Two-Photon Excitation Study of Peridinin in Benzene and in the Peridinin Chlorophyll *a*-Protein (PCP)

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Peridinin chlorophyll *a*-protein (PCP) is a unique light harvesting protein found in dinoflagellates, that contains a large amount of the carotenoid peridinin. Carotenoids have unusual spectroscopic properties due to their approximate C_{2h} symmetry, which makes transitions from their ground states to their S_1 (S_2) states one-photon forbidden (allowed). To gain information about one-photon forbidden electronic states in peridinin, fluorescence excitation spectra were measured after two-photon excitation for peridinin in benzene and in the PCP. The samples were excited using 920−1320 nm light. Fluorescence of the isolated peridinin S_1 state was then measured at 750 nm. In PCP, the excited peridinin transfers energy to chlorophyll whose fluorescence was monitored at 670 nm. 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. 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 peridinin's polar groups, symmetry breaking, and possible mixing of electronic states lead to two-photon character of the normally one-photon allowed S_0−S_2 transition.

Introduction

Dinoflagellates, the photosynthetic algae responsible for the red tide, contain a unique light harvesting protein called peridinin chlorophyll *a*-protein (PCP).[1] PCP has a trimer structure, each monomer consisting of two identical chromophore complexes of four peridinin molecules and one chlorophyll *a* (Chl), giving PCP an uncommonly large carotenoid-to-Chl ratio.[2] PCP is also unusual in being water-soluble; so it is probably associated extrinsically with the thylakoid membrane rather than within the membrane itself. Peridinin in PCP absorbs solar energy and transfers the electronic excitation to Chl, which in turn transfers it predominantly to photosystem II (PS II) located in the thylakoid membrane.[3] Once excitation energy reaches the reaction center, photosynthesis, the conversion of light into chemical energy, can begin.

Peridinin is an unusual light harvesting carotenoid, whose structure is shown in Figure 1. Its conjugated chain length is shorter ($N = 8$) than most light harvesting carotenoids ($N = 9$−13). Carotenoid S_1 energy levels increase rapidly as their conjugation lengths decrease. The S_2 energies also increase, but only slightly with shorter conjugation lengths. This leads to smaller S_1 to S_2 energy gaps in short carotenoids. Internal conversion rates between S_2 and S_1 increase with smaller energy gaps, which causes carotenoids with less than seven conjugated double bonds ($N < 7$) to fluoresce from their S_1 states. Mid-length carotenoids ($N = 7$−9) typically exhibit dual fluorescence from both their S_1 and S_2 states, while longer carotenoids
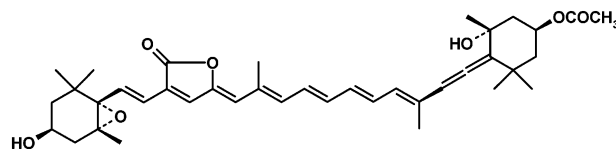

Figure 1. Structure of Peridinin.

fluoresce exclusively from their S_2 states. Peridinin ($N = 8$), however, behaves as a shorter carotenoid by fluorescing exclusively from its S_1 state; therefore, an S_1 energy higher than that predicted from its conjugated chain length might be expected. Similar behavior is seen for fucoxanthin ($N = 8$ plus a carbonyl group in conjugation with its carbon backbone), in the light harvesting complex of the algal diatom *Phaeodactylum tricornutum.*[4] The structure of peridinin is also more asymmetric than the average light harvesting carotenoid, which is typically described by the C_{2h} symmetry point group (Figure 1). Peridinin contains a symmetry-breaking lactone ring along its polyene backbone. Bautista et al. and Frank et al. have made extensive studies of the effect of solvent polarity on carotenoid energy levels.[5,6] The S_1 energy level of peridinin (as determined from the fluorescence spectrum) was found to decrease slightly with increases in solvent polarity, while the S_2 energy remained relatively unchanged. Going from nonpolar to polar solvents, the S_1 fluorescence quantum yield decreases from 10^{-3} to 10^{-5} and the S_1 lifetime decreases from 170 ps to 7 ps.[5,6] These observations can be explained by the existence of a charge-transfer state, whose energy is strongly solvent dependent such that it becomes lower in energy than the S_1 state in high polarity environments. The existence of a low-lying charge-transfer state is made likely by the presence of a carbonyl group in conjugation with the carbon−carbon double bond system along the peridinin backbone[6] (Figure 1).

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Despite the structural asymmetry of peridinin, the C_{2h} point group is generally used to describe its energy levels. Using this description, the peridinin ground, first, and second excited singlet states possess A_g^-, A_g^-, and B_u^+ symmetries, respectively. Recently, in other carotenoids, evidence was found for the existence of an excited singlet state[7,8] that lies between the S_1 ($2A_g^-$) and S_2 ($1B_u^+$) state and this was assigned to the theoretically predicted $1B_u^-$ state.[9]

Transitions are symmetry forbidden between two g states or two u states,[10] while alternancy forbids transitions between two + states or two − states.[11] Therefore, one-photon transitions from the ground state ($1A_g^-$) to the S_1 ($2A_g^-$), and to the $1B_u^-$ state, are both forbidden, while the ground state ($1A_g^-$) to S_2 ($1B_u^+$) transition is fully allowed. With this symmetry scheme, a two-photon transition, however, is allowed only for the ground state-to-S_1 transition. This phenomenon can be used to selectively excite the S_1 state of carotenoids. For example, the energetic positions of the carotenoid S_1 states have been determined via two-photon excitation (TPE) spectroscopy in bacterial (LH2) and plant (LHCII) light-harvesting complexes.[12−16]

The efficiency of electronic energy transfer from peridinin to Chl in PCP is approximately 88% with transfer through the peridinin S_1 state being the major route of energy transfer to Chl.[17−19] Akimoto et al.[17] found the peridinin S_2 fluorescence lifetime (∼190 fs) to be identical in PCP and solvent environments. This result indicates that little, if any, energy transfer occurs directly from the S_2 state, since energy transfer from S_2 would have appeared as a shorter S_2 lifetime. Bautista et al.[18] found the S_1 lifetime of peridinin to match the onset of the Chl bleach in PCP through pump−probe experiments, again indicating S_1 as the primary energy transfer route. However, more recent results have suggested that the S_2 and charge-transfer states of peridinin may play important energy transfer roles. Krueger et al.[19] found 25−50% of the peridinin-to-Chl energy transfer taking place via the S_2 state on a 100 fs time scale through polarized species associated decay spectra. Zigmantas et al.[20] performed near infrared transient absorption experiments and found the 2.5 ps decay of the peridinin charge-transfer state to match the rise of the Chl bleach, indicating that the charge-transfer state is also involved in energy transfer in PCP.

To elucidate the energy transfer pathways, more must be learned about the electronic states involved. At this point, the absorption line shapes of the dark states (i.e. the $2A_g^-$ (S_1), $1B_u^-$, and charge-transfer states of peridinin) are not known in solution or in PCP. In addition, the effect of symmetry breaking on the electronic properties of peridinin is poorly understood. To aid in answering these questions, fluorescence excitation spectra were measured after two-photon excitation (TPE) of peridinin in benzene and in PCP.

Experimental Section

Pigments were extracted from washed thylakoid membranes of *Amphidinium carterae* by the procedure of Martinson and Plumley,[21] except that 1-butanol was used in place of 2-butanol. Peridinin was separated by HPLC on a C18 Econosil column, 250 × 10 mm (Alltech), using a two-solvent gradient. Solvent A was 50% water/25% methanol/25% acetonitrile, and solvent B was 50% methanol/50% acetonitrile. The column was equilibrated with 80% B/20% A. The sample was applied at a flow rate of 1.5 mL/min, which was held for 5 min, and then B was gradually increased to 99% over 20 min. Finally, the flow rate was increased to 3 mL/min until Chl-*a* eluted. The eluent was monitored at 440 nm, and the fractions containing peridinin were dried under vacuum in darkness and stored at −20 °C.
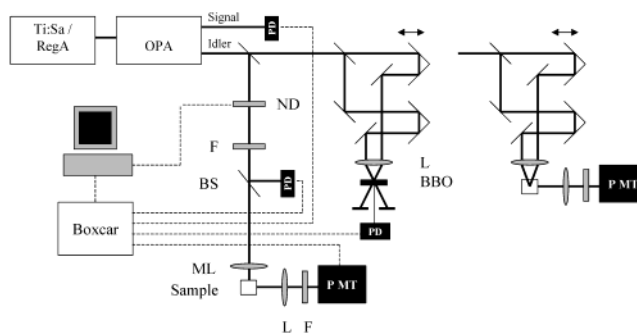

Figure 2. Experimental setup. ND: neutral density filter wheel; F: filter; BS: beam splitter; ML: microscope lens; L: lens; PD: photodiode. For the two-photon auto-correlation experiment, the BBO crystal was replaced by the sample and detection setup (see text for details).

Chl-*c* emerged close to the void volume, peridinin eluted at 17.4 min, and Chl-*a* at 39.8 min.

The experimental arrangement is shown in Figure 2. The idler of an OPA (optical parametric amplifier, Coherent 9450) was used for excitation, pumped by a home-built 2 kHz regenerative amplifier system described elsewhere.[22] The idler pulses were tuned in the wavelength range between 960 and 1320 nm and had a spectral fwhm of about 20 nm, pulse duration of about 95 fs, and maximum pulse energy of 20 nJ. Several long pass filters (Schott RG850, CVI LPF-110, FLN 4-8) were placed into the excitation beam to suppress any light other than the OPA idler. The intensity of the excitation beam was varied continuously by a computer-controlled reflecting filter wheel. A portion of the excitation light was sent to a photodiode (Thorlabs DET410) to monitor the excitation power before focusing the beam via a microscope objective (Nikon E10, N. A. 0.25, focal length 6 mm) into a 3 mm quartz cuvette, providing a focus of about 50 μm. Emitted light was collected at 90 degrees by a 5 cm lens and focused onto a photomultiplier (Hamamatsu R928 red-extended). Band-pass filters (PCP: 670 ± 20 nm, Coherent 42.7757; peridinin: 700 ± 20 nm, Cheshire 700IF40) were used to ensure that only fluorescence light was detected. In the case of PCP, fluorescence from Chl *a* was detected; whereas for peridinin, the emission of the pigment itself was monitored following two-photon excitation. The outputs of the photodiode and photomultiplier were preamplified (EG&G Ortec 9306) and measured by two boxcar integrators (Stanford Research SR250). The synchronization of the boxcars was provided by the signal beam of the OPA. The boxcars integrated over 10 laser pulses, and their outputs were recorded by a computer that also controlled the rotation of the filter wheel.

Alternatively, the excitation beam could be sent into an autocorrelator, which was used to measure the pulse width before each measurement as well as to determine the spectral shape of the pulse by doubling it in the autocorrelator BBO crystal. The same autocorrelator system was used for the TPE autocorrelation experiment described later.

Each data point was measured according to the following procedure. After determining the pulse width and spectral shape of the excitation light with the autocorrelator, the photodiode was calibrated by placing a power meter (Coherent Fieldmaster) in front of the microscope lens and varying the power by rotating the filter wheel. The power dependence of the TPE fluorescence was then measured twice and the curves were fitted with a polynomial of second order, taking into account offsets by the boxcar integrators. The amplitude of the second-order term gives the relative two-photon absorption intensity.
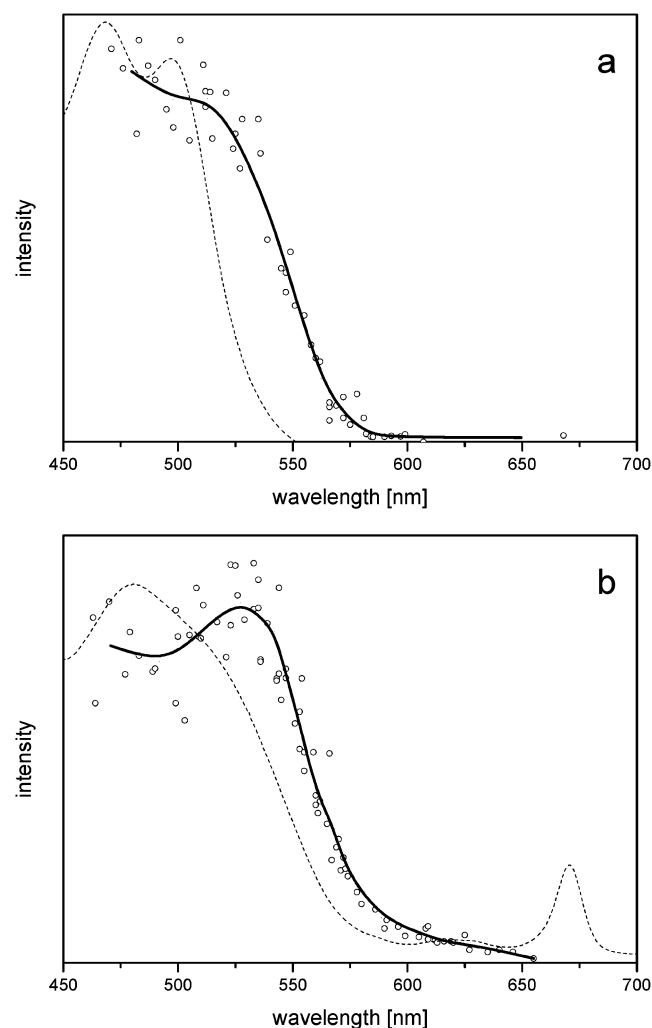
Figure 3. Fluorescence excitation spectrum of (a) peridinin in benzene, and (b) PCP in buffer following two-photon excitation. The circles display the original data points, and the bold line the averaged spectrum. For comparison, the corresponding one-photon absorption spectrum is shown (dashed line) and the TPE data are plotted over the double excitation wavelength.

To attain the smoothed two-photon excitation spectra, all data points were ordered by increasing wavelength, and every five data points were averaged. This procedure yielded one point with an averaged wavelength and an averaged two-photon absorption intensity for every five raw data points. The resulting points were then plotted.

Results

The fluorescence excitation spectra for peridinin in benzene and PCP were measured after two-photon excitation (Figure 3). As expected, the signal intensity was much higher for the PCP sample compared to the peridinin sample due to the much higher fluorescence quantum yield of chlorophyll (0.32 in methanol[23]) compared to peridinin (10^{-3} in benzene[5]). In contrast to previous work on light harvesting carotenoids,[12−16] a distinct S_1 absorption band was not found, but rather there was two-photon absorption in both the S_2 region and to the red of this allowed transition. For peridinin in benzene (Figure 3a), two-photon absorption begins at 600 nm (double excitation energy), leading to a shoulder peaking at 505 nm (half-maximum of the rise at 545 nm), and a further intensity increase to the blue. Measurement of two-photon absorption was attempted at several points in the 600−670 nm region; however, no fluorescence signal
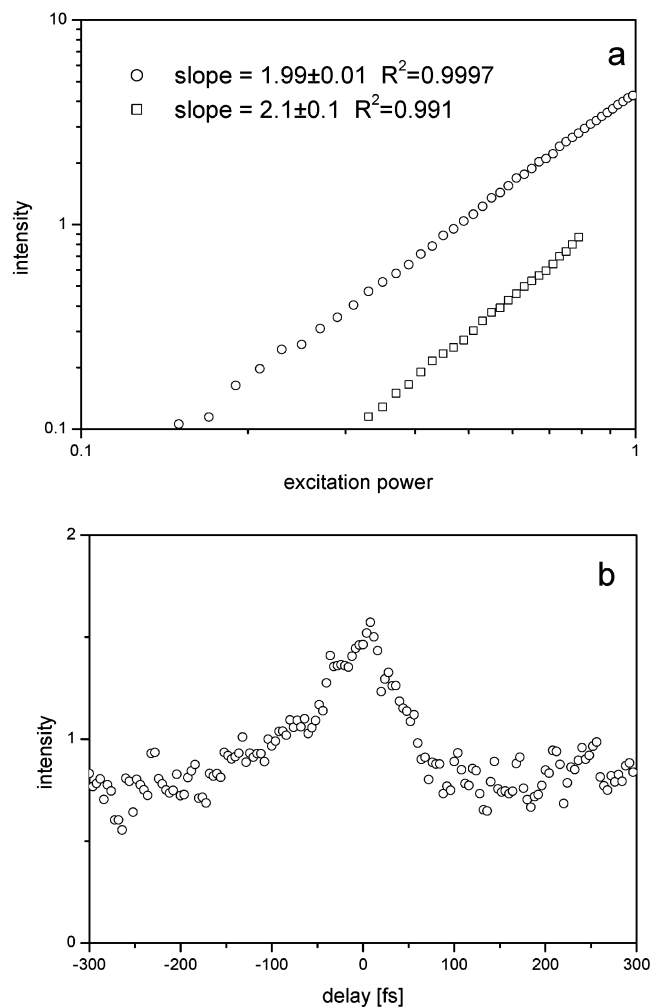

Figure 4. Tests of the TPE origin of the signal: (a) power dependence of the TPE fluorescence for Rhodamine B (circles, λ_{ex} = 1040 nm, λ_{em} = 580 nm) and PCP (squares, λ_{ex} = 1100 nm, λ_{em} = 670 nm), (b) autocorrelation signal of the PCP sample for excitation at 1060 nm.

above the ambient noise level was detected. For the PCP measurement (Figure 3b), two-photon absorption begins at 655 nm and reaches a maximum at 527 nm, followed by a second smaller maximum at 475 nm. The half-maximum of the rise lies at 560 nm. The excitation spectrum of peridinin in PCP is therefore red-shifted compared to that for peridinin in benzene. The peridinin one-photon absorption in PCP is also red-shifted compared to peridinin in benzene.

Given the difference between this result and our earlier data on the bacterial complex, LH2,[12,13] and the plant complex, LHCII,[14−16] a number of tests were performed to verify the results. First, the power dependence of the signal was measured, which should be quadratic for TPE. Figure 4a shows the result for Rhodamine B in methanol as a standard[24] and a typical PCP measurement. An almost perfect power squared dependence was found for both samples, supporting the TPE origin of the signal. Furthermore, the ratio of the absorptivities $\Omega = \delta_{cir}/\delta_{lin}$ using circular and linear polarized light was determined for the PCP sample. Ω equals one for one-photon absorption but is in the range of 0 to 1.5 for TPE.[25] This ratio was found to be 0.84 for β-carotene.[16] A value of $\Omega = 0.78$ was found for excitation of PCP at 1144 nm. However, moving into the spectral region of one-photon absorption, the Ω value increases to about 1 for excitation at 1050 nm.

Due to the latter result, the possibility of a one-photon excitation of the sample was considered. In using near infrared

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(NIR) excitation light, any piece of glass (including the sample cell) could generate second harmonic radiation (SHG), creating excitation light in the visible region (VIS). The S_2 state of peridinin could then be directly one-photon excited. The fluorescence intensity would still depend on the excitation power squared, as in true two-photon excitation. To test for SHG, a visible cutoff filter (600 nm) was placed on the inside of the sample cuvette. If second harmonic light were generated anywhere along the excitation path, it would be attenuated prior to reaching the sample. The use of the cutoff filter did not change the ratio of the fluorescence signal intensity at the blue side ($\lambda = 970$ and 1050 nm) compared to that at the red side ($\lambda = 1124$ nm) of the spectrum, indicating that the signal at the blue edge of the excitation spectra was not due to SHG. The same conclusion was drawn from an autocorrelation test of the TPE signal, the experimental setup for which is shown in Figure 2. The excitation light was split into two beams. One beam was sent to a translation stage so that its arrival time at the sample could be changed relative to the other beam. The beams travelled noncollinearly through the sample cell. An enhanced signal that depends on both spatial and temporal overlap of the excitation beams and corresponds to the second order autocorrelation function of the pulse is expected only for nonlinear processes in the sample itself. So, two-photon excitation would produce an autocorrelation signal, whereas any second harmonic radiation generated outside the focus would not. An autocorrelation signal was detected for excitation wavelengths of 1060, 1100, and 1140 nm (Figure 4b), further verifying that TPE was achieved.

The only remaining possibility for creating VIS light that excites the S_2 state of peridinin via one-photon absorption is in the NIR focal region inside the cuvette. Again, SHG, resulting in a pseudo power squared dependence of the signal, or other nonlinear processes that generate VIS light might occur in the focal region. In this case, the efficiency of VIS light generation should strongly depend on the size of the focus and rapidly decrease if the beam diameter is increased. To test for this, the microscope lens was replaced by lenses with 2.5 and 5 cm focal lengths. This reduced the signal by a significant amount but did not change the shape of the two-photon excitation spectrum.

We conclude that the origin of the signal over the whole spectral range results from two-photon excitation.

Discussion

For a single beam experiment, the two-photon transition moment M_{gf} from state g to state f is given by:[26,27]

$$M_{gf} = \sum_{i(\neq g, \neq f)} \left\{ \frac{2}{\hbar} \frac{(\vec{\mu}_{gi} \cdot \vec{e})(\vec{\mu}_{if} \cdot \vec{e})}{(\omega - \omega_{ig})} \right\} - \frac{2(\Delta \vec{p}_{gf} \cdot \vec{e})(\vec{\mu}_{gf} \cdot \vec{e})}{\omega} \quad (1)$$

where g, i, and f are the ground, intermediate, and final states of the two-photon transition, $\hbar\omega_{ig}$ is the energy difference between g and i, $\vec{e}$ and ω are the electric field vector and frequency of the excitation light, $\vec{\mu}_{ab}$ is the transition dipole moment from state a to b, and $\Delta\vec{p}_{gf}$ is the change in static dipole moment between the ground and final states. According to eq 1, two-photon transitions can occur by two distinct mechanisms: (i) via (virtual) intermediate states (1st term in eq 1), or (ii) via a change in static dipole moment between the ground and final states (2nd term in eq 1).

For nonpolar molecules that carry no static dipole moment, TPE is possible only via the first mechanism. In this case, only excited states of the same symmetry as the ground state can be populated via two-photon absorption. The one-photon forbidden S_1 state in nonpolar carotenoids, for example, can be selectively populated by TPE since both the ground and first excited states are of A_g^- symmetry in the C_{2h} point group, whereas the B_u^- and B_u^+ states cannot be populated by this mechanism. For polar molecules, however, two-photon absorption can also occur via the second mechanism, where a large change in dipole moment occurs upon excitation of the ground state.[26] Only one-photon allowed states are accessed by this mechanism since the two-photon transition moment is proportional to the one-photon transition dipole in this case. For a polar carotenoid in the approximate C_{2h} point group, the S_2 (B_u^+) state, which is strongly one-photon allowed, may also have a two-photon absorption via this mechanism, while the dark A_g^- (S_1), charge transfer, and B_u^- states will not if they have no detectable oscillator strength in the one-photon absorption spectrum.

The observation that peridinin exhibits two-photon absorption both in the region of the S_2 state and to another state to the red of S_2, while light-harvesting carotenoids investigated in previous two-photon excitation studies have shown absorption exclusively to the S_1 region,[12,13,15,16] is consistent with the polar character of peridinin. In fact, the blue side of the TPE spectrum resembles the one-photon absorption spectrum. However, the lack of a distinct band on the red side shows that the state responsible for the TPE in this region lies close to the S_2 state and overlaps the strong S_2 two-photon absorption. The polarization ratios of 1.0 and 0.8, measured on the blue and red sides of the excitation spectrum, also support the interpretation of the TPE spectrum as arising from two states. The value of 0.8 is found in the region of two-photon absorption that happens via the usual TPE mechanism and is very similar to the ratio found for S_1 states of other nonpolar carotenoids.[13,28] The value of 1.0 then presumably reflects the fact that two-photon absorption in the S_2 region occurs via a different mechanism. We therefore suggest that the TPE signal originates from two different electronic transitions, most likely transitions to the S_1 (A_g^-) and S_2 (B_u^+) states. Different polarization ratios are expected for transitions to states described by different symmetry groups, while alternancy symmetry is not expected to affect the ratio. Therefore, two-photon transitions to the peridinin B_u^+ and B_u^- states are not expected to have different polarization ratios.[29] No attempt was made to decompose the spectrum into two spectral profiles since this would be too speculative.

Our interpretation is supported by the CNDO-CI calculations of Dick et al.[26] who calculated the two-photon absorption cross sections of a series of substituted, linear, conjugated polyenes with increasingly polar substituents. They found that the two-photon absorption intensity to the S_1 state changed little, while the two-photon absorption to S_2 gained considerable intensity with increasing polarity. In fact, in the most polar cases, the two-photon absorption to S_2 becomes more intense than the symmetry allowed two-photon absorption to S_1. Furthermore, the polar groups in carotenoid molecular structures affect the S_1 and S_2 energy levels. The $2A_g^-$ (S_1) energy increased substantially with the addition of more polar groups, while the $1B_u^+$ (S_2) energy increased only slightly, thus reducing the energy gap between S_1 and S_2.

In peridinin, the polar keto group (Figure 1) conjugated with the polyene backbone should allow two-photon excitation to both the S_1 and the S_2 states. In addition, the decrease of the energy gap between S_1 and S_2, caused by the polar groups, might explain the lack of a distinct S_1 two-photon absorption band. Mixing of the close lying S_1 and S_2 states of peridinin may also account for the two-photon character of the S_2 state. Besides

the effect of the polar groups, any deviation from C_{2h} symmetry can lead to two-photon excitation to the S_2 state. Peridinin, with its ring structures on both ends, an epoxy ring along its backbone, and an allene group, is more asymmetric than typical light-harvesting carotenoids. Symmetry may also be broken by the protein environment, and by vibrations. Furthermore, thermal isomerization in solution may lead to structures with enhanced two-photon absorption to the peridinin S_2 state, though this would not apply to peridinin in the PCP complex.

However, because of the unusual nature of the TPE spectrum, it is appropriate to discuss other possible assignments for the two-photon signal to the red of the S_2 band. For example, the presence of the B_u^- state in the two-photon spectrum should be considered. If alternancy symmetry is preserved, the transition from the ground state to the B_u^- state is one-photon forbidden. Contributions from the second term in eq 1 require a transition moment between the ground and final state, and in this case B_u^- should be two-photon forbidden, whereas B_u^+ should be two-photon allowed. A ground-state transition to the B_u^- state could be very weakly one-photon allowed, but then the dipole moment change would have to be very large for the two-photon intensity (via the second term) to be similar to that from the B_u^+ transition.

Of course, alternancy symmetry may not hold in such an asymmetric carotenoid as peridinin, and the B_u^- state may have a one-photon allowed transition from the ground state. In this case, presumably ground-state transitions to both the B_u^+ and B_u^- states contribute to the one-photon spectrum. However, we see two-photon intensity to the red of any one-photon absorption, and so the conclusion that we see a transition to a state that is not B_u^+ or B_u^- seems inescapable.

The presence of the charge-transfer[26] state in the two-photon absorption spectra should also be considered. The charge-transfer state has a large permanent dipole moment change upon excitation, which would make two-photon absorption possible with any small amount of one-photon oscillator strength via the second term in eq 1. However, even if we assume a small oscillator strength for the charge-transfer state that is undetectable in the one-photon absorption spectrum, the position of the charge-transfer state is expected to be strongly solvent dependent and should appear at very different energies in the TPE spectra for PCP and peridinin in benzene. In contrast our TPE spectra are quite similar in the two experiments, and this leads us to conclude that the signal to the red of the one-photon spectrum most likely arises from the A_g^- state.

The origin of the S_0–S_1 transition for carotenes with conjugated chain lengths of 7 and 8 were measured as 520 and 570 nm, respectively.[30] Bautista et al. predicted the position of the S_0–S_1 0–0 transition to be about 600 nm for peridinin in hexane.[18] However, fucoxanthin ($N = 8$ plus a carbonyl group) has a significantly higher energy S_1 state than would be predicted from its conjugation length alone, with a two-photon absorption maximum of about 530 nm.[31] As in the two-photon excitation spectra of other carotenoids we have studied,[12,15,16] the 0–0 band of the two-photon absorbing state of peridinin is relatively weak and the maximum in the TPE spectrum is substantially blue-shifted from the origin, making an assignment of the S_1 origin difficult. However, as a best estimate, we suggest that the S_1 origin of peridinin lies at about 550–560 nm in PCP and at around 530–540 nm in benzene.

The red-shift in PCP compared to benzene solution probably arises from the different polarities of PCP and the solvent. Bautista et al. and Frank et al. have carried out extensive studies of the effect of solvent polarity on peridinin photophysics.[5,6]

They have shown that the S_1 energy level of peridinin decreases slightly with increase in solvent polarity. The conjugated parts of the pigments (peridinins and chlorophylls) pack closely with each other and with the lipids in PCP. Their environment is apolar, and contacts to protein residues mainly involve hydrophobic residues. In contrast, one finds a higher number of polar residues in the region of the lactone rings, and the peridinin headgroups are even incorporated in a hydrogen-bonding network. The sum of these influences is that peridinin is in a somewhat polar environment. Spectroscopically, PCP appears to provide an environment with a polarity similar to that of methanol, based on the fluorescence and absorption wavelengths of peridinin.[5] Since the two-photon excitation of peridinin was measured in benzene, the PCP spectrum can be expected to be red-shifted in comparison. Bautista and Frank, however, found that the S_2 energy was independent of solvent polarity.[5,6] The one-photon absorption spectrum of PCP also appears to be red-shifted in comparison to peridinin in benzene. This apparent red-shift could result from broadening of the peridinin band as a result of the distinct protein environments at each binding site. The two-photon absorption spectra measured in this work are highly reminiscent of those of the retinal chromophore in solution and in protein.[32] Like peridinin, retinal and retinol have polar groups, possess approximate C_{2h} symmetry, and show two-photon absorption to both their A_g^- and B_u^+ states. Retinal has a larger change in dipole moment than retinol, and thus exhibits stronger two-photon absorption. Furthermore, bacteriorhodopsin has a 13.5 D and a 9.1 D change in dipole moment upon excitation to its B_u^+ and A_g^- states, respectively.[33] Bacteriorhodopsin accordingly shows stronger two-photon absorption to its B_u^+ state than to its A_g^- state.

Concluding Remarks

The two-photon excitation spectrum of peridinin in benzene solution and in PCP differs significantly from that found for other light harvesting carotenoids. We suggest that the polar groups and structural asymmetry make two-photon transition to the S_2 ($1B_u^+$) state strongly allowed. Two-photon absorption observed to the red of the one-photon S_2 band is assigned to the S_1 ($2A_g^-$) state, although no maximum is observed for this state, the origin of the transition appears to be around 530–540 nm in benzene and around 550–560 nm in PCP. Thus the S_1 state appears strongly overlapped with S_2, and it does not seem possible to extract an S_1 line-shape from the data in Figure 3 since the relative S_1 and S_2 contributions are unknown.

The higher energy of the S_1 state and its strong overlap with the S_2 state complicates, and perhaps renders moot, the discussion of the relative importance of S_2 and S_1 states in energy transfer to chlorophyll in PCP. For example, it would not be easy to distinguish S_2 and S_1 in decay associated spectra,[19] and the potential mixing of the two states may modify expectations based on the pure symmetries of the two levels.

To what extent is the high lying S_1 state of peridinin of functional significance? A clear answer to such a question will probably have to wait for electronic structure calculations for peridinin, the quantitative investigation of coupling strengths, and spectral overlaps between peridinin and chlorophyll molecules. Such calculations will need to include the revised energy of the S_1 state, the putative charge transfer state,[5,6] thought to lie below S_1 in polar environments and play a role in energy transfer in PCP, and the influence of excitonic interactions between the S_2 states of the peridinin molecules.[34] Preliminary calculations by Hsu and co-workers,[35] based on time-dependent density functional theory, find vertical transitions to an A_g^- like

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state with low oscillator strength (S_1), a state with a strongly allowed transition from the ground state (S_2), and a state characterized by considerable transfer of charge from the lactone and epoxide ring region to the polyene chain (S_{CT}). Although the vertical excitation energy of this charge-transfer state lies above the S_2 state at the geometry of the vacuum calculation, inclusion of solvent and relaxation of the geometry are likely to lower the energy of the state substantially. It will also be of interest to measure the lifetime(s) of the state(s) directly formed by two-photon absorption in a manner analogous to the work of Walla et al.[13,14,16]

References and Notes

(1) Prezelin, B. B. Photosynthetic Physiology of Dinoflagellates. In *The Biology of Dinoflagellates*; Taylor, M., Ed.; Blackwell Publishing: New York, 1987; pp 174−223.

(2) Hofmann, E.; Wrench, P. M.; Sharples, F. P.; Hiller, R. G.; Welte, W.; Diederichs, K. *Science* **1996**, *272*, 1788−1791.

(3) Mimuro, M.; Tamai, N.; Ishimaru, T.; Yamazaki, I. *Biochim. Biophys. Acta* **1990**, *1016*, 280−287.

(4) Mimuro, M.; Nagashima, U.; Takaichi, S.; Nishimura, Y.; Yamazaki, I.; Katoh, T. *Biochim. Biophys. Acta* **1992**, *1098*, 271−274.

(5) Bautista, J. A.; Connors, R. E.; Raju, B. B.; Hiller, R. G.; Sharples, F. P.; Gosztola, D.; Wasielewski, M. R.; Frank, H. A. *J. Phys. Chem. B* **1999**, *103*, 8751−8758.

(6) Frank, H. A.; Bautista, J. A.; Josue, J.; Pendon, Z.; Hiller, R. G.; Sharples, F. P.; Gosztola, D.; Wasielewski, M. R. *J. Phys. Chem. B* **2000**, *104*, 4569−4577.

(7) Sashima, T.; Nagae, H.; Kuki, M.; Koyama, Y. *Chem. Phys. Lett.* **1999**, *299*, 187−194.

(8) Sashima, T.; Koyama, Y.; Yamada, T.; Hashimoto, H. *J. Phys. Chem. B* **2000**, *104*, 5011−5019.

(9) Tavan, P.; Schulten, K. *J. Chem. Phys.* **1986**, *85*, 6602.

(10) Hudson, B. S.; Kohler, B. E.; Shulten, K. Linear Polyene Electronic Structure and Potential Surfaces. In *Excited States*; Academic Press: New York, 1982; Vol. 6, pp 1−93.

(11) Damjanovic, A.; Ritz, T.; Shulten, K. *Phys. Rev. E* **1999**, *59*, 3293−3311.

(12) Krueger, B. P.; Yom, J.; Walla, P. J.; Fleming, G. R. *Chem. Phys. Lett.* **1999**, *310*, 57−64.

(13) Walla, P. J.; Linden, P. A.; Hsu, C.-P.; Scholes, G. D.; Fleming, G. R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 10808−10813.

(14) Walla, P. J.; Linden, P. A.; Fleming, G. R. Fs-Transient Absorption and Fluorescence Upconversion after Two-Photon Excitation of Carotenoids in Solution and in LHC II. In *Ultrafast Phenomena*; Elsaesser, T., Mukamel, S., Murnane, M. M., Scherer, N. F., Eds.; Springer: New York, 2000; pp 671−673.

(15) Walla, P. J.; Yom, J.; Krueger, B. P.; Fleming, G. R. *J. Phys. Chem. B* **2000**, *104*, 4799−4806.

(16) Walla, P. J.; Linden, P. A.; Ohta, K.; Fleming, G. R. *J. Phys. Chem.*, accepted.

(17) Akimoto, S.; Takaishi, S.; Ogata, T.; Nishimura, Y.; Yamazaki, I.; Mimuro, M. *Chem. Phys. Lett.* **1996**, *260*, 147−152.

(18) Bautista, J. A.; Hiller, R. G.; Sharples, F. P.; Gosztola, D.; Wasielewski, M.; Frank, H. A. *J. Phys. Chem. A* **1999**, *103*, 2267−2273.

(19) Krueger, B. P.; Lampoura, S. S.; van Stokkum, I. H. M.; Papagiannakis, E.; Salverda, J. M.; Gradinaru, C. C.; Rutkauskas, D.; Hiller, R. G.; van Grondelle, R. *Biophys. J.* **2001**, *80*, 2843−2855.

(20) Zigmantas, D.; Polivka, T.; Hiller, R. G.; Yartsev, A.; Sundstrom, V. *J. Phys. Chem. A* **2001**, *105*, 10296−10306.

(21) Martinson, T. A.; Plumley, G. F. *Anal. Biochem.* **1995**, *228*, 123−130.

(22) Agarwal, R.; Rizvi, A.; Prall, B.; Fleming, G. R. *J. Phys. Chem. B*, in press.

(23) Weber, G.; Teale, F. W. J. *Trans. Faraday Soc.* **1957**, *53*, 646−655.

(24) Xu, C.; Webb, W. W. *J. Opt. Soc. Am. B* **1996**, *13*, 481−491.

(25) Drucker, R. P.; McClain, W. M. *J. Chem. Phys.* **1974**, *61*, 2609−2615.

(26) Dick, B.; Hohlneicher, G. *J. Chem. Phys.* **1982**, *76*, 5755−5760.

(27) Abe, S. *Chem. Phys.* **2001**, *264*, 355−363.

(28) Holtom, G. R.; McClain, W. M. *Chem. Phys. Lett.* **1976**, *44*, 436−439.

(29) McClain, W. M.; Harris, R. A. Two-Photon Molecular Spectroscopy in Liquids and Gases. In *Excited States*; Lim, E. C., Ed.; Academic Press: New York, 1977; Vol. 3.

(30) Andersson, P. O.; Gillbro, T. *J. Chem. Phys.* **1995**, *103*, 2509−2519.

(31) Shreve, A. P.; Trautman, J. K.; Owens, T. G.; Albrecht, A. C. Carotenoid to Chlorophyll *a* Singlet Energy Transfer: Direct Evidence for Involvement of the Carotenoid 'A' State in an Algal Light-Harvesting System. In *Current Research in Photosynthesis*; Baltscheffsky, M., Ed.; Kluwer Academic Publishers: Amsterdam, 1990; Vol. 2, pp 293−296.

(32) Birge, R. R. Energy Levels of the Visual Chromophores by Two-Photon Spectroscopy. In *Methods in Enzymology*; Academic Press: New York, 1982; Vol. 88, pp 522−533.

(33) Birge, R. R.; Zhang, C. F. *J. Chem. Phys.* **1990**, *92*, 7178−7195.

(34) Damjanovic, A.; Ritz, T.; Schulten, K. *Biophys. J.* **2000**, *79*, 1695−1705.

(35) Hsu, C. P.; Vaswani, H. M.; Head-Gordon, M.; Fleming, G. R. Unpublished Results.