



Nature of low-energy exciton levels in light-harvesting complex II of green plants as revealed by satellite hole structure

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Abstract

Persistent non-photochemical hole burning at 4.2 K is an efficient experimental tool to unravel position and nature of low-energy excitonic states in pigment-protein complexes. This is demonstrated here for the case of the trimeric chlorophyll (Chl) *a/b* light-harvesting complexes of Photosystem II (LHC II) of green plants, where previous work (Pieper et al. *J Phys Chem B* 103:2412, 1999a) reported a highly localized lowest energy state at 680 nm. At that time, this finding appeared to be consistent with the contemporary knowledge about the LHC II structure, which mainly suggested the presence of weakly coupled Chl heterodimers. Currently, however, it is widely accepted that the lowest state is associated with an excitonically coupled trimer of Chl molecules at physiological temperatures. This raises the question, why an excitonically coupled state has not been identified by spectral hole burning. A re-inspection of the hole burning data reveals a remarkable dependence of satellite hole structure on burn fluence, which is indicative of the excitonic coupling of the low-energy states of trimeric LHC II. At low fluence, the satellite hole structure of the lowest/fluorescing ~ 680 nm state is weak with only one shallow satellite hole at 649 nm in the Chl *b* spectral range. These findings suggest that the lowest energy state at ~ 680 nm is essentially localized on a Chl *a* molecule, which may belong to a Chl *a/b* heterodimer. At high fluence, however, the lowest energy hole shifts blue to ~ 677 nm and is accompanied by two satellite holes at ~ 673 and 663 nm, respectively, indicating that this state is excitonically coupled to other Chl *a* molecules. In conclusion, LHC II seems to possess two different, but very closely spaced lowest energy states at cryogenic temperatures of 4.2 K.

Keywords Photosynthesis · Light-harvesting complex II · Excitation energy transfer

Abbreviations

Car	Carotenoid
Chl	Chlorophyll
EET	Excitation energy transfer
FLN	Fluorescence line-narrowing
LHC II	Light-harvesting complex II
SHB	Spectral hole burning

Introduction

Light-harvesting and subsequent excitation energy transfer (EET) to reaction center complexes occur in dedicated pigment-protein complexes referred to as antennae (Mirkovic et al. 2017). The light-harvesting complex of photosystem II (LHC II) is the major antenna complex of green plants and one of the best studied photosynthetic pigment-protein complexes in general. The structure of LHC II (Liu et al. 2004; Standfuss et al. 2005) was investigated by high-resolution X-ray diffraction and is available at nearly atomic resolution (see Fig. 1). LHC II forms a trimeric complex with each monomer binding 14 chlorophyll (Chl) molecules and four carotenoid (Car) molecules. It was possible to unambiguously assign 8 Chl *a* and 6 Chl *b* molecules, which are arranged in Chl *a*–*a* and Chl *b*–*b* homodimers as well as in one Chl *a*–*b* heterodimer (see schematic representation in Fig. 1). The closest center-to-center distances of 8 to 12 Å within the latter clusters suggest complex effects due to pigment–pigment interactions.

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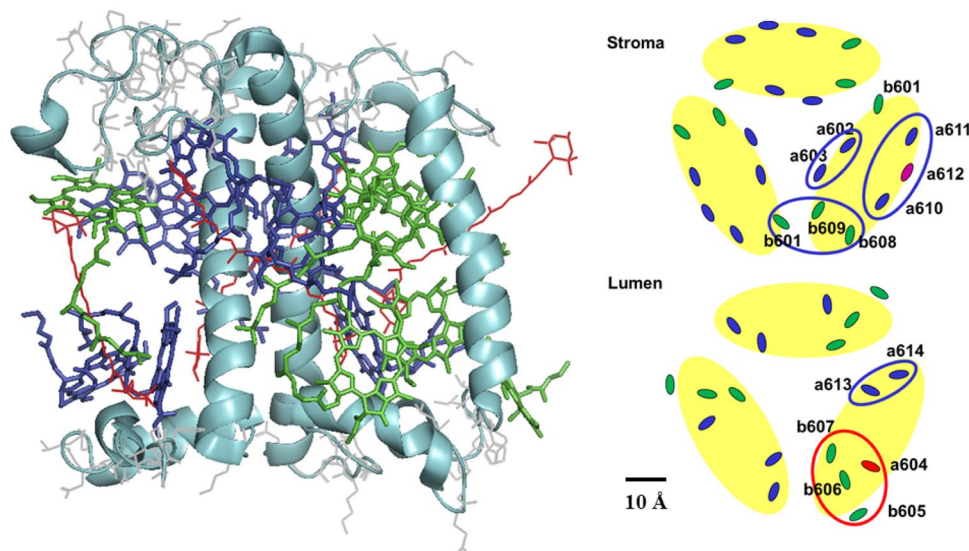


Fig. 1 Left panel: crystal structure of a monomeric subunit of LHC II according to Liu et al. (2004) (pdb code 1RWT). Chl *a* molecules are shown in blue, Chl *b* in green, and carotenoids in purple color. Right panel: Schematic representation of the arrangement of Chl molecules at the stromal (top) and luminal side (bottom) of trimeric LHC II and their nomenclature according to Liu et al. (2004). The Chl molecules

are again shown in blue and green for Chl *a* and Chl *b*, respectively. Clusters of strongly coupled Chl molecules are encircled by blue and red lines. Chl *a*612 suggested to carry the lowest excitonic state at room temperature is shown in purple, while Chl *a*604 which may correspond to the lowest state at 679.6 nm at 4.5 K is shown in red

This is important because a previous structure (Kühlbrandt et al. 1994) indicated rather weak excitonic delocalizations within Chl heterodimers (Voigt et al. 1996). The LHC II trimer can be constituted by three different proteins referred to as Lhcb1-3 with slightly distinct spectroscopic properties (Standfuss and Kühlbrandt 2004).

The specific structural arrangement of pigment molecules in LHC II provides the framework for efficient, ultrafast EET. A comprehensive overview about steady-state properties and singlet and triplet EET dynamics in LHC II is provided by van Amerongen and van Grondelle (2001). A number of more recent time-resolved absorption and 2D spectroscopy studies have directly revealed time constants in the order of femto- to picoseconds for Chl-to-Chl (Lewis et al. 2016; Ramanan et al. 2017; Akhtar et al. 2019) and Car-to-Chl EET (Gradinaru et al. 2000; Croce et al. 2001). Several theoretical studies have modeled spectroscopic data in LHC II and thus provided deeper insight into the specific pathways of ultrafast EET (Novoderezhkin et al. 2005; van Grondelle and Novoderezhkin 2006; Müh et al. 2010; Martiskainen et al. 2011; Zucchelli et al. 2012). Under high-light conditions, LHC II is also involved in non-photochemical quenching (NPQ) and thus in protection against excess energy (Demming-Adams et al. 2014). The terminal excitonic state is often associated with the cluster of strongly coupled Chl *a* molecules *a* 610-*a* 611-*a* 612 in both experimental and theoretical studies, see above, which is also consistent with

site-directed mutagenesis studies (Remelli et al. 1999; Rogl et al. 2002).

In the frequency domain, spectral hole burning (SHB) contributes valuable information on excited state positions, homogeneous and inhomogeneous broadening as well as on electron-phonon coupling to the overall understanding of EET in pigment-protein complexes at low temperatures (Jankowiak et al. 2011). LHC II has also been intensively studied using SHB (Reddy et al. 1994; Pieper et al. 1999a, 2009) and complementary site selective methods like fluorescence line-narrowing (FLN) (Pieper et al. 2001) and fluorescence hole burning (Gibasiewicz et al. 2009).

Previous detailed hole burning studies of trimeric LHC II (Reddy et al. 1994; Pieper et al. 1999a) have established that the lowest excitonic energy level lies at ~ 680 nm at 4.2 K. The 680 nm state is characterized by moderate electron-phonon coupling with a Huang-Rhys factor $S \sim 0.9$ as well as by a strongly asymmetric one-phonon profile with a mean phonon frequency ω_m of ~ 18 cm^{-1} and a width of ~ 105 cm^{-1} (Pieper et al. 2004).

It is remarkable that the 680 nm state was reported to be highly localized at cryogenic temperatures by all above SHB studies, although Chl *a* 612 inferred as carrying the lowest state is part of a cluster of strongly coupled Chl molecules, see above. Thus, there seems to be an apparent contradiction between previous SHB results and current structural assignments of the lowest energy states of LHC II. This has led us to reconsider the data of Pieper et al. (1999a) in the present

paper and discuss the observed satellite holes in terms of the actual high-resolution X-ray structures of LHC II (Liu et al. 2004; Standfuss et al. 2005).

Materials and methods

Sample preparation The LHC II preparation used was described in Pieper et al. (1999a). Briefly, LHC II was purified from spinach Photosystem II membrane fragments by sucrose density gradient centrifugation in the presence of *n*- β -dodecylmaltoside as described in detail previously (Irrgang et al. 1988). Analysis of the protein composition of LHC II revealed no PsaA and Lhcb4, PsaS contributed less than 1%, and Lhcb 5/6 less than 5% to the total amount of protein. The relative contributions of Lhcb 1, 2, and 3 were 40, 40, and $20 \pm 10\%$, respectively. The Chl *a/b* ratio (w/w) was determined to be 1.38 ± 0.5 ($n = 22$). At the employed detergent concentration of 0.025% w/v *n*- β -dodecylmaltoside, the trimeric form was highly stable as described in Pieper et al. (1999b). The Chl concentration of the samples used for hole burning measurements was 4 $\mu\text{g/ml}$.

Experimental setup The hole burning apparatus for lower-resolution measurements ($> 0.5 \text{ cm}^{-1}$) was described in Pieper et al. (1999a). Briefly, the burn laser was a Coherent CR 699–21 dye laser (linewidth of $\sim 0.05 \text{ cm}^{-1}$) pumped by a 6 W Coherent Innova Ar ion laser. The sample temperature was maintained at 4.2 K using a Janis 8-DT convection cooling liquid helium cryostat. Burn intensities (I_B) and times (τ_B) or burn fluences ($I_B \tau_B$) as well as read resolutions are given in the figure captions. A Bruker IFS 120 h Fourier transform spectrometer was used to record (read) the pre-burn and postburn absorption spectra.

Results and discussion

Assignment of the lowest energy state We will first discuss typical hole burning spectra of trimeric LHC II, which were obtained with burn wavelengths outside of the lowest energy states, i.e., under non-resonant conditions. The 4.2 K Q_y -absorption and selected hole burning spectra of trimeric LHC II as obtained using a burn wavelength of 660 nm and fluences of 8 to 234 J/cm^2 in Pieper et al. (1999a) are shown in Fig. 2. As discussed in the latter study, the 4.2 K Q_y -absorption spectrum exhibits only four prominent bands at 676.1, 671.3, 661.4, and 649.5 nm although excitonic states of up to 42 Chl molecules can be expected to contribute in trimeric LHC II. This is mainly due to significant inhomogeneous broadening of the energetically closely spaced absorption bands of the latter 42 pigment molecules.

Persistent spectral hole burning is capable to reveal more structure within the broad and featureless absorption

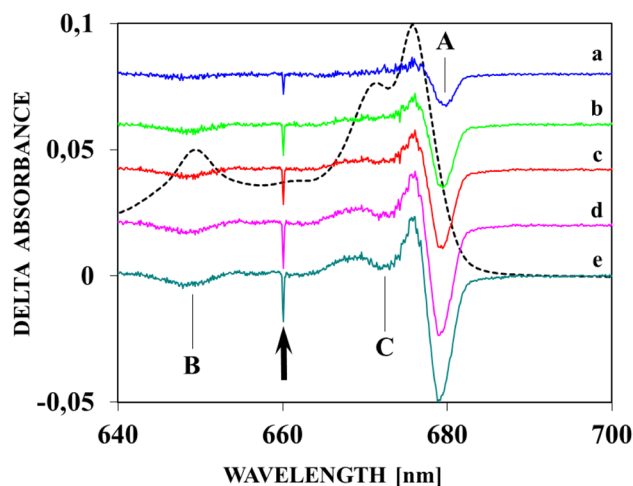


Fig. 2 4.2 K absorption (black dashed line) and hole burned spectra of trimeric LHC II. The spectra were obtained using a burn wavelength of 660 nm, a burn intensity of 130 mW/cm^2 , and burn times (top to bottom) of 1, 5, 10, 20, and 30 min. The read resolution was 4.0 cm^{-1} . The burn wavelength is labeled by a black arrow, letters A, B and C label the broad satellite holes at 679.6, 648.8, and 673.0 nm. The hole spectra are separated by a ΔA of 0.02 for ease of inspection. The data are taken from Pieper et al. (1999a) with permission, copyright 1999 American Chemical Society

spectrum. The hole burned spectra were generally found to show three main features: (i) a narrow hole coincident with the burn wavelength (660 nm in Fig. 2), (ii) an intense hole in the vicinity of 680 nm (hole A) which appears as a result of efficient EET to low-energy trap states, and (iii) a weak satellite hole (hole B) at about 649 nm. Another weak satellite hole at $\sim 673 \text{ nm}$ is emerging at fluences higher than 70 J/cm^2 .

The observation of these spectral features has established that the lowest excitonic energy level lies at $\sim 680 \text{ nm}$ at 4.2 K based on the low-energy hole A (see Fig. 2). This means that the lowest energy level of trimeric LHC II is located about 4 nm to the red of the main absorption band at $\sim 676 \text{ nm}$. This has first been reported by Reddy et al. (1994) using partly aggregated samples and was later confirmed by Pieper et al. (1999a) for non-aggregated LHC II. More recently, fluorescence and SHB studies were used to verify the assignment of the lowest Q_y -state (Gibasiewicz et al. 2009).

In Pieper et al. (1999a), the lowest state at 680 nm was interpreted as being highly localized on one Chl molecule mainly because of the weak satellite hole structure, i.e., the Chl molecule was found to be weakly excitonically coupled to others. One satellite hole at $\sim 673 \text{ nm}$ could not be consistently interpreted. It has to be emphasized, however, that the data of Pieper et al. (1999a) were interpreted based on a structure of LHC II with a resolution of 3.4 \AA (Kühlbrandt et al. 1994) that favored close spacings within Chl *a*–Chl *b*

heterodimers. This structural assignment appeared to be in line with the relatively high degree of localization of the 680 nm state of LHC II and the weak satellite hole structure observed by SHB. That is, the energetic difference between the site energies of the closely spaced Chls had to be assumed to be much larger than their interaction energy. This is also the reason why the satellite hole structure of the hole burning spectra shown in Fig. 2 did not attract much attention, when the initial SHB study was published (Pieper et al. 1999a). However, it is the satellite hole structure that contains information about the excitonic coupling in a pigment-protein complex, see below.

Heterogeneity of the lowest energy states A closer inspection of the hole burning data shown in Fig. 2 reveals that the spectra depend on burn fluence or burn time, respectively. As mentioned above, hole C appears at high fluence only. Moreover, the position of hole A blue-shifts from ~ 680 nm at a relatively low fluence (spectrum a in Fig. 2 to ~ 679 nm in the saturated spectrum (spectrum e in Fig. 2). This effect is better visible in Fig. 3, which shows a magnification of the data of Fig. 2 in the spectral region of the 680 nm hole. The steep rise of the high-energy wing of the low-energy hole at high fluence is due to interference with the closely spaced antihole. This overlap may lead to a seeming red-shift of hole A at high fluence so that its real position may be further blue-shifted than 679 nm. In (Pieper et al. 1999a), this shift was assumed to be due to a heterogeneity involving three energetically inequivalent low-energy states. Each of these three states was inferred to be widely localized on a Chl *a* molecule of one monomeric subunit.

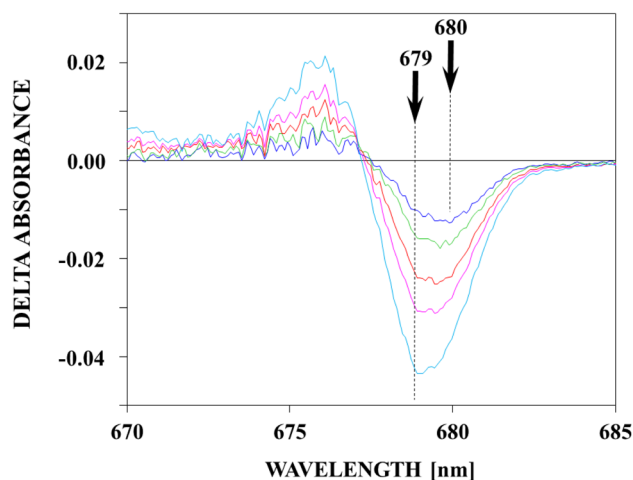


Fig. 3 Magnification of the hole burning spectra of trimeric LHC II of Fig. 2 emphasizing the low-energy region around the intense satellite hole in the vicinity of 680 nm. The spectra were obtained using a burn wavelength of 660 nm, a burn intensity of 130 mW/cm², and burn times (top to bottom) of 1, 5, 10, 20, and 30 min. The color code is the same as in Fig. 2

Indeed, it was shown later that the spectral position of the lowest energy states of the individual Lhcb1-3 proteins varies in the same order of magnitude although only Lhcb3 exhibits a significant blue-shift of its lowest state (Standfuss and Kühlbrandt 2004). Thus, it appeared reasonable to identify the three closely spaced low-energy states with the lowest states of the Lhcb1-3 proteins constituting the LHC II trimer. Nevertheless, the satellite hole structure associated with hole A was not considered, thus casting some doubts on the latter interpretation.

Nature of low-energy states In addition to assignment of spectral positions, SHB can also contribute valuable information on the nature of the low-energy states of LHC II by the evaluation of the satellite hole structure. Excitonic satellite holes have been observed e.g., in the cases of WSCP (Pieper et al. 2011) and phycobiliproteins of *A. marina* (Gryliuk et al. 2014; Pieper et al. 2017).

In Fig. 2, it is visible that the hole burning process in hole A produces satellite holes B and C in the Chl *a*- and Chl *b* spectral ranges, respectively. As mentioned before, however, hole C appears at higher fluence only. This suggests that the appearance of both satellite holes at different fluences may be related to the fluence-dependent shift of the low-energy hole A discussed above. This is a fact that has not been noted by Pieper et al. (1999a). To address this question, Fig. 4 shows two hole burning spectra of LHC II trimers: (i) one spectrum obtained at low fluence within the first 2 min of burn time (see blue line in Fig. 4) and (ii) one spectrum representing high fluence corresponding to the last ten minutes before saturation of the hole structure,

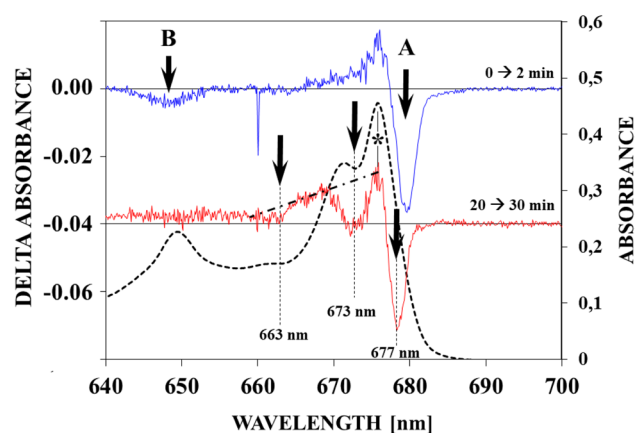


Fig. 4 Effect of fluence on 4.2 K hole burned spectra of trimeric LHC II. The hole spectrum obtained at low fluence (blue line) is identical to spectrum a) in Fig. 2. The high-fluence spectrum (red line) is the difference between spectra e) and d) in Fig. 2 and thus reflects only the effect of the last 10 min of burning before saturation of the burn process. Satellite holes are labeled by arrows. The approximate background level in the range of satellite holes is very roughly indicated by a dashed-dotted line. A 4.2 K Q_y-absorption spectrum of trimeric LHC II is shown for comparison (dashed line)

which is obtained as the difference between the last two spectra shown in Fig. 2 (see red line in Fig. 4). It is apparent that both spectra look essentially different. This means that the latter (high-fluence) spectrum contains information about hole burning in an excited state that became effective only when burning in the lowest excited state at 4.2 K was saturated.

The low-fluence spectrum (see blue line in Fig. 4) resembles the situation discussed by Pieper et al. (1999a) and Pieper et al. (2009) with a low-energy state at 680 nm in the Chl *a* region and a satellite hole B at 649 nm indicating the presence of a Chl *a*–Chl *b* heterodimer. Most importantly, however, the high-fluence spectrum (see red line in Fig. 4) looks essentially different from the low-fluence case. The separation of the fluence effects reveals that at high-fluence burning in the lowest energy, 680 nm state is saturated so that an energetically closely spaced state at about 677 nm becomes visible. This is the same effect shown in Fig. 3 where it was not separated for different burn times/burn fluences. An inspection of the satellite hole structure in Fig. 4 also reveals important differences between the low- and high-fluence cases. While the Chl *b* satellite hole B is no longer visible, two other holes in the Chl *a* spectral range at 663 and 673 nm, respectively, appear to be related to the 677 nm hole in the low-energy region. In this assignment, one has to take into account that both satellite holes are burned into the antihole, which appears as a positive difference in absorption and is expected to have an asymmetric shape. The latter effect leads to an effective shift of the baseline, which—in a crude estimate—is indicated as a dashed-dotted line in Fig. 4. This means that the satellite hole structure can be interpreted as the signature of a Chl *a* homotrimer. In summary, the observation of a different satellite hole structure of the 680 and 677 nm states, respectively, suggests an assignment of the low-energy states to different clusters of pigment molecules within the LHC II monomer.

It is instructive to compare these characteristics of the 680 and 677 nm states established by SHB to the currently available crystal structures (Liu et al. 2004; Standfuss et al. 2005) and more recent excitonic calculations for LHC II (Novoderezhkin et al. 2005; Müh et al. 2010; Martiskainen et al. 2011; Zucchelli et al. 2012). Low-temperature (77 K), time-resolved, and frequency-domain spectroscopic data were simulated using a modified Redfield approach (Novoderezhkin et al. 2005), which accounted reasonably well for many features of 77 K time domain, CD, absorption, and fluorescence data. In this study, the fs kinetics are attributed to fast EET within excitonically coupled clusters of Chl *b* and Chl *a* molecules, respectively, while the slower ps components are ascribed to relatively long-lived monomeric electronic states which are mainly found in the intermediate wavelength region of 660–670 nm. In contrast, Chl *a*604 appears to be weakly coupled to other Chl *a* molecules. The

terminal (fluorescent) excitonic state was assigned to a cluster of strongly coupled Chl *a* molecules *a*610–*a*611–*a*612. As mentioned above, this appears to be in line with site-directed mutagenesis studies, which found the lowest excitonic state of LHC II to be located on Chl *a*612 at room temperature (Remelli et al. 1999; Rogl et al. 2002). The positions of the satellites holes reported here are similar within a few nanometers to those reported by Novoderezhkin et al. (2005), which is an encouraging result given the experimental uncertainties. Later, simulations of 2D time-resolved spectroscopic data have confirmed the basic features of the latter assignments of site energies including the location of the lowest energy state at the strongly coupled trimer of Chl *a* molecules *a*610–*a*611–*a*612.

However, comparison with the SHB results discussed above suggests that only the high-fluence hole burning spectrum (red line in Fig. 4) exhibits a satellite structure expected for the Chl *a* homotrimer *a*610–*a*611–*a*612. That is, the latter homotrimer is present in the low-energy region of LHC II, but not in the lowest energy state at 4.2 K. Instead, the satellite hole structure produced by hole burning at low fluence within the ~ 680 nm state is weak with only one satellite hole located at ~ 649 nm (see blue line in Fig. 2), which is almost coincident with the major Chl *b* absorption band of trimeric LHC II. Consequently, it appears that the lowest energy state should be weakly coupled to other Chl *a* molecules. Excitonic coupling between a Chl *a* in the vicinity of ~ 680 nm and Chl *b* is also in line with results of nonlinear polarization spectroscopy at room temperature for trimeric LHC II (Krikunova et al. 2002) and for the highly homologous minor antenna complex CP29 (Voigt et al. 2002). Based on these arguments, Chl *a* 604 has been proposed to carry the lowest energy state of LHC II as an alternative possibility (Pieper et al. 2009). The observation of multiple low-energy states in the present study is generally consistent with recent results of 2D electronic spectroscopy at 77 K, which reported even three-terminal exciton states at 675, 677–678, and 680–681 nm, respectively, which are weakly coupled to each other (Do et al. 2019). It is reasonable to assume that SHB resolves two of the three low-energy states of the latter study. Non-resonantly burned SHB spectra frequently exhibit intense blue-shifted antiholes as also observed here in the case of LHC II. Thus, it is likely that any hole feature corresponding to the third low-energy state at 675 nm reported by Do et al. (2019) may be covered by the latter antihole.

Finally, it has to be added that the identity of the lowest energy states may depend on temperature because the position of the absorption band of Chl *a*612 has been shown to shift to the red with increasing temperature when LHC II switches from a rigid state below 77 K to a state exhibiting conformational flexibility at higher temperatures (Vrandečić et al. 2015; Golub et al. 2018). This red-shift surpassing the

position of the 680 nm state at physiological temperatures may explain the observation of the lowest state on Chl *a*612 in mutagenesis studies. In summary, LHC II seems to possess different, spectrally closely spaced low-energy states, which are located on separate clusters of pigment molecules probably involving Chl *a*604 on the lumenal and Chl *a*612 on the stromal sides of the thylakoid membrane, respectively. This could be an advantage for LHC II function because EET to minor and reaction center core antenna complexes can proceed directly and more efficiently in both lumenal and stromal layers of pigment molecules.

Conclusion

We have re-inspected 4.2 K hole burning data of LHC II with special emphasize on the satellite hole structure, which is indicative of the excitonic coupling of the low-energy states. At low fluence, we observe the lowest/fluorescing excitonic state at ~ 680 nm state in the Chl *a* spectral range and one shallow satellite hole at 649 nm in the Chl *b* spectral range. These findings indicate the presence of a Chl *a/b* heterodimer associated with the lowest energy excitonic state at 4.2 K. At high fluence, the lowest energy hole shifts blue to ~ 677 nm exhibiting two satellite holes located at 673 and 663 nm, respectively, indicating a Chl *a* homotrimer. In summary, LHC II appears to possess two spectrally closely spaced low-energy states at 4.2 K, which are located on distinct clusters of pigment molecules and may involve Chl *a*604 on the lumenal and Chl *a*612 on the stromal sides of the thylakoid membrane, respectively. The positions of the lowest energy states of LHC II appear to depend on temperature above 77 K because of conformational flexibility. This may explain the observation of the lowest state on Chl *a*612 at physiological temperatures in mutagenesis studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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