Selective Interaction between Xanthophylls and Chlorophylls in LHCII Probed by Femtosecond Transient Absorption Spectroscopy

Claudiu C. Gradinaru,[†,‡] **Rienk van Grondelle,**[†] **and Herbert van Amerongen**[*,†,§]

Department of Biophysics and Physics of Complex Systems, Division of Physics and Astronomy, Faculty of Sciences, Vrije Universiteit, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands, and Laboratory of Biophysics, Department of Agrotechnology and Food Sciences, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

We have performed femtosecond transient absorption measurements on trimeric light-harvesting complex II from spinach. Either chlorophyll (Chl) *a* (675 nm) or Chl *b* (650 nm) was excited, and the spectral response was probed for wavelengths longer than 470 nm. Excitation of Chl *b* led to instantaneous bleaching of two distinct Chl *b* bands with absorption peaks at 473 and 486 nm, corresponding to different Chl *b* subpopulations. The latter band probably also contains a contribution from a neoxanthin molecule, which is strongly coupled to Chl *b*. Most of the subsequent energy transfer from Chl *b* to Chl *a* occurs well within a picosecond, but energy transfer from the Chl *b* subpopulation with an absorption peak at 473 nm occurs faster (several hundreds of femtoseconds). Excitation of Chl *b* does not lead to a detectable instantaneous response of the lutein molecules with absorption peaks at 494 and 510 nm. This is in contrast with excitation of Chl *a*, which in turn does not lead to a detectable response of the neoxanthin or Chl *b* molecules. Excitation of Chl *a* leads to concomitant small bleachings of both lutein molecules at 494 and 510 nm (2% and 1% respectively of the Chl *a* bleaching) which we ascribe to excitonic mixing of the S_1 states of the lutein molecules and the Q_y states of the Chl *a* molecules.

Introduction

Light-harvesting complex II (LHCII) is a photosynthetic antenna protein that binds more than 50% of the pigments in photosystem II of green plants and algae.[1,2] Its xanthophylls are efficient quenchers of chlorophyll triplets that can originate on Chl (chlorophyll) *a* molecules[3]. These Chl *a* triplets are potentially dangerous because they can lead to the formation of highly reactive singlet oxygen. In addition, these xanthophylls can "deactivate" singlet oxygen as a sort of backup protection mechanism[3]. Moreover, LHCII is presumably a key player in the mechanism of nonphotochemical quenching (NPQ) that shortens the excited-state lifetimes in photosystem II in order to minimize photoinhibition.[4]

The structure of the trimeric LHCII complex was determined at 3.4 Å resolution, and it shows 12 chlorophylls and 2 xanthophylls per monomeric subunit. These two xanthophylls, located in the center of the subunit near helices A and B, are lutein molecules.[1] In trimeric LHCII, the absorption maximum of the 0−0 $S_2 \leftarrow S_0$ optical transition of one lutein is at 494 nm, whereas that of the other is near 510 nm.[5,6] The energy transfer efficiency from these luteins to Chl is close to 100% for LHCII from spinach.[7] It was recently demonstrated by Gradinaru et al.[8] that this transfer takes almost exclusively place toward Chl *a* molecules and not to Chl *b* molecules, in agreement with earlier measurements with somewhat lower time resolution.[9] Croce et al.[10] studied the same process for recon-

stituted monomeric LHCII and concluded that also some energy transfer toward Chl *b* takes place in these complexes. A third xanthophyll that was not observed in the crystal structure is neoxanthin, and it was found to be located near helix C, next to at least two Chl *b* molecules.[11,12] Its absorption maximum is at 486 nm, and it is somewhat less efficient in transferring energy toward Chl.[7] It was found by Gradinaru et al.[8] that this neoxanthin transfers its energy almost exclusively toward Chl *b*. A fourth xanthophyll, namely violaxanthin, is loosely bound at the periphery of LHCII,[13] and it does not transfer excitation energy toward the chlorophylls.[6] Energy transfer from the xanthophylls occurs predominantly from the S_2 state on a time scale of ca. 100 fs, but ~20% of the transfer occurs via the S_1 state with a time constant of ~1 ps.[8] It was calculated in the same paper that the latter corresponds to a coupling strength of roughly 100 cm^{-1}. It was argued by van Amerongen and van Grondelle[14] that this leads to excitonic mixing of the xanthophyll S_1 state and the chlorophyll Q_y state, which will shorten the excited-state lifetime of LHCII as compared to that of isolated Chl *a*. A lifetime shortening (by 40%) has indeed been observed before.[15] It was hypothesized[14] that an increase of the xanthophyll−chlorophyll coupling strength might be (partly) responsible for the lifetime shortening that occurs during NPQ. A much larger amount of lifetime shortening occurs upon aggregation of LHCII, and it was recently demonstrated that this aggregation is accompanied by a change in xanthophyll−chlorophyll interaction[16] although it could not be proven that the change in interaction is responsible for this decrease. Therefore, a study of the xanthophyll−chlorophyll interactions and the excitonic mixing of the aforementioned states is of interest.

The strong xanthophyll−chlorophyll interactions are also manifested by efficient triplet transfer from Chls *a* to xantho-

* Corresponding author. Phone: +31-317-482634. Fax: +31-317-482725. E-mail: Herbert.vanAmerongen@wur.nl.
† Vrije Universiteit.
‡ Present address: Rijksuniversiteit Leiden, Department of Biophysics, Huygens Laboratory, Leiden University, Niels Bohrweg 2, 2300 RA Leiden, The Netherlands.
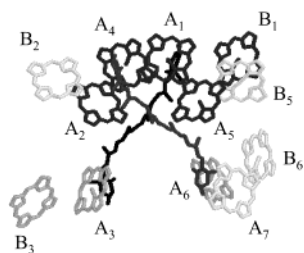§ Wageningen University.

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Figure 1. Organization of the pigments in a monomeric subunit of LHCII. The binding sites are numbered according to Kühlbrandt et al. (ref 1). Dark pigments are presumably Chl *a* molecules, gray ones can be either Chl *a* or Chl *b*, and white ones are Chl *b* (see text for additional explanation).

phylls. It was demonstrated with triplet-minus-singlet (TmS) measurements[7,17,18] that this transfer predominantly takes place toward the xanthophylls that are responsible for the absorption peaks at 494 and 510 nm and which are now known to be luteins.[5,19] No evidence for any triplet transfer to neoxanthin could be found. A remarkable observation was the fact that the presence of a triplet on a xanthophyll led to a change in absorption (mainly a bleach) in the Chl *a* region, indicating again the interaction of the luteins and Chl *a* molecules.[7,17,18,20,21] A similar change in the Chl *b* absorption was not observed. Both the femtosecond transient absorption measurements and the triplet-minus-singlet spectroscopy therefore demonstrate the close interactions between luteins and Chls *a*. It is of interest to note that from studies on reconstituted monomeric LHCII in which chlorophyll binding sites were mutated it was concluded that not all chlorophylls that are in van der Waals contact with the two central xanthophylls (Chls at sites A1−A7, see Figure 1) are Chl *a* molecules.[22] Whereas it was concluded that the Chls on positions A1, A2, A4, and A5 are indeed Chl *a* molecules, sites A3 and A6 did not show any preference for Chl *a* or Chl *b* (similar binding affinity), and site A7 appeared to bind Chl *b*. Despite an average number of two Chl *b* molecules per monomer being in contact with the central xanthophylls in those preparations, we have not detected any spectroscopic evidence for these contacts in the case of trimeric LHCII from spinach.[7,8,9,17] It might be that this is (partly) due to a difference in structure/composition of our isolated trimeric LHCII complexes and the monomeric reconstituted complexes.[22] The fact that Croce et al.[10] detected some energy transfer from lutein to Chl *b*, although the amount was small, might point in such a direction. Alternatively, it is possible that the coupling strength between the central xanthophylls and the Chls at positions A1, A2, A4, and A5 is much larger than that between the Chls at sites A3, A6, and A7 and these xanthophylls, making it difficult to spectroscopically probe interactions between the latter molecules. The latter explanation is supported by the fact that recent photon-echo experiments in our lab confirm the presence of strongly coupled Chl *b* pairs in trimeric LHCII from spinach, implying that also at least one of the sites A1−A7 can also bind a Chl *b* molecule.[23,24]

In analogy with the TmS spectroscopy measurements[7,17] in which the presence of xanthophyll triplet states led to a change in the absorption spectrum of LHCII in the Chl *a* region, we have now performed femtosecond pump−probe spectroscopy in order to investigate whether excitation of the Chl *b* or Chl *a* lowest singlet states leads to a spectral response in the xanthophyll region. This will shine additional light on specific chlorophyll−xanthophyll contacts and may also provide valuable information for estimating the coupling strength. Note that we performed a similar, preliminary study before on a different

experimental setup with longer excitation pulses and higher excitation intensities.[25]

Materials and Methods

Sample Preparation. Trimeric LHCII was purified from spinach according to the method described elsewhere,[17] using anion-exchange chromatography and the detergent *n*-dodecyl β,D-maltoside (DM) for solubilization of the complexes. Since the measurements were performed at 77 K (Oxford cryostat, DN1704), LHCII was diluted in a buffer containing 20 mM Hepes (pH 7.5), 0.06% (w/v) DM, and 70% (v/v) glycerol. For pump−probe experiments, the absorption at the Q_y maximum was between 0.7 and 1 in a 1-mm cuvette.

Laser Setup. The transient absorption spectra were recorded on a 1 kHz home-built femtosecond spectrometer as described elsewhere.[8] For selective excitation of the Chl *a* and *b* Q_y states, the output of the noncollinear optical parametric amplifier was tuned to 650 and 675 nm, respectively. Because the amplified pulses owned a spectral width in the range of 50 nm, additional interference filters were used to limit the width to ∼20 nm, before these pulses excited the sample. Their temporal width, estimated from autocorrelation traces, was 100−120 fs. A small fraction of the regenerative amplifier 800 nm output was sent through a variable delay line (minimal steps of 0.1 μm) and then focused on a sapphire plate in order to generate a white light continuum as probe light. This light was split into two almost identical beams, labeled signal and reference, as the former overlapped with the pump and the latter did not. All three beams, i.e., pump, signal, and reference, are focused to a spot size of ∼120 μm in the sample. The polarization of the pump beam was set to the magic angle (MA) relative to that of the probe beams, by using a Berek prism compensator in order to eliminate anisotropic contributions to the signal.

For each excitation wavelength, two separate sets of data were combined to yield a final probe window between 460 and 710 nm. Between 200 and 400 delays were sampled, up to a maximum delay of a few hundreds of picoseconds. Pump intensities of 30−100 nJ were generally used, corresponding to excitation densities of 10^{14}−10^{15} photons/pulse/cm^2. The maximum absorption changes in our experiments were around 60 × 10^{-3}, with a noise level of less than 0.5 × 10^{-3}.

Data Analysis. The time-gated spectra were analyzed with a global fitting program as described.[26] Species-associated difference spectra (SADS) were determined assuming a sequential, irreversible model A → B → C → D. The arrows symbolize increasingly slower monoexponential processes, with time constants that can be regarded as the species lifetimes. This picture enables us to visualize clearly the evolution of the excited states in the system, although the calculated SADS need not be associated with "pure states". The instrument response function, fitted with a Gaussian profile, had fwhm values of 100−120 fs. The probe pulse dispersion was fitted to a third-order polynomial function of the wavelength, and the obtained parameters agreed very well with those found for several other experiments performed under similar conditions.

Results and Discussion

Spectral Response of Xanthophylls upon Excitation of Chl *a* Molecules in LHCII. In order to observe the xanthophyll spectral response following Chl *a* excitation, we excited Chl *a* at 675 nm with pulses of ∼100 fs and recorded the absorption difference spectra between 475 and 600 nm. The data were globally analyzed, and three exponentials were needed to obtain a satisfactory fit of the data. The corresponding species-
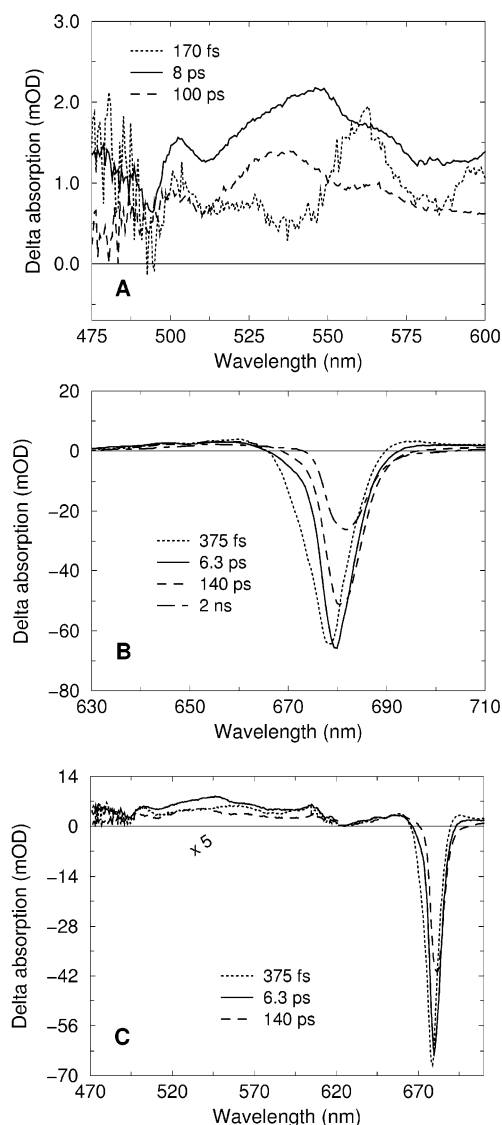
Figure 2. (a)−(c) Species-associated difference spectra that are obtained from fitting the 77 K transient absorption data for LHCII in the indicated wavelength interval, excitation wavelength is 675 nm.

associated difference spectra (SADS) are shown in Figure 2a. The instantaneously observed spectrum transforms very rapidly with a time constant of 170 fs into the next SAD spectrum, which is replaced with a time constant of 8 ps by the third one. This spectrum then disappears with a long time constant, and the corresponding decay time was fixed to 100 ps in the fit. The measuring time window did not allow an accurate estimate of the corresponding decay time, and in reality, this time is longer (see later). The SADS are all positive, which should be ascribed to excited-state absorption (ESA) of the Chl *a* molecules. In the wavelength region where the xanthophylls absorb (between 475 and 525 nm), there are two very clear negative dips built on the positive ESA background, which are particularly clear for the second and third SADS. The first SAD spectrum is rather noisy, which is due to the fact that the corresponding lifetime is rather short, and therefore, its shape is only determined by the very first experimental spectra. The positions of the dips can most accurately be determined for the second SADS, which contains the least noise, and they turn out to be at 494 and 511 nm. These features are ascribed to the lutein molecules (see later). There are also some negative dips above 550 nm, and they must most probably be ascribed to the bleaching of Chl Q_x and vibronic bands of Chl *a,* since the

xanthophylls do not absorb in this wavelength region. The decrease in intensity of the transient absorption spectrum upon going from the second to the third SADS is mainly due to singlet−singlet annihilation, which leads to the disappearance of excited states on this time scale.[27−29] The presence of annihilation can be observed much better in the Q_y region. Figure 2b shows the spectral evolution in the Q_y region after excitation at 675 nm. In this case, four SADS are needed to obtain a satisfactory fit of the data. The large signals in this wavelength region provide a much better signal-to-noise ratio, which is partly responsible for the fact that more kinetic details can be observed than in the absorption region below 600 nm. One should, however, realize that energy transfer steps between Chl *a* spectral forms that differ in the Q_y region are not necessarily observable in the region below 600 nm. The spectral evolution in the Q_y region shows characteristics of energy transfer (i.e., a red shift) on a femtosecond and picosecond time scale in agreement with previous studies.[27,28] In addition, it is observed that the intensity of the bleaching decreases, which should be ascribed to singlet−singlet annihilation.

It is of interest to compare the size of the bleaching of Chl *a* and the size of the negative dips which are ascribed to luteins. Therefore, we performed a global analysis over the entire wavelength region from 470 to 710 nm after normalizing to the pump intensities. The result is given in Figure 2c, where 3 SADS were used to obtain a reasonable fit. We did not fit with 4 components because this leads to a lower signal-to-noise ratio for the individual SADS, thereby giving less accurate values for the relative sizes of the Chl and xanthophyll signals, whereas the exact time constants are not essential for obtaining these ratios. The most accurate ratios can be obtained from the SADS with lifetime 6.3 ps. It should be noted that the shape of this spectrum is not significantly influenced by singlet−singlet annihilation, the amount of which possibly differs for both wavelength regions. The Q_y bleaching/SE (stimulated emission) band has a magnitude of -64×10^{-3} (-64 mOD, OD = optical density). The size of the 494 and 511 nm dips can only roughly be determined because of the ESA background. We estimate a size of -0.6×10^{-3} for the 494 nm band and -0.3×10^{-3} for the 511 nm band, corresponding to ratios of 0.02 and 0.01, respectively, with respect to the Q_y bleaching of Chl *a* (the blue side of the spectrum was scaled up by a factor of 5, and the true bleaching was assumed to be half of the negative Q_y band, the other half being due to stimulated emission[30]).

Spectral Response of Xanthophylls upon Excitation of Chl *b* Molecules in LHCII. In order to obtain the xanthophyll spectral response after Chl *b* excitation, we excited Chl *b* at 650 nm with pulses of ∼100 fs and recorded the absorption difference spectra. The absorption difference spectrum was probed between 460 and 580 nm. The data were globally analyzed, and four exponentials were needed to obtain a satisfactory fit of the data. The corresponding species-associated difference spectra (SADS) are shown in Figure 3a. The instantaneously observed spectrum transforms very rapidly with a time constant of 220 fs into the next SADS, which is replaced with a time constant of 750 fs by the third SADS. Finally, this spectrum transforms into the fourth SADS with a time constant of 14.8 ps. This spectrum then disappears with a long time constant, and the corresponding decay time was fixed to 1 ns in the fit. The first two SADS are negative below 500 nm, mainly because of bleaching of the originally excited Chl *b* molecules, which absorb in this wavelength region. Above 500 nm, ESA can be observed. The two SADS at later times reflect the situation after most of the excitations have been transferred
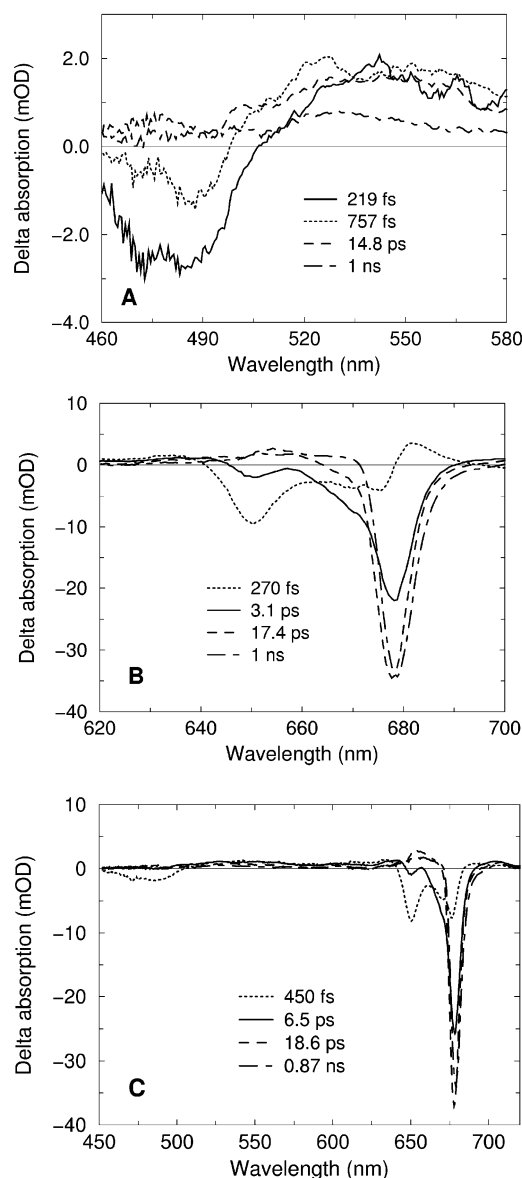
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Figure 3. (a)−(c) Species-associated difference spectra that are obtained from fitting the 77 K transient absorption data for LHCII in the indicated wavelength interval, excitation wavelength is 650 nm.

to Chl *a*. It is well-known that most of the transfer rates corresponding to this process are below 1 ps.[27] The third SAD spectrum resembles indeed the SAD spectrum of Figure 2a, although the intensities are lower in this case, because of lower pump intensities. In the wavelength region where the xanthophylls and Chl *b* molecules absorb (between 460 and 525 nm), two negative features can be observed for the first and second SADS. The positions of the negative features are at 473 and 486 nm in the first SAD spectrum. The position of the second feature is approximately 487 nm in the second SAD spectrum. The position of the first feature cannot accurately be determined, but it is clear that bleaching is still present around 473 nm, albeit that the amount of bleaching has decreased.

Figure 3b shows the spectral evolution in the Q_y region after excitation at 650 nm. Also in this case, four SADS are needed to obtain a satisfactory fit of the data. The spectral evolution in the Q_y region shows that the largest fraction of Chl *b* excitations are transferred to Chl *a* within 1 ps, whereas a much smaller fraction is transferred with a time constant of several ps, in agreement with previous studies.[27,31,32]

It is also of interest to compare the size of the bleaching/SE in the Q_y and Soret region. Therefore, we performed again a global analysis of the entire wavelength region from 450 to 710 nm after normalizing to the pump intensities. The result is given in Figure 3c, where four SADS were needed to obtain a reasonable fit. The intensities of the negative features at 473 and 486 nm have approximately equal size (−1.7) whereas the size of the Chl bleaching/stimulated emission around 650 nm is −8.0. Assuming again that half of the latter signal is due to stimulated emission, the ratio of the bleaching of the either Soret band and the Q_y band is ∼0.4.

Absorption Characteristics of LHCII Pigments in the Soret Region. In order to interpret the optical response in the Soret region upon femtosecond excitation in the Chl Q_y region, the absorption characteristics of the various pigments in LHCII need to be addressed. Analysis of the absorption spectrum is a difficult issue, because of the strong spectral overlap of the various chromophores. An elaborate room temperature study was performed by Croce et al.[33] They compared the absorption properties of reconstituted monomeric LHCII complexes for which the pigment composition was varied in a controlled manner in different ways: either mutants were used which were lacking specific Chl-binding amino acids, or the pigment composition in the reconstitution mixture was changed. It was concluded that there are at least three spectral forms present for Chl *b* with absorption peaks at 467, 476, and 482 nm. Chl *a* only shows a weak absorption tail above 460 nm. Neoxanthin has its main 0−0 S_2 absorption peak at 488 nm. For the luteins, they are located at 489 and 495 whereas violaxanthin has a peak at 492 nm. The peak positions are not at all identical to those of our trimeric LHCII complexes from spinach, measured at 77 K. These preparations show clear xanthophyll absorption peaks near 486, 494, and 510 nm.[7] The former one was assigned to neoxanthin, and the peak position is close to the one for reconstituted monomeric LHCII.[33] Also, Ruban et al.[5] found that the maximum of neoxanthin is located at 486 nm for trimeric LHCII from spinach. The 510 nm peak is the weakest one of the three, and it was originally assigned to violaxanthin because the amount of violaxanthin was significantly less than that of the other xanthophylls.[7] Finally, the 494 nm band was assigned to lutein. However, it was recently shown by Ruban et al.[5] that the 510 nm band is due to one of the two luteins, and this was confirmed by Caffarri et al.[6] The apparently low intensity of the absorption band was explained by a relatively large width of the band.[19] The absorption maximum of the other lutein was found to be located at 495 nm. Two different populations of violaxanthin molecules were found in thylakoid preparations with maxima at 488 and 497 nm, and it is unclear which one most likely corresponds to the violaxanthin molecule in our preparation. However, this uncertainy is irrelevant for the results in the present study because violaxanthin in isolated LHCII does not appear to be in close contact with any chlorophylls since no energy transfer from violaxanthin to chlorophyll takes place.[6]

In conclusion, only the neoxanthin and lutein molecules of LHCII are in contact with chlorophylls, and in the spinach trimeric preparation, their absorption maxima are located at 486 nm (neoxanthin), 494−495 nm (lutein), and 510 nm (lutein). Chl *a* does not show appreciable absorption in the wavelength region of interest (above 460 nm), whereas several Chl *b* spectral forms are probably present.

Origin of the Spectral Changes in the Soret Region upon Excitation of Chlorophyll Molecules. Excitation of Chl *a* in LHCII in the Q_y region leads to an instantaneous response in

the Soret region, notably to bleaching at 494 and 511 nm. As pointed out already, these wavelengths correspond to the maxima of the two lutein molecules. No optical response of the neoxanthin molecule (maximum at 486 nm) could be detected. This is in line with the literature results which were pointed out in the Introduction: (1) Both luteins transfer singlet excitations exclusively to Chl *a*, not to Chl *b*, whereas the reverse is the case for neoxanthin. (2) Triplets that are formed on Chl *a* molecules are transferred to both luteins, not to neoxanthin. (3) Triplets that are located on luteins lead to an optical response (bleaching) of Chl *a* molecules, not of Chl *b* molecules. All these results indicate that Chl *a* molecules are strongly coupled to lutein molecules, not to neoxanthin, whereas lutein molecules are only strongly coupled to Chl *a* molecules and not to Chl *b* molecules, at least within the signal-to-noise ratios of our experiments. This seems to be at odds with the findings of Remelli et al.[22] that on average two of the seven Chls that are in contact with the central luteins are Chl *b* molecules. One possible explanation could be that the Chls at positions A1, A2, A4, and A5 are much more strongly coupled to the central luteins than the ones at positions A3, A6, and A7 (note that Chl *b* molecules can only bind at these sites, not at the former ones), although all of them appear to be in van der Waals contact with the luteins. In that case, it is not only the edge-to-edge distance between lutein and Chl molecules that determines the interaction strength but also the precise parts of the respective molecules that are in contact and their relative orientations. At this point, theoretical calculations seem to offer the most likely way for resolving this problem. It is of interest to note that Herek et al.[34] performed a femtosecond transient absorption study on LH2 from *Rhodobacter sphaeroides* that is similar to the one that is described here. They also detected an optical response of the carotenoids in LH2 upon excitation of BChl *a*. However, in that case, the response was not instantaneous and showed a rise time of 100−200 fs, in contrast to our results, in which the response is instantaneous or at least well within the instrument response time of 100−120 fs. In principle, it is conceivable that an inaccuracy in the dispersion correction could have led to an error in estimating time zero in the Soret region. However, the fact that the Chl *b* bleaching in the Soret region upon Chl *b* excitation at 650 nm is instantaneous rules out this possibility (see later). Therefore, it seems most likely that the optical response of the xanthophylls in LHCII has a different origin than that of the carotenoids in LH2, although this cannot be concluded with absolute certainty.

It is found that excitation of Chl *b* molecules at 650 nm leads to strong bleaching signals in the Soret region with peaks at 473 and 486 nm. It is unavoidable that direct excitation of Chl *b* leads to immediate bleaching of the Chl *b* absorption in the Soret region. Therefore, the observed bleaching should largely be ascribed to Chl *b*. Also, the size of the bleaching supports this conclusion. The amount of bleaching at 473 and 486 nm is equal to approximately 21% for both bands as compared to the amount of bleaching/SE at 650 nm, although this percentage may be even higher due to the ESA background. The amount of bleaching at 650 nm is approximately equal to 50% of the signal; the remaining part should be ascribed to stimulated emission. Therefore, the integrated amount of bleaching in the Soret region is close to that at 650 nm. Given the fact that the Chl *b* Soret band is more intense than the Q_y absorption band, and the fact that some ESA occurs in the region below 500 nm thereby lowering the apparent amount of bleaching, we conclude that the optical response in the Soret region is in this case largely due to direct bleaching of Chl *b*. Therefore, it appears that Chl

b in trimeric LHCII from spinach is responsible for at least two distinct absorption bands in the Soret region at 473 and 486 nm. These values differ significantly from those of reconstituted monomeric LHCII where Chl *b* absorption peaks were found at 467, 476, and 482 nm.[33]

However, we cannot rule out the possibility that also some bleaching of the Neo occurs, which is known to be in contact with several Chl *b* molecules (see a preceding discussion) and which is at least partly responsible for an absorption peak at 486 nm, coincident with the maximum amount of bleaching. It is in fact almost unavoidable that strong excitonic interactions are present, given the fact that the strongly allowed transitions of Chl *b* and neoxanthin in this wavelength region are so close together and the fact that neoxanthin transfers its excitation energy extremely rapidly to Chl *b*.[8] Upon transfer of the excitations from Chl *b* to Chl *a*, the bleaching in the Soret region does not disappear uniformly. The Chls that are responsible for the 473 nm bleaching transfer their excitations more rapidly than the ones that are responsible for the 486 nm bleaching. Finally, it is worth mentioning that following Chl *b* excitation there is no evidence for any optical response at 494 and 510 nm (luteins), in agreement with the stated conclusion that no strong interactions can be observed between lutein and Chl *b* molecules.

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