Conformational dynamics of photosynthetic Light-Harvesting Complex II in a native membrane environment

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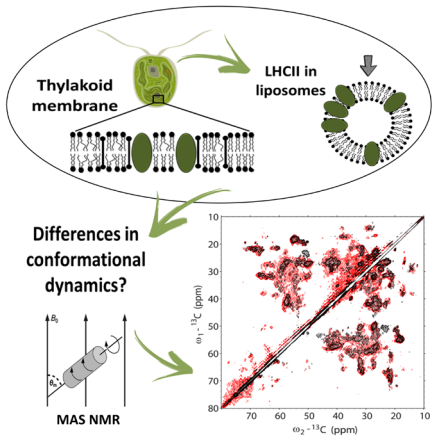
ABSTRACT Photosynthetic light-harvesting antennas (LHCs) of plants, moss and green algae can undergo dynamic conformational transitions, which has been correlated to their ability to adapt light harvesting and excitation-quenched, dissipative states. Herein, we demonstrate the application of *in situ* solid-state NMR spectroscopy on wild type, heterogeneous thylakoid membranes of *Chlamydomonas reinhardtii* (*Cr)* and on *Cr* Light-Harvesting Complex II (LHCII) in lipid bilayers to detect LHCII conformational dynamics in a native membrane environment. We show that membrane-reconstituted LHCII contains selective sites that undergo fast, large-amplitude motions, including the phytol tails of two chlorophylls. Protein plasticity is observed in the N- and C-terminal stretch and in the helix edges facing the lumen. In thylakoid membranes, the dynamics of those protein and pigment sites is significantly reduced. This suggests that *in situ*, the protein complexes are locked in specific conformational states.

TOC GRAPHICS



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Multi-pigment protein complexes in plants, moss, and photosynthetic algae perform delicate photo-physical and chemical tasks. To maintain a homeostatic balance in fluctuating sunlight conditions, these processes are tightly regulated by dynamic membrane responses to prevent photodamage [1-2]. The most abundant light-harvesting complex, Light Harvesting Complex II (LHCII) has been studied extensively for its molecular role in light-harvesting regulation. LHCII has the capacity to switch between light-harvesting and photoprotective, excitation-quenched states [2-12], and "fluctuating antenna" theoretical models have been proposed to describe excitation energy migration in photosynthetic thylakoid membranes [13-14]. The intrinsic dynamics of LHCII proteins has been studied in crystals or detergent solutions [4, 10, 15-16], which conditions however are different from the native thylakoid membranes where the proteins are held in specific arrangements within LHCII-Photosystem II supercomplexes [17] and where xanthophylls and lipids further control their conformational dynamics [18].

Until now, no structure-based methods have been presented to detect the molecular structure and dynamics of LHCs inside a membrane environment. We here apply solid-state NMR (Nuclear Magnetic Resonance) spectroscopy to study the conformational dynamics of LHCII in lipid bilayers and in native thylakoid membranes. Solid-state NMR spectroscopy is a powerful tool for atomistic detection of membrane proteins and in some cases, NMR signals of membrane proteins could even be detected in native membrane or cellular environments, using overexpression of the target proteins in prokaryotic and eukaryotic host-expression systems [19-25]. We take advantage of the fact that our target protein is already present at a high abundance in thylakoid membranes under native conditions and recorded NMR spectra of thylakoid membranes that were isolated from wild type *U*-^{13}C *Chlamydomonas reinhardtii* (*Cr*) cells. In

addition, we recorded spectra of isolated LHCIIs that were reconstituted in lipid bilayers prepared from native-like lipid mixtures.

A biochemical analysis of our thylakoid preparations (Fig. S1 and S2) shows that the LHCII trimers are the most abundant pigment-containing complexes in the thylakoid membranes. Different molecular-weight bands are distinguished for LHCII due to the fact that the LHCII trimers are isomers consisting of different polypeptides of which the most abundant types are Lhcbm1, Lhcbm2/7 (Lhcbm2 and Lhcbm7 have identical mature peptide sequences) and Lhcbm3 [26]. Our LHCII proteoliposomes contain the lipids MGDG (monogalactosyldiacylglycerol), DGDG (digalactosyldiasylglycerol), SQDG (sulfoquinovosyl diacylglycerol) and the phospholipid PG (phosphatidylglycerol) at a protein to lipid ratio of 1:55 (*mol/mol*) to mimic the protein density and lipid composition of the native thylakoid membranes [27]. The 77K fluorescence spectrum of the proteoliposomes shows a prominent band at 700 nm (Fig. S3), which is characteristic for LHCII aggregates. Aggregation of LHCII in membranes is associated with the formation of quenching states [28-29]. Indeed, a fluorescence lifetime analysis reveals an average lifetime of 0.7 ns for LHCII proteoliposomes compared to a lifetime of 3.5 ns for LHCII in α-DM detergent (Table S1 and Fig. S4) indicating that LHCIIs in the proteoliposomes are in a strongly quenched state.

Two complementary types of solid-state NMR experiments were employed, selective for rigid or highly dynamic, molecules, respectively. In Cross-Polarization (CP) based experiments, ^{1}H-^{13}C or ^{13}C-^{15}N magnetization is transferred via the dipolar interaction. Dipolar-based transfer experiments become inefficient for dynamic molecules that display sub-microsecond motions, as the dipolar couplings are averaged due to fast tumbling. In INEPT based experiments,

magnetization is transferred via *J* couplings. In the presence of strong motions, where orientation dependent interactions are sufficiently averaged out by magic-angle spinning and fast molecular tumbling, the combination of *J*-based pulse sequences can be employed for screening very flexible parts of large biomolecules that undergo large-amplitude motions on ps-ns time scales [30]. Membrane proteins typically contain large transmembrane stretches with low flexibility, which are predominantly detected in dipolar-based experiments.

Dipolar-based ^{13}C-^{13}C (CC) and ^{15}N-^{13}C (NC) experiments of LHCII in proteoliposomes (Fig. 1) verify the incorporation of isotope labels. Resonance signals of specific amino-acids can be identified and classified as helix or coil based on their unique chemical-shift patterns that agree with the known crystallographic structure of plant LHCII. In addition to signals originating from the protein content, Chl and carotenoid signals can be identified in regions of the CC spectrum where no protein signals are expected. Also a single set of lipid glycerol carbon resonances is observed. It is well-known that each monomer unit of LHCII contains one phosphatidyl-glycerol (PG) lipid, which forms the ligand for Chl611 (nomenclature Liu et al. [15]). Based on their chemical shifts, we attribute the detected lipid resonances to the head carbons of LHCII-bound PG lipid molecules.

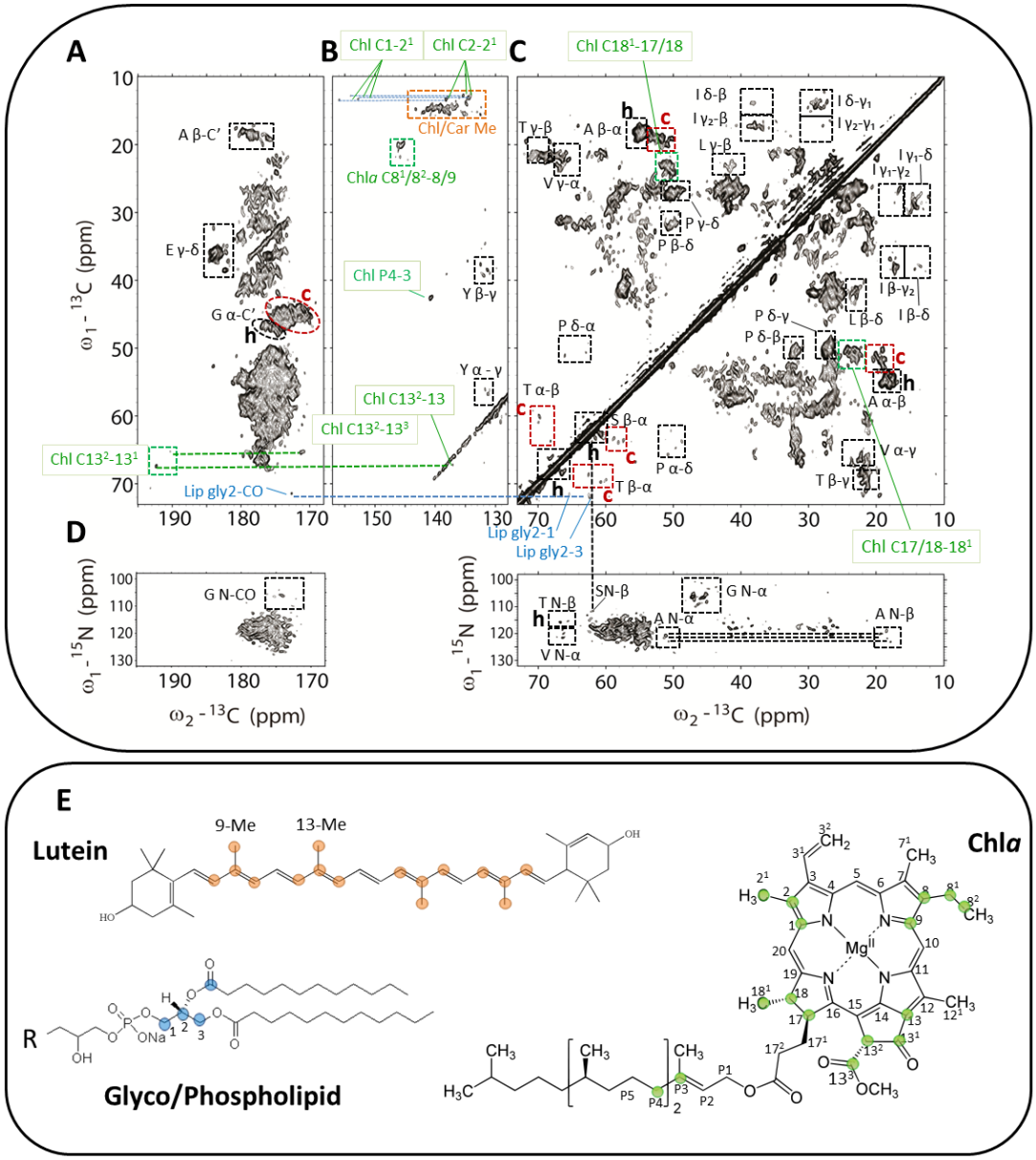

Figure 1. NMR spectra of LHCII proteoliposomes. *A-C: ^{13}C-^{13}C CP-PARIS spectrum; D: ^{15}N-^{13}C NCACX spectrum; E: Chemical structures of lutein, Chl* a *and of a glyco-or phospholipid. Helix (h) and coil (c) correlations of Thr, Ser, Ala and Gly are indicated in black and red in spectra A-D and Chl, carotenoid and lipid correlations are indicated in green, orange and blue. Their attributed carbon atom types are color-coded in panel E.*

Fig. 2A shows the dipolar-based CC spectrum of LHCII (black) overlaid with the spectrum of thylakoid membranes (red). The strong overlap of the two spectra demonstrates, in agreement with the biochemical analysis, that the most abundant signals in the thylakoid spectrum arise from LHCII. Many of the additional peaks in the thylakoid spectrum can be attributed to lipids and in particular, resonance signals accumulated between 70-80 ppm are from the galactosyl heads.

We use correlation signals of Ala, Thr and Ser as reporters for the protein secondary structure. Those amino-acid types are chosen because of the clear spectral separation of their Cα-Cβ correlation signals. The Ala, Thr and Ser amino acids are distributed over the LHCII polypeptide helical and coil region, and their resonance signals report on the folds of the transmembrane helices as well as of the connecting loops and terminal stretches. The insets of panel 2B, C and D show Ala, Thr and Ser selective regions. The helical (h) and coil (c) signals fall into separate regions as indicated with the red and black boxes.

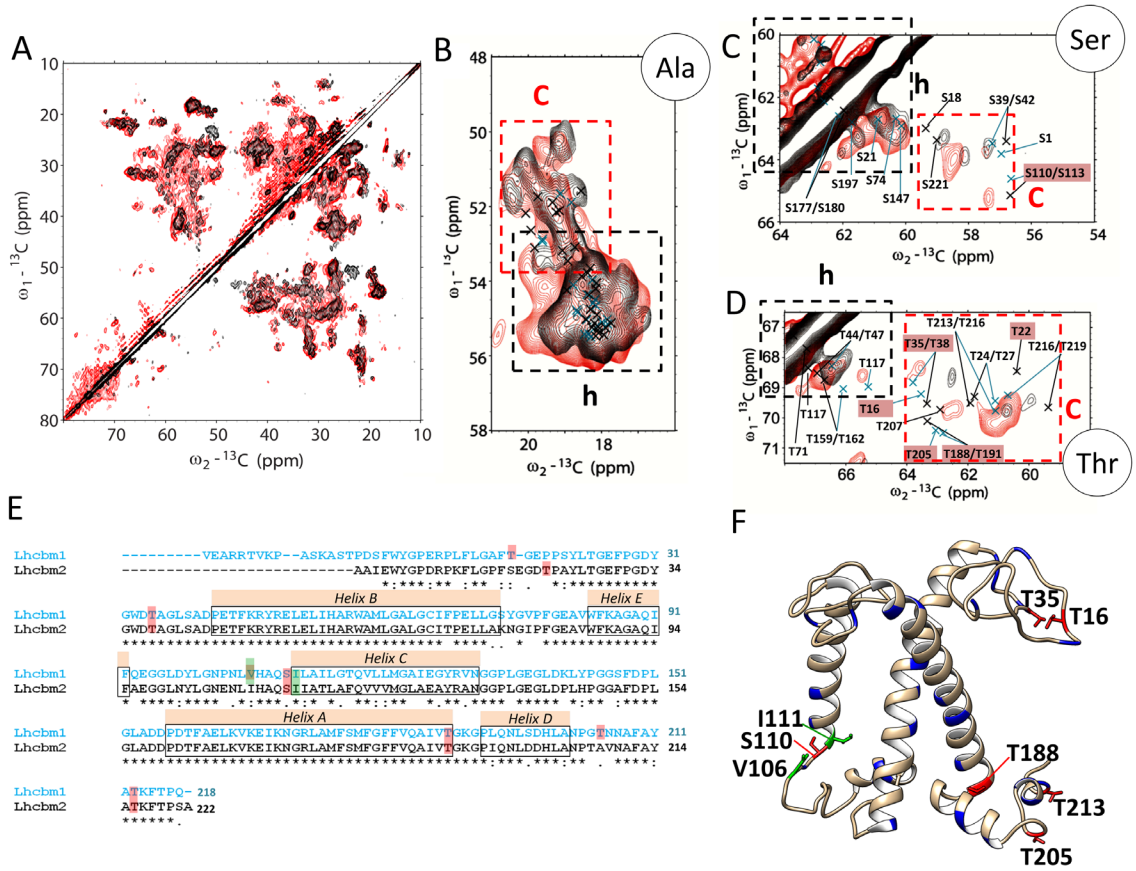

Figure 2. NMR comparison of LHCII proteoliposomes and thylakoid membranes and structural analysis. *A-D: ^{13}C-^{13}C CP-PARIS spectrum of thylakoid membranes (red) with the LHCII spectrum (black) overlaid. The insets show Ala (B), Thr (C) and Ser (D) spectral regions overlaid with chemical-shift predictions of Lhcbm1 (cyan crosses) and Lhcbm2 (black crosses). Predicted shifts that significantly deviate from experimental correlations are highlighted in red. Helix (h) and coil (c) regions are indicated by black and red boxes. E: Lhcbm1 and Lhcbm2 sequences, highlighting residues for which experimental NMR chemical shifts deviate from their predictions in LHCII CC (red) or NC (green) spectra. F: Homology model of Lhcbm1, highlighting Thr, Ser and Ala residues with matching chemical shifts in blue and residues with deviating NMR chemical shifts in red or green.*

In panel 2B, C and D the experimental resonances of Ala, Thr and Ser for isolated LHCII are shown in black. To compare the experimental shift correlations with the crystallography-based *Cr* LHCII structure, three steps were undertaken: (i) models were built for Lhcbm1 and Lhcbm2, the two most abundant LHCII polypeptides in *Cr*, based on the spinach LHCII crystal structure; (ii) Cα and Cβ chemical shifts predictions were generated from the Lhcbm1 and Lhcbm2 homology models using the program SHIFTX2 [31] and (iii) simulated CC correlations were generated from the predicted chemical shifts using the program FANDAS [32]. The resulting Cα-Cβ chemical shift predictions are overlaid in the inset spectra with cyan crosses for Lhcbm1 and with black crosses for Lhcbm2. The full spectrum with all the predictions overlaid can be found in Fig. S5. Comparing the experimental resonances with the structure-based predictions, most of the predictions match well with experimental correlations. However, for several Ser and Thr residues in the coil regions the signals are weak, which suggests that the residues are dynamic or adapt multiple conformations. Furthermore, it is remarkable that three strong coil Ala peaks that are not predicted by the structure-based models appear in the experimental spectrum. Those Ala might involve amino acids in the N terminus. The latter is indeed not resolved in the LHCII crystal structures and no predictions have been generated.

Deviations between predicted (ω pred) and experimentally observed (ω exp) Cα-Cβ or N-C protein backbone correlations are considered significant if $\sqrt{[(\omega_1 \text{ exp} - \omega_1 \text{ pred})^2 + (\omega_2 \text{ exp} - \omega_2 \text{ pred})^2]} \geq 1.2$ ppm. The involved residues are indicated in 2E and 2F in red (deviations in the CC spectrum) or in green (deviations in the NC spectrum, see Fig. S6). They highlight protein sites that are dynamic or for which the conformation differs from the X-ray structure. The pattern shows that these are located in the N-terminal stretch and the CE loop, the edge of helix A and the C terminus facing the lumen. According to the LHCII crystal structures, none of the

highlighted residues are in Van der Waals contact with pigment ligands, excluding protein-pigment interactions as the cause of chemical-shift anomalies.

We now compare the data of LHCII proteoliposomes to the LHCII signals in native thylakoid membranes (red spectrum). The insets show that the thylakoid CC spectrum, in contrast to the proteoliposome spectrum, contains strong Thr and Ser coil correlations that match well with their predicted shifts. This indicates that the Lhcbm structure models are representative the protein structure in native membranes and suggests that the Thr and Ser coil regions of LHCII have constrained dynamic in native membranes. Several peaks are close to predictions of non-conserved amino acids, for instance of T117 that can be found in the sequence of Lhcbm1 but not Lhcbm2 and of S221 that is found in Lhcbm2 but not conserved in the sequence of Lhcbm1.

To further explore the presence of dynamic protein sites, we proceeded with *J*-based (INEPT-TOBSY) experiments that are exclusively selective for molecules with strong dynamics and large-amplitude motions. Fig. 3 presents the 2D INEPT-TOBSY spectrum of the proteoliposomes (black) overlaid on the INEPT-TOBSY spectrum of thylakoid membranes (red). As expected by the fact that the majority of the protein correlations are detected in the dipolar-based spectra, only a limited set of protein signals are detected that we refer to as "*J*" amino acids. The overlaid spectra clearly show that many more *J* signals are detected in the INEPT-TOBSY spectrum of LHCII (black) than in the spectrum of thylakoid membranes (red). We could assign the *J* residues in the LHCII spectrum to Ala, Thr, Ser, Phe, Pro, Val, Lys, Ile, Glu, Asn and Leu amino acid types (see Table S2 and Fig. 3 and Fig. S7). Because *J* residues represent amino acids that display fast and large-amplitude motions, we expect them to be located in the non-helical stretches and in the N- and C-termini. Indeed, the N terminus of the most abundant LHCII polypeptides, Lhcbm1, 2/7 and 3, contain several Thr, Ser and Ala amino

acids and many of the observed *J* amino-acid types can be found in the C-terminal stretch of Lhcbm1 starting from A212 (ATKFTPQ) or the equivalent C stretch in Lhcbm2/7 and Lhcbm3 (ATKFTPSA). Interestingly, three distinct peaks are observed in the proteoliposome spectrum for Ala, for Thr and for Ser, whereas their *J*-based correlations usually tend to strongly overlap. The INEPT-TOBSY spectrum of the proteoliposomes also contains two sets of Chl phytol chain signals (Fig. 3 and Table S3), revealing that two Chls have dynamic tails. In the LHCII crystal structures, the phytol chain structures of Chl*b* 605 and 606 (nomenclature according to Liu et al. [15]) are not observed, while the other Chl phytol tails are resolved and tightly involved in stabilizing pigment-protein interactions. We thus attribute the two sets of Chl phytol resonances to the dynamic tails of Chls 605 and 606. Those Chls are close to residues V106, S110 and I111 of which deviating shifts have been observed in the dipolar-based spectra. Chl605 and 606 are located at the exterior of the complex and their tails may point outward towards the lipid bilayer, having freedom to move in the lateral plane of the membrane.

The INEPT TOBSY spectrum of LHCII contains many signals of *J* amino-acids that are not observed in the thylakoid spectrum, which indicates, in agreement with the analysis of the CP-based spectra, that selective sites of LHCII are more dynamic in liposomes than in native thylakoid membranes. In particular, the dynamic Chl tails that are observed in the LHCII INEPT spectrum suggest that the LHCIIs are less tightly packed in the reconstituted membranes.

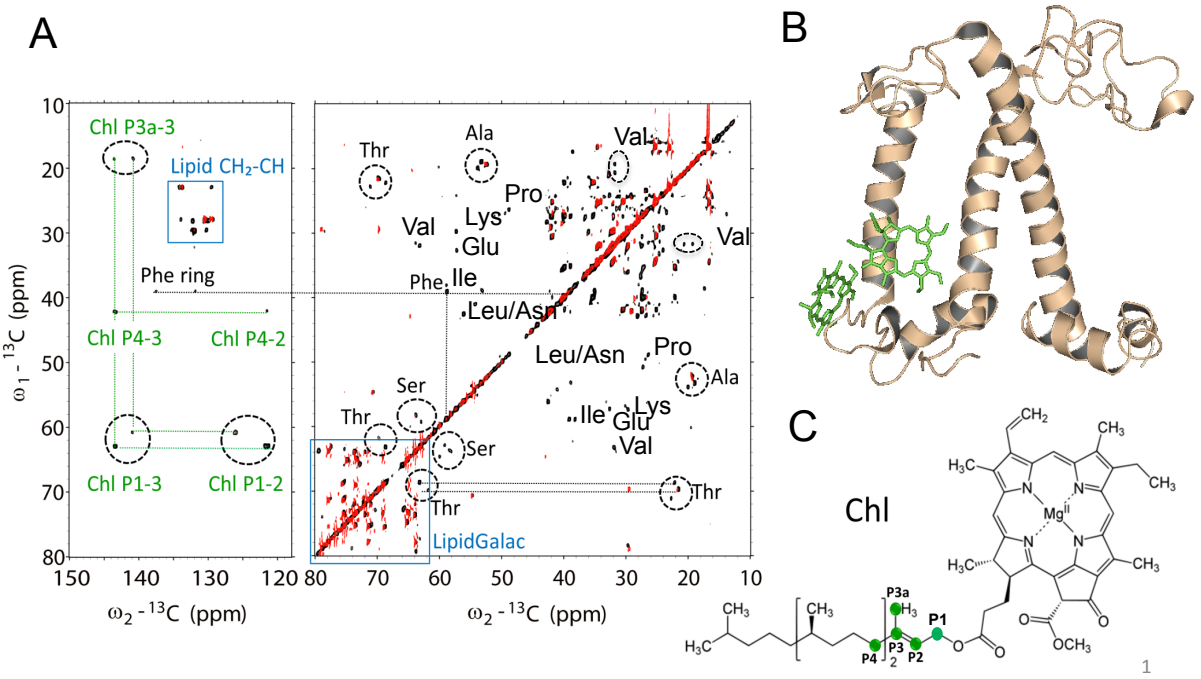

Figure 3. J-analysis of LHCII proteoliposomes and thylakoid membranes*. A: Overlay of the INEPT-TOBSY spectrum of LHCII (black) and of thylakoid membranes (red). Resonance signals of Chl phytol chains, lipids and Ala, Thr, Ser, and Phe amino acids are indicated. B: LHCII structure with Chl605 and Chl606 in green. C: chemical structure of Chl, with the attributed carbon atom types color coded.*

The lipid signals in the NMR spectra of LHCII allow us to identify the nature of the LHCII-associated lipid molecules. The ^{13}C lipid NMR signals must originate from the original thylakoid lipids that remained associated with the LHCII complex after purification, because the reconstituted lipids are not isotope labeled. A phospholipid, attributed to PG, is detected in the dipolar-based CC spectrum (Fig. 1) as mentioned, indicating that this lipid molecule is strongly bound to LHCII. MD simulations of a LHCII monomer in a lipid bilayer showed dynamic fluctuations of PG, which could modulate Chl611-Chl612 excitonic interactions [9], but the NMR

data does not give any evidence for such dynamics. No other lipids are detected in the CP-based spectra of LHCII proteoliposomes, but galactolipids are detected in the INEPT TOBSY spectrum that can be assigned to MGDG or DGDG [33] (Table S4 and Fig. S7). Those are likely from annular lipids that are not strongly bound and may have exchanged with the liposome lipids upon reconstitution of LHCII.

The lipid signals in the thylakoid spectra provide a unique molecular picture of lipid dynamics inside the thylakoid membrane. Lipid signals are detected in the *J*-based and in the dipolar-based CC spectrum, indicating that the thylakoid membrane contains mobile lipids with strong dynamics and low segmental order, as well as immobilized lipids with low dynamics, in agreement with previous work [34], [35]. We can distinguish different lipid types and assign CH-CH_2 correlations of the unsaturated lipid tails. The CH-CH_2 correlations appear in the INEPT spectrum but not in the CP-based spectrum, showing that, despite crowding and overall rigidity of the thylakoid membranes, unsaturated lipids in the thylakoid membrane have highly mobile tails.

The NMR analysis demonstrates that selective fast, large-amplitude motions in the loops and tails of LHCII are present in the proteoliposomes that are suppressed in thylakoid membranes. *Cr* thylakoid membranes form stacks that are stabilized by transversal salt bridges between the stromal sites of LHCIIs [36]. Cryo-electron microscopy (cryo-EM) structures of *spinach* PSII-LHCII show that Chl605 and the LHCII luminal loops are in close contact with the minor antennas [17, 37] and in the low-resolution structures of *Cr* PSII-LHCII, *Cr* LHCII are arranged next to CP29 and CP26 [26] so that these protein regions could have restricted dynamics. Thylakoid membranes in addition contain a pool of free LHCIIs. The thylakoid INEPT TOBSY spectrum,

which has excellent signal to noise, does not show weak *J* signals from a subset of LHCIIs, suggesting that also those have reduced dynamics in native thylakoid membranes.

Our findings call to question whether or not spontaneous fluctuations of individual LHCIIs between different conformational states, as has been observed by single-molecule spectroscopy and has been suggested by MD simulations, do occur *in vivo*. For isolated LHCII, conformational fluctuations may be strong enough to occasionally cross the energetic barriers between distinct states and according to our data, membrane-reconstituted LHCII has selective mobile sites that potentially could be involved in a molecular switch. LHCII in thylakoid membranes does not contain highly mobile sites, which makes it unlikely that LHCIIs will undergo spontaneous conformational fluctuations in their native setting. The thylakoid membrane, however, is not a static architecture but in living organisms actively responds to changes in the light environment. Thylakoid remodeling under light stress involves membrane unstacking, super-complex reorganization and xanthophyll exchange, all of which effects may modify the protein landscape of LHCII and (temporarily) reduce the energetic transition barriers between distinct protein states.

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