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Charge-Transfer Character of the Low-Energy Chl *a* Q_y Absorption Band in Aggregated Light Harvesting Complexes II

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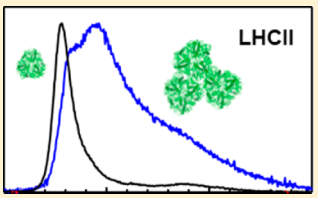
ABSTRACT: One of the key functions of the major light harvesting complex II (LHCII) of higher plants is to protect Photosystem II from photodamage at excessive light conditions in a process called "non-photochemical quenching" (NPQ). Using hole-burning (HB) spectroscopy, we investigated the nature of the low-energy absorption band in aggregated LHCII complexes - which are highly quenched and have been established as a good *in vitro* model for NPQ. Nonresonant holes reveal that the lowest energy state (located near 683.3 nm) is red-shifted by ~4 nm and significantly broader (by a factor of 4) as compared to nonaggregated trimeric LHCII. Resonant holes burned in the low-energy wing of the absorption spectrum (685−710 nm) showed a high electron−phonon (el-ph) coupling strength with a Huang−Rhys factor *S* of 3−4. This finding combined with the very low HB efficiency in the long-wavelength absorption tail is consistent with a dominant charge-transfer (CT) character of the lowest energy transition(s) in aggregated LHCII. The value of *S* decreases at shorter wavelengths (<685 nm), in agreement with previous studies (J. Pieper et al., *J. Phys. Chem. B* **1999**, *103*, 2422−2428), proving that the low-energy excitonic state is strongly mixed with the CT states. Our findings support the mechanistic model in which Chl−Chl CT states formed in aggregated LHCII are intermediates in the efficient excited state quenching process (M. G. Müller et al., *Chem. Phys. Chem.* **2010**, *11*, 1289−1296; Y. Miloslavina et al., *FEBS Lett.* **2008**, *582*, 3625−3631).



1. INTRODUCTION

The major light-harvesting complex in Photosystem II (PSII), called LHCII, together with the minor light-harvesting complexes (CP24, CP26, and CP29), absorbs sunlight and transfers the excited state energy to the reaction centers of PSII where it is converted to chemical energy.[1,2] *In vivo*, the LHCII complexes exist as trimers and bind more than half of the pigments connected to PSII. Each LHCII monomer subunit contains eight chlorophyll (Chl) *a*, six Chl *b*, and four carotenoid (Car) molecules.[3−5] Among the four Car molecules, two are lutein (Lut) molecules, one is neoxanthin (Neo), and one is a xanthophyll-cycle Car (Xanc).[4] Figure 1 shows the structure of the LHCII trimer based on the X-ray crystal structure (PDB ID: 1RWT).[4] Chls in each monomeric subunit form several clusters of strongly excitonically coupled pigments, and intramonomer excitation energy transfer rates are very fast.[6,7]

Besides its function in light-harvesting, another important property of LHCII is the regulation of light-harvesting efficiency by quenching of Chl excited states. This latter function is called "non-photochemical quenching" (NPQ), an important reversible regulation mechanism present in plants and other photobionts. NPQ protects the photosystems from severe damage under excess light conditions by converting excited state energy into heat.[8−10] It has been proposed that quenching depends on the trans-thylakoid membrane pH gradient (ΔpH),[9,11] the conversion of the Xanc pigments violaxanthin to zeaxanthin,[12,13] and the action of the PsbS protein.[14] Aggregated LHCII, formed when the detergent is removed from a suspension of isolated LHCII trimers in detergent, has been established as a good *in vitro* model for the

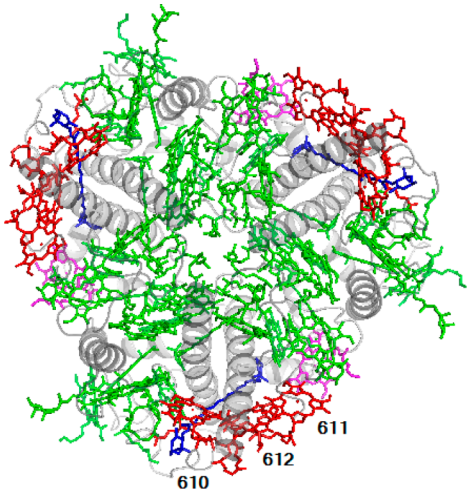


Figure 1. Structure of the LHCII trimer based on recent X-ray data.[4] Pigments highlighted in red (*a*610. *a*611, and *a*612) contribute to the lowest energy absorption band of the LHCII trimer.[2,22−24] Lut 620 is shown in blue, and another externally located Chl, *a*614, is shown in pink.

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so-called *qE* quenching located in LHCII in intact organisms and, as has been noted previously, this type of quenching is associated with the development of far-red emitting Chl states.[15−17]

Although NPQ has been studied extensively in the past two decades, the quenching site(s) and molecular mechanism(s) are still under debate (see, e.g., ref 18 for a recent discussion). It should be pointed out that the molecular quenching mechanism may differ in different antenna complexes (i.e., minor versus major light-harvesting complexes). The key ideas regarding the quenching mechanism in LHCII are (i) Chl-Car singlet energy transfer[19] where the LHCII complex undergoes a change to a conformational state, which allows transfer of Chl excited state energy to the carotenoid's lowest energy excited state; (ii) enhancement of Car/Chl exciton coupling that would mix their excited states and thus control the deactivation via the S_1-state of Car;[20] and (iii) formation of Chl−Chl charge-transfer (CT) states as a pathway for Chl excited state deactivation.[15,16,21] In the present work, we characterize the spectroscopic properties of aggregated LHCII and test the model of aggregation-induced Chl−Chl CT state formation as the deactivation pathway for excited state Chls.

2. EXPERIMENTAL METHODS

2.1. Sample Isolation Procedure and Preparation of Aggregated LHCII Complexes. The trimeric LHCII complexes were extracted from spinach thylakoids as previously described[16,21] and were dispersed in 5 mM tricine buffer (pH = 7.5) with detergent (0.1 M sucrose and 0.06% n-dodecyl *β*-D-maltoside [*β*-DM]). Removal of the detergent by several treatments with Biobeads SM-2 (Bio-Rad) formed large, highly quenched aggregates (called "large aggregates" throughout this paper). In an attempt to form smaller and more homogeneous aggregates, some samples were partially solubilized with a low concentration of detergent (0.008% *β*-DM) and measured before or after ultrasonication.

2.2. Spectroscopic Methods. Details about the measurement setup were described elsewhere.[25] Here, only a brief description is given. A Bruker HR125 Fourier transform spectrometer was used to measure the absorption and hole burned (HB) spectra. In absorption and nonresonant HB, the resolution was set at 4 cm^{-1}. For resonant HB a spectral resolution of 0.5 cm^{-1} was used. The laser source for nonresonant HB was 496.5 nm produced from a Coherent Innova 200 argon ion laser. In the resonant HB experiments, the tunable wavelengths came from a Coherent CR699 ring dye laser pumped by a Millenia 10s diode-pumped solid state laser at 532 nm from Spectra-Physics. With laser dye LD 688 (Exciton), a spectral range of 650 to 720 nm was available with a line width of 0.07 cm^{-1}. The power from the ring laser output was stabilized with a Laser Power Controller (Brockton Electro-Optics Corp.). The laser power in the experiments was precisely set by a continuously adjustable neutral density filter. All the experiments were performed at 5 K inside a Janis 8-DT Super Vari-Temp liquid helium cryostat. The sample temperature was read and controlled with a Lakeshore Cryotronic model 330 temperature controller. Low-temperature (77 K) fluorescence spectra characterizing the quenched aggregated samples were measured using a home-built fluorometer consisting of an Ocean-Optics spectrometer with CCD detection and blue LED excitation (455 nm peak wavelength) with samples frozen in an Oxford cryostat (sample thickness of 0.1 mm).

3. RESULTS AND DISCUSSION

3.1. 77 K Emission Spectra of LHCII Proteins. The 77 K emission spectra of trimeric LHCII, as well as "large aggregates" and "small aggregates" of LHCII, are shown in Figure 2. The spectrum of the "large aggregate" sample is typical for highly quenched LHCII aggregates, showing only a small shoulder at the regular trimer emission peak (~678 nm) and a dominant emission band at 694.5 nm, which is specific for quenched LHCII aggregates.[16,26,27]

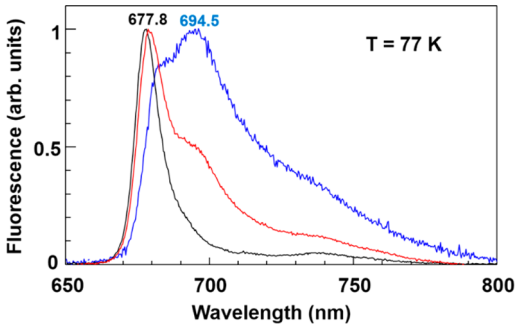

Figure 2. 77 K fluorescence spectra of trimeric LHCII (black curve), "large aggregates" of LHCII (detergent-free, blue curve), and "small aggregates" of LHCII obtained by partial solubilization of "large aggregates" with 0.008% *β*-DM (red curve). Measurements were carried out in 0.1 mm sample cells with front face excitation, thus avoiding self-absorption effects.

3.2. Low-Temperature Absorption and Nonresonant Persistent HB Spectra of Aggregated LHCII Complexes. The 5 K absorption spectra of LHCII aggregates are shown in Figure 3. Spectrum 1, shown in frame A (sample 1), corresponds to normal LHCII aggregates (i.e., "large aggregates", *vide supra*). In an attempt to homogenize the aggregates and obtain small oligomers, the "large aggregates" sample was partially solubilized with a buffer containing 0.008% of *β*-DM (sample 2). Frame B shows absorption spectra of

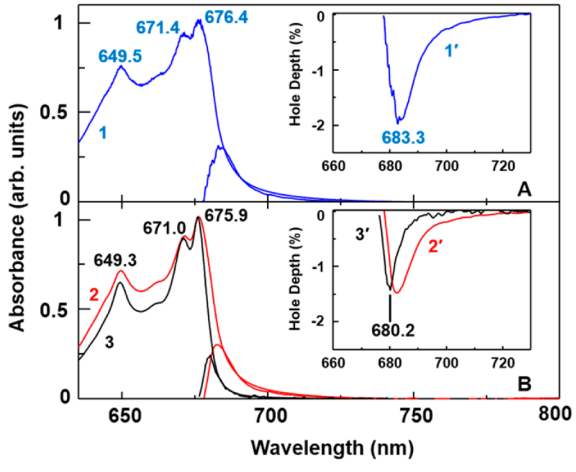

Figure 3. Frame A: Curves 1 and 1′ (inset) are the absorption/nonresonant HB spectra of "large aggregates" of LHCII (OD ~ 1) without detergent. Frame B: Curves 2/2′ and 3/3′ (inset) are the absorption and HB spectra of "small aggregates" containing 0.008% *β*-DM, before and after ultrasonication, respectively. Note that inverted and normalized holes fit very well the low-energy absorption wing proving that the extended long-wavelength absorption does not originate from scattered light.

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sample 2 frozen directly (spectrum 2) and after being treated additionally by ultrasonication (sample 3, spectrum 3). Absorption spectra of samples 1 and 2 are similar and have three clearly resolved bands near 676.4, 671.4, and 649.5 nm with one apparent shoulder near 661 nm. Note that addition of 0.008% β-DM detergent alone without ultrasonication (sample 2) did not disaggregate the sample, while subsequent ultrasonication (sample 3) had a pronounced effect on the spectra. This is reflected by the different positions and bandwidths of the corresponding low-energy state, as revealed via the nonresonant holes shown in the inset of Figure 3B. All nonresonant (persistent) HB spectra discussed in this section were obtained with a burn wavelength (λ_B) of 496.5 nm and fluence f = 90 J/cm^2 ($f = I^*t$, where I and t correspond to the laser intensity and time, respectively). The hole obtained for sample 1 (curve 1′ with the full-width-at-half-maximum, fwhm, of 200 cm^{-1}) is located at 683.3 nm. The shape of the nonresonant holes clearly indicates that the long-wavelength absorption wing undergoes HB following transfer of excitation energy from the initially excited excitonic states(s) to states that have predominant CT character. Antiholes are not shown for simplicity, as we focus only on the position and bandwidth of the lowest energy hole. Spectroscopic features similar to the data shown here for sample 1′ were observed for many independent samples studied; though occasionally broader, even more red-shifted holes (with the hole-minimum shifted up to 685 nm) were also observed. Such behavior clearly indicates that there is a broad distribution of aggregate sizes, as has been characterized previously with atomic force microscopy data (unpublished data). Note that the lowest energy state in aggregates is significantly broader (fwhm ∼200 cm^{-1}) and red-shifted by 3.9 nm, as compared to LHCII trimers for which the hole was observed at 679.4 nm (with a fwhm of ∼55 cm^{-1}).[28]

The nonresonant hole of sample 2 (curve 2′ in Figure 3B) is slightly narrower and blue-shifted as compared to hole 1′, though both inverted (and normalized) holes fit well the low-energy wing of the corresponding absorption spectra. All three samples have similar hole depths of 1−2% under the same fluence (f = 90 J/cm^2 with λ_B = 496.5 nm); *vide infra*. All samples consistently revealed a very low HB efficiency, which is characteristic of strong mixing of low-energy exciton states with likely formed CT-state(s);[28−30] *vide infra*. It is not surprising that HB efficiency is low, since the quenching in these aggregates is very efficient, and fluorescence lifetimes observed for aggregated LHCII trimers are much shorter than those of LHCII trimers.[16,26]

The absorption spectrum shown in Figure 3B (curve 3), and its corresponding nonresonant hole (curve 3′), were obtained for sample 2 after sonication for several minutes as an attempt to decrease LHCII aggregate size. This is indeed observed, as the HB spectrum narrows and shifts blue, more closely resembling the nonresonant hole obtained for isolated LHCII trimers. Thus, ultrasonication after the addition of detergent dissolves the aggregates partly, probably reforming detergent micelles. This is supported by the similarity of the spectra compared to those observed previously for LHCII trimers.[30] However, in spectrum 3′ there is still a pronounced contribution present characteristic of aggregates. That is, the main absorption peak in the sonicated sample is blue-shifted by 0.2−0.5 nm only, depending on the extent of sonication, but has a significantly narrower lowest exciton band (∼90 cm^{-1}). This tailing is ascribed to partial aggregation.

3.3. Resonant HB Spectra of Aggregated LHCII.

Resonant HB was performed in order to further characterize the spectroscopic properties of the aggregates and to reveal the strength of the el−ph coupling. Figure 4 shows resonant HB

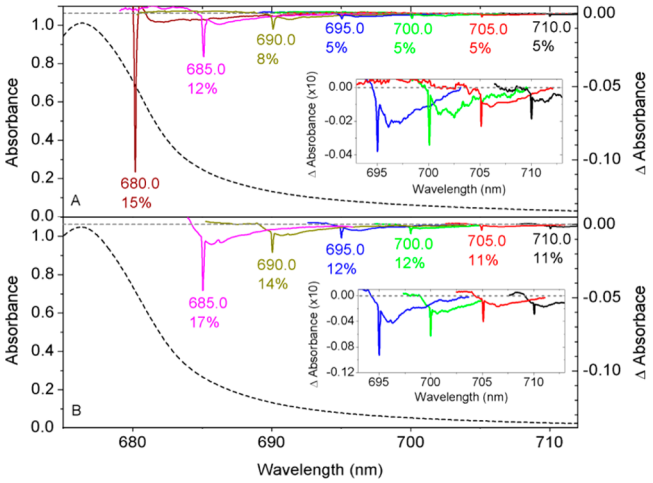

Figure 4. Resonant HB spectra of LHCII aggregates without detergent (frame A) and with 0.008% of β-DM detergent (frame B) compared with their respective low-energy parts of the absorption spectra (dashed curves).

spectra obtained for samples 1 (large LHCII aggregates) and 2 (aggregates with 0.008% β-DM detergent present), respectively. Holes were burned at the low-energy absorption wing (in the 680−710 nm spectral range) with a much higher fluence than previously used,[28,30] i.e., f = 200 J/cm^2, within the lowest energy absorption band. The insets show expanded holes burned at 695.0, 700.0, 705.0, and 710.0 nm. Note that resonant holes burned in sample 2 (frame B) have somewhat larger hole depths (in %) than those for sample 1 (frame A). The latter is due to a different overlap of red-shifted (long-lived) trimer states with aggregation induced CT-states. The widths of the zero phonon holes (ZPHs) are resolution limited.

Careful inspection of the resonant holes in Figure 4 reveals that narrow ZPHs are accompanied by structures different from "common" phonon side bands (PSBs), i.e., there is a considerable tailing at high energies, indicating an overlap of the lowest energy trimer state with aggregation induced CT states. We emphasize that previous resonant HB spectra reported for aggregated LHCII trimers were obtained with a much smaller fluence of only 6 J/cm^2.[28] This is why the constant-fluence ZPH action spectrum in ref 28 did not reveal any ZPHs for λ_B > 683 nm but showed the site-distribution function (SDF) characteristic of the lowest exciton level of trimers. Nevertheless, the latter work first identified a red-shifted low-energy absorption centered near 684 nm with a large width of 240 cm^{-1}, which was assigned to the red-emitting states, but its nature has not been established.

Finally, we note that recently it has been commented that the enhancement of PSBs and absence of sharp ZPHs can be produced by selective coupling between CT states and vibration excited exciton states, rather than by strong local Huang−Rhys (HR) factors for each site.[31] In this regard, it should be emphasized that HB experimentally probes the line-shape of the absorption spectrum and the distribution of these line-shapes across an absorption band. Whether the broad line-shapes (and absence of ZPHs) observed in HB measurements

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are interpreted in terms of strong el−ph coupling or in terms of displacement between the nuclear coordinates of CT and exciton states is essentially unimportant from a HB perspective, as these measurements probe the final results (the actual absorption spectrum).

3.4. Electron−Phonon Coupling Strength in Aggregated LHCII. The strength of the el−ph coupling (in the low temperature limit) can be estimated by the following relation: $\alpha = \exp(-S) = I_{ZPH}/(I_{ZPH} + I_{PSB})$, where α is the Debye−Waller factor, S is the HR factor, and I_{ZPH} and I_{PSB} are the integrated areas under the ZPH and PSB, respectively. A detailed description of the HR factor can be found in ref 32, only a brief description is needed here. In the weak-coupling limit $S \rightarrow 0$ and, conversely, in the strong-coupling limit S is large. When $S > 1$, the el−ph coupling is considered "strong." The HR factors were calculated based on data shown in Figure 4. The S factor is plotted in Figure 5 as a function of λ_B for samples 1 and 2. The error bars were estimated by averaging data from different measurements; small uncertainties are caused by the noisy PSB holes. Note that the el−ph coupling strength is generally large and S increases with longer λ_B. This large S factor of ~3.5 for $\lambda_B > 690$ nm indicates that there is a strong mixing of the lowest energy Q_y-exciton state(s) with CT state(s) in aggregated LHCII. For LHCII trimers the S factor is much weaker, i.e., $S = 0.8$.[30]

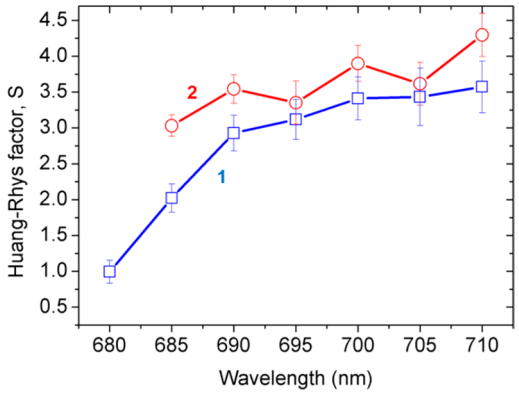

Figure 5. HR factor plotted as a function of λ_B. Curves 1 and 2 were obtained from data shown in Figure 4.

These results strongly suggest an overlap of excitonic trimer states with aggregation-induced Chl-Chl CT states. It is likely that the formation of these CT states in aggregates induces efficient Chl excited state deactivation. This conclusion from HB results is in agreement with conclusions based on ultrafast fluorescence and transient absorption data on aggregated LHCII complexes,[16,21] as well as the results from Stark fluorescence effects on LHCII aggregates.[33] All of these data indicated the formation of Chl−Chl CT states as intermediates in the quenching process in LHCII aggregates.

4. CONCLUSION

We have demonstrated that Chl−Chl CT states associated with the long-wavelength absorption band undergo low-yield HB following transfer of excitation from the initially excited excitonic states. Although the CT states are, in the first order approximation, Franck−Condon forbidden in a ground-state transition (i.e., large value of S), we were able to burn weak ZPHs upon selective excitation in the low-energy absorption wing. The large S factor indicates that the el−ph coupling in the quenching state(s) is very strong, a characteristic feature of CT states.[29]

In our view there exists no reasonable alternative interpretation for the characteristics of these bands. Their interpretation as deriving from Chl−Chl CT states is also in agreement with the results of our time-resolved data.[16,21] The large S values of the long-wavelength bands also excludes more localized Chl excited states as their origin, resulting, e.g., from partially detached/disturbed Chl-protein binding. Even if such states were present, albeit we have no evidence for their existence at all, they could not explain these large S values.

It is interesting here to make some comparison with the properties of CT states observed in reaction centers. The Krausz group has directly observed a low-energy CT absorption feature[34] and has more recently shown emission from this state, in Photosystem II core preparations.[35] In this case, the Stokes shift seems to be even larger than in the states observed here in LHCII aggregates. We note, however, that the exact value of the Stokes shift will depend on many factors regarding coupling and environment. It is interesting nevertheless, that quite similar states can be observed in antennae complexes as are clearly present in reaction centers.

An interesting question that has not been answered as of yet pertains to the internal changes that actually afford the CT state formation and the ensuing quenching. According to recent results from LHCII trimers in a gel, the quenching is not induced *per se* by the aggregation, but actually by the removal of the detergent micelle around the LHCII complex.[26] Thus, aggregation is rather a byproduct and a consequence of detergent removal, rather than the prime factor causing CT-state formation and thus quenching. Our general idea for the switching mechanism required to activate the CT state formation is the creation of a polar environment around the excitonically coupled Chls at the reactive site. A polar environment could lower the energy of the CT state below that of the localized excited state(s). In the case of LHCII aggregates, this would be caused by polar groups (water, ions) getting access near the quenching center upon detergent removal. Under *in vivo* conditions, that polar switching would be caused by binding of the psbS protein. However, a detailed mechanistic molecular understanding of this switching process has not yet been achieved.

Finally, we note that recently assembled solar cells (with a thin TiO_2 barrier layer sensitized with aggregated LHCII from spinach) showed strongly enhanced photocurrent most likely due to the formation of Chl−Chl CT states that are effectively coupled with the TiO_2 surface and inject electrons into the TiO_2 conduction band (unpublished data). The assembled solar cells demonstrated remarkable stability in both aqueous buffer and acetonitrile electrolytes over 30 days.

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Notes

The authors declare no competing financial interest.

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