Molecular Origin of Photoprotection in Cyanobacteria Probed by Watermarked Femtosecond Stimulated Raman Spectroscopy

Yusaku Hontani,[†,⊥] Miroslav Kloz,[†,‡,⊥] Tomáš Polívka,[§] Mahendra K. Shukla,[∥] Roman Sobotka,[∥] and John T. M. Kennis*,[†]

[†]Department of Physics and Astronomy, Vrije Universiteit, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands
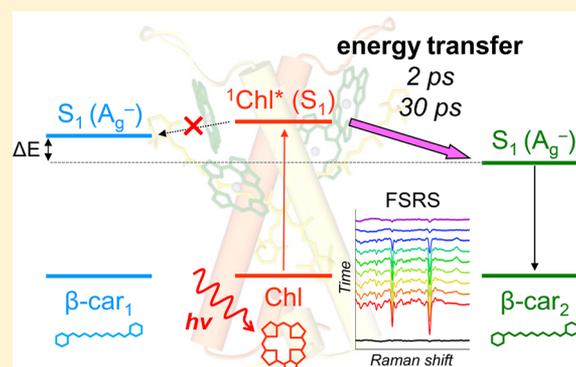[‡]ELI-Beamlines, Institute of Physics, Na Slovance 2, 182 21 Praha 8, Czech Republic
[§]Institute of Physics and Biophysics, Faculty of Science, University of South Bohemia, 370 05 České Budějovice, Czech Republic
[∥]Centre Algatech, Institute of Microbiology, Academy of Sciences of the Czech Republic, Třeboň, 379 81, Czech Republic

S *Supporting Information*

ABSTRACT: Photoprotection is fundamental in photosynthesis to avoid oxidative photodamage upon excess light exposure. Excited chlorophylls (Chl) are quenched by carotenoids, but the precise molecular origin remains controversial. The cyanobacterial HliC protein belongs to the Hlip family ancestral to plant light-harvesting complexes, and binds Chl a and β-carotene in 2:1 ratio. We analyzed HliC by watermarked femtosecond stimulated Raman spectroscopy to follow the time evolution of its vibrational modes. We observed a 2 ps rise of the C=C stretch band of the $2A_g^-$ (S_1) state of β-carotene upon Chl a excitation, demonstrating energy transfer quenching and fast excess-energy dissipation. We detected two distinct β-carotene conformers by the C=C stretch frequency of the $2A_g^-$ (S_1) state, but only the β-carotene whose $2A_g^-$ energy level is significantly lowered and has a lower C=C stretch frequency is involved in quenching. It implies that the low carotenoid S_1 energy that results from specific pigment−protein or pigment−pigment interactions is the key property for creating a dissipative energy channel. We conclude that watermarked femtosecond stimulated Raman spectroscopy constitutes a promising experimental method to assess energy transfer and quenching mechanisms in oxygenic photosynthesis.

Oxygenic photosynthetic organisms need to protect themselves from the consequences of excess sunlight, as the photosynthetic machinery easily gets overloaded even at moderate light intensities. To this end, elaborate photo-protection mechanisms have evolved, collectively known as nonphotochemical quenching (NPQ).[1,2] NPQ involves the active dissipation (quenching) of singlet excited states in the light harvesting antenna before they reach the reaction centers for photochemical conversion, and manifests itself in distinct ways in various oxygenic photosynthetic organisms. In plants and algae, NPQ involves specific interactions between carotenoids and chlorophylls in the light-harvesting complex (LHC) family, where the lifetime of Chl singlet excited states is quenched to hundreds of picoseconds. The mechanism by which this process occurs has been controversially discussed in the literature:[3] energy transfer,[4−8] electron transfer,[9−11] excitonic coupling,[12,13] and Chl−Chl charge transfer interactions[14] have been proposed.

Cyanobacterial photosynthesis is ancestral to that of plants and algae, and although cyanobacteria do not use the plant-like LHC antenna system for light harvesting, they contain so-called high-light inducible proteins (Hlips) that are homologues to first and third helices of p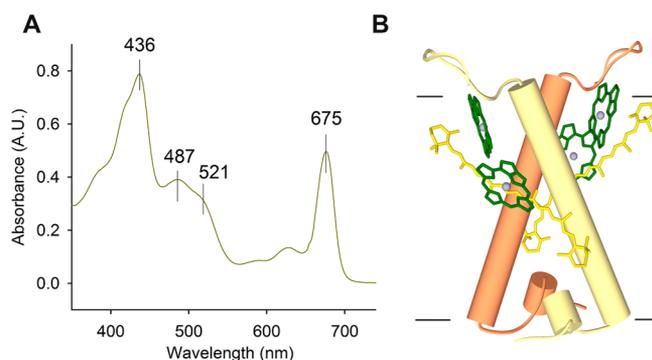lant LHC proteins. Hlips are small single-helix polypeptides (5−7 kDa) ubiquitous in cyanobacteria, which play an important role during assembly and repair of photosystem II, particularly under stress conditions.[15] So far, only two members of the Hlip family, HliC and HliD, have been isolated and biochemically characterized.[5,16] Both these proteins, isolated from the cyanobacterium *Synechocystis* 6803, form oligomers, and bind four Chl a (HliC) or six Chl a (HliD) and 2 β-carotenes per a putative dimer.[5,16]

Figure 1 shows the absorption spectrum and a structural model of HliC.[5] Despite the apparent 2-fold symmetry in the proposed structure, resonance Raman spectroscopy demon-strated that two distinct β-carotene conformers exist in HliC and also in HliD:[17] β-car$_1$ absorbs at higher energy and exhibits a higher C=C stretch frequency at 1525 cm^{-1}, whereas β-car$_2$ absorbs at lower energy and has a lower C=C stretch frequency at 1515 cm^{-1}. Strikingly, ultrafast transient absorption spectroscopy showed that the HliD protein was highly quenched, with dominant Chl a lifetimes of only 2 and 30 ps, and a minor unquenched fraction.[5] Moreover, it was
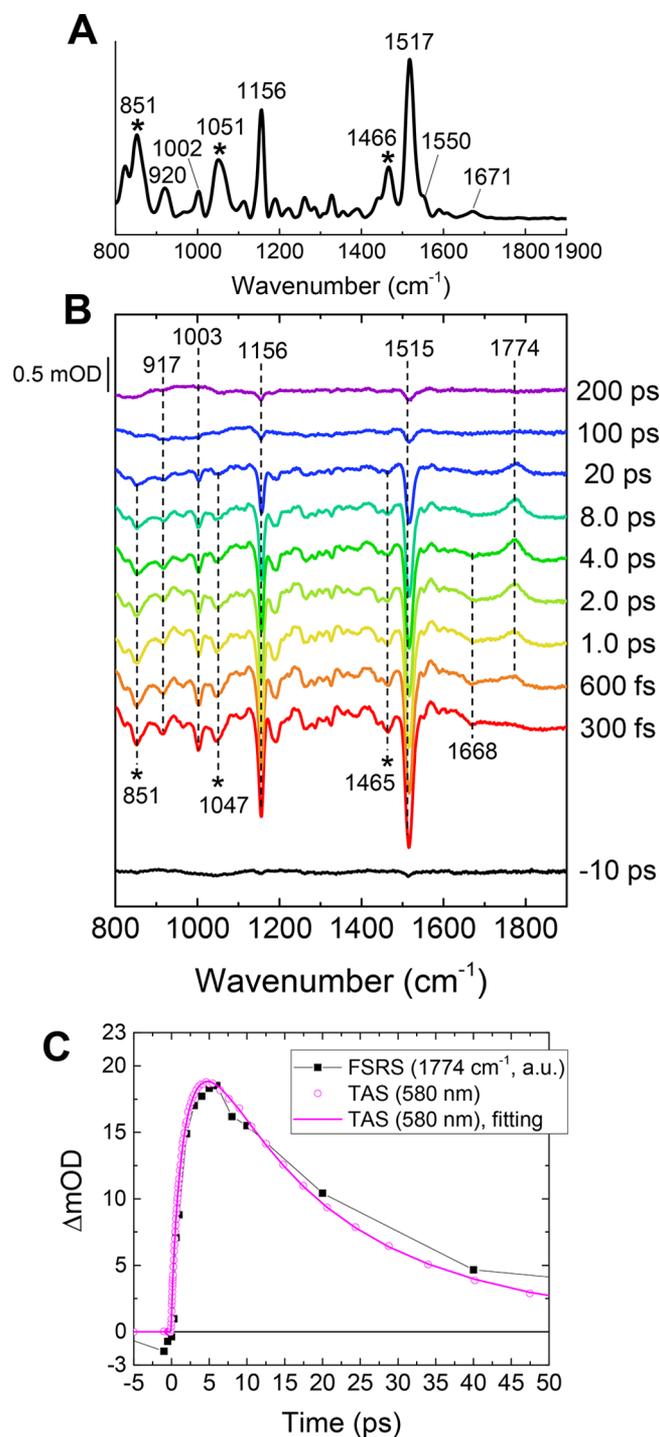
Figure 1. Steady-state absorption and a structural model of HliC. (A) Room-temperature absorbance spectrum of the purified HliC protein. (B) Structural model of the putative HliC dimer depicted as a side view along the membrane plane (modified from ref 16).

shown that the quenching of the Chl *a* excited state proceeded via energy transfer to the optically forbidden S_1 (A_g^-) state of β-carotene.[5] This observation posed an important conundrum: close Chl-carotenoid positioning that is a common motif in light-harvesting proteins is necessary to promote triplet−triplet transfer from Chl to carotenoid upon Chl intersystem crossing. Yet, in most antenna complexes, the Chl singlet excited state is not quenched at all. Hence, unresolved questions remain about the quenching mechanisms in photosynthetic light harvesting complexes with regard to electronic coupling to optically forbidden states and the energetics of the states involved.[18] The latter is especially pressing because the energy level of the optically forbidden S_1 state of carotenoids is largely insensitive to polarity and polarizability of the environment.[19]
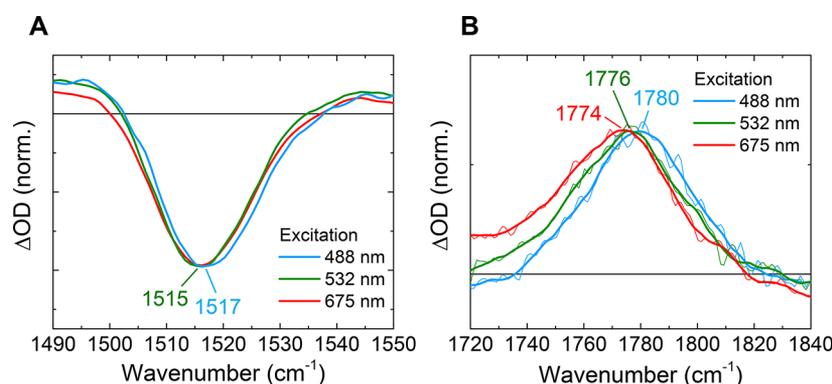
Femtosecond stimulated Raman spectroscopy (FSRS) is a powerful method to gain detailed molecular information through transient vibrational spectra.[20] It features a high temporal resolution of <100 fs, high spectral resolution, and high sensitivity with respect to time-resolved resonance Raman methods. Recently, we have developed a watermarked FSRS method involving shot-to-shot Raman pump wavelength modulation and wavelet transformation to successfully suppress the large and unpredictable baseline fluctuations that have dogged the FSRS method since its inception more than a decade ago.[21−23] Through this method, baseline issues due to nonresonant nonlinear contributions, transient absorption, pump−dump−probe, and pump−repump probe signals are successfully suppressed without any biased human intervention. Watermarked FSRS seems particularly suited to study pigment−protein complexes of oxygenic photosynthesis since its Raman pump at 800 nm is conveniently preresonant with the main pigment absorption bands. In this work, we make use of the ability of FSRS to follow specific molecular vibrations with sub-100 fs time resolution to assess the mechanism, pathways, and energetics of excited-state energy quenching in HliC. To our knowledge, this work represents the first demonstration of energy transfer processes in a photosynthetic light harvesting complex probed with FSRS and lays the groundwork for general application of the watermarked FSRS method in photosynthesis research.

Figure 2A shows the stimulated Raman spectrum of the HliC ground state with preresonant 800 nm pump. The two strongest bands at 1517 and 1156 cm^{-1} are due to the β-carotene C═C (ν_1) and C−C stretches (ν_2), respectively.[16,17] The bands at 1002 and 920 cm^{-1} belong to the ν_3 (methyl in-

Figure 2. FSRS of HliC upon 675 nm excitation. (A) Ground-state Raman spectrum of HliC. (B) Selected time traces of difference spectra of FSRS. Asterisks (*) indicate signals originated from glycerol. (C) Transient absorption kinetic trace at 580 nm (magenta open dots) with a fitting curve (magenta line) overlapped with FSRS data at 1774 cm^{-1} (black closed squares).

plane rocking) and ν_4 (hydrogen-out-of plane rocking) vibrations of β-carotene, respectively.[16,17] It furthermore features a shoulder near 1550 cm^{-1} and a band at 1671 cm^{-1}, which are both due to Chl *a*.[16] The amplitude of the β-carotene bands is much higher than those of Chl *a* even though the 800 nm Raman pump is more preresonant with the Chl *a* Q_y band than the β-carotene S_2 band, which relates to the higher Raman

Figure 3. Comparison of FSRS bands of HliC at 4 ps upon excitation at different wavelengths. (A) The bleaches of the C=C stretch in the ground state and (B) the C=C stretch of the S_1 state of β-carotene. Signals upon excitation at 488, 532, and 675 nm are shown in cyan, green, and red, respectively. In panel B, a 21 point smoothing (over 1 cm^{-1} intervals) was applied (thick lines) with Savitzky−Golay filtering. The thin lines show the watermarked data without smoothing.

cross section of the latter pigment. The bands at 1466, 1051, and 851 cm^{-1} are due to glycerol, which was added to stabilize the sample and are conveniently used as an internal marker for the FSRS experiments. The spectral width of the HliC stimulated Raman bands is approximately 20 cm^{-1}, which is larger than the bandwidth expected from convolution with the spectral width of the Raman pump (~10 cm^{-1}). Hence, the observed bandwidths do not result from the experimental conditions but are inherent to the HliC protein at room temperature.

Figure 2B shows FSRS spectra at selected delays upon Chl a excitation at 675 nm. At very short delays (300 fs), large bleaches of the β-carotene C=C stretch at 1515 cm^{-1} and C−C stretch at 1156 cm^{-1} are observed, as well as a bleach of the Chl a band at 1668 cm^{-1}. Importantly, the glycerol solvent bands at 1465, 1047, and 851 cm^{-1} are bleached as well, which indicates that these are likely spurious signals caused by partial absorption of the 800 nm Raman pump by Chl a excited-state absorption (ESA),[24,25] i.e., an inner filter effect that diminishes the Raman pump intensity and hence results in a lower signal.[26] Thus, the bleaching signals associated with β-carotene stretches at early delay times do not imply population of β-carotene excited states. This was confirmed by a FSRS experiment on a Chl a−β-carotene mixture in organic solvent, where no close interactions exist between the pigments. Here, selective Chl a excitation and population also lead to bleach of Chl a, β-carotene, and solvent modes (Figure S1), confirming the inner filter effect. We did not observe any positive bands that arise from the Chl a excited state, which may seem surprising given the resonance of the Raman pump with the Chl a ESA. However, the Chl a ESA has a rather low amplitude, and the strong electronic transition to the Q_y state at 670 nm, which is preresonant with the Raman pump in the ground state, entirely disappears in the excited state. At the same time, β-carotene has a very high Raman cross section. Apparently, these combined effects result in Chl a excited-state Raman bands that are unobservably weak with respect to those of the β-carotene ground state.

In the ensuing evolution on the picosecond time scale, we observe the rise of a positive band at 1774 cm^{-1}. This band is a unique marker of the optically forbidden S_1 ($2A_g^-$) state of carotenoids, as it represents an upshifted C=C stretch frequency that results from strong vibronic coupling between the S_0 ($1A_g^-$) and S_1 ($2A_g^-$) states.[27] Thus, FSRS data gives direct evidence of energy transfer from the excited Chl a to the

optically forbidden S_1 state of β-carotene in 2 ps, consistent with transient absorption measurements (Figures 2C, S2, and S3) where the β-carotene S_1 state has a prominent absorption at 560 nm. The same quenching pathway has also been earlier reported by ultrafast transient absorption experiments on HliD.[5] The 1774 cm^{-1} band disappears on a time scale of 10 ps, which is assigned to the S_1−S_0 internal conversion (IC) of β-carotene.[19] In addition, a minor slow phase of 30 ps is observed in the β-carotene S_1 decay (1774 cm^{-1}) in transient absorption as well as in stimulated Raman. The Chl a bleach signal mainly decayed in 2.5 and 20 ps (Figure S2). Given that the IC time constant of β-carotene can hardly be longer than 15 ps due to the properties of its conjugated π-electron system,[19] we interpret this to result from a slow 30 ps phase in the energy transfer process from Chl a to β-carotene, which through inverted kinetics (i.e, when a state is populated slower than it is depopulated, it rises with its decay time, and decays with its rise time[7]) shows up as a 30 ps lifetime component of the β-carotene S_1 state. A nondecaying phase in the Chl a transient absorption is assigned to a minor fraction of loosely bound or unbound Chl a (Figure S2B, magenta line).

Given the (low) Chl a ESA around 800 nm, it might be anticipated that Chl a could be promoted to a higher-up excited state due to the combined actions of actinic and Raman pumps. However, the FSRS time evolution closely follows that of the TA experiments (Figures 2C and S3), indicating that such processes, if they occur at all, do not affect the FSRS experiment in any appreciable way.
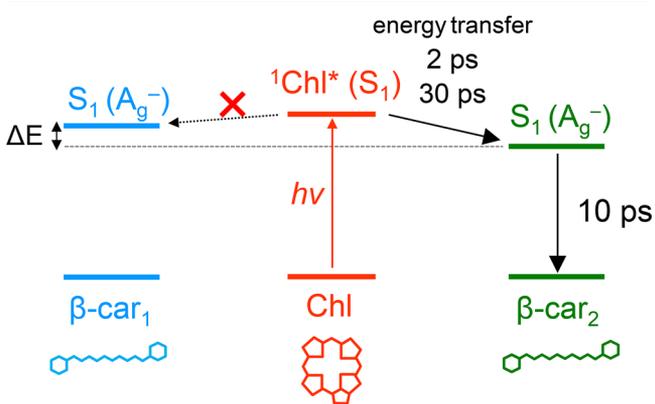
To gain additional information about the energy transfer processes and pathways, we performed FSRS experiments with direct excitation of β-carotene at 488 and 532 nm (Figure S4). Here, the two β-carotene conformers, β-car$_1$ and β-car$_2$, are to a certain extent selectively excited. We observe that, for both data sets upon 488- and 532 nm excitation, the high-frequency β-carotene S_1 marker band around 1775 cm^{-1} rises in about 300 fs and upshifts with approximately the same time constant, which is assigned to ultrafast IC from the optically allowed S_2 state, followed by intramolecular vibrational cooling.[21,28] For both data sets, the β-carotene S_1 marker band decays in ~10 ps. Figure S5 shows the results of transient absorption experiments with 488 and 532 nm excitation.

Although the spectral evolution is very similar between the two data sets, significant spectral differences are observed. Figure 3 shows an overlap of the FSRS spectra at 4 ps in the C=C stretch regions of the S_0 state (panel A) and S_1 state

(panel B) with excitation at 488 nm (cyan), 532 nm (green), and 675 nm (red). Strikingly, in the S_1 state (Figure 3B) a 4 cm^{-1} difference in the band maxima is observed with 488 and 532 nm excitation, which demonstrates that the β-car$_1$ and β-car$_2$ conformers have distinct C=C stretch frequencies in the S_1 state. Note that selectivity is not 100% with either excitation wavelength,[17] so the difference in S_1 state frequency of β-car$_1$ and β-car$_2$ is probably larger than 4 cm^{-1}. Smaller, but observable shifts were observed in the ground state C=C stretch manifested as the S_0 state bleaching signal (Figure 3A). Figure S6 shows overlapped FSRS spectra at various time delays.

If we now compare the FSRS data with Chl *a* excitation at 675 nm (Figure 3B, red) with that at 488 nm excitation (Figure 3B, cyan), we find that the former has a frequency of 1774 cm^{-1}, which is lower by 6 cm^{-1} than the latter (Figure 3B). This observation demonstrates that the β-car$_2$ conformer, and not β-car$_1$ acts as the energy acceptor that quenches the excited Chl *a*. In HliC and HliD, on the basis of the linear relationship between the ground state C=C stretch frequency and effective conjugation length,[29] the conjugation length of β-car$_2$ was estimated to be ~10.5, as opposed to ~9.6 for β-car$_1$,[16] which would result in an up to 800 cm^{-1} (0.1 eV) energy difference between β-car$_1$ and β-car$_2$.[19] The 6 cm^{-1} shift of the C=C stretch frequency in the S_1 state observed here by FSRS corroborates this finding, although the precise relationship between the C=C stretch frequency in the S_1 state and effective conjugation has not been established yet. The S_1 C=C frequency of carotenoids is determined through the combined effects of the effective π-electron conjugation length and the vibronic coupling with the S_0 state.[27] Under the assumption that the vibronic coupling with S_0 is the same for β-car$_1$ and β-car$_2$, this result implies that the S_1 energy of β-car$_2$ indeed is lower than that of β-car$_1$. This finding implies that specific carotenoid–protein interactions induces asymmetry between the β-carotene molecules in HliC, making the β-car$_2$ the quenching site.

Figure 4 summarizes our findings. Application of FSRS allowed to follow the specific vibrational mode in the S_1 state, revealing that the β-carotene S_1 energy level tuning provides a key property in creating dissipative energy transfer pathways in closely confined Chl–carotenoid geometries. The carotenoid S_1 state energy is largely insensitive to environmental polarity and polarizability,[19] which implies that specific pigment–protein or pigment–pigment interactions must be invoked to tune the S_1 energy. One promising avenue is provided by specific in-plane tuning of the β-carotene β-rings, which bring them in conjugation with the π-electron system of the polyene backbone, thereby lowering the overall energy levels of the electronic excited states.[17] The ramifications of these observations are very important indeed, as the same type of carotenoid molecule, β-carotene in this case, may assume a quenching and a nonquenching role in the same Hlip, a mechanism that has long been hypothesized for plant LHCs.[2,4,30] Here, watermarked FSRS has revealed the vibrational signature of the β-carotene quenching state in HliC, and the technique may play an important role in elucidating quenching mechanisms in various types of LHCs that are less clear-cut and harder to assess by traditional means.

◼ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.8b00663.

> Additional experimental details, global fitting analysis, and transient absorption spectra (PDF)

◼ AUTHOR INFORMATION

Corresponding Author
*E-mail: j.t.m.kennis@vu.nl.

ORCID Ⓘ
Tomáš Polívka: 0000-0002-6176-0420
John T. M. Kennis: 0000-0002-3563-2353

Author Contributions
[⊥]Y.H. and M.K. contributed equally.

Notes
The authors declare no competing financial interest.

◼ ACKNOWLEDGMENTS

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Figure 4. Energy transfer model of HliC upon excitation of Chl. After excitation of Chl, excited-state energy transfer occurs specifically to the lower energy β-carotene (β-car$_2$) in 2 and 30 ps. The decay of the S_1 state β-car$_2$ proceeds in 10 ps. The higher-energy β-car$_1$ is not populated because of unfavorable energetics. See text for details.

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