

Insights into the Photoprotective Switch of the Major Light-harvesting Complex II (LHCII)

A PRESERVED CORE OF ARGININE-GLUTAMATE INTERLOCKED HELICES COMPLEMENTED BY ADJUSTABLE LOOPS*

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Background: The peripheral light-harvesting complexes of photosystem II (LHCII) switch between light energy capturing and dissipative states.

Results: An NMR analysis is presented of the arginines in LHCII prepared in its two states.

Conclusion: The protein core of arginine-glutamate interlocked helices is preserved, whereas moderate changes occur in the stromal loop region.

Significance: This work reveals consequences for the molecular switching of LHCII.

Light-harvesting antennae of the LHC family form transmembrane three-helix bundles of which two helices are interlocked by conserved arginine-glutamate (Arg-Glu) ion pairs that form ligation sites for chlorophylls. The antenna proteins of photosystem II have an intriguing dual function. In excess light, they can switch their conformation from a light-harvesting into a photoprotective state, in which the excess and harmful excitation energies are safely dissipated as heat. Here we applied magic angle spinning NMR and selective Arg isotope enrichment as a noninvasive method to analyze the Arg structures of the major light-harvesting complex II (LHCII). The conformations of the Arg residues that interlock helix A and B appear to be preserved in the light-harvesting and photoprotective state. Several Arg residues have very downfield-shifted proton NMR responses, indicating that they stabilize the complex by strong hydrogen bonds. For the Arg C_α chemical shifts, differences are observed between LHCII in the active, light-harvesting and in the photoprotective, quenched state. These differences are attributed to a conformational change of the Arg residue in the stromal loop region. We conclude that the interlocked helices of LHCII form a rigid core. Consequently, the LHCII conformational switch does not involve changes in A/B helix tilting but likely involves rearrangements of the loops and helical segments close to the stromal and luminal ends.

The elementary step in photosynthesis is the capture of solar energy by the light-harvesting antenna. In eukaryotic organisms, this step is performed by a family of pigment-binding proteins called light-harvesting complexes (LHCs)² that absorb

sunlight and transfer the excitation energy toward the reaction centers, where charge separation takes place (1). The Lhc antenna proteins of higher plants and moss and Lhcbm antenna proteins of LHCs in green algae contain three membrane-spanning helices A, B, and C, and their tertiary structure is typified by two crossing A/B helices interconnected by Arg-Glu ion pairs that form ligation sites for Chls.

The most abundant LHC in plants and green algae is the major light-harvesting complex II (LHCII), which captures about 50% of all land-bound chlorophylls. The LHCII complex is trimeric, and each monomer forms a scaffold for eight Chla and six Chlb molecules, two luteins (Lut), one neoxanthin, and one violaxanthin, which is reversibly replaced by zeaxanthin upon de-epoxidation during the xanthophyll cycle (2–4).

In addition to their light-harvesting function, LHCII complexes are involved in several regulatory mechanisms that balance the incoming excitation energies and prevent photodamage (1, 4–6). Under high sunlight conditions, the photosynthetic antenna can rapidly switch from light-harvesting into a photoprotective state in which excess light energy is safely dissipated as heat (7). This photoprotective mechanism is called non-photochemical quenching (NPQ) and protects oxygenic organisms against photooxidative damage. The major component of NPQ, *qE*, depends on the transmembrane proton gradient ΔpH . In plants, a decrease in luminal pH triggers protonation of PsbS (8, 9) and of LHCs in photosystem II (10) and activates the conversion of the LHC-bound xanthophyll cycle carotenoid from violaxanthin to zeaxanthin (11). In green algae, PsbS is absent, and the NPQ state is triggered by LhcSR, a pigment-binding complex with a short fluorescence lifetime that senses the decrease in luminal pH (12–14). In algae, LHCII also participates in quenching, in particular the Lhcbm1 com-

quenching; MAS, magic angle spinning; CP-MAS, cross-polarization-MAS; HetCor, heteronuclear correlation; β -DM, β -dodecylmaltoside; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DFT, density functional theory.

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² The abbreviations used are: LHC, light-harvesting complex; LHCII, light-harvesting complex II; Chl, chlorophyll; Lut, lutein; NPQ, non-photochemical

ponent (15, 16). On a supramolecular scale, a reorganization of the thylakoid membrane takes place in which the LHCII complexes dissociate from photosystem II and self-associate or associate with PsbS (17–20). The supramolecular rearrangements, xanthophyll exchange, and decrease in luminal pH were proposed to promote subtle conformational changes inside the photosystem II light-harvesting proteins, by which altered chromophore configurations create a dissipation channel for the incoming light energy (5).

To date, the molecular basis for the photophysical process of NPQ is under much debate (22–28), and it is unclear how the LHCs respond to environmental changes and energy dissipative channels are formed. The LHCII complexes of plants have been studied extensively and were shown to reversibly switch their conformation between active, unquenched and photoprotective quenched conformational states (24, 29, 30). In the photoprotective state, Chl excitations in LHCII are quenched by Chl-Lut energy transfer (24) or by low lying Chl-Lut excitonic states (23), whereas Chl-Chl charge transfer states have also been proposed (25).

There is a controversy whether the LHCII x-ray structures represent the active or quenched form of the protein (22, 31, 32). LHCII complexes reconstituted in lipid nanodiscs retained their fluorescent state but showed small spectral changes when compared with LHCII in detergent micelles (33), suggesting that the protein can adopt slightly different conformations within its active state. The quenched state is associated with a twist in the configuration of the LHCII-bound neoxanthin that may promote conformational changes at the Lut L1 site (24). Protonation of specific acidic residues under low luminal pH conditions could trigger conformational changes of the short helix D and the BC loop (2), and these residues are important for stabilizing the complex at different pH values (34). Under NPQ conditions, changes in the thickness of the thylakoid membrane have been observed, and it was proposed that LHCII adopts a more condensed structure (35).

In this work, we applied MAS-NMR techniques in combination with selective Arg isotope labeling as a noninvasive method to obtain high-resolution structural information of LHCII in its active and photoprotective states. Photosynthetic light-harvesting proteins are accessible for NMR via uniform or selective isotope enrichment of the photosynthetic organisms (36). This way, pigment-protein interactions could be detected in atomic detail inside intact purple bacterial light-harvesting oligomers (37–39). More recently, we performed a MAS-NMR analysis of LHCII from uniformly ^{13}C -enriched *Chlamydomonas reinhardtii* green algae cells (40, 41). The *C. reinhardtii* Lhcbm sequences have a high similarity with Lhcb sequences of higher plants (40), making *C. reinhardtii* LHCII a suitable *in vitro* model system for the structural flexibility of plant Lhcb and algae Lhcbm proteins.

Here we analyze LHCII complexes from an Arg-auxotrophic strain of *C. reinhardtii* that was supplied with $^{13}\text{C}_6$ - $^{15}\text{N}_4$ Arg. One-dimensional ^{13}C and ^{15}N CP-MAS and two-dimensional ^1H - ^{13}C dipolar heteronuclear correlation (HetCor) Arg NMR spectra are presented of LHCII in quenched and unquenched states. The Arg residues that stabilize the interlocked helix pair in LHCII are identified because of Chl ring current-induced

shifts of their NMR responses. We show that these protein sites are preserved in the photoprotective state, confirming a structural view in which the flexible loop regions of the LHCII polypeptides are complemented by a relatively rigid scaffolding of the protein interior (42).

EXPERIMENTAL PROCEDURES

Isotope Enrichment and Purification of LHCII Trimers—*C. reinhardtii* strain cc424, an Arg auxotrophic strain obtained commercially from the *Chlamydomonas* Connection, was grown in liquid TAP (Tris-acetate-phosphate) medium (43) with 50 mg/liter Arg used (44). Cells were cultured at room temperature under continuous illumination at 60 microeinsteins of flux. Arginine was added separately to the cell cultures, and for selective Arg enrichment, it was substituted by [$^{13}\text{C}_6$, $^{15}\text{N}_4$]Arg purchased from Silantes GmbH. The cells were harvested in mid-log phase by centrifugation (4000 rpm, 6 min, 4 °C), and thylakoids were prepared as described in Ref. 45 with a few modifications described in Ref. 46. Thylakoids were separated from other materials on a discontinuous gradient (24,000 rpm, 1 h, 4 °C) in a TST-41.14 swing-out rotor. Thylakoid membranes were washed two times, first with 10 mM HEPES, pH 7.5, and 5 mM EDTA and finally with 10 mM HEPES, pH 7.5. Thylakoids were resuspended in solubilization buffer (10 mM HEPES, pH 7.5) to a Chl concentration of 1 mg/ml, and equal amounts of β -dodecylmaltoside (β -DM) were added to get a final concentration of 0.6% β -DM. The suspension was vortexed a few seconds and centrifuged (15,000 rpm, 10 min, 4 °C) to remove unsolubilized material, and the supernatant was loaded on a sucrose density gradient, prepared by 0.65 M sucrose, 10 mM Tricine, pH 7.8, 0.03% β -DM and ultracentrifuged for 17 h at 37,000 rpm. The top band of the sucrose gradient contained LHCII trimer complexes with more than 90% purity, verified by FPLC.

For obtaining LHCII in its quenched state, isolated LHCII trimers in 0.03% β -DM were dialyzed for 72 h against detergent-free buffer. The quenched state of the detergent-depleted LHCII aggregates was verified by low temperature fluorescence spectroscopy, whereas the unquenched, light-harvesting state of the LHCII trimers in β -DM was verified by time-resolved fluorescence spectroscopy.

Fluorescence Experiments—Steady-state fluorescence excitation and emission spectra were measured with a commercial spectrophotometer (HORIBA Scientific, FluoroLog). For 77 K fluorescence emission measurements, the samples were diluted in 40% HEPES/ β -DM buffer and 60% glycerol (v/v) and cooled in a nitrogen-bath cryostat to 77 K.

Time-resolved fluorescence emission measurements were performed at room temperature with a Streak camera setup. The sample was measured front-face using a 1-mm quartz cuvette. To minimize the effects of photodamage, about 10 spectra of maximum 1 min per scan were acquired. It was verified that within this time period of illumination, no degradation of sample occurred. Excitation pulses of 400 nm (\sim 100 fs) with vertical polarization were generated using a titanium:sapphire laser (Coherent Vitesse) with a regenerative amplifier (Coherent, MIRA seed, and RegA) that was used to pump an optical parametric amplifier (Coherent, OPA). The repetition

MAS-NMR Analysis of [^{15}N - ^{13}C]Arg-LHCII

rate was 50 kHz with pulse energies of ~ 0.2 nJ. A 10-fold increase or 2-fold decrease of the excitation pulse energy did not affect the fluorescence lifetimes, confirming that experiments were performed in the annihilation-free regime. The obtained streak data were analyzed with Glotaran3 (47).

Solid-state NMR Experiments—One-dimensional ^{13}C and ^{15}N and two-dimensional ^1H - ^{13}C frequency-switched Lee-Goldburg heteronuclear correlation experiments were performed with a Bruker AV-750 spectrometer equipped with a 4-mm triple resonance MAS probe head, using a ^{13}C radio frequency of 188.6 MHz. The temperature was lowered to 220–240 K under slow spinning of the sample. For the NMR experiments, spinning frequencies of 13 kHz were used. The chemical shift scale was calibrated from an FSLG spectrum of solid tyrosine HCl salt.

Density Functional Theory Calculations—Density functional theory (DFT) calculations were performed in vacuum within the DFT framework and using the Gaussian 03 package (48). The BLYP exchange correlation function (49–51) was applied to produce the NMR chemical shifts (48, 52). The geometric arrangements of Arg-70, Glu-180, and Chl610 were extracted from the 2BHW crystallographic structure of pea LHCII (3). The phytyl group of the Chl610 was truncated at the ester group and replaced by a hydrogen atom. The truncation had no effect on the electronic structure of porphyrin ring. The geometries were partially optimized, preserving the planar structure of the Chl macrocycle and of the backbone of Arg-70 and Glu-180. The ^1H , ^{13}C , and ^{15}N NMR chemical shieldings were calculated by using the gauge-independent atomic orbital (54–57) on the whole complex, and subsequently, NMR calculations were performed. For both partial optimization and NMR chemical shift calculations, the BLYP/6-311G** basis set was used.

RESULTS

Fluorescence Conditions of LHCII in Detergent-solubilized and Aggregated State—LHCII was prepared in its active, light-harvesting state by solubilizing the LHCII complexes in β -DM buffer. Time-resolved fluorescence experiments on LHCII complexes concentrated in β -DM buffer solution verified that the light-harvesting state of LHCII was retained in the highly concentrated form required for the NMR experiments. Fig. 1 shows the decay-associated spectra obtained by a global analysis fitting of the streak camera images of LHCII concentrated in β -DM solution. The average fluorescence lifetime ($\langle\tau_f\rangle$) of this sample is 3.0 ns and verifies that the concentrated sample of LHCII in β -DM buffer retained its unquenched state. LHCII aggregates that reflect the photoprotective state of LHCII were produced by dialysis against detergent-free buffer. For the aggregate sample, the fluorescence signal was below the detection limit of the Streak camera setup, and its quenched state was verified by a 77 K steady-state fluorescence emission spectrum. Fig. 2 presents a 77 K fluorescence spectrum of LHCII in β -DM buffer (*dashed spectrum*) and after dialysis against detergent-free buffer (*solid spectrum*). Significant fluorescence quenching of LHCII aggregates formed upon dialysis is confirmed by the 10-fold decrease and 4-nm red shift of the fluorescence peak at 675 nm and the appearance of an additional fluorescence band

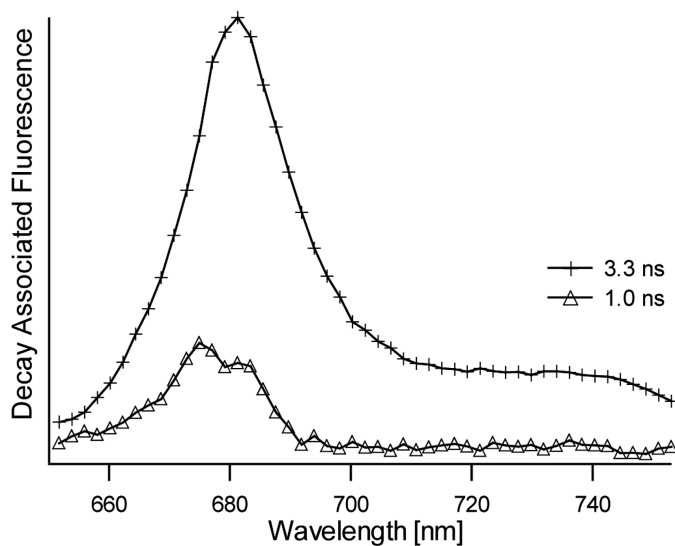


FIGURE 1. Decay-associated fluorescence spectra and associated lifetimes of LHCII concentrated in β -DM buffer solution.

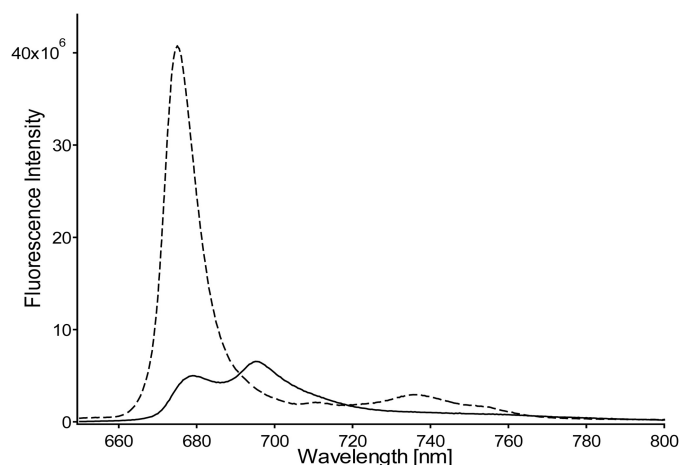


FIGURE 2. 77 K fluorescence spectra of LHCII. *Dashed spectrum*, LHCII in β -DM buffer. *Solid spectrum*, LHCII in detergent-free buffer after extensive dialysis for removal of the β -DM.

at 695 nm, which has been associated with quenching and aggregation of LHCII (30).

Arg NMR Responses for LHCII in Its Active and Dissipative State—Fig. 3 shows the one-dimensional ^{13}C (*panel A*) and ^{15}N (*panel B*) CP-MAS spectra of quenched (*solid*) and unquenched (*dashed*) LHCII. In *panel C*, the second derivative of the ^{15}N spectra is drawn. The Arg chemical structure is drawn with the spectra in *panel A*. The natural abundance ^{13}C NMR responses of the β -DM detergent molecules is denoted with *asterisks* in the ^{13}C spectrum of unquenched LHCII. Selective [$^{13}\text{C}_\epsilon$, $^{15}\text{N}_4$]Arg enrichment of the LHCII samples is confirmed by the characteristic C_ζ Arg peaks of ~ 158 ppm and N_η and N_ϵ responses of ~ 72 and ~ 82 ppm, respectively. Both samples show splitting of NMR responses for the Arg C_ζ , C_δ , and C_α and for the N_η , N_ϵ , and nitrogen atoms.

Fig. 4 shows an overlay of ^1H - ^{13}C HetCor spectra of quenched (*red*) and unquenched (*blue*) LHCII in the Arg $^{13}\text{C}_\zeta$ region (*left panel*) and $^{13}\text{C}_\delta$ region (*right panel*). The *left panel* presents the correlations between C_ζ and $\text{H}_{\eta_1}/\text{H}_\epsilon$, whereas the *right panel* presents the correlations between C_δ and H_δ (pro-

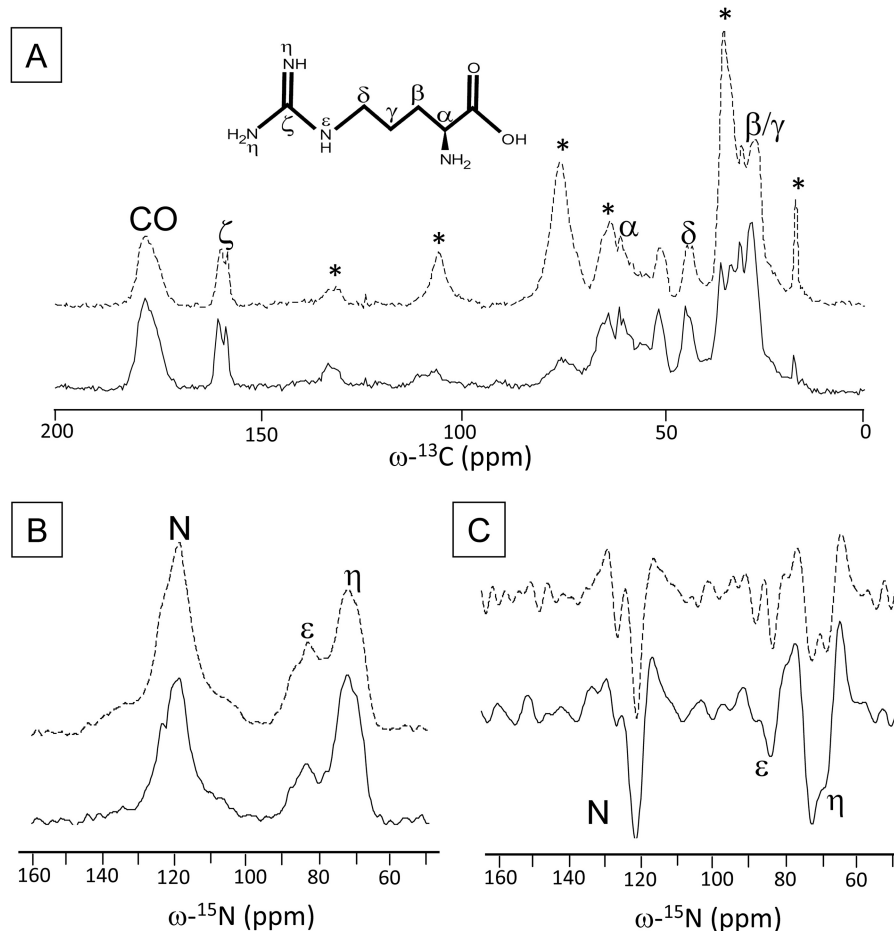


FIGURE 3. ^{13}C CP-MAS spectra of unquenched (dashed line) and quenched (solid line) LHCII. A, detergent peaks in unquenched LHCII are denoted with asterisks. The chemical structure of Arg is also shown. B, ^{15}N CP-MAS spectra of unquenched (dashed line) and quenched (solid line) LHCII. C, second derivatives of the ^{15}N spectra in B.

ton range 3–5 ppm) and between C_δ and H_ϵ (proton range 6–8.5 ppm). Because the H_ϵ proton signals (right panel) are in the range of 6.5–8.5 ppm, the proton responses in the left panel between 8.5 and 10.5 ppm are attributed to the H_{η_i} protons. The left panel also shows very peculiar well resolved correlation signals of narrow C_ζ responses at 156.8 ppm ^{13}C associated with very upfield shifted proton responses centered around 4.2 ppm ^1H .

Fig. 5 shows the ^1H - ^{13}C HetCor spectra in the region of the Arg $^{13}\text{C}_\alpha$ responses (panel A, unquenched LHCII, and panel B, quenched LHCII). The correlation signals in the range 62–64 ppm are attributed to ^{13}C natural abundance chemical shift signals of the detergent molecules. The Arg C_β and C_γ responses are strongly obscured by overlap with the detergent signals in the range 15–35 ppm and could not be resolved (data not shown). A comparison of Fig. 5, A and B, shows that the C_α correlation peak signal at 57 ppm ^{13}C and 3.2 ppm ^1H in the spectrum of unquenched LHCII (Fig. 5A) apparently shifts considerably downfield in the ^1H dimension and produces a doubled response for quenched LHCII (Fig. 5B).

DISCUSSION

The LHCII trimers of *C. reinhardtii* are isomers composed of different Lhcbm polypeptides. These Lhcbm polypeptides contain 6 Arg residues that are conserved in the Lhcb sequences

of *Arabidopsis thaliana* (40). The Lhcbm1, Lhcbm5, and Lhcbm10 polypeptides contain additional Arg residues (2 residues for Lhcbm1 and 10 and 1 residues for Lhcbm5) very close to the N terminus, in a flexible part of LHCII that was not resolved in the LHCII x-ray structures. Most likely, the NMR responses of these additional Arg are also unresolved in our NMR datasets because they are only present in a subpopulation of the LHCII isomers. Moreover, their NMR responses are likely to be weakened due to intrinsic disorder of the N-terminal part, which causes dynamic broadening of the NMR lines and poor cross-polarization. For example, in MAS NMR datasets of purple bacterial antenna proteins, the terminal ends of the α - and β -polypeptides were not resolved (58).

Fig. 6A shows the homology structure of the *C. reinhardtii* Lhcbm1 monomer, based on the pea LHCII x-ray structure (2BHW) (3), with the 6 conserved Arg residues highlighted. Five Arg residues reside in the α -helical part of the protein, whereas the 6th Arg is located in the stromal loop region. The arginines that form ion pairs with Glu and ligate Chls, Arg-70, Arg-185, and Arg-160, are indicated in the figure, as well as Arg-25 in the loop region. Fig. 6B shows the arrangement of one of the stabilizing Arg-Glu pairs in detail: Arg-70 and Glu-180 that link helix A and B and ligate Chl610.

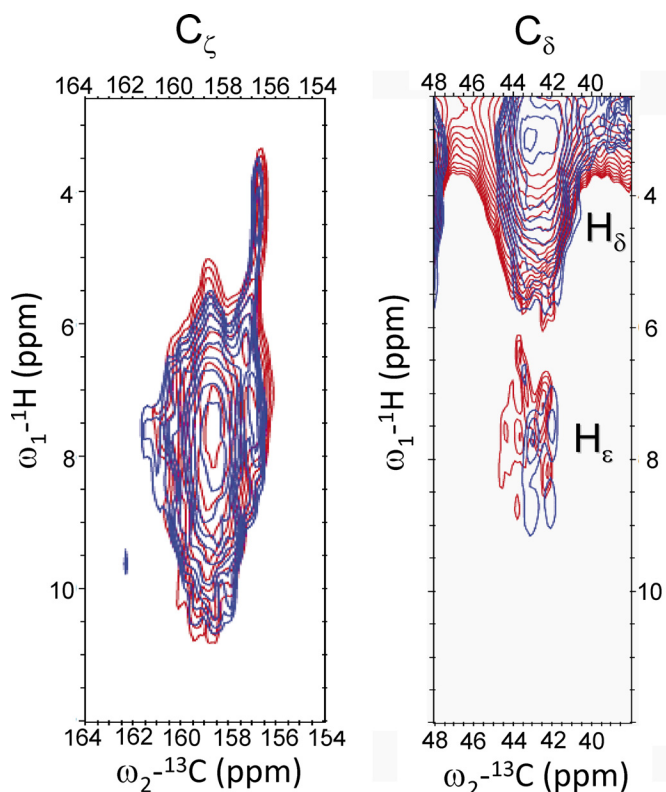


FIGURE 4. Heteronuclear ^1H - ^{13}C correlation spectra of unquenched (blue) and quenched (red) LHCII in the Arg $^{13}\text{C}_\zeta$ region (left) and in the $^{13}\text{C}_\delta$ region (right).

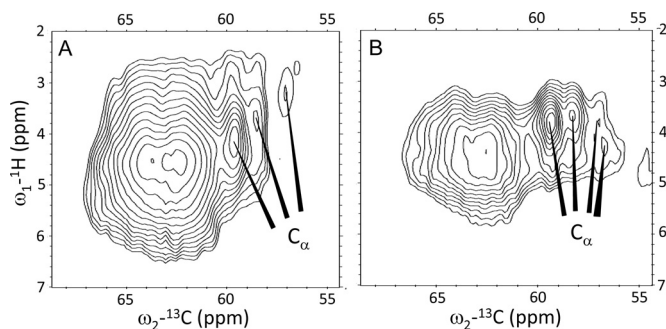


FIGURE 5. Heteronuclear ^1H - ^{13}C correlation spectra of unquenched (A) and quenched (B) LHCII in the Arg $^{13}\text{C}_\alpha$ region.

According to the plant LHCII x-ray structures, the 6 Arg residues are capable of forming hydrogen bonds via their side chain H_ϵ or H_η amides. The strong downfield NMR shifts of the Arg proton responses in the HetCor spectrum in Fig. 4, left panel, suggest that in *C. reinhardtii* LHCII, strong hydrogen bonds are also formed to the Arg residues that involve the NH_η amide protons.

Upfield Shifted NMR Responses Are Explained by Chl Ring Current Shifts for Arg-70 and Arg-185—In the HetCor spectrum in Fig. 4, left panel, the $^{13}\text{C}_\zeta$ response at 156.8 ppm correlates with unusual upfield shifted proton responses at 4.2 ppm that are ~ 3 ppm shifted relative to the average values (7.4 and 6.8–6.9 ppm, respectively) found for Arg H_ϵ and H_η in the Biological Magnetic Resonance Bank (59). To find an explanation for these unusual shifts, we estimated the ring current effects that are produced by the Chl macrocycle rings. Chl ring

currents can induce large shifts for the NMR responses of atoms in close vicinity to the ring center distances (60). Close inspection of the LHCII x-ray structures shows that both Arg-70 and Arg-185 have their side chains hanging over the macrocycle planes of the ligating Chls (Chl602 and Chl610), which positions the Arg side chain atoms close to the Chl rings. The third Arg residue involved in Chl ligation, Arg-160, forms an ion pair with a Glu residue of the same helix (helix C) and has its side chain oriented perpendicular to the macrocycle plane of its ligating Chl (Chl609). For Arg-160, only one proton is positioned close enough to the Chl ring center that it could experience significant ring current effects.

The geometric arrangements of Arg-70, Glu-180, and Chl610 as shown in Fig. 6B were taken from the 2BHW crystal structure and partially optimized as described under “Experimental Procedures.” Quantum-mechanical DFT calculations were performed for three structure models: 1) Arg-70, 2) Arg-70...Glu-180, and 3) Arg-70...Glu-180...Chl610 to estimate the magnitude of Chl ring current and H-bonding-induced shifts of the Arg-70 NMR responses. Table 1 shows how the chemical shifts of Arg-70 (model 1) are affected by interaction with Glu-180 (model 2) and by combined interaction with Glu-180 and Chl610 (model 3). The effects of a partial geometry optimization were most critically obvious for the calculated chemical shifts of the Arg-70 H_ϵ and H_{21} atoms. For these protons also, non-optimized calculated shifts values are presented in which the geometries were taken directly from the x-ray structure.

The results in Table 1 show that for both the unmodified and the partially optimized structures, the presence of the Chl causes strong ring current shifts for the Arg-70 H_ϵ and H_{21} responses of ~ -5 and ~ -3.5 ppm (*i.e.* the differences between the NMR responses calculated for models 2 and 3), which we attribute primarily to the ring currents in the Chl610 that counteract the large downfield shifts due to H-bonding of these protons to the Glu-180 carboxyl. For H_ϵ , the H-bond interaction with the Glu-180 carboxyl is weakened when a ligand to magnesium is formed via the same oxygen atom, which may also affect the H_ϵ chemical shift.

The net effect of H-bonding and ring current shifts in our calculations are that the H_ϵ and H_{21} NMR responses are shifted between +3.5 and -1 ppm, depending on the optimization procedure that was applied. The net effects for the other amide protons H_{11} , H_{12} , and H_{22} are shifts between -1.5 and 2 ppm, whereas the side chain carbon responses of C_ζ and C_δ are shifted -2.7 and -2.4 ppm, respectively.

Although the exact geometries of the Arg-Glu-Chl structures may differ in *C. reinhardtii* LHCII from the plant LHCII x-ray structures, the trends of the calculated chemical shift changes match with the experimental observations of (i) C_ζ carbon responses that are shifted 2–3 ppm upfield from the bulk of C_ζ signals and that correlate with (ii) proton responses that are shifted 0–2 ppm downfield in addition to (iii) proton responses with significant upfield shifts because for the H_{11} , H_{12} , and H_{22} , the interaction with Glu-180 and Chl610 produces shifts of -1.5 to -2 ppm. Hence, according to the DFT chemical shift calculations, the upfield shifted ^1H - $^{13}\text{C}_\zeta$ correlations that are well resolved in Fig. 4, left panel, could originate from Arg-Glu-

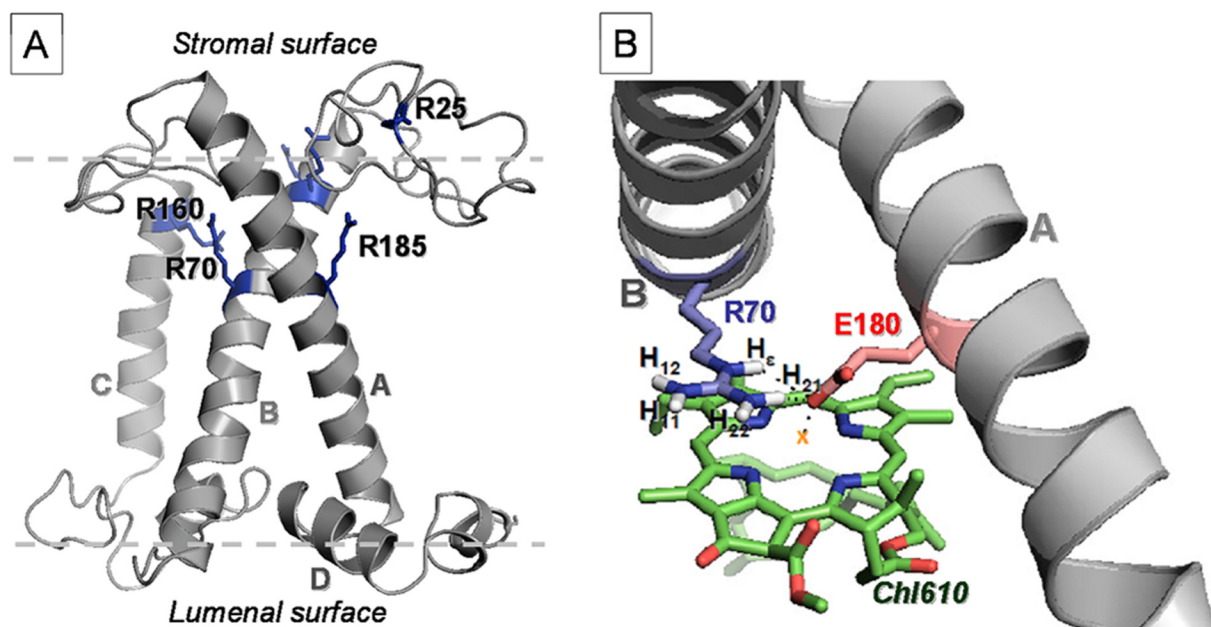


FIGURE 6. *A*, homology structure of *C. reinhardtii* LHCII highlighting the Arg residues. *B*, the geometric arrangements of Arg-70, Glu-180, and Chl610 taken from the 2BHW (3) LHCII x-ray structure.

TABLE 1

DFT-predicted NMR chemical shift displacements for Arg-70

The NMR chemical shifts of R70 were calculated using model 1 (Arg-70), model 2 (Arg-70→Glu-180), and model 3: (Arg-70→Glu-180→Chl610).

Atom	Arg-70→Glu-180 (model 2) ($\Delta\sigma$) ^a	Arg-70→Arg-180→Chl610 (model 3) ($\Delta\sigma$) ^b
	ppm	ppm
H _e	+6.0	+1.0
H _e	+3.5 ^c	-1.1 ^c
H ₁₁	-1.1	-1.5
H ₁₂	-0.9	-1.7
H ₂₁	+7.0	+3.7
H ₂₁	+3.0 ^c	-0.6 ^c
H ₂₂	-0.8	-2.1
C _ε	-1.2	-2.7
C _σ	-1.2	-2.4
N _ε	-3.3	+1.3
N ₁	+11.6	+9.5
N ₂	-8.1	-6.8

^a Chemical shift differences between model 2 and model 1.

^b Chemical shift differences between model 3 and model 1.

^c Non-optimized.

Chl interactions involving the two Arg residues that form an important structural motif by locking helix A and B.

Our limited models do not take into account hydrogen bonds to other parts of the protein. In the LHCII x-ray structures, one amide proton of Arg-70 is very close to the C13 keto carbonyl of Chl608. This proton is too far from the Chl ring centers to experience any ring current shift. A strong H-bond of this proton to the Chl608 carbonyl should induce a strong downfield shift of its NMR response. Instead, no strong downfield shifts are observed in the ¹H dimension for the C_ε signal at 156.8 ppm. This suggests that the H-bonding patterns to the Chl side chains in *C. reinhardtii* LHCII differ from plant LHCII. In fact, in an earlier study on uniform ¹³C-labeled *C. reinhardtii* LHCII, we estimated that the number of Chls with H-bonded keto carbonyls is lower in *C. reinhardtii* LHCII than in plant LHCII (40).

When comparing the C_ε regions in the HetCor spectra for quenched and unquenched LHCII (Fig. 4), no significant changes are observed, and the spectra are almost identical. In

particular, the unusual correlation signal with very upfield proton shifts that we attribute to the helix-connecting Arg residues is preserved. The data imply that the Arg-Glu-Chl geometric arrangements are preserved in the quenched state, relative to the unquenched state. Because the Arg-Glu pairs interlock helix A and B, the conserved chemical shift patterns suggest that the orientations of these two transmembrane helices, which define the tertiary structure, are also preserved in the two states. In addition, the Arg hydrogen-bonding patterns appear to be preserved in the two forms of LHCII.

The DFT-calculated nitrogen chemical shifts in Table 1 predict displacements of the Arg amide ¹⁵N NMR responses when a hydrogen bond is formed to Glu (model 2), which breaks the symmetry of the N_{ηi} responses and induces an upfield shift of the N_ε response. The splitting of the Arg ¹⁵N_η signal (Fig. 3, B and C) therefore can be explained by the induced asymmetry when a hydrogen bond is formed to one of the two N_η atoms, whereas splitting of the Arg N_ε signal is explained by heterogeneity of the Arg structures with respect to H-bonding of their side chain N_ε atoms.

In Quenched LHCII, Arg-25 Changes Its Backbone Conformation—The Arg ¹³C_α chemical shift responses reflect moderate conformational changes between the quenched and unquenched forms of LHCII. In the spectrum of unquenched LHCII, two C_α correlation signals appear at 59.5 and 58.5 ppm, and a smaller peak appears at 57 ppm. In the spectrum of quenched LHCII, the latter peak is split into two weaker signals.

The LHCII x-ray structures 2BHW (3) and 1RWT (2) were used to predict the Arg backbone chemical shifts using the SHIFTX2 server. The structure-predicted Arg C_α shifts range between 58 and 60 ppm for the residues in the α-helical stretches, whereas the Arg residue in the stromal loop region, Arg-25, has a predicted C_α shift of 56 ppm. In the NMR spectrum of unquenched LHCII (Fig. 5A), the relative signal intensities of the C_α peaks at 59.5, 58.5, and 57 ppm are roughly in the

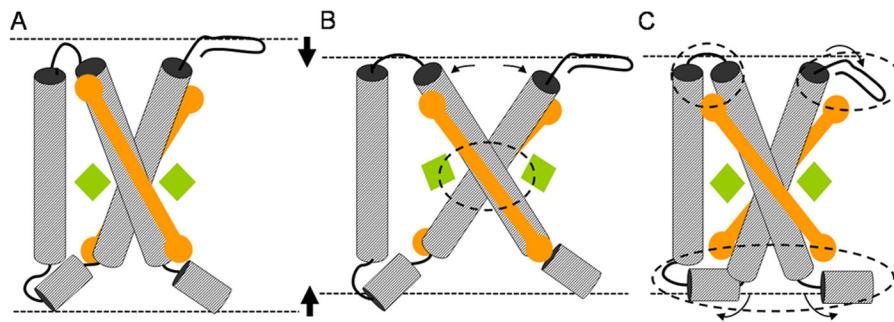


FIGURE 7. **Graphic illustrating the possible effects of membrane thinning on LHCII.** Compression of the LHC structure in A is achieved by (i) increased tilting of the transmembrane helices, causing structural changes in the Arg-Glu interlocked core (B; the affected core region is *encircled*) or (ii) reorientation of protein segments close to the water interface, causing structural changes in the regions near the luminal and stromal sites (C; the affected end regions are *encircled*). A structural change as depicted in B will affect the orientations of Chl602 and Chl610 (*green diamonds*) that are ligated to the Arg-Glu interlocked core. However, such a structural rearrangement is unlikely according to the preserved NMR chemical shifts of the Chl-ligating Arg. The structural change depicted in C could reorient the luteins (*orange rods*) that have their head groups bound to the affected protein end regions.

order of 3:2:1, matching with 5 Arg residues located in the α -helical stretches that have C_{α} shifts in the range 58–60 ppm and one Arg residue in the loop region with a more upfield shifted C_{α} response. Therefore we tentatively assign the response at 57 ppm to the C_{α} of Arg-25 in the stromal loop region. We cannot exclude that the response at 57 ppm $^{13}\text{C}_{\alpha}$ includes the additional Arg residues at the N-terminal site, appearing in a subpopulation of LHCII. The backbone NMR chemical shifts of the additional Arg residues will likely fall in the chemical shift range for random coil structures, *i.e.* 54–58 ppm.

Apparently, the backbone conformation of Arg-25 (and/or of the additional Arg at the N-terminal ends) is changed in the photoprotective state (Fig. 5B). Arg-25 is in the water-exposed stromal region of the protein and this part of the protein is probably affected by a change in hydrophobicity or by protein-protein interactions inside the LHCII aggregates.

Stability of the Interlocked A and B Helices in the LHCII Core and Implications for Possible Conformational Changes—Early studies of Murakami and Packer (61) showed a thinning of the thylakoid membrane bilayer upon illumination together with an increase in membrane hydrophobicity, and similar effects were observed in electron microscopy micrographs of granal thylakoid membranes (17, 19). Johnson *et al.* (35) performed an extended analysis and demonstrated that apparent changes in the appressed membrane zone upon illumination correlated with structural changes in membrane thickness. The authors suggested that the observed decrease in membrane thickness reflects conformational changes of LHCII, forming a more condensed state (35). A similar condensed state is proposed to form upon aggregation *in vitro* due to protein-protein interactions and changes in hydrophobicity (5). Here we elaborate on this theory and reason that a hypothetical compressed conformation of LHCII can be established in two ways.

First, it can be established by increased helix tilting, an effect that is a common response of membrane helices to hydrophobic mismatch (53, 62). The LHCII monomers can be compressed by increasing the angles of the membrane-spanning helices A and B with respect to the membrane normal. This is illustrated in Fig. 7B. The LHC structure in Fig. 7A is compressed by increased tilting of the interlocked helices, resulting in the structure drawn in Fig. 7B. However, such a mechanical

movement would modify the geometric arrangements of the Glu and Arg residues linking the two helices and of the Arg-Glu ligated Chls (Fig. 7B, *encircled area*). According to our results in Fig. 4, *left panel*, this scenario is unlikely. The NMR data show that the responses of the involved Arg residues are almost identical for the quenched and unquenched forms of LHCII, whereas our modeling results predict that these responses are very sensitive to the specific orientations of the interacting Glu, Arg, and Chls. Our findings match with the results of Dockter *et al.* (42), which show that the LHCII polypeptides have low flexibility in the core region.

Second, alternatively, a condensed state of LHCII can be created by rearrangement of protein segments located near the water interface. This is illustrated in Fig. 7C, where segments at the stromal and luminal ends of the structure in Fig. 7A are refolded (*encircled areas*) to create a more compact conformation. Such conformational changes are in line with the NMR data and can explain the variability of the $^{13}\text{C}_{\alpha}$ Arg response, which we tentatively attribute to rearrangement of Arg-25 in the stromal region (Fig. 5). Although under our labeling conditions there are no residues reporting at the LHCII luminal site, it has been proposed that changes in luminal pH under NPQ conditions could enhance local refolding of the luminal loop region by protonation of acidic residues (2, 34).

Photophysical quenching models have been proposed based on altered carotenoid-Chl interactions, in specific between lutein Lut620 and Chl_a molecules in the terminal emitter domain (Chl610, Chl611, and Chl612) (23, 24). According to our data results here on [^{13}C - ^{15}N]Arg LHCII, the ring current effects of Chl610 and Chl602 acting on Arg-70 and Arg-185 do not change in the quenched state. This is a strong indication that the orientations of Chl610 and of Chl602, which are in close distance with either of the two luteins in LHCII, are preserved. The position of Chl612 is also likely to be conserved because its ligand Asn-183 is close to the Arg-Glu interlocked helical core. Thus, photophysical quenching models based on altered lutein-Chl_a interactions must involve a change in the orientation of a lutein chromophore with respect to the fixed positions of the adjacent Chls. Indeed, the quenched state of LHCII has been associated with a conformational change of the Lut620 carotenoid (21). A change in the orientation of a lutein could affect the NMR responses of closely spaced Chl carbons,

explaining the quenching-related changes of specific LHCII Chla NMR responses that were observed in Ref. 41. Lut620 is stabilized by protein interactions via helix D and the loop segment connecting helix A and C. In a hypothetical, mechanistic model, changes in pH and hydrophobicity may reorient these protein segments while maintaining the position of the interlocked helices A and B, resulting in a more compact protein structure as illustrated in Fig. 7C and moving Lut620 with respect to the fixed positions of Chl610 and Chl612, creating a photophysical quencher state.

Conclusion—We used a noninvasive method to selectively probe the structure and environment of the Arg residues in *C. reinhardtii* LHCII without the need for recombinant approaches. Our approach shows that solid-state NMR is a powerful method to determine the molecular structure of light-harvesting proteins while controlling their functional states. The conformations of the Arg residues in the α -helical regions near the stromal site and in the interlocked core are preserved in the light-harvesting and photoprotective states. In contrast, moderate changes are observed for the Arg in the stromal loop region. The results fit into a mechanistic picture where conformational changes of the LHCII end segments under NPQ conditions may reorient a carotenoid with respect to the fixed positions of the Chls in the terminal emitting domain, rendering a photophysical response for dissipation of harmful excitation energies.

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