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► **To cite this version:**

Manuel J. Llansola-Portoles, Andrew A. Pascal, Manuel J. Llansola-Portoles, Fei Li, Pengqi Xu, et al.. Tuning antenna function through hydrogen bonds to chlorophyll a. *Biochimica biophysica acta (BBA) - Bioenergetics*, 2019, 1861 (4), pp.148078. 10.1016/j.bbabi.2019.148078 . hal-02281193

HAL Id: hal-02281193

<https://hal.science/hal-02281193>

Submitted on 18 Nov 2020

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Tuning antenna function through hydrogen bonds to chlorophyll a

Manuel J. Llansola-Portoles ^a, Fei Li ^{b,c}, Pengqi Xu ^d, Simona Streckaite ^a, Cristian Iliaia ^a, Chunhong Yang ^b, Andrew Gall ^a, Andrew A. Pascal ^{a*}, Roberta Croce ^d, and Bruno Robert ^{a*}

^aInstitute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Saclay, F-91198, Gif-sur-Yvette cedex, France

^bKey Laboratory of Photobiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, PR China

^cUniversity of Chinese Academy of Sciences, Beijing 100049, PR China

^dDepartment of Physics and Astronomy, Faculty of Sciences, VU University Amsterdam, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands

Corresponding authors:

* Bruno ROBERT e-mail : bruno.robert@cea.fr

* Andrew A. PASCAL e-mail : andrew.pascal@i2bc.paris-saclay.fr

Keywords: Chl-*a*, hydrogen bonds, light-harvesting, energy regulation, oxygenic photosynthesis

Abstract

We describe a molecular mechanism tuning the functional properties of chlorophyll *a* (Chl-*a*) molecules in photosynthetic antenna proteins. Light-harvesting complexes from photosystem II in higher plants – specifically LHCII purified with α - or β -dodecyl-maltoside, along with CP29 – were probed by low-temperature absorption and resonance Raman spectroscopies. We show that hydrogen bonding to the conjugated keto carbonyl group of protein-bound Chl-*a* tunes the energy of its Soret and Q_y absorption transitions, inducing red-shifts that are proportional to the strength of the hydrogen bond involved. Chls-*a* with non-H-bonded keto C13¹ groups exhibit the blue-most absorption bands, while both transitions are progressively red-shifted with increasing hydrogen-bonding strength – by up 382 & 605 cm^{-1} in the Q_y and Soret band, respectively. These hydrogen bonds thus tune the site energy of Chl-*a* in light-harvesting proteins, determining (at least in part) the cascade of energy transfer events in these complexes.

Introduction

Molecules of the chlorophyll (Chl) family are involved in the first steps of photosynthesis, during which solar energy is converted into chemical potential energy. Chls are responsible for i) absorption of solar photons (principally within light-harvesting proteins), ii) transfer of the resulting excitation energy towards reaction centres, and iii) primary charge separation within these reaction centres, which ultimately leads to the stabilisation of the harvested solar energy as a chemical potential. During these processes, the excitation energy is first stabilised on the lower singlet excited state (S_1) of Chl-*a*, and the subsequent transfers occur between Chl S_1 states with a quantum efficiency close to one [1]. The individual S_1 energies of Chl molecules within this energy transfer cascade are thus an essential factor in determining this efficiency. Understanding the parameters involved in the regulation of the S_1 energetic levels of Chls is therefore a crucial step in disentangling the molecular mechanisms responsible for the exceptionally high quantum yield of photosynthetic energy transfer.

In purple bacterial antenna proteins, tuning of the S_1 excited state energy of bacteriochlorophyll (BChl) molecules has been extensively studied. In these complexes, BChls are involved in particularly strong excitonic interactions, producing a red-shift in the position of their Q_y transition of up to 400 cm^{-1} [2], whereas the electrostatic properties of their binding pockets may modify the energy of this electronic level by up to 150 cm^{-1} [3]. Furthermore, the molecular structure

of BChls contains a conjugated acetyl carbonyl group that plays a major role in tuning the energy of this excited state, via the formation of hydrogen bonds from the surrounding environment (amino acid residues or water molecules). In light-harvesting complexes (LH), the formation of a hydrogen bond to this acetyl group can shift the position of the BChl S_1 by as much 225 cm^{-1} , the extent of the effect being dependent on the strength of the H-bond [3-6]. Figure 1 shows the molecular structures of BChl-*a* and Chl-*a*, as well as a detail from the crystal structure of LH2 from *Rhodoblastus (Rbl.) acidophilus* [7] featuring BChls-*a* 305 & 304 and their H-bonds with Trp α 44 and Tyr α 45, respectively. It is worth noting that modification of the hydrogen bonding state of the conjugated keto carbonyl in position C13¹ does not appear to influence the electronic properties of BChl-*a* [8].

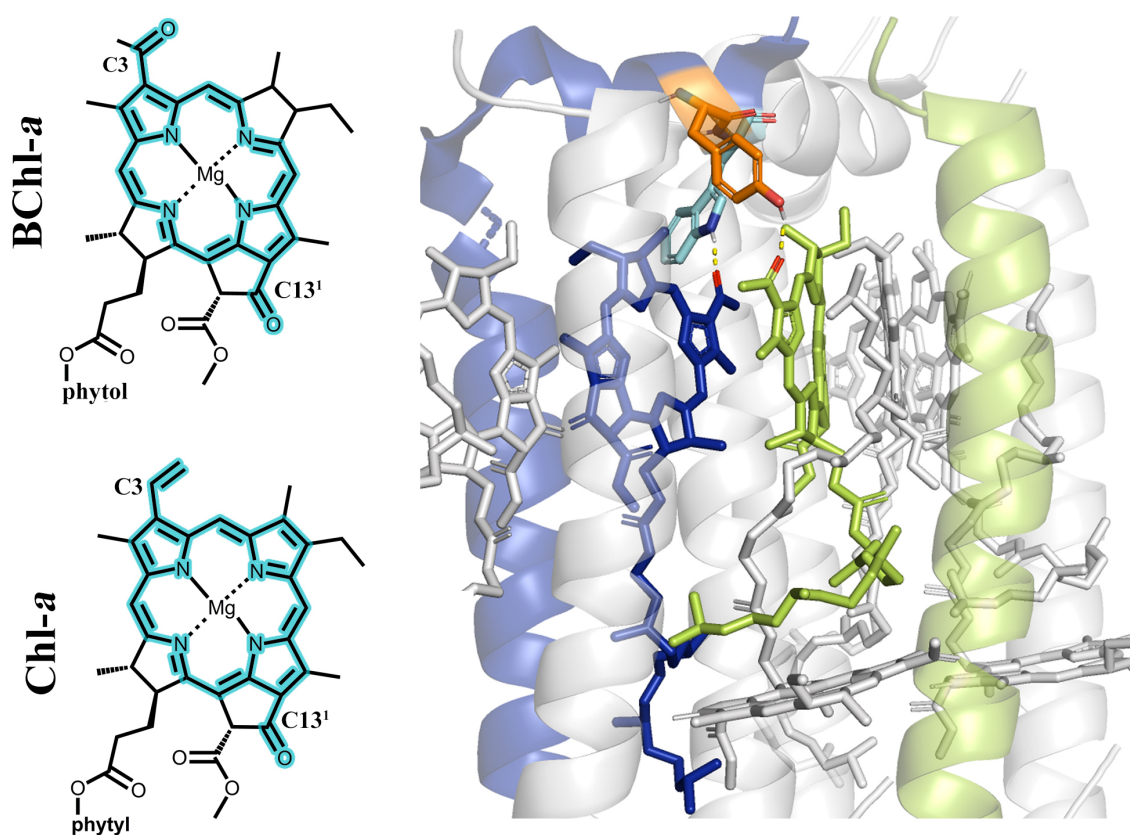


Figure 1/Left: Molecular structure of BChl-*a* and Chl-*a*, highlighting the conjugated part of the macrocycle in cyan, and the C3 and C13¹ carbon atoms. **Right:** Partial view of the crystal structure of light-harvesting complex II from *Rbl. Acidophilus* PDB ID: 1nkz [7], featuring an $\alpha\beta$ heterodimer with its bound B850 and B800 BChls-*a* (α , β subunits in blue, green, respectively). Residues Trp α 44 (orange) and Tyr α 45 (cyan) form H-bonds, shown as dotted yellow lines, to B850 BChls-*a* 304 and 305 (green, blue), respectively.

Oxygenic phototrophs (plants, algae and cyanobacteria), which are responsible for the majority of the primary production on our planet [9], use Chl molecules – instead of the closely-related BChls – as the main light-harvesting pigment. As for the BChls in purple photosynthetic bacteria, the position of the absorption transitions of Chls in photosynthetic proteins is modulated over a wide range. Protein-bound Chls-*a* display absorption transitions in the range 425 to 444 nm (Soret band) and 658 to 725 nm (Q_y band), representing energy differences of 1007 and 1405 cm^{-1} , respectively [10-13]. The molecular structure of Chl-*a* does not possess the C3 acetyl carbonyl group implicated in tuning the position of the Q_y electronic transition for BChl-*a*, and the only conjugated chemical group capable of accommodating hydrogen bonds from the environment is the ketone at position C13¹ – which does not play a significant role in tuning BChls [8]. Therefore, it is difficult to predict the precise origin of the tuning of Chl absorption properties – which may reach 442 cm^{-1} in the absence of strong excitonic interactions, *e.g.* from 663 to 683 nm in photosystem II [10]. Interestingly, experiments performed on Chl-*a* *in vitro* suggest that the energy of the Chl-*a* S_1 excited state is sensitive to both the polarizability and the electrophilicity of the solvent used [14-16] – the latter of which includes the ability to form hydrogen bonds.

The vibrational properties of Chls and BChls *in vitro* and *in vivo* can be studied by resonance Raman spectroscopy, a highly selective technique capable of revealing the precise H-bonding state of each carbonyl group conjugated to the tetrapyrrole ring [17, 18]. In the high-frequency region of the Raman spectra, the major contributions arise from stretching vibrations largely localised on these carbonyl groups. The stretching frequency of a non-H-bonded keto group in a non-polar environment is observed around 1700 cm^{-1} . This frequency downshifts by up to 45 cm^{-1} according to the strength of hydrogen bonds that these groups are involved in [19], while smaller downshifts (up to 10 cm^{-1}) are induced when the keto group is present in a polar environment [20]. The position of the Q_y electronic transition and the Raman mode of the C13¹ keto group have been determined for a wide range of solvents. Lower Q_y transition energies and lower-frequency vibrational modes were observed in highly polarizable and/or protic solvents – the latter being those capable of forming hydrogen bonds. For example, at room temperature Chl-*a* in diethyl ether exhibits a Q_y band at 660.6 nm and a keto mode at 1700 cm^{-1} , while for Chl-*a* in phenol these values are 673.1 nm and 1662 cm^{-1} , respectively [16]. However, the precise nature of each of these effects could not be inferred directly.

The Chls-*a* in oxygenic photosynthetic organisms absorb over a large spectral range [21]. Their protein binding pockets may present very different environments, and so the specific influence of hydrogen bonds on the chlorophyll Q_y transition may be blurred by other contributory factors such as local polarizability and/or excitonic interactions. A definitive description of the role of H-bonds in tuning the electronic properties of photosynthetic Chls has therefore remained elusive. In this work, we demonstrate that the hydrogen-bonding strength to the Chl-*a* C13¹ keto group does indeed play an important role in tuning the absorption properties of the protein-bound pigment, and we estimate the extent of the effect that these interactions may induce. In order to obtain such precise conclusions, we combine low-temperature absorption, evaluating the bathochromic shifts undergone by the Chls-*a* under study, with resonance Raman spectroscopy, to relate these shifts to the H-bonding state of their keto carbonyl group.

Materials and Methods

α DM- and β DM-LHCII were isolated from spinach as described by Xu et al [22]. In brief, thylakoids at Chl concentration of 1 mg/mL were solubilized by adding the same volume of buffer containing 1.2 % α DM or 2 % β DM, respectively. The mixture was gently vortexed for a few seconds, and unsolubilized material was removed by centrifugation at 17000 g for 10 minutes. The supernatant was then loaded onto a sucrose density gradient containing 10 mM Hepes pH 7.5, 0.03 % α DM or 0.06 % β DM, respectively, and 0-0.1 M sucrose. The LHCII band was collected after overnight ultracentrifugation at 280000 g. The CP29 protein was obtained from spinach by column chromatography of Tris-washed photosystem II [23], using a modification of the procedure described by Henrysson et al [24].

UV-Vis Absorption spectra were measured using a Varian Cary E5 Double-beam scanning spectrophotometer with a 1.0 cm pathlength cuvette. Low temperature measurements were performed in a helium bath cryostat (Maico Metriks, Tartu, Estonia); 60 % glycerol (v/v) was added to the sample to prevent devitrification. Absorption difference spectra were calculated to minimise global changes and yield a conservative shape (*i.e.* with approximately equal positive and negative peak areas), by varying the normalization factor used.

Resonance Raman spectra were recorded at 77 K using an LN₂ flow cryostat (Air Liquide, France). Laser excitations at 406.7 & 413.1 nm were obtained with a Coherent Kr⁺ (Innova 90) laser. Output laser powers of 10–100 mW were attenuated to < 5 mW at the sample. Scattered light was

collected at 90° to the incident light, and focused into a Jobin-Yvon U1000 double-grating spectrometer (1800 grooves/mm) equipped with a red-sensitive, back-illuminated, LN₂-cooled CCD camera. Sample stability and integrity were assessed based on the similarity between the first and last Raman spectra. Difference spectra were calculated by normalization to the methine bridge stretching mode of Chl-*a* (*ca.* 1612 cm⁻¹ for five-coordinated Mg), which is not directly affected by the state of the keto group [25, 26].

Results

To study the influence of hydrogen bonds on the electronic properties of Chl-*a*, we have chosen the most abundant plant antenna protein LHCII, a trimer which contains 8 Chl-*a*, 6 Chl-*b* and 4 carotenoids per monomer [27]. Depending on the purification protocol – and more specifically, on the use of either α - or β -dodecyl-maltoside (α DM or β DM, respectively) to solubilise the thylakoid membrane – the absorption properties of Chl-*a* molecules in this protein are slightly altered [28] (Figure 2a). The 4.2 K absorption spectrum of LHCII solubilised with α -dodecyl-maltoside (α DM-LHCII) displays a Chl-*a* Q_y absorption peak at 675.3 nm with a shoulder at 669.9 nm (14808 & 14928 cm⁻¹, respectively), while the Soret region exhibits an envelope of absorption bands with a maximum around 431 nm (23202 cm⁻¹; blue spectrum in Figure 2a). The absorption spectrum of LHCII solubilized with β -dodecyl-maltoside (β DM-LHCII) is red-shifted in both Q_y and Soret bands relative to α DM-LHCII – the main Q_y peak (also at 675.3 nm) increases in intensity relative to the 669.9 nm shoulder, and an additional shoulder is observed on the red side at 676.4 nm (14784 cm⁻¹). The Soret band peaks at 440 nm (22727 cm⁻¹; red spectrum in Figure 2a). The typical S-shaped difference spectra “ β DM-LHCII-minus- α DM-LHCII” (Fig 2b&c) clearly indicate a red-shift for β DM-LHCII relative to α DM-LHCII in both Q_y and Soret regions (658 to 674.7 nm and 430 to 441.5 nm, respectively), for a sub-population of Chl-*a* molecules. We estimate that one or two Chls-*a* are involved in this shift, based on the relative area of the difference peaks.

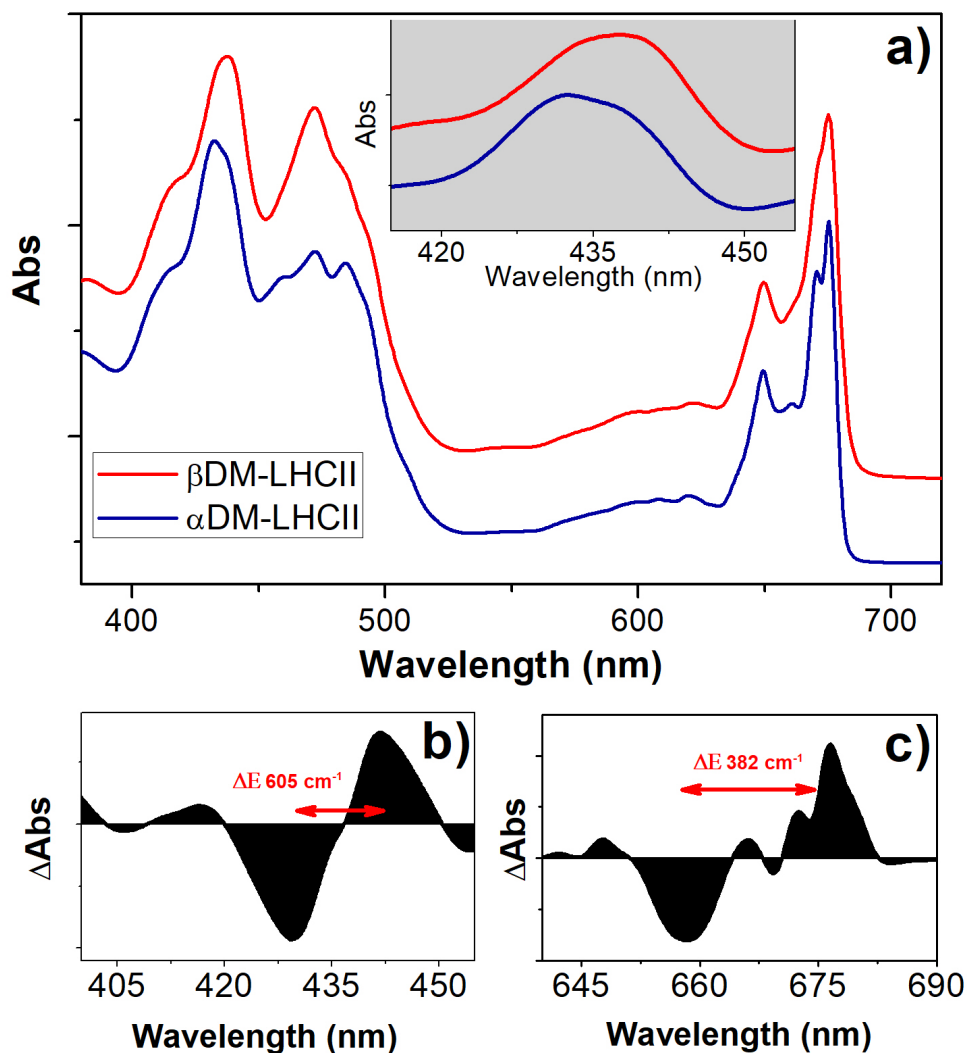


Figure 2/ a) Absorption spectra at 4.2 K of α DM-LHCII (blue) and β DM-LHCII (red). **b)** & **c)** Difference absorption spectra “ β DM-LHCII minus α DM-LHCII” in the Soret and Q_y regions, respectively.

In order to investigate the origin of this absorption red-shift, we measured the resonance Raman spectra of the two LHCII preparations at 77 K, using excitations enhancing the Chl-*a* signal at 406.7 and 413.7 nm (Figure 3). In both samples, the region corresponding to keto vibrational modes ($1650\text{--}1700 \text{ cm}^{-1}$) can be divided into three main groups of contributions – as discussed previously in the literature for β DM-LHCII [29]. The spectra exhibit a low frequency contribution at 1659 cm^{-1} , corresponding to Chls-*a* involved in strong hydrogen bonds; a broad envelope around $1670\text{--}1680 \text{ cm}^{-1}$, corresponding to Chls-*a* involved in medium-strength H-bonds; and two bands at 1685 and 1700 cm^{-1} due to weakly- or non-H-bonded Chls-*a*. When comparing the two preparations, β DM-LHCII

shows a more intense contribution around 1670 cm^{-1} , indicating an increase in the population of Chl-*a* with medium-strength H-bonded keto groups with respect to α DM-LHCII. This is complemented by a reduction in intensity for the two peaks at 1685 and 1700 cm^{-1} , corresponding to two Chl-*a* molecules with keto carbonyl groups that are weakly- or non-H-bonded. In order to analyze the differences between the two samples more precisely, we calculated their difference spectra “ β DM-LHCII-minus- α DM-LHCII” (Figure 3c & d), using the methine bridge modes around 1612 cm^{-1} for normalization. The S-shaped spectra confirm that two bands at 1685 and 1700 cm^{-1} in α DM-LHCII are shifted to $\sim 1670\text{ cm}^{-1}$ in β DM-LHCII – corresponding to a decrease in weakly-bonded or free keto groups, concomitant with an increase in keto groups involved in medium-strength hydrogen bonds. These results also show non-negligible differences between the resonance Raman spectra obtained at each of the two excitation energies (406.7 & 413.1 nm). We observe at 406.7 nm excitation, that the increase in intensity at lower energies (below 1670 cm^{-1}) is relatively small with respect to the decrease at higher energies (1685 and 1700 cm^{-1}). The situation is inverted for 413.1 nm excitation – a greater increase in intensity is observed at 1670 cm^{-1} , with a relatively smaller reduction at 1685 and 1700 cm^{-1} .

Additionally to the H-bonding state of its conjugated carbonyl group, macrocycle distortions are expected to have a significant effect on the absorption properties of Chl-*a*, and such distortions are easily probed by resonance Raman. A number of modes have been shown to be sensitive to these distortions [25, 30, 31], the most sensitive of which are in the high frequency region (1500 - 1615 cm^{-1}). The band ca. 1550 cm^{-1} , attributed to complex vibrational modes of the macrocycle, is primarily sensitive to macrocycle distortions - for example, it is located at 1550 and 1540 cm^{-1} when the central Mg is five- or six-coordinated, respectively, and can downshift to 1517 cm^{-1} upon large distortions. A second band, mainly arising from methine-bridge stretching modes, appears around 1600 cm^{-1} when the central magnesium is six-coordinated, and can shift to 1615 cm^{-1} when the macrocycle is strained [25, 30]. As these two modes are insensitive to the detergent used to solubilize LHCII, in both position and bandwidth (data not shown), it may be safely concluded that the differences observed between α DM- & β DM-LHCII do not result from distortion of the Chls-*a* involved.

Altogether, we conclude the following from these observations: i) the hydrogen-bonding pattern of two Chl-*a* molecules in the LHCII structure is altered according to the detergent used for solubilisation, being weakly- or non-H-bonded in α DM-LHCII, but involved in medium-strength H-bonds in β DM-LHCII; ii) the formation of these hydrogen bonds is accompanied by red-shifts of 382

& 605 cm^{-1} (ca. 17 & 11.5 nm) in the Q_y and Soret band of Chl-*a*, respectively; iii) excitation at 406.7 nm preferentially favors the vibrational modes of Chl-*a* molecules whose carbonyls are weakly or non-H-bonded, while 413.1 nm favors those with strongly-H-bonded keto groups – consistent with this Soret red-shift.

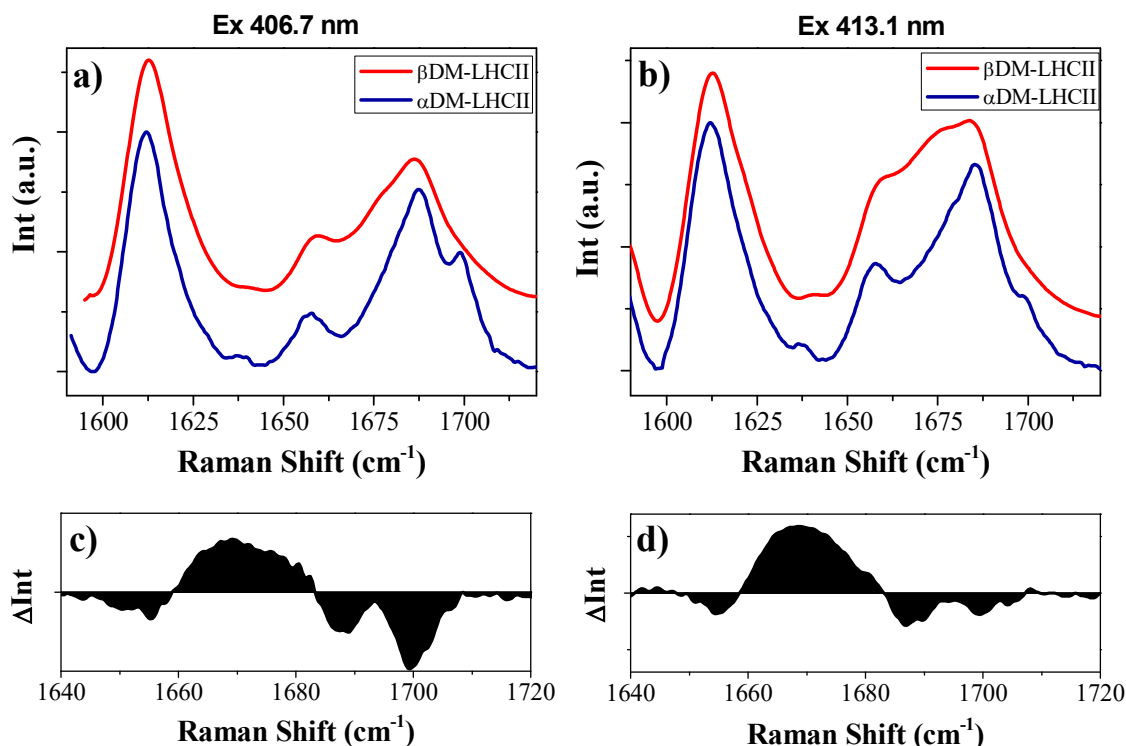


Figure 3 | Resonance Raman spectra of α DM-LHCII (blue) and β DM-LHCII (red) at 77 K in the $1590 - 1720\text{ cm}^{-1}$ region (a, b), and their differences “ β DM-LHCII-minus- α DM-LHCII” in the $1640 - 1720\text{ cm}^{-1}$ region (c, d), for excitations at 406.7 nm (a, c) and 413.1 nm (b, d).

We have tested whether this apparent relationship, between the strength of hydrogen bonds to the Chl-*a* keto and the position of the electronic absorption transitions, can be extended to other Chl-*a*-containing proteins. CP29 is one of the minor light-harvesting complexes of photosystem II. The most recent structure in the photosystem II supercomplex C2S2M2 from peas reveals the presence of 10 Chl-*a*, 4 Chl-*b*, and 3 carotenoid molecules [32]. This structure is very similar to an LHCII monomer, resulting from their high degree of sequence homology [33], and this similarity extends to the microenvironments of (most of) the Chl binding pockets. This minor LHC protein is therefore an excellent candidate to test our hypothesis.

The absorption spectra of β DM-LHCII and CP29 [23] at 4.2 & 13 K, respectively, are compared in Figure 4a. Based on previous reports of the evolution of CP29 absorption with temperature [23, 34], we do not expect any significant change in the absorption spectrum of CP29 between 13 and 4.2 K. CP29 exhibits a Chl-*a* Soret region with a quasi-symmetrical band peaking *ca.* 438 nm, and a Chl-*a* Q_y region with a single band peaking *ca.* 674 nm accompanied by a small absorption tail below 660 nm. In order to observe the absorption changes relative to β DM-LHCII, we calculated the difference absorption spectrum “CP29-minus- β DM-LHCII” (Figure 4b). While the large negative component in the Chl-*b* Q_y band (*ca.* 649 nm) reflects the higher Chl-*a/b* ratio for CP29, the negative shoulder at 656 nm should rather be attributed to Chl-*a*. Together with the positive feature on the red side of the Chl-*a* Q_y band, around 673 nm, this indicates that CP29 has a greater proportion of red-absorbing Chls-*a* than does LHCII.

The 77 K resonance Raman spectra of CP29 [29] in the high-frequency region is shown in Figure 4c for excitation at 406.7 & 413.1 nm. At 406.7-nm excitation, a principal contribution is observed for CP29 at 1675 cm⁻¹ with a pronounced shoulder at 1658 cm⁻¹. At 413.1 nm excitation, the major contribution is observed at 1670 cm⁻¹ with a small shoulder around 1680 cm⁻¹ and a larger one at *ca.* 1659 cm⁻¹. The CP29 signal at both excitations exhibits little or no contributions around 1690 – 1700 cm⁻¹, indicating that few or no Chls-*a* are present in CP29 with free or weakly-H-bonded keto groups. Most of the Chls-*a* are involved in medium-strength H-bonds (contributions at 1670-1680 cm⁻¹), while a smaller number are strongly H-bonded (1658 cm⁻¹). As observed for β DM-LHCII, excitation at 406.7 nm enhances the modes corresponding to weakly- or non-H-bonded keto groups selectively, whereas excitation at 413.1 nm favors the medium-to-strongly H-bonded keto groups. The resonance Raman signal of CP29 with respect to β DM-LHCII shows clear differences for both 406.7 & 413.1 nm excitations (Figure 4c). About 30 % of the keto contributions for β DM-LHCII are observed around 1680 – 1700 cm⁻¹, whereas CP29 exhibits negligible contributions in this region. Thus CP29 exhibits a greater proportion of medium/strong H-bonds than does β DM-LHCII.

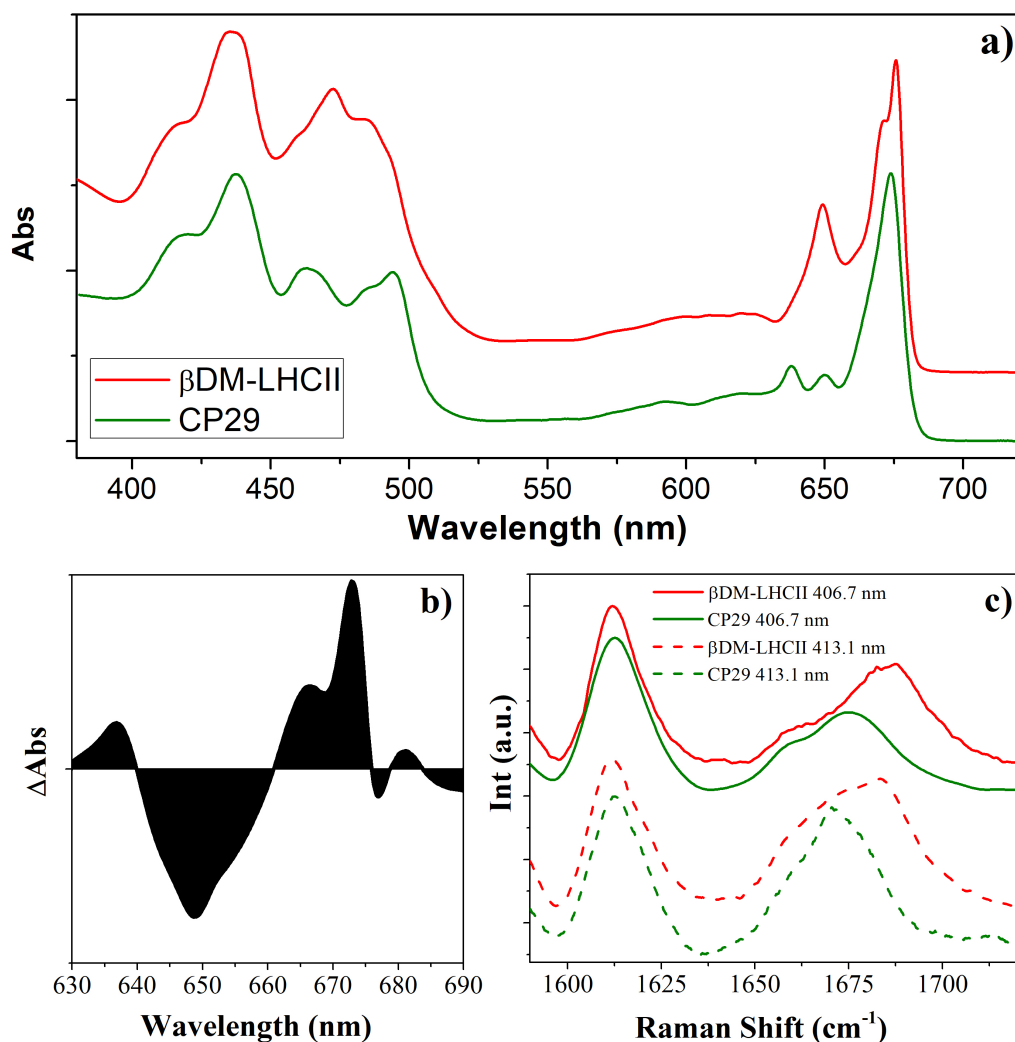


Figure 4 | **a)** Absorption spectra at 4.2 K of β DM-LHCII (red) and CP29[23] (green). **b)** Difference absorption spectra “CP29-minus- β DM-LHCII” in the Q_y spectral region. **c)** 77 K resonance Raman spectra of β DM-LHCII (red) and CP29[29] (green) excited at 406.7 nm (solid line) and 413.1 nm (dashed line).

Discussion

Our results first show that, in resonance Raman spectra of Chl-*a*, excitation at 406.7 nm enhances the contributions of Chls-*a* whose keto groups are weakly-/non-hydrogen-bonded, whereas 413.1 nm excitation favors Chls-*a* with strongly-hydrogen-bonded keto groups. This phenomenon is very clear in the spectra of all the proteins studied here, as well as for all other LHC proteins in the literature [35-40]. We explain this observation in terms of the resonance effect [41], where a higher intensity Raman signal, in defined conditions of resonance, is observed for a molecule

possessing a more intense absorption coefficient at the considered excitation wavelength. Our observations thus indicate that Chls-*a* with weakly-interacting or unbound keto carbonyl groups absorb relatively more at 406.7 nm, while those with hydrogen-bonded carbonyls tend to absorb more intensely at 413.1 nm. The natural conclusion is that the Soret transitions of Chls-*a* with strongly-hydrogen-bonded keto groups are red-shifted relative to those of Chls-*a* with free keto groups – consistent with the observed shifts in the Soret transition between the LHC proteins measured here. The literature indicates that isolated Chl-*a* *in vitro* exhibits parallel shifts in the Soret and Q_y transitions [14, 15, 42], and so it is tempting to conclude that the presence of hydrogen bonds to the Chl-*a* carbonyl will also red-shift the position of this Q_y band. Comparison of the spectra of LHCII obtained by two different purification methods confirms this conclusion. In LHCII purified in β-dodecyl-maltoside, about two Chls-*a* are red-shifted relative to LHCII in α-dodecyl-maltoside - in both the Soret region (430 → 441.5 nm; ~ 605 cm⁻¹) and the Q_y band (658 → 675 nm; ~ 382 cm⁻¹). These absorption shifts correlate with the formation of hydrogen bonds to the carbonyl groups of at least two Chls-*a*, as observed in resonance Raman spectra. In addition, a comparative analysis of the spectra of CP29 is fully consistent with these conclusions. Resonance Raman spectra of CP29 exhibit a major contribution from Chl-*a* keto groups involved in medium-to-strong hydrogen bonds, and a smaller contribution (~30 % of the total intensity) from strongly-H-bonded keto groups – no modes are observed corresponding to free or weakly-H-bonded carbonyls. This fits well with the Q_y band observed in the absorption spectrum, which exhibits a quasi-symmetrical peak with a maximum at 673.8 nm, as expected for Chls-*a* involved in medium to relatively strong hydrogen bonds. This example is therefore consistent with a specific correlation between the strength of H-bonding to the keto carbonyl of Chl-*a* and the position of the Q_y transition of this molecule – as observed for LHCII. The available structures of LHCII [27] and CP29 [43] show that ca. 50 and 66 % of the Chl-*a* keto groups, respectively, are involved in medium/strong H-bonds – in general agreement with our conclusions. In addition, analysis of the vibrational structure of the terminal emitter chlorophylls in LHCII and CP29 by Fluorescence Line Narrowing confirm, for both proteins, that the Chl(s)-*a* with lower Q_y energy (*i.e.* the most red-shifted) have their C13¹ keto group strongly H-bonded [34, 44]. Indeed, a global analysis of all the published resonance Raman spectra for LHC proteins tends to support our conclusions [35-40], although the differences in pigment stoichiometries, as well as the presence of pigment-pigment and pigment-protein interactions in some cases, render a definitive comparison somewhat complex.

In conclusion, we propose that the energy levels of Chl-*a* are, at least in part, tuned through the formation of hydrogen bonds to their C13¹ keto group within the protein binding pocket. Our observations all converge towards the same conclusion – that the formation of an H-bond at the level of the keto carbonyl of Chl-*a* tunes (and more precisely red-shifts) the position of the Q_y transition, and it is likely that the extent of this absorption red-shift correlates with the strength of the formed H-bond. The observed effect of a hydrogen bond to the C13¹ keto group is significantly larger in these photosynthetic proteins than that described for Chl-*a* in solvents (382 cm⁻¹ here, *cf.* 60 cm⁻¹ *in vitro* [15]). It is possible that, in contrast to the isotropic environment in solvents, the protein is able to fine-tune the relative position and orientation of the Chl-*a* molecule and the surrounding amino acids, in order to maximize the effect of this H-bond.

Acknowledgements

This work was supported by the ERC funding agency (PHOTPROT project), the French Infrastructure for Integrated Structural Biology (FRISBI) ANR-10-INBS-05, the Dutch Organization for scientific research via TOP grant (No 84713002) and the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 675006 (SE2B).

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