the *bT* and *bM* spectra is comparable to that of ‘native’ LHCII; however, the Chl *a* Q_y region differs from that of ‘native’ LHCII due to the strongly reduced Chl *a* content in the *bT* complexes. The difference in the Chl *a* Q_y region between *bM* and *bT* complexes indicates that at least some Chl *a* is bound at a binding place that is affected by the aggregation state of the complex. This is in agreement with the fact that the presence of some Chl *a* seems to be crucial for the formation of trimeric *b* complexes (Hobe and Paulsen, unpublished).

The intensity of the CD signal at 650 nm is 1×10^{-3} for both *bM* and *bT* complexes with an OD at 650 nm of 1. This is somewhat lower than the CD intensity of ‘native’ LHCII, which is $\sim 1.6 \times 10^{-3}$ with an OD of 1 at 650 nm. However, since the contribution to the CD signal at 650 nm of Chl *b* molecules binding at Chl *a* binding sites is not known, these numbers are difficult to compare. It can be concluded that the CD signal of *bM* and *bT* complexes is (i) much larger than that of unbound pigments and (ii) of the same order of magnitude as that of ‘native’ LHCII.

Linear Dichroism. The room temperature LD spectrum (dashed) of *bT* complexes in the Q_x – Q_y region is shown in Figure 4. Also shown are the OD spectrum (solid) and the reduced LD spectrum (dotted). The Q_y region of the LD

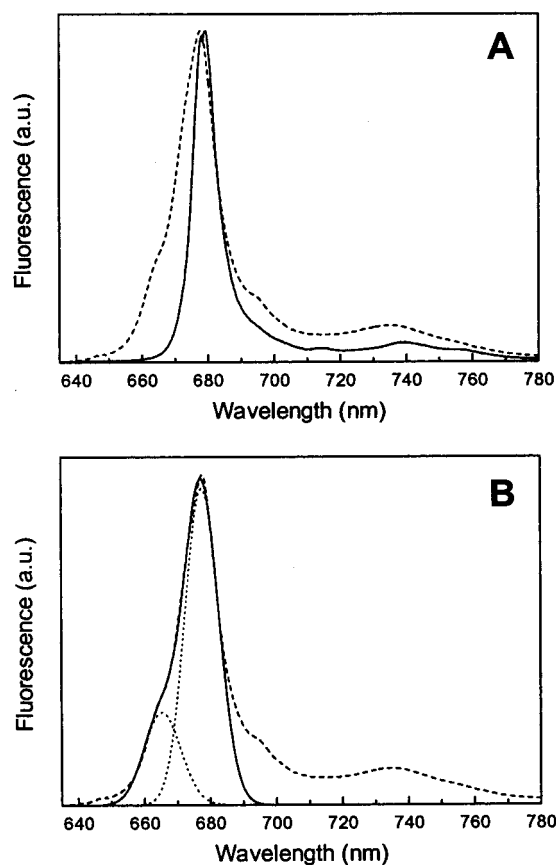


FIGURE 5: (A) Fluorescence emission spectrum of *bT* (dashed) and *abT* (solid) complexes at 77 K. Excitation was at 475 nm. (B) Gaussian-fit (dashed) of *bT* fluorescence emission spectrum in order to estimate the relative amounts of Chl *b* and Chl *a* fluorescence. The solid line shows the sum of the two Gaussian bands.

spectrum is characterized by a positive band at 660 nm, a negative band at 646 nm, and a ‘positive’ band at 640 nm.

In the LD spectrum of ‘native’ (reconstituted) LHCII trimers, the most positive LD band is caused by the red-most Chl *a* molecules (absorbing at 676 nm) (11, 26). For the LD spectrum of the *bT* complexes, the strongest LD band is caused by the red-most Chl *b* pigments (660–662 nm). This strongly suggests that in the *bT* complexes Chl *a* molecules have been replaced by Chl *b* molecules while the orientation of the pigments has remained the same. The rest of the spectrum is hard to interpret due to the large overlap of many absorption bands.

Fluorescence. In Figure 5A the fluorescence emission spectra of *bT* and *abT* complexes at 77 K are shown as obtained upon excitation at 475 nm, at which wavelength Chl *a* absorption is negligible. The maximum of the fluorescence is located at 678 nm for the *bT* complexes and at 679 nm for the *abT* complexes, which is in both preparations characteristic for Chl *a* fluorescence. This shows that also in the case of the *bT* complexes, energy transfer has occurred from Chl *b* to Chl *a*. The fluorescence peak of the *bT* complexes (FWHM 13 nm) is broader than that of the *abT* complexes (FWHM 8 nm). This is caused by some Chl *b* fluorescence, leading to a shoulder near 665 nm. The *abT* fluorescence spectrum is in good agreement with the spectrum of similar complexes presented in (11). In Figure 5B a Gaussian fit of the short-wavelength part of the fluorescence emission spectrum of the *bT* complexes is

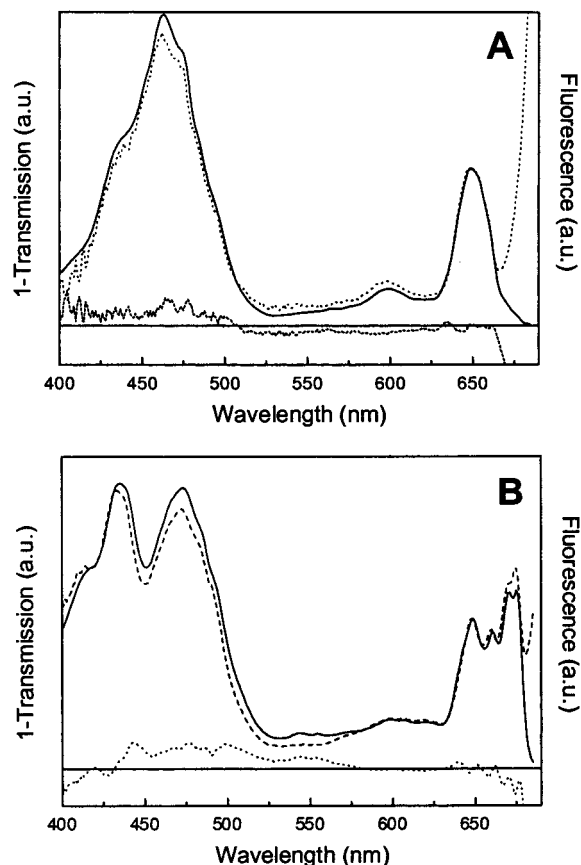


FIGURE 6: (A) Fluorescence excitation spectrum (dash), 1-T spectrum (solid), and difference spectrum (dotted) at 77 K of the *bT* complexes, $\lambda_{\text{det}} > 690$ nm. (B) As in (A), of *abT* complex.

shown, which will be used for the estimation of the relative amounts of Chl *a* and Chl *b* fluorescence (see Discussion).

The fluorescence emission spectrum of *bT* complexes in a buffer containing 0.06% w/v DM showed a peak at ~ 695 nm (data not shown), indicative for the formation of aggregates (23). At a DM concentration of 0.1%, this peak practically disappears (see Figure 5). Therefore, all experiments have been performed at a DM concentration of 0.1% w/v.

In order to further study singlet energy transfer, fluorescence excitation measurements were performed at 77 K. Mainly Chl *a*, but also some Chl *b*, fluorescence was detected with the 690 nm high-pass filter. In Figure 6, fluorescence excitation spectra, 1-T spectra, and the difference of these spectra are shown for *bT* and *abT* complexes. The 1-T and fluorescence excitation spectra are normalized at 650 nm in order to quantify Xan to Chl singlet energy transfer. Since the 1-T and fluorescence excitation spectra are roughly the same both in the Chl *b* Q_y region and in the 400–550 nm region, the amounts of Chl *b* to Chl *a* and of Xan to Chl *a* (possibly via Chl *b*) transfer have to be the same (at least $\sim 45\%$, see Discussion).

Figure 6B shows the spectra of the *abT* complex which have been normalized in the Chl *b* absorption region (650 nm). In the Chl *a* Q_y region, the fluorescence excitation spectrum is higher than the 1-T spectrum. The difference spectrum shows some structure, due to some incomplete Chl *b* to Chl *a* energy transfer combined with the contribution of some scattered excitation light. The ratio of the fluores-

cence excitation and the 1-T spectrum has been determined in the 665–680 nm region, showing that the singlet Chl *b* to Chl *a* transfer efficiency is $\sim 90\%$ (also see Discussion). Since moreover the fluorescence yield of Chl *b* is much lower than that of Chl *a*, Chl *b* hardly contributes to the total fluorescence. Finally, the Xan to Chl *a* transfer efficiency also appears to be $\sim 90\%$.

Triplet Minus Singlet Absorption Difference Spectroscopy. The triplet energy transfer was studied using T-S absorption difference spectroscopy. After selective excitation of chlorophyll at 590 nm with a light flash of several nanoseconds, the transient changes in absorption were measured at many wavelengths. The kinetic traces contained two lifetime components: $\sim 10 \mu\text{s}$ and $\sim \text{ms}$. Due to the short experimental time base used (100 μs), the latter lifetime could not be determined more accurately. With the use of global analysis (19), the corresponding decay-associated difference spectra (DADS) were obtained, and the results are given in Figure 7.

Figure 7A,B shows the spectra of the 10 μs and $\sim \text{ms}$ lifetime components of the *bT* complexes. The shapes of the T-S spectra of the *bM* complexes are similar to those of the *bT* complexes (data not shown). In the Soret region, the spectrum of the 10 μs component shows peaks at 515 and 478 nm and a minimum at 496 nm. Apparently, Chl triplets are quenched effectively by the xanthophylls such as in 'native' LHCII (20), giving rise to xanthophyll triplets (^3Xan) with a lifetime of $\sim 10 \mu\text{s}$. In the Q_y region, also some features with the same lifetime are visible, which will be discussed below. The $\sim \text{ms}$ DADS with minima at 444, 472, and 651 nm is due to the bleaching of Chl *b* molecules in the triplet state. Apparently not all Chl *b* triplets are transferred to Xans. On the contrary, no Chl *a* triplets ($\sim \text{ms}$ lifetime, bleaching 670–680 nm) could be detected. This is striking since it was concluded from the fluorescence spectra that singlet excitation energy transfer from Chl *b* to Chl *a* is rather efficient. Besides the Xan triplets (43%, see Table 2), a significant amount of Chl *b* triplets (57%) is observed which have not been transferred to either Chls *a* or Xans.

In Figure 7C,D the spectra of the *abT* complexes are shown. The 10 μs Xan component shows peaks at 507 and 473 nm, a minimum at 494 nm, and a positive shoulder at ~ 527 nm. This shoulder is also found in the T-S spectrum of 'native' trimeric LHCII whereas it is not observed for 'native' monomeric LHCII. It was tentatively assigned to violaxanthin (3). In the Q_y region, the 10 μs component shows a peak at 677 nm. Besides minima at 472 and 649 nm (Chl *b* triplets), the $\sim \text{ms}$ component shows a minimum at 674 nm, meaning that some of the triplets formed on Chl *a* have not been transferred to the xanthophylls. The estimated percentage of triplets on Xan, Chl *a*, and Chl *b* are 62, 24, and 14%, respectively.

The meaning of the bleaching in the Q_y region with a $\sim 10 \mu\text{s}$ lifetime as observed here and in 'native' LHCII complexes is not yet fully understood. It is not due to chlorophyll triplets but must be ascribed to absorption changes of some of the xanthophylls induced by the triplets on nearby xanthophylls (20). In 'native' LHCII, these signals are observed in the Chl *a* region and not in the Chl *b* region around 650 nm. The 10 μs DADS (open diamonds) in Figure 7B shows a small absorption change at ~ 675 nm. However, the spectrum is too noisy to resolve whether there is also some signal at

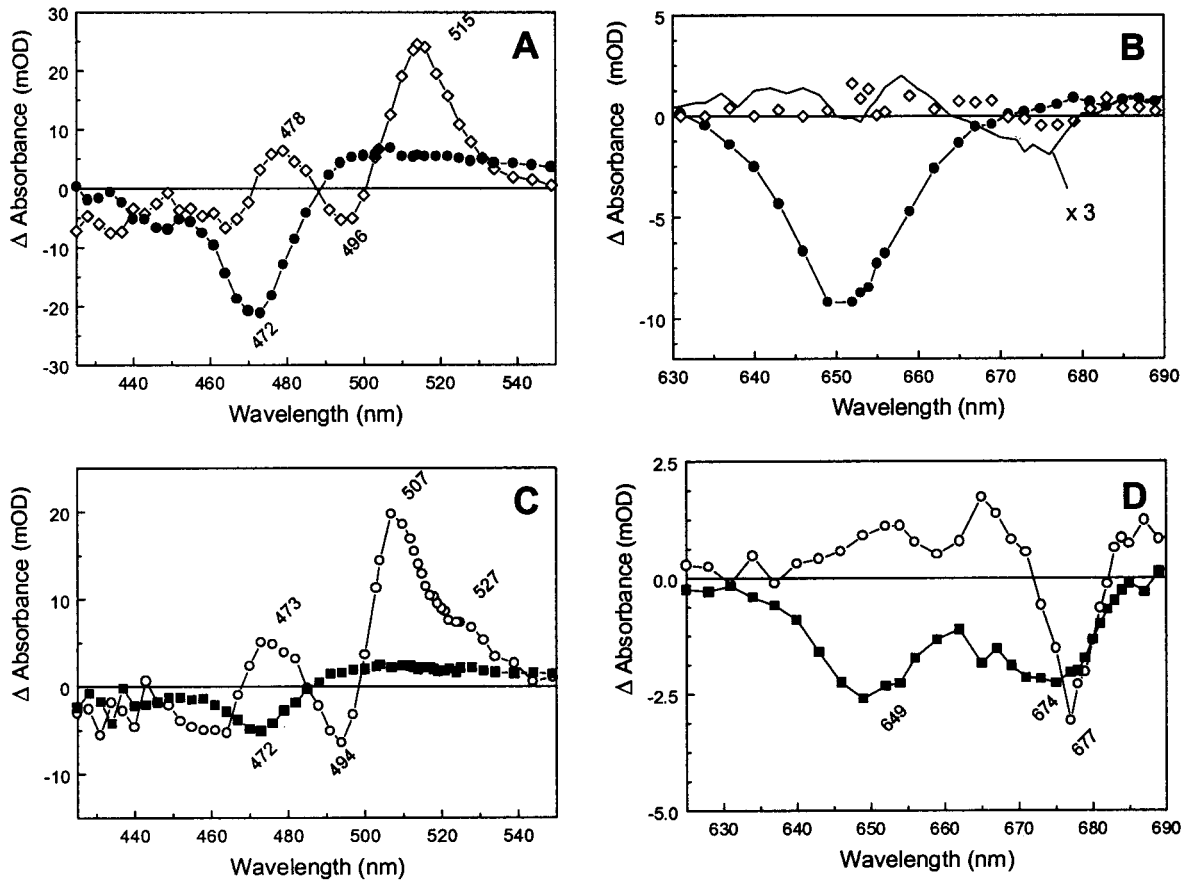


FIGURE 7: (A, B) T-S spectrum of the 10 μ s (open diamonds) spectral component (3 Xan) and the ms (filled circles) spectral component (3 Chl) of *bT* complexes measured at 77 K. The solid line in (B) shows the average 10 μ s spectral component of three reconstituted complexes with very similar pigment compositions (scaled to the peak at 515 nm and subsequently multiplied by 3). (C, D) T-S spectrum of the 10 μ s (open circles) spectral component (3 Xan) and the ms (filled squares) spectral component (3 Chl) of *abT* complexes measured at 77 K.

Table 2: Estimated Relative Amounts of Triplets Located on Different Pigments Calculated on the Basis of the DADS Shown in Figure 7 and the Extinction Coefficients Given under Materials and Methods^a

pigments	<i>bT</i>	<i>abT</i>
% Xan	43	62
% Chl <i>b</i>	57	24
% Chl <i>a</i>	—	14

^a Shown are the numbers for *bT* and *abT* complexes.

~650 nm. Therefore, we have in addition plotted the average of the DADS's of previous T-S measurements on similar complexes (scaled to the peak at 515 nm and subsequently multiplied by 3). The small signal visible at ~650 nm in the averaged spectrum possibly indicates some contact between one or more Chl *b* molecules and xanthophyll(s) which is absent in 'native' LHCII. A plausible explanation for this difference is that in *bT* complexes Chl *b* molecules are binding at sites which are occupied by Chl *a* molecules in 'native' LHCII.

DISCUSSION

Structural and Biochemical Consequences of Replacement of Chl a by Chl b. Here we report on the spectroscopic properties of reconstituted LHCII of which the Chl *a*/Chl *b* ratio has been largely reduced by using small Chl *a/b* ratios in the reconstitution procedure. The formation of reconsti-

tuted LHCII with such varied Chl *a/b* ratios seems to contradict earlier observations on the pigment conditions for in vitro reconstitution since in (6) it was reported that only reconstituted complexes with a 'native' pigment composition could be isolated. The major difference between the present results and the earlier investigations is the level of stringency with which the complexes are isolated after their formation. The rapid removal of dodecyl sulfate by potassium precipitation with parallel exchange by octyl glucoside enables the formation of reconstituted complexes with a much larger flexibility in their pigment composition albeit at a lowered complex stability. Thus, monomeric LHCII can be reconstituted that does not contain any Chl *a* whereas trimers only form when at least one Chl *a* per trimer is present (unpublished data). A more detailed biochemical analysis of such complexes is presently underway.

Spectroscopic evidence as well as biochemical data proves that the isolated complexes with largely reduced Chl *a/b* ratio indeed are in the trimeric state. The formation of pigment-protein complexes is confirmed by the CD spectra of *bT* and *bM* complexes (Figure 3) which are in the Chl *b* and xanthophyll region very similar to that of 'native' trimeric LHCII (13) and far more intense than those of free Chl. Comparison of the CD spectra of *bT* and *bM* complexes reveals a peak at 478 nm in the case of the *bT* complexes. This confirms that trimers are formed since monomeric and trimeric 'native' (reconstituted) LHCII show the same characteristic difference (11, 13).

Furthermore, *bT* complexes show exactly the same DP at 24 kDa as 'native' LHCII when assayed with thermolysin (Figure 1B). It has been shown before that the formation of trimeric complexes leads to a largely protected apoprotein, giving rise to an N-terminally shortened LHCP of 24 kDa, whereas in monomeric LHCII a cleavage site further into the apoprotein can be reached by thermolysin, resulting in the characteristic DP* at 20 kDa (22). The fact that *bT* complexes hardly produce any DP*, which in turn is the main product of *bM* complexes, proves that *bT* and *bM* complexes are folded in a 'native' like manner and indeed are in trimeric and monomeric states, respectively. Although digestion conditions were relatively mild (note some DP in monomeric samples), we still see a small portion of DP* in *bT* complexes indicating a reduced stability as compared to 'native' trimeric LHCII where DP* is completely absent. Also the complete degradation to DP strongly suggests that *bT* complexes are not in an unspecific aggregation state. Aggregation would shield participating complexes and lead to a portion of nondegraded LHCP.

The trimeric complexes studied here, containing Chl *a/b* ratios of respectively 0.7 and 0.03, are characterized by a Chl/protein ratio of 10.5 ± 1.5 and a Xan/Chl ratio of ~ 0.28 , indicating that nearly the full number of Chls and Xans is bound. The fact that neither the Xan composition nor the overall Xan/Chl ratio changes drastically upon the variations in the Chl *a/b* ratio also indicates a more or less correct three-dimensional folding. Furthermore, it suggests (i) a rather specific binding of xanthophylls and (ii) binding of Chl *b* to sites where Chl *a* is supposed to bind. The fact that the Chl *a/b* ratio is increased by a factor of 3 upon complex formation (0.01 vs 0.03) while at the same time several chlorophyll positions can obviously be occupied by either Chl *a* or Chl *b* indicates that binding sites differ with respect to their relative affinity for one of the pigments. This is also confirmed by competition experiments during complex formation, covering a wide range of Chl *a/b* ratios (unpublished experiments).

The red-most Chl *a* molecules show the strongest LD in 'native' LHCII complexes (26), while in *bT* complexes the red-most Chl *b* molecules show the strongest LD, indicating that they have very similar molecular orientations as the Chl *a* molecules at these binding sites in 'native' LHCII. It is possible that the phytol chains of the Chls force the Chls in a specific orientation at each binding site.

Singlet and Triplet Energy Transfer in bT Complexes. It is evident that in *bT* complexes both singlet and triplet energy transfer occur. Excitation at 475 nm, where Chls *b* and xanthophylls absorb but Chl *a* absorption is nearly absent, leads to mainly Chl *a* fluorescence, indicating efficient singlet energy transfer. We tried to estimate the $[\text{Chl } a^*]/[\text{Chl } b^*]$ ratio in the singlet excited state using the 77 K fluorescence spectrum of the *bT* complexes (the asterisk refers to the singlet excited state). In Figure 5B, a Gaussian fit of the short-wavelength part of this spectrum is shown. The maxima of the two Gaussian bands are located at 665 nm (FWHM 13 nm) and 678 nm (FWHM 12 nm), respectively. We ascribe the band at 665 nm to Chl *b* fluorescence. The integrated areas of both bands are then a measure for the relative amounts of Chl *a* and Chl *b* fluorescence which gives 25% of Chl *b* fluorescence and 75% of Chl *a* fluorescence. The fluorescence quantum yields for unbound Chl *a* and Chl

b are 0.22 and 0.06–0.1, respectively, in alcohols or alcohol-containing mixtures (27). When we take the values 0.22 and 0.06 for Chl *a* and Chl *b*, respectively, this leads to 45% of the excitations on Chl *a* and 55% on Chl *b*, giving an upper limit for the relative amount of excitations on Chl *b*. Using a fluorescence quantum yield of 0.1 for Chl *b*, we find that 58% of the excitations are localized on Chl *a* and 42% on Chl *b*. Although this is only a rough estimate, it seems safe to conclude that about half of the singlet excitations on Chl *b* are transferred to Chl *a*.

At this stage we cannot conclude why the other half is not being transferred. It might be that excitations are trapped on Chl *b* because the energy transfer rate of some Chls *b* toward the single Chl *a* in each trimer is of a similar magnitude as or even smaller than the excited state decay rate. This might be tested with time-resolved (polarized) measurements. If the decrease of energy transfer was due to structural differences, they would have to be so subtle that they do not influence the inaccessibility of large parts of the protein to protease. We cannot exclude, however, that binding of Chl *b* at Chl *a* sites causes some reorientation of Chl molecules which then renders some of the Chl *b* molecules less effective in terms of energy transfer to the single Chl *a*. In principle it is also possible that in 50% of the trimers no Chl *a* is present. However, this is inconsistent with the observation that trimers cannot be formed in vitro in the absence of Chl *a*.

From the T–S measurements, it was concluded that $\sim 43\%$ of the triplets which were formed on the chlorophylls (because of selective chlorophyll excitation) are transferred to the xanthophylls. No Chl *a* triplets are observed, and probably they have been transferred to xanthophylls like in 'native' LHCII where the transfer efficiency is $\sim 90\%$ at 77 K (20). In contrast, many Chl *b* triplets are observed, and apparently these triplets cannot be transferred to the xanthophylls. The triplet yield of unbound Chl *b* is slightly higher [0.88, (28)] than that of unbound Chl *a* [0.64, (28)]. Based on the $[\text{Chl } a^*]/[\text{Chl } b^*]$ ratio calculated above, one would expect $[\text{}^3\text{Chl } a]/[\text{}^3\text{Chl } b]$ to be approximately 37%/63% if no xanthophylls would accept the triplets (the superscript 3 refers to molecules in the triplet state). If all Chl *a* triplets would be transferred to xanthophylls, one would expect $[\text{}^3\text{Xan}]/[\text{}^3\text{Chl } b]$ to be 37%/63% (or 50%/50% in case a fluorescence quantum yield for Chl *b* of 0.1 would be used). Since the observed amount of Chl *b* triplets is 57%, the amount of triplet transfer from Chl *b* to Xan is probably very low if present at all. So apparently those Chls *b* which do not transfer their singlet excitations to Chl *a* also do not transfer their triplets to Xan. These might be one or a few "trap" Chls *b* at the periphery of the complex.

Based on the observed pigment stoichiometry, the CD and LD spectra, and the reasonable amount of singlet and triplet transfer, it seems likely that the *bT* complexes adopt a structure which is to a significant extent similar to that of 'native' LHCII. In 'native' LHCII, at least seven chlorophylls are in close contact with the central xanthophylls (4), indicating that in the *bT* complexes many Chl *b* molecules are located next to the xanthophylls. The small features in the Chl *b* absorption region of the average 10 μs T–S DADS spectrum indeed might confirm this. Also in the Chl *a* absorption region (670–680 nm) clear features are observed, pointing to a contact between Chl *a* and xanthophylls which

is required for the apparently very efficient triplet transfer. It should be noted that for 'native' LHCII such signals were also observed for Chl *a* but not for Chl *b* (20).

Singlet and Triplet Energy Transfer in abT Complexes. In the *abT* complexes, efficient Chl *b* to Chl *a* singlet energy transfer occurs (90%). Fluorescence excitation spectra also show efficient singlet energy transfer from Xan to Chl. The amount of triplets transferred to Xan is significantly higher in the *abT* complexes than in the *bT* complexes, namely, 62% vs 43%. The triplet transfer efficiency is lower than in 'native' LHCII monomers and trimers where it is 80% and 90%, respectively, at 77 K (3). In 'native' LHCII, these nontransferred triplets are only formed on Chl *a*. In *abT* complexes, besides Chl *a* triplets also Chl *b* triplets are formed. If triplet transfer to Xan only takes place from Chl *a* and not from Chl *b* (as suggested by the results on the *bT* complexes), the transfer efficiency from Chl *a* is 82%, rather similar to what is found for 'native' LHCII at 77 K. The Chls *b* on which triplets are formed do not transfer their singlet excitations to Chl *a*. One would therefore expect to see some Chl *b* fluorescence. Using the triplet quantum yields for free Chl *a* and *b* (28) one can calculate that 17% of the singlet excitations would be located on Chl *b* and 83% on Chl *a*. From the fluorescence excitation measurements, we estimated the singlet transfer efficiency to be roughly 90%, in reasonable accordance.

Exchange of Chl a with Chl b and Its Functional Implications. A particularly interesting feature in the *bT* complexes is the single Chl *a* molecule that, on the average, is present per trimer. This unique Chl *a* molecule should be more easily characterized spectroscopically than Chls in 'native' LHCII since its signals are not obscured by other Chl *a* molecules. However, the second derivative of the absorption spectrum suggests that there are two distinct signals in the Chl *a* absorption region, indicating that there are Chl *a* molecules in at least two different environments. This is surprising, as we expect one Chl *a* to be bound per trimeric LHCII rather homogeneously since we have been unable to reconstitute trimeric complexes in the absence of Chl *a* and conclude that one Chl *a* per trimer is needed for stability. The heterogeneous absorption signal then means that the single Chl *a* molecule either occupies different binding sites or is bound to complexes adopting different conformational states. The fact that this Chl *a* is required for LHCII trimer stability suggests that it is located near the interface between monomers, at the periphery of a monomeric complex. This view is supported by the fact that the Chl *a* CD differs significantly for monomers and trimers. On the other hand, this molecule shows 100% efficient triplet energy transfer to xanthophylls; therefore, it must be in close contact to one of the xanthophyll molecules. It cannot be excluded that this Chl *a* binding site is not present in monomeric LHCII but formed only upon trimer formation. Then the Chl *a* would most likely be in contact with one of the more peripherally bound xanthophylls that are not seen in the LHCII crystal structure (4). If, on the other hand, this single Chl *a* is bound to one of the Chl positions visible in the LHCII crystal structure, the *a5* binding site (notation as in ref 4) would fulfill both requirements of being close to the monomer interface and to one of the central xanthophylls. It is worth mentioning that the 100% efficient triplet energy transfer from this Chl *a* to Xan is even higher than for

'native' LHCII at 77 K and also higher than for the *abT* complexes in the present study. This illustrates that the single Chl *a* molecule does not arbitrarily bind at one of the available Chl binding sites but has a preference for one or more of these sites.

The results presented in this paper suggest that it is possible to replace most of the Chl *a* molecules in LHCII by Chl *b* without destroying the structure of the complex. However, the spectroscopic data also clearly show that a decrease in the Chl *a/b* ratio strongly affects singlet and triplet energy transfer efficiencies within LHCII. Thus proper functioning of the light-harvesting complex seems to require the correct Chl *a/Chl b* stoichiometry.

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