Chlorophyll *a* and Carotenoid Triplet States in Light-Harvesting Complex II of Higher Plants

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ABSTRACT Laser-flash-induced transient absorption measurements were performed on trimeric light-harvesting complex II to study carotenoid (Car) and chlorophyll (Chl) triplet states as a function of temperature. In these complexes efficient transfer of triplets from Chl to Car occurs as a protection mechanism against singlet oxygen formation. It appears that at room temperature all triplets are being transferred from Chl to Car; at lower temperatures (77 K and below) the transfer is less efficient and chlorophyll triplets can be observed. In the presence of oxygen at room temperature the Car triplets are partly quenched by oxygen and two different Car triplet spectral species can be distinguished because of a difference in quenching rate. One of these spectral species is replaced by another one upon cooling to 4 K, demonstrating that at least three carotenoids are in close contact with chlorophylls. The triplet minus singlet absorption (T-S) spectra show maxima at 504–506 nm and 517–523 nm, respectively. In the Chl Q_y region absorption changes can be observed that are caused by Car triplets. The T-S spectra in the Chl region show an interesting temperature dependence which indicates that various Car's are in contact with different Chl *a* molecules. The results are discussed in terms of the crystal structure of light-harvesting complex II.

INTRODUCTION

In photosynthesis, excitation of pigments (chlorophylls (Chl) or carotenoids (Car)) leads efficiently to a free-energy stabilizing charge separation. This charge separation competes with several loss processes of the singlet excitations: fluorescence, internal conversion, and intersystem crossing. Intersystem crossing, which occurs with a quantum yield of about 4.5% in plants (Siefermann-Harms, 1987), leads to the formation of unwanted and potentially dangerous chlorophyll triplet excited states (^{3}Chl). These states are so high in energy that they can react with oxygen (a triplet in its ground state) to form singlet oxygen. Singlet oxygen is very reactive and can cause severe damage to the organism. All wild-type photosynthetic organisms are equipped with the same protection mechanism against the dangers of singlet oxygen: carotenoids. Carotenoids can in principle perform this task in two ways (Siefermann-Harms, 1987). First, they can react with singlet oxygen, yielding (ground state) triplet oxygen and (excited state) ^{3}Car. Second, and more importantly (Siefermann-Harms, 1987), they can prevent singlet oxygen formation by accepting triplets from Chl molecules. The lowest triplet energy level of Car's with nine or more double bonds (all Car's in plant chloroplasts belong to this class) is lower in energy than singlet oxygen, and therefore the triplet state of one of these Car's cannot react with oxygen yielding singlet oxygen (Siefermann-Harms, 1987; Cogdell and Frank, 1987, and references therein). The phenomenon of triplet quenching has been studied extensively for both bacteria (for a review see Cogdell and Frank, 1987) and plants (Siefermann-Harms, 1987).

In the light-harvesting chlorophyll *a/b* protein associated with photosystem II of plants (LHCII) different types of Car are present: lutein (two molecules per monomer), neoxanthin (one molecule per monomer), and violaxanthin (in variable, substoichiometric amounts) (Ruban et al., 1994; Jansson, 1994). The recently published model for the structure of LHCII at 3.4 Å resolution (Kühlbrandt et al., 1994; Kühlbrandt, 1994) revealed interesting features with respect to the Car's. Two Car molecules (assigned to be lutein because of stoichiometry and symmetry) are present in the center of the monomer, stabilizing the complex. This important structural role is in line with the finding that the Car's are essential for reconstitution of the complex (Plumley and Schmidt, 1987; Paulsen et al., 1990). The luteins are in Van der Waals contact with several Chl's, which is advantageous both for efficient light harvesting and efficient photoprotection (Siefermann-Harms, 1987). For light harvesting singlet excitations are transferred from Car to Chl via the exchange (Dexter) mechanism or the Coulombic mechanism (Owens et al., 1992; Nagae et al., 1993). For photoprotection triplets are transferred from Chl to Car via the exchange mechanism (Siefermann-Harms, 1987). Whatever mechanism is assumed, both singlet and triplet energy transfer between Car's and Chl's requires very short donor-acceptor distances (<1 nm) to be efficient. In LHCII mainly Chl *a* triplets are being formed because Chl *b* to Chl *a* excitation transfer is much faster (~ps; Du et al., 1994; Kwa et al., 1992a; Bittner et al., 1994, 1995) than triplet formation (on the order of 10 ns; this value can be calculated from the triplet yield, 64% (Bowers and Porter, 1967) and the excited state lifetime, 6 ns (Seely and Connoly, 1986), of Chl *a* in polar solution). This means that only Chl *a* triplets need to be quenched by Car's, which led Kühlbrandt and

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co-workers to identify 7 of the 12 Chl molecules per monomer that are closest to the two central carotenoids as Chl *a*. It should be noted that only two Car's have been resolved in the three-dimensional maps; the location of the other(s) is unknown.

Nechustai and co-workers (1988) performed flash-induced transient absorption measurements on LHCII at room temperature under anaerobic conditions. They found very efficient Chl *a* to Car triplet transfer (100%). The Car triplet showed a decay time of 10 μs and a peak at 510 nm in the triplet minus singlet absorption (T-S) spectrum. Unfortunately, the wavelength resolution of the measurements was too low to determine how many Car's contribute. Moreover, the measurements were not performed under aerobic conditions, which prevail in vivo. Because this study was performed at room temperature, a comparison with other studies using magnetic resonance techniques at temperatures below 4 K is difficult (Carbonera et al., 1989; 1992; Van der Vos et al., 1991; Carbonera and Giacometti, 1992; Van der Vos et al., 1994). The most important findings of these studies are summarized below:

i) Three different Car triplets were observed with absorbance detected magnetic resonance (ADMR) (Van der Vos et al., 1994) and fluorescence detected magnetic resonance (FDMR) (Carbonera and Giacometti, 1992). In the T-S spectra these triplets showed maxima at 505 nm and 523 nm and were assigned (Van der Vos et al., 1994) to lutein (505 nm and a shoulder at 523 nm), neoxanthin (523 nm), and violaxanthin (523 nm). This assignment is based on the assumptions that the population of triplets over the various Car's is proportional to their relative occurrence and that factors like population and decay rates of the sublevels and the optical extinction coefficients are the same for different Car's. An important factor not taken into consideration is that at 1.2 K strong localization of singlet excitations on the lowest Chl *a* state occurs (Reddy et al., 1994; Savikhin et al., 1994), leading to a population of specific Chl triplets that might transfer their triplet to a specific Car. Another complicating factor is the method of excitation. With white light excitation (ODMR measurements) Car triplets may be formed not only from Chl triplets but also directly via singlet fission, as occurs in some bacterial antennae (Kingma et al., 1985).

ii) The T-S spectra of the Car triplets show prominent absorption changes in the Chl *a* Q_y region. These observed bleachings at 670 nm and 677 nm were assigned to changes in Chl *a* absorption due to the presence of a nearby Car triplet (Van der Vos et al., 1991).

iii) With FDMR a Chl *a* triplet not quenched by Car's was observed. Whether this triplet is due to uncoupled Chl remained unclear (Carbonera and Giacometti, 1992).

iiii) Both the FDMR (Carbonera and Giacometti, 1992) and ADMR (Van der Vos et al., 1994) signals showed a strong dependence on the LHCII aggregation state. These measurements showed that the earlier ADMR measurements (Van der Vos et al., 1991) were performed on aggregated LHCII.

A lot of questions about triplets in LHCII remain unanswered. These concern the temperature dependence of the triplet characteristics, the influence of the presence of oxygen at room temperature, singlet fission as a possible mechanism for Car triplet formation, the efficiency of Chl to Car triplet transfer, the presence of Chl triplets not quenched by Car's, the amount and type of contributing Car's, and the interactions between Chl (*a* and *b*) and the Car's. To address these questions we performed laser-flash-induced absorption difference measurements on Car and Chl triplets in trimeric LHCII at different temperatures ranging from 4 K to room temperature. In a forthcoming article we will present data of other LHCII preparations (monomers and aggregates) and of polarized and energy selective T-S measurements on trimeric LHCII, to determine which Car's are contributing to the spectra.

MATERIALS AND METHODS

Trimeric LHCII was isolated from spinach thylakoids according to a novel method (F. Calkoen and J. P. Dekker, manuscript in preparation). In short: BBY membrane fragments were precipitated by spinning to remove salt. The precipitate was resolubilized and extra *n*-dodecyl-β,D-maltoside was added. The nonsolubilized material was spun down and trimeric LHCII was purified from the supernatant using a mono Q ion-exchange FPLC column (Pharmacia). Samples were diluted in a buffer containing 20 mM HEPES (pH 7.5), 0.06% *n*-dodecyl-β,D-maltoside, and (except for the 298 K measurements) 70% (v/v) glycerol to an optical density of 0.5–1.0 cm^{-1} at 675 nm at room temperature, and put in acrylic cuvets with a path length of 1 cm. The temperature of the samples was obtained by an Oxford Instruments DN1704 liquid nitrogen cryostat (for measurements at 77 K) or CF1204 helium flow cryostat in conjunction with an Oxford Instruments ITC-4 temperature controller. At room temperature oxygen-free conditions were obtained by flushing the sample with argon.

Absorption spectra were measured on a Cary 219 spectrophotometer with a bandwidth of 0.5 nm. Laser-flash induced T-S absorption difference measurements were performed on a home-built spectrometer as described earlier (Van Mourik et al., 1991). In short, pulses (~8 ns full width at half-maximum (FWHM), ~10 mJ) of a dye laser (Quanta Ray PDL2) at 590 (dye: Rhodamine 610), 610 (Sulforhodamine 640), 462, or 480 nm (both Coumarin 480) pumped by a frequency doubled (tripled in case of Coumarin 480) Nd:YAG laser (Quanta Ray DCR2) were used for excitation. Excitation at 610 and 590 nm was used for selective Chl excitation (no excitation of the Car's), and excitation at 462 and 480 nm was used for partial Car excitation (Chl *b* was also excited). Unless stated otherwise excitation at 590 or 610 nm (both leading to exactly the same results) was used. Detection light was provided by a pulsed xenon lamp, detected (continuously during the pulse) via a 1/4 m monochromator (PTI) with a photomultiplier (Hamamatsu R928). The response time of the setup was less than 0.5 μs. Wavelengths were calibrated with an argon calibration lamp (Oriel) and were determined with a bandwidth of 1 nm in the 400–560-nm region and 2 nm in the 650–700-nm wavelength region. Traces (averages of 8–32 shots at 0.1 Hz) measured at ~100 different detection wavelengths were analyzed globally (Van Stokkum et al., 1994). No degradation of LHCII was observed during the measurements.

The efficiency of the Chl to Car triplet transfer was estimated by dividing the amount of Car triplets by the total (Car + Chl) amount of triplets. As a measure for the amount of triplets the amplitudes in the maxima of the T-S spectra were taken, corrected for the triplet (peak) extinction coefficient. As difference extinction coefficient for Chl *a* triplets (showing a bleaching of the Chl *a* Q_y absorption) a value of 1.0×10^5 $M^{-1}cm^{-1}$ (the Q_y extinction coefficient) at 670–680 nm was assumed. To estimate the amount of Car triplets formed the extinction coefficient of the

$T_n \leftarrow T_1$ absorption of β-carotene, $2.4 \times 10^5\ M^{-1}cm^{-1}$ in the 500–540 nm region, was taken (Bensasson et al., 1983).

RESULTS

Absorption spectra

In Fig. 1 the absorption spectrum of trimeric LHCII is shown at room temperature and 4 K (in Fig. 1 *C* also at 77 K). The spectra are the same as published before (Hemelrijk et al., 1992; Kwa et al., 1992a, b). In contrast to the spectra at 4 K and 77 K hardly any fine structure is resolvable at room temperature. The spectra at 4 K and 77 K are very similar, and bands at 676, 671, 662, and 649 nm can clearly be distinguished (Fig. 1 *C*). In the region where the Car's have their absorption maxima (Fig. 1 *B*) bands at 510, 494, 486, 473, and 459 nm can be observed, clearly visible as minima in the second derivative spectrum (it should be noted that the Soret bands of Chl *b* are located here as well).

Room temperature, anaerobic

At room temperature measurements on trimeric LHCII were performed under aerobic and anaerobic conditions. Under anaerobic conditions a typical carotenoid T-S spectrum was observed peaking at 508 nm (see Fig. 2 *A*) with a lifetime of 9 μs (see traces a and b in Fig. 3), similar to what was measured by Nechushtai and co-workers (1988). Interesting is the presence of the bleaching centered around 674 nm (see Fig. 2 *B*), in the Chl *a* Q_y region, decaying with the same time constant as the Car triplet. Van der Vos and co-workers observed similar signals in their ADMR measurements at 1.2 K (Van der Vos et al., 1991). At room temperature no longer lived (~ms) Chl triplet states could be observed, indicating complete triplet transfer from Chl to Car.

Room temperature, aerobic

In the presence of oxygen the decay of the (Car) triplet state is faster and biexponential. Two components could be resolved (see Fig. 3 and Fig. 4), one with a decay time of 2 μs (peaking at 504 nm) and the other with a decay time of 4 μs (peaking at 518 nm). The shape of the sum of the two T-S spectra (not shown) is equal to the spectrum measured under anaerobic conditions, suggesting that no ^{3}Chl quenching by oxygen (leading to $^1O_2$) takes place. Both decay components show features in the Chl *a* Q_y region (not shown), but these are too noisy to allow meaningful conclusions. The increase in decay time is probably due to the enhancement of intersystem crossing of the ^{3}Car's by oxygen. In this process no singlet oxygen is formed (Porter and Wright, 1959; Truscott et al., 1973). The quenching of ^{3}Car's by oxygen has been observed before in the alga *Chlorella pyrenoidosa* (Duysens et al., 1972; Den Haan, 1976) and spinach chloroplasts (Mathis et al., 1979). The presence of two spectral components points to the contribution of (at least) two different Car's to the T-S spectra in trimeric LHCII. The difference in decay times of both components might be caused by a difference in the accessibility for oxygen, in structure (chemical or conformational) and/or in triplet energy levels of the Car's.

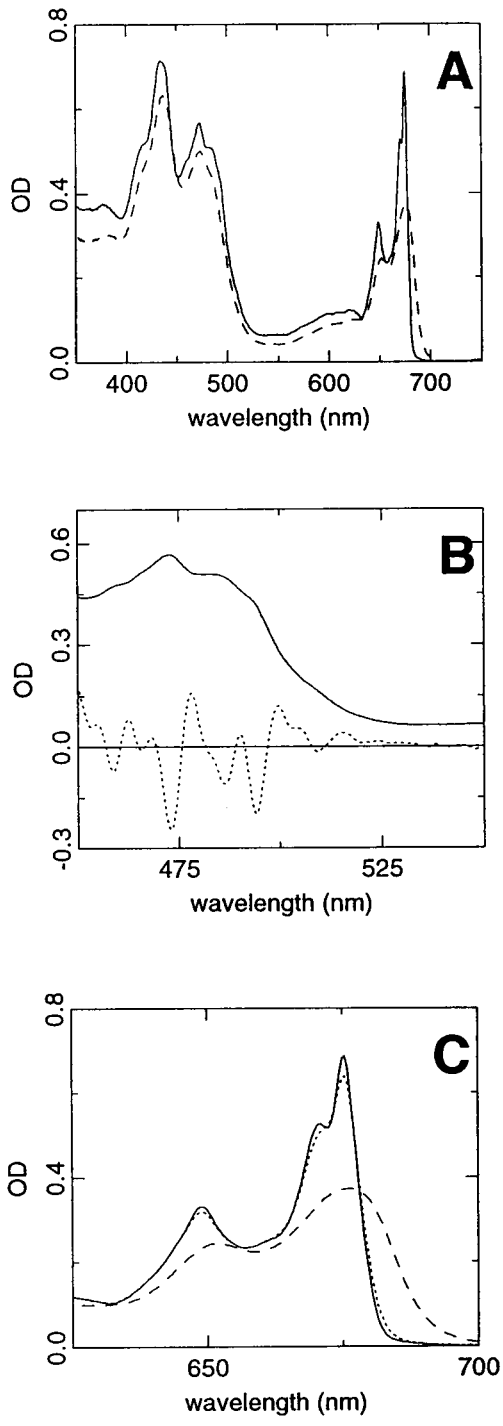
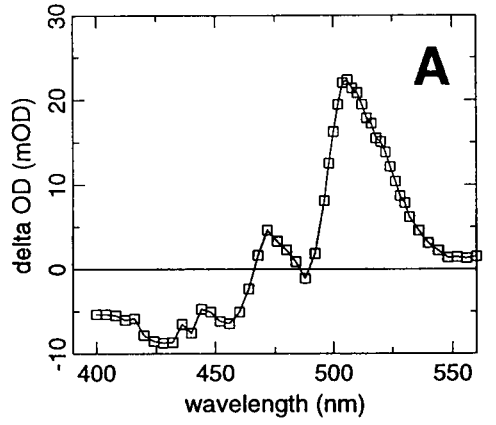


FIGURE 1 Absorption spectra of trimeric LHCII from 350 to 750 nm (*A*), in the Car S_2 region (*B*) (2nd derivative is shown *dotted*), and in the Chl Q_y region (*C*) at 4 K (*solid line*), 77 K (*dotted*, only in *C*), and room temperature (*dashed*).



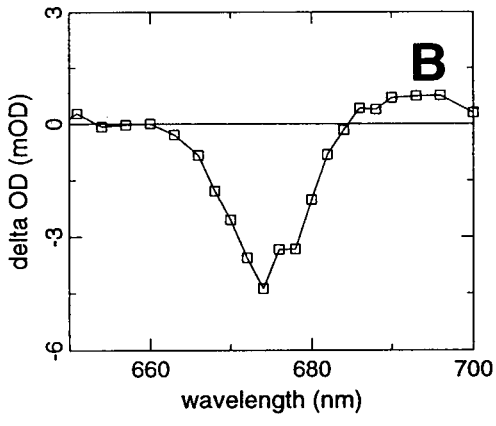


FIGURE 2 T-S spectra of LHCII trimers at room temperature, under anaerobic conditions: spectrum of the 9 μs component obtained with global analysis, in the Car region (*A*) and the Chl Q_y region (*B*). The absorption changes in *A* and *B* are directly comparable.

77 K

At 77 K only one Car lifetime component (12 μs) as well as a small, slow component with ^{3}Chl *a* characteristics (lifetime in the millisecond time range) were needed to fit the kinetics in the wavelength range from 400 to 700 nm for trimeric LHCII. In Fig. 5, *A* and *B*, the spectra of both components are shown. The Car component shows two (clear) maxima at 525 and 506 nm. In the Chl Q_y region again a 12-μs change in Chl absorption is observed, with a minimum at 676 nm, a shoulder at 672 nm, and maxima at 654, 666, and 687 nm. The slow component shows a broad (18 nm FWHM) bleaching centered at 673 nm. The spectrum and the lifetime of this component indicate that it represents triplet states located on Chl *a*, showing that the Chl *a* to Car triplet transfer is not complete at 77 K. Assuming one Chl *a* triplet and two Car (peaking at 525 and 506 nm) states contributing, the efficiency of the triplet transfer can be estimated to be 94 ± 2%. At 77 K also, excitation at 480 and 462 nm was used to check whether direct excitation of the Car's could lead to ^{3}Car via singlet

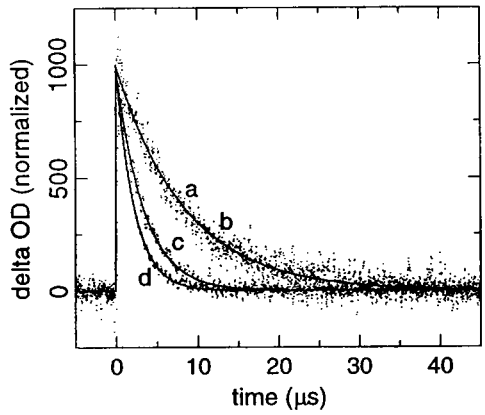


FIGURE 3 Normalized transient absorption traces of trimeric LHCII at room temperature, induced by laser excitation. The traces are recorded at 528 (*a* and *c*) and 506 nm (*b* and *d*) under anaerobic (*a* and *b*) and aerobic (*c* and *d*) conditions. Note: traces *a* and *b* are identical.

fission. If this process would occur different T-S spectra could be expected when exciting the Car's instead of exciting the Chl's. However, no difference was observed (data not shown), so no indication can be found for singlet fission to occur.

4 K

At 4 K the kinetics of the flash-induced absorption changes are more complex. Because of the absence of spin-lattice relaxation at this low temperature the triplet sublevels are uncoupled, and in contrast to the measurements at 77 K and room temperature decays from the different sublevels could be partially resolved (as, for example, also observed for CP47 by Groot et al., 1995). The best fit of the kinetics in the wavelength range from 400 to 700 nm was obtained with two spectral components, one (^{3}Car) with two decay times (8 (70%) and 40 μs (30%)), the other (^{3}Chl *a*) with one decay time (in the millisecond time range). The spectra

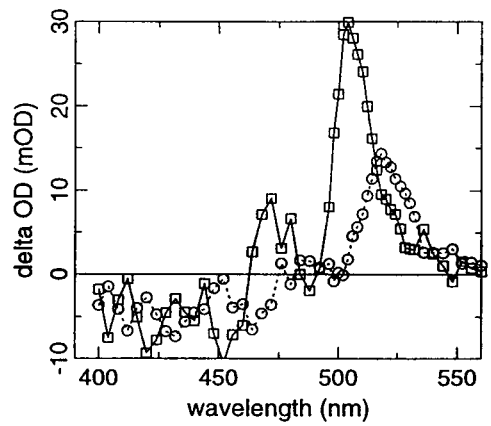


FIGURE 4 T-S spectra of LHCII trimers at room temperature, under aerobic conditions: spectra of the 2 μs (*solid line, squares*) and the 4 μs (*dashed line, circles*) spectral component.

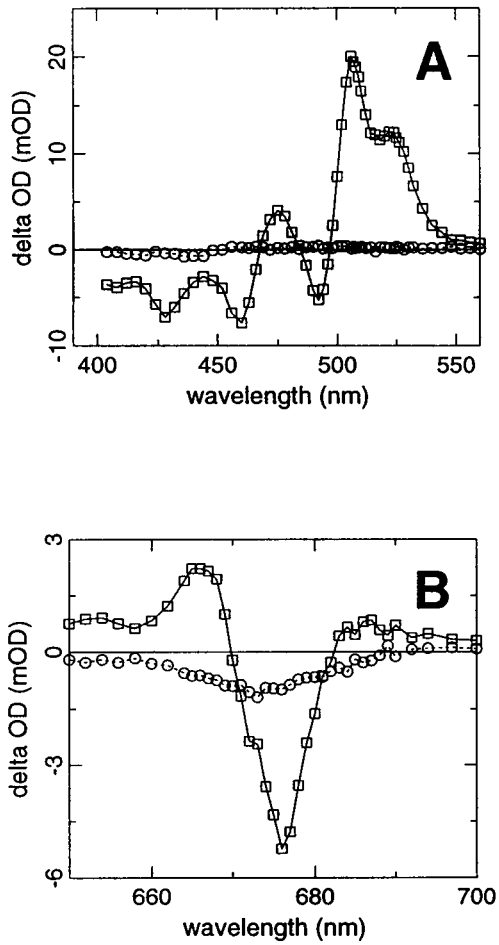


FIGURE 5 T-S spectra of LHCII trimers at 77 K: spectrum of the 12 μs (*solid line, squares*) and the ~ms (*dashed line, circles*) spectral components, in the Car region (*A*) and the Chl Q_y region (*B*). The absorption changes in *A* and *B* are directly comparable.

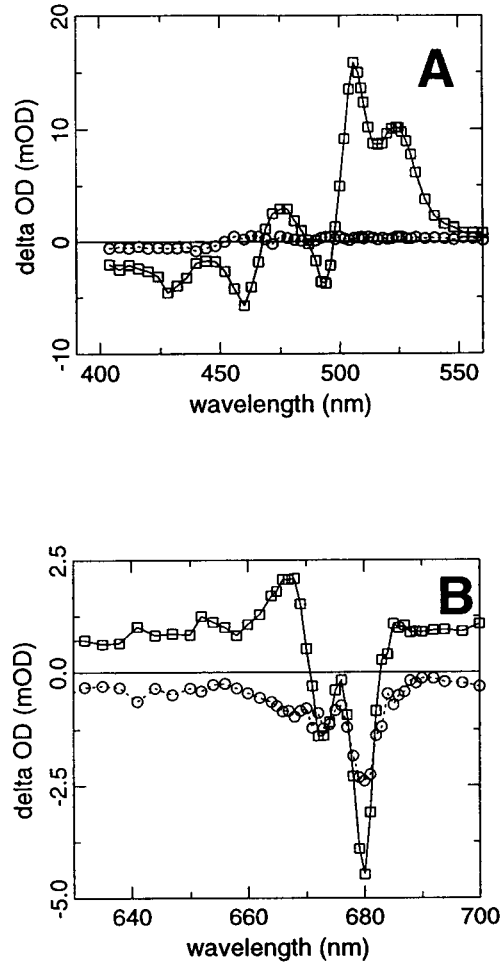


FIGURE 6 T-S spectra of LHCII trimers at 4 K: spectrum of the 8 and 40 μs (*solid line, squares*) and the ~ms (*dashed line, circles*) spectral components, in the Car region (*A*) and the Chl Q_y region (*B*). The absorption changes in *A* and *B* are directly comparable.

of both components are shown in Fig. 6, *A* and *B*. The spectrum of the Car triplet component is very similar to the one at 77 K. The Car T-S spectrum shows maxima at 524 and 506 nm and a minimum at 494 nm. In the Chl Q_y region again absorption changes with the same decay time as the Car triplets are visible. In this region the spectrum shows maxima at 652 and 665 nm and a minimum at 672 nm as at 77 K, but the main minimum has shifted from 676 to 680 nm. The T-S spectrum measured by Van der Vos and co-workers with ADMR at 1.2 K (Van der Vos et al., 1991) is globally comparable to ours, but a closer look reveals several clear differences. In the Car region the ADMR spectrum shows peaks at similar positions, but the 525 nm band is about twice as large as the 506 nm band (in our case it is about two-thirds). In the Chl Q_y region the ADMR spectrum is globally the same (two bleaching features, separated by about 7 nm, flanked by two positive bands on the short wavelength and one positive on the long wavelength side), but the ratio between the bleaching signals is different (about 1:1 blue:red for ADMR and about 1:3 in our case) and the ADMR spectrum is shifted 3 nm to the blue (bleachings at 677 and 670 nm). It seems unlikely that this is caused by the small temperature difference. More probably this originates from differences in measuring method and/or sample preparation (the ADMR measurements were performed on aggregates). The Chl *a* triplet state in the present study shows a narrow (6-nm FWHM) bleaching at 680 nm together with a broader bleaching at about 673 nm, similar to the one observed at 77 K. The position and width of the 680-nm band in both the Car and Chl *a* T-S spectrum are in good agreement with those obtained from holeburning (Reddy et al., 1994) and fluorescence (Peterman et al., 1994) measurements at 4 K. Both studies revealed a narrow red-most absorption band at 680 nm (5.5 nm FWHM inhomogeneous width, according to the holeburning data) in trimeric LHCII. At low temperatures all excitations are transferred to this (lowest) excited state (Savikhin et al., 1994). Consequently this state is the starting point for fluorescence and intersystem crossing. The Chl *a* triplet state in trimeric LHCII at liquid helium temperatures has been observed with FDMR (Carbonera et al., 1992) but not with

ADMR (Van der Vos et al., 1991). Assuming that two Car (peaking at 524 and 506 nm) and two Chl *a* (peaking at 680 and 673 nm) triplet states contribute to the observed spectral features, the Chl to Car triplet transfer can be estimated to have an efficiency of about 82 ± 7%.

40 K

To gain more insight into the differences between the Chl and Car T-S spectra in the Chl Q_y region at 4 K and 77 K we also performed measurements at 40 K. The kinetics over the wavelength range from 650 to 700 nm were fitted with two components, one characteristic for ^{3}Car with a decay time of 12 μs, and the second reflecting ^{3}Chl *a* with a decay time in the millisecond time range. The decay-associated spectra are shown in Fig. 7, *A* and *B*, together with the spectra at 4 K and 77 K. The Car T-S spectrum (Fig. 7 *A*) shows a major bleaching at 678 nm, a shoulder at 673 nm, and maxima at 652, 667, and 685 nm. The spectra at 4, 40, and 77 K show an isosbestic point at 678 nm, from which it can be concluded that upon raising the temperature from 4 K to 77 K the 680 nm band transfers its intensity to a band at 676 nm. It argues against the shift of the 680 nm band to 676 nm. At 40 K the Chl T-S spectrum (Fig. 7 *B*) shows a broad (17 nm FWHM), asymmetric bleach, with a minimum at 679 nm, intermediate between the broad Chl T-S spectrum obtained at 77 K and the sharp spectrum obtained at 4 K.

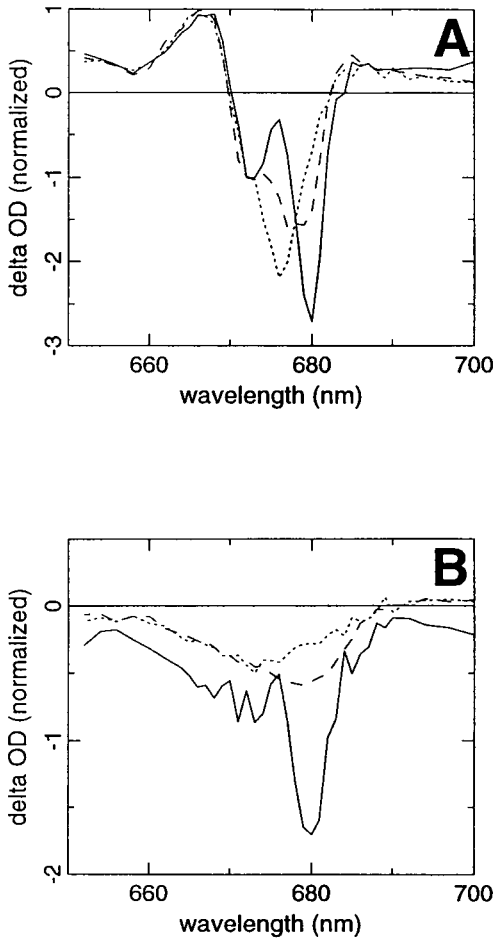


FIGURE 7 T-S spectra of LHCII trimers at 4 K (*solid line*), 40 K (*dashed line*), and 77 K (*dotted line*) of the μs spectral components (^{3}Car) in the Chl Q_y region (*A*) and of the ms spectral components (^{3}Chl) in the same region (*B*). The specta are normalized at the 672 nm shoulder of the μs spectral components (^{3}Car) and corrected for the baseline.

DISCUSSION

Interactions of Chl *a* and *b* with Car's

After a short flash changes in absorption in the Chl Q_y region are observed that decay with the same kinetics as the Car triplet. Recently similar signals were reported for several purple bacterial antenna systems (Angerhofer et al., 1995). An explanation for these signals could be that they arise from a fast (compared to the lifetime of ^{3}Car) equilibrium between Car and Chl triplet states. In this model the triplet would decay from the fastest decay channel: intersystem crossing from the Car triplet state. However, this seems very unlikely. The energy of the lowest triplet level of the Car's present in LHCII (about 6000 cm^{-1}) is much lower than that of Chl *a* (about 10,500 cm^{-1}) (Siefermann-Harms, 1987), so a fast equilibrium where a large fraction of the ^{3}Car resides on the Chl can be excluded. Another explanation might be that a fraction of the Chl's have a slow Chl to Car triplet transfer. But there is no evidence for this in our data and data fits: the ingrowth of the ^{3}Car signals were within the instrumental response (<0.5 μs) and all data could be fitted with parallel decaying spectral species. More likely is the explanation given by Van der Vos and co-workers (1991) that the fast Chl kinetics are induced by a change in interaction between the Chl and a nearby Car when this Car is in the triplet state. It has been shown (Kuki et al., 1989) in calculations on polyenic model systems of Car's that in the triplet state the distribution of the π-electrons over the molecule is significantly different from the ground state, which will have an effect on the polarizability and charge distribution of the molecule. Consequently Chl molecules that are in close contact with the Car's (for optimal singlet and triplet excitation transfer) will experience a different environment, which can lead to changes in absorption (bandshifts, changes in intensity, changes in bandwidth). Another change of interaction might be a small delocalization of the Car triplet wavefunction over an adjacent Chl molecule (this is the explanation given for the signals in the BChl region of the T-S spectrum of several bacterial antenna systems (Angerhofer et al., 1995)). This mixing of ^{3}Chl character in the ^{3}Car state would provide a straightforward explanation for the shape of the signals: bleaching of a Chl Q_y band and a broad featureless absorption increase in this region, as is to be expected for a ^{3}Chl T-S spectrum (see, for example, Groot et al., 1995, for Chl *a* triplet states in CP47). A problem, however, is the size of the signals in our spectra. The intensity of the bleaching

signals in the Chl Q_y region is on the order of 30% of the main Car absorption changes (at 4 K; see Fig. 6, *A* and *B*). Considering the extinction coefficients (see above) of both species this implies that the presence of a triplet state on a Car bleaches about 75% of the (peak) absorption equivalent of one Chl *a*. This would mean that the ^{3}Car in LHCII contains a large amount of ^{3}Chl character, which seems very unlikely, keeping in mind the large difference in energy between both states. Another type of Car-Chl interaction that is changed upon ^{3}Car formation may be the exchange or Coulombic interactions between the Chl *a* Q_y state and the S_1 or S_2 level of the nearby Car. A strong interaction between these levels is to be expected because of the efficient Car to Chl singlet energy transfer in LHCII (Siefermann-Harms, 1987). Further measurements and calculations are necessary to decide which mechanism is responsible for Chl signals in Car T-S spectrum.

Independent of the mechanism that is assumed, bleachings in the Chl Q_y region of the T-S spectrum signify the position of the original absorption bands. The main bleaching features of the T-S spectra (Figs. 7 *A* and 6 *B*) in the 600–700 nm region are at 680 nm (4 and 40 K), 676 nm (40 and 77 K), and 672 nm, all belonging to absorption bands in the Chl *a* region. The positions of the bleachings are in agreement with bands identified with holeburning (680 and 671 nm; Reddy et al., 1994), circular and linear dichroism (676 nm; Hemelrijk et al., 1992; Nussberger et al., 1994), and absorption (676 and 671 nm; Hemelrijk et al., 1992; Nussberger et al., 1994; this work). In the Chl *b* region only very small negative features are present (at 658 and 650–644 nm), about eight times less intense than the 680 or 676 nm bleachings. The observation that mainly the absorption in the Chl *a* region is influenced by ^{3}Car's was made by Van der Vos and co-workers using ADMR (Van der Vos et al., 1991), which supported Kühlbrandt and co-workers in their assignment of the Chl's closest to the Car's to Chl *a* (Kühlbrandt et al., 1994). However, it should be noted that the absence of clear changes in Chl *b* absorption connected to Car triplets does not rule out close distances between some Chl *b* molecules and Car's. Kuki and co-workers (1989) showed in their calculations that the main changes in bond order of Car model systems going from ground to triplet state occur in the central part of the polyene chain (the so-called triplet-excited region). These calculations were performed to give an explanation for the isomerization behavior of β-carotene in the triplet state. It might be that only Chl molecules close to this region are influenced by the ^{3}Car. In the structural model of Kühlbrandt and co-workers (1994) two Chl *a* molecules (*a*2 and *a*5) are located near the triplet-excited region of the two central luteins, whereas the other Chl's are located near the ends of the luteins or further away from the luteins (note that some of these might be located near another Car that so far is not resolved in the crystal structure). It can be speculated that mainly the absorption spectra of these two Chl *a* molecules (*a*2 and *a*5) are influenced. This leads to the conclusion that Chl *a*2 and *a*5 contribute significantly to the 672, 676, and 680 nm absorption bands.

The number of Car triplets contributing to spectral changes

From the aerobic room temperature T-S data it is clear that at least two Car triplets contribute, one showing a T-S absorption maximum at 518 nm and one at 504 nm. At lower temperatures (4 and 77 K) the features in the Car T-S spectrum are sharper, showing maxima at 525 and 506 nm. Although the Car T-S spectrum in the 400–560 nm region hardly changes upon going from 77 to 4 K (Figs. 5 *A* and 6*A*), the changes in the Chl region (induced by Car triplets; Fig. 7 *A*) are very pronounced. The bleaching at 676 nm is no longer observed at 4 K, whereas a sharp minimum around 680 appears. Apart from some general narrowing of the bands at 4 K there is no change in the Chl *a* Q_y absorption spectrum going from 77 to 4 K (Fig. 1 *C*). Therefore, the most likely explanation for the changes in the T-S spectra with temperature is that at 4 K another Car triplet is formed with the same T-S spectrum in the 400–560 nm region as the Car triplet observed at 77 K. At 4 K only the lowest Chl singlet state at 680 nm will be populated (Reddy et al., 1994; Savikhin et al., 1994; Peterman et al., 1994; this work). At 4 K triplets are only formed from this state and will be transferred to a Car near the Chl molecule(s) responsible for the 680 nm state. When this Car is in the triplet state it influences the 680 nm state, leading to bleaching at 680 nm. At higher temperatures, however, the much more intense 676 nm band is more populated and triplets will mainly be formed from the corresponding state(s), leading to a Car triplet that influences the 676 nm band (it should be noted that the 680 nm state corresponds to an oscillator strength of approximately one Chl *a* per LHCII trimer (Reddy et al., 1994), whereas the 676 nm state contains an oscillator strength of approximately 12 Chl *a* molecules per trimer (Hemelrijk et al., 1992)). This implies that at 40 K at least three Car's contribute to the T-S spectra. Two of these show a very similar T-S spectrum (in the 450–550 nm region no change in the T-S spectrum was observed upon variation of the temperature between 4 and 77 K). It is not clear from the data which of the peaks in the T-S spectrum, 525 or 506 nm, is associated with these two Car's. At room temperature, the Car T-S spectrum shows maxima at 517 and 504 nm. The spectrum is much broader than at low temperatures. The absorption spectrum (Fig. 1 *B*) shows that at room temperature the Car fine structure has disappeared because of broadening of the absorption bands. This broadening of the absorption bands might be accompanied by a shift in the peak positions in the Car T-S spectrum (from 525 to 517 nm and 506 to 504 nm). In conclusion: we find evidence that triplets are being transferred to at least three Car's.

The nature of the Car triplet signals

Because a Car T-S spectrum can be represented by a shift of the Car S_2 ground state absorption bands to longer wavelengths upon triplet formation (Bensasson et al., 1983; Aust et al., 1991), bleaching in the T-S spectrum reflects the position of a maximum in the Car (ground state) absorption spectrum. In our low-temperature spectra a correlation seems to be present between the 494 nm shoulder in the absorption spectrum (Fig. 1 *B*) and the bleaching at the same position in the T-S spectra (Figs. 5 *A* and 6 *A*). It is clear from the aerobic room temperature T-S spectrum (Fig. *4*) that this bleaching is connected to the (T-S) maximum at 506 nm. In this spectrum a minimum at 500 nm connected to the 517 nm (T-S) maximum is also present. A similar minimum, but now connected to the 525 nm maximum, cannot be distinguished in the low-temperature T-S spectra. The minimum should be observed at about 505–515 nm, but probably it is masked by the intense peak at 506 nm. In the low-temperature absorption spectrum, however, a clear shoulder is present at 510 nm. We correlate the 525 nm T-S shoulder (517 nm at room temperature) to the shoulder in the absorption spectrum at 510 nm. The data presented do not give straightforward evidence to assign the Car T-S spectra to the different types of Car's present in the complex. In a forthcoming article (Peterman et al., manuscript in preparation) we will present T-S data of monomeric and aggregated LHCII and polarized and energy-selective T-S measurements of trimeric LHCII, which provide evidence on the chemical nature of the Car's appearing in the T-S spectra.

The efficiency of the Chl to Car triplet transfer

As we excited only Chl's, all Car triplets observed are formed via triplet transfer from Chl's. Even when we directly excited the Car's singlet fission could be excluded as a possible mechanism for Car triplet formation. At room temperature no Chl triplets could be observed, indicating complete Chl to Car triplet transfer. At low temperatures, however, the transfer efficiency decreased (94 ± 2% at 77 K and 82 ± 7% at 4 K), and Chl triplets could be observed. The error margins are due to uncertainties in the extinction coefficients of Car $T_n \leftarrow T_1$ absorption and difficulties in determining the triplet contributions because of varying linewidths and overlap of positive and negative peaks. Nevertheless, the trend is clear. The reason for the decrease in efficiency may be twofold. Either the efficiency of the Chl to Car triplet exchange mechanism decreases with temperature, or at low temperatures singlet and consequently triplet excitations end up in other (lower lying) states, which might be less efficient in transferring their triplets to Car's. In spinach chloroplast and subchloroplast particles it has been shown (Kramer and Mathis, 1980) that the ingrowth of the ^{3}Car is slower at 4 K (35–40 ns, depending on the particle) than at room temperature (10–20 ns, depending on the particle). In the reaction centers of the bacterium *Rhodobacter sphaeroides* the BChl to Car triplet transfer efficiency decreases sharply at temperatures below 35 K, which was explained by an energetic barrier formed by the accessory BChl in the inactive branch, which is an intermediate in the triplet transfer from the special pair to the Car (Schenck et al., 1984; Cogdell and Frank, 1987). There is no evidence for a mechanism like this (Chl to Chl triplet triplet transfer before transfer to a Car) in trimeric LHCII, but it cannot be excluded.

^{3}Car quenching by oxygen at room temperature

At room temperature we observed quenching of the Car triplets by oxygen. It is very likely that this quenching will also occur in natural circumstances. In the chloroplast, where oxygen is formed, LHCII will sense a relatively high oxygen concentration. It is unclear whether this phenomenon of enhanced intersystem crossing plays a biological role. Car triplets seem to be rather harmless; they cannot react with oxygen to produce singlet oxygen. Therefore, it seems unnecessary for the organism to loose the Car triplets even quicker then their relatively short lifetime.

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