Transient Absorption Study of Peridinin and Peridinin−Chlorophyll *a*−Protein after Two-Photon Excitation†

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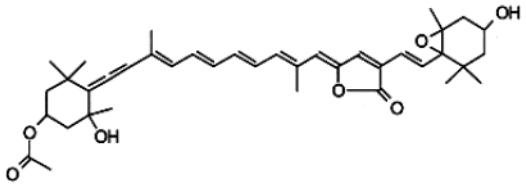
Two-photon excited pump−probe spectroscopy was carried out on the peridinin−chlorophyll *a*−protein complex (PCP) and on the peridinin molecule in methanol solution. Our data are consistent with earlier two-photon fluorescence excitation spectra[1,2] and lead to the conclusion that, for peridinin in methanol solution, a separate two-photon-allowed transition exists just to the red of the strongly allowed $S_0 - S_2(B_u^+)$ band. In the PCP complex, two-photon excitation at 1150 nm can also be interpreted as preparing a new state. In both cases, the new state is most likely the $S_1(A_g^-)$ state. It is not yet possible to conclude experimentally whether peridinin possesses a single low lying state with solvent dependent properties or two distinct states, the A_g^- and the charge transfer states.

Introduction

Peridinin is a carotenoid found in the light harvesting apparatus of dinoflagellates, called peridinin−chlorophyll *a*−protein (PCP).[3] In contrast to most photosynthetic light-harvesting complexes, the carotenoid peridinin, and not the chlorophyll, is the main light-absorbing pigment in PCP. Peridinin is an unusual carotenoid, being the only one known with three full rings in its structure (see Scheme 1). Furthermore, it contains an allene group, an epoxy group, and several carbonyl groups, including a lactone ring. Previous studies have shown that peridinin's unique structure significantly alters the character of its excited states. In addition to the usual S_1 and S_2 states, strong evidence has emerged for the existence of a low lying intramolecular charge transfer (ICT) state in polar environments and in the PCP complex.[4−6] After light absorption by peridinin, the excitation energy is transferred to chlorophyll and the efficiency of this energy transfer step is rather high. Previous studies have shown that the majority of the energy is transferred through peridinin's S_1 state.[5,7] However, more recent studies also indicate that the S_2 and ICT states are also involved.[8,9] Here, we seek to clarify the role of the low lying singlet states in energy transfer.

Our previous work investigated the energy and line shape of the S_0-S_1 transition.[1] According to the C_{2h} symmetry point group, which adequately describes most carotenoids, transitions from ground state (S_0) to the lowest singlet excited state (S_1), both of A_g^- symmetry, are one-photon forbidden, whereas transitions to the second excited state (S_2) of B_u^+ symmetry are one-photon allowed. Two-photon excitation provides a direct method of populating the S_1 state, thereby enabling a determination of its energy and subsequent dynamics.[10,11] We therefore used 920−1320 nm light to measure the fluorescence excitation spectrum after two-photon excitation for peridinin in benzene and in PCP.[1] Fluorescence of the isolated peridinin S_1 state was then measured at 750 nm. In PCP, the photoexcited peridinin transfers energy to chlorophyll whose fluorescence was monitored at 670 nm. The results are shown in Figure 1 for reference with the data obtained in this work. Surprisingly, two-photon absorption was observed in both the peridinin S_1 and S_2 regions, with the spectrum slightly red-shifted in the protein sample. Two-photon absorption observed to the red of the one-photon S_2 band was assigned to the S_1 ($2A_g^-$) state; although no separate absorption maximum is observed for this state, the origin of the transition appeared to be around 530−540 nm in benzene and around 550−560 nm in PCP. The peridinin S_1 energy was found to be higher than that of typical light harvesting carotenoids, making its S_1 state very close in energy to its S_2 state. We suggest that the polar groups and structural asymmetry make two-photon transition to the S_2 ($1B_u^+$) state strongly allowed. Similar results were obtained by Shima et al.[2]

SCHEME 1: Molecular Structure of Peridinin



Ground state transitions to the ICT state, on the other hand, are neither one-photon nor two-photon allowed. They may also be strongly Franck−Condon disfavored if the ICT state has an equilibrium geometry significantly different from that of the ground state. The existence of this dark, low-lying ICT state in peridinin was concluded from the strong solvent polarity dependence of the S_1 fluorescence lifetime.[12] Electronic structure calculations have not reached consensus as to whether the ICT state can be regarded as a distinct state[4] or as sufficiently mixed

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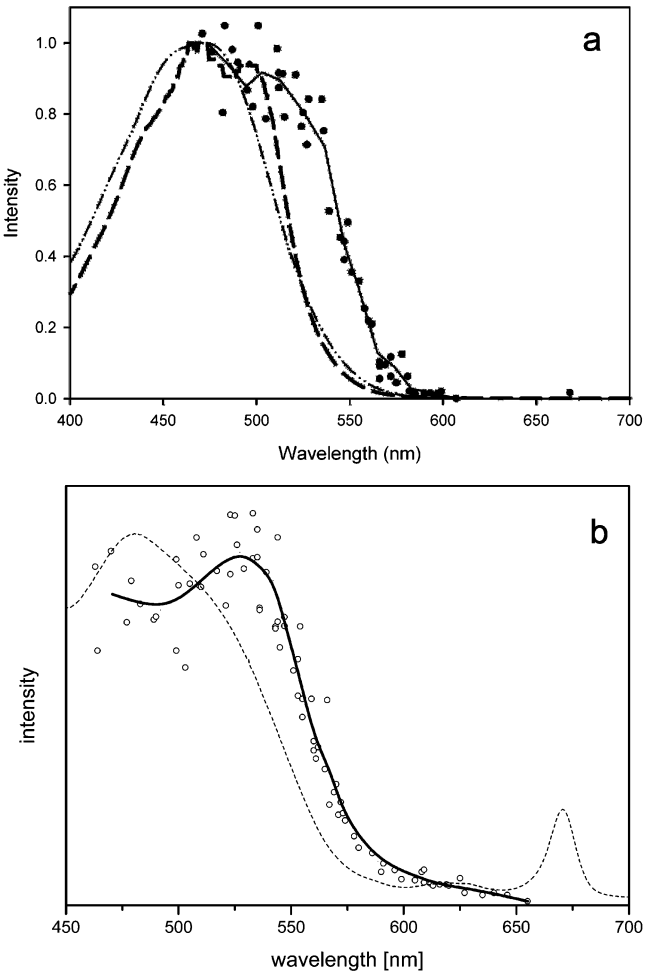

Figure 1. Two-photon fluorescence excitation spectrum of (a) peridinin in benzene, and (b) PCP in buffer following two-photon excitation. The circles display the original data points, and the bold line the averaged spectrum. For comparison, the corresponding one-photon absorption spectra are shown in benzene (dashed line) and methanol (dot−dash line) (a) and in PCP (dashed line) (b) and the TPE data are plotted over the excitation wavelength halved.

with S_1 that only a single lower state exists.[2,6] Femtosecond pump−probe experiments have been interpreted in terms of a collective S_1/ICT state.[13] Pump−probe measurements for peridinin in methanol identified an excited state absorption (ESA) band at 590 nm[6] and a stimulated emission (SE) band centered at 950 nm,[14] which have been attributed to the ICT character of this S_1/ICT state. In this model, the amount of ICT character in this collective S_1/ICT state increases with solvent polarity and is further enhanced by hydrogen bonding in protic solvents.[13] The same SE band at 950 nm was identified in PCP after photoexcitation of peridinin,[8] suggesting significant ICT character of the S_1 state in PCP as in polar, protic solvents such as methanol. The fact that only a single time constant is found suggests the involvement of a degenerate, mixed S_1/ICT state in the peridinin-to-chlorophyll energy transfer.

To complement our two-photon excitation spectra of peridinin and of PCP,[1] we have carried out two-photon pump−probe studies using tunable near-infrared pump pulses. The kinetics were probed at wavelengths of 550 and 580 nm, which have been suggested by Zigmantas et al.[13] to be sensitive to excited-state absorption (ESA) to a higher S_n state manifold from areas of the lowest excited-state surface with S_1 and ICT character, respectively.

Experimental Section

Samples of peridinin and PCP were prepared as described by Zimmermann et al.[1] Femtosecond transient absorption measurements were carried out on peridinin in methanol and in PCP. Peridinin and PCP were two-photon excited using pump wavelengths between 1000 and 1200 nm and one-photon excited using a 510 nm pump beam. The near-IR excitation beam was provided by the idler and the visible beam by the signal beam of an OPA (Coherent 9450), pumped by an amplified 250 kHz Ti:sapphire laser system (Coherent 9000). Typical pulse energies of the near-IR beam were 20 nJ with a pulse width of 80−90 fs. Excited state absorption (ESA) was then probed at either 550 or 580 nm. A white light continuum was generated for the 550 and 580 nm probe wavelengths by focusing an 800 nm beam into a sapphire window. The pump and probe beams were focused collinearly into the sample with a spot size of <100 μm. Solutions of $\sim$3 OD at 475 nm for PCP and 460 nm for peridinin in methanol were prepared in a 1 mm path length cuvette, which was moved up and down during the measurement to refresh the excitation volume. Bandpass filters with a 10 nm fwhm were used before and after the sample to select the desired probe wavelength, while short wavelength pass filters were used after the sample to cut the pump light. The probe light was detected by a diode connected to a lock-in amplifier (EG&G 5209) while chopping the pump beam.

For each peridinin/PCP measurement, methanol/buffer was measured under the same conditions. A constant background, determined as the average signal level at negative times, was subtracted from each data set. The solvent response was then subtracted from the peridinin data set to get the true peridinin response, which was then fit. The data were fit with multiexponentials using a least-squares fitting method. Best fits were determined by χ^2 values.

Fluorescence upconversion experiments utilized the same laser system. Excitation was at 490 nm and fluorescence was detected at 560 nm for both peridinin and PCP. The instrument response function had a width of $\sim$110 fs. Further details are given in the work of Holt et al.[15] For PCP, lifetimes were independent of excitation energy from 2.5 nJ/pulse to 6.0 nJ/pulse.

Results

Peridinin in Methanol. Figure 2a shows the normalized pump−probe kinetics of peridinin in methanol for three different pump wavelengths, probed at 580 nm. A striking feature of the two-photon data, at pump wavelengths between 1000 and 1150 nm, is an initial spike with pulse width limited decay. This spike was not observed in one-photon pump−probe experiments. Concentration-dependent measurements verified that the spike is due to peridinin rather than a solvent artifact since the normalized decay spectra for samples of different peridinin concentrations are identical (data not shown). However, power-dependent measurements showed a change in the relative amplitude of the initial signal compared to the slower component. The spike maximum shows a linear power dependence, whereas the slower component has a clear power-squared dependence (Figure 3). Obviously, the initial signal is not due to a transient probe process, for which a power-squared dependence would be expected. We suggest that the initial signal originates from a two-color two-photon excitation to a higher excited state of peridinin, using one photon from the pump and one photon from the probe pulse. This explains the linear pump-power dependence as well as the concentration dependence of the initial sig-

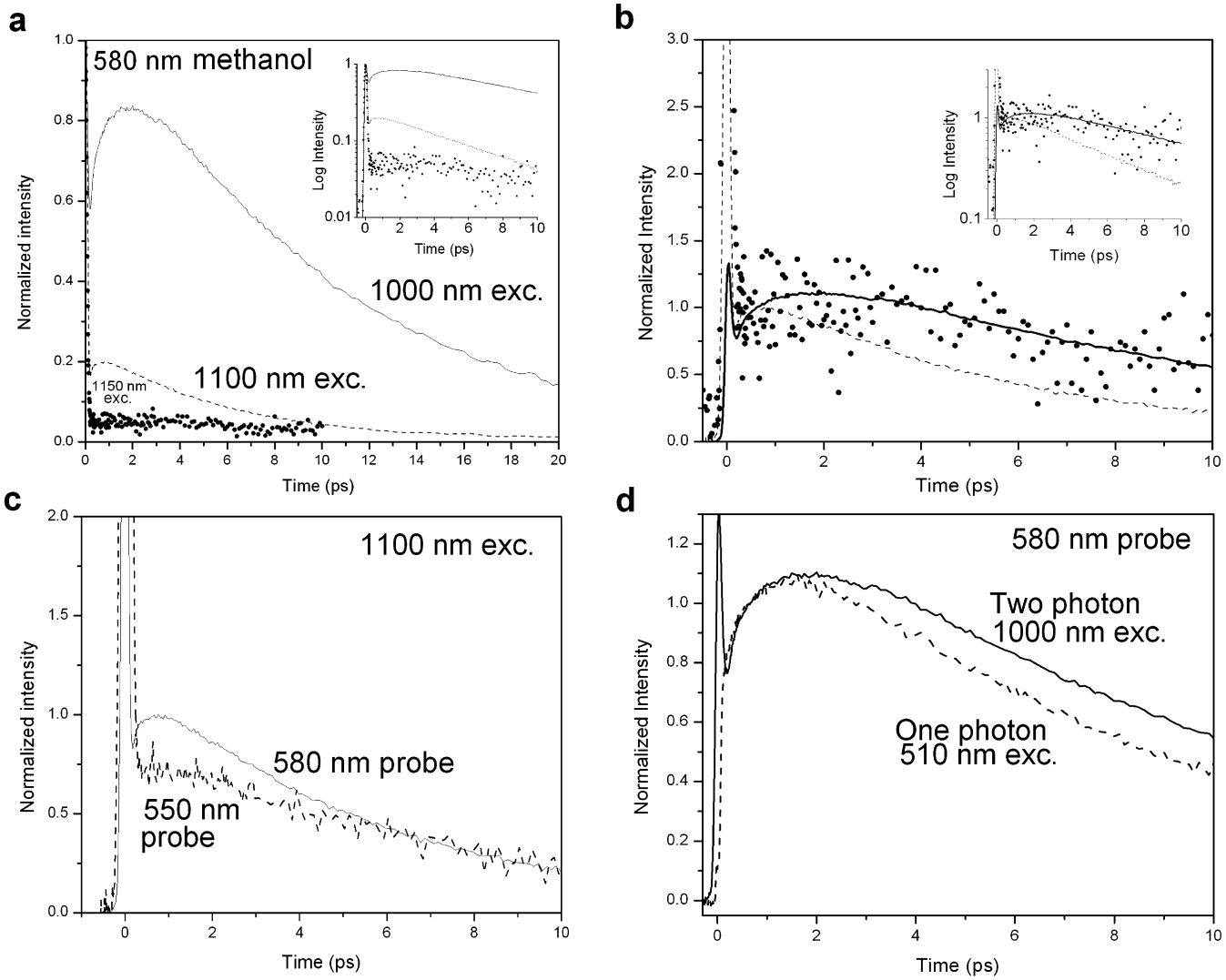

Figure 2. (a) Two-photon pump−probe signals for peridinin in methanol. (a) Excitation at 1000 nm (solid line), 1100 nm (dashed line), and 1150 nm (dots); probing at 580 nm. Inset same data with logarithmic intensity scale. (b) The same data as (a) but scaled to the same amplitude at 670 fs. (c) Excitation at 1100 nm, probing at 580 nm (solid line) and 550 nm (dashed line). (d) Comparison of one-photon (λ_{ex} = 510 nm) (dashed line) and two-photon (λ_{ex} = 1000 nm) (solid line) pump−probe data. Probe wavelength is 580 nm and the curves are normalized at 670 fs.

nal, which can be interpreted as a cross-correlation signal between the pump and probe pulses. We will therefore not consider the initial spike for the fitting of the data. Fit results beginning after the decay of the initial spike (after 200−380 fs) are shown in Table 1.

The data can be fit with two exponentials: a well-defined rise component of ∼1 ps appearing well after the initial spike, and a decay component of ∼10 ps that is the characteristic S_1 lifetime. Although the magnitude of the overall signal decreases at longer excitation wavelengths, giving larger errors to the fits, particularly for the 1150 nm pump wavelength, two trends clearly appear in the data. (i) The amplitude of the rise component decreases as the pump wavelength is increased from 1000 to 1100 nm. The same trend was observed probing at 550 nm. The 1150 nm data is quite noisy, and rather than trying to compare fitting results with the 1000 and 1100 nm data, in Figure 2b we show the three 580 nm probe wavelength data sets scaled to the same amplitude at 670 fs. The 1000 and 1150 nm data are identical within experimental error, while the 1100 nm data clearly decay more quickly. (ii) The decay component has a 9.6 ps time constant upon excitation at 1000 nm, which is characteristic of the S_1 state lifetime in methanol. However, the decay time shortens to 6−8 ps at 1100 nm pump wavelengths, probing at 580 nm. No such change was observed for probing at 550 nm, but the time constant (∼8 ps) is again shorter than the 10 ps observed for one-photon excitation. Figure 2c compares data for 1100 nm excitation at probe wavelengths of 550 and 580 nm. The rising component is clearly less prominent at 550 nm, and the overall differences between the curves are consistent with the one-photon excited time resolved spectra of Zigmantas et al.[14] This point is further emphasized by Figure 2d, in which one-photon excitation at 510 nm is compared with two-photon excitation at 1000 nm, probing at 580 nm. The slightly faster decay observed for 510 nm excitation is consistent with the shortening of the 950 nm stimulated emission signal lifetime as excitation wavelength is increased.[13]

PCP. Figure 4 shows the normalized pump−probe kinetics of PCP for different pump wavelengths for a probe wavelength of 580 nm. As with peridinin in methanol, two-photon excitation generated an initial spike that was not fitted, and the signal intensity at longer times decreased with increasing excitation wavelength. Probing PCP at 580 nm after one-photon (510−535 nm) or two-photon excitation (1000−1150 nm) yielded multiexponential decays with three decay times (0.8 ps, 3.7 ps, and

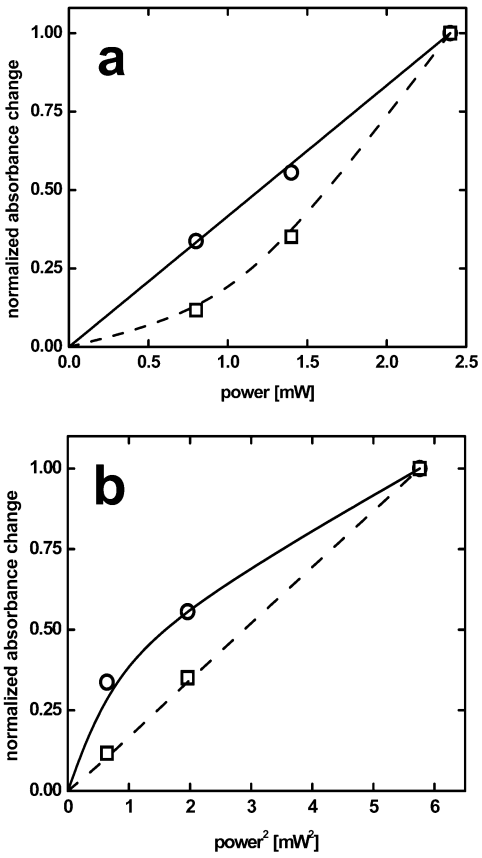

Figure 3. Power dependence of the initial spike and the slow component in the two-photon pump−probe measurements (1050 nm pump, 550 nm probe) of peridinin in methanol (circles: amplitude of initial spike; squares: amplitude of slow component, solid line: linear power dependence, dashed line: power squared dependence).

TABLE 1: Fit Results for Peridinin in Methanol

λ_{pump}	λ_{probe}	τ_1 [ps]	a_1	τ_2 [ps]	a_2
one-photon excitation					
480 nm[a]	600 nm	1.0	−0.35	9.7	1.0
510 nm	580 nm	0.9	−0.43	8.4	1.0
two-photon excitation					
1000 nm	580 nm	1.28	−0.52	9.6	1.0
1100 nm	580 nm	0.35	−0.47	5.8	1.0
1150 nm	580 nm			7.7	1.0
1050 nm	550 nm	0.66	−0.55	8.1	1.0
1100 nm	550 nm	0.82	−0.23	7.6	1.0

[a] From ref 18.

a long time component). The picosecond decay times were not fixed during the fitting procedure, but best fits yielded these average decay components as shown by the fitting results in Table 2. The long time component was fixed to the Chl-*a* lifetime of 3.7 ns.[16]

Fluorescence Up-Conversion Measurements on Peridinin and PCP. The existence of the spike complicates the determination of which state is initially prepared by two-photon excitation. To determine the S_2 lifetime directly, we carried out fluorescence up-conversion measurements, detecting fluorescence at 560 nm (Figure 5). The fluorescence lifetime of peridinin in methanol is 130 ± 10 fs. Akimoto et al.[7] previously reported a lifetime for S_2 of 192 ± 7 fs in methanol. The spike in the two-photon data may contain a contribution from S_1/ICT relaxation, in addition to the two-color two-photon absorption process described in the previous sections.

For PCP, the S_2 lifetime is very short: 66 ± 6 fs. This value is much shorter than the value of 195 ± 24 fs reported by Akimoto et al.,[7] but as Figure 5 shows, the time resolution of the measurement is clearly adequate to determine such a short fluorescence lifetime.

Discussion

Peridinin in Solution. There is, as yet, no consensus on the electronic structure of the low-lying excited singlet states of the peridinin molecule. For example, Shima et al.[2] used modified neglect of differential overlap using pseudo-spectral singles and doubles configuration interaction (MNDO−PSDCI) to calculate the electronic structure of peridinin. These authors found three low-lying states: an A_g^--like state, a B_u^+-like state, and a B_u^--like state. They conclude that the A_g^--like state is the charge transfer state discussed by various authors. On the other hand, Vaswani et al.,[4] using time dependent density functional theory, found a distinct charge transfer state in addition to the A_g^--like and B_u^+-like states.

From an experimental perspective, strongly mixed S_1(A_g^--like) and ICT states have been invoked by Frank et al.[6] to explain the remarkable shortening of the "S_1" lifetime in polar solvents such as methanol (10 ps) compared with the 170 ps lifetime found in nonpolar solvents. They propose that the progressive stabilization of the ICT state in increasingly polar solvents leads to a decrease in the energy of S_1 and enhanced S_1-S_0 internal conversion as a result of the smaller energy gap. Zigmantas et al. carried out an extensive series of one-photon pump probe studies of peridinin in various solvents and with various excitation wavelengths (425−570 nm). In the most recent work[13] they conclude that their results are best described by a model in which S_1 and the ICT state are strongly coupled to form a single "collective" S_1/CT state with a single lifetime that is determined by the degree of ICT character. However, different regions of the potential surface, corresponding to A_g^- or ICT character, have different absorption and emission spectra in this model. This model is further elaborated by suggesting that hydrogen bonding leads to the formation of a new ("red") peridinin species with different properties in both the ground and excited states.

A key aspect of these discussions is the appearance, in polar solvents, of a stimulated emission (SE) band with maximum at 950 nm. As far as we are aware, the steady state fluorescence spectrum of peridinin has been recorded only out to 850 nm,[5,12,17,18] and has a peak at ∼725 nm. There is no evidence in the published spectrum for a second peak at longer wavelengths. At 730 nm, the spontaneous fluorescence decay in methanol, measured with a streak camera, has a time constant of 10.6 ps.[14] Fluorescence up-conversion measurements in the 727−827 nm region reveal, in addition to a ∼10 ps decay, a 1 ps rise whose amplitude progressively increases as the detected emission wavelength increases. A portion of the fluorescence rise was reported to be instantaneous. In one-photon studies,[14] the time constants observed in the 980 nm SE and the 590 nm ESA were identical: a 1 ps rising component and a 10 ps decay component. Our own one-photon excitation (510 nm pump, 580 nm probe) is in good agreement with these results (Table 1). Zigmantas et al.[13] went on to study the excitation wavelength dependence of these timescales in methanol and found that the rise in the 950 nm SE signal became faster and less pronounced as the excitation wavelength was tuned to the red (0.4 ps for 550 nm excitation vs 1.5 ps for 425 nm excitation). In addition, the decay component at 950 nm shortened from 11 ps for λ_{ex} = 425 nm to 6 ps for λ_{ex} = 550 nm. These changes were not

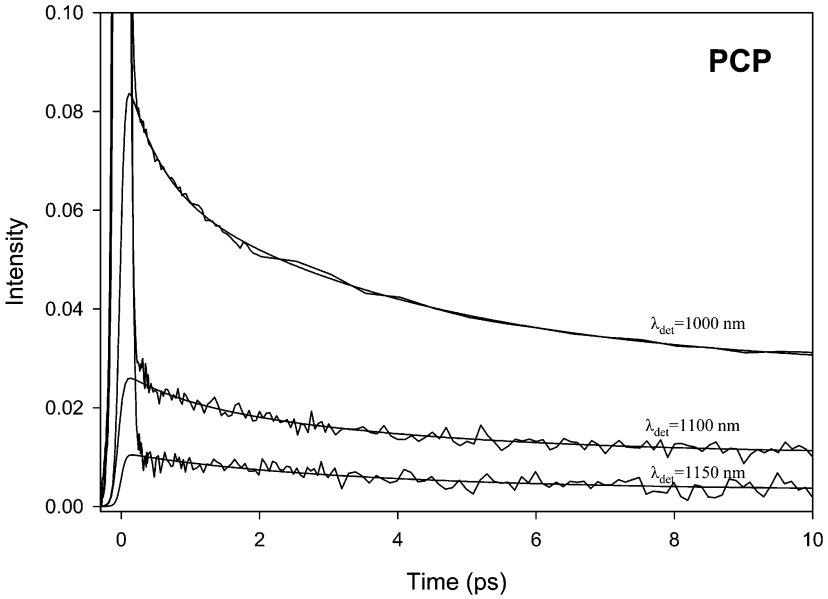

Figure 4. Two-photon pump−probe data for the PCP complex (probe wavelength 580 nm) as a function of two-photon excitation wavelength. λ_{ex} = 1000, 1100, and 1150 nm. Noisy curves: data; smooth curves: fit. See Table 2 for fitted values.

TABLE 2: Fit Results for PCP

λ_{pump}	λ_{probe}	τ_1 [ps]	a_1	τ_2 [ps]	a_2	τ_3 [ns]	a_3
one-photon excitation							
510 nm	580 nm	0.80	0.13	3.4	0.42	3.7	0.45
535 nm[a]	580 nm	0.79	0.13	3.9	0.48	3.7	0.39
two-photon excitation							
1000 nm	580 nm	0.75	0.26	3.6	0.44	3.7	0.30
1100 nm	580 nm	0.83	0.16	3.9	0.47	3.7	0.37
1150 nm	580 nm	0.79	0.05	3.9	0.62	3.7	0.27

[a] From ref 16.

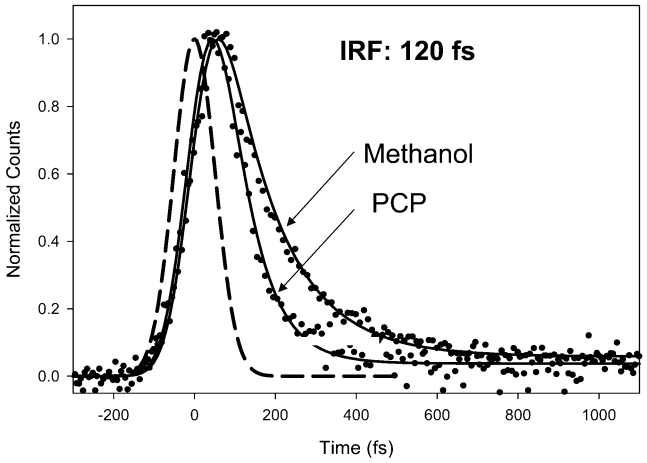

Figure 5. Fluorescence up-conversion data for peridinin in methanol solution and PCP. The data are shown by solid circles, instrument reponse by the dashed line, and best fits to the data by the solid lines.

observed in polar aprotic solvents, and hydrogen bonding was invoked to suggest the formation of new ("red") peridinin species with different ground and excited-state properties. Indeed the excitation wavelength dependent data can be fit with two lifetimes of 4.5 and 10.5 ps and varying amplitudes,[13] suggesting the existence of two ground-state species.

Our two-photon excitation data, probing at 550 or 580 nm, exhibit very similar trends. Excitation at 1000 nm yields (for a single decaying exponential fit) 9.6 ps (vs 9.6 ps for 500 nm excitation[13]), while 1100 nm gives 5.8 ps (vs 5.9 ps for 550 nm one-photon excitation[13]). Unfortunately, we do not have a two-photon excitation spectrum for peridinin in methanol, as the much reduced fluorescence yield makes the measurement more difficult than for benzene solution (Figure 1). The one-photon absorption spectrum in methanol is broader on the red side than the benzene spectrum, but by 550 nm the molecule absorbs weakly and by 575 nm the absorption is negligible. At 550 nm we expect substantial two-photon cross section, and even at 575 nm the two-photon cross section should still be reasonable. Of course, if all the two-photon absorption was coming from the B_u^+ (S_2) state, the similarity of our data and those of Zigmantas et al.[13] would hardly be surprising. This is, however, not the conclusion of Zimmermann et al.[1] or Shima et al.[2] who both concluded that two-photon absorption at wavelengths on the red edge of the one-photon spectrum excites the A_g^--like state. In particular, we would expect the 1150 nm excitation to be dominated by two-photon absorption to the A_g^--like state. The signal is noisy but clearly different from the 1100 nm signal. In fact, if the 1000 nm pump data are scaled to match the initial amplitude of the 1150 data, the two curves are clearly very similar, while the 1100 nm signal decays more rapidly (Figure 2b). This seems to be strong evidence that a different state is being prepared by 1150 nm excitation than by one-photon excitation: Table 2 and Figure 3 of Zigmantas et al.[13] show that, in polar solvents, the average lifetime decreases monotonically as the one-photon excitation wavelength is increased and thus should produce a decay on the order of 5.5 ps or so for excitation at 1150/2 = 575 nm. Thus the results in Figures 2 and 3 agree with the conclusions of Zimmermann et al.[1] and Shima et al.[2] that the two-photon absorption cross section in the region of strong one-photon absorption comes from the S_2 (B_u^+-like) state, but the two-photon excitation to the red of this band prepares a different state, most likely the (A_g^--like) state.

The shortening of the rise component (Table 1) at 1100 nm (0.35 ps) as compared to 1000 nm (1.3 ps) is also consistent with the one-photon data of Zigmantas et al.[13] (1 ps at 500 nm, 0.4 ps at 550 nm). These workers connected this component with reorientation of the carbonyl group resulting in an enhancement of the ICT component of the lowest excited state.

PCP Complex. Our two-photon excitation wavelength dependent data for the PCP complex behave rather differently, both with respect to one-photon data, and as a function of

wavelength as compared to the peridinin in solution data. First, the form of the two-photon excited, 580 nm probed data depends less strongly on excitation wavelength than does the peridinin in methanol data.

In Table 2, the 3.7 ns decay is fixed at the Chl excited-state lifetime. Following Zigmantas et al., we assign the 3.6−3.9 ps component to the main S_1 or S_1/CT energy transfer timescale to Chl, analogous to the 2.5 ps rising component seen in bleaching/SE of Chl in one-photon experiments.[8] Most intriguing is the 0.8 ps component, observed in both one- and two-photon experiments. Zigmantas et al.[8] suggested this may arise from hot ICT/Chl energy transfer or from spectral relaxation in the S_1/ICT state.

Looking at the data in Table 2, the 510, 535, and 1100 nm excitation experiments give very similar fits, suggesting that 1100 nm prepares mostly S_2 and that energy transfer proceeds both via the S_2 and S_1/ICT states. In contrast, the amplitude of the 3.7 ns component, which represents excited-state absorption of chlorophyll, is reduced relative to the 3.9 ps component for excitation at 1150 nm, as is the amplitude of the 0.8 ps component. This is consistent with two-photon excitation preparing a significant amount of S_1 directly, with a decreased overall Chl yield coming from the lack of the S_2 contribution and/or hot transfer from S_1/ICT. Although the presence of the spike makes definitive fitting difficult, the amplitude of the 0.8 ps component is always larger for good fits of the 1000 nm pump data. This is consistent with the 0.8 ps component relating to either hot transfer or spectral evolution as a result of cooling. What is not clear is why this component should be larger than is observed for a 510 nm one-photon excitation, unless 1000 nm excitation prepares significant amounts of S_1 directly. Further speculation on these results is probably not warranted because of the heterogeneity of the eight peridinin molecules in PCP. For example, it has been suggested that the blue absorbing peridinins are excitonically coupled in PCP, while the redder absorbers are not.[19]

The overall efficiency of energy transfer from peridinin to Chl for one-photon excitation is estimated to be 88%.[5] The fraction of energy transfer coming from S_2 has been estimated at 25%[8] and 25%−50%.[9] Our up-conversion measurements support the higher number. Steady-state spectroscopy suggests that methanol provides a reasonable approximation to the peridinin environment in PCP.[12] Assuming that the S_2 lifetime in PCP, in the absence of energy transfer, is the same as in methanol, the efficiency of energy transfer from S_2 to Chl is ∼50%. For the efficiency to be 25%, the S_2 lifetime in PCP without energy transfer would have to be ∼260 fs, a value that seems too long to be realistic. If 3.8 ps indeed represents the S_1/ICT lifetime in PCP, then using 9.6 ps as the S_1 lifetime, absent energy transfer, leads to 60% yield of energy transfer from S_1, giving a total yield of 80%, in reasonable agreement with the 88% obtained from steady-state measurements.

Concluding Comments

Our two-photon excited pump−probe data on peridinin in methanol are consistent with the conclusions of Zimmermann et al.[1] and Shima et al.[2] that a separate two-photon-allowed transition exists just to the red of the strongly allowed S_0-S_2-(B_u^+) band. Excitation at 1150 nm in methanol clearly prepares a different state than at 1100 nm, most likely the A_g^--like state. Following Zigmantas et al.[13] we have discussed the excitation wavelength dependence in both one- and two-photon data in terms of ground state conformers, in addition to the B_u^+-like and A_g^--like states. Another possibility is that excitation wavelength dependent branching to two distinct excited states occurs after initial excitation. At the present state of knowledge, the ground state conformer model (in polar protic solvents) seems the simplest explanation, but our data do not appear to rule out the excited state branching model. It thus remains an open question whether the lowest excited state(s) of peridinin is a single state with polarity and hydrogen-bond-dependent charge transfer character, or whether two low-lying states with differing lifetimes exist. Answering such a question via electronic structure calculation appears to be at, or somewhat beyond, the current state-of-the-art. We hope the results presented here will provide a spur to further development in this area.

Likewise, for PCP, understanding the peridinin−peridinin coupling and the site heterogeneity is a necessary prerequisite to any definitive unraveling of the S_2 and S_1/ICT contributions to energy transfer, although our data are consistent with a new, two-photon-accessible state lying just below S_2 in the protein complex as well as in solution.

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