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Highly efficient energy transfer from a carbonyl carotenoid to chlorophyll *a* in the main light harvesting complex of *Chromera velia*

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ABSTRACT

We report on energy transfer pathways in the main light-harvesting complex of photosynthetic relative of apicomplexan parasites, *Chromera velia*. This complex, denoted CLH, belongs to the family of FCP proteins and contains chlorophyll (Chl) *a*, violaxanthin, and the so far unidentified carbonyl carotenoid related to isofucoxanthin. The overall carotenoid-to-Chl-*a* energy transfer exhibits efficiency over 90% which is the largest among the FCP-like proteins studied so far. Three spectroscopically different isofucoxanthin-like molecules were identified in CLH, each having slightly different energy transfer efficiency that increases from isofucoxanthin-like molecules absorbing in the blue part of the spectrum to those absorbing in the reddest part of spectrum. Part of the energy transfer from carotenoids proceeds via the ultrafast S_2 channel of both the violaxanthin and isofucoxanthin-like carotenoid, but major energy transfer pathway proceeds via the S_1/ICT state of the isofucoxanthin-like carotenoid. Two S_1/ICT-mediated channels characterized by time constants of ~0.5 and ~4 ps were found. For the isofucoxanthin-like carotenoid excited at 480 nm the slower channel dominates, while those excited at 540 nm employs predominantly the fast 0.5 ps channel. Comparing these data with the excited-state properties of the isofucoxanthin-like carotenoid in solution we conclude that, contrary to other members of the FCP family employing carbonyl carotenoids, CLH complex suppresses the charge transfer character of the S_1/ICT state of the isofucoxanthin-like carotenoid to achieve the high carotenoid-to-Chl-*a* energy transfer efficiency.

1. Introduction

The primary function of light-harvesting complexes of photosynthetic organisms is to collect light and transfer the absorbed energy to the reaction centers [1]. The key light-harvesting pigments are chlorophylls (Chl) or bacteriochlorophylls (BChl), but especially in photosynthetic microorganisms, the importance of the accessory pigments, carotenoids, as the light-harvesting agents increases [2]. Carotenoids effectively cover the spectral region that is not accessible to (B)Chl, and due to the large structural variability of carotenoids found in nature, they are able to tune their spectral properties to a much larger extent than (B)Chl [3]. This, in combination with their ability to transfer energy very efficiently to (B)Chl, makes carotenoids crucial components of light-harvesting proteins of many photosynthetic microorganisms, which utilize carotenoids to develop various light-harvesting strategies that are optimized to the light conditions of their ecological niches [2].

Chromera (*C.*) *velia* is a photosynthetic unicellular alveolate, a supposed symbiont of stony corals, and together with the recently described *Vitrella brassicaformis*, they are the closest known photosynthetic relatives of apicomplexan parasites such as *Plasmodium* which causes malaria [4]. Ancestors of apicomplexan parasites have acquired their plastid bounded by a four-membrane envelop through secondary endosymbiosis of free-living photosynthetic red alga [5]. The photosynthetic apparatus of *C. velia* is interesting as it utilizes three different and evolutionary distant types of light-harvesting antenna complexes: one bound to PS I reaction center that it is related to the PS I light-harvesting complex of red algae [6], a second which is a far-red antenna complex absorbing above 700 nm [7,8], and a third complex which shows sequential similarity to fucoxanthin chlorophyll *a*/*c* binding light-harvesting complex (FCP) [9].

The FCP-like antenna complex of *C. velia*, which is a subject of this study, belongs to the family of FCP proteins that may be found in diatoms or kelps [10]. Although FCPs are related to the well-known *Cab*

Abbreviations: acpPC, chlorophyll-*a*–chlorophyll-c_2–peridinin protein complex; BChl, bacteriochlorophyll; CLH, *Chromera* light-harvesting; EADS, evolution-associated difference spectrum; FCP, fucoxanthin chlorophyll *a*/*c* binding light-harvesting complex; HPLC, high performance liquid chromatography; ICT, intramolecular charge transfer; Ifx-l, isofucoxanthin-like carotenoid; LHC, light-harvesting complex; PCP, peridinin chlorophyll *a* protein; UV–VIS, ultraviolet visible absorption spectroscopy; XLH, *Xanthonema* light-harvesting complex

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protein family from higher plants [11], the structures of FCPs remain unknown. No atomic structure of a member of the FCP family was resolved so far, and our knowledge relies solely on comparing the spectroscopic data taken for the FCP family proteins with those recorded for LHCII whose structure is known to a great detail [12]. It is therefore expected that the central luteins known from the LHCII structure are likely replaced by two fucoxanthins in FCP from diatoms [13].

The FCP-like light-harvesting complex of *C. velia*, denoted as *Chromera* light-harvesting (CLH) complex, was studied by electron microscopy and circular dichroism, suggesting its close relation to the XLH antenna from xanthophytes [6]. Yet, while the XLH complex contains only non-carbonyl carotenoids, diatoxanthin and diadinoxanthin [14,15], the CLH complex also accommodates a carbonyl carotenoid (carotenoid containing the conjugated C = O group). Thus it is spectroscopically and functionally closer to FCP proteins. Besides the Chl *a* and carotenoid violaxanthin, the CLH complex contains a carbonyl carotenoid yet to be identified. Based on HPLC, UV–VIS, electrospray mass spectroscopy, and chemical analysis, this unknown carotenoid must be related to isofucoxanthin. It contains all the functional groups of isofucoxanthin, and it is characterized by $C_{42}H_{58}O_6$ composition [16]. The absorption spectrum of this carotenoid in methanol is, however, red-shifted by about 13 nm compared to isofucoxanthin, indicating that conjugation length of the unknown carotenoid is longer than that of isofucoxanthin. We therefore denote this carotenoid as isofucoxanthin-like (Ifx-l). The pigment composition of CLH exhibits a Chl *a*:Vio:Ifx-l ratio of approximately 10:1:4 [6]. A later report, using both gel filtration and anion exchange for purification of CLH, demonstrated that while the Chl *a*:Ifx-l ratio of 5:2 is constant, violaxanthin content may vary from 5% to 20% of Chl *a* depending on purification method, suggesting that violaxanthin is likely loosely bound in CLH and may be partially removed during purification [8].

Even though the molecular structure of Ifx-l remains unknown, it clearly belongs to the family of carbonyl carotenoids whose spectroscopic properties are largely affected by environment [17,18]. When embedded in a polar environment, these carotenoids often have an intramolecular charge transfer (ICT) state in their excited-state manifold. The ICT state is readily identified in transient absorption spectra due to its characteristic excited-state absorption (denoted as an ICT-like band) in the 600–700 nm spectral region as well as the stimulated emission in the near infrared [19,20]. The ICT state is believed to be coupled to the S_1 state, which is usually denoted S_1/ICT state [20]. Depending on polarity, the degree of the charge transfer character of the S_1/ICT state varies, and this variation can be monitored by the magnitude of the ICT-like band. Importantly, for some carbonyl carotenoids the S_1/ICT state exhibits lifetime dependence on solvent polarity [17,18]. Then, because the intrinsic carotenoid lifetime is a crucial parameter in determining the energy transfer efficiency [2], if a carbonyl carotenoid is bound to a light-harvesting protein, the local polarity of the binding protein may tune the energy transfer efficiency as it was demonstrated for synthetic systems containing carbonyl carotenoids [21].

Besides the possibility of tuning the energy transfer efficiency by polarity of the protein environment, there are two other properties that make the carbonyl carotenoids vital constituents of light-harvesting proteins. First, the symmetry breaking caused by the conjugated carbonyl group increases the dipole moment of the S_1/ICT state, which enhances the donor–acceptor coupling. Second, the conjugated carbonyl group decreases the energy of the S_2 state while keeping the S_1/ICT energy sufficiently high to enable transfer to the Q_y state of Chl *a* [18]. This allows extension of absorption into the green-yellow region (520–580 nm) even for oxygen-evolving photosynthetic organisms utilizing Chl *a*.

The importance of the ICT state for maintaining a high light-harvesting capacity has been demonstrated in a number of antenna containing carbonyl carotenoids. Peridinin chlorophyll *a* protein (PCP) has been widely used as a model system, because its structure is known to atomic resolution [22,23]. A number of studies [24–31] provided a basic understanding of light-harvesting strategies utilizing carbonyl carotenoids. The S_1/ICT state of peridinin in PCP gains significant CT character, and the pathway through the S_1/ICT becomes dominant. This behavior is mirrored in light-harvesting complexes from the FCP family utilizing carbonyl carotenoids. The dominant S_1/ICT pathway in one carbonyl carotenoid having a substantial CT character of its S_1/ICT state was also reported for both FCP [32–35] and a chlorophyll-*a*–chlorophyll-c_2–peridinin protein complex (acpPC) of *Amphidinium carterae* [36–38]. Yet, it was found that in the complexes from the FCP family there are also peridinins or fucoxanthins with less CT character of the S_1/ICT state and that these carotenoids are only marginally (if at all) involved in energy transfer via the S_1/ICT state [34,37,38]. The importance of the S_1/ICT state is also manifested by comparison of FCP and acpPC with XLH, another complex from the FCP family. XLH does not bind any carbonyl carotenoid; the overall efficiency of carotenoid-to-Chl-*a* transfer is markedly diminished compared to FCP and acpPC [15].

Here we show time-resolved study of yet another light-harvesting complex from the FCP family, CLH, which comes from an evolutionarily old organism. Even though the exact molecular structure of the carotenoid in CLH remains unknown, the aim of this study was to compare spectroscopic properties of CLH with those reported earlier for other complexes from the FCP family and to test whether the light-harvesting strategy of utilizing the S_1/ICT state with enhanced CT character is employed also by *C. velia*, a photosynthetic relative of apicomplexan parasites.

2. Materials and methods

The cells of *C. velia* were grown in 5 l Erlenmeyer flasks at 28 °C in modified f2 medium and bubbled with filtered air. The irradiance was 100 μmol photons m^{-2} s^{-1}, provided by a metal halide lamp (Osram Powerstar HQI 250 W/D PRO, Osram GmbH, Germany). The light regime was 15 hour light–9 hour dark. The antenna complex isolation was performed as described earlier [6,8]. Briefly, thylakoid membranes solubilized by β-dodecyl maltoside were pre-purified on sucrose gradient, the zone containing antenna complexes was collected and further purified by anion exchange chromatography on DEAE Sepharose CL-6B (Sigma-Aldrich, St. Louis, MO, USA) using a linear NaCl gradient, to eliminate minor antenna components [8]. Fractions with highest Ifx-l:Chl *a* ratio (estimated as A_{540}/A_{674}) were pooled, desalted and stored at −80 °C. As described earlier [8], certain amount of violaxanthin was eluted in the protein-free fraction during the chromatographic step, indicating its loose binding to protein complexes.

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. Optical density ~0.6/mm at 677 nm for femtosecond transient absorption experiments and 0.07/cm at 677 nm for fluorescence measurements were used.

Transient absorption spectra were measured at room temperature using a femtosecond spectrometer employing Ti:sapphire amplifier (Integra-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 470 to 720 nm by detecting the dispersed white light by double-diode array after excitation with ~130 fs laser pulses centered at the desired excitation wavelength. Using neutral-density filters, the intensity of excitation in all experiments was kept at $\sim 5.0 \times 10^{13}$ photons $pulse^{-1}$ cm^{-2}.

Femtosecond transient absorption data collected by diode array detectors were fitted globally using either DAFit software (Pascher Instruments) or Glotaran package (glotaran.org). Both software packages fit the data to a sum of exponentials, including numerical deconvolution of the response function, and a polynomial describing the chirp. The fitting procedure used either general linear regression (DAFit) or single-value decomposition (Glotaran) to fit the data. To visualize the excited-state dynamics, we assumed that the excited CLH complex evolves according to a sequential, irreversible scheme A → B, B → C, C → D… The arrows represent increasingly slower monoexponential processes and the time constants of these processes correspond to lifetimes of the individual excited-state species A, B, C, D… in the sequential scheme. The spectral profile of each species is called evolution-associated difference spectrum (EADS). Although EADS obtained from the sequential model do not correspond to individual excited states in a complex system such as the CLH complex studied here, they help to visualize excited-state processes and carry important information about excited-state dynamics [39].

3. Results

3.1. Steady-state spectroscopy

Absorption, fluorescence, and fluorescence excitation spectra of CLH complex are shown in Fig. 1. The absorption spectrum has a shape typical for an antenna from the FCP family, consisting of sharp Soret and Chl *a* bands of Chl *a* and broad carotenoid absorption in the 450–570 nm spectral region. Closer inspection of the CLH absorption spectrum, however, reveals certain differences from other complexes. While there are no significant differences between the antenna complexes in the Soret region, the Q_y band of Chl *a* in the CLH complex peaks at 677 nm, which is red-shifted from other members of the FCP family: 670 nm for the FCP complex from *Cyclotella meneghiniana* [32–34], 672 nm for the acpPC complex from *A. carterae* [36], and 675 nm for the XLH complex from *Xanthonema debile* [15]. Carotenoid absorption in the CLH complex is rather featureless and extending even beyond 550 nm, which is often observed for the light-harvesting complexes containing carbonyl carotenoids [24–38].

The CLH complex, when excited at 435 nm, exhibits characteristic Chl *a* fluorescence band with maximum at 683 nm. This again is red-shifted from the Chl *a* emission reported for other complexes from the FCP family, reflecting the red-shift observed in the absorption spectra. The fluorescence excitation spectrum, detected at the emission maximum, shows that carotenoids contribute significantly to the emission, and represents clear evidence of energy transfer from carotenoids to Chl *a*. Based on the comparison of absorption (1-T) and fluorescence excitation spectra, the overall efficiency of carotenoid–Chl *a* energy transfer is close to 100% in the 500–550 nm region, and it drops slightly to values 82–85% in the 450–480 nm range. The decrease of the efficiency in the blue part of the carotenoid absorption band is likely due to violaxanthin, which is expected to absorb in this region. Comparing the carotenoid–Chl *a* energy transfer efficiency with other antenna complexes from the FCP family, the CLH complex is clearly the most efficient.

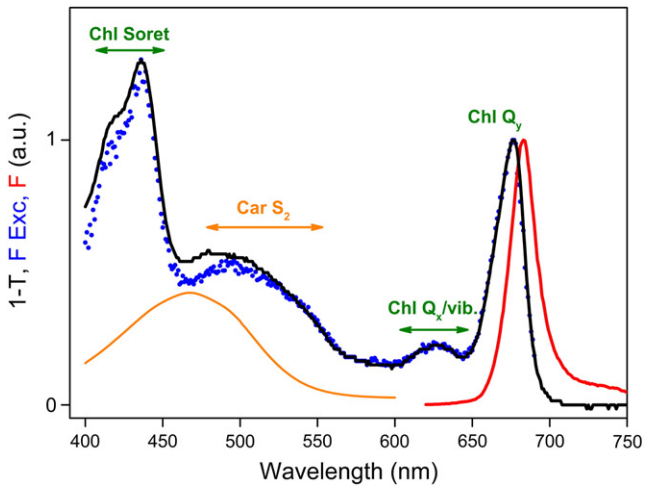

Fig. 1. Absorption (black), fluorescence (red) and fluorescence excitation (blue) spectra of CLH complex. The absorption spectrum is shown as 1-T (T is transmission) spectrum to allow direct comparison with fluorescence excitation spectrum. Absorption spectrum of Ifx-l in acetonitrile is also shown (orange). All spectra are normalized to maximum of Q_y band of Chl *a*. Fluorescence spectrum was obtained after excitation at 435 nm, detection wavelength for fluorescence excitation spectrum was 685 nm.

The spectral dependence of the energy transfer efficiency provided a basis for our choice of excitation wavelengths in transient absorption experiment. We have excited the CLH complex into the red edge of the absorption spectrum, at 540 nm, where the efficiency is largest, and we should selectively excite the lowest vibrational band of the S_0–S_2 transition of the Ifx-l that was identified by circular dichroism spectroscopy [6]. For the second excitation wavelength we have chosen 480 nm where the absorption ratio of violaxanthin/Ifx-l molecules should be most favorable for violaxanthin. Thus, after 480 nm excitation we should be able to track excited-state dynamics of violaxanthin.

3.2. Transient absorption spectra

Transient absorption spectra measured after excitation at 540 nm are shown in Fig. 2a. Already, 100 fs after excitation, the transient absorption spectrum contains a clear signal from the Chl *a* bleaching centered at 677 nm. Since there is essentially no Chl *a* absorption at 540 nm, the population of Chl *a* must be achieved via energy transfer from carotenoid. Also, excited-state absorption around 600 nm indicates that the S_1/ICT state of the carotenoid is already partly populated for such a short delay after excitation. This observation implies that the S_2 state of the carotenoid excited at 540 nm is swiftly depopulated, both via energy transfer to Chl *a* and internal conversion to the S_1/ICT state. At 0.5 ps, the Chl *a* bleaching further increases. At longer delays, both the carotenoid ground state bleaching and S_1/ICT excited-state absorption decrease with a concomitant rise of the Chl *a* signal, a process associated with another energy transfer channel from the S_1/ICT state to the Q_y state of Chl *a*. The spectrum measured at 20 ps delay has no carotenoid signal and is purely due to Chl *a* that is populated via energy transfer from carotenoid.

In order to quantitatively describe the dynamics of CLH after excitation at 540 nm, Fig. 2b shows the EADS extracted from global fitting the data. To obtain reasonable fits, three time components were needed. The first EADS is due to the excited S_2 state of Ifx-l and decays in less than 100 fs to the second EADS whose shape is characteristic of the S_1/ICT state of the carotenoid. In addition, a clear Chl *a* bleaching signal indicates a fast S_2-mediated energy transfer pathway. The second EADS decays in 530 fs. It is characterized by decay of the S_1/ICT excited-state absorption, which peaks at 595 nm, decay of the carotenoid ground state bleaching, and rise of the Chl *a* bleaching at 677 nm. The third EADS, decaying in 4.2 ps, is almost identical in shape to the second EADS, but has smaller amplitude. This EADS is therefore associated with another energy transfer channel via the S_1/ICT state. However, the vibrational structure of the carotenoid bleaching is markedly less resolved in the 4.2 ps EADS which is likely due to a different carotenoid pool associated with this energy transfer pathway. The final EADS that does not decay within the time window of the experiment is due to Chl *a*.

Transient absorption spectra measured after excitation at 480 nm are depicted in Fig. 3a. As for the 540 nm excitation, already at 100 fs after excitation, at 480 nm we see significant Chl *a* bleaching and S_1/ICT excited-state absorption, thus the fast S_2 channel is active also after excitation of the carotenoids absorbing in the blue part of the carotenoid absorption

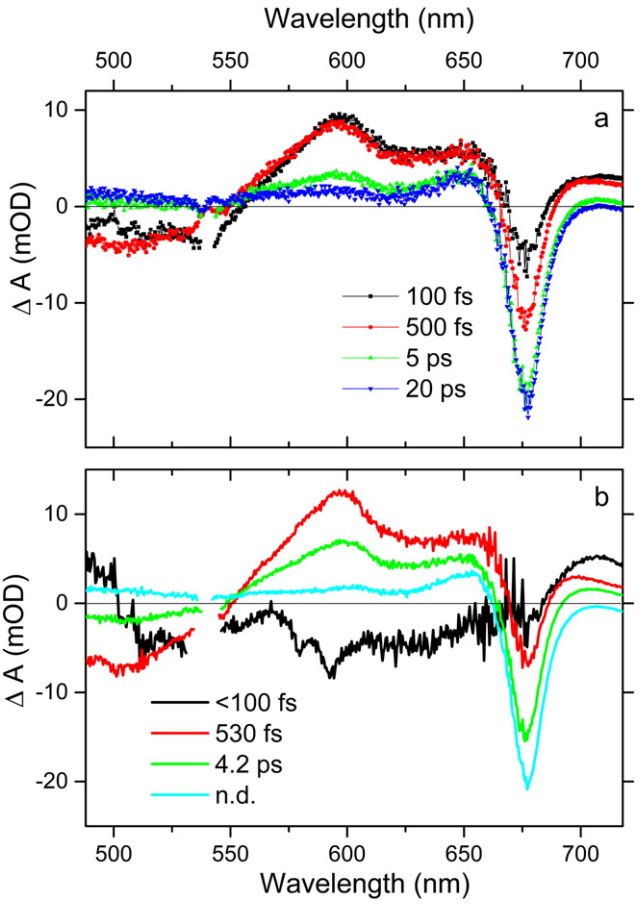

Fig. 2. (a) Transient absorption spectra of CLH complex measured after different time delay after excitation at 540 nm. (b) EADS extracted from global fitting. n.d. — non-decaying component.

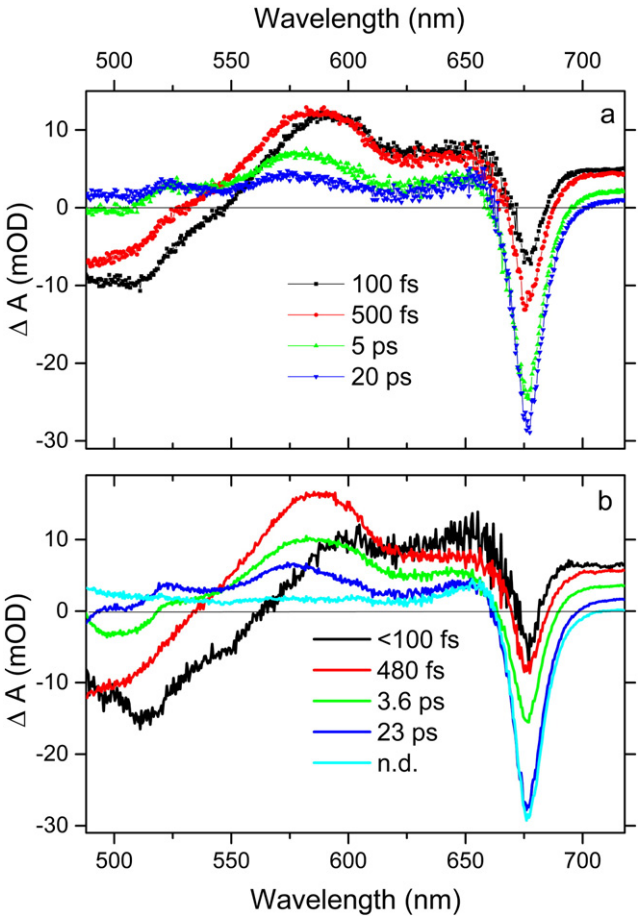

Fig. 3. (a) Transient absorption spectra of CLH complex measured after different time delay after excitation at 480 nm. (b) EADS extracted from global fitting. n.d. — non-decaying component.

in CLH. Further dynamics is comparable to that after 540 nm excitation with exception to longer delays. Energy transfer is evidenced by comparison of transient absorption spectra measured at 0.5 and 5 ps. The part of the signal associated with carotenoid (ground state bleaching below 540 nm and excited-state absorption in the 550–650 nm region) decays while the Chl *a* signal at 677 nm gains amplitude. Major differences between 540 and 480 nm excitations occur at longer delays; the transient absorption spectrum taken at 20 ps after excitation at 480 nm contains weak positive bands at 520 and 575 nm that are not detected in the experiment using the 540 nm excitation. These two bands are most likely associated with 'slow' Ifx-l (575 nm) and violaxanthin (520 nm).

The results of fitting the data globally for excitation at 480 nm are shown in Fig. 3b. Due to the differences at longer delays, one extra component is needed to fit the data measured after 480 nm excitation. In contrast to 540 nm excitation, the first EADS contains some Chl *a* bleaching indicating that the S_2 pathway is faster than for the 540 nm excitation as energy transfer from the S_2 state of carotenoids excited at 480 nm occurs essentially within the excitation pulse. We again extract two energy transfer channels via the S_1/ICT state. These two channels are associated with the second and third EADS which have very similar shape and time constants of 480 fs and 3.6 ps, respectively. These two energy transfer pathways are comparable to those found after 540 nm excitation. It must be noted, however, that the maximum of the S_1/ICT signal in the second and third EADS is at 585 nm and is thus blue-shifted by about 10 nm as compared to the S_1/ICT signal observed after 540 nm excitation (Fig. 2b). This clearly shows that even though the dynamics of the two energy transfer channels are similar for both excitations, different carotenoids associated with these two channels are excited at 480 and 540 nm. The fourth EADS has a lifetime of 23 ps and is obviously unrelated to any dynamics in the Chl *a* region. Instead, this EADS is associated with decay of the two bands located at 520 and 575 nm that should be therefore associated with S_1 bands of carotenoids in CLH that do not transfer energy to Chl *a*. The non-decaying component is due to Chl *a* which does not exhibit any decay within the time window of the experiment.

Fig. 4 compares kinetics measured at the maximum of Chl *a* bleaching after excitation at 480 and 540 nm. It is obvious that energy transfer dynamics, monitored as arrival of excitations to Chl *a* molecule in CLH, do not differ much for the two excitation wavelengths. The fast and slow channels are clearly distinguished in the kinetics and the major difference between 480 and 540 nm is the ratio between the fast and slow channels. The slow channel dominates after 480 nm excitation while the fast channel becomes more important when CLH is excited into the red edge of the carotenoid absorption at 540 nm.

For proper assessment of energy transfer dynamics we need to know the excited-state properties of the isolated carotenoids that are bound to CLH. While the spectroscopic properties of violaxanthin are well known from previous reports [40,41], Ifx-l has never been studied by time-resolved spectroscopy, and the only spectroscopic property known for Ifx-l is the absorption spectrum [16]. Since Ifx-l has a conjugated carbonyl group and its molecular structure is related to that of fucoxanthin [16], some dependence of its excited-state properties on polarity can be expected. To test whether Ifx-l exhibits polarity-dependent behavior we measured the transient absorption spectra and kinetics in the non-polar *n*-hexane and polar acetonitrile. The results are shown in Fig. 5.

The transient absorption spectrum in *n*-hexane taken at 1 ps after excitation at 510 nm is dominated by a single S_1–S_n band peaking at

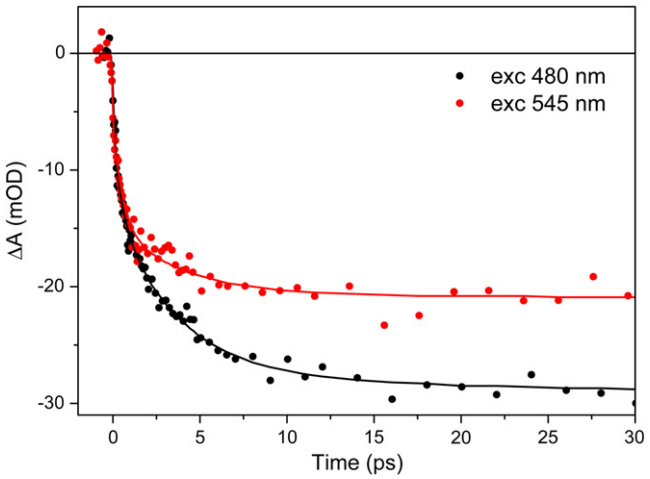

Fig. 4. Comparison of rise of the Chl *a* signal after excitation of carotenoid at 480 nm (black) and 545 nm (red). Detection wavelength is 680 nm.

555 nm with a distinct shoulder at 520 nm that is reminiscent of the S* state [42,43]. When dissolved in polar acetonitrile, the transient absorption spectrum at 1 ps exhibits significant changes. The S_1–S_n band shifts to 565 nm and the S*-like shoulder disappears. A new spectral band centered at 650 nm appears exclusively in acetonitrile. This band, whose magnitude is comparable to that of the S_1–S_n transition, is known from previous reports on other carbonyl carotenoids [17,18]. It is due to ICT–S_N transition, and it is a marker of significant charge transfer character of the S_1/ICT state. The intensity ratio of the ICT-like and S_1-like transitions is often taken as the measure of the CT character [20]. For Ifx-l this ratio is ~1, larger than for fucoxanthin, for which this ratio is ~0.78 in acetonitrile [18]. Because Ifx-l must have a longer conjugation than fucoxanthin (Fig. S1), this is a surprising observation as it is known that the charge transfer character of the S_1/ICT state usually decreases with increasing conjugation length [17,18,44].

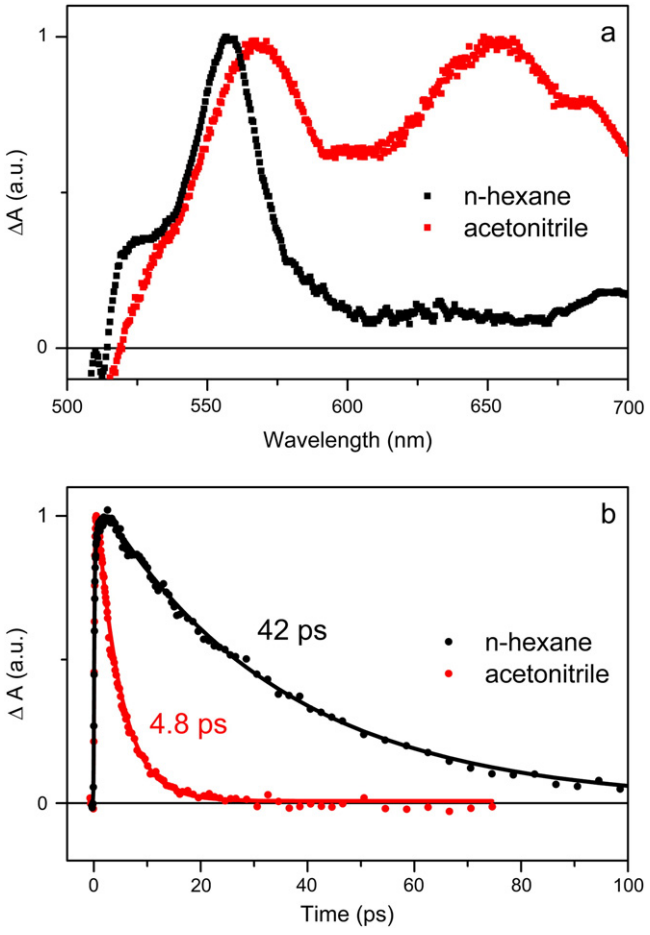

Fig. 5. (a) Transient absorption spectra of Ifx-l measured at 1 ps delay in *n*-hexane (black) and acetonitrile (red). (b) Kinetics (symbols) and fits (lines) of Ifx-l in *n*-hexane (black) and acetonitrile (red). Probing wavelengths were 555 nm (*n*-hexane) and 565 nm (acetonitrile). All data are normalized to maximum. Excitation at 510 nm.

The considerable polarity-dependent behavior of Ifx-l is further evidenced by the markedly different lifetimes of the S_1/ICT state in *n*-hexane and acetonitrile (Fig. 5b). The lifetime decreases by an order of magnitude from 42 ps in *n*-hexane to 4.8 ps in acetonitrile. This is again a much larger change than for shorter fucoxanthin whose S_1/ICT lifetimes are 60 ps in *n*-hexane and 30 ps in acetonitrile [18]. Thus, although the exact molecular structure of Ifx-l remains unknown, its polarity dependent behavior is unexpectedly strong. This fact has important consequences for the light-harvesting function of Ifx-l in CLH.

4. Discussion

The overall energy transfer efficiency between carotenoids and Chl *a* in CLH is very high as evidenced by the fluorescence excitation spectrum shown in Fig. 1. It reaches nearly 100% in the 500–560 nm spectral region, and it exceeds efficiencies reported for other light-harvesting complexes from the family of FCP-like proteins [10,15,32–35]. Yet, all FCP-like proteins do better than LHCII from higher plants, an antenna protein exhibiting close homology with FCP-like antenna proteins [9,11]. Thus, it is obvious that diatoms, dinoflagellates, and other photosynthetic microorganisms utilize specific light-harvesting strategies to maximize the light-harvesting capacity of carotenoids. This is clearly achieved by employing carbonyl carotenoids, because XLH, an antenna protein from the FCP family that does not utilize carbonyl carotenoids, has the lowest efficiency of carotenoid-to-Chl-*a* energy transfer from the FCP-like antenna complexes studied so far [15]. This underscores the advantage of carbonyl carotenoids whose excited-state properties may be tuned by environment [17,18,45], which in turn can tune efficiency of energy transfer as demonstrated in synthetic peridinin- and fucoxanthin-pyropheophorbide dyads [21,46]. Since there is still some variability between complexes utilizing peridinin (acpPC, [36–38]), fucoxanthin (FCP, [32–35]), Ifx-l (CLH) or siphonaxanthin (SPC, [47]), it means that each of these organisms employ slightly different tools to achieve large efficiency of carotenoid-to-Chl-*a* energy transfer.

In CLH, the spectral region with highest energy transfer efficiency is covered by Ifx-l, a carotenoid with unknown molecular structure, which must be related to isofucoxanthin (Fig. S1). The other carotenoid in CLH, violaxanthin, has a relatively short conjugation ($N = 9$) and lacks the conjugated carbonyl group (Fig. S1). This molecular structure prevents violaxanthin from extending its S_0–S_2 transition beyond 520 nm. Moreover, violaxanthin is loosely bound to CLH as some violaxanthin can be removed during purification [8]. The violaxanthin content in CLH also varies with growing conditions, and a recent report indicates that it has likely a photoprotective role in CLH [8]. Thus, the key light-harvesting carotenoid in CLH is Ifx-l.

As for other light-harvesting complexes from the FCP family, also in CLH there are three distinct channels that are utilized to transfer energy from carotenoid to Chl *a*. First, there is the ultrafast S_2-mediated pathway that is observed in essentially all photosynthetic antenna studied so far by means of ultrafast methods [2]. The rate constant cannot be resolved in our experiment due to limited time resolution (~100 fs). The first EADS corresponding to the initially excited species in CLH contains significant Chl *a* signal. This initial Chl *a* bleaching constitutes ~50% and ~30% of total Chl *a* signal after 540 and 480 nm excitations, respectively (Figs. 2b and 3b). Such a large signal cannot be explained by direct excitation of Chl *a*, because, especially at 540 nm, Chl *a* has virtually zero absorption [48]. Thus, regardless of the excitation wavelength, a significant fraction of Chl *a* is populated via energy transfer from Ifx-l within the time duration of our excitation pulse, most likely on a time

scale significantly shorter than 100 fs. For the 480 nm excitation, involvement of violaxanthin in this ultrafast S_2-mediated channel cannot be excluded; thus, both carotenoids can in principle serve as energy donors in this channel.

The other two energy transfer channels proceed via the S_1/ICT state. For both excitation wavelengths we observe nearly identical time constants of ~0.5 ps for the fast channel and ~4 ps for the slow channel, but the ratio between the two channels depends on excitation wavelength. While for the 540 nm excitation both channels contribute nearly equally, when carotenoids are excited at 480 nm the slow channel dominates. Even though the energy transfer rates are essentially the same for both excitation wavelengths, different excitation wavelengths must excite different Ifx-l molecules in CLH. Comparison of EADS corresponding to the fast and slow channels (Fig. 6) reveals the spectral inhomogeneity in the Ifx-l pool. As for FCP [33] or acpPC [36–38], there are at least two spectrally distinct Ifx-l molecules having S_1/ICT-S_n peaks at 585 and 595 nm, respectively. These two Ifx-l pools are selectively excited at 480 and 540 nm. Whereas different Ifx-l molecules can be readily assigned to different excitation wavelengths, the Ifx-l molecules involved in the fast and slow channel after the same excitation are spectrally very similar; the EADS corresponding to the fast and slow channels for each excitation wavelength have the same maxima (Figs. 2b and 3b).

The data recorded after excitation at 480 nm also revealed spectral bands associated with carotenoids that do not transfer energy to Chl *a*. The fourth EADS in Fig. 3b has a lifetime of 23 ps and contains distinct excited-state absorption bands centered at 520 nm and 575 nm. The weak band at 520 nm can be assigned to violaxanthin. Comparable S_1–S_n maxima were observed for either reconstituted or mutated LHCII containing predominantly violaxanthin [49,50], and even the lifetime of 23 ps matches the lifetime of violaxanthin in solution [40]. The other band at 575 nm is too red-shifted to be associated with violaxanthin and must therefore be due to another spectral form of Ifx-l that does not transfer energy to Chl *a*. Thus, there must be at least three spectral forms of Ifx-l that have their S_1/ICT maxima at 575, 585 and 595 nm. The first one is exclusively excited at 480 nm and does not transfer energy to Chl *a*, the second one is also excited only at 480 nm but transfer energy from the S_1/ICT state via both fast and slow channels, and the third one is exclusively excited at 540 nm and again is active in both fast and slow energy transfers via the S_1/ICT state. Such spectral inhomogeneity is also known from another member of the FCP family, the FCP complex from diatom *C. meneghiniana*, for which also three distinct fucoxanthin pools were identified [51]. Simplified scheme of major energy transfer pathways between carotenoids and Chl *a* in the CLH complex is depicted in Fig. 8.

The presence of a non-transferring Ifx-l in CLH provides information about intrinsic S_1/ICT lifetime of Ifx-l. Assuming that all Ifx-l molecules in CLH have approximately the same intrinsic lifetime, the S_1/ICT lifetime of the non-transferring Ifx-l (23 ps) can serve as a basis for calculation of energy transfer efficiency. Taking into account the 0.5 and 4 ps lifetimes of the S_1/ICT state that transfers energy to Chl *a* via a fast and a slow channel, the estimated energy transfer efficiency is 98% and 83% for the fast and slow channels, respectively. For the 540 nm excitation, where fast and slow channels are about equally weighted, this gives the average efficiency of the S_1/ICT pathway over 90%, which together with a significant fraction (~50%) going via the S_2 route is the reason for the highly efficient energy transfer in the 500–560 nm spectral region evidenced by fluorescence excitation spectra (Fig. 1). In the blue spectral region the overall efficiency is lower due to a few reasons. First, the S_2 route is less efficient as evidenced by the smaller fraction of the Chl *a* signal in the first EADS that may account only for up to ~30% of total energy transfer (Fig. 3b). Second, the slow S_1/ICT channel dominates after 480 nm excitation (Fig. 4), decreasing the average efficiency of this pathway compared to the 540 nm excitation. Finally, in the blue part of the carotenoid absorption in CLH there are at least two carotenoids that do not transfer energy via the S_1/ICT route, violaxanthin having the S_1–S_n transition at 520 nm and Ifx-l with S_1/ICT maximum at 575 nm (Fig. 3b).

The question is how the Ifx-l molecules in CLH achieve such efficiency. It is assumed that the charge transfer character of the S_1/ICT state increases the dipole moment of the S_1/ICT state thereby facilitating the coupling to Chl *a* resulting in enhanced efficiency of the S_1/ICT route [24]. Indeed, a number of experiments exploring the excited-state

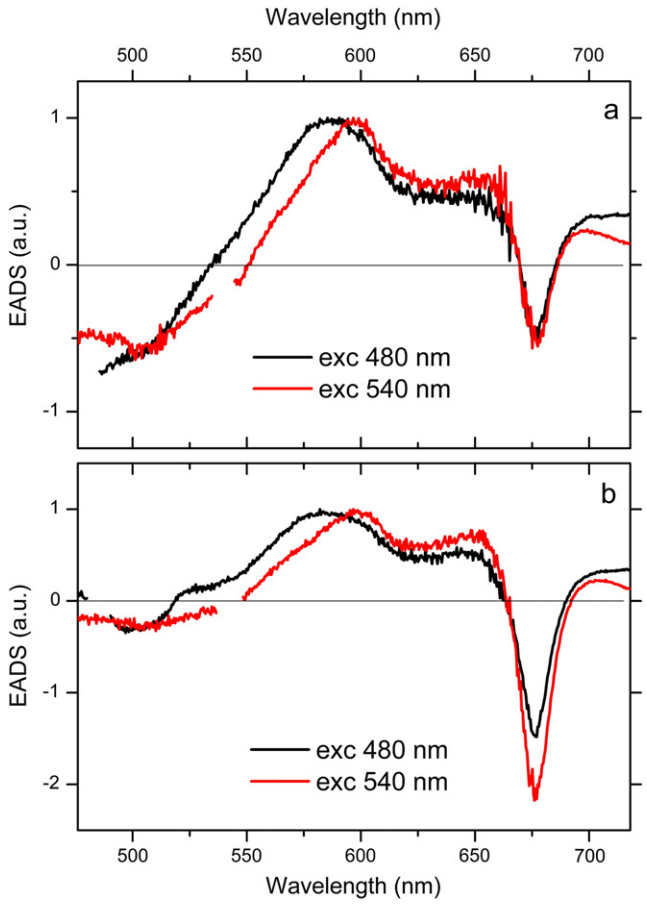

Fig. 6. EADS corresponding to the fast (a) and slow (b) energy transfer channels. EADS were obtained from fitting the data measured after excitation at 480 nm (black) and 540 nm (red). All EADS are normalized to maximum.

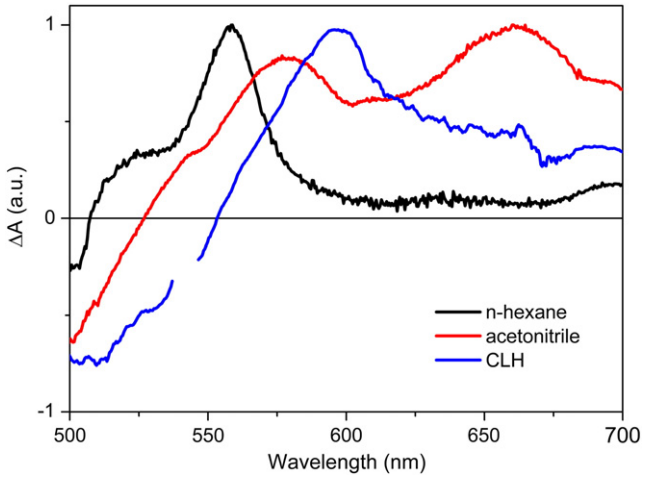

Fig. 7. Comparison of EADS corresponding to the S_1/ICT lifetime of Ifx-l in *n*-hexane (black), acetonitrile (red) and in CLH (blue). For Ifx-l in CLH, the EADS is the one associated with the fast energy transfer channel (0.53 ps) after 540 nm excitation. The Chl *a* contribution was removed by subtracting the scaled non-decaying EADS that corresponds solely to Chl *a*.

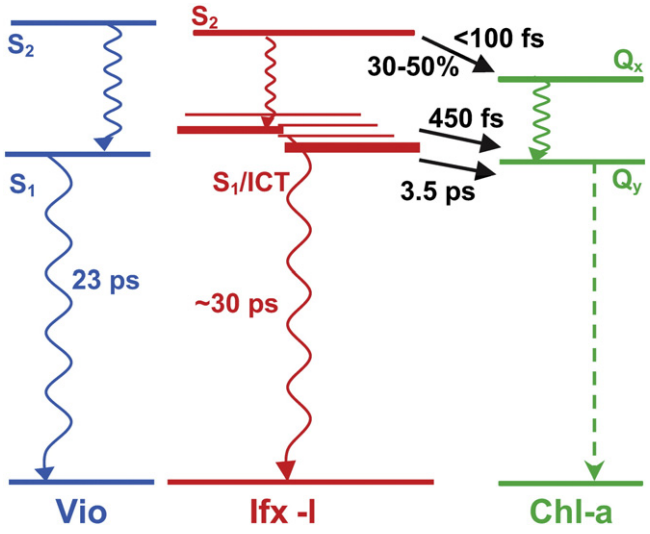

Fig. 8. Scheme of energy transfer pathways between carotenoids and Chl *a* in CLH complex. The pathways are denoted as black solid arrows labeled by corresponding time constants. Wavy arrows correspond to internal conversion processes, dashed arrow denotes Chl *a* fluorescence. See text for details.

dynamics of peridinin and fucoxanthin in PCP, acpPC, and FCP support this hypothesis as in all these complexes, both peridinin and fucoxanthin exhibit behavior similar to that in polar solvents. The ICT-like band in transient absorption spectra, whose magnitude scales with increasing degree of charge transfer character of the S_1/ICT state, is observed in all these complexes [24,32–36]. One may thus expect to see the same behavior for Ifx-l too. To test this, Fig. 7 compares EADS associated with the S_1/ICT lifetime of Ifx-l in *n*-hexane, acetonitrile and CLH. For Ifx-l in CLH, the EADS of the slow S_1/ICT channel is chosen, and, to allow for direct comparison with spectral properties in solution, the Chl *a* contribution in CLH is subtracted. It is apparent that EADS of Ifx-l in CLH do not contain any significant ICT bands, suggesting that the S_1/ICT state of Ifx-l in CLH has only weak charge transfer character and its spectroscopic properties are close to those in non-polar solvent. This is in sharp contrast with other complexes containing carbonyl carotenoids, such as PCP [24], FCP [34,35] or acpPC [36].

The reason for this behavior can be traced to the specific excited-state properties of Ifx-l. While fucoxanthin always has even in the most polar environment the S_1/ICT lifetime always longer than 15 ps [17,18,52], if the S_1/ICT state of Ifx-l possesses a significant charge transfer character (as in acetonitrile, see Fig. 5) its lifetime can be as short as 4.2 ps. With such short intrinsic S_1/ICT lifetime the energy transfer channel could hardly compete with the S_1/ICT-S_0 relaxation. It seems that the intrinsic S_1/ICT lifetime in the range of 15–20 ps is ideal for proper balancing of the energy transfer rate and S_1/ICT-S_0 relaxation. In PCP for example, the intrinsic S_1/ICT lifetime of peridinin was estimated to be 16 ps [24], and that is also the limit that could be theoretically achieved for fucoxanthin if it resides in polar environment. Thus, to maintain high efficiency, the CLH complex prevents the S_1/ICT state of Ifx-l from gaining a significant charge transfer character. Comparing the 23 ps S_1/ICT lifetime of the non-transferring Ifx-l in CLH with that in *n*-hexane (42 ps), we may conclude that Ifx-l resides in CLH in a weakly polar environment. This is consistent with the EADS shown in Fig. 7 as the Ifx-l in CLH clearly has more signal in the 630–700 nm region than Ifx-l in *n*-hexane, indicating weak charge transfer character of the S_1/ICT state of Ifx-l in CLH.

We conclude that the CLH complex is a light-harvesting system utilizing yet another light-harvesting strategy to achieve high efficiency of carotenoid-to-Chl-*a* energy transfer. Since *C. velia* is a symbiont of corals thriving in shallow waters, harvesting the photons in the 500–560 nm spectral region is of high importance [53]. To extend the absorption into the green-yellow region, CLH complex binds predominantly the carbonyl carotenoid Ifx-l, because, regardless of polarity, the conjugated carbonyl group shrinks the S_2-S_1/ICT gap, resulting in shifting the S_2 state to the 500–560 nm region while keeping the S_1/ICT state high enough to transfer energy to Q_y of Chl *a* [18]. However, contrary to other light-harvesting complexes utilizing carbonyl carotenoids, CLH suppresses the charge transfer character of the S_1/ICT state to keep the high efficiency of energy transfer.

It is interesting to compare the light-harvesting properties of CLH with other complexes from the FCP family and also with PCP, the most studied antenna complex utilizing carbonyl carotenoid. First of all, the fast and slow S_1/ICT channels are present in all complexes, yet the contribution of these two channels varies significantly among the complexes. In CLH, the fast and slow channels contribute nearly equally to the S_1/ICT route, and a comparable contribution of the fast channel was reported for acpPC complex [36]. It should be noted, though, that the fast channel in acpPC was attributed to a transfer from hot S_1/ICT state which is not the case for CLH. In FCP the contribution of the fast channel is less [32–35], and in PCP the fast channel is negligible, and essentially all transfer via the S_1/ICT route in PCP proceeds with ~3 ps time constant [24,28,30]. All complexes also contain non-transferring carotenoids with the S_1/ICT lifetime in the 15–30 ps range [28,32–36].

The most intriguing observation is the ubiquitous presence of the energy transfer channel characterized by the 2.5–4 ps time constant. This channel is present (and in most cases it is also dominant) in all complexes with Chl *a* acceptors even though they have completely different carotenoid composition and a completely different structure as in the case of PCP. A 3.5 ps energy transfer component was also found in the siphonaxanthin chlorophyll protein (SCP) from *Codium fragile* [47]. In this complex, the final assignment of the 3.5 ps component is complicated by the fact that this complex contains also Chl *b*, and it was studied only by time-resolved fluorescence spectroscopy. Thus, the 3.5 ps component could also be due to Chl *b*-to-Chl *a* transfer as suggested by Akimoto et al. [47]. Moreover, even the XLH complex belongs to the FCP family but has no carbonyl carotenoids, yet it has the S_1 route active and characterized by a 3.5 ps time constant [15]. These observations suggest that despite different structures and different carotenoid composition, time constants of the major energy transfer channel via the S_1/ICT route converge to a 2.5–4 ps value, providing the Q_y band of Chl *a* is the acceptor. The role of the acceptor is likely crucial in this convergence as changing the acceptor molecule by reconstitution of PCP complex with different chlorophylls significantly alters this rate constant [30].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.06.001.

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