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Two-Photon-Sensitized Fluorescence and Excitation Spectra of Photosystem I of *Thermosynechococcus elongatus*

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In this work, we present the two-photon excitation spectrum and two-photon-sensitized fluorescence spectra of photosystem I (PS I) of *Thermosynechococcus elongatus*. The two-photon excitation spectrum of PS I agrees well with known spectra of the carotenoid (Car) S_1 state of β-carotene in solution. Only a small intensity of the PS I two-photon spectrum around its 0–0 transition indicates hot-state Car $S_1 \rightarrow$ chlorophyll (Chl) energy transfer. The two-photon-sensitized fluorescence spectrum of PS I shows no major difference of the intensities of bulk- and red-chlorophyll fluorescence in comparison to the fluorescence spectrum observed after nonselective one-photon excitation of the PS I chlorophylls. Fluorescence spectra measured after selective excitation of red chlorophylls show a decreased fluorescence of bulk chlorophylls around 685 nm, indicating that the thermal equilibration of the excitations between bulk and red chlorophylls is not complete prior to emission. Taking these results together, there seems to be almost no preference for Car $S_1 \rightarrow$ red chlorophyll energy transfer, even though this could have been energetically favorable. We conclude that the small number of red chlorophylls is not sufficient to act as major acceptors for Car S_1 energy. The fact that no increased red-chlorophyll emission is observed after two-photon excitation also provides a strong indication that no two-photon-allowed excitonic states can be formed from chlorophyll–chlorophyll or carotenoid–chlorophyll interactions. A comparison of the measured two-photon data with experimental and theoretical chlorophyll energies yields a fraction of ~40% carotenoids that are potential Car S_1 donors, neglecting any hot-state energy transfer. Spectral overlap calculations yield estimated vibrational ground-state Car $S_1 \rightarrow$ Chl energy-transfer time constants of 3.1–4.4 ps.

Introduction

The photosystem I (PS I) complex of *Thermosynechococcus elongatus* has been the subject of many investigations since its complete structure was resolved at 2.5-Å resolution.[1–15] One reason for the attractiveness of PS I *T. elongatus* is that it is the first high-resolution crystal structure of a photosynthetic complex that contains the reaction center and light-harvesting pigments inseparably in one unit. With the high-resolution structure, detailed theoretical calculations could be carried out which provided good agreement with experimental results.[2–4,15] Spectroscopic data[16] and the X-ray structure of plant PS I at 4.4-Å resolution[17] provide strong evidence that PS I from *T. elongatus* is quite similar to the photosystem I core complex of higher plants[18] and green algae. It represents a large, multisubunit membrane-integral protein–pigment complex that converts light energy into chemical energy. After primary charge separation initiated by excitation of the chlorophyll dimer P700, the electron passes along the electron-transfer chain consisting of a monomeric Chl *a* cofactor (A_0), a phylloquinone (A_1), and three Fe_4S_4 clusters (F_X, F_A, F_B). At the stromal side, the electron is given by cluster F_B to ferrodoxin (or flavodoxin) and then transferred to $NADP^+$ reductase. The reaction cycle is completed by rereduction of $P700^+$ by cytochrome c_6 (or plastocyanin) at the luminal side of the membrane.[1]

Photosystem I of *Thermosynechococcus elongatus* binds about 96 chlorophyll *a* (Chl *a*) and 22 β-carotene molecules per monomeric unit.[1] The arrangement of the chlorophylls within each monomer consists of six Chl *a* molecules assigned to the reaction center (RC) and the remaining Chl *a* molecules packed in a bowllike structure closely surrounding the RC, plus two peripheral domains, where the Chl *a* molecules are organized in two discrete layers near the stromal (cytoplasmic) and luminal (inner) sides of the membrane. In the core antenna of PS I there exist Chl *a* molecules with a longer transition wavelength than the primary electron donor P700, the so-called "red" pigments (for reviews, see refs 10 and 19). The amounts and energies of these low-energy chlorophylls are highly species-dependent.[20] For *T. elongatus*, the absorption spectrum and a corresponding energy diagram are shown in Figure 1. The function and role of the red chlorophylls is still not well understood and intensively investigated in many research groups.

As can be seen from the crystal structure, carotenoids also play a crucial role in the photosynthetic apparatus. The Car molecules work hand in hand with the Chl *a* molecules: they quench Chl *a* triplet states to prevent the creation of dangerous

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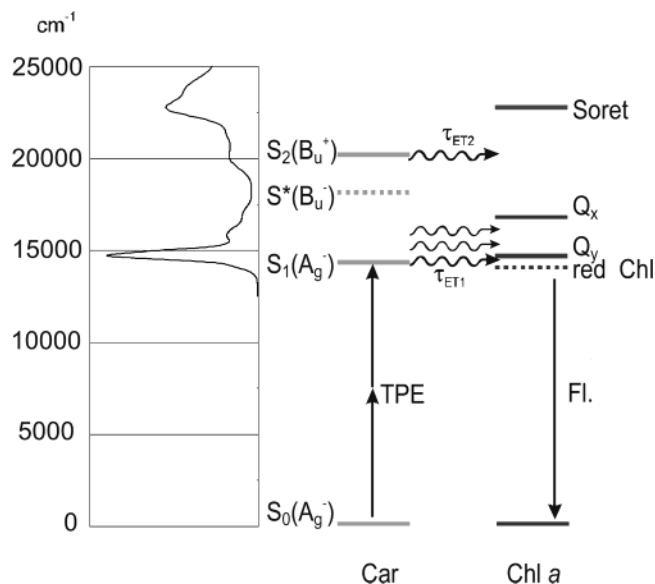
Figure 1. Left: Absorption of PS I. (Please note that the intensities of spectra presented on a wavenumber scale are different from the intensities on a wavelength scale. This is due to differences in the densities of states at various energies in both representations.) Right: Energetic diagram for PS I. Car, β-carotene; Chl a, chlorophyll *a*; TPE, two-photon excitation; Fl, fluorescence. For details, see the text.

singlet oxygen; they play an important structural role; and, of course, they assist in the light-harvesting process. In light-harvesting complex II of higher plants and green algae (LHC II), for example, which probably collects more than 50% of the light used for photosynthesis on earth, carotenoids contribute about 20−30% of light harvesting. The way in which carotenoids might play a crucial role in the dissipation of excessive excitation energy under high-light conditions is also still under discussion.

However, despite intensive investigation of the underlying mechanisms, many aspects of the function and role of the carotenoids are still poorly understood (for a recent review, see ref 21). It is a very good approximation to describe the electronic states of carotenoids by analogy to polyenes[22] in the idealized C_{2h} point group (Figure 1). Because of the A_g^- symmetry of the ground state S_0 and the first excited state S_1 of the carotenoids, the only route for harvesting sunlight with carotenoids is excitation to the B_u^+ state. According to the selection rules for optical transitions, only the $S_0 \leftrightarrow S_2$ transition is optically dipole-allowed and contributes significantly to the absorption spectrum of carotenoid molecules. However, after excitation into the S_2 state, carotenoid molecules undergo rapid internal conversion (IC) to the S_1 state. The corresponding time constant, τ_{IC}, varies for β-carotene from 120 fs (quinoline) to 177 fs (hexane) in different solvents.[23] The ground state (S_0) and first excited state (S_1) both exhibit A_g^- symmetry. Therefore, the $S_1 \leftrightarrow S_0$ transition is dipole-forbidden, and it still remains a challenge to elucidate mechanisms such as light harvesting, energy transfer, and dissipation of excess excitation energy that potentially involve the optically forbidden first excited state Car S_1. The situation has become even more complicated since another dark state has been identified between the S_1 and S_2 states of Car molecules.[24] This state has B_u^- symmetry and could play an important role in facilitating the Car $S_2 \rightarrow$ Car S_1 IC.

An elegant way to populate S_1 directly is by two-photon excitation (TPE) because transitions between states of A_g symmetry are two-photon-allowed.[25] Evidence was found for Car $S_1 \rightarrow$ BChl excitation energy transfer (EET) in various bacterial light-harvesting complexes by measuring the steady-

state BChl fluorescence and time-resolved transient Car S_1 absorption after Car TPE excitation.[26,27] By varying the two-photon excitation wavelength, the spectrum of the Car molecule states that transferred energy to Chl molecules was observed. Similar results have been obtained in the light-harvesting complex LHC II of higher plants and green algae.[28] In addition, very good agreement was obtained between experimentally determined and calculated coupling constants for the previously unknown mechanism of Car $S_1 \rightarrow$ Chl energy transfer.[26]

Recently, de Weerd et al. found strong indications that there is indeed energy transfer from both carotenoid states, Car S_1 and Car S_2, in PS I of *T. elongatus*.[6] Therefore, we expected that the spectrum of the Car S_1 state could be resolved by two-photon excitation monitoring of the Chl fluorescence. In the present work, we applied two-photon spectroscopy to photosystem I of *Thermosynechococcus elongatus* for two reasons. First, we wanted to learn more about the role of the carotenoids in PS I and about the participation of the optical dark state in light harvesting, energy transfer, and dissipation of excess excitation energy. Second, we wanted to elucidate the role of the red chlorophylls. Their low-lying electronic states would be better suited as energy acceptors for the low-lying optical dark state of β-carotene than normal chlorophyll energy states. In addition excitonic interactions can be imagined between the red-chlorophyll states and the optical dark state of β-carotene. Even though a direct Chl monomer excitation as a significant contribution to the observed fluorescence after two-photon excitation could be excluded, it was not clear whether two-photon-allowed excitonic states could be formed either from chlorophyll states or from carotenoid and chlorophyll states. Because of the presence of dominant red-chlorophyll emission that is due to excitonic interactions, PS I is an ideal system for testing this hypothesis.

Materials and Methods

Trimeric photosystem I core complexes (PS I) were isolated from the thermophilic cyanobacterium *Thermosynechococcus elongatus* (formerly known as *Synechococcus elongatus*) as described elsewhere. [29] For the emission measurements, the samples were diluted to a concentration of ∼10 μM with a buffer containing 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 6.5, 20 mM KCl, 20 mM CaCl$_2$, and 0.02% dodecyl-β-D-maltoside (β-DM). The resulting optical density was OD ≈ 0.3 at $\lambda = 680$ nm for a 1-mm path length. To elucidate the effects of the strong absorption of water in the region between 1400 and 1500 nm, some samples were diluted with a corresponding D$_2$O buffer or the water was replaced by D$_2$O by reconcentration of the samples with a protein-impermeable filter.

For the realization of the two-photon excitation, we used an amplified femtosecond laser system consisting of a Femtolite-A-10-SP fiberlaser (IMRA), which generates 48 MHz, 16.1 mW, 783 nm pulses, and a home-built Ti:Sa amplifier, which was pumped by 527 nm pulses of 6.5 mJ pulse energy at a repetition rate of 1 kHz generated by a Nd:YLF laser (Quandronix model 527 DP−H) (Figure 2). A traveling-wave optical parametric amplifier of superfluorescence (TOPAS) (Light Conversion) was pumped by the amplifier (1 kHz, 700 mW, 780 nm) to produce varying wavelengths. To separate the signal and idler pulses, we used a polarization beam splitter (Newport, 05FC16PB.9) with a separation of p to s polarization of >500:1 in the wavelength range of 1200−1600 nm. Remaining visible light was blocked by an IR filter (OD = 4 for $\lambda < 1100$ nm) to avoid population via one-photon excitation. A filter wheel in
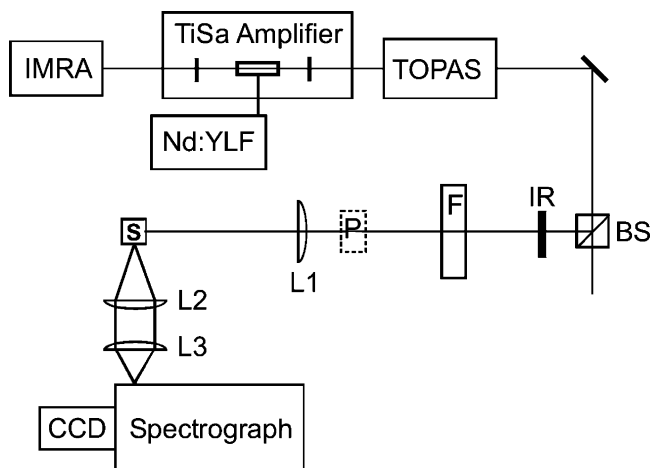
Figure 2. Experimental setup for the two-photon experiments. BS, polarizing beam splitter; IR, infrared filter; F, variable OD filter; P, power meter; S, sample cell.

combination with a broadband power meter (13PEM001, Melles Griot) was used to display the power dependency of the excitation. To obtain high efficiency in two-photon excitation, the light was focused into the sample cuvette (flow cell with a path length of 1 mm) using a lens with $f = 200$ mm. Fluorescence was collected at 90° using a lens with $f = 50$ mm and focused into a CHR-20-00001 spectrograph (500 IS/SM, Optilas GmbH) using a lens with $f = 20$ mm. The spectrum of the fluorescence light was detected using an AT200 CCD camera (Photometrics) with exposure times of 2 s for the TPE spectrum and 30 s for the fluorescence spectra. Much attention was paid to avoid any effects caused by reabsorption of fluorescence light by red chlorophylls.

One-photon absorption spectra of the samples were collected with a Cary 5E UV−vis−NIR spectrograph (Varian). One-photon fluorescence spectra of the samples were collected with a Cary Eclipse fluorescence spectrometer.

Results

Integrity of the Samples. We tested spectral changes as an indication of the integrity of the PS I samples during measurements by purposely heating them to 80 °C in steps of 10 °C and 2-h duration. It turned out that the minor change in the absorption spectrum provides little information on the current status of the sample, whereas the change in the fluorescence spectrum is much more significant (Figure 3 and 4). The untreated ("fresh") trimeric PS I complex exhibits an emission maximum at about 720 nm. A shoulder is present between 680 and 690 nm. Heat treatment strongly increases the fluorescence peaking at 680 nm. Similar changes of the fluorescence spectrum have been observed in a dependence on the detergent concentration.[30] The band around 680 nm, which becomes significantly stronger upon sample degradation, can be attributed to the fluorescence of treatment-induced uncoupling of Chl molecules from the antenna network. Therefore, we used the intensity of the fluorescence band at 680 nm as an indication of sample integrity.

Two-Photon Excitation Measurements. As in previous publications, we used the quadratic dependence of the observed signal as evidence for two-photon excitation (Figure 5). We fitted the observed power dependence to the sum of a linear and a quadratic term and interpreted the prefactor of the quadratic term as the two-photon cross section at the corresponding wavelength.
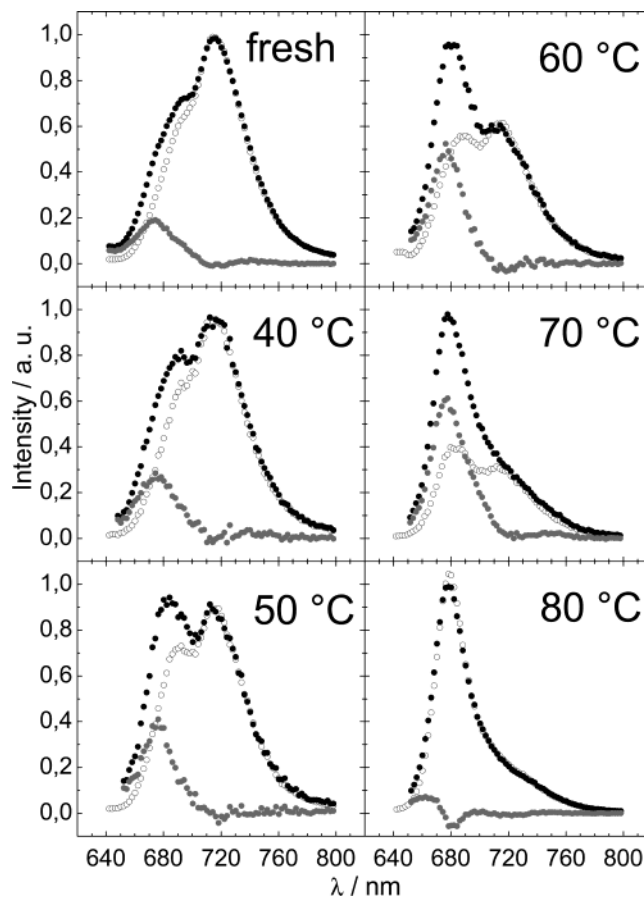
Figure 3. Spectral changes of the fluorescence of PS I due to sample degradation. Shown are the fluorescence spectra observed after excitation at 640 nm (black circles), 500 nm (open circles), and their difference spectra (gray circles) from a fresh sample and the same sample sequentially heated for 2 h at increasing temperatures.
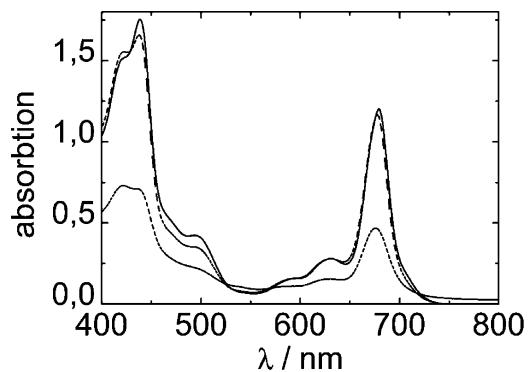

Figure 4. Spectral changes of the absorption of the fresh (solid line) and 70 °C (dashed line) and 80 °C (short dashed line) heated samples from Figure 3. Note the small difference up to 70 °C in comparison to the large change in the fluorescence spectra.

The entire spectrum was constructed by repeating the procedure at various excitation wavelengths accessible with our setup. Because water has a strong absorption around 1400−1500 nm, we repeated the measurements with PS I samples dissolved in D_2O buffer. A comparison of spectra from the samples in D_2O buffer with spectra from the samples dissolved in H_2O buffer, which were corrected for the water absorption, showed no significant difference. In Figure 6, the average results of four independent measurements are shown with the corresponding one-photon absorption and emission of PS I.

The fluorescence spectra observed after two-photon excitation with various excitation wavelengths are shown in Figure 7. No

Two-Photon Spectra of PS I of *T. elongatus*

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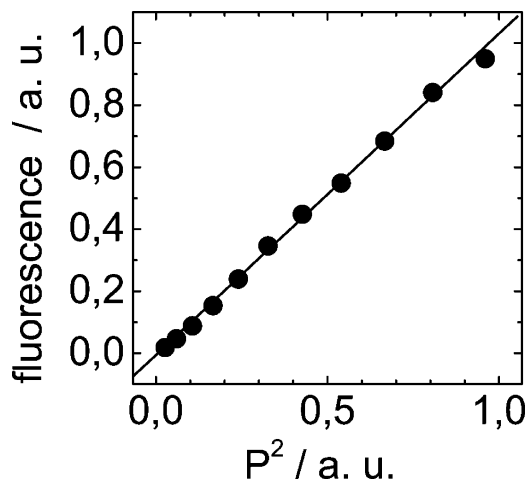
Figure 5. Example of the dependence of the fluorescence intensity of PS I on the two-photon excitation power at 1400 nm.
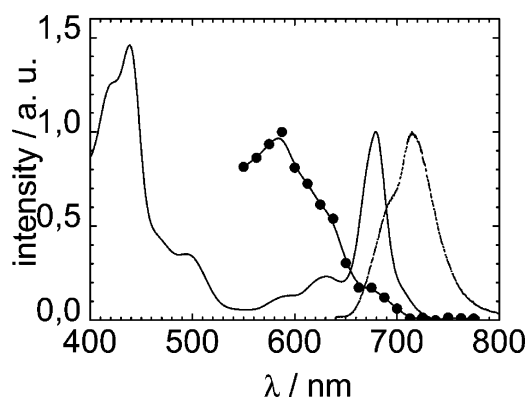
Figure 6. Two-photon excitation spectrum of PS I (circles with solid line). Solid line, one-photon absorption; dashed line, fluorescence observed after one-photon excitation at 500 nm. To reflect the actually excited energies the TPE intensities are plotted versus $\lambda_{exc}/2$.

significant difference was found between samples dissolved in D_2O buffer or H_2O buffer.

Fluorescence Spectra after Red-Chlorophyll Excitation. To determine the extent of thermal equilibration between red chlorophylls and bulk chlorophylls prior to fluorescence emission we measured the fluorescence spectra of a sample of PS I at various wavelengths. These wavelengths were in the spectral region of the Car S_2 state (500 and 508 nm), of the chlorophylls (630 nm), and of the red chlorophylls (680−720 nm). The excitation and emission parameters of the spectrometer where chosen so that perturbation of the detected emission in the red chlorophyll region by scattering of the excitation light was kept to a minimum. The results of these measurements are shown in Figure 8.

Discussion
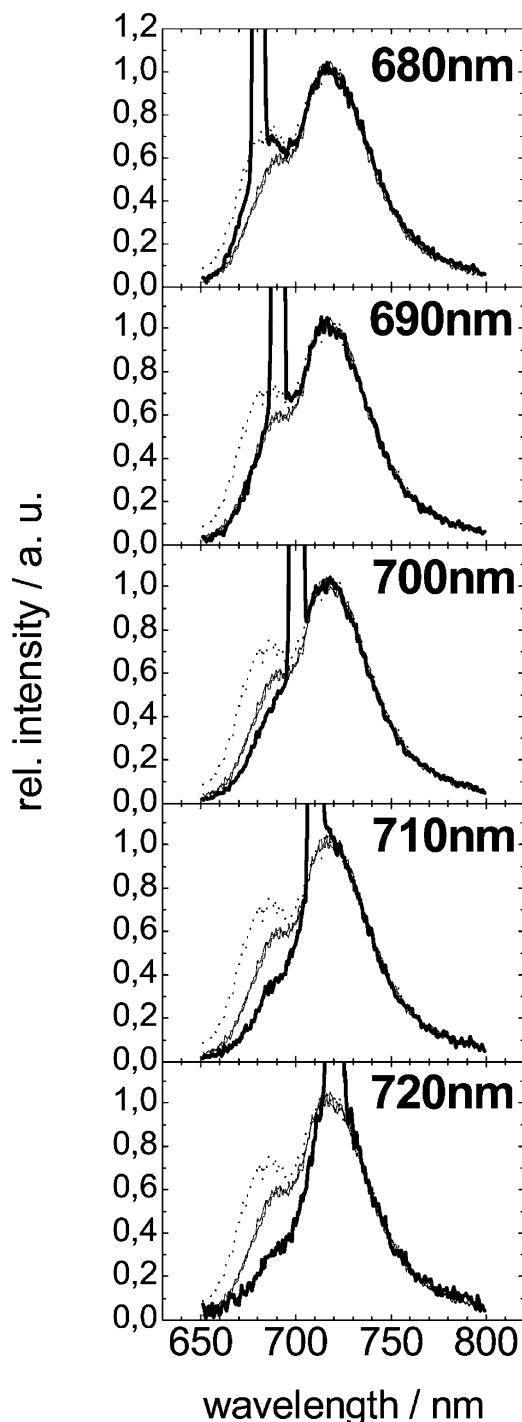
Two-Photon Spectrum and Hot-State Energy Transfer. Figure 6 shows the two-photon excitation spectrum observed with trimeric photosystem I from Thermosynecoccocus elongatus. A strong contribution of β-carotene two-photon absorption to the sensitized chlorophyll fluorescence is expected because it is known from the literature[6] that, in PS I from *T. elongatus*, there is at least a 30% efficient Car S_1 → Chl energy transfer. Fortunately, a considerable amount of data on β-carotene with which we can compare our two-photon excitation spectrum is available in the literature. Onaka and co-workers[31] were able to measure the Car S_1 emission spectrum of β-carotene in
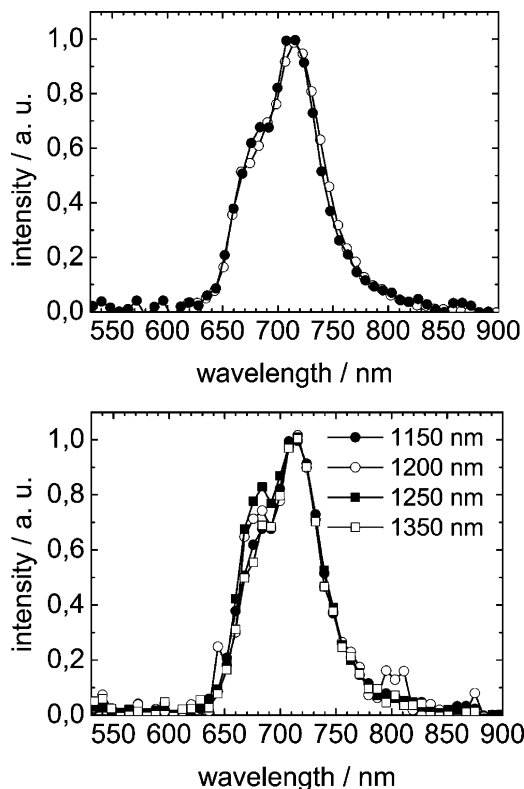
Figure 7. Top: Two-photon-sensitized ($\lambda_{exc} = 1150$ nm) fluorescence spectrum of PS I (black circles) in comparison to the fluorescence spectrum observed after one-photon excitation at 600 nm (open circles) with the exact same sample and setup. Bottom: Two-photon fluorescence spectra sensitized at various excitation wavelengths.

n-hexane at 170 K. In this work, a 0−0 transition of 14500 cm^{-1} for the Car S_1 state of β-carotene was estimated. For a comparison we used this wavenumber to generate a mirror image of the two-photon spectrum of PS I (Figure 9) and assumed a small Stokes shift of ∼150 cm^{-1} as suggested by Onaka et al.[31] The Stokes shift vanishes almost entirely when a typical difference of about 100 cm^{-1} between the spectral origins of two- and one-photon spectra of octatetraenes is considered.[32,33] The two-photon spectrum of PS I was collected using a TOPAS, which provides computer-controlled tuning of the excitation wavelength and high pointing stability. This is important for the measurement of accurate two-photon spectra as they are extremely sensitive to any variations in the excitation conditions. Because of this accuracy, we believe that a fit of vibronic bands to the measured spectrum is justified. For the fit of several Gaussian bands, we fixed the 0−0 transition at 14500 cm^{-1} and allowed only identical vibronic bandwidths to be fitted. All other parameters were allowed to vary freely. As can be seen in Table 1, remarkable agreement with the data of Onaka et al.[31] is obtained. These fitting results do not change much when small shifts are applied to the mirror image.
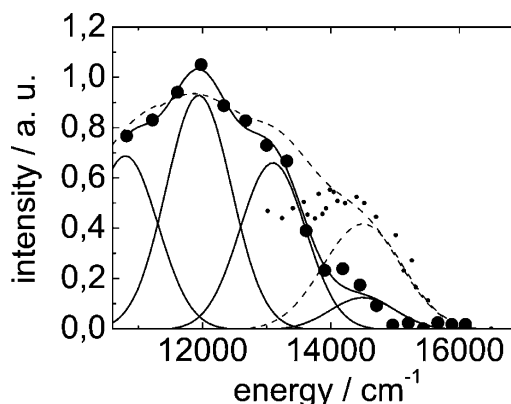
Only at wavelengths very close to the 0−0 transition is the intensity of the mirror-image PS I TPE spectrum significantly smaller than the Car S_1 emission of β-carotene in solution. Also, a direct comparison with the mirror-image two-photon spectrum of β-carotene[34] shows that the PS I TPE spectrum has significantly less intensity around the 0−0 transition (Figure 9). However, no perfect overlap of the β-carotene TPE spectrum with the TPE of PS I can be achieved. This might be due to either a solvent shift of ∼300 cm^{-1}, different Franck−Condon factors of the 0−0 transition, or other unknown uncertainties. An artificial decrease of the PS I TPE 0−0 transition due to the strong water absorption around 1400−1500 nm was

Figure 9. Mirror-image two-photon excitation spectrum (large circles) with fitted Gaussian bands and resulting spectrum (thick lines). Dashed line: spectrum constructed using the data reported by Onaka et al.[31] for the Car S_1 emission of β-carotene in solution. Small circles: Mirror-image two-photon excitation spectrum of β-carotene in solution overlapped with the latter spectrum.[34] Note the imperfect overlap which could be either due to solvent shifts (~ 300 cm^{-1}) or a larger amplitude of the 0−0 transition of the TPE spectrum β-carotene in solution.

Chl energy transfer given that the vibrationally cooled Car S_1 state is very close or even below the chlorophyll states. As a result, an excitation of "hot" Car S_1 states would result in a more efficient overall energy transfer in addition to the unfavorable cold Car S_1 state energy transfer, and hence, a lower intensity of the Car S_1 0−0 transition is then obtained in the excitation spectra. From the currently available data, it is hard to quantify the extent to which Car S_1 energy could be transferred via hot states. Assuming that the lower amplitude of the PS I TPE 0−0 transition, $A_{PSI}^{0-0\ transition}$, in comparison with the 0−0 transition amplitude of β-carotene in solution, $A_{\beta\text{-carotene}}^{0-0\ transition}$, reflects the additional fraction of hot-state Car S_1 → Chl energy transfer in PS I, Φ_{hot}, a value of even

$$\Phi_{hot} \approx \frac{A_{\beta\text{-carotene}}^{0-0\ transition} - A_{PSI}^{0-0\ transition}}{A_{\beta\text{-carotene}}^{0-0\ transition}} = \frac{0.49 - 0.14}{0.49} = 71\%$$

could be estimated. Nevertheless, we believe that the uncertainties in comparing the available spectra do not allow for an accurate estimate and that a determination of the significance of hot-state energy transfer in PS I requires additional data. Recently, de Weerd et al.[6] concluded from transient absorption that there might be only very little hot energy transfer. We want to stress, however, that it is hard to observe a rapidly transferring hot-state Car S_1 component in transient absorption measurements when the Car S_1 state itself is solely populated via the Car S_2 → Car S_1 internal conversion. Because the Car S_2 → Car S_1 internal conversion itself occurs on a fast time scale, the Car S_1 population corresponding to rapid hot-state transfer will always remain small and probably undetectable in such a situation. The fact that only an overall Car → Chl energy quantum efficiency of 70% can be deduced from the measured kinetic data of de Weerd et al.[6] is an indication that some energy-transfer pathways could be missed. An estimate of the overall Car → Chl energy quantum efficiency based on fluorescence upconversion measurements yielded greater values of up to $\sim$90% for PS I.[5] The difference of about 20% could be at least partially due to hot-state energy transfer. de Weerd et al. already considered this possibility in their work.[6]

A dominant contribution of pure (nonexcitonic) Chl a states to the PS I two-photon spectrum can definitively be ruled out, because two-photon excitation spectra of chlorophylls in solution

Figure 8. Fluorescence spectrum of PS I after excitation at 630 nm (dotted lines) at 500 and 508 nm (thin lines), and at various wavelengths in the spectral region of red chlorophylls (thick lines).

excluded by a careful comparison with measurements of PS I dissolved in D$_2$O.

The decreased intensity in the region of the 0−0 transition provides an indication for Car S_1 hot-state energy transfer.[34] In many situations involving energy transfer, the vibrational energy in the donor state is almost entirely dissipated prior to energy transfer. Therefore it is often assumed that energy transfer occurs exclusively from the vibrationally cooled excited state of a donor. However, fast energy transfer might occur from vibrationally excited states (thin wavy arrows in Figure 1) prior to vibrational cooling even when energy transfer from the vibrationally cooled excited state would correspond to an unfavorable uphill energy transfer. This situation might apply for Car S_1 →

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TABLE 1: Fitted Lineshape Parameters (Figure 8)

	vibrational energy (cm^{-1})		relative amplitude (cm^{-1})		fwhm (cm^{-1})	
transition[a]	this work[b]	Onaka et al.[c]	this work[b]	Onaka et al.[c]	this work[b]	Onaka et al.[c]
S$_1$ 0→0	14500	14500	0.14	0.49	1280	1460
S$_1$ 1→0	13100	13200	0.71	0.79		
S$_1$ 2→0	11950	12000	1	0.89		
S$_1$ 3→0	10800	10900	0.74	0.76		

[a] 0-0 transition: 14500 cm^{-1}. [b] Mirror image of the data in the present work. [c] Data taken from Figure 1b in Onaka et al.[31]
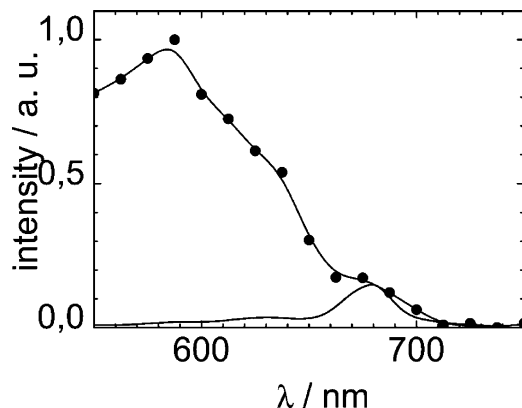
Figure 10. Two-photon spectra of PS I (circles with line) and maximum contribution of Chl *a* two-photon absorption of 8% (line). For details see text.

have a very different spectral shape. Two-photon excitation spectra of chlorophylls in solution are very similar to chlorophyll one-photon absorption spectra with respect to band positions, widths, and intensities.[25] By scaling the 0−0 transition of the Chl *a* absorption in PS I to the corresponding TPE intensity of PS I at 680 nm, a maximum contribution of 8% Chl *a* two-photon excitation to the total area of the PS I TPE spectrum can be estimated (Figure 10). The possibility of two-photon-allowed excitonic chlorophyll states is addressed in a later section.

Red Chlorophylls as Energy Acceptors from the Carotenoids. One motivation for the present work was the possibility to learn more about the so-called red chlorophylls present in PS I. Chl *a* fluorescence of PS I sensitized by Car S$_1$ excitation originates purely from Car S$_1$ → Chl energy trnsfer (ET). Hence, it was expected that information would be gained about specific Chl molecules that are in close proximity to Car molecules. The pool of red chlorophylls could be especially well suited as energy acceptors, because the density of isoenergetic donor−acceptor states (spectral overlap) should be at least slightly better than that for bulk Chl molecules (Figure 1). In addition, most of the red pool chlorophylls proposed in the literature are in very close proximity to β-carotene molecules. Especially the pair A32−B07 (assignment according to Jordan et al.[1]) is proposed by most studies. According to the crystal structure,[1] A32−B07 has up to six β-carotene molecules in close proximity. Because A32−B07 is located in the trimerization region of PS I, this would sum to a total of 18 β-carotene molecules. In addition to A32−B07, semiempirical INDO/S calculations of Damjanovic et al.,[2] for example, suggest that strong interactions between B24−B25 and A26−A27 result in red antenna states. Jordan et al.[1] suggest excitonic interactions in the chlorophyll pairs A32−B07, A38−A39, B37−B38, and B31−B32−B33 based on Mg center and interplanar distances. Sener et al.[4] are suggesting A32−B07, A33−A34, A24−A35, and B22−B34 based on semiempirical PPP calculations. Finally, Byrdin[3] et al. suggested A32−B07, A38−A39, B37−B38, and perhaps A12−A14 and B31−B32−B33 based on fitting spectra and

TABLE 2: Proposed Red Chlorophylls from the Literature and nearby Carotenoids

chlorophyll pairs assigned by at least two groups to be red chlorophylls	carotenoids in close proximity
A32−B07[a−d]	C17, C18, C19, C20
B37−B38[b,d]	C17, C18, C19, C20
A24−A35[b,c]	C7, C8
A38−A39[b,d]	C11, C12, C14, C15
B22−B34[b,c]	C9, C10

[a] Damjanovic et al.[2] [b] Jordan et al.[1] [c] Sener et al.[4] [d] Byrdin et al.[3]

dynamics. From all of these suggestions, the five pairs A32−B07, B22−B34, B37−B38, A38−A39, and A24−A35 have been assigned to be red chlorophylls by at least two groups. All five pairs are in close proximity to at least two carotenoids (Table 2). Interestingly, all five pairs are situated in a ring close to the reaction-center chlorophylls.

To determine whether there is indeed a preferential Car S$_1$ → red chlorophyll energy transfer, we resolved the two-photon-sensitized bulk-chlorophyll (∼680 nm) and red-chlorophyll (∼720 nm) fluorescence of PS I spectrally. However, it turned out that, regardless of the two-photon excitation wavelength, there was no increased red chlorophyll fluorescence compared to the fluorescence spectrum observed after one-photon excitation (Figure 7). At some excitation wavelengths, there even seemed to be an increased intensity of the bulk chlorophyll fluorescence. This indicates that there is no preference for carotenoid → red chlorophyll energy transfer even though this could be energetically favorable.

The question arises why no such preference was found. From the semiempirical INDO/S calculations of Damjanovic et al.,[2] it follows that about 17 (18%) of the 96 calculated excitonic Chl states are below 14500 cm^{-1} corresponding to the 0−0 transition of β-carotene in PS I. A very similar value can be observed by simply comparing the relative areas of chlorophyll Q$_y$ absorption in PS I above and below 14500 cm^{-1}. We now assume that a carotenoid transfers its energy only when it is in van der Waals contact with at least one chlorophyll corresponding to an excitonic state with an energy below 14500 cm^{-1}. From the crystal structure, it is known that 60 chlorophylls are in van der Waals contact with the 22 carotenoids. Therefore, on average, one carotenoid is in van der Waals contact with 2.7 chlorophylls. The probability that one of these chlorophylls has a state energy below 14500 cm^{-1} is then

$$P = 1 - (P_{\geq 14500 cm^{-1}})^{2.7} = 1 - (0.82)^{2.7} = 41.5\%$$

where $P_{\geq 14500 cm^{-1}}$ is the probability for an excitonic chlorophyll state to have an energy higher than 14500 cm^{-1}. Note that this estimate is valid only for energy transfer from the vibrational ground state and neglects any hot-state energy transfer. Our estimate is in reasonable agreement with the results of de Weerd et al.,[6] who found that about 30% of the β-carotene S$_1$ state is transferred to the chlorophylls with a time constant of 3 ps. According to the semiempirical INDO/S calculations of Damjanovic et al.,[2] only four of the 17 excitonic states with energies

TABLE 3: Parameters for the Car S$_1$ → Chl Energy-Transfer Time Constant Estimate

complex used for estimate	$\dfrac{J_{rel}^{PSI}}{J_{rel}^{complex}}$	$\dfrac{n_{Chl}}{n_{Car}}$	experimental τ_{ET1} (ps)	upper limit estimate		lower limit estimate		average of estimates
				$\tau_{S_1}^{PSI}$ (ps)	τ_{ET1}^{PSI} (ps)	$\tau_{S_1}^{PSI}$ (ps)	τ_{ET1}^{PSI} (ps)	$\tau_{S_1}^{PSI}$ (ps)
LHC II[a]	0.053	3.5	0.25	3.1	4.7	1.6	1.9	2.4
PCP[b]	0.364	1	3.1	4.4	8.8	2.5	3.5	3.5
PCP[c]	0.364	1	2.5	3.9	6.9	2.1	2.7	3.0
PS I[d]	1	2.7	3.0	(3.0)	(4.5)	(3.0)	(4.5)	(3.0)

[a] Walla et al.[28]   [b] Bautista et al.[37]   [c] Krueger et al.[39]   [d] de Weerd et al.[6]

below 14500 cm^{-1} correspond to red chlorophylls. Experimental results suggest a very similar distribution of the chlorophyll energies below 14500 cm^{-1}.[3] In summary, the fluorescence spectrum sensitized by two-photon excitation of the Car S$_1$ state seems to be identical to the spectrum observed after nonselective one-photon excitation because there are too few open Car S$_1$ → red chlorophyll pathways. This result is also supported by the findings of de Weerd et al.[6] From these time-resolved measurements, no indication for a major Car S$_1$ → red chlorophyll pathway could be found.

Two-Photon-Allowed Excitonic States? As mentioned earlier, PS I is also very well suited for learning more about the possibility of two-photon-allowed excitonic Chl *a* states, which do not contribute to two-photon spectra of diluted chlorophyll solutions.[25] Nevertheless, excitonic states are potentially present in all photosynthetic complexes because of the very dense arrangement of the pigments. In PS I, excitonic states seem to be especially significant because its emission is clearly dominated by red chlorophylls. Red chlorophyll states, in turn, are at least partially formed through excitonic interactions. Therefore, a dominant two-photon excitation of excitonic chlorophyll states should lead to an increased red-chlorophyll emission in PS I. However, the fact that the two-photon-sensitized fluorescence spectrum and the fluorescence spectrum observed after one-photon excitation does not show any increased red-chlorophyll fluorescence is a very strong indication that no two-photon-allowed excitonic states contribute to the TPE spectrum of PS I. Because the PS I fluorescence is even dominated by red chlorophyll emission, we believe that, in general, two-photon-allowed excitonic states from chlorophyll–chlorophyll or carotenoid–chlorophyll interactions can be neglected.

Thermal Equilibration between Red and Bulk Chlorophylls. We have to consider the possibility that the similarities of the one- and two-photon-sensitized fluorescence spectra are due to a thermal equilibration between red and bulk chlorophylls prior to emission. Schlodder and co-workers[35] found direct evidence that, at room temperature, the quantum yield of primary charge transfer in the PS I reaction center is always the same when red chlorophylls are excited regardless of the excitation of wavelength in a range from 690 to 750 nm. This indicates that thermal activation is always sufficient to surpass the energetic gap between red chlorophylls ($\lambda_{max} \approx 719$ nm $\hat{=}$ 13900 cm^{-1}) and P700 ($\sim$14200 cm^{-1}). Nevertheless, it was not clear whether a total equilibration also occurs between red and bulk chlorophylls (absorption maximum at $\sim$14700 cm^{-1}) before PS I of *T. elongatus* fluoresces.[18,36] Therefore, we carefully compared fluorescence spectra of PS I observed after excitation at various wavelengths in the carotenoid (500 and 508 nm) and chlorophyll (630 nm) regions with fluorescence spectra observed after selective excitation in the red-chlorophyll spectral region (690–720 nm). To elucidate whether there is a decrease in the relative amount of bulk chlorophyll emission after red-chlorophyll excitation, the spectra are best compared with the

fluorescence sensitized by dominant Car S$_2$ excitation. This is because the contribution of free chlorophyll fluorescence should be the lowest when the PS I emission is dominantly sensitized by Car → Chl energy transfer. As can be seen from Figure 8, at some red chlorophyll excitation wavelengths, almost no bulk chlorophyll emission can be observed. This provides strong evidence that at least no complete thermal equilibration occurs between red and bulk chlorophylls prior to emission.

"Cold" Car S$_1$-to-Chlorophyll Energy-Transfer Time Constant. To estimate the energy-transfer efficiency from the Car S$_1$ vibrational ground state, i.e., neglecting any hot-state energy transfer, we calculated a relative spectral overlap integral, J_{rel}, by using the mirror image of the measured Car S$_1$ TPE spectra, $f_{Car}(\nu)$; the normalized absorption spectra of the acceptor, $\epsilon_{Chl}(\nu)$; and the stoichiometric ratio of chlorophylls to carotenids in van der Waals contact, n_{Chl}/n_{Car}

$$J_{rel} = \frac{n_{Chl}}{n_{Car}} \times \frac{\int f_{Car}(\nu) \times \epsilon_{Chl}(\nu)\, d\nu}{\int f_{Car}(\nu)\, d\nu \times \int \epsilon_{Chl}(\nu)\, d\nu}$$

in a similar manner as was done by Walla et al.[28] for light-harvesting complex II of higher plants and green algae (LHC II) and the peridinin Chl *a* protein (PCP) from *Amphidinium carterae*.[37–40] As in Walla et al., we assume that the electronic couplings, V, are similar because there is a large but comparable spatial and orientational disorder of interacting donors and acceptors in all complexes. Therefore, the spatial and orientational dependence of the electronic couplings should be roughly averaged out in a similar manner. In addition, all complexes contain the same acceptor molecules (Chl *a*). With these simplifying assumptions, the obtained energy-transfer rates in the complexes should be approximately proportional to the relative spectral overlap integral, J_{rel}, because these provide an estimate for the density of isoenergetic donor–acceptor states, J_{DA}, according to Fermi's golden rule

$$k_{ET} = \frac{2\pi}{\hbar}|V|^2 J_{DA}$$

For the ratio $J_{rel}^{PSI}/J_{rel}^{LHCII}$, we obtained a value of 0.053 using $n_{Chl}/n_{Car} = 3.5$ for LHC II[28] and $n_{Chl}/n_{Car} = 2.7$ for PS I.

n_{Chl}/n_{Car} for PS I was estimated from the ratio of chlorophylls that are in van der Waals contact with carotenoids, 60/22. For the ratio $J_{rel}^{PSI}/J_{rel}^{PCP}$, we obtained a value of 0.364 using $n_{Chl}/n_{Car} = 1$ for PCP.[28] The resulting data are summarized in Table 3. The estimated values for τ_{ET1}^{PSI} range from 4.7 to 8.8 ps. Considering the internal conversion time constant, $\tau_{S_1 \to S_0}$, for the Car S$_1$ state of β-carotene of about 9 ps, estimated Car S$_1$ lifetimes of $\tau_{S_1}^{PSI} = (\tau_{S_1 \to S_0}^{-1} + \tau_{ET1}^{PSI}{}^{-1})^{-1} \approx 3.1$–4.4 ps can be obtained.

These spectral overlap calculations do not consider the fact that, according to the experimental data[6] and the estimate in the previous section, only approximately 30–40% of the

carotenoids are in van der Waals contact with Chl states, allowing effective energy transfer. Hence, the calculated values of $\tau_{S_1}^{PSI} \approx 3.1-4.4$ ps represent all carotenoids together and provide only an upper limit for the subpopulation of energy transferring carotenoids. The ~60−70% Car S_1 states that do not transfer their energy efficiently have a smaller density of accepting states in the spectral region below ~14500 cm^{-1}. Thus, to calculate the time constants for the subpopulations of energy-transferring carotenoids, the density of accepting states below ~14500 cm^{-1} must be increased accordingly. Assuming that the 60% nontransferring carotenoids are in contact with almost no accepting Chl states, the density of states for the remaining 40% must be increased by a factor of 100%/40% = 2.5 to yield the same average intensity as the normalized PS I absorption spectrum. Therefore, we repeated the calculation with the acceptor spectrum increased by this factor. Again, this is only an approximation, because now, the density of nonaccepting states ($\gtrsim$14500 cm^{-1}) is overrepresented. Nevertheless, the spectral overlap is small in the corresponding spectral region. Therefore, the calculation now provides a reasonable lower limit for the time constants of $\tau_{S_1}^{PSI} \approx 1.6-2.5$ ps.

The average values of the lower and upper limits for τ_{S_1} are ~2.4 and 3.5 ps, respectively. These values are in very good agreement with the experimental time constant of 3 ps determined by de Weerd et al.[6] Although the comparison with LHC II is potentially complicated because of hot-state energy transfer, the estimated value is still in good agreement with the experimental data. In PCP, no such difficulties are expected because the S_1 energy of peridinin is well above the Chl *a* energies.[40] These spectral overlap calculations show that the large number of potential Chl acceptors in PS I does not result in a higher time constant τ_{ET1}^{PSI} as in LHC II because the β-carotene Car S_1 state provides only a poor spectral overlap even with red chlorophylls.

What Could be the Role of the Red Chlorophylls? The data in the present work show that there seems to be only a very slight preference for Car $S_1 \rightarrow$ red chlorophyll energy and that no strong two-photon-allowed excitonic interactions between chlorophylls or carotenoids are present. Hence, the exact role of the red chlorophylls still remains unclear. The function of the red chlorophylls in photosynthesis might differ depending on their location in the antenna system and, therefore, on the distance between the red chlorophylls and P700. It has been proposed that the red chlorophylls might be located close to the reaction center to facilitate trapping by focusing the excitation energy toward P700.[41] However, recent data have shown that red chlorophylls slow the trapping rate.[10]

Long-wavelength chlorophylls compete with P700 for excitation energy (especially at low temperature) and, therefore, might affect the efficiency of charge separation. At room temperature, it has been shown that the quantum yield of photochemistry, i.e., P700 oxidation, is independent of the wavelength of the excitation, even at wavelengths of up to 760 nm, as a result of uphill energy migration to bulk chlorophylls and then to P700.[42]

The main purpose of the red chlorophylls is probably the extension of the spectral range for light harvesting to longer wavelengths to increase the efficiency of the antenna system. It has been suggested that the utilization of far-red light for photochemistry is the result of the adaptation of cyanobacterial dense suspensions to low-light conditions.

Red chlorophylls might also be involved in protecting the PS I complex against excess excitation light energy. Triplet states in the antenna system are most probably formed by intersystem crossing of excited red chlorophylls because the

excitation energy is localized to a large extent on these red pigments before trapping. Therefore, quenching of Chl *a* triplet states in the antenna already occurs efficiently if carotenoids are located in close vicinity to the red chlorophylls.

Another possible function for the long-wavelength chlorophylls located at the periphery of the core complex could be to provide "entrance points" of the excitation energy from external antenna complexes to the core antenna of PS I.

Conclusion

The two-photon excitation spectrum of PS I agrees well with known spectra of the carotenoid S_1 state of β-carotene in solution.[31] This provides a strong indication that the observed two-photon excitation spectrum originates almost exclusively from carotenoids. Only a smaller intensity of the PS I two-photon spectrum around its 0−0 transition suggests hot-state Car $S_1 \rightarrow$ Chl energy transfer. This could by a reason that kinetic data from detailed femtosecond transient absorption studies[6] indicate only an overall Car $\rightarrow$ Chl quantum efficiency of ~70%. Estimates based on fluorescence upconversion experiments on PS I yielded higher values of ~90%.[5] de Weerd et al.[6] already considered this possibility. In their work, Car S_1 was populated via Car $S_2 \rightarrow$ Car S_1 internal conversion, which probably does not allow for an accurate determination of hot-state energy transfer. A comparison with β-carotene Car S_1 emission data[31] and β-carotene two-photon data[34] indicates that, indeed, an amount of hot-state energy transfer of up to 20% of the overall Car $\rightarrow$ Chl energy transfer is conceivable. However, more data are needed to determine reliably the true significance and exact amount of hot energy transfer in PS I.

Regardless of the two-photon excitation wavelength, the two-photon-sensitized fluorescence spectrum of PS I does not show any increased red-chlorophyll fluorescence in comparison to one-photon excitation. To exclude the possibility that this is due to a thermal equilibration between red and bulk chlorophylls prior to emission, we carefully measured the PS I fluorescence after direct excitation of the red chlorophylls. These measurements show that there is no complete equilibration between bulk and red chlorophylls prior to emission. This provides very strong evidence that there is very little preference for Car $S_1 \rightarrow$ red chlorophyll energy transfer, even though it could be energetically favorable. We conclude that the small number of potential red chlorophylls is the reason for the acceptance of the major part of the transferred Car S_1 energy by bulk chlorophylls. A comparison of chlorophyll energies calculated by Damjanovic et al.[2] with the measured 0−0 transition of β-carotene in PS I yields a fraction of ~40% carotenoids that can transfer their energy via Car S_1, ignoring hot-state energy transfer. This result is in good agreement with the data published by de Weerd et al.,[6] who found a carotenoid fraction of ~30% that can transfer their energy via Car S_1 on a time scale of about 3 ps.

Simple spectral overlap calculations yielded estimated Car S_1 lifetime constants ranging from 3.1 to 4.4 ps for energy transfer from the vibrational ground state. These values are also in good agreement with the experimental data observed by de Weerd et al.[6] The calculations show that the large number of potential Chl acceptors in PS I does not lead to a potentially fast Car $S_1 \rightarrow$ Chl energy-transfer time constant as in LHC II because the low-lying β-carotene Car S_1 state gives only a poor spectral overlap even with the red chlorophylls.

Finally, the identity of the two-photon-sensitized fluorescence spectrum and the fluorescence spectrum observed after one-photon excitation rules out the possibility of two-photon-allowed excitonic states due to chlorophyll−chlorophyll or carotenoid−

chlorophyll interactions. An excitation of an excitonic red-chlorophyll state clearly should lead to a more dominant red-chlorophyll emission.

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