# Identifying the Pathways of Energy Transfer between Carotenoids and Chlorophylls in LHCII and CP29. A Multicolor, Femtosecond Pump–Probe Study

Claudiu C. Gradinaru,<sup>\*,†</sup> Ivo H. M. van Stokkum,<sup>†</sup> Andy A. Pascal,<sup>‡</sup> Rienk van Grondelle,<sup>†</sup> and Herbert van Amerongen<sup>†</sup>

Faculty of Sciences, Division of Physics and Astronomy, Department of Biophysics and Physics of Complex Systems, Vrije Universiteit Amsterdam, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands, and Universitá di Verona, Facoltá di Scienze MM.FF.NN., Biotechnologie Vegetali, Strada Le Grazie, I-37134 Verona, Italy

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Spectral and kinetic information on energy transfer from carotenoids (Cars) to chlorophylls (Chls) within light-harvesting complex II (LHCII) and CP29 was obtained from femtosecond transient absorption study by using selective Car excitation (489 and 506 nm) and detecting the induced changes over a wide spectral interval (460-720 nm). By examining the evolution of entire spectral bands rather than looking at a few single traces, we were able to identify the species (pigments and/or electronic states) which participate in the energy flow, as well as the lifetimes and quantum yields of individual processes. Hence, it was found that the initially excited Car S<sub>2</sub> state decays very fast, with lifetimes of 70–90 fs in CP29 and 100  $\pm$  20 fs in LHCII, via two competing channels: energy transfer to Chls (60-65%) and internal conversion to the lower, optically forbidden S<sub>1</sub> state (35-40%). In CP29, the energy acceptors are exclusively Chls a, while in LHCII, this is only valid for lutein and violaxanthin. In the latter case, neoxanthin transfers energy mostly to Chls b. In both complexes, ca. 15–20% of the initial Car excitations are transferred to Chls a via the  $S_1$  level, with a time constant of around 1 ps, thus bringing the total Car-Chl transfer efficiency to ca. 80%. Given the yield of this process and the large difference between the transfer time and the intrinsic S<sub>1</sub> lifetime ( $\sim$ 20 ps), it seems that lutein is the only species active on this pathway. From the measured transfer rates, we estimated that a coupling of 280-330 cm<sup>-1</sup> drives the transfer via the S<sub>2</sub> route, while a coupling value of around 100 cm<sup>-1</sup> was estimated for the  $S_1$  transfer. The Car  $S_2$  state is coupled to both  $Q_x$  and  $Q_y$  states of the Chl through a Coulombic mechanism; from the available structural information, we estimated the dipole-dipole contribution to be  $450-500 \text{ cm}^{-1}$ . The S<sub>1</sub> state is coupled to the Chl a Q<sub>y</sub> transition via an exchange and/or a Coulombic mechanism.

## Introduction

In photosynthetic organisms, solar energy is collected by (bacterio)chlorophylls (Chls) and carotenoids (Cars) bound to proteins. These pigment-protein complexes serve as antennae for another class of proteins, the reaction centers, to which the excitation energy is delivered to drive the primary reactions of photosynthesis.<sup>1</sup> The most abundant antenna protein of higher plants is the light-harvesting complex II (LHCII), which usually transfers energy to the photosystem II (PSII) reaction center. In its native form, LHCII is a trimeric membrane protein, and chemical analysis has shown that each monomer binds two types of Chls-7-8 Chls a and 5-6 Chls b-and three types of Cars-2 luteins (Lut), 1 neoxanthin (Neo), and ca. 0.3 violaxanthin (Vio).<sup>2,3</sup> CP29 is another member of the LHC family, which occupies an intermediate location between LHCII and the core PSII region.<sup>4</sup> It is a monomeric complex, and its protein sequence is highly homologous to that of LHCII, binding fewer chromophores—6 Chls a, 2 Chls b, and 2 Cars.<sup>5,6</sup>

Besides their role in increasing the cross section of absorption in the blue-green spectral window,<sup>2</sup> the Cars in these antennae are essential to photoprotection. They quench Chl triplet states very efficiently,<sup>7,8</sup> thus preventing the formation of potentially dangerous singlet oxygen. Non-photochemical quenching mechanisms, which control the energy flow to and from the reaction center, are also known to involve the xanthophyll cycle Cars, i.e., Vio and zeaxanthin.<sup>9</sup> Finally, the carotenoids are vital for stabilizing the structure and are an absolute requirement for the reconstitution of both LHCII and CP29.<sup>3,10</sup>

The optical properties of the carotenoids are determined by the conjugated  $\pi$ -electron backbone of their polyene chain. It has been shown by both theory and experiment that the Cars possess two low-lying singlet states in the visible range, denoted  $S_1$  and  $S_2$ , which play a major role in the photochemistry of the Cars.<sup>11–13</sup> The lower of these states,  $S_1$ , has the same inversion symmetry  $(A_{e})$  as the ground-state S<sub>0</sub> in the idealized  $C_{2h}$  point group. Consequently, electronic transitions between these two states are forbidden by symmetry. However, electronic transitions between the ground state and the S<sub>2</sub> state are allowed, since  $S_2$  has a different inversion symmetry  $(B_u)$ . The strong absorption in the 350-550 nm region characteristic of all polyenes and Cars is attributed to the  $S_0 \rightarrow S_2 (1^1A_g \rightarrow 1^1B_u)$ transition. The energy of the  $S_2$  state can be readily obtained from the absorption spectrum, and it is strongly dependent on the polarizability of the solvent through dispersion interaction.<sup>14</sup> Its lifetime is very short, usually between 100 and 200 fs, as it relaxes via internal conversion (IC) to the S1 state. The energy

<sup>\*</sup> Corresponding author. Tel.: (+31) 20 4447931. Fax: (+31) 20 4447999. E-mail: klaus@nat.vu.nl.

<sup>&</sup>lt;sup>†</sup> Vrije Universiteit Amsterdam.

<sup>&</sup>lt;sup>‡</sup> Universitá di Verona.

of the  $S_1$  state can be directly measured for short Cars (with fewer than nine carbon–carbon double bonds) from the (weak) fluorescence emission spectra. This becomes increasingly difficult for longer, naturally occurring Cars because of the increased rate of the  $S_1 \rightarrow S_0$  IC, which reduces the fluorescence quantum yield to ca.  $10^{-6}$ .<sup>11,12</sup> Alternatively, the  $S_1$  energy can be estimated from its lifetime, measured in transient absorption experiments, using the "energy-gap law".<sup>15</sup> Unlike the  $S_0 \rightarrow S_2$  transition, the  $S_0 \rightarrow S_1$  transition is hardly affected by the environment because of its near-zero associated dipole.

As a result of its involvement in the xanthophyll cycle, Vio has been the most studied carotenoid among the species occurring in LHCII and CP29. The  $S_1$  lifetime is 23–25 ps, as measured by transient absorption spectroscopy of the  $S_1 \rightarrow S_N$ transition in hexane<sup>16</sup> and the  $S_1 \rightarrow S_2$  transition in methanol.<sup>17</sup> Frank et al. estimated the Vio  $S_1$  level to be at 15 120 cm<sup>-1</sup> by using an empirical "energy-gap" formula deduced for shorterchain carotenoids,<sup>16</sup> but direct measurement of the  $S_1 \rightarrow S_2$ transition energy by Polivka et al. led to a significantly lower value, 14 470 cm<sup>-1</sup>.<sup>17</sup> More recently, a value of 14 880 cm<sup>-1</sup> was deduced from fluorescence spectroscopy.18 Time constants of the IC from S<sub>1</sub> to the ground state were also reported for Lut and Neo, 14.6 and 35 ps, respectively.<sup>20,21</sup> The lifetime of the S<sub>2</sub> state in the absence of energy transfer was measured only for Vio in solution ( $\sim$ 320 fs<sup>17</sup>). However, while the S<sub>1</sub> lifetimes in the protein are expected to be very close to those determined in solution, this is probably not the case for the  $S_2$  state (see above).

Several time-resolved studies performed on LHCII have shown that all the energy absorbed by the Chl b molecules is transferred very efficiently to Chls *a* in less than 5 ps.<sup>22–25</sup> Therefore, only these pigments should be protected against triplet formation by being in close contact with the Cars. This was the argument which led Kühlbrandt and co-workers to assign the Chls in their 3.4 Å structural model: the seven closest neighbors to the two central Cars were assigned to Chls a, while the remaining five were assigned to Chls b.<sup>26</sup> The Car molecules were assigned to the two Luts identified by HPLC analysis, although recent studies on reconstituted complexes have shown that only one of these sites is occupied exclusively by Lut while the other can accommodate both Lut and Vio<sup>3</sup> (but with a strong preference for Lut<sup>28</sup>). Moreover, there is evidence for the existence of two other (peripheral) Car binding sites, which exhibit high affinity for Neo and Vio.27-29 Analysis of triplet spectra has also demonstrated that there are several Car species involved in the triplet quenching process.<sup>7,8</sup> Together, all these facts casted serious doubt on the original pigment assignment in LHCII.

One of the motivations for studying Car–Chl excitation energy transfer (EET) is to improve our knowledge of pigment arrangement and function in LHCII. Two time-resolved studies which deal with this issue are available in the literature, but they lead to contradicting conclusions. Connelly et al.<sup>30</sup> found that the energy absorbed by Cars is preferentially transferred to Chls *b* with a time constant of ca. 150 fs, and they suggested that the conventional assignment should be modified to accommodate about two Chls *b* near the central Luts. No such evidence was found in a similar study by Peterman et al.,<sup>31</sup> who argued that Chls *a*, not Chls *b*, are the primary acceptors, and thus, the results do not conflict with the original assignment.

In this contribution, we will deal with the issue of singlet energy transfer from Car to Chl in LHCII by using multicolor pump-probe spectroscopy. A new laser setup (see below) enabled us to examine the complex evolution of the states

formed by excitation at around 500 nm, as transient absorption data over a wide spectral interval were recorded with femtosecond resolution. By examining the evolution of entire spectral bands rather than looking at a few individual traces, we were able to single out the species (pigments and/or electronic states) which participate in this process, and thereby, we obtained much more reliable lifetimes. In our approach, we also investigated the CP29 complex, most likely containing a subset of the chromophores bound to the LHCII protein.<sup>5,6</sup> There is some evidence that CP29 binds Cars only at the two central sites<sup>5</sup> and that the Chls *b* are not located near them.<sup>5,32</sup> Therefore, we expect that the Chls b are not implicated in the Car-Chl EET pathway, unlike in LHCII, where such a possibility may arise. Our goal is to determine the involvement of the Car states  $(S_1,$  $S_2$ ) in the energy flow, the identity of the Chl acceptors, and the quantum yields of the individual processes.

#### **Materials and Methods**

**Sample Preparation.** Trimeric LHCII was purified from spinach<sup>7</sup> using anion-exchange chromatography and the detergent *n*-dodecyl  $\beta$ ,D-maltoside (DM) for solubilization of the complexes. This preparation is essentially free of monomeric, minor LHC complexes (CP29, CP26, and CP24), as confirmed by gel electrophoresis. CP29 was prepared using a protocol<sup>6</sup> based on column chromatography of Tris-washed PSII membranes. The homogeneity was tested by a combination of gel electrophoresis and immunological analysis. Since all the measurements were performed at 77 K (Oxford cryostat, DN1704), both LHCII and CP29 were 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<sub>v</sub> maximum was 0.6–0.7 in a 1 mm cuvette.

Laser Setup. The transient absorption spectra were recorded on a 1 kHz home-built femtosecond spectrometer. A diodepumped Nd:YAG laser (2W Verdi Coherent) was used for pumping a Ti-sapphire oscillator (Mira-Seed Coherent), which produces 50 fs pulses at 800 nm. The pulse energy is amplified from nanojoules to millijoules using a kilohertz amplifier system (Alpha-1000 US, B.M. Industries) based on the principle of chirped-pulse amplification. First, the pulse duration is enlarged to about 200 ps in the stretcher module by using a single grating with an Öfner triplet as the afocal device. The special design of the optical components in the stretcher ensures that the large spectral bandwidth (ca. 45 nm) is preserved during this step. The regenerative amplifier module consists of a Ti-sapphire crystal as a gain medium, pumped at 527 nm by a 621-D CWpumped, frequency-doubled Nd:YLF laser (B.M. Industries). This laser functions in Q-switched regime at a repetition rate of 1 kHz (available range, 1-5 kHz), producing 0.5 µs pulses with a stable, high energy of about 10 mJ/pulse. Only one Pockels cell is used in the regenerative amplifier as a polarization switch to control both the injection of the low-energy seed pulse (ca. 0.5 nJ) and the ejection of the high-energy amplified pulse (ca. 1 mJ) into and out of the amplifier cavity, respectively. Finally, a pulse duration of 40 fs (typically 50-60 fs) can be achieved after compression, resulting in an energy of 0.4-0.5 mJ/pulse.

Less than 10% of the output is sent to a variable delay line (minimal steps of  $0.1 \,\mu\text{m}$ ) and then focused on a 2 mm sapphire plate to generate a white light continuum as the probe. This light passes through a short-pass filter (700 nm) to reject the (intense) infrared wavelengths before being split into signal and reference beams. The other portion of the laser output is used in a home-built, single-pass optical parametric amplifier (OPA)

based on a noncollinear phase-matching geometry to generate tunable excitation pulses.<sup>33</sup> Briefly, a second-harmonic beam (400 nm) is generated in a 2 mm BBO crystal (Casix) and then focused just before being passed through the OPA crystal (BBO. 2 mm, cut angle of 32°, Casix). For small angles of incidence, a bright superfluorescence cone is seen behind the crystal. As seed light, a single-filament white-light continuum was used, generated in a 2 mm sapphire plate, which overlaps with the 400 nm pump in the OPA crystal. The angle between the seed and the pump is kept at around  $6^{\circ}$ . For a certain incidence angle on the OPA crystal, the white-light seed overlaps with the superfluorescence cone, so by simply tuning the delay between the two pulses, we can change the color of the amplified light from 470 to 750 nm. Typically, pulse energies on the order of a few microjoules are obtained, while the spectral bandwidth (fwhm) varies from 10-15 nm at 500 nm to 50-60 nm at 700 nm. To excite the Car S<sub>2</sub> transitions in LHCII and CP29, we tuned the OPA output to 489 and 506 nm (fwhm of 13-15 nm). The chirp of the amplified pulses was compensated by using a pair of prisms (LaLK21, Melles Griot) to achieve pulse widths of 40-50 fs, as measured in a home-built, backgroundfree autocorrelator. The polarization of the pump was set to a magic angle relative to that of the probe by using a Berek compensator (New Focus, 5540).

The two probe beams were focused on the entrance slit of a spectrograph which disperses approximately 140 nm of their spectra onto a home-built dual photodiode array (PDA) detection system. Each array contains 256 photodiodes (Hamamatsu, S4801-256Q) with superior characteristics such as low dark current, high saturation charge, good linearity, and wide spectral response (200-1000 nm). Each millisecond a frame of 1 kB is transferred to the computer host, containing the readout of the dual image sensor converted by a 16 b ADC. For every laser shot, the spectra of the signal and reference are recorded together with the pump intensity, monitored separately by a photodiode and fed into the PDA system via an analogue input. To account for static differences between the signal and reference beams, we mechanically chopped the pump beam. This allows the spectra to be classified as pumped (energy within 10% of the average) and dark (energy less than 10% of the average), while the rest are discarded (usually, less than 5% of the shots). Moreover, a spectrum selection is also performed on-line by removing those that do not fall within the standard deviation from an average spectrum. Typically, the selection ratio was around 80%, and ca. 5000 difference absorption spectra were averaged per time delay. For every excitation wavelength, two separate sets of data were combined to yield a final probe window between 460 and 720 nm. Between 200 and 400 delays were sampled, with a maximum delay of a few hundred picoseconds. Pump intensities of 20-30 nJ were generally used, corresponding to excitation densities on the order of 10<sup>14</sup> photons pulse<sup>-1</sup> cm<sup>-2</sup>. The maximum changes in absorption in our experiments were 15-20 mOD, with a noise level of ca. 0.2 mOD.

**Data Analysis.** The time-gated spectra were analyzed with a global fitting program.<sup>34</sup> Species-associated difference spectra (SADS) were determined assuming the sequential, irreversible model  $A \rightarrow B \rightarrow C \rightarrow D$ . 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". This requires a more detailed analysis of selected spectral windows. The instrument response function, fitted with a



**Figure 1.** 77 K absorption spectra of LHCII (solid line) and CP29 (dashed line) normalized at their  $Q_y$  peaks. The arrows indicate the excitation wavelengths, 489 and 506 nm (fwhm = 13–15 nm, see text).

Gaussian profile, had fwhm values of 100-120 fs, similar to the values obtained from the analysis of the induced birefringence in CS<sub>2</sub>. The probe pulse dispersion was fitted to a thirdorder polynomial function of the wavelength, and the obtained parameters agreed very well with those found for several experiments under similar conditions.

## Results

The low-temperature absorption spectra of LHCII trimers and CP29 are displayed in Figure 1. The Car  $S_2$  transitions (0-0) corresponding to Neo, Lut, and Vio in LHCII are probably located at 486, 494, and 510 nm, respectively,<sup>2,7</sup> although a slightly different assignment has been inferred from recent spectroscopic measurements.<sup>35</sup> Unlike in bacterial LHC's, the exact contribution of Cars to the blue-green absorption is difficult to estimate due to overlap with the intense Chl Soret bands (437 nm for Chl a and 473 nm for Chl b). For CP29, only two features likely to be associated with Car absorption have previously been identified, at 483 and 496 nm.<sup>6</sup> However, they are more distinctive than in LHCII because of the relative decrease of the Chl *b* intensity at around 465 nm. In the  $Q_{y}$ spectral region (630-690 nm), the CP29 spectrum shows two single-Chl b bands at 638 and 650 nm and a composite Chl a band peaking at 675 nm, with a shoulder around 670 nm. LHCII binds more pigments than CP29, but it does not show more fine structures: 7-8 Chl *a* molecules give rise to three bandsaround 676, 670, and 662 nm-while 5-6 Chls b all contribute to a single band, centered at 650 nm.36

CP29 489 nm Excitation. Three exponential decays were minimally required to fit the evolution of the absorption changes detected at 254 different wavelengths between 450 and 590 nm. The global analysis program estimated lifetimes (75  $\pm$  15 fs,  $1.1 \pm 0.2$  ps, and  $24 \pm 1$  ps) and spectra of the associated intermediates (SADS), presented in Figure 2. In the first spectrum, which reflects the time-zero signal, the features are obviously related to the initially populated Car S<sub>2</sub> state: the bleaching of the  $S_0 \rightarrow S_2$  transition around 495 nm and, to the red region, the stimulated emission (SE) associated with the S2  $\rightarrow$  S<sub>0</sub> fluorescence. The SE profile agrees quite well with the steady-state S<sub>2</sub> fluorescence spectra of xanthophylls-e.g., Vio<sup>18</sup> and Neo<sup>37</sup>-showing vibronic peaks near 530 and 575 nm. Despite a poorer signal-to-noise ratio, the blue side of the main bleaching exhibits a broad shoulder around 470 nm, indicative of direct Chl *b* excitation (see also below).

The second spectrum replaces the first one in less than 100 fs. During this step, the  $S_2$  bleaching shifts to 493 nm and



**Figure 2.** SADS for 489 nm excitation in CP29. The species generated by the laser pulse (dotted spectrum), with a time constant of  $75 \pm 15$  fs, is replaced by the next one (solid), which in turn decays in  $1.1 \pm 0.2$  ps into the dashed spectrum. This species decays to zero with a lifetime of 24 ps.

decreases by ca. 60%. The relative decrease of the Chl bbleaching around 470 nm indicates that part of the initially excited Chls b have already transferred their energy to the Chls a. The SE is replaced by a large positive signal at wavelengths longer than 505 nm, clearly resembling the excited-state absorption (ESA) spectrum of the Car S<sub>1</sub> state previously measured in the picosecond studies on various Cars.<sup>21</sup> Hence, this ultrafast process is associated with the depopulation of the  $S_2$  state, which can take place via two channels: IC to the optically forbidden S<sub>1</sub> state and energy transfer to the Chls. The final spectrum is formed with a time constant of  $1.1 \pm 0.2$  ps, and it displays the  $S_2$  bleaching reduced to 5–10% of its initial amplitude, reflecting a fraction of badly connected carotenoids. The absence of the blue shoulder indicates that the Chl  $b \rightarrow$ Chl a EET is now complete. A smaller decrease is observed for the induced absorption mainly in the red region (540-590 nm), which leads to a blue-shift of the isosbestic point from 505 to 500 nm. The lifetime of this species is 24 ps, close to the literature values for the  $S_1$  lifetime.<sup>16,17,21</sup>

Between 590 and 710 nm, the spectral evolution is described by at least four species (SADS shown in Figure 3A) interconnected by the following lifetimes: 70-80 fs, 450-500 fs, 7.5  $\pm$  0.2 ps, and >700 ps. In the initial spectrum, the negative signal below 620 nm corresponds to the tail of the short-lived Car  $S_2$  emission detected in the blue region (Figure 2). At the same time, both Chls b are in an excited state, resulting in the presence of the bleaching/SE at 638 and 650 nm. Since the Car S<sub>2</sub> state has not yet been depopulated, this most likely is not the outcome of Car  $\rightarrow$  Chl *b* EET, but it may be due to direct excitation of these pigments in their Soret band. To diminish the global analysis influence of Car or Chl a on Chl b kinetics, we examined the Chl *b* region (630-660 nm) separately. Four lifetimes were required to describe the spectral evolution, 220  $\pm$  20 fs, 2.2  $\pm$  0.2 ps, 9.8  $\pm$  0.2 fs, and  $\geq$  200 ps (instrument response of  $\sim 100$  fs). The obtained SADS (Figure 3B) demonstrate (a) that there is no "ultrafast" rise of the Chl bbleaching, which is instead instantaneous at both 638 and 650 nm, (b) that the EET from the "blue" Chl b to Chl a is mainly fast, while that from the "red" Chl b is slow, in excellent agreement with our previous study of the Chl  $b \rightarrow$  Chl a energy transfer in CP29,<sup>32</sup> and (c) that for each Chl b pool, the transfer exhibits multiexponential kinetics with the following ampli-



**Figure 3.** SADS and lifetimes characterizing the spectral evolution in the entire  $Chla/b Q_y/Q_y$  region of CP29 after 489 nm excitation (panel A). An analysis restricted to the Chl *b*  $Q_y$  region is presented in panel B, while an analysis of the Chl *a*  $Q_y$  region is shown in panel C.

tudes: (1) at around 638 nm, 60% (220 fs), 20% (2.2 ps), and 20% (9.8 ps); (2) at around 650 nm, 80% (2.2 ps) and 20% (9.8 ps).

The presence of several EET rates for one spectral pool supports, in principle, the idea of mixed occupancies of some Chl a/b binding sites in CP29 put forward by Bassi et al.<sup>5</sup> Biochemical analysis of a family of mutant CP29 proteins lacking individual chromophore-binding sites allowed these authors to conclude that two pairs of sites have this property: A3 (0.7 Chl a/0.3 Chl b) paired with B3 (0.3 Chl a/0.7 Chl b) and B5 (0.6 Chl a/0.4 Chl b) paired with B6 (0.4 Chl a/0.6 Chl b). For instance, assuming zero correlation between the oc-



**Figure 4.** SADS and associated lifetimes estimated from the transient absorption data recorded in the red part of the CP29 absorption after excitation at 506 nm.

cupancy of the sites in one pair, we can estimate that  $\sim 25\%$  of the Chl  $b \rightarrow$  Chl *a* EET involving the A3/B3 pigments (corresponding to transfer from the 640 nm Chl  $b^{5,32}$ ) takes place on a picosecond time scale (Chl *b* at both sites), while the remaining 75% is much faster (Chl *b* at only one site). However, we cannot exclude that the multiexponential behavior in our pump-probe kinetics is caused by sample inhomogeneity or by the particular excitation conditions.

From the global analysis (Figure 3A), the second SADS exhibits a large Chl a bleaching at 675 nm, while on the blue side, the signal becomes positive, resembling the Chl ESA absorption<sup>32</sup> rather than the Car  $S_1 \rightarrow S_N$  absorption.<sup>21</sup> This would therefore represent the decay of the Car S<sub>2</sub> state in 70-80 fs through both the EET to the Chl  $a Q_x/Q_y$  states and the IC to the lower S<sub>1</sub> state. Superimposed on this is the Chl  $b \rightarrow$ Chl a transfer (see above), which is also present during the next step (ca. 0.5 ps lifetime). The Car  $S_1$  ESA contribution, dominant between 590 and 630 nm, decays in two distinct phases: 0.45-0.5 ps (ca. 30%) and 7.5  $\pm$  0.2 ps (ca. 70%). Roughly, this corresponds to the fast and slow S1 decay times measured around 550 nm (Figure 2) and points to an energy transfer via the Car  $S_1$  state with a lifetime of ca. 1 ps (see Discussion). The energy transfer is finished after 7-8 ps, when excitation reaches the Chl *a* with the lowest  $Q_{y}$  transition energy in CP29. The SADS of this last species peaks at 678 nm, lacks Chl b bleaching, and displays the characteristic ESA shape of Chl a on the blue side. The analysis performed on the Chl a Qy region alone (665-695 nm), which includes the final energy acceptors in this system, leads to the lifetimes and SADS in Figure 3C. Note that the fastest rise time is now 180-200 fs, slower than the decay of the Car S<sub>2</sub> bleach/SE but faster than Chl  $b \rightarrow$  Chl a kinetics. The delay compared to the rate of the Car decay ( $\sim$ 80 fs) could be explained by the mixing of the Chl  $b \rightarrow$  Chl a and  $Car \rightarrow Chl a$  kinetics or by the involvement of an intermediate state in the Car  $S_2 \rightarrow$  Chl *a*  $Q_y$  EET (see Discussion).

**CP29 506 nm Excitation.** In general, the evolution of the transient absorption for this excitation wavelength is very similar to the case discussed above. The characteristic lifetimes and the corresponding SADS for the Chl  $Q_x/Q_y$  region are shown in Figure 4. The first spectrum again contains an instantaneous bleaching of both Chl *b* bands and, on the blue side, the SE from the Car S<sub>2</sub> state. The signal between 670 and 700 nm may be a manifestation of an electrochromic shift of the Chl *a* 

absorption induced by the excited Cars, known to possess a large dipole in their S<sub>2</sub> excited state.<sup>38</sup> Alternatively, direct excitation of the Chls *a* at 506 nm overlapping with Car  $S_2$ ESA may be the cause of this feature. Similar to the 489 nm data, the rise of the Chl *a* bleaching/SE (time constant,  $150 \pm$ 20 fs) appears to be correlated with the fast decay of the Car bleaching around 500 nm in 70-90 fs (data not shown). A 0.8  $\pm$  0.2 ps phase of the Car S<sub>1</sub> decay is also detected in the blue region, which manifests itself in Figure 4 as the reduction of the ESA between the second and the third SADS (590-630 nm, 1.1 ps time constant). As in the previous case, the main features associated with the Chl kinetics in CP29 were observed, namely, the spectral separation of the fast and slow phases of the Chl  $b \rightarrow$  Chl *a* transfer and the Chl *a* equilibration process in  $12 \pm 2$  ps.<sup>32</sup> The final spectrum for both excitation wavelengths is virtually identical.

**LHCII 489 nm Excitation.** Simultaneous analysis of the transient absorption data acquired in both blue and red probing intervals led to the SADS presented in Figure 5. The inset displays decay profiles recorded at a few characteristic wavelengths, i.e., 495 nm (Car S<sub>2</sub>), 565 nm (Car S<sub>1</sub>), 653 nm (Chl *b*), and 680 nm (Chl *a*). Even though three decay components fitted the Car kinetics very well (lifetimes of  $100 \pm 10$  fs,  $1.1 \pm 0.1$  ps, and 28 ps; spectra not shown), the evolution in the Chl Q<sub>y</sub> region required at least four components. The lifetimes associated with the global four-component fit are 95 ± 10 fs,  $580 \pm 40$  fs,  $8.2 \pm 0.1$  ps, and  $\gtrsim 100$  ps.

The species formed by the excitation pulse (primarily Car  $S_2$ ) shows strong bleaching around 495 nm and a structured negative spectrum between 520 and 600 nm, most likely due to SE. In the red region, this spectrum contains a broad, positive signal, where sharper features occur at 650 nm for Chl *b* and 672 and 679 nm for Chl *a*. This indicates that a significant amount of the initial excitation energy was deposited on Chl *b* and, to a much smaller extent, on Chl *a*. The quantitative description of the initial population of excited pigments for each pumping wavelength will be left for the next section. The origin of the induced absorption must be related to the ESA properties of the Car  $S_2$  and/or Chl *b* Soret states.

After ca. 100 fs, the  $S_0 \rightarrow S_2$  bleaching decreases by 60– 65% and shifts 2 nm to the blue region, while the SE is replaced by a large ESA with a maximum near 540 nm and a long, red tail. The shape of the spectrum suggests that the Cars are now in their S<sub>1</sub> state, following fast IC from the initially excited state, S<sub>2</sub>. Competing with this channel for the S<sub>2</sub> depopulation are the EET processes toward the Chls. A major negative signal appears during this step in the Chl *a* Q<sub>y</sub> region, with the maximum at 679 nm and a shoulder at around 673 nm, indicating direct energy transfer from Car mainly to redabsorbing Chl *a*. Despite the fact that some Chl *b*  $\rightarrow$  Chl *a* EET processes occur on a similar time scale,<sup>23-25</sup> a relatively smaller increase of the bleaching/SE around 650 nm is also observed, indicating energy flow toward Chls *b* (see below).

The 600 fs process is primarily associated with the Chl  $b \rightarrow$ Chl *a* energy transfer; roughly 40% of Chl  $b \rightarrow$  Chl *a* EET is known to occur with such a time constant in LHCII.<sup>22–25</sup> This event is clearly illustrated by the decrease of the Chl *b* signal around 650 nm in the third SADS, coupled with an increase of the bleaching/SE of Chl *a* above 675 nm. In the blue region, the Car S<sub>0</sub>  $\rightarrow$  S<sub>2</sub> bleaching and S<sub>1</sub>  $\rightarrow$  S<sub>N</sub> absorption are significantly reduced, thus implying that at least 50% of the Car S<sub>1</sub> states have a considerably shorter lifetime than in vitro. We interpret this as an energy transfer in which the donor is the Car S<sub>1</sub> state. Most likely, the acceptors are Chl *a* Q<sub>y</sub> states,





**Figure 5.** SADS and associated lifetimes resulting from a global fit of the data obtained over the whole probing interval after 489 nm excitation in LHCII. The inset displays the traces constructed from the data at a few characteristic wavelengths together with their fits: 495 nm (S<sub>2</sub> bleach), 565 nm (S<sub>1</sub>–S<sub>N</sub> excited-state absorption), 653 nm (Chl *b*  $Q_y$  bleach), and 680 nm (Chl *a*  $Q_y$  bleach). On the *x*-axis, the time is given in ps, while on the *y*-axis, the change of absorption is given in mOD.

but at this point, we cannot exclude the possibility that two Chls b, transferring excitation to Chl a in less than 200 fs,<sup>23,24</sup> may act as intermediates in this pathway.

Hence, the third species describes the system after the Car  $\rightarrow$  Chl transfer from both the short-lived S<sub>2</sub> state and the longlived S1 state has been completed. Around 490 nm, this SADS contains 10-15% of the initial signal, which is in agreement with the efficiency of the Car to Chl a energy transfer found by steady-state spectroscopy.<sup>7</sup> The evolution toward the last SADS takes about 8 ps, which describes the  $S_1 \rightarrow S_0$  IC lifetime of the nontransferring carotenoids and the slow phase of the Chl  $b \rightarrow$  Chl a transfer together. Indeed, the signal in the blue region becomes very small in the last spectrum, and no residual Chl b bleaching is found around 650 nm. This lifetime is also characteristic for the energy equilibration among Chls a,<sup>39</sup> as reflected by the disappearance of the blue shoulder, which gives a red-shift of the Chl a bleaching/SE to 681 nm. The overall loss of amplitude is due to singlet-singlet annihilation, expected to occur in LHCII over a wide time range.24,39 However, lifetimes longer than a few tens of picoseconds are poorly determined by our data, which do not extend beyond a 40 ps delay.

By restricting the fit to the Chl *a*  $Q_y$  region (665–695 nm), we found four characteristic lifetimes, 150–180 fs, 850 fs, 10.5 ps, and  $\gtrsim 100$  ps. The estimated SADS are shown in Figure 6. The first two processes are associated with energy transfer from Car (S<sub>2</sub> and S<sub>1</sub> states) and from Chl *b*; the third rate describes the "slow" Chl *b*  $\rightarrow$  Chl *a* transfer and Chl *a* equilibration kinetics, while the fourth is associated with the decay of the equilibrated state.

**LHCII 506 nm Excitation.** At this excitation wavelength, less direct excitation of Chl b is expected, along with increased sensitivity for Vio kinetics and negligible contribution from Neo. This is perfectly illustrated in Figure 7, where the SADS and the characteristic lifetimes are shown. In the Car absorption region, the initial spectrum is negative due to the bleaching of



**Figure 6.** SADS and associated lifetimes resulting from a sequential analysis of the transient absorption after 489 nm excitation in LHCII, restricted to the Chl  $a Q_y$  region.

the  $S_0 \rightarrow S_2$  transition and due to the SE from the excited state (Figure 7A). The maximum signal is recorded at 498–500 nm, roughly 5 nm to the red region compared to the earlier excitation wavelength. The reduced population of directly excited Chls *b* is evident from Figure 7B, where the amplitude of the Chl *b* bleaching/SE is smaller than in the previous case (see Discussion).

In the blue region after ca.  $110 \pm 20$  fs, the difference absorption spectrum becomes bipolar, i.e., negative below 510 nm and positive for longer wavelengths. During this step, the bleaching diminishes by approximately 65% and shifts to the blue (peak at 492 nm) region. In the red region, the Chl *a* bleaching/SE at both 673 and 679 nm rises with a similar time constant,  $140 \pm 20$  fs. On the other hand, the signal around 650 nm remains almost constant on this time scale. Careful fitting of the Chl *b* region alone revealed neither an ultrafast



**Figure 7.** Global analysis results (SADS and connecting lifetimes) in the case of 506 nm excitation of LHCII for the blue (panel A) and red probing windows (panel B).

rise component (on the order of the 50 fs lifetime) nor significant kinetics in the 100–150 fs range (spectra not shown). In principle, this supports the idea that the Cars excited at 506 nm—namely, Lut and Vio—transfer their S<sub>2</sub> energy preferentially, if not exclusively, to Chl a.

The next spectral species is formed with time constants of  $1.0 \pm 0.1$  ps in the blue region and  $0.9 \pm 0.1$  ps in the red region. Analogous to the 600 fs component detected for 489 nm excitation, this step accounts for both the Chl  $b \rightarrow$  Chl aEET and the Car  $\rightarrow$  Chl EET, the latter occurring from the lowest excited state of the Car. Thus, a major increase of the Chl a bleaching/SE at wavelengths longer than 675 nm is coupled with the decay of donor features (Chl b bleaching at 650 nm and Car bleaching/ESA in the blue). For the blue region, the third lifetime  $(15 \pm 1 \text{ ps})$  describes the nonradiative relaxation of the Car S1 state to the ground state. This accounts for the fraction of the 506 nm light absorbed by the Cars in LHCII which is not transferred to Chls and therefore not used in photochemistry. In the red region, a 8.0 ps process concludes the EET among Chls, and a long-lived ( $\gtrsim$  300 ps lifetime) equilibrated state is formed, exhibiting bleaching/SE centered at 681 nm and a small positive signal at shorter wavelengths. The annihilation level was lower in this case than for 489 nm because of lower excitation densities and because the extinction coefficients of the carotenoids at 506 nm are significantly lower.

## Discussion

**Fractions of Initially Excited Pigments.** To estimate the lifetimes and the yields of Car to Chl energy transfer as well as



**Figure 8.** A. Deconvolution of the room-temperature (RT) absorption spectrum of LHCII in the blue-green spectral window using the spectra of chlorophylls and xanthophylls measured in organic solvents.<sup>2</sup> Relative contributions were imposed to fit the stoichiometric data,<sup>2</sup> while literature values were used for the extinction coefficients.<sup>39,40</sup> B. The procedure for LHCII was applied to the room-temperature absorption spectrum of CP29, given the pigment stoichiometry.<sup>6</sup> For both panels, the inset displays the residuals of the fit.

the species involved in this process, we need to know the distribution of excited states immediately after the samples are excited. First, this implies a decomposition of the absorption spectra of both CP29 and LHCII into the spectra of the individual pigments (Figure 8). The Chl spectra were measured in acetone and those of the Cars in 3-methylpentane,<sup>2</sup> and the fitting routine made use of the global analysis library.<sup>34</sup> The accuracy achieved is remarkable, given the fact that the in vivo spectral properties should be somewhat different from those found in vitro. Thus, by merely estimating the contribution of each pigment to the absorption spectrum from the known pigment stoichiometry<sup>2,6</sup> and the extinction coefficients,<sup>40,41</sup> we obtained positions of the Chl and Car bands, which are virtually identical to those derived from combining several spectroscopic techniques.<sup>2,6,7</sup> The pump profile was then convolved with each contribution in the absorption spectra to yield the fractions of initially excited species shown in Table 1. For our purposes, these estimates should be regarded as lower limits for Car and upper limits for Chls because they are valid at room temperature, while at 77 K, the individual spectra are sharper. However, it is evident that the Chls b will absorb a significant percentage

 TABLE 1: Fractions of Excited Pigments in LHCII and CP29<sup>a</sup>

sample/exc.	$\Sigma$ Car	Lut	Neo	Vio	Chl b	Chl a
LHCII/506 nm	0.65	0.46	0.04	0.15	0.25	0.10
LHCII/489 nm	0.45	0.28	0.13	0.04	0.50	0.05
CP29/506 nm	0.67	0.27	0.05	0.35	0.20	0.13
CP29/489 nm	0.55	0.22	0.16	0.17	0.40	0.05

<sup>*a*</sup> The fractions were estimated by convoluting the pump spectrum with each component of the spectral deconvolution in Figure 8. Room-temperature spectra were used, and at low temperature, higher proportions of Cars and lower proportions of Chls are expected.

of the pump light, particularly at 489 nm ( $\sim$ 40% in LHCII and  $\sim$ 30% in CP29). In all cases, a sizable amount of excitation occurs on Lut at time zero, while the other two carotenoids are each favored by one excitation wavelength, Neo by 489 nm and Vio by 506 nm. The exception is CP29 pumped at 489 nm, where an even distribution of excitation among Car species is expected.

**Energy Transfer from the S<sub>2</sub> State.** It is known from the in vitro studies that the intrinsic lifetime of the Car S<sub>2</sub> state in the absence of energy transfer is  $200 \pm 100$  fs.<sup>17,42</sup> In the protein environment, this value may be different due to dispersive interactions which bring the S<sub>2</sub> energy level closer to the S<sub>1</sub> state and hence speed the IC. Consequently, to effectively compete with deactivation, the energy transfer to Chls might be expected to occur even faster. In our experiments, we can accurately measure the decay of the S<sub>2</sub> state by examining the corresponding SE signals between 520 and 620 nm. Concomitantly with the depopulation of the S<sub>2</sub> state, this negative signal is converted either to a large positive signal (Car S<sub>1</sub> ESA) or to a small ESA due to Chls.

In CP29, the SE is replaced by a signal bearing the signature of the  $S_1$  ESA spectrum in 70–90 fs, irrespective of the excitation wavelength. In principle, lifetimes as short as 40-50 fs could have been resolved in these experiments, but no faster event was identified by the global analysis routine. The decay of the SE is accompanied by a substantial decrease of the negative signal around 490-500 nm. Fluorescence studies of S2-emitting Cars in organic solvents at room temperature have shown that the emission and the absorption spectra are well separated, with their maxima tens of nanometers apart.18,37 At 77 K, this separation is probably even more pronounced, so the contribution of the SE at waves shorter than 500-510 nm can be considered negligible. Therefore, the decay around 490 nm is caused by the Car  $\rightarrow$  Chl energy transfer, since the S<sub>0</sub>  $\rightarrow$  $S_2$  transition is expected to remain bleached upon IC to the  $S_1 % \left( {{{\mathbf{S}}_{1}}} \right)$ state. Next, we will assume the area under the spectrum in the region between 480 and 510 nm to be a measure of the density of Car excited states, either  $S_2$  or  $S_1$ . The  $S_1 \rightarrow S_N$  ESA also contributes to this region, leading to some inaccuracy in this estimation. This is included in the uncertainty level of the results given below.

The area of Car bleaching diminished by 60-65% during the 70-90 fs step (see, e.g., Figure 2), thus indicating that almost two-thirds of the energy absorbed by the Cars bound to CP29 is transferred to Chls from their S<sub>2</sub> state. In terms of kinetic rates, this can be expressed as

$$k_{\rm T2} + k_{\rm IC2} = (70 - 90 \text{ fs})^{-1}$$
 (1)

$$k_{\rm T2}/(k_{\rm T2} + k_{\rm IC2}) = 60 - 65\% \tag{2}$$

Solving these equations, we find an energy transfer  $k_{T2}^{-1}$  of 130  $\pm$  20 fs and an IC  $k_{IC2}^{-1}$  from S<sub>2</sub> to S<sub>1</sub> state of 210  $\pm$  20 fs.

TABLE 2: Evolution of Excited States in the SADS Spectra<sup>a</sup>

	А	В	С	D				
CP29/506 nm								
[Car*]	$3.3 \pm 0.2$	$1.1 \pm 0.1$	$0.7 \pm 0.1$	_				
[Chl <i>b</i> *]	$1 \pm 0.05$	$0.60 \pm 0.05$	$0.35 \pm 0.05$	—				
[Chl a*]	$\sim 0.2$	$2.0 \pm 0.1$	$2.5\pm0.1$	$\sim 2.5$				
LHCII/506 nm								
[Car*]	$5.7 \pm 0.3$	$1.9 \pm 0.2$	$\sim 1$	_				
$[Chl  \vec{b}^*]$	$1 \pm 0.05$	$0.75\pm0.05$	$0.28 \pm 0.03$	-				
$[Chl a^*]$	$\sim 0.4$	$1.53\pm0.05$	$2.73\pm0.05$	$\sim 2.8$				
LHCII/489 nm								
[Car*]	$5.5 \pm 0.3$	$2.4 \pm 0.3$	$0.8 \pm 0.1$	_				
$[Chl b^*]$	$1 \pm 0.05$	$2.0 \pm 0.1$	$0.62\pm0.03$	_				
$[Chl a^*]$	$\sim 0.2$	$2.1 \pm 0.1$	$2.9 \pm 0.2$	$\sim 2.0$				

<sup>*a*</sup> The concentration of excited states on each pigment was estimated from the spectra of the species shown in Figures 2–7 (see text for details). For each experiment, the amount of excited states was normalized to the initially excited Chls *b*. Generally, the transition between the A and B species takes ~100 fs, between the B and C species takes ~1 ps, between the C and D species takes 8–10 ps in the red and 20–30 ps in the blue, while D is a long-lived species (see Results for precise values in each case).

These should be seen as average values for the three Cars, since more than one species is excited at a time. The absence of excited Neo for the 506 nm pump (Table 1) does not significantly affect the results, suggesting that both  $k_{T2}$  and  $k_{IC2}$ for this Car are very close to the average values. It was argued recently that Lut, Neo, and Vio compete for two central binding sites, the CP29 analogues of those identified in the LHCII structure.<sup>5</sup> In our experiments, the decay pattern of the S<sub>2</sub> state seems to be independent of the Car species in terms of both amplitude and lifetime, suggesting that it is mainly determined by the (Chl) environment, which is the same for all Cars.

Xanthophylls in CP29 Transfer Excitations to Chl a. The energy acceptors in CP29 are quite obviously Chl a molecules, as proven by the fast rise of bleaching/SE around 675 nm (Figures 3C and 4). No transfer to Chls b was detected (see Figures 3B and 4), the involvement of these pigments in the observed spectra being largely due to direct excitation. To quantify the possible amount of Chl b involved in ultrafast («100 fs) EET, we checked the initial SADS for the contributions of Car, Chl b, and Chl a and compared them to those obtained from spectral decomposition. For quantification, we used the area under the spectrum in the 480-510, 630-660, and 660-700 nm regions for Car, Chl b, and Chl a, respectively, after correcting for different extinction coefficients  $-\epsilon_{Car}:\epsilon_{Chlb}:$  $\epsilon_{\text{Chla}}$  is (1.4–1.5):(0.65–0.7):(1.0–1.1).<sup>40,41</sup> The intramolecular relaxation for Chls b excited directly at 489 or 506 nm was assumed to have occurred much faster than any intermolecular energy transfer event. Otherwise, knowing the EET transfer time from the 650 nm Chl b to be around 2 ps,<sup>32</sup> we should have noticed the addition of SE to the initial bleaching in the second SADS (see, e.g., Figure 3B). The resulting amounts for each pigment are listed in Table 2 (column A), and they agree reasonably with the expected values (see, e.g., Table 1), arguing against an ultrafast, unresolved Car  $\rightarrow$  Chl b EET. Note that the amount of excitation on Cars is somewhat higher in the initial spectra than expected from the decomposition of the absorption spectra, probably because the SE contribution around 500 nm was neglected in the first case. However, this does not significantly affect our conclusions.

In LHCII Lut and Vio Transfer to Chl a and Neo Transfers to Chl b. In LHCII, a similar approach was used to examine the pattern of the  $S_2$  decay. The following points are worth mentioning: (a) according to the spectral deconvolution, the

Car excitation vectors, defined as ([Lut\*], [Neo\*], [Vio\*]), are (2, 1, 0) for 489 nm excitation and (3, 0, 1) for 506 nm excitation; (b) the  $S_2$  lifetime in the presence of energy transfer is 95  $\pm$  10 fs for the first case and 110  $\pm$  20 fs for the second; and (c) the total [Car\*] population decreases in both cases by ca. two-thirds, quite similar to the behavior in CP29. Equations 1 and 2 yield for the blue excitation  $k_{T2}^{-1} = 120 - 150$  fs and  $k_{IC2}^{-1}$ = 250-300 fs, while for the red excitation, values of 150-170 and 300-350 fs were obtained. Although Lut dominates the kinetics in both cases, we can conclude regarding the S<sub>2</sub> transfer rates and efficiencies that the Car species bound to LHCII show much similarity. This is an intriguing result, since there is some evidence that they are bound in rather different environments within the LHCII protein, unlike CP29. Recently, Croce et al. proposed that the central sites are occupied either by Lut alone (site L1) or by both Lut and Vio (site L2) and that Neo is associated with the helix C domain,<sup>3</sup> whereas according to other reports, Vio is weakly bound to a peripheral site.29,43

The most remarkable feature of energy transfer from the  $S_2$  state in LHCII is that there are different acceptors for the two excitation wavelengths. Hence, both Chl *a* and *b* molecules receive excitation energy absorbed by Lut and Neo at 489 nm, while Lut and Vio transfer exclusively to Chl *a* in the case of 506 nm pumping. To understand this better, we extracted from the SADS determined by global analysis (Figures 2–7) the relative concentrations of excited pigments at each intermediate step (Table 2). These quantities were calculated as described above for the initial bleach spectrum of CP29, and for a given experiment, they were normalized to the number of initially excited Chls *b*. For the EET from the Car S<sub>2</sub>, we consider only the first two species, the first SADS (A) and the one formed after ca. 100 fs (B).

Let us first examine the case of pumping at 506 nm because this shows a higher selectivity for Car excitation-ca. 80%, according to the bleaching spectrum A, and at least 65%, according to the decomposition results (Table 1). It is obvious that the decrease of S<sub>2</sub>-excited Cars in species B versus species A is correlated with the increase of excitation on Chl a. The mismatch between the loss and the gain is most likely due to imprecision in estimating [Car\*], given that in the second species, the ESA from  $S_1$  interferes with our estimation procedure. However, it is very important that no increase of [Chl  $b^*$ ] is observed during the first 110 fs of the dynamics. On the contrary, the population of excited Chls b is diminished by 20-25%. From several pump-probe studies, it is known that when Chl b is excited at around 650 nm, the transfer to Chl *a* occurs with the following lifetimes:  $^{22-25}$  0.15–0.20 ps  $(\sim 40\%)$ , 0.5–0.6 ps  $(\sim 40\%)$ , and 4–9 ps  $(\sim 20\%)$ . So it follows that the decrease of Chl b bleaching is ascribed to the fast phase of the Chl  $b \rightarrow$  Chl a transfer. Ultimately, these data may be taken to show that none of the Cars excited by the 506 nm pulse-namely, Lut and Vio-are connected to Chls b to allow singlet transfer from their S<sub>2</sub> state.

This outcome contradicts the work of Connelly et al., who asserted that both central Luts transfer predominantly to Chl b,<sup>30</sup> but it generally agrees with the conclusion of Peterman et al. that most, if not all, of the Car excitation is delivered to Chl a.<sup>31</sup> At the same time, it discards the modifications of Kühlbrandt's Chl assignment—namely, swapping two Chl a positions with two Chl b positions—proposed by the first authors and carried out by Trinkunas et al..<sup>44</sup> The disagreement with Connelly et al. originates mainly from differences in estimating the amount of directly excited Chl b. These authors argued, for

instance, that for a 490 nm pump, only 10% of the excited pigments at time zero are Chls *b*, while in our case, the decomposition of both the OD and time-zero spectra suggests a significantly higher value (Tables 1 and 2). The lifetime of the Car  $\rightarrow$  Chl *b* transfer published by Connelly et al. (140 fs) is approximately 3 times longer than that from our temporal resolution, so it should have been detectable in the current measurements. Moreover, it is not likely that we missed an ultrafast Car  $\rightarrow$  Chl *b* component, as can be inferred from careful analysis of the Chl *b* Q<sub>y</sub> region alone.

An increase of bleaching/SE attributable to both Chl b and Chl a is observed 100 fs after excitation with a 489 nm pulse, correlated to the decrease of the Car signal. Approximately 60% of Car\* energy is transferred to these pigments in the following proportions:  $\frac{2}{3}$  to Chl *a* and  $\frac{1}{3}$  to Chl *b* (Table 2). At this excitation wavelength, we expect that 2/3 of the [Car\*] are Luts and 1/3 are Neos immediately following excitation (Table 1). In light of the previous discussion, it is straightforward to assign the fraction reaching Chl a as transferred from Lut and that arriving to Chl b as from Neo. This is in line with the recently proposed Neo binding site near the helix C domain,<sup>3</sup> in the vicinity of two Chl b molecules, denoted b5 and b6 in the structural model.26 A special interaction between Neo and Chl b was also traced in our earlier femtosecond experiments,<sup>46</sup> in which an instantaneous bleaching response around 485 nm was detected when LHCII samples were excited with 650 nm light at 77 K. This signal decays with the same lifetime (0.5-0.6)ps) as the intermediate phase of the Chl  $b \rightarrow$  Chl a energy transfer.

On the other hand, the Luts are likely to be bound to the central sites proposed in the conventional model,26 where they are surrounded by many Chls a (see below). It seems that most of the acceptors have their  $Q_{v}$  transition at 678 nm, but a certain fraction of them peak at shorter wavelengths (672–673 nm). Although Vio is not accessible to selective excitation, the relatively higher 672 nm bleaching/SE in the 506 nm compared to the 489 nm data shows that this Car is coupled to a "blue" Chl a. This preferential coupling—blue Car with "red" Chl a and "red" Car with "blue" Chl a-has previously been observed for both singlet<sup>31</sup> and triplet<sup>2,7,8</sup> transfers, and it suggests that in LHCII the Vio is bound to a site different from those of the two Luts. In addition, the idea of Vio binding to a peripheral versus a central site is supported by its selective loss when monomeric LHCII is prepared from trimeric particles<sup>2</sup> or even when the trimers are undergoing mild detergent treatment.<sup>29</sup>

Estimating the Coupling between the Car S<sub>2</sub> State and the Chl Excited States. Given the estimated time constant of EET,  $130 \pm 30$  fs, we will examine the magnitude and the mechanism of the coupling governing the S2 transfer channel. For both CP29 and LHCII, the rise of the Chl a bleaching/SE is slower than the decay of the Car bleaching/SE, i.e., 150-200 fs versus 80-110 fs. This can be explained in part by the mixing of the Car  $\rightarrow$  Chl EET kinetics with the Chl  $b \rightarrow$  Chl a transfer, which takes place independently and has distinct lifetimes. In addition, the Car S<sub>2</sub> states in CP29 and LHCII transfer energy to the higher Chl excited states ( $Q_x$  and/or vibrational  $Q_y$ ), which subsequently relax to the lowest-energy level. Four Chl-binding sites, A1, A2, A4, and A5,5,45 symmetrically arranged around the central 2-fold axis in the 3-dimensional structure,<sup>26</sup> were found to be occupied by Chl a in both CP29 and LHCII (see Figure 9). In terms of center-to-center distances, these chlorophylls are also the closest neighbors of the two central Cars: A1 and A2 for the xanthophyll in site L1 (10.6 and 6.6 Å, respectively) and A4 and A5 for the xanthophyll in site L2 (10.2



**Figure 9.** Chromophore arrangement in the LHC2 monomer: black, Luts; gray, Chls. The assignment and the notation of each molecule are taken from Kühlbrandt et al.<sup>26</sup> The dashed lines connect the centers of the molecules, and the distances are given in Å.

and 7.5 Å, respectively). Given the considerable  $S_2$  transition dipole, ~15 D,<sup>14</sup> the Coulombic interaction is expected to drive the Car  $\rightarrow$  Chl *a* energy transfer. Moreover, several authors have recently examined the role of exchange coupling in purple bacterial LHC's and in the PCP complex, where the Car–Chl distances are comparably short; they found that in all cases, this term was negligible compared to the Coulombic coupling.<sup>47,48</sup> Next, we will investigate to what extent the dipole– dipole term, estimated using the available structural information, is responsible for the rate of excitation transfer from the Car S<sub>2</sub> state to Chl *a* states.

In the weak-coupling limit, the rate of EET is given by<sup>49</sup>

$$k_{\rm DA} = \frac{1}{\hbar^2 c} |V_{\rm DA}|^2 J_{\rm DA} \tag{3}$$

where  $\hbar$  is the Planck constant (divided by  $2\pi$ ), *c* is the speed of light,  $V_{\text{DA}}$  is the coupling energy between the donor and the acceptor electronic transitions, and  $J_{\text{DA}}$  represents the overlap on the frequency scale between the donor emission and the acceptor absorption spectra

$$J_{\rm DA} = \frac{\int \frac{f_{\rm D}(\nu)}{\nu^3} \frac{\epsilon_{\rm A}(\nu)}{\nu} \,\mathrm{d}\nu}{\int \frac{f_{\rm D}(\nu)}{\nu^3} \,\mathrm{d}\nu \int \frac{\epsilon_{\rm A}(\nu)}{\nu} \,\mathrm{d}\nu} \tag{4}$$

The dipole-dipole interaction can be written as

$$|V_k|^2 = C \frac{\mu_{\rm D}^2 \mu_{\rm A}^2}{(4\pi\epsilon_0)^2} \sum_i \frac{\kappa_{ik}^2}{R_{ik}^6}$$
(5)

where k = L1 and k = L2 are the two central Luts and i = 1and i = 2 are two Chl *a* acceptors, i.e., A1 and A2 for L1 and A4 and A5 for L2. We estimated the dipole strength of the Lut S<sub>2</sub> transition,  $\mu_D^2$ , to be around 200 D<sup>2</sup> using the absorption spectrum in 3-methylpentane<sup>2</sup> scaled to the extinction coefficient of 150 mM<sup>-1</sup> cm<sup>-1</sup> in the maximum.<sup>41</sup> From the atomic model of LHCII,<sup>26</sup> we can extract both the donor-acceptor distances,  $R_{ik}$  (see above), and the orientation factors,  $k_{ik}^2$ , assuming the S<sub>2</sub> transition dipole to be parallel to the polyene backbone and the Chl *a* Q<sub>y</sub> dipoles oriented as in ref 39. Normally, the Q<sub>x</sub> and Q<sub>y</sub>



**Figure 10.** Chl *a* absorption spectrum in acetone (RT, solid line), the 77 K Car  $S_2$  emission spectrum at estimated using the initial SADS from Figure 2 (dotted line, see text for details), and the Vio  $S_1$  emission reconstructed from steady-state fluorescence data<sup>18</sup> (dashed line, RT). The normalization of the spectra is arbitrary.

transitions must be considered separately, since both show comparable contributions to the Chl *a* absorption in the overlap region (Figure 10) and the associated dipoles are orthogonal. However, the corresponding |k| factors are very similar (e.g., for the L2–A5 pair the values are 0.58 for Q<sub>y</sub> and 0.82 for Q<sub>x</sub>), and thus, the entire absorption profile can be regarded as one electronic transition for our purposes. Using the Chl *a* spectrum measured in acetone (Figure 10), we estimate the total dipole strength,  $\mu_D^2$ , to be ca. 45 D<sup>2</sup> (from 500 to 700 nm) and the overlap integral,  $J_{DA}$ , to be 0.7 × 10<sup>-4</sup> cm. The *C* factor in eq 5 is related to the difference in the refractive indices between the organic solvents and the protein environment,<sup>50</sup> which, given  $n_{\text{prot}} = 1.55-1.6$ ,<sup>39,43,50</sup> ranges between 0.35 and 0.4.

With these assumptions, the dipole-dipole couplings of (1.65  $\pm 0.15$ ) × 10<sup>5</sup> cm<sup>-2</sup> ( $|V_{L1}|^2$ ) and (2.9  $\pm 0.2$ ) × 10<sup>5</sup> cm<sup>-2</sup> ( $|V_{L2}|^2$ ) were calculated, which gave an average value per Lut of 450- $500 \text{ cm}^{-1}$  for  $|V_{\text{dip}-\text{dip}}|$ . On the other hand, from the rate equation (eq 3), a somewhat smaller value was obtained for  $|V_{DA}|$ , i.e., 280-330 cm<sup>-1</sup>. The agreement between the two values is quite satisfactory, considering the level of approximation used in the analysis. Calculations using pigment geometries from the LH2 complex of Rhodopseudomonas (Rps.) acidophila have shown that the dipole-dipole approximation overestimates the total Coulombic coupling by a factor of 2 for Car-BChl pairs separated by ca. 10 Å.<sup>51</sup> In both LHCII and CP29, the twisted configuration of the central Cars may give rise to important contributions of the higher-order terms in the multipole expansion. However, a structure with an improved resolution is needed before any detailed calculations can be performed to estimate various contributions to the total Car-Chl coupling in LHCII. At the moment, on the basis of our results, it can only be concluded that the dipole-dipole term plays the major role in the energy transfer from the S2 state to "hot" vibronic states of both  $Q_x$  and  $Q_y$  transitions of Chl *a*. The acceptor molecules, labeled A1, A2, A4, and A5 in the structural model,<sup>26</sup> receive more or less equal amounts of the excitation as the result of an average Coulombic coupling of about 200 cm<sup>-1</sup> to the xanthophylls L1 and L2.

**Energy Transfer from the S<sub>1</sub> State.** The efficiency of the Car to Chl energy transfer was estimated by steady-state fluorescence excitation spectroscopy to be in the range of 80–90% for LHCII.<sup>2</sup> A value around 80% was obtained for the CP29 complex in 77 K measurements performed in our lab.<sup>19</sup> We have shown that for both systems, approximately 60–65% of the Car excitation is transferred to the Chl Q<sub>x</sub> state and/or

 $Q_y$  state directly from the S<sub>2</sub> state in ca. 100 fs. The difference, i.e., 15–20%, must be delivered from the optically forbidden S<sub>1</sub> state after IC from S<sub>2</sub>. Indeed, this is best seen in 506 nm excitation for CP29 and LHCII due to very low singlet—singlet annihilation levels. Relatively more excited states are produced by the 489 nm pump, and therefore, the loss of excitation interferes with EET.

For all four experiments presented in this work, we detect a lifetime of  $1.0 \pm 0.2$  ps associated with the decay of both the Car S<sub>2</sub> bleaching between 480 and 500 nm and the characteristic  $S_1 \rightarrow S_N$  spectrum between 520 and 620 nm. This component has an amplitude of 10-30% depending on the excitation wavelength and on the system under investigation. The in vitro S<sub>1</sub> lifetimes are much longer than this-14.6 ps for Lut,<sup>20</sup> 35 ps for Neo,<sup>21</sup> and 23–25 ps for Vio<sup>16,17</sup>—while the S<sub>2</sub> lifetimes are much shorter.<sup>17,42</sup> No faster  $S_1 \rightarrow S_0$  IC component was identified for these xanthophylls in organic solvents. Although the in vivo  $S_1$  lifetimes should be very similar to the in vitro values, distortions of the polyene chain or close range interaction with Chl molecules may speed the intramolecular relaxation in the protein environment. However, in the data collected for 506 nm excitation, a clear increase of the total concentration of Chl excited states occurs on the same time scale: 30-35% for LHCII and around 10% for CP29 (see Table 2, columns B and C). This provides strong evidence that the fast depopulation of the S<sub>1</sub> state is an energy transfer event toward the Chl neighbors. Since this rate is many times faster than the intrinsic S<sub>1</sub> lifetime and only part of the S<sub>1</sub> energy reaches the Chls, it seems that not all Cars possess this ability, which may depend on the individual molecular properties and/or the binding site.

First, we will address the question of the identity of S<sub>1</sub> donors and acceptors for CP29. As mentioned above, this antenna system contains two carotenoids, probably bound at the central sites. Lut, Neo, and Vio compete for these binding sites, which means that any pair of the two (six possibilities) can occur with equal probability. Our data show a decrease of 30-40% of the  $S_1 \rightarrow S_N ESA$  signal (area under the spectrum between 510 and 590 nm) for both excitation wavelengths, which matches the increase of the bleaching in the Chl region (see, e.g., Table 2 for 506 nm excitation). At this moment, several explanations can account for this effect (for instance, Lut alone is an  $S_1$ donor), but an unambiguous identification of the S<sub>1</sub> donors in CP29 will ultimately rely on similar studies on reconstituted complexes with a controlled Car content. However, in the SADS in Figure 2, some spectral evolution may be noticed to accompany the reduction of the overall amplitude of the ESA. It seems that species B contains several spectral forms while the spectrum of species C shows less inhomogeneity (see, e.g., the vibrational features) after significant decay of the red signal. In principle, the Chls b could act as primary acceptors for the  $S_1$  energy. However, we do not see any evidence for this in the evolution of the Chl b bleaching/SE (see also the information below).

In LHCII, ca. 50–60% of the S<sub>1</sub> energy is delivered to the Chls within 1 ps after excitation of the sample at 489 and 506 nm (Table 2). Although the ESA spectrum is less well defined, some spectral evolution is observed concomitantly with the EET step (see Figures 5 and 7A). The lifetimes of the next species, attributable to nontransferring S<sub>1</sub> states, are quite long, i.e., 20– 30 ps, close to the values measured for Vio and Neo in vitro.<sup>16,17,21</sup> This makes the central Luts the best candidates for successful S<sub>1</sub> transfer in LHCII and the four "highly conserved" Chls *a* (A1, A2, A4, and A5) the most likely acceptors. The analogy with CP29 suggests that it is not the binding position,

but its electronic properties, that decides if a certain Car is capable of S<sub>1</sub> transfer. In a recent work, Walla et al. found a sub-picosecond rise of the Chl *a* emission upon two-photon excitation of Car S<sub>1</sub> states in LHCII, thus exposing the presence of S<sub>1</sub>-mediated EET to Chl *a*.<sup>52</sup> In those experiments, selective excitation was used ( $\sim 2 \times 1200$  nm), while in our case, all the Car species are S<sub>1</sub>-populated, thus allowing a much more global view of the transfer process. However, in the present work, there are other events, which occur on a similar time scale and interfere with the transfer via the S1 state. This may be the origin of some differences in the lifetimes and yields estimated in the two studies.

Recently, due to considerable improvements of the detection sensitivity, the extremely weak emission from the S<sub>1</sub> state of Vio has been measured by Frank et al. at room temperature in hexane.<sup>18</sup> These authors decomposed the experimental spectrum into Gaussian bands corresponding to the  $0 \rightarrow 0, 1, 2, 3$  vibronic bands of the  $S_1 \rightarrow S_0$  emission. The spectrum generated by the fitting procedure is presented in Figure 10. On the low-energy side, it overlaps with the Chl  $a O_v$  absorption band, while the intensity is negligible at energies less than 16 000 cm<sup>-1</sup> in the region of the Chl  $b Q_y$  transition. No data are available for Lut yet, but we can assume that the S<sub>1</sub> emission of Lut is very similar to that of Vio, given that their intrinsic  $S_1$  lifetimes are quite close.16,17,20 Because of the significant width of the spectrum, small shifts of fluorescence do not matter much in estimating the spectral overlap (eq 4). Neglecting both the temperature and the environment effects on the S<sub>1</sub> emission, we obtained a value of 0.764  $\times$  10<sup>-4</sup> cm for J<sub>DA</sub>. By introduction of this in eq 3 together with the measured EET rate of 1.0  $\pm$  0.2 ps, the electronic coupling term,  $|V_{DA}|$ , was estimated to lie between 95 and 120 cm<sup>-1</sup>. Assuming at least two acceptors per xanthophyll (see above), we derive an average coupling strength on the order of 50 cm<sup>-1</sup> between the Lut S<sub>1</sub> and Chl  $a Q_{y}$  states. Due to the vanishingly small dipole associated with the transition to the Car S1 state, we would conclude that excitation transfer from this state proceeds through the exchange mechanism.<sup>53</sup> However, assuming this type of coupling between the lycopene  $S_1$  and BChl *a*  $Q_v$  (B850) states in *Rhodosprillum* (*Rs.*) molischianum, the estimated rate of excitation transfer differs from the experimental value by 2 orders of magnitude.<sup>48</sup> Other authors have emphasized the role of different orbital overlap terms, such as the polarization and charge-transfer mechanisms, in the energy transfer involving the Car S<sub>1</sub> state.<sup>47</sup> In addition, the optically forbidden  $S_1$  state might become slightly allowed after symmetry breaking and/or intensity borrowing through the vibrational modes of the Car  $S_2$  state or through the  $Q_y$  state of a neighboring Chl a. This would further enhance the Coulombic contribution in the overall coupling. Evidence for such an effect has come from pump-probe experiments on LHCII, in which instantaneous Lut bleaching at 495 nm was detected for 670-680 nm excitation.<sup>46</sup> While the questions regarding the mechanism remain to be answered by future experiments, better structural data, and improved theoretical treatment, our work shows that there is a considerable transfer of excitation via the S<sub>1</sub> route in LHCII and CP29 on a time scale which is very similar to those determined for PCP and LH2 complexes.54-56

#### Conclusions

The results presented in this multicolor pump-probe study show that the energy absorbed by carotenoids in the antenna complexes coupled to the PSII reaction center (LHCII and CP29) is delivered to neighboring chlorophylls via both the initially excited  $S_2$  state and the low-lying, optically forbidden  $S_1$  state,



**Figure 11.** Diagram of the states relevant to EET from Car to Chls in LHCII and CP29. The energy of the  $S_1$  level is not precisely known (dashed line). The solid arrows indicate the excitation transfer processes, while the dotted arrows represent IC steps. The 0.13 ps represents the sum of transfers to the Chl  $Q_x$  and  $Q_y$  states.

the latter after the IC from  $S_2$  (see, e.g., Figure 11). In CP29, the observed decay time of the  $S_2$  state is 70–90 fs for both 489 and 506 nm excitation. The data show that ca. 60–65% of the  $S_2$  excitations are transferred to Chls *a* while the rest (35– 40%) remain localized on Cars in their lower  $S_1$  state. Hence, we estimated the time constant of Car to Chl *a* EET via the  $S_2$ pathway to be 130 ± 20 fs and the Car  $S_2$  lifetime in the absence of energy transfer to be 190–230 fs. Furthermore, these experiments show that the transfer from the  $S_2$  state is the same for all Car species bound to CP29 in terms of lifetime, amplitude, and acceptors. This strongly suggests that the determining factor must be the (Chl) environment, since Lut, Vio, and Neo are competing among themselves for two binding sites.

Previous studies on Car to Chl EET kinetics in LHCII have focused on the rise of the Chl Q<sub>y</sub> bleaching/SE, where only one transfer time was found in the range of 150 or 220 fs.<sup>30,31</sup> By a simultaneous analysis of data collected over a wide spectral range (450-720 nm) with an improved temporal resolution, we were able to identify two separate phases of the energy transfer from Cars to Chls. The fast phase reflects the transfer directly from the  $S_2$  state, with virtually identical amplitudes, i.e., 60-65%, for both complexes. The lifetimes measured in LHCII are somewhat slower, around 90 and 110 fs for 489 and 506 nm excitations, respectively. This yields slightly different time constants of S<sub>2</sub> transfer for the two pumping wavelengths: 120-150 fs and 150-170 fs, respectively. Since the Car binding in LHCII is much more heterogeneous than in CP29, 3,28,29 such a spread in lifetimes is quite reasonable. Moreover, we find that Neo is delivering excitation to Chls b, in line with the recently proposed location of this Car,<sup>3</sup> while both Lut and Vio are connected to Chls a. In agreement with earlier time-resolved studies,<sup>7,8</sup> the current data show that energy acceptors from Lut are mostly "red" Chls a (677-680 nm), whereas the "blue" Chls a (670-673 nm) are coupled to Vio, thus supporting the idea of different binding sites for these two carotenoids in LHCII. However, our results conflict with neither the classical model<sup>26</sup> nor the latest pigment assignments.<sup>45</sup> The coupling between the Lut  $S_2$  state and the Chl *a*  $Q_x$  and  $Q_y$  states was estimated to be  $280-330 \text{ cm}^{-1}$  from the EET rate, while an average dipole-dipole interaction of 450-500 cm<sup>-1</sup> per donor

molecule was calculated from the 3-dimensional structure. This supports the idea that the transfer from  $S_2$  is mediated by a Coulombic mechanism, with higher multipole terms showing significant negative contribution to the coupling. The Chl *a* acceptors are probably A1 and A2 for the lutein L1 and A4 and A5 for the lutein L2, with both their  $Q_x$  and the  $Q_y$  states receiving excitation energy from the Lut  $S_2$  state.

Approximately 15–20% of the initial Car excitations are transferred to Chls *a* with a time constant around 1 ps from the S<sub>1</sub> level. This is beautifully demonstrated for 506 nm pumping as an increase in the overall area of Chl bleaching in both LHCII and CP29, although the S<sub>1</sub>-driven energy transfer seems to be more pronounced in LHCII. This is a new result for plant antennae since previous measurements have shown the S<sub>1</sub> state to be involved in EET only in PCP<sup>54</sup> and in LH2 from purple bacteria.<sup>55,56</sup> The total yield of the Car to Chl energy transfer for LHCII and CP29 is 80 ± 10%, in good agreement with the steady-state value.<sup>2,19</sup> It seems that Lut specifically is capable of transfer via its S<sub>1</sub> state, given the yield of this pathway and the large difference between the EET and the IC time constants, i.e., 1 versus 15–35 ps.

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