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Role of Carotenoids in Light-Harvesting Processes in an Antenna Protein from the Chromophyte *Xanthonema debile*

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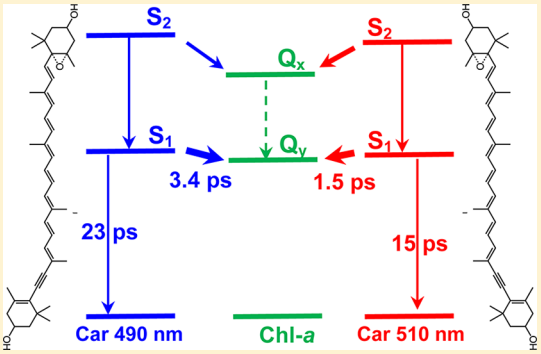
Supporting Information

ABSTRACT: Chromophytes are an important group of microorganisms that contribute significantly to the carbon cycle on Earth. Their photosynthetic capacity depends on efficiency of the light-harvesting system that differs in pigment composition from that of green plants and other groups of algae. Here we employ femtosecond transient absorption spectroscopy to study energy transfer pathways in the main light-harvesting complex of *Xanthonema debile*, denoted XLH, which contains four carotenoids—diadinoxanthin, heteroxanthin, diatoxanthin, and vaucheriaxanthin—and Chl-*a*. Overall carotenoid-to-chlorophyll energy transfer efficiency is about 60%, but energy transfer pathways are excitation wavelength dependent. Energy transfer from the carotenoid S_2 state is active after excitation at both 490 nm (maximum of carotenoid absorption) and 510 nm (red edge of carotenoid absorption), but this channel is significantly more efficient after 510 nm excitation. Concerning the energy transfer pathway from the S_1 state, XLH contains two groups of carotenoids: those that have the S_1 route active (~25%) and those having the S_1 pathway silent. For a fraction of carotenoids that transfer energy via the S_1 channel, energy transfer is observed after both excitation wavelengths, though energy transfer times are different, yielding 3.4 ps (490 nm excitation) and 1.5 ps (510 nm excitation). This corresponds to efficiencies of the S_1 channel of ~85% that is rather unusual for a donor−acceptor pair consisting of a noncarbonyl carotenoid and Chl-*a*. Moreover, major carotenoids in XLH, diadinoxanthin and diatoxanthin, have their S_1 energies in solution lower than the energy of the acceptor state, Q_y state of Chl-*a*. Thus, binding of these carotenoids to XLH must tune their S_1 energy to allow for efficient energy transfer. Besides the light-harvesting function, carotenoids in XLH also have photoprotective role; they quench Chl-*a* triplets via triplet−triplet energy transfer from Chl-*a* to carotenoid.



1. INTRODUCTION

Light-harvesting processes are of vital importance for all photosynthetic organisms.[1] Energy transfer between carotenoids and (bacterio)chlorophylls ((B)Chl) is an important process that extends the light-harvesting capacity of many photosynthetic systems because carotenoids absorb light in spectral region not accessible to (B)Chls.[2] In various light-harvesting systems, there are two major pathways of energy transfer from carotenoids to (B)Chl: from the strongly allowed S_2 state, which is responsible for the strong absorption of carotenoids in the blue-green spectral region, and from the lowest, forbidden S_1 state, which is populated via internal conversion from the S_2 state on a 100−300 fs time scale.[3]

The key parameter determining whether the energy transfer from either S_2 or S_1 state is active is energy of the S_2 and S_1 states which must be high enough to allow transfer to Q_x and Q_y states of (B)Chls. The energies of carotenoid excited states depend on conjugation length, N.[3] Since energy of the S_2 state is for all naturally occurring carotenoids always higher than the Q_x state of (B)Chls, the S_2 pathway is active in nearly all photosynthetic antennae.[2] On the other hand, activity of the S_1 route strongly depends on the carotenoid structure as for many carotenoids the S_1 energy drops below the Q_y state of (B)Chls.

The S_1 route is often active in bacterial antenna systems because the Q_y state of BChls is significantly lower than that of Chls.[4−7] On the contrary, in plants and algae carotenoids that have their S_1 energies approximately equal to or lower than the Q_y band of Chl-*a*, the S_1 route plays a negligible role in energy transfer,[8−10] except for a few cases where hot S_1 state was found as energy donor.[11−13] Instead, back-energy transfer from Q_y to carotenoid S_1 state is proposed as a photoprotective quenching mechanism.[14] Thus, the energies of the carotenoid S_1 state and Q_y state of the acceptor are crucial for optimizing overall efficiency of energy transfer.

Another light-harvesting strategy was developed in chromophytes and dinoflagellates. These photosynthetic microorgan-

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isms often contain Chl-*c* in addition to Chl-*a* and employ carotenoids with conjugated carbonyl group,[15] which activates an intramolecular charge transfer (ICT) state.[16,17] The ICT state is coupled to the S_1 state, resulting in significant consequences for energy transfer pathways and efficiencies.[18] Carbonyl carotenoids have the S_2 state shifted to lower energies compared to their noncarbonyl counterparts,[17] which extends their absorption far beyond 500 nm and allows to efficiently collect green light. At the same time, the S_1/ICT state is maintained high enough to enable efficient energy transfer to Chl-*a* via the S_1/ICT route.[18,19] Thus, the S_1/ICT pathway becomes the major channel as evidenced in light-harvesting systems of dinoflagellates utilizing carbonyl carotenoid peridinin[18−21] or chromophytes having carbonyl carotenoid fucoxanthin.[22,23]

The class Xanthophyceae differs from other chromophytes because it lacks the carotenoid fucoxanthin. A recently characterized organism from this class, soil chromophytic alga *Xanthonema debile*, contains only non-carbonyl carotenoids and no Chl-*c*.[24] *X. debile* has an antenna system denoted XLH (Xanthophytes' light harvesting) complex that contains carotenoids diadinoxanthin, diatoxanthin, heteroxanthin, and vaucheriaxanthin (see Figure 1 for structures) in 11:5:4:1

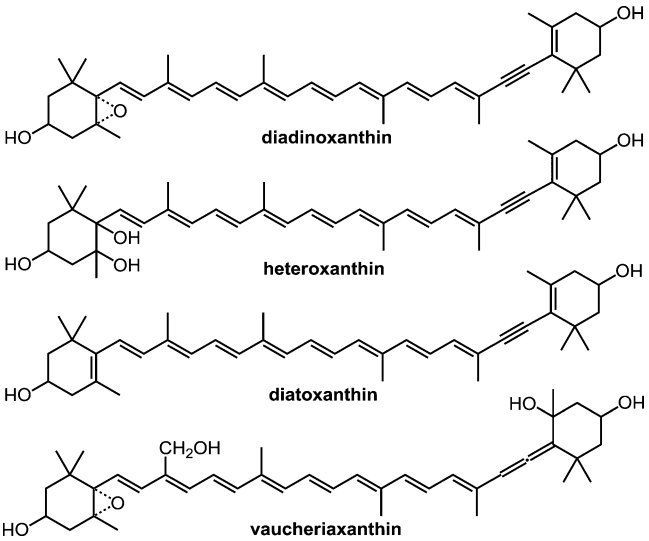

Figure 1. Molecular structures of carotenoids in XLH.

ratio.[24] It should be noted that vaucheriaxanthin in Xanthophyceae is often esterified,[25] but esterification does not affect the structure of the conjugated chain and consequently does not alter spectroscopic properties. Although diadinoxanthin is often found in dinoflagellates or diatoms, energy transfer routes in these organisms are usually associated with peridinin or fucoxanthin, and no significant energy transfer from diadinoxanthin was found so far.[20,23,26] Instead, the presence of diadinoxanthin and diatoxanthin in these organisms is usually related to photoprotective processes.[27,28] However, in XLH of *X. debile* diadinoxanthin is the major carotenoid and therefore should be active in energy-transfer processes between carotenoids and Chl-*a*.

Here we show the results of femtosecond transient absorption spectroscopy that aim to disclose the energy transfer pathways and efficiencies between carotenoids and chlorophylls in XLH complexes. The results show that although carotenoids in XLH have their S_1 energies in solution lower than energy of the Q_y band of Chl-*a* in XLH, a fraction of these carotenoids are still capable of transferring energy from their S_1 states with high efficiency.

2. MATERIALS AND METHODS

XLH complexes were prepared from *X. debile* as described previously.[24] The fraction obtained from sucrose gradient was further desalted, concentrated (Centricon 10000 molecular weight cutoffs, Amicon, Millipore), and finally dissolved in a buffer (10 mM HEPES, pH 7.4, 2 mM $MnCl_2$, 2 mM $MgCl_2$, 10 mM KCl, 0.03% *n*-dodecyl-*β*-D-maltoside) to yield optical density ∼0.6/mm at 675 nm for femtosecond transient absorption experiments, ∼0.7/cm at 675 nm for microsecond transient absorption experiments, and 0.07/cm at 675 nm for fluorescence measurements. Absorption spectra were measured on UV-300 (Spectronic Unicam, Cambridge, UK) spectrophotometer; fluorescence spectra were recorded on Fluorolog 2 (Spex) spectrofluorometer. A 1 cm path length cuvette was used for steady-state absorption and room-temperature fluorescence measurements. By varying the sample concentration, we have tested that the concentration of samples for fluorescence spectroscopy was always low enough to prevent reabsorption.

Transient absorption spectra were measured at room temperature using a femtosecond spectrometer employing Ti:sapphire amplifier (Intregra-i, Quantronix) as a primary source of femtosecond pulses. Excitation pulses were generated in an optical parametric amplifier (TOPAS, Light Conversion), while a white-light single filament continuum generated in a 2 mm sapphire plate was used as a probe. The mutual orientation of the excitation and probe beams polarization was set to the magic angle (54.7°). A 1 mm path length rotating quartz cuvette spinning at a rate to ensure that each excitation pulse hits a fresh sample was used for transient absorption measurements. Time-resolved absorption changes were measured in a broad spectral range from 474 to 716 nm by detecting the dispersed white light by double-diode array after excitation with ∼130 fs laser pulses centered at either 490 or 510 nm. Using neutral-density filters, the intensity of excitation at both wavelengths was kept at $\sim 6.0 \times 10^{13}$ photons pulse^{-1} cm^{-2}.

Femtosecond transient absorption data collected by diode-array detectors were fitted globally. To visualize the excited-state dynamics, we assume that the excited XLH complex evolves according to a sequential, irreversible scheme, in which the extracted time constants correspond to lifetimes of the individual excited-state species in the sequential scheme. The spectral profile of each species is called evolution-associated difference spectrum (EADS). It must be noted that EADS obtained from the sequential model do not correspond to individual excited states in a complex system such as the XLH complex studied here. Instead, the EADS will inevitably contain a mixture of excited-state species due to branching processes that occur in XLH. Yet, EADS help to visualize excited-state processes and carry important information about excited-state dynamics.[29]

Transient spectra in the microsecond time domain were measured with a pump-and-probe based instrument described in detail elsewhere.[30] Xenon flash lamps were used for both pump and probe; sample and reference rays were dispersed in an imaging spectrometer and detected by two 38-element photodiode arrays. The spectral resolution of the instrument was 2.1 nm per pixel. Red long-pass filter (Corning RA61) was used on the pump flash lamp. Cuvettes with 1 cm optical path

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length were used. The sample was cooled to 5 °C. By changing the delay between pump and probe flashes, kinetics of absorption changes were measured in the range of 3−40 μs.

3. RESULTS

Steady-State Spectroscopy. Absorption, fluorescence, and fluorescence excitation spectra of the XLH complex are shown in Figure 2. The absorption spectrum is dominated by

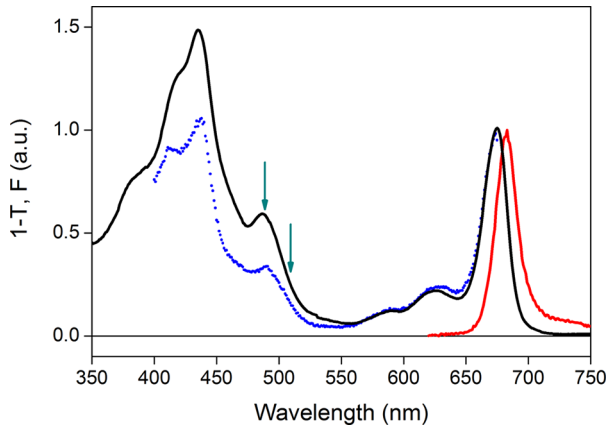

Figure 2. Absorption (black), fluorescence (red), and fluorescence excitation spectra (blue) of XLH complex from *X. debile*. Absorption spectra are plotted as $1 - T$ (T is transmittance) to allow estimation of energy transfer efficiencies by comparison with fluorescence excitation spectra. Fluorescence spectra were obtained after excitation at 435 nm. Detection wavelength for the fluorescence excitation spectra was 682 nm. The arrows denote excitation wavelengths used in transient absorption experiment, 490 and 510 nm.

absorption bands due to Chl-*a*; the Soret band peaks at 435 nm, and Q_y state has a maximum at 675 nm. The weaker bands at 590 and 625 nm are due to Q_x transition of Chl-*a* and higher vibrational band of the Q_y transition of Chl-*a*, respectively. The distinct absorption band peaking at 488 nm is due to carotenoids. After excitation of Chl-*a* Soret band at 435 nm, XLH complex exhibits a typical Chl-*a* fluorescence band peaking at 682 nm that constitutes a mirror image of the Q_y absorption of Chl-*a*. The fluorescence excitation spectrum, detected at the fluorescence maximum, shows that carotenoids contribute substantially to the observed signal and represents clear evidence of energy transfer between carotenoids and Chl-*a*, a finding that is later confirmed by kinetic data. Based on the comparison of absorption (1-T) and fluorescence excitation spectra, the overall efficiency of carotenoid-Chl-*a* energy transfer is ∼60%, though it varies slightly over the spectral region of the carotenoid absorption (450−550 nm).

The carotenoid absorption band consists of contributions from the four different carotenoids present in XLH complex. However, closer inspection of the carotenoid structures (Figure 1) tells that the conjugated chains of diadinoxanthin and heteroxanthin are the same. Consequently, these two molecules are spectroscopically identical because the two additional hydroxyl groups of heteroxanthin located at carbons C5 and C6 (Figure 1) will not alter the spectroscopic properties. Thus, hereafter we will use solely the name diadinoxanthin, but it is meant to describe excited-state properties of both diadinoxanthin and heteroxanthin in XLH. A slightly shorter effective conjugation could be expected for vaucheriaxanthin (Figure 1), but since this carotenoid constitutes less than 10% of total carotenoid composition,[24] we may neglect the contribution of this carotenoid to the spectroscopic properties described below. On the other hand, diatoxanthin, which is formed by de-epoxidation of diadinoxanthin,[31] has its conjugation extended by one C=C bond (Figure 1), resulting in lower energy of the S_2 state as compared to diadinoxanthin.[28,32] Thus, the peak at 488 nm is due to 0−0 band of the S_0−S_2 transition of diadinoxanthin, which is very close to the position of this band for diadinoxanthin in acetone.[32] Since diatoxanthin S_0−S_2 transition is in acetone shifted by about 5 nm to the red as compared to diadinoxanthin,[32] diatoxanthin may contribute to the very red edge of the carotenoid band. Accordingly, in attempt to distinguish these carotenoids and their roles in energy transfer to Chl-*a*, we have chosen two excitation wavelengths: 490 nm which should predominantly excite the S_2 state of diadinoxanthin and 510 nm which should selectively excite the lowest vibrational band of the S_0−S_2 transition of diatoxanthin.

Excitation at 490 nm. Transient absorption spectra after excitation at 490 nm are shown in Figure 3a. At wavelengths

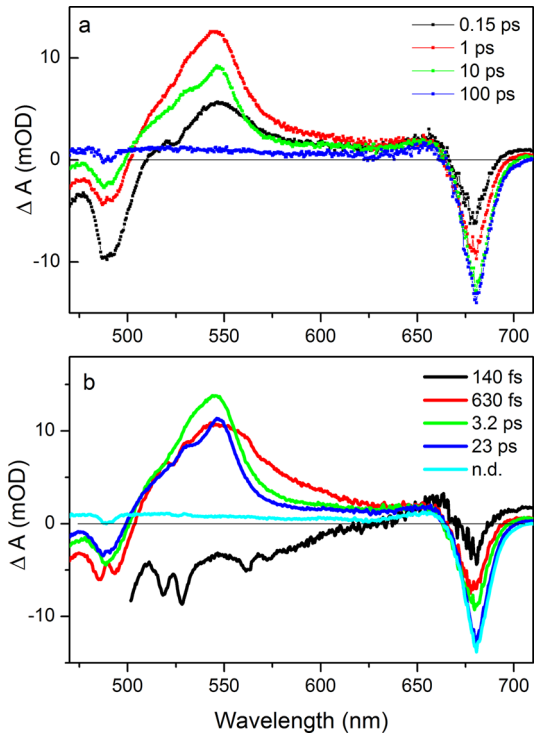

Figure 3. (a) Transient absorption spectra of XLH complex excited at 490 nm. (b) EADS from global fitting of data obtained after 490 nm excitation of the XLH complex. The first EADS below 500 nm is omitted due to scattering at excitation wavelength. n.d. = nondecaying component.

shorter than 500 nm, negative signal appearing immediately after excitation is due to bleaching of the S_0−S_2 band of diadinoxanthin as evidenced by a distinct peak at 488 nm that mirrors the 0−0 band of the S_0−S_2 transition in absorption spectrum (Figure 2). The positive signal, excited-state absorption, is typical of S_1−S_n transition of carotenoids. In this case, the major contribution is expected from diadinoxanthin. The S_1−S_n transition of diadinoxanthin peaks at 545 nm in XLH, thus at lower energy than diadinoxanthin in acetone solution (537 nm).[32] Therefore, while the S_0−S_2 transition of diadinoxanthin in XLH occurs at nearly the same wavelength as

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in acetone solution,[32] the S_1-S_n transition is red-shifted in XLH, indicating that protein environment in XLH affects the S_0-S_2 and S_1-S_n transitions of diadinoxanthin differently. Decay of the S_0-S_2 bleaching and S_1-S_n excited-state absorption of diadinoxanthin is accompanied by appearance of bleaching at 680 nm that is clearly due to excited Chl-*a* populated by energy transfer from diadinoxanthin.

To extract time constants associated with various excited-state processes in XLH, we have fitted the whole data set globally, and results are depicted as EADS in Figure 3b. To obtain reliable fits, four time components were needed to fit the transient absorption spectra. The initial EADS (black) has a shape typical of the carotenoid S_2 state spectrum and decays within 140 fs, suggesting energy transfer from the S_2 state of diadinoxanthin as evidenced by increase of Chl-*a* bleaching during the 140 fs process. However, the presence of Chl-*a* bleaching in the first EADS suggests that part of energy absorbed by carotenoids is transferred to Chl-*a* within our time resolution (~120 fs). The second EADS is reminiscent of spectrum of a hot S_1 state of diadinoxanthin.[32] It decays in 630 fs, which is somewhat faster than for diadinoxanthin in solution.[32] This, together with a slight increase of Chl-*a* bleaching, suggests that even the hot S_1 state may be involved in energy transfer between diadinoxanthin and Chl-*a* as reported earlier in other light harvesting systems.[11−13] Yet, based on the very small increase of the Q_y bleaching during the 630 fs process, this channel plays only a minor role.

The next two EADS, characterized by time constants of 3.2 and 23 ps, have essentially identical shape and correspond to a spectrum of relaxed S_1 state of diadinoxanthin. Although EADS associated with these two processes are of similar shape, it is obvious that while the 3.2 ps process is accompanied by an increase of Chl-*a* bleaching, no dynamics in the Chl-*a* region is related to the 23 ps process. Consequently, the 3.2 ps process must reflect energy transfer from the relaxed S_1 state of a certain subpopulation of diadinoxanthin, whereas the slower decay is the S_1 lifetime of diadinoxanthin that do not transfer energy to Chl-*a* via the S_1 state because the 23 ps lifetime matches the S_1 lifetime of diadinoxanthin in solution.[28,32] Our conclusion that the 3.2 ps component is associated with energy transfer from diadinoxanthin S_1 state to Chl-*a* is supported by comparing the kinetic of Chl-*a* bleaching at 680 nm with that of S_1-S_n signal of diadinoxanthin at 537 nm (Figure 4). It is clear that S_1-S_n decay matches the rise of Chl-*a* bleaching, confirming the S_1-mediated energy transfer. Final EADS is due to Chl-*a* that does not exhibit any decay within the time window of the experiment (300 ps).

Excitation at 510 nm. Excitation at 510 nm excites the very red edge of carotenoid absorption spectrum in XLH and holds promise to selectively excite diatoxanthin which, due to its longest conjugation, should absorb at the longest wavelengths of all carotenoids in XLH. Transient absorption spectra recorded at a few delay times after excitation at 510 nm are depicted in Figure 5a. At first glance, the spectra are remarkably similar to those obtained after 490 nm excitation; Q_y bleaching peaks at 680 nm and maximum of the S_1-S_n band is at 545 nm, thus the same as measured after 490 nm excitation (Figure 3a). However, closer inspection reveals a few differences. First, it is obvious that the magnitude of the Q_y bleaching measured after 510 nm excitation at early delay times is significantly larger than after excitation at 490 nm, suggesting more efficient energy transfer from the S_2 state of the carotenoid excited at 510 nm. Second, decay of the S_1-S_n band is somehow faster after 510 nm excitation as evidenced by comparison of magnitudes of the S_1-S_n signal measured after 1 and 10 ps for both excitations.

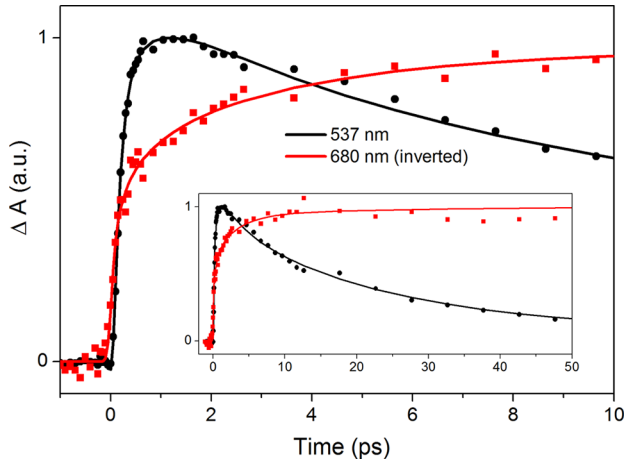

Figure 4. Kinetics of the S_1-S_n decay (black) monitoring depopulation of the carotenoid S_1 state and inverted kinetics of Chl-*a* rise (red) showing appearance of excitations at Chl-*a* measured after excitation of XLH complex at 490 nm. Inset shows longer time window of both kinetics. All kinetics are normalized to maximum.

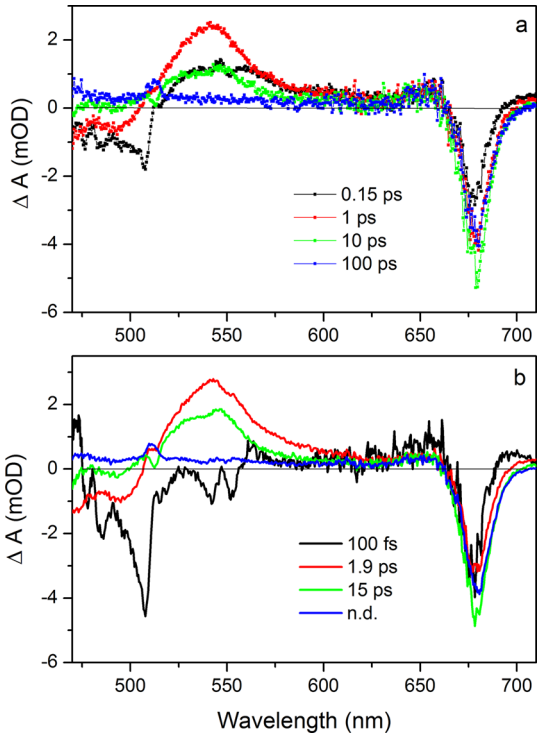

Figure 5. (a) Transient absorption spectra of XLH complex excited at 510 nm. (b) EADS from global fitting of data obtained after 510 nm excitation of the XLH complex. n.d. = nondecaying component.

The differences between data obtained after 490 and 510 nm excitation are further disclosed when global fitting is applied. First, contrary to the 490 nm excitation, the data recorded after 510 nm excitation require only three time components to obtain good fits. Resulting EADS are shown in Figure 5b, and it is obvious that spectrum corresponding to a hot S_1 state is missing in data recorded after 510 nm excitation. It should be noted that we can include a fourth component with 400−700 fs time constant, which has a spectrum reminiscent of a hot S_1

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state, but amplitude of this additional component is negligible (not shown). This suggests that relaxation processes induced by excitation of the carotenoid absorbing at the very red edge do not involve hot S_1 state.

Besides the missing hot S_1 state, another significant difference between the 490 and 510 nm excitations is the magnitude of Chl-*a* bleaching in the initial EADS. While the initial EADS after 490 nm excitation contains Chl-*a* bleaching that reaches about 30% of full magnitude of Chl-*a* bleaching (Figure 3b), after 510 nm excitation this magnitude reaches ~80% of the total bleaching magnitude. This clearly demonstrates that carotenoid excited at 510 nm has significantly faster and more efficient S_2 channel. The second (red) and third (green) EADS have comparable shape and correspond to relaxed S_1 state of two populations of carotenoids, each having different S_1 lifetime. This is qualitatively the same as observed after 490 nm excitation, but time constants obtained after 510 nm excitation are different. The faster component, again accompanied by an increase of Chl-*a* bleaching and thus associated with energy transfer, has a lifetime of 1.9 ps. The slower component of 15 ps has no corresponding rise of Chl-*a*, and it is therefore the S_1 lifetime of carotenoids that do not transfer energy to Chl-*a*. It is worth mentioning that the 15 ps component matches well the S_1 lifetime of diatoxanthin in solution (13 ps).[32] It should be also noted that besides the 15 ps decay in the S_1-S_n spectral region, there is also some decay of Chl-*a* bleaching associated with the 15 ps component. However, this is solely due to lower S/N ratio of the data excited at 510 nm, resulting in poor fit at longer time scales. As shown in Supporting Information (Figure S1), when the Chl-*a* spectral region (600−720 nm) is fitted separately, the 15 ps component is not needed. As for the 490 nm excitation, the nondecaying EADS is due to Chl-*a* representing the final trap in isolated XLH complex. Moreover, the shape of the final EADS obtained after 510 and 490 nm excitation are essentially identical (Supporting Information, Figure S2), indicating that the final Chl-*a* state is the same regardless the excitation wavelength.

Triplet Spectra in the Microsecond Time Domain. To test whether carotenoids in XLH also have a photoprotective role, we have recorded triplet-minus-singlet spectra at the microsecond time scale. Figure 6 shows transient absorption spectrum measured at 3 μs. The sample was excited by Xe flash lamp with 615 nm long-pass filter to ensure selective excitation of Chl-*a*. The T-S spectrum consists of sharp positive band peaking at 513 nm, which is clearly due to the T_1-T_n transition of carotenoids, carotenoid bleaching at wavelengths below 470 nm, and Chl-*a* bleaching at 675 nm. Population of carotenoid triplet indicates that carotenoids quench Chl-*a* triplets via triplet−triplet energy transfer. However, the presence of Chl-*a* bleaching suggests that a fraction of Chl-*a* triplets coexist with the carotenoid triplet, a phenomenon observed in other light-harvesting systems containing Chl-*a*[33−36] and explained by delocalization of triplet excitation over carotenoid and Chl-*a*.[37] Decay of carotenoid triplet can be fitted by a monoexponetial function resulting in a time constant of 9 μs (Figure 6, inset).

4. DISCUSSION

Data described in the previous section unequivocally prove that carotenoids in XLH transfer energy to Chl-*a*. However, it is obvious that excitation close to the peak of carotenoid absorption in XLH produces different pattern of energy transfer pathways than that obtained after excitation at the very red edge of carotenoid absorption in XLH, indicating that a different set of carotenoids is excited at 490 and 510 nm. The question is whether different carotenoid, e.g. diatoxanthin, is excited at 510 nm or it is the same carotenoid as that excited at 490 nm (diadinoxanthin/heteroxanthin) but with spectroscopic properties altered by interaction with protein.

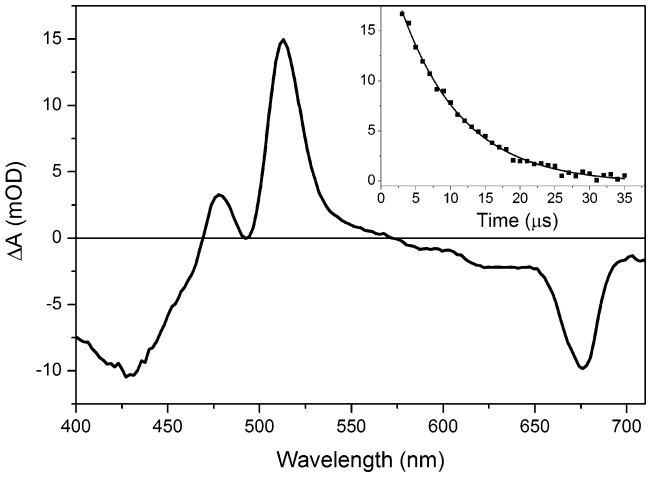

Figure 6. Triplet-minus-singlet spectrum of XLH detected at 3 μs delay between pump and probe. To maintain spectral resolution, the T-S spectrum is constructed from five separate spectra measured with different grating positions. Inset shows kinetics at 513 nm monitoring the decay of the carotenoid triplet state.

The first clue to answer this question comes from global fitting that shows that at both excitation wavelengths a significant fraction of excited carotenoids does not transfer energy to Chl-*a*, revealing intrinsic S_1 lifetime of these carotenoids in XLH. Since carotenoids in XLH excited at 490 nm have the intrinsic S_1 lifetime of 23 ps, matching the S_1 lifetime of diadinoxanthin/heteroxanthin in solution, but those excited at 510 nm have markedly shorter S_1 lifetime of 15 ps in XLH, it is tempting to conclude that 490 nm excites predominantly diadinoxanthin/heteroxanthin in XLH while 510 nm rather excites diatoxanthin whose S_1 lifetime in solution is 13 ps.[32] This conclusion could be also supported by the fact that diatoxanthin has longer conjugation length (Figure 1); thus, its S_0-S_2 transition should be shifted to longer wavelengths.

Comparing energy transfer via the S_2 pathway, it is clearly more efficient after 510 nm excitation as evidenced by a large contribution of Chl-*a* bleaching in the initial EADS (Figure 5b) that must be due to depopulation of the S_2 state via energy transfer in less than 100 fs. While such fast channel also exists for carotenoids excited at 490 nm, its contribution is smaller, and an additional, slower S_2-mediated transfer occurring with a time constant of 140 fs is found exclusively after 490 nm excitation. This suggests that while at 510 nm we rather excite a specific carotenoid having very efficient S_2 channel, there are at least two distinct carotenoids excited at 490 nm, each transferring to Chl-*a* from the S_2 state with different energy transfer rate. The significant difference in efficiency of the S_2-mediated energy transfer pathway is shown in Figure 7 that compares ratio of Chl-*a* bleaching and S_1-S_n magnitudes at different delay times. It shows that magnitude of Chl-*a* bleaching at early delay times is significantly larger after 510 nm excitation, confirming the efficient S_2 pathway taking place exclusively after 510 nm excitation. Assuming that S_2 pathway

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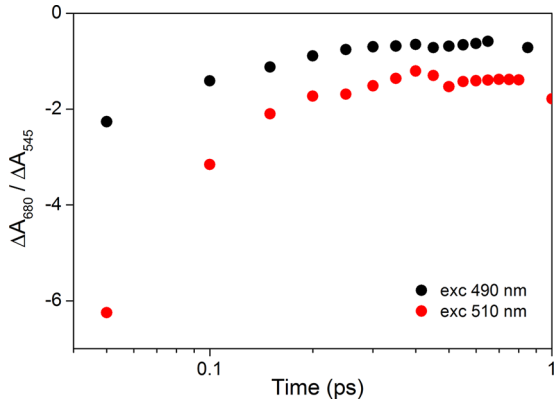

Figure 7. Ratio of maximal amplitudes of Chl-*a* bleaching (at 680 nm) and S_1–S_n excited-state absorption (at 545 nm) at different delay times for XLH complex excited at 490 nm (black) and 510 nm (red).

proceeds via the Förster-type mechanism as it is in other carotenoid-containing systems,[2] the faster energy transfer rate observed after 510 nm excitation could be either due to a better spectral overlap between S_2 emission of the carotenoid excited at 510 nm and absorption of the acceptor (Q_x state of Chl-*a*) or due to more favorable donor–acceptor orientation and/or distance.

If we turn our attention to the S_1 channel, the situation is comparable to the S_2-mediated pathway. The S_1 pathway is active regardless of the excitation wavelength as demonstrated in Figures 4 and 8 that compare kinetics of the S_1 decay and Chl-*a* rise measured after 490 and 510 nm excitations. However, Figure 8 also visualizes the results of global fitting which show that the S_1-mediated energy transfer is slower after excitation at 490 nm, as the S_1 lifetime yields 3.2 ps whereas 1.9

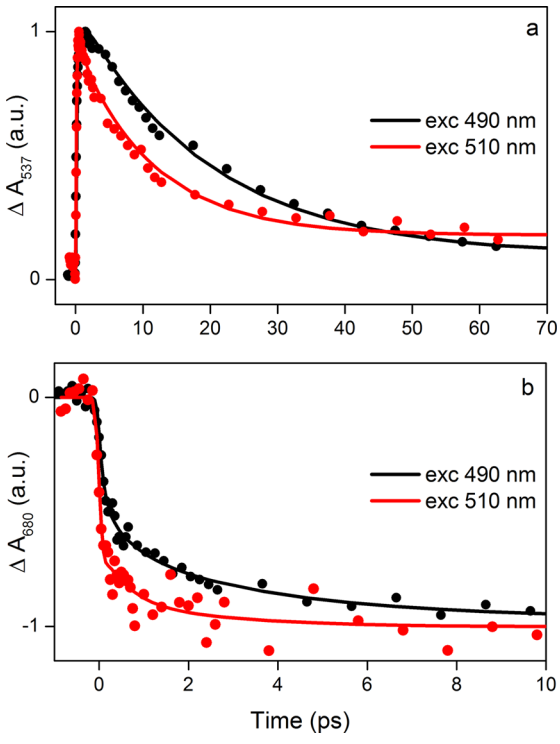

Figure 8. Kinetics of carotenoid S_1 decay (a) and Chl-*a* rise (b) recorded after excitation of XLH complex at 490 nm (black) and 510 nm (red).

ps is obtained after 510 nm excitation. Knowledge of the intrinsic S_1 lifetimes of carotenoids in XLH, 23 ps for the carotenoid excited at 490 nm and 15 ps for those excited at 510 nm, allows for calculation of energy transfer time and efficiency of the S_1 channel. Energy transfer times yield 3.7 and 2.2 ps for S_1 channels generated by 490 and 510 nm excitation, respectively. The corresponding efficiencies of energy transfer via the S_1 channel are 0.84 and 0.85. Thus, even though the energy transfer rates of the S_1 pathway differ markedly for the two excitation wavelengths, efficiency of this pathway is independent of the excitation wavelength.

Energy transfer efficiency of the S_1 channel higher than 80% is surprising for donor–acceptor pair consisting of carotenoid and Chl-*a*. While such high efficiencies of the S_1 pathway are often observed in purple bacterial antenna having BChl-*a* as acceptor,[4–7] in Chl-*a* containing light-harvesting systems of plants and algae the S_1 pathway is mostly suppressed because energy of the S_1 state of carotenoids in these systems is too low to transfer energy to the Q_y state of Chl-*a*. Efficiencies reported for the transfer of energy from the S_1 state of carotenoids did not exceed 20% in these light-harvesting complexes.[2,9–11] On the other hand, light-harvesting strategy that evolved in Chl-*a*-based antenna of dinoflagellates or diatoms containing carbonyl carotenoids peridinin[18–21] or fucoxanthin[22,23,26] allows for efficient S_1-mediated transfer. Carbonyl carotenoids have spectroscopic properties dependent on polarity of the environment, and their S_1 state is coupled to an ICT state.[16,17] This shifts their S_1 energy higher than for their non-carbonyl counterparts with the same effective conjugation length,[17] making the donor–acceptor energy difference favorable for energy transfer. Moreover, the S_1–ICT coupling facilitates interaction with Q_y state of Chl-*a*, resulting in efficiencies of the S_1 channel of 80–90%.[18–23]

In XLH, the efficiency and energy transfer rate of carotenoid-to-Chl-*a* transfer via the S_1 state are comparable to that observed in peridinin-chlorophyll protein (PCP) or fucoxanthin-chlorophyll protein (FCP), even though carotenoids in XLH do not have conjugated carbonyl group thus the ICT-driven enhancement of the S_1 channel is not possible. It should be also noted that energy of the acceptor state (Q_y band of Chl-*a*) is essentially the same in XLH and PCP/FCP (~14 800 cm^{-1}). In PCP, to achieve ~85% efficiency and ~3 ps energy transfer time, the S_1/ICT state of peridinin must be >16 000 cm^{-1};[19] thus, comparable S_1 energies could be also expected for carotenoids active in S_1 channel in XLH. But the S_1 energies of diadinoxanthin and diatoxanthin in solution were recently determined from measurements of spectral profiles of the S_1–S_2 transition, and the resulting values are 14 120 ± 80 and 13 930 ± 80 cm^{-1}.[32] These values are significantly lower than those of peridinin or fucoxanthin[17,38,39] and are rather comparable with the S_1 energies of xanthophylls found in light-harvesting complexes of plants and algae.[40–42]

Taking into account the measured S_1 energies of diadinoxanthin and diatoxanthin in solution, the observed fast and efficient S_1 energy transfer in XLH is unexpected because S_1 energies of carotenoids in XLH should be lower than energy of the acceptor state. It must be noted however that efficient S_1-mediated channel in XLH is observed only for a fraction of carotenoids as evidenced by EADS shown in Figures 3 and 5 and by clear double-exponential decay of kinetics monitoring the S_1 decay (Figure 8). It thus shows that, contrary to PCP and FCP, there are two groups of carotenoids in XLH: those that transfer energy via their S_1 states to Chl-*a* with efficiency of

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~85% (about 25% of total carotenoid content in XLH) and those having the S_1 pathway inactive.

Based on spectroscopic data reported earlier, possible candidates for the carotenoids active in S_1-mediated energy transfer in XLH are cis-isomers. The cis-carotenoids have their S_1 energies systematically higher than their all-trans counterparts; depending on carotenoid species, the S_1 energy of the cis-isomer is 300−700 cm^{-1} higher than for the trans-configuration.[42,43] Moreover, the energy difference between cis- and trans-isomers increases as the conjugation length decreases. The largest differences are observed for carotenoids with $N \leq 10$,[42,43] which matches the conjugation length of the carotenoids in XLH (Figure 1). Thus, the carotenoid S_1 energy in XLH may be tuned by the carotenoid configuration, and those in cis-configuration have their S_1 energies high enough to allow efficient energy transfer to Chl-*a*.

To test this hypothesis, we carried out an HPLC analysis of carotenoids in XLH (Supporting Information, Figure S3). Comparison of chromatogram detected in the cis-peak region (340 nm) with that measured at 450 nm shown in Figure S3 suggests that cis-isomers are not present in XLH. Thus, the observed efficient S_1 channel in XLH complex cannot be due to cis-isomers. On the other hand, the carotenoid binding site usually forces carotenoids into configurations that are not stable in solution. While in most cases these configurations deviate only slightly from the expected solution structure, in some cases the protein binding site alters the configuration in a way that results in significant changes in spectroscopic properties.[44,45] Since quantum chemical calculations on various carotenoid structures suggest that energies of excited states of certain configurations may differ significantly from those of stable configurations,[46] it is in principle possible that certain carotenoid binding sites in XLH render the S_1 energy markedly higher than in solution, making the energy transfer via the S_1 state possible. These configurations are unstable in solution and only high-resolution structure of XLH, which is not yet available, can provide structure of diadinoxanthin and diatoxanthin in their respective binding sites.

This brings us back to the question whether diatoxanthin is selectively excited at 510 nm. Because of its longer conjugation (Figure 1), diatoxanthin should have the S_1 energy lower than diadinoxanthin, and this assumption was confirmed by direct measurements of S_1 energies in solution.[32] Even though the S_1 energy of diatoxanthin in solution is only ~200 cm^{-1} lower than that of diadinoxanthin,[32] it seems unlikely that a carotenoid with lower S_1 energy would have larger energy transfer rate, challenging the hypothesis of diatoxanthin being selectively excited at 510 nm. This conclusion is also supported by comparing the S_1-S_n bands recorded at 1 ps after excitation at 490 and 510 nm (Figure 9). Although carotenoids excited at these two wavelengths exhibit different energy transfer rates to Chl-*a* and even different intrinsic S_1 lifetimes, they have nearly identical S_1-S_n spectra. The transient absorption spectra differ in the carotenoid bleaching region confirming population of different sets of carotenoid at these two excitation wavelengths, but their S_1-S_n maxima differ by only 2 nm, with redder carotenoid having bluer S_1-S_n maximum. This is in sharp contrast with the S_1-S_n bands of diadinoxanthin and diatoxanthin in solution; the S_1-S_n maximum of the redder carotenoid, diatoxanthin, is red-shifted by almost 20 nm from the S_1-S_n peak of diadinoxanthin, and the S_1-S_n band of diadinoxanthin is markedly broader.[32] Thus, similarity of the S_1-S_n bands shown in Figure 9 rather indicates that they

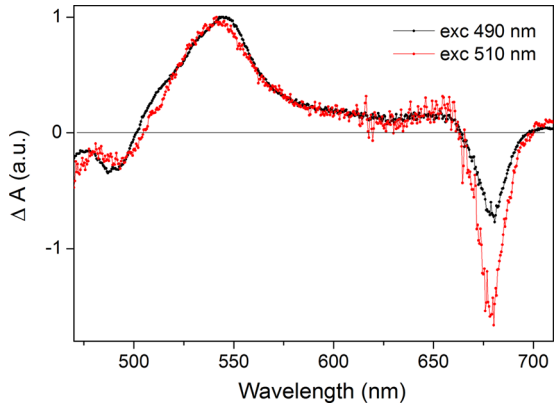

Figure 9. Transient absorption spectra of XLH complex measured at 1 ps after excitation at 490 nm (black) and 510 nm (red). Spectra are normalized to maximal intensity of the S_1-S_n band.

originate from the same carotenoid, diadinoxanthin, and the slight difference in S_1-S_n bands shown in Figure 9 is due to diadinoxanthin molecules with different excited-state properties being excited at 490 and 510 nm.

The notion of diadinoxanthin being the only carotenoid involved in energy transfer processes described above is also favored by XLH pigment composition. The XLH pigment composition reported recently[24] gives pigment molar ratio of 28 Chl-*a*:11 diadinoxanthin:5 diatoxanthin:4 heteroxanthin:1 vaucheriaxanthin. This is 49 pigments in total, and taking into account recent pigment analysis of the closely related FCP from diatom *Cyclotella meneghiniana*,[47,48] this value matches the expected number of pigments per trimer, based on estimation of ~16 pigments per monomer as identified in FCP.[47,48] Then, assuming that diadinoxanthin and heteroxanthin are spectroscopically identical, this translates into XLH monomer containing ~5 molecules of diadinoxanthin/heteroxanthin and ~1−2 molecules of diatoxanthin per monomer while vaucheriaxanthin is not present in each monomer. Then, it is impossible to have ~25% diatoxanthins transferring energy from their S_1 states as implied by the global analysis shown in Figure 5 because on average there are less than 2 diatoxanthins in each monomer. This again supports the hypothesis that it is rather red-shifted diadinoxanthin that is excited at 510 nm. This conclusion is also in agreement with recent report on FCP that contains ~8 fucoxanthins per monomer that are distributed into three groups absorbing in blue, green, and red parts of carotenoid absorption band in FCP.[47]

Thus, we conclude that diadinoxanthin/heteroxanthin is the key carotenoid responsible for efficient energy transfer between carotenoids and Chl-*a* in XLH. Although we cannot distinguish spectroscopically diadinoxanthin and heteroxanthin, it is possible that differences in functional groups at the terminal ring of these two carotenoids may be crucial for binding in XLH (e.g., hydrogen bonding via the hydroxyl groups of heteroxanthin), resulting in stabilization of the proposed configurations with higher S_1 energy.

The only observation that remains to be explained is the difference between intrinsic lifetimes of diadinoxanthin/heteroxanthin molecules excited at 490 and 510 nm, which yields 23 and 15 ps, respectively. This difference is rather unusual because significant changes in S_1 lifetimes due to interaction with environment are observed only for carbonyl carotenoids,[16,17] while non-carbonyl carotenoids are known to have the S_1 lifetimes constant regardless the environment.[3]

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Binding of carotenoid to protein can under certain conditions alter the S_1 lifetime, but this occurs exclusively for carotenoids with conjugation extended to terminal rings. Twisting the ring by the binding pocket may alter the effective conjugation resulting in the S_1 lifetime shorter[44,45] or longer[49] than in solution. However, this is not the case of diadinoxanthin whose conjugated chain is linear. Yet, the shorter intrinsic S_1 lifetime of diadinoxanthin may result from a specific carotenoid configuration. We have already pointed out that certain configurations may have higher S_1 energy that can promote the S_1-mediated energy transfer. Similarly, it is known that a change of carotenoid configuration may lead to significant change in S_1 lifetime.[42,43] Different configurations of diadinoxanthin would then also explain the difference in intrinsic lifetimes of diadinoxanthin excited at 490 and 510 nm.

Finally, we discuss the role of carotenoid triplet states in XLH. Significant population of carotenoid triplet observed after Chl-*a* excitation (Figure 6) provides evidence for triplet−triplet energy transfer between carotenoid and Chl-*a*. As in many other light-harvesting systems, this process has key photoprotective function because it prevents triplet sensitization of singlet oxygen. Moreover, coexistence of the carotenoid triplet and Chl-*a* bleaching suggests that there are either unquenched long-living Chl-*a* triplets or the triplet state is delocalized over carotenoid and Chl-*a* as suggested for other light-harvesting systems.[33−37] The latter case would imply that XLH contains strongly interacting carotenoid−Chl-*a* pair(s) such as found for example in PCP,[50] but lack of detailed structural knowledge prevents assignment which carotenoids are those responsible for the effective triplet quenching.

5. CONCLUSIONS

XLH complex from the Chromophyte *X. debile* is a unique light-harvesting system that is closely related to FCP complexes of diatoms, but it does not contain fucoxanthin and Chl-*c*. Instead, XLH has only one chlorophyll type, Chl-*a*, and four carotenoids, diadinoxanthin, heteroxanthin, diatoxanthin, and vaucheriaxanthin. Our results show that carotenoids in XLH complex efficiently transfer energy to Chl-*a*. Scheme of energy transfer pathways is depicted in Figure 10. Of the four carotenoids presented in XLH, the major energy donors are diadinoxanthin and heteroxanthin, two carotenoids that are spectroscopically indistinguishable. Carotenoids utilize both S_2 and S_1 channels as energy donors with efficiencies varying slightly across the spectral region of carotenoid absorption. Energy-transfer efficiency is ∼65% at 510 nm, with significant contribution proceeding via the S_2 channel. At 490 nm, the efficiency drops below 60%, most likely due to less efficient S_2 channel. At both excitation wavelengths, about 25% of diadinoxanthin/heteroxanthin molecules in XLH transfer energy to Chl-*a* also via their S_1 states with efficiency exceeding 80%. Such large efficiency of the S_1 channel, which has not been observed so far in systems with Chl-*a* acceptor except those containing carbonyl carotenoids, is explained by specific configuration of diadinoxanthin/heteroxanthin that is proposed to occur in XLH. This configuration likely makes the S_1 energy of some of diadinoxanthin/heteroxanthin in XLH higher than in solution, enabling efficient energy transfer from the S_1 state to Chl-*a*. Moreover, carotenoids in XLH are capable of effective quenching of Chl-*a* triplet states.

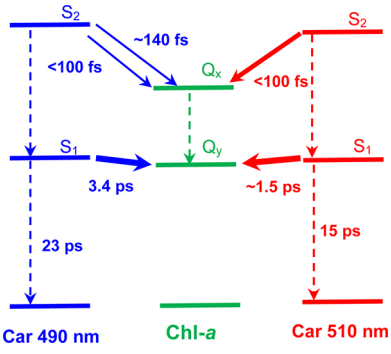

Figure 10. Scheme of excitation wavelength dependent energy transfer pathways between carotenoids and Chl-*a*. The pathways are denoted as solid arrows labeled by corresponding time constants. Thickness of the arrow is proportional to efficiency of energy transfer. Dashed arrows correspond to internal conversion processes.

The XLH complex thus represents another example of large variability of light-harvesting strategies occurring among various photosynthetic microorganisms. Contrary to diatoms and dinoflagellates that utilize specific properties of carbonyl carotenoids to achieve efficient energy transfer from the lowest excited state of carotenoid to Chl-*a*, XLH most likely employs so far unknown carotenoid−protein interaction to modify carotenoid structure, resulting in an increase of S_1 energy in protein that makes the efficient carotenoid-Chl-*a* energy transfer via the S_1 state possible.

■ ASSOCIATED CONTENT

Supporting Information

Results of global fitting of the Chl-*a* bleaching region, comparison of final EADS obtained from data measured after 490 and 510 nm excitation, pigment analysis demonstrating that no cis-carotenoids are present in XLH. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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