Quenching of Chlorophyll Fluorescence by Triplets in Solubilized Light-Harvesting Complex II (LHCII)

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ABSTRACT The quenching of chlorophyll fluorescence by triplets in solubilized trimeric light harvesting complexes was analyzed by comparative pump-probe experiments that monitor with weak 2-ns probe pulses the fluorescence yield and changes of optical density, ΔOD, induced by 2-ns pump pulses. By using a special array for the measurement of the probe fluorescence (Schödel R., F. Hillman, T. Schrötter, K.-D. Irrgang, J. Voight, and G. Renger, 1996. *Biophys. J.* 71:3370–3380) the emission caused by the pump pulses could be drastically reduced so that even at highest pump pulse intensities, I_P, no significant interference with the signal due to the probe pulse was observed. The data obtained reveal: a) at a fixed time delay of 50 ns between pump and probe pulse the fluorescence yield of the latter drastically decreased with increasing I_P, b) the recovery of the fluorescence yield in the μs time domain exhibits kinetics which are dependent on I_P, c) ΔOD at 507 nm induced by the pump pulse and monitored by the probe pulse with a delay of 50 ns (reflecting carotenoid triplets) increases with I_P without reaching a saturation level at highest I_P values, d) an analogous feature is observed for the bleaching at 675 nm but it becomes significant only at very high I_P values, e) the relaxation of ΔOD at 507 nm occurs via a monophasic kinetics at all I_P values whereas ΔOD at 675 nm measured under the same conditions is characterized by a biphasic kinetics with τ values of about 1 μs and 7–9 μs. The latter corresponds with the monoexponential decay kinetics of ΔOD at 507 nm. Based on a Stern-Volmer plot, the time-dependent fluorescence quenching is compared with the relaxation kinetics of triplets. It is shown that the fluorescence data can be consistently described by a quenching due to triplets.

INTRODUCTION

The capability of adaptation to different illumination conditions is most important for both high efficiency of solar radiation exploitation and survival of cyanobacteria and plants under light stress. To satisfy these demands, photosynthesizing organisms have developed a variety of antenna systems. They consist of pigment-protein complexes that are either incorporated into the membrane as integral proteins or bound as extrinsic units (Sidler, 1994; Thornber et al., 1991). The most abundant light harvesting complex (LHC) is LHCIIb, which binds about 50% of the total chlorophyll content of the thylakoid membrane. This complex is normally isolated in trimers which are assumed to be also the predominant form of LHCIIb in vivo. Together with minor pigment-protein complexes (each containing $\leq$5.5% of the total Chl), LHCII constitutes an oligomeric system in the thylakoid membrane and builds up the peripheral and proximal antenna of photosystem II (PS II) in green plants (Kühlbrandt, 1994a; Jansson, 1994; Zucchelli et al., 1994; Paulsen, 1995; Green and Durnford, 1996; Bassi et al., 1997).

The structure of LHCII has been resolved to 3.4 Å by electron diffraction of two-dimensional crystals (Kühlbrandt et al., 1994b). It was shown that 12 chlorophylls (7 Chl *a*, 5 Chl *b*) are ordered in two layers near the stroma and lumen exposed surfaces of the membrane-bound pigment-protein complexes. Another striking structural feature is the internal crossbrace of two luteins in the center of the complex and the close distance (van der Waals contact) between nearest chlorophylls and carotenoids. This information is important for a deeper understanding of efficient photon collection and protection against harmful light reactions. The former goal is achieved by ultrafast singlet-singlet excitation energy transfer (sub-picosecond and picosecond time domain) from carotenoids to chlorophyll (Peterman et al., 1997; Connelly et al., 1997a) from Chl *b* to Chl *a* and among different Chl *a* molecules (Bittner et al., 1994, 1995; Connelly et al., 1997b). Among the protective functions a fast quenching of chlorophyll triplets is of key relevance to carotenoids in order to prevent the formation of singlet oxygen as a reactant of photodynamic destruction. Carotenoids play a key role in rapid triplet-transfer from ^{3}Chl to Car and simultaneously as efficient quenchers of singlet oxygen (Siefermann-Harms, 1987; Yamamoto and Bassi, 1996). A recent study revealed that the triplet transfer rate constant is much higher ($k_{TR} \geq (500\ \text{ps})^{-1}$) than reported so far, thus keeping any population of ^{3}Chl at an extremely low level (Schödel et al., 1998).

Under natural illumination conditions the excited singlet states are efficiently transferred from the antenna to the photochemically active pigment of the reaction center and trapped with high quantum yield by charge separation (Renger, 1992; van Grondelle et al., 1994). A small fraction of the absorbed light is emitted as fluorescence. Therefore, measurements of time-resolved fluorescence decay provide

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a powerful analytic tool to analyze processes of excited energy transfer and photochemical trapping in PS II (Holzwarth, 1989, and references therein). As previously shown by Nordlund and Knox (1981), the fluorescence kinetics of LHCII remains virtually unaffected by high laser pulse intensities (Liu et al., 1993), thus indicating a practically instantaneous annihilation if more than one photon is absorbed during the excitation pulse (for discussion see Mauzerall, 1976, 1978; Gülen et al., 1986; Schödel et al., 1996).

Excited singlet states are quenched not only by interaction with other singlets, but also by interaction with triplet states (Breton et al., 1979; Breton and Geacintov, 1980; Mathis and Paillotin, 1981; Paillotin et al., 1983; Kolubajev et al., 1985 and references therein). Even short pulses at high repetition rates lead to a drastic increase of the triplet population in photosynthetic membranes (Geacintov et al., 1978).

The lifetime of triplets exceeds that of singlets by orders of magnitude. Therefore, the possible interference by triplet quenching has to be taken into account in studies where transient changes of the relative fluorescence yield are used as an analytical tool to monitor redox reactions in PS II. An illustrative example provides the transient rise of the relative fluorescence yield in the domain of a few microseconds. This rise originates from at least two processes, i.e., reduction of $P680^{+\bullet}$ and decay of ^{3}Car (for recent discussions see Reifarth et al., 1997; Christen et al., 1998 and references therein).

The present study focuses on the analysis of processes of triplet quenching in the relatively simple and well defined system of solubilized trimeric LHCII. Comparative pump probe experiments with 2-ns pulses were performed to monitor the population of triplet states of carotenoids and chlorophylls by measuring flash-induced changes of the optical density at 507 and 675 nm, respectively, and simultaneously to detect the quenching of fluorescence due to the probe pulse. The most interesting result emerging from the experimental data and analyses of them is the conclusion that, apart from the major route of chlorophyll triplet formation via intersystem crossing from the first excited singlet state, another pathway is opened at high photon densities. In this case, triplets can be formed either via singlet-singlet interaction or intersystem crossing from higher excited singlet states.

MATERIALS AND METHODS

Sample material

PS II membrane fragments were isolated from spinach according to the method of Berthold et al. (1981) with some modifications as outlined in Völker et al. (1985). Isolation of LHCII was performed in the presence of β-dodecylmaltoside as described in detail (Irrgang et al., 1988). The LHCII preparations were characterized by room temperature absorption spectroscopy using a Shimadzu UV 3000 spectrophotometer (Shimadzu Co., Kyoto, Japan). Absorption maxima in the red were localized at 652 ± 1 and 675 ± 1 nm. Pigment concentrations and Chl *a*/*b* ratio were determined using the method of Porra et al. (1989). The latter value was found to be 1.35 ± 0.05. The polypeptide composition of the LHCII preparation has been checked by SDS/urea/PAGE using the method described in Irrgang et al. (1988). The LHCII preparation was diluted with a buffer containing 30 mM MES-NaOH, pH 6.5, 10 mM $CaCl_2$, 20% w/v sucrose, and 0.025% w/v β-dodecylmaltoside. The total chlorophyll concentration of the sample was 0.2 mg Chl/cm^3.

The content of β-carotene and xanthophylls has been spectrophotometrically determined by measuring the absorbance at 470 nm in 80% v/v acetone. The calculations were carried out using the equation described in Wellburn and Lichtenthaler (1984). The carotenoid composition of the samples was analyzed by RP-HPLC using a Nucleosil 100 C-18 column (Knauer, Berlin, Germany) (25 cm × 4.0 mm; i.d. 5 μm) in combination with methanol/tetrahydrofuran 9/1(v/v) as solvent. The pigments were separated at a flow rate of 1 ml/min. Elution followed at 450 nm. Chl *a* (Fluka, Deisenhofen, Germany), lutein (Sigma, Deisenhofen, Germany), and β-carotene (Fluka) were applied as external and internal standards. The average pigment composition of ten different batches of LHCII preparations was investigated using HPLC analysis resulting in the following: 0.2 lutein, 0.078 violaxanthin, 0.078 neoxanthin, and traces (0.015) of β-carotene per Chl *a* (mol/mol). These values are similar to those described by Peterman et al. (1997) and Bassi et al. (1993) for LHCII of maize except that in those preparations a lower violaxanthin content and no β-carotene was found. The highest variability in the content of carotenoids was found for neoxanthin. Some samples did not contain any neoxanthin; they have not been used for this study. The lability of the binding of neoxanthin has also been established by Kühlbrandt et al. (1994b). Possibly neoxanthin is located in the periphery of the pigment protein complex.

The LHCII preparation contains solubilized nonaggregated trimeric complexes consisting of Lhcb1/2 and minor amounts of Lhcb3, Lhcb5, and Lhcb6 as analyzed by immunoblotting with monoclonal/polyclonal antibodies and silver staining of SDS/urea/polyacrylamide gels (see also Vasil'ev et al., 1997a). Two different lines of experimental evidence strongly support the presence of trimeric complexes and the absence of aggregates. First, the fluorescence emission spectra at 77 K show a narrow band at 681 ± 1 nm with a full width at half maximum of 9 nm, which is characteristic for the trimeric form (Vasil'ev et al., 1997a; Peterman et al., 1996; Hemelrijk et al., 1992). Second, no additional fluorescence emission bands could be observed at wavelengths of 712 or 738 nm, indicating that no aggregated pigment complexes were present. Aggregates can easily be obtained after dialysis of the complexes to remove the detergent used for isolating the complex (β-dodecylmaltoside) (Vasil'ev et al., 1997a).

Anaerobic conditions were obtained by exposure of the sample to a stream of wet nitrogen gas at room temperature.

Experimental setup

A schematic description of home-built equipment is shown in Fig. 1.

Pump pulses at 645 nm (repetition, 10 Hz) were generated from a Nd^{3+}-YAG laser-pumped dye laser (LAS LDL 105). The probe pulses were provided by a compact N_2/dye laser module (VSL-dye, Laser Sci-

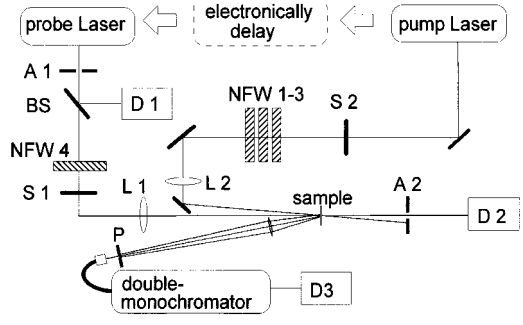

FIGURE 1 Scheme of the optical arrangement of home-built equipment, described in the text. A, aperture; BS, beam splitter; D, detector; L, lens; NFW, wheels with neutral density filters; P, Pinhole; S, automatic shutter.

ence, Inc., Newton, MA) that was synchronized to the pump laser by external triggering. Both pump and probe pulses are about 2 ns in duration (for details see Schödel et al., 1996, 1998). The delay between pump and probe pulses was adjusted from 20 ns up to the millisecond time range via a home-built programmable electronically triggered time delay (time resolution ± 10 ns). The measurements of ΔOD at 507 and 675 nm were performed as described in Schödel et al. (1998). The spot produced by the probe beam was about 70 μm in diameter, whereas the pump spot was considerable larger (about 300 μm). The spatial intensity distribution of both spots was carefully analyzed as described in Schödel et al. (1996). The intensity of the pump pulses was adjusted to the desired value by using neutral density filters. The intensity of the probe pulses used for the ΔOD measurements was about 10^{12} photons cm^{-2} $pulse^{-1}$ (low intensity limit).

Changes of the fluorescence yield induced by the pump pulses were determined by monitoring the fluorescence due to weak probe pulses (with 10^{13} photons cm^{-2} $pulse^{-1}$) at detector D3 (see Fig. 1). In order to obtain reliable results, it was indispensable to collect the photons only from an area with almost homogeneous (pump) excitation. This goal could be achieved by using a spatial separation as outlined in detail in Schödel et al. (1996). The principle of this method is the projection of the cuvette surface onto a pinhole (see Fig. 1). The diameter of the latter was adjusted to the spot size of the pump pulse in the plane of the sample cuvette (optical pathway 0.2 mm for the fluorescence measurements). This permitted the detection of photons that were collected exclusively from the selected area of almost homogenous intensity around the center of the spot illuminated by the pump pulse.

An additional advantage of this method is a drastic decrease of the fluorescence owing to the pump pulse, because the fluorescence yield of the pump pulse was found to decrease drastically with increasing I_P when measured at proper spatial selection. At 10^{18} photons cm^{-2} $pulse^{-1}$, values of about 10^{-3} were found for the normalized pump fluorescence yield (see Fig. 3 of Schödel et al., 1996). Accordingly, the total pump pulse fluorescence signal levels off for I_P values $\geq 10^{16}$ photons cm^{-2} $pulse^{-1}$. Therefore, a careful selected optical geometry permits the suppression of electronic disturbance owing to oversaturation of the detector system by the pump pulse fluorescence up to the highest I_P values (see below).

The time delay between the pump and probe pulses (each of 2 ns duration) was at least 50 ns. Based on the lifetime of about 4 ns for solubilized LHCII (Vasi'lev et al., 1997b), the remaining fluorescence emission caused by the pump pulse decayed at $t \geq 50$ ns to a level of less than 10^{-5} of its original value at $t = 0$. Therefore, the fluorescence of the probe pulse is superimposed by only very small contributions from the pump pulse. In spite of this effect and the above mentioned spatial suppression of the pump pulse fluorescence, the latter still causes some effects on the detector electronics. This gives rise to an additional signal at very high I_P which has been eliminated by a suitable difference method. The relative change of the probe fluorescence, F/F_0, was then obtained by the following relation:

$$\frac{F(I_P,\, t \geq 50\ \text{ns})}{F_0} = \left.\frac{S(\text{pump}\uparrow\ \text{probe}\uparrow) - S(\text{pump}\uparrow\ \text{probe}\downarrow)}{S(\text{pump}\downarrow\ \text{probe}\downarrow) - S(\text{pump}\downarrow\ \text{probe}\downarrow)}\right|_{I_P,\, t\geq 50\ \text{ns}} \quad (1)$$

where $\uparrow$ and $\downarrow$ symbolize the state of the shutters (see Fig. 1, pump $\uparrow/\downarrow$: S2 = on/off, probe $\uparrow/\downarrow$: S1 = on/off) and S the time-integrated signals obtained with a Boxcar Integrator (BCI 280, synchronized to the delayed probe pulse with a gate width of 10 ns). In this way contributions due to the pump pulse could be eliminated.

In order to assure that electronic artifacts do not affect the data obtained according to Eq. 1, the following check experiments were performed. The sample was replaced by a scattering surface and the same wavelength was used for the pump pulse, the probe pulse and the detection wavelength of the double monochromator. The electronic signals at detector D3 were adjusted to those that were monitored (and additionally proved with a Tektronix TDS 220 oscilloscope, Tektronix, Inc., Wilsonville, OR) for the fluorescence signals of the LHCII even at highest I_P. As a result of these check experiments the signal ratio obtained according to Eq. 1 was found to be independent of I_P and t (data not shown), thus indicating that under the experimental conditions of the probe fluorescence measurements the results are not affected by distortions of the detector system.

RESULTS AND DISCUSSION

Measurements of ΔOD at 507 nm and 675 nm

The change of the optical density was measured at a delay time of 50 ns between pump and probe pulses. This time is long enough to achieve a virtually complete formation of triplets but sufficiently short to avoid effects caused by their decay. The experiments were performed at 507 nm, where the difference spectrum of carotenoid triplet (^{3}Car) formation exhibits the peak of its most prominent positive band, and at 675 nm, where a Chl bleaching is observed (Peterman et al., 1995). Control experiments confirmed that the results are not affected by a possible sample modification owing to irreversible damage effects even at highest pump pulse intensities. Fig. 2 shows the normalized flash induced changes of the relative optical densities $\Delta OD/OD_0$ at 507 and 675 nm as a function of the pump pulse intensity. An inspection of the data obtained reveals two interesting features: $\Delta OD/OD_0$ at 507 nm markedly increases with increasing pump pulse intensity and a comparatively minor bleaching arises at 675 nm that could be detected only at relatively high pump intensities.

For the sake of direct comparison with fluorescence measurements performed in this study another effect has to be taken into account. The ΔOD experiments were performed

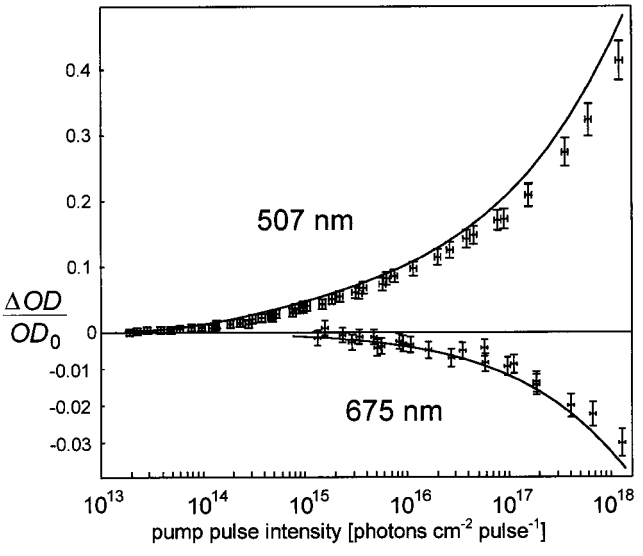

FIGURE 2 Relative change of $\Delta OD/OD_0$ at 507 and 675 nm measured 50 ns after the pump pulse as a function of the pump pulse intensity. The error bars indicate the measured data (at 36% transmittance at the pump wavelength of 645 nm). The solid lines represent the dependencies of $\Delta OD/OD_0$ at the surface of the cuvette. For details, see text.

with a 1-mm cuvette (see Materials and Methods). Thus, at a chlorophyll concentration of 0.2 mg/ml in the sample, the pump pulse intensity at 645 nm decreased to about 36% inside the cuvette. As a consequence any population of species formed by the actinic flash exhibits a gradient and the probe pulse monitors an average value. Therefore, the data points with the error bars shown in Fig. 2 symbolize average ΔOD values. On the other hand, the fluorescence measurements mainly reflect the properties at the surface of the sample in a cuvette of 0.2 mm (see Materials and Methods). Therefore, the surface ΔOD values were calculated from the experimental data as it is outlined in the Appendix. The results obtained are represented by unbroken curves in Fig. 2.

An inspection of Fig. 2 reveals that the extent of ΔOD at 507 nm markedly increases with increasing I_P without showing any tendency of saturation. Accordingly, questions arise on the origin of this phenomenon. To address this point, possible routes of triplet formation will be briefly discussed.

Pathways of triplet generation

The following analysis is based on two prerequisites. First, at a pump pulse wavelength of 645 nm any carotenoid triplet is necessarily produced via the indirect pathway through ^{3}Chl formation from excited singlets and subsequent triplet-transfer to Car. Second, the ΔOD values at 507 nm are a linear measure of the carotenoid triplet population at all pump pulse intensities used in this study. The first point is self-evident because only chlorophylls can absorb light of 645 nm in LHCII. The second assumption is based on the finding that the monoexponential relaxation of ΔOD at 507 nm remains invariant to I_P (Schödel et al., 1998). Therefore, the normalized population of carotenoid triplets, x_T^{Car}, can be obtained from the data of $\Delta OD/OD_0$ at 507 nm as it is outlined in the Appendix.

For the sake of simplicity, at first the ^{3}Car population at comparatively low I_P values will be considered. In this case ^{3}Chl is formed exclusively from the first excited singlet state ^{1}Chl*(S_1) via intersystem crossing and the normalized population of carotenoid triplets at 50 ns after the pump pulse can be calculated on the basis of a comparatively simple model as it is shown in Schödel et al. (1998). The following relation is obtained:

$$x_T^{Car}(t = 50\ \text{ns}, I_P) \cong N^{Chl}/N^{Car} \cdot k_{ISC} \cdot \int_{-\infty}^{t=50\ \text{ns}} x_1^{Chl}(t', I_P)dt' \tag{2}$$

where $x_1^{Chl}(t', I_P)$ is the normalized population of ^{1}Chl*(S_1) at the time t' and I_P. In order to test whether or not this relation is satisfied over the whole range of I_P values, the value of the integral must be measured by an independent method. The first excited singlet state ^{1}Chl*(S_1) not only decays via intersystem crossing but also gives rise to fluorescence emission. At 50 ns after the pump pulse the ^{1}Chl*(S_1) population completely decayed into the ground state because the lifetime was found to be about 4 ns in solubilized LHCII (Vasil'ev et al., 1997a). Accordingly the integrated pump pulse fluorescence can be written as

$$F^{pump}(I_P) = k_F \cdot N^{Chl} \cdot \int_{-\infty}^{50\ \text{ns}} x_1^{Chl}(t', I_P)dt' \tag{3}$$

A comparison with Eq. (2) readily reveals that at low I_P values a linear relation should exist between ΔOD at 507 nm measured at the surface of the cuvette and the integral pump pulse fluorescence. The surface ΔOD values are calculated as described in the Appendix and the integrated pump pulse fluorescence at different I_P is gathered from the data presented in Schödel et al. (1996). After suitable scaling of the data the double logarithmic plot in Fig. 3 *A* shows that up to a threshold of about 10^{15} photons cm^{-2} pulse^{-1} the expected linear relation is satisfied. At higher I_P values marked deviations are obtained. As outlined in the Appendix, the difference between both curves (dotted line in Fig. 3 *B*) can be explained by the existence of an additional channel for chlorophyll triplet formation from excited chlo-

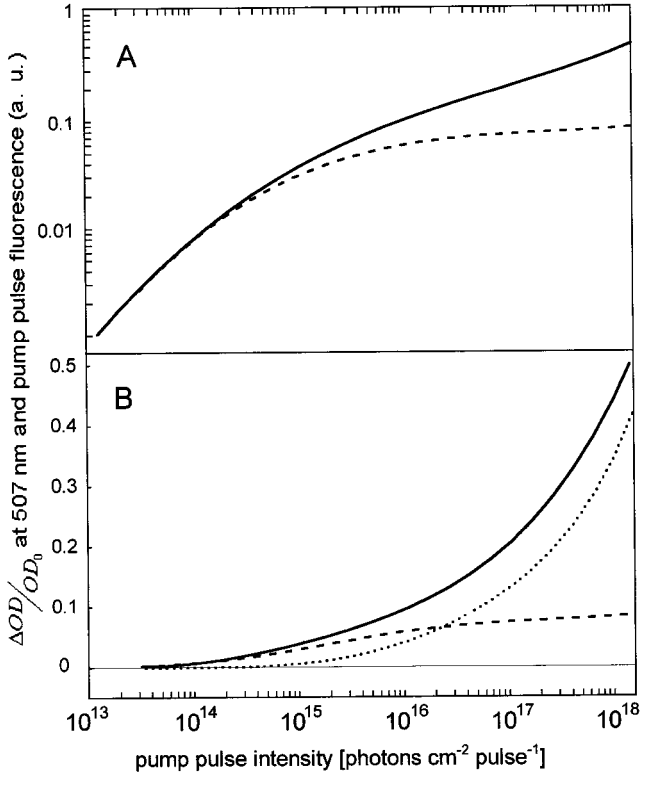

FIGURE 3 (*A*) Double logarithmic plot of $\Delta OD/OD_0$ at 507 nm (*solid line*, corresponding to Fig. 2, *solid line*) together with the data of the (integrated) pump fluorescence (*dashed line*, calculated from the data of Schödel et al., 1996) as a function of the pump pulse intensity. The pump fluorescence data are scaled to fit with $\Delta OD/OD_0$ at 507 nm at low I_P. (*B*) Plot of $\Delta OD/OD_0$ at 507 nm (*solid line*), the scaled pump fluorescence (*dashed line*), and the difference of both (*dotted line*).

rophyll singlets that are not fluorescent (further increase of $x_T^{Car}(t = 50\ ns, I_P)$ at saturated $F^{pump}(I_P)$ at higher I_P, see Fig. 3 *A*):

$$D \cdot \int_{-\infty}^{50\ ns} x_2^{Chl}(t', I_P)dt' = x_T^{Car}(t = 50\ ns, I_P) - C \cdot F^{pump}(I_P) \quad (4)$$

where C is the scaling factor between $x_T^{Car}(t = 50\ ns, I_P)$ and $F^{pump}(I_P)$ obtained from the above low I_P limit and D is an arbitrary constant. This difference is shown in the semilogarithmic plot of Fig. 3 *B* as dotted line. It represents an integrated chlorophyll excited singlet population that is not fluorescent and increases with increasing I_P.

Measurements of the probe fluorescence

Triplets are known to quench chlorophyll fluorescence efficiently (see Introduction). Based on the information gathered from the data above it is possible to analyze this quenching in detail by performing comparative fluorescence measurements. Therefore, the ratio of the relative yield of the probe pulse fluorescence $F(t \geq 50\ ns, I_P)/F_0$ was determined at different pump pulse intensities as a function of the delay time t of the probe pulse. Special care was taken to eliminate any effect of the fluorescence emission caused by the pump pulse (see Materials and Methods).

Fig. 4 shows in a double logarithmic plot the values of $F(t = 50\ ns, I_P)/F_0$ as a function of the pump pulse intensity I_P. At high I_P a drastic decrease of the probe fluorescence was observed (logarithmic scale!).

Fig. 5 shows the recovery of $F(t \geq 50\ ns, I_P)/F_0$ with increasing delay time when pump pulses of different intensities I_P are used. In the following this time dependent quenching will be analyzed in detail.

Fluorescence quenching in the microsecond domain

Because the fluorescence data depicted in Figs. 4 and 5 are not affected by contributions due to the emission caused by the pump pulse, the data can be directly used for the calculation of the quenching efficiency of triplets. The fluorescence quantum yield of the probe pulse in the absence of a pump pulse, Φ_F^0, is given by $\Phi_F^0 = (k_F/k)$, where k_F is the rate constant of radiative emission and k is the reciprocal lifetime of the excited singlet state which was found to be $k^{-1} = (4.3 \pm 0.2)$ ns in solubilized LHCII (Liu et al., 1993; Vasil'ev et al., 1997a,b). If, in addition, the actinic flash leads to formation of quenchers with lifetimes much longer than those of the singlet states, the fluorescence quantum yield, Φ_F, decreases down to lower values. Φ_F is given by $\Phi_F = (k_F/\{k + \Sigma_i k_q^i \cdot x^i\})$, where k_q^i are the quenching constants assigned to the relative population, x^i, of the fluorescence quenching species i. This leads to the Stern-Volmer equation:

$$\frac{\Phi_F^0}{\Phi_F} = 1 + \sum_i \frac{k_q^i}{k} \cdot x^i \quad (5)$$

In the following the sum $\Sigma_i(k_q^i/k) \cdot x^i(t)$ will be symbolized by $Q_{tot}(t)$. The fluorescence quantum yield is defined by $\Phi_F = F/I_{abs}$ where I_{abs} is the number of absorbed photons (at the probe pulse wavelength of 430 nm, see Experimental Setup). It was found that the change of the optical density owing to the pump pulse is rather small at this wavelength ($\Delta OD/OD_0 \approx -0.01$ at 10^{17} photons cm^{-2} $pulse^{-1}$) (Schödel et al., 1998) whereas $F(t = 50\ ns, I_P)/F_0$ is much larger ($\approx$0.3, see Fig. 5). Accordingly, the number of photons absorbed by the sample during the probe pulse (I_{abs}) remains almost unaffected by the pump pulse after a delay of 50 ns and Φ_F^0/Φ_F can be replaced by $F_0/F(t \geq 50\ ns, I_P)$. The latter ratio can be obtained directly as the reciprocal values of the results depicted in Fig. 5. Therefore, from the experimental data of the fluorescence yield one obtains

$$Q_{tot}(t) = \frac{F_0}{F(t \geq 50\ ns, I_P)} - 1 \quad (6)$$

The results gathered from the data of Fig. 5 by using Eq. 6 (normalized to unity) are shown in Fig. 6.

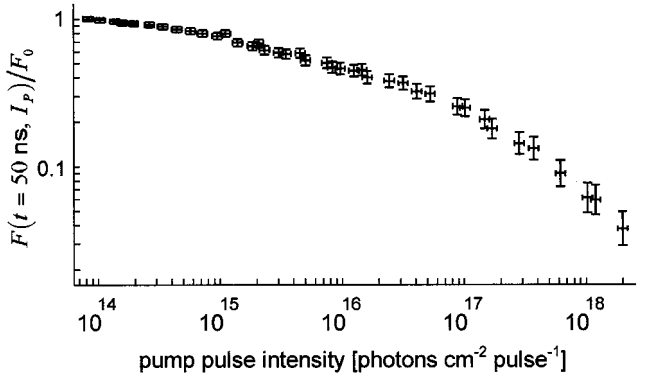

FIGURE 4 Double logarithmic plot of the ratio $F(t = 50\ ns, I_P)/F_0$ as a function of the pump pulse intensity, I_P, in solubilized LHCII.

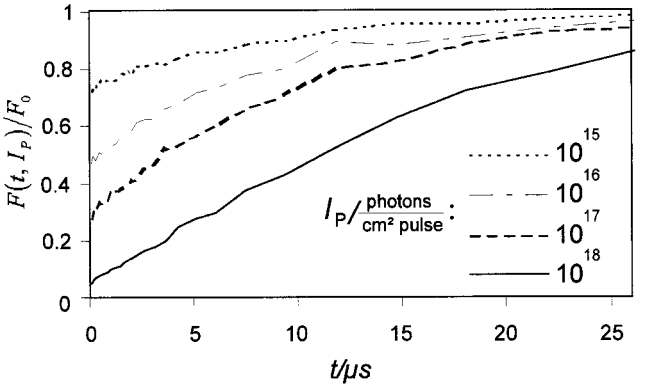

FIGURE 5 Ratio $F(t, I_P)/F_0$ as a function of the delay time (starting at 50 ns) at different pump pulse intensities, I_P, in solubilized LHCII.

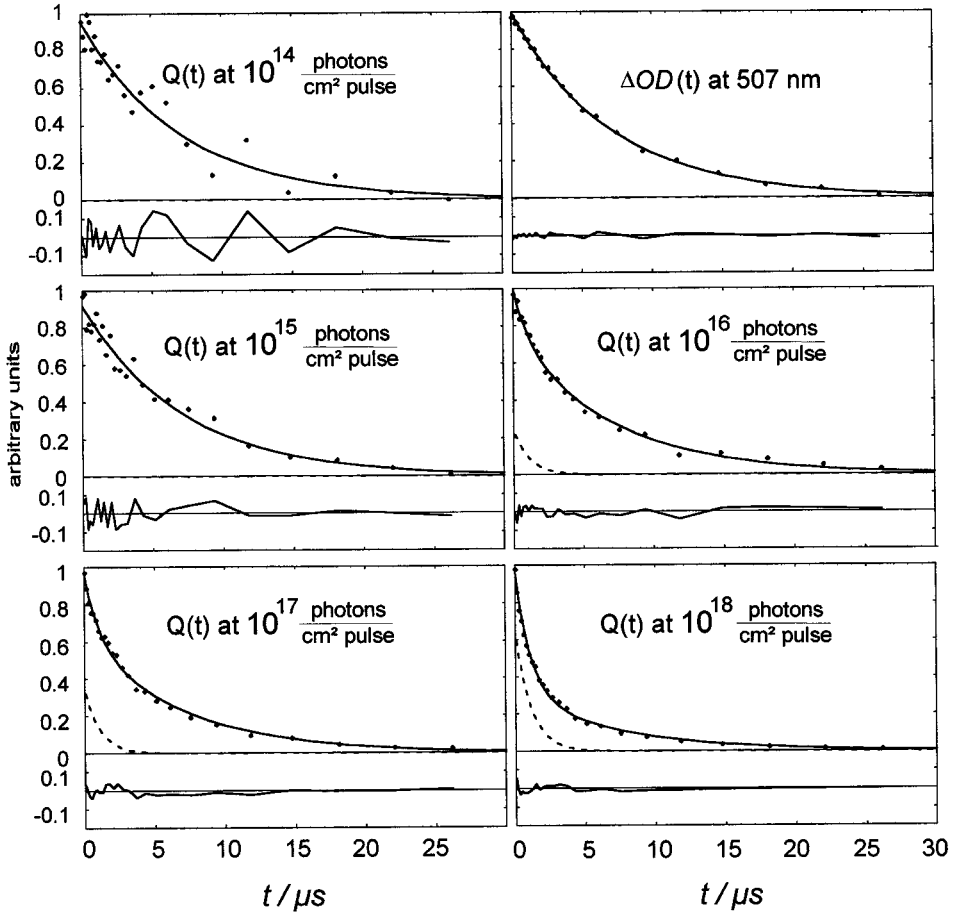

FIGURE 6 Normalized total quencher population in solubilized LHCII at different pump pulse intensities. The data points are gathered from Fig. 5 by using Eq. 6. For comparison, the curve on the right top shows the decay kinetics of carotenoid triplets measured in the same sample as used for the experiments of Fig. 5. The dashed lines reflect the extent of the faster (1.1-μs) decay component. For further details, see text.

Fig. 6 reveals that the decay kinetics of $Q_{tot}(t)$ at (low) pump intensities up to 10^{15} photons cm^{-2} pulse^{-1} can be satisfactorily described by a monoexponential kinetics with a lifetime of about 7 μs. These kinetics are virtually identical with those obtained for the carotenoid triplet decay measured at the same sample (see trace on the top, right side of Fig. 6) that was found to be independent of I_P, see Schödel et al., 1998). Thus, at comparatively low pump pulse intensities carotenoid triplets appear to be the only quenchers of Chl *a* fluorescence in the μs time domain in LHCII and $Q_{tot}(t)$ is given by: $Q_{tot}(t) = (k_q^{^3Car}/k) \cdot x_T^{Car}(t)$. A very interesting feature emerges at higher pump pulse intensities. In this case the decay of $Q_{tot}(t)$ comprises at least two decay kinetics. A kinetic analysis reveals that the data can be well described by a two-exponential kinetics with $\tau_1 \cong 1.1$ μs and $\tau_2 \cong 7.1$ μs as shown by the full lined curves in Fig. 6. The normalized extent of the fast component increases at the expense of the slower one with increasing pump pulse intensity I_P (see dotted curves in Fig. 6). The corresponding values of the 1.1-μs decay component were found to be $A^{1.1\,\mu s} = 0.25, 0.35, 0.65$ at $I_P = 10^{16}$, 10^{17}, 10^{18} photons cm^{-2} pulse^{-1}, respectively.

The invariance of the ΔOD relaxation at 507 nm with respect to I_P and the kinetic properties of $Q_{tot}(t)$ raises questions on the origin of the faster 1.1-μs component. Because at all delay times $\geq$50 ns singlets can be excluded as quenching states, it is most reasonable to assume that another quencher is formed at higher pump pulse intensities that is most likely a chlorophyll triplet. In principle, fluorescence quenching could also arise by interaction with cation radicals (Breton et al., 1979, 1980). It seems unlikely that increasing pump pulse intensities give rise to $Chl^{+\bullet}$ formation in LHCII.

Although the bleaching observed at 675 nm (see Fig. 2) is very small, it could support the idea that a fraction of chlorophyll triplets is formed at high I_P. In order to analyze this feature, the decay kinetics of ΔOD at 675 nm was measured. Fig. 7 shows the results obtained together with the decay at 507 nm (representing the carotenoid triplet decay measured at the identical sample of LHCII). The carotenoid triplet decay exhibits a monoexponential kinetics with $\tau \cong 7$ μs that is typical for almost anaerobic samples. The curve at 675 nm was measured at a high pump pulse intensity of $6 \cdot 10^{17}$ photons cm^{-2} pulse^{-1}. It shows a biexponential decay with a fast component of about 1 μs in addition to a longer component of about 7 μs. At lower intensities the noise of the curves measured at 675 nm was too high for an unambiguous kinetic analysis. The striking similarities of the fast decay component strongly supports the idea that the 1-μs kinetics measured at ΔOD at 675 nm is in close correlation with the 1-μs component obtained for the kinetics of the probe fluorescence quencher and that the

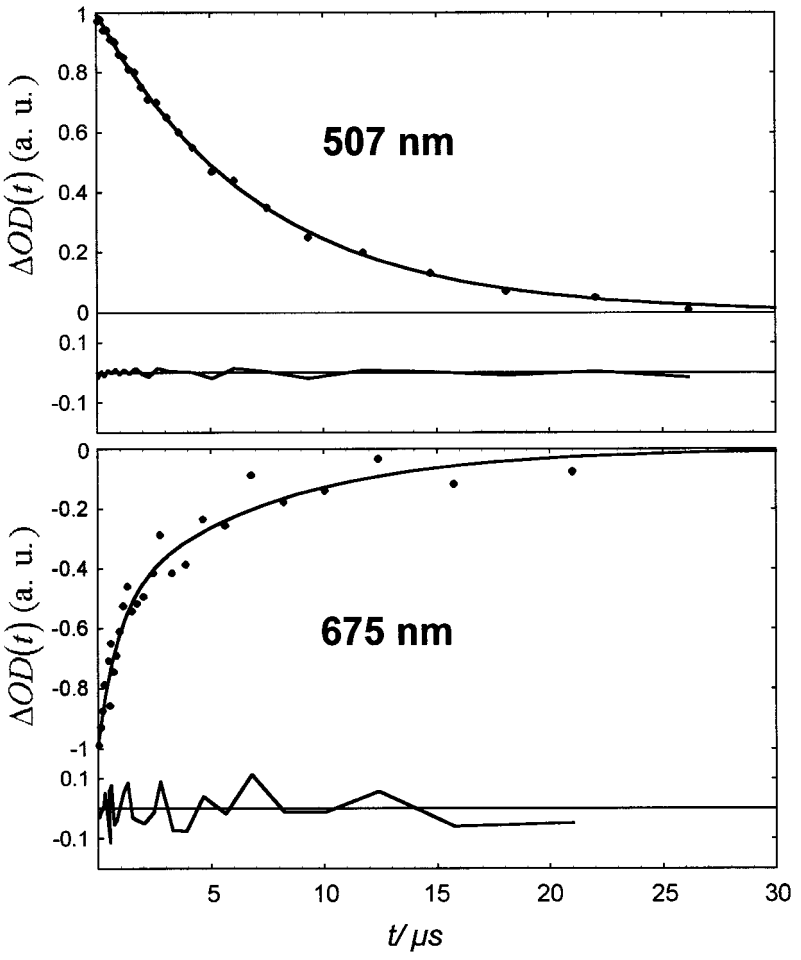

FIGURE 7 Normalized flash-induced ΔOD at 507 nm (*top*) and 675 nm (*bottom*) as a function of delay time between pump and probe pulse in solubilized LHCII under unaerobic conditions. Pump pulse intensity: $6 \cdot 10^{17}$ photons cm^{-2} $pulse^{-1}$. The full lined curves represent the best fit of the data with a monoexponetial kinetics for the top trace at 507 nm: $\tau = 7.1$ μs and a biexponential kinetics for the bottom trace: $a_1 = -0.48$; $\tau_1 = 0.9$ μs; $a_2 = -0.52$; $\tau_2 = 7.1$ μs.

additional quencher is a chlorophyll triplet. Consequently the total quencher population is given by

$$Q_{tot}(t) = \frac{k_q^{^3Car}}{k} \cdot \left[x_T^{Car}(t) + \frac{k_q^{^3Chl}}{k_q^{^3Car}} \cdot x_T^{Chl}(t) \right] \qquad (7)$$

where $k_q^{^3Car}$ and $k_q^{^3Chl}$ are the rate constants for the quenching of the probe fluorescence due to a population of carotenoid triplets, x_T^{Car}, and chlorophyll triplets, x_T^{Chl}, respectively. The latter are given in relative amounts.

Determination of rate constants for chlorophyll fluorescence quenching by triplets

Carotenoid triplet-induced fluorescence quenching at low pump pulse intensities

Based on the kinetic coincidence of the carotenoid triplet decay (see Fig. 7, top trace) and the fluorescence quenching at $I_P \leq 10^{15}$ photons cm^{-2} $pulse^{-1}$ (see Fig. 6) the latter can be fully accounted for dissipation of chlorophyll singlets by carotenoid triplets in the time domain of 50 ns to 30 μs at this (relatively) low pump pulse intensities.

Accordingly, Eq. 6 simplifies to:

$$\left.\frac{F(t, I_P)}{F_0}\right|_{I_P \leq 10^{15}} = \frac{1}{1 + \frac{k_q^{^3Car}}{k} \cdot x_T^{Car}(t, I_P)} \qquad (8)$$

At a delay time of 50 ns the singlet state population generated by the pump pulse is very close to zero (the singlet lifetime in solubilized LHCII is 4.3 ns, see Liu et al., 1993, Vasil'ev et al., 1997a,b), whereas x_T^{Car} attains practically its maximum value because the decay of ^{3}Car is considerably slow ($\tau \approx 7$ μs). As a consequence, $F(t = 50$ ns, $I_P)/F_0$ reflects the maximum quenching by triplets at a particular I_P. Therefore the ratio $k_q^{^3Car}/k$ can be determined via a best fit of the data of $F(t = 50$ ns, $I_P)/F_0$ as a function of I_P in the range of $I_P \leq 10^{15}$ photons cm^{-2} $pulse^{-1}$. This analysis leads to a value of $k_q^{^3Car}/k = 7 \pm 2$. Fig. 8 shows that the experimental results can be satisfactorily described by Eq. 8 up to $I_P = 10^{15}$ photons cm^{-2} $pulse^{-1}$. If one accepts that the rate constant $k_q^{^3Car}$ is independent of the pump pulse intensity, the progressing deviation at higher I_P values (dashed line in Fig. 8) can only be explained by the formation of an additional fluorescence quencher that is most likely a chlo-

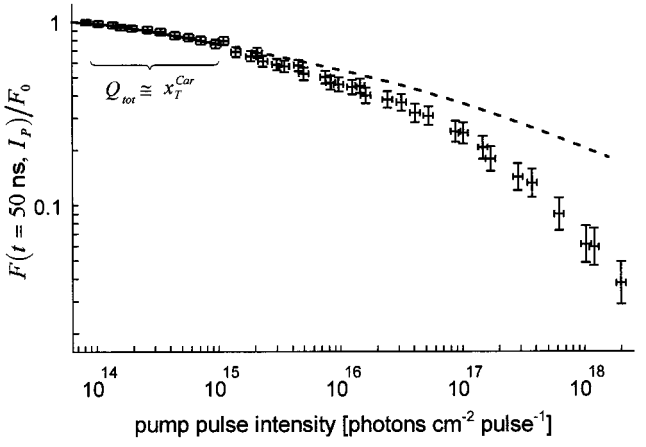

FIGURE 8 Ratio of $F(t = 50\ \text{ns}, I_P)/F_0$ (data points) and calculated ratio for $k_q^{3Car}/k = 7$ (*dashed and solid lines*), assuming only carotenoids to act as quenchers (Eq. 8). At comparable low pump pulse intensities the calculated curve fits well with the experimental data (*solid line*) whereas at intensities exceeding 10^{15} photons cm^{-2} pulse^{-1} a growing deviation is obtained (*dashed line*).

rophyll triplet (see above). In the following section the rate constant for this quenching reaction will be determined.

Chlorophyll triplet-induced probe fluorescence quenching

The biphasic decay of ΔOD at 675 nm (see Fig. 2) is interpreted as a composite of two contributions, i.e., an electrostatic effect due to interaction between ^{1}Chl(S_0) and ^{3}Car (van der Vos, 1991) which exhibits the same decay with $\tau \approx 7\ \mu$s as ^{3}Car and a second fraction due to formation of Chl fluorescence quenchers which are characterized by a relaxation rate of about 1 μs. Accordingly, only the latter fraction is used for calculation of k_q^{3Chl} (see below). Rearrangement of Eqs. 5 and 6 leads to the following expression:

$$\frac{k_q^{3Chl}}{k_q^{3Car}} = \frac{\left(\dfrac{F_0}{F(t, I_P)} - 1\right) \cdot \dfrac{k}{k_q^{3Car}} - x_T^{Car}}{x_T^{Chl}} \qquad (9)$$

At $t = 50$ ns the maximum of the relative carotenoid triplet population, x_T^{Car}, at a given pump pulse intensity is obtained from $\Delta OD/OD_0$ measured at 507 nm (see Appendix) and $F_0/F(t = 50\ \text{ns}, I_P)$ from the reciprocal value of the data depicted in Fig. 4 at $6 \cdot 10^{17}$ photons cm^{-2} pulse^{-1}. The relative population of chlorophyll triplets can be estimated in the following way: at the pump pulse intensity of $6 \cdot 10^{17}$ photons cm^{-2} pulse^{-1}, the fraction of the fast component of $\Delta OD/OD_0(t)$ measured at 675 nm is about 0.5 (see Fig. 7). Because this fast component is a measure of x_T^{Chl} it remains to calculate its value from ΔOD at 675 nm. As is outlined in the Appendix, the relative population of chlorophyll triplets results to $x_T^{Chl}(t = 50\ \text{ns}, I_P = 6 \cdot 10^{17}$ (photons/cm^2 pulse)) $\approx 0.015 \pm 0.005$.

A rather high value of 100 ± 20 is obtained for the ratio k_q^3Chl/k_q^3Car when using Eq. 9 and the numbers calculated for $F_0/F(t = 50\ \text{ns}, I_P)$, x_T^{Car}, and x_T^{Chl} at $I_P = 6 \cdot 10^{17}$ photons cm^{-2} pulse^{-1} and the k/k_q^3Car value of 0.15 from the analysis of the results at $I_P \leq 10^{15}$ photons cm^{-2} pulse^{-1}. This finding indicates that the Chl-triplet species with an unusually short lifetime, generated only at high pump pulse intensities (see Pathways of triplet generation), are very efficient quenchers of Chl fluorescence.

CONCLUSIONS

The present study provides a detailed analysis of the quenching of Chl fluorescence by triplet states in solubilized LHCII. Three basic conclusions can be gathered from the experimental results and their data analysis. First, excitation with 2-ns laser pulses does not lead to formation of fluorescence quenchers with lifetimes exceeding 7–9 μs. Second, at comparatively weak pump pulses of $I_P \leq 10^{15}$ photons cm^{-2} pulse^{-1}, the fluorescence quenching in the microsecond time domain is entirely due to ^{3}Car. Third, excitation with strong pump pulses opens a new road for triplet formation, which comprises the generation of an additional quencher that is most likely a ^{3}Chl with unusual properties (see below).

The first two conclusions imply that in our solubilized trimeric LHCII preparation (for a more detailed biochemical and biophysical characterization, see Vasil'ev et al., 1997a; Schödel et al., 1998) all chlorophyll triplets which are formed via intersystem crossing from ^{1}Chl*(S_1) very rapidly decay via efficient triplet-transfer to carotenoids. This idea is in line with previous conclusions (Peterman et al., 1995, 1997b; Schödel et al., 1998). Taking into account the rather high value of at least $\geq$(500 ps)$^{-1}$ for the rate constant of ^{3}Chl quenching by Car (Schödel et al., 1998), LHCII appears to be a highly optimized operational unit for light harvesting and protection of superfluous excitation energy under natural illumination conditions of plants. The carotenoid triplet valve not only provides an almost perfect suppression of chlorophyll triplets as sensitizers for the formation of dangerous singlet oxygen, but the transiently formed Car-triplets also open a channel for radiationless decay of superfluous excited singlet states. The population of ^{3}Chl with unusual properties is practically zero even in bright sunlight.

The third conclusion is the most spectacular. It is a consequence of the unexpected phenomena that arise when the population of fluorescent chlorophyll singlet states ^{1}Chl*(S_1) approaches a saturation level at pump pulse intensities of about 10^{16} photons cm^{-2} pulse^{-1} (see dashed curves in Fig. 3). The explanation of the striking features emerging above this intensity threshold for kinetics and amplitudes of ΔOD at 507 nm and 675 nm (see Figs. 2 and 7) and of fluorescence quenching (see Figs. 4, 5, and 6) requires two assumptions: 1) the opening of an unconven-

tional pathway for triplet formation and 2) the population of chlorophyll triplets with unusual kinetic properties. The lifetime of the latter species is shorter by at least two orders of magnitude ($\approx$1 μs) than those of ^{3}Chl in solution (Chibisov, 1969; Mathis and Setif, 1981; Durrant et al., 1990) or how it would be expected from protein-bound chlorophyll that is lacking triplet-transfer to carotenoids (Breton et al., 1979). This result is in contrast to the previously reported formation of long living (millisecond time domain) quenching species in isolated chloroplasts that were also ascribed to Chl triplets (Breton et al., 1979). The nature of the 1-μs relaxation channel remains to be clarified in future studies. At present we can only speculate that a partial depletion of the carotenoid ground state may prevent a complete triplet-transfer from ^{3}Chl to Car at sufficiently high pump pulse intensities. It must be emphasized that the extent of this particular chlorophyll triplet population (decaying with 1 μs) remains very low compared to the population of carotenoid triplets, even at the highest pump pulse intensities. A most interesting question arises about possible underlying mechanism(s) of the unconventional triplet formation pathway.

As the rate constants of both radiative emission (k_F) and intersystem crossing (k_{ISC}) from the first excited singlet states ^{1}Chl*(S_1) are expected to be independent of I_P, the drastic increase of carotenoid triplets with increasing pump pulse intensities (see Fig. 2) can only be explained by either singlet-singlet pair combination into a triplet-triplet pair or by intersystem crossing from higher excited singlet states. Recently, the latter mechanism has been reported to take place in Zn-phthalocyanines and the rate constant estimated to be of the order of $(100\ \text{ps})^{-1}$ (Rückmann et al., 1997). Of course, LHCII and its pigments establish a markedly different system, but the general possibility of the existence of ISC channels from higher excited singlet states is quite an interesting idea that deserves further analysis. Some implications will be outlined in a forthcoming paper.

APPENDIX

Calculation of ΔOD at the surface of the sample

The change of the optical density in the LHCII sample depends on the intensity of the pump pulse. This value decreases along its optical pathway and therefore the signal monitored by the probe pulse represents an average value that is given by:

$$\overline{\Delta OD} = \frac{1}{(1-T)\cdot I_P}\cdot\int_{T\cdot I_P}^{I_P}\Delta OD(I'_P)\cdot dI'_P \qquad (A1)$$

where I'_P is the actual pump pulse intensity at any point of the optical pathway and T the sample transmittance. The latter value is 0.36 for the experimental conditions used in this study (Chl concentration 0.2 mg/ml, optical path length 1 mm, pump pulse wavelength 645 nm, $I_P \le 10^{18}$ photons cm^{-2} pulse^{-1}). Based on Eq. A1, the value of $\Delta OD(I_P)$ can be obtained in the following way via the first derivative of the average ΔOD:

$$\begin{aligned}\frac{d}{dI_P}\overline{\Delta OD}(I_P) &= \frac{1}{1-T}\cdot\frac{d}{dI_P}\left[\frac{1}{I_P}\cdot\int_{T\cdot I_P}^{I_P}\Delta OD(I'_P)\cdot dI'_P\right]\\ &= \frac{1}{1-T}\cdot\left\{-\frac{1}{I_P^2}\cdot\int_{T\cdot I_P}^{I_P}\Delta OD(I'_P)\cdot dI'_P\right.\\ &\quad\left.+\frac{1}{I_P}\cdot\frac{d}{dI_P}\left[\int_{T\cdot I_P}^{I_P}\Delta OD(I'_P)\cdot dI'_P\right]\right\}\\ &= \frac{1}{I_P}\cdot\left\{-\overline{\Delta OD}(I_P)+\frac{1}{1-T}\cdot[\Delta OD(I_P)\right.\\ &\quad\left.-T\cdot\Delta OD(T\cdot I_P)]\right\}\end{aligned}$$

Algebraic rearrangement of the latter expression leads to:

$$\Delta OD(I_P) = T\cdot\Delta OD(T\cdot I_P)+(1-T)\cdot\left[I_P\cdot\frac{d}{dI_P}\overline{\Delta OD}(I_P)+\overline{\Delta OD}(I_P)\right] \qquad (A2)$$

Eq. A2 permits a stepwise calculation of $\Delta OD(I_P)$ from the average values $\overline{\Delta OD}(I_P)$ that are experimentally determined at $T \cong 36\%$. In the first step a continuous function of $\overline{\Delta OD}(I_P)$ was generated from the data points. The calculation of $\Delta OD(I_P)$ started at the low pump intensity limit where $\Delta OD(0.36\cdot I_P)$ was estimated from $\overline{\Delta OD}(I_P)$.

Estimation of the carotenoid triplet population from ΔOD at 507 nm in solubilized LHCII

At a delay time of 50 ns virtually all chlorophyll singlets formed by the pump pulse are relaxed into their ground state. Therefore, any change of the absorption coefficient, α, at 507 nm is due to contributions of triplets. Recently it was found that even at the highest pump pulse intensities used in this study the decay kinetics of ΔOD at 507 nm remains invariant to I_P (Schödel et al., 1998). This observation favors the idea that ΔOD at 507 nm reflects only carotenoid triplets. Based on this assumption, the absorption coefficient α is given by $\alpha = \sigma_0^{Car}\cdot(N^{Car}-n_T^{Car}) + \sigma_T^{Car}\cdot n_T^{Car} + \sigma_0^{Chl}\cdot N^{Chl}$, where σ_0^{Car} and σ_T^{Car} are the cross-sections of ground and triplet states assigned to carotenoids, n_T^{Car} is the concentration of Car triplets, N^{Car} and N^{Chl} are the total concentrations of carotenoids and chlorophylls, respectively, and σ_0^{Chl} is the absorption cross-section of ground states assigned to chlorophyll where $\sigma_0^{Chl}\cdot N^{Chl}$ is a term reflecting the constant fraction of chlorophyll ground states absorbing at 507 nm. The linear absorption coefficient is given by $\alpha_0 = \sigma_0^{Car}\cdot N^{Car} + \sigma_0^{Chl}\cdot N^{Chl}$. Therefore, with $\Delta\alpha = \alpha - \alpha_0$ the change of α normalized to α_0 is given by $(\Delta\alpha/\alpha_0) = n_T^{Car}\cdot(\sigma_T^{Car}-\sigma_0^{Car})/(N^{Car}\cdot\sigma_0^{Car} + \sigma_0^{Chl}\cdot N^{Chl})$. According to the data reported by Siefermann-Harms (1985), the ratio $\sigma_0^{Chl}\cdot N^{Chl}/\sigma_0^{Car}\cdot N^{Car}$ is about 1/3 at 507 nm. With this value the experimentally determined ratio $\Delta OD/OD_0$ at 507 nm is given by

$$\frac{\Delta OD}{OD_0} = \frac{\Delta\alpha}{\alpha_0} \approx \frac{3}{4}\cdot x_T^{Car}\cdot\left(\frac{\sigma_T^{Car}}{\sigma_0^{Car}}-1\right) \qquad (A3)$$

where x_T^{Car} is the normalized carotenoid triplet population n_T^{Car}/N^{Car}.

The results of Fig. 2 clearly show that it is impossible to saturate the carotenoid triplet population even at extremely high pump pulse intensities, i.e., $x_T^{Car} < 1$. Therefore, from Eq. A3 the following relation is obtained:

$$x_T^{Car} = \frac{\Delta OD}{OD_0} \cdot \frac{1}{\frac{3}{4} \cdot \left(\frac{\sigma_T^{Car}}{\sigma_0^{Car}} - 1 \right)} < 1.$$

At $I_P = 10^{18}$ photons cm^{-2} pulse^{-1} the experimental value of $\Delta OD/OD_0$ is about 0.4. Accordingly it follows that the ratio $\sigma_T^{Car}/\sigma_0^{Car}$ exceeds a value of about 1.5. If one assumes a carotenoid population of 0.5 at $I_P = 10^{18}$ photons cm^{-2} pulse^{-1} the resulting ratio of the optical cross sections $\sigma_T^{Car}/\sigma_0^{Car}$ is about 3. Therefore, taking into account the uncertainties of x_T^{Car}, a value of about 2.3 for $\sigma_T^{Car}/\sigma_0^{Car}$ at 507 nm appears to be a reasonable estimate. This value is used for the calculation of the relative occupation of carotenoid triplets from the experimental data of $\Delta OD/OD_0$ at 507 nm as a function of the pump pulse intensity. Eq. A3 leads to:

$$x_T^{Car}(I_P) \approx \frac{\Delta OD(507\text{ nm}, I_P)}{OD_0(507\text{ nm})}.$$

Estimation of the chlorophyll triplet population from ΔOD at 675 nm in solubilized LHCII

In contrast to the blue-green spectral region, carotenoids do not absorb in the red, that is, at 675 nm (for review see Siefermann-Harms, 1985). In that case, the absorption coefficient α is given by $\alpha = \sigma_0^{Chl} \cdot (N^{Chl} - n_T^{Chl}) + \sigma_T^{Chl} \cdot n_T^{Chl}$, where σ_0^{Chl} and σ_T^{Chl} are the absorption cross-sections of ground and triplet states assigned to chlorophyll, $N^{Chl} - n_T^{Chl}$ is the concentration of remaining chlorophyll ground states, and n_T^{Chl} is that of chlorophyll triplets. Analogous to the above considerations for carotenoid triplets, the relative change of the optical density caused by chlorophyll triplets is given by $\Delta OD/OD_0 = x_T^{Chl} \cdot (\sigma_T^{Chl}/\sigma_0^{Chl} - 1)$ at 675 nm. At a pump pulse intensity of $I_P = 6 \cdot 10^{17}$ photons cm^{-2} pulse^{-1} the normalized amplitude of the fast decay component of ΔOD at 675 nm is about 0.5. Because the latter was assigned to ^{3}Chl population one can estimate that chlorophyll triplets are responsible for $\Delta OD/OD_0$ of about -0.01 (see Fig. 2). For the ratio $\sigma_T^{Chl}/\sigma_0^{Chl}$ an approximate value can be gathered with Chl *a* in solution. From the T – S-difference spectrum of Chl *a* and the corresponding absorbance spectrum we obtained $\sigma_T^{Chl}/\sigma_0^{Chl} = 0.15$ in the red absorbance maximum (data not shown). The use of this value together with the above equation and $\Delta OD/OD_0 \approx -0.01$ leads to the relation:

$$x_T^{Chl}(50\text{ ns}, I_P = 6 \cdot 10^{17}\text{ (photons/cm}^2\text{ pulse)}) \approx 0.015 \pm 0.005.$$

Chlorophyll singlets that populate triplets

Without specifying the mechanism, the additional pathway for ^{3}Chl formation at high I_P-values will be symbolized by $k_2 \cdot x_2^{Chl}$, where x_2^{Chl} is the normalized population of nonfluorescent chlorophyll singlets that generate ^{3}Chl via a rate constant k_2 (without relying on the particular mechanism). Accordingly the rate equations for triplet state turnover are given by

$$\frac{d}{dt} x_T^{Chl} \cong k_{ISC1} \cdot x_1^{Chl} + k_{ISC2} \cdot x_2^{Chl} - k_{TT} \cdot x_T^{Chl} \quad \text{(A4a)}$$

$$\frac{d}{dt} x_T^{Car} \cong N^{Chl}/N^{Car} \cdot k_{TT} \cdot x_T^{Chl} \quad \text{(A4b)}$$

where triplet relaxation terms have been ignored, because Eqs. A4 are only relevant for $t \leq 50$ ns. The normalized population of chlorophyll triplets, x_T^{Chl}, and thus its derivative, are approximately zero even at highest I_P (otherwise $\Delta OD/OD_0$ at 675 nm would not remain vanishingly small compared to 507 nm). Therefore, Eq. A4a can be set to zero. This leads to

$$x_T^{Car}(I_P) = k_{ISC1} \cdot N^{Chl}/N^{Car} \cdot \int_{-\infty}^{50\text{ ns}} x_1^{Chl}(t')dt' + k_{ISC2} \cdot N^{Chl}/N^{Car} \cdot \int_{-\infty}^{50\text{ ns}} x_2^{Chl}(t')dt' \quad \text{(A5a,b)}$$

from which Eq. 4 is obtained.

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