Rigid Core and Flexible Terminus

STRUCTURE OF SOLUBILIZED LIGHT-HARVESTING CHLOROPHYLL a/b COMPLEX (LHCII) MEASURED BY EPR

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Background: Structural changes of LHCII may be required for its roles beyond light harvesting.

Results: EPR distance mapping reveals a high flexibility of the N-proximal protein domain.

Conclusion: The N-terminal domain in LHCII adopts various conformations in an aqueous environment.

Significance: The flexibility of hydrophilic domains in LHCII is compatible with potential structural changes of the protein between its various functional states.

The structure of the major light-harvesting chlorophyll a/b complex (LHCII) was analyzed by pulsed EPR measurements and compared with the crystal structure. Site-specific spin labeling of the recombinant protein allowed the measurement of distance distributions over several intra- and intermolecular distances in monomeric and trimeric LHCII, yielding information on the protein structure and its local flexibility. A spin label rotamer library based on a molecular dynamics simulation was used to take the local mobility of spin labels into account. The core of LHCII in solution adopts a structure very similar or identical to the one seen in crystallized LHCII trimers with little motional freedom as indicated by narrow distance distributions along and between α helices. However, distances comprising the lumenal loop domain show broader distance distributions, indicating some mobility of this loop structure. Positions in the hydrophilic N-terminal domain, upstream of the first trans-membrane α helix, exhibit more and more mobility the closer they are to the N terminus. The nine amino acids at the very N terminus that have not been resolved in any of the crystal structure analyses give rise to very broad and possibly bimodal distance distributions, which may represent two families of preferred conformations.

The light-harvesting complex LHCII6 largely increases the efficiency of the photosynthetic process in green plants by collecting light energy and conducting it to a photosynthetic reaction center where light-driven charge separation takes place. LHCII is accumulated at high amounts in plant chloroplast membranes, making it the most abundant membrane protein on earth (1). A total of 18 chromophores are noncovalently bound to the apoprotein and warrant absorption of solar radiation over much of the visible spectrum. These chromophores are eight chlorophyll (Chl) a, six Chl b, and four carotenoid molecules. The crystal structure of the functional, trimeric form of LHCII is known in atomic detail (2, 3). Each monomer possesses three transmembrane helices H1, H3, and H4 spanning the thylakoid membrane and creating a central scaffold where the chromophores are bound in their specific positions at a high density (Fig. 1A). Only a segment of the N-terminal domain remains elusive in the atomic structures (2, 3).

Besides its main light-harvesting function, LHCII is involved in several regulatory processes to optimize photosynthesis and to prevent photo-damage of the photosystems (PS) in changing light conditions. It is assumed by some but not by others that the switch from one functional state to another may require a conformational change of some domains of the apoprotein. Under high light conditions, LHCII is involved in the short term protection of the photosynthetic system, which is referred to as the energy-dependent component (qE) of nonphotochemical quenching or feedback de-excitation (4). Triggered by the acidification of the thylakoid lumen, the carotenoid violaxanthin is converted to zeaxanthin by a lumenal de-epoxidase, and the excess energy is dissipated as heat. The protonation of lumen-exposed acidic residues under low pH conditions has been suggested to trigger a conformational change of helix H3 and the lumenal loop domain, resulting in an active energy-trapping

6 The abbreviations used are: LHCII, light-harvesting chlorophyll a/b complex; DEER, double electron-electron resonance; PROXYL, 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinolox; Chl, chlorophyll; PS, photosystem; ESEEM, electron spin echo envelope modulation; PDB, Protein Data Bank; PG, phosphatidylglycerol.
Site composed of xanthophyll cycle carotenoids and a Chl a
dimer (2). According to another model of nonphotochemical
quenching, LHCIIT interacts with the protein PsbS in the pho-
tosynthetic membrane at low lumen pH, which results in a con-
formational change of LHCIIT, an altered configuration of its
pigments, and consequently, a switch to the dissipative state (5).
Ruban et al. (6) postulated an alternative model for energy dis-
sipation in LHCCIIT. Based on the twist in the configuration of
the LHCIIT-bound carotenoid neoxanthin, the structure of LHCIIT is
changed into the dissipative state, and excess energy is trans-
ferred from Chl a to a lower energy carotenoid lutein.

To date, the structural basis of nonphotochemical quenching
in LHCIIT is unclear and controversially discussed. Kühlbrandt
and co-workers (3) proposed alternative models for quenching
mechanisms without conformational changes of LHCIIT. First,
the simple replacement of violaxanthin by zeaxanthin in the
binding pocket of LHCIIT itself can cause an effective quenching
of excess energy without any structural changes. Second, the
interaction between LHCIIT and PsbS monomers creates a
quenching unit of transiently bound pigments that then func-
tion as a channel for the safe dissipation of energy (7). Milo-
slavina et al. (8) proposed another model involving a Chl-Chl
charge transfer state as a quenching unit. The prerequisite for
their model is the formation of inter-trimer Chl-Chl pairs at the
interface of LHCCIIT oligomers. Recently, Avenson et al. (9) sug-
gested a carotenoid radical cation model for energy dissipation.
Minor LHCCs, positioned between LHCIIT and PSII, provide the
quenching sites by binding zeaxanthin. Electrons can be trans-
ferred from Chl to zeaxanthin, which then undergoes charge
separation.

Besides this energy-dissipating function, LHCIIT is involved in
the balanced distribution of excitation energy between the pho-
tosystems by dissociating from PSII and binding to PSI in high
light conditions. This short term process is referred to as state
transition and is regulated by phosphorylation of the N-termi-
nal protein domain in LHCIIT by the thylakoid kinase STN7
(10–12). Different binding specificities of phosphorylated
LHCIIT for both photosystems have been proposed, but the
molecular basis of the redistribution of LHCIIT complexes
between PSII and PSI remains unknown as yet (13). Among
several hypotheses that have been put forward, a conforma-
tional switch of the N-terminal conformation has been dis-
cussed (14). A light-induced conformational change of the
N-terminal region was shown by an increase of its accessibility
to tryptic cleavage and was also suggested in connection with
the phosphorylation process (15).

Yet another function of LHCIIT is the organization of chloro-
plast membranes in chloroplast grana stacks, which is thought
to be mediated by interactions between N-terminal LHCIIT
domains. The recently published cryoelectron tomography of
thylakoid membranes supports a Velcro-like interaction of
LHCCIIT trimers in grana thylakoids (16) as it was suggested ear-
erlier (3). According to the model, grana stacks are held together
by the electrostatic interaction between the positively charged
N-terminal domains of one trimer with the negatively charged
stomal surface of the opposite trimer. For such an interaction,
some flexibility in the N-terminal domain, including the posi-
tive charge cluster, would be advantageous but was never con-
firmed experimentally. A flexible N terminus in LHCIIT has also
been proposed to explain the low resolution in this segment of
the LHCIIT crystal structure (16).

Structural information on proteins in solution, their flexibil-
ity, and their conformational changes can be gathered by vari-
ous techniques of EPR in combination with site-directed spin
labeling. The apoprotein of LHCIIT is recombinantly expressed
in Escherichia coli (17) and therefore can easily be spin-labeled
in specific positions (18). Adding pigments to the apoprotein in
detergent solution induces the spontaneous assembly of mono-
meric (17, 19, 20) or trimeric LHCIIT (21). Double electron-elec-
tron resonance (DEER) spectroscopy allows the measurement
of distances between two spin-labeled residues in the range of
2–8 nm (22, 23). This technique is especially useful for assess-
ning structure dynamics. The distribution of protein conforma-
tions observed in a frozen sample represents the variety of con-
formations that are accessible to the protein at physiological
temperatures (24–27). Distance mapping of individual protein
domains in LHCIIT revealed a two-state structural behavior of
the N-terminal domain in monomers and trimers (24). DEER
recently provided insight into the refolding process of LHCIIT in vitro (20). Additional information about the accessibility of sin-
gle residues in various domains of LHCIIT monomers was
obtained by comparison of conventional continuous wave and
pulsed EPR measurements (18).

In this study, we used DEER for a refined and extended
distance mapping to obtain information about LHCIIT structure in
solution, particularly the positioning and flexibility of the
N-terminal domain. Spin-labeled versions of LHCIIT were refolded
and purified in their monomeric or trimeric form, and distances
were monitored between spin label pairs in the core
region and the N-terminal domain of LHCIIT. Based on the EPR
distance map, we compared the structure of LHCIIT in detergent
solution with the structure predicted from crystal data (3),
including a PROXYL rotamer library based on a molecular
dynamics simulation (28).

EXPERIMENTAL PROCEDURES

Protein Preparation and Site-directed Spin Labeling—Pro-
teins used in this study were C-terminally His₆-tagged deriva-
tives of the Lhcb1*2 (AB80) gene from pea (Pisum sativum) (29)
with its single Cys in position 79 replaced with Ser. In EPR
measurements, different mutant versions were used, each con-
taining either one or two Cys replacing Ser at positions 3, 11, 12,
14, 29, 34, 59, 106, 123, and 160; Ala at positions 4 and 10; Lys at
position 7, or Val at positions 9, 22, 90, and 196. All derivatives
have either been described in earlier studies (18, 20, 24) or were
constructed by using the QuikChange mutagenesis kit (Strat-
egene). Bacterial expression of the derivatives (17) and labeling
of the protein with PROXYL spin labels (3-(2-iodoacetamido-
PROXYL, Aldrich) were performed as described in the supple-
mental material.

Reconstitution of Spin-labeled LHCIIT with Pigments—Plant
pigments for reconstitution of spin-labeled LHCIIT were
extracted from pea thylakoids according to Ref. 17. All spin-
labeled versions of LHCIIT were reconstituted in their mono-
meric form using the detergent-exchange procedure as
described previously (30), except that 10 mM β-mercaptoethanol was used as a reductant.

Singly labeled versions of LHCII were immobilized on nickel-chelating Sepharose via a C-terminal His<sub>6</sub> tag to prepare trimERIC LHCII complexes (24), and doubly labeled LHCII samples were purified in their monomeric form. Purification of monomeric and trimeric LHCII was performed by ultracentrifugation on 0.1–1 m sucrose density gradients containing 0.1% (w/w) n-dodecyl-β-D-maltoside (Merck) and 5 mM Tris-HCl (pH 7.8) (Serva). After spinning for 16 h at 230,000 × g at 4 °C, the bands containing monomeric or trimeric LHCII were collected and concentrated in centrifugal filter units (30 kDa) (Millipore). LHCII samples exhibiting a protein concentration of about 600 μM were mixed at a 1:1 volume ratio with 80% glycerol as a cryoprotectant, loaded onto EPR tubes, and flash-frozen in liquid nitrogen. The quality of EPR samples was checked by fluorescence emission spectroscopy (31) and CD spectroscopy in the visible domain (21), as described in the supplemental material. High glycerol concentrations did not alter the functional structure of LHCII as shown previously (20).

**EPR Spectroscopy**—X-band (9 GHz) pulse EPR measurements were performed on a Bruker Elexsys E580 EPR spectrometer using a Bruker Flexline split-ring resonator ER 4118X_MS3 overcoupled to Q ~100. Sample temperature was kept constant at 50 K with liquid helium cooling using an Oxford CF935 cryostat with an Oxford ITC4 temperature controller.

The four-pulse DEER experiments were performed as described by Jeschke et al. (24) with an interpulse delay τ<sub>2</sub> between 2000 and 2500 ns depending on the transverse relaxation times, an optimum repetition time between 3 and 6 ms depending on saturation behavior, and a total measurement time of ~8 h. The quality of EPR samples was checked by electron spin echo measurements and T<sub>1</sub> relaxation measurements as described previously (18).

Three-pulse ESEEM measurements were performed as described by Volkov et al. (18), using a home-written Bruker Pulse SPEL program for accessibility measurements that is available on request. A sequence (π/2) − τ − (π/2) − T − (π/2) − τ−echo was used with fixed τ = 344 ns, corresponding to a proton blind spot, and π/2 pulse lengths of 16 ns. The variable time T was incremented in steps of 8 ns starting at T<sub>0</sub> = 80 ns. ESEEM traces of 1024 points with a total length of 8.184 μs were acquired. To eliminate echo crossings, a ((+x,+x) − (−x,+x) − (+x,−x) + (−x,−x)) phase cycle was applied to the final two π/2 pulses.

**Data Analysis**—DEER data were analyzed using the “DeerAnalysis” program (32), which is available on line. A background function was fitted to the data at dipolar evolution times τ > τ<sub>0</sub>. Starting time τ<sub>0</sub> was determined automatically as described by Jeschke et al. (32). After background correction of the data by an exponential decay function, the distance distribution was determined by Tikhonov regularization with an optimum regularization parameter determined by the L curve criterion. Significance of features at long distances was checked with the validation tool in “DeerAnalysis.” ESEEM data were analyzed as described in Volkov et al. (18) using a home-written Matlab program that is available on request.

**Structure of Solubilized LHCII Measured by EPR**

Rotamer Library Simulations—Distance distributions were predicted from the crystal structure (PDB code 2BHW (3)) with rotamer library modeling of spin label conformations (28) as implemented in the software MMM, which is available on line. The DEER window in MMM was used to check to what extent three-spin effects (33) influence analysis in terms of distance distributions at the observed modulation depths. For the qualitative and semi-quantitative discussions in this work and at the reduced modulation depths compared with samples with 100% labeling efficiency, such an influence was found to be negligible. The DEER window in MMM was also used to test whether DEER data simulated from the crystal structure were in agreement with experimental data.

**RESULTS**

Distances between Positions Near the LHCII Core—The LHCII structure in detergent solution was analyzed by pulsed EPR distance measurements and compared with that in stacked two-dimensional crystals (3). EPR distances were defined by the nitroxide of PROXYL spin labels attached to engineered Cys residues in positions 59, 90, 106, 123, 160, or 196 as shown in Fig. 1, B and C. Except for positions 59 and 106, these labeling sites were located within the hydrophobic core of the complex (18) and thus inside the detergent micelle. The labeling sites were chosen in the periphery of the LHCII core, facing toward the LHCII surface to avoid steric clashes and structural perturbations. Spin labeling of unfolded apoproteins was optimized as described previously and found to be higher than 90% for mutants containing a singular Cys (supplemental Fig. S1). The actual labeling efficiency was somewhat smaller due to partial reduction of the labels under the conditions of LHCII reconstitution. A reduced spin labeling efficiency will only affect the intensity of the expected DEER signal but not give rise to false signals.

Spin-labeled apoproteins of LHCII were reconstituted with pigments according to the standard procedure (30), and the purified and concentrated complexes in their monomeric or trimeric form were compared with those reconstituted with the unlabeled WT protein. Neither CD spectra in the visible range nor the intramolecular energy transfer from Chl b to Chl a showed any difference, demonstrating that spin labeling of the chosen sites did not affect the structure or function of LHCII (18, 20). When LHCII samples were thawed after EPR measurements, they gave fluorescence and CD spectra very similar to those obtained before the measurements (supplemental Figs. S2–S4), indicating that neither the addition of glycerol as cryoprotectant (40% (w/v) final concentration) nor the shock freezing step affected the functional structure of the pigment-protein complexes. A 1:1 mixture of two singly labeled monomers did not result in any appreciable DEER signal (supplemental Fig. S5) (20, 24) proving the notion that DEER signals originate from dipole–dipole interaction between electron spins within the same protein in doubly labeled complexes or between singly labeled monomers in trimeric samples but not from intermolecular spin-spin interactions due to aggregation of the complexes. However, one of the control samples (monomers labeled in position 59, supplemental Fig. S5B) exhibited a minor but significant DEER signal with a modulation depth of 0.17,
indicating that unwanted protein aggregation or oligomerization to a minor extent cannot be excluded in the sample preparation procedure (see "Discussion").

The core structure of detergent-solubilized LHCII monomers was assessed by measuring two distances parallel to the trans-membrane helices, one alongside helix H1 (59/90) and one between the luminal and stromal loops (106/160, dotted lines, Fig. 1B) as well as several distances parallel to the luminal surface of the complex (90/123, 90/196, and 123/196), providing information on the position of the trans-membrane helices to each other (dashed lines, Fig. 1B). Additionally, distances within trimeric LHCII were measured between monomers spin-labeled either in position 59 (stromal side, Fig. 1C) or 106 (luminal side). Three spin-spin distances are present in each of these trimers; however, because of the 120° symmetry in the trimeric structure, these three distances are expected to be the same.

To obtain interspin distances from the raw DEER data, these (Fig. 2B, black lines) were background-corrected and used to calculate spin-spin distance probability distributions (Fig. 2C, black lines). The width of the distance distributions reflects the unknown conformation distribution of the protein domains carrying the spin labels and the conformation distribution of the spin labels themselves. The latter adds a distance uncertainty between two given points in the protein of \( \pm 0.5 \) nm per spin label, i.e. \( \pm 1 \) nm uncertainty for each spin-spin distance, assuming complete conformational freedom for the nitroxide labels. In most cases, this freedom will be restricted due to steric hindrance by the protein and pigment environment of the labels. This restricted conformation distribution of the spin labels has been modeled into the crystal structure (28) identifying allowed orientations and estimating their probability by a rotamer library (Fig. 2A). The resulting predicted distance distributions, taking into account only conformation distribution of the label but no flexibility in the protein domains (dotted lines, Fig. 2C), were used for simulating the expected EPR dipolar evolution signals (gray curves, Fig. 2B). Those distances in our analysis that exhibit a larger deviation from the crystal structure than what is expected because of spin label conformational distribution can be detected by significant differences between measured and simulated dipolar evolution function and distance distributions (Fig. 2, B and C, respectively).

The distance distribution obtained with the spin pair on either end of helix H1 (59/90) is very narrow, consistent with the expected low protein flexibility in an \( \alpha \) helix, and it coincides very well with the one resulting from the rotamer library (maxima of 4.9 and 4.8 nm, respectively). The measured distance distribution is even narrower than the one predicted from the rotamer library, indicating that the simulation overestimates the rotational freedom of the spin labels. The parallel distance between the stromal and luminal loops (106/160) also yielded a distance distribution very similar to the predicted one, although in this case the EPR-measured distribution is somewhat broader. This may reflect some mobility of the loop domains, which is expected to be higher than that of the \( \alpha \) helices (7).

The distance measurements on the luminal surface of LHCII yielded a similar agreement between the EPR-measured data and those predicted from modeling. The measurement between helices H1 and H4 (90/196) resulted in a very narrow main peak, as expected for the rigid labeling positions in the helices. The same is true for the separation between helices H1 and H3 (90/123), where the measured distance distribution again is narrower than the modeled one and the H3-H4 separation (123/196). These three distance distributions contain, next to the main peaks corresponding to the expected separation, additional peaks on the shorter and longer distance sides. The 59/90 measurement also yielded an additional peak at shorter distances compared with the main distance peak. These may reflect artifacts due to protein aggregation and will be dis-
cussed below. Taken together, these data show that the hydrophobic core domain of LHCII in detergent-solubilized monomers adopts a structure very similar or identical to the one seen in crystallized LHCII trimers.

Reasonable agreement between the EPR-measured distances and the crystal structure was also seen when trimeric LHCII was analyzed with spin labels attached to position 59, i.e. to the N-proximal end of helix H1 (59 + 59 + 59 in Fig. 2). The sharp peak at 2.1 nm overlaps with the range of spin-spin distances resulting from the rotamer library, but the latter predicts a distance of 2.6 nm as the most frequently adopted one. The distance distribution between the three luminal loops in the trimeric structure (106 + 106 + 106) shows a broad major peak between 2.5 and 5.3 nm. Part of this broad distribution may stem from some mobility of the loop structure, but some of it is due to a larger mobility of the spin label as compared with the one seen in the α-helical labeling positions, as deduced from the broader modeled distance distribution. Although the peak maxima of the measured and predicted data coincide, the predicted range of distances is shifted to larger values (3.5–6 nm). The EPR measurement of LHCII labeled in the three luminal loop domains yielded extra peaks on the shorter and longer distance side (1.9 and 6.2 nm, respectively). Again, these may be artifacts (see “Discussion”).

**Distances Extending to N-terminal Domain**—The N terminus of LHCII up to residue 10 has not been resolved even in the latest crystal structure, which has been interpreted to indicate a certain degree of flexibility of this domain (7). Two series of experiments were performed to investigate the structure of the N-terminal domain in LHCII monomers and trimers. In one line of experiments, we measured distances between parts of the N-terminal protein domain and the rigid LHCII core. In the other series, the positioning of the three N termini relative to each other in trimeric LHCII was assessed.

The five N-terminal distances 29/59, 14/29, 3/59, 3/29, and 3/14 in LHCII monomers were assessed by DEER EPR (Fig. 3). In the case of 29/59, the predominant distances calculated from the DEER data correlate with the prediction based on the rotamer library between 2.5 and 3.8 nm. In addition, shorter and longer distances are detected indicating either a higher degree of label delocalization or peptide flexibility at position 29. In sample 14/29, hardly any correlation exists between DEER data and prediction. Here, a narrow distribution of spin-spin distances between 1.5 and 2.5 nm is expected from the simulation, although a broad range of distances between 1.5 and 5.5 nm results from the DEER experiment. The extension of the measured distance distribution toward longer distances cannot be explained by an underestimation of conformation distribution of the label, as was checked with the “Any rotamers?” feature of the software MMM, which demonstrates that distances longer than 3 nm cannot be reconciled with the back-

![Figure 2](image-url)

**FIGURE 2.** DEER results for monomeric and trimeric LHCII samples labeled in core positions and in the stromal and luminal loops. A, crystal structure (PDB entry 2BHW) of monomeric LHCII with possible acetamido-PROXYL rotamers. B, normalized dipolar evolution (black line) and rotamer library simulation (gray line). C, distance distribution calculated from the data in B with a regularization parameter λ = 10 and a dipolar evolution time of 2.5 μs (black line) and determined from a rotamer library-based simulation (dotted line).
bone coordinates of residues 14 and 29 in the crystal structure. Hence, monomeric samples in solution display conformations of the N-terminal domain that deviate from the known crystal structures (2, 3).

Broad distributions are also found for distances 3/59, 3/29, and 3/14. Residue 3 is the only one among these labeling positions that is not resolved in the crystal structure. Therefore, a distance prediction between residue 3 and residues 14, 29, and 59, and thus a comparison with the DEER distance distribution, is not possible. However, the broad distance distributions again cannot be explained by only conformation distribution of the label and thus indicate a considerable flexibility of the domain. Again, a side peak at ∼5 nm is detected in all monomeric samples labeled at N-terminal positions (see “Discussion”).

The second series of DEER experiments assessed 12 different interspin distances between the three N-terminal domains of trimeric LHCII as displayed in Fig. 4. Here, an extensive Cys walk toward the N terminus was performed, to detect possible deviations between the structures of LHCII in solution and in crystals and to assess the flexibility of individual segments of this domain. As the fixed point in these measurements, position 59 at the N-proximal end of helix H1 was chosen as part of the rigid core of LHCII.

The consistency between measured and predicted data remains fairly good going from position 59 to position 12. The only exception is position 22 where both the measurement and the structure simulation yield narrow distance distributions, but the peak maxima (at 5 and 6 nm, respectively) differ by 1 nm. The rotamer library prediction for this site is rather insensitive to repacking of the side groups in the LHCII structure with SCWRL4 (34); however, the “Any rotamers?” feature of MMM can reconcile the experimentally observed and simulated distance distributions, except for the minor noise-related peak at about 3 nm. Remarkably, in the 12 + 12 + 12 measurement, the EPR distance measurement yields a main peak that is narrower than the predicted distribution. The measured data do include an additional peak at 3.5 nm, which was found to be insignificant by validation and is probably noise related.

Up to position 12, the simulations can still be reconciled with experimental data considering the combined uncertainties; starting at residue 11 this is clearly no longer possible. The measured distance distribution becomes much broader than those distributions seen at the more distal labeling points and also broader than the predicted distribution. Moreover, the peak maximum in the measured distance distribution is shifted toward shorter distances in comparison with that of the predicted data (5 and 6.5 nm, respectively). This shift is even more pronounced in the 10 + 10 + 10 measurement (5.1 and 7.1 nm). In positions 10 and 11, the rotamer simulations possibly predict distributions that are somewhat too broad, as the spin label conformations may be restricted by side groups of the first nine

FIGURE 3. DEER results for monomeric LHCII doubly labeled in the N-terminal domain. A, stromal top view of monomeric LHCII (PDB entry 2BHW) with interspin distances measured in the N-terminal domain (dotted lines). B, normalized dipolar evolution (black line) and rotamer library simulation (gray line). C, distance distribution calculated from the data in B with a regularization parameter $\alpha = 10$ and a dipolar evolution time of 2.5 μs (black line) and determined from a rotamer library-based simulation (dotted line).
residues that are not resolved in the structure and thus disregarded in the simulations. However, the strong discrepancy between simulated and experimental data for these sites cannot be explained by this uncertainty, as the difference in the mean distances is larger than the total width of the conformational distribution for an unrestricted label, as tested with the “Any rotamers?” feature of MMM. Distances shorter than 4.7 nm for site 11 and shorter than 5.2 nm for site 10 are inconsistent with the backbone coordinates of these residues in the crystal structure. Possible suppression of long distances in the experimental

FIGURE 4. DEER results obtained for trimeric LHCII labeled in the N-terminal domain. A (1st column and continues in 3rd column), normalized dipolar evolution (black line) and rotamer library simulation (gray line). B (2nd column and continues in 4th column), distance distributions calculated from the data in A with a regularization parameter δ = 10 and a dipolar evolution time of 2.5 μs (black line) and determined from a rotamer library-based simulation (dotted line).
distribution by background correction cannot explain the results either, because the presence of the short distances that are inconsistent with the crystal structure can be inferred already from the fast decay of the initial part of the uncorrected dipolar evolution data at times smaller than 1 μs. Clearly, this part of the N-terminal domain is shifted toward the symmetry center of the LHCII trimer in comparison with the crystal structure. For the amino acid positions closer to the N terminus (positions 9, 7, 4, and 3), no comparison is possible with data predicted from the crystal structure because this protein segment has not been resolved crystallographically. The measured data clearly show that the distance distributions become even broader, indicating an increasing protein mobility toward the N terminus. Moreover, the maxima in the distributions further shift toward smaller distances, indicating that in the most frequently adopted structure the N termini extend toward the symmetry center of the trimer.

Water Accessibility to N-terminal Amino Acid Residues—ESEEM EPR was used to localize the N-terminal residues in monomers and trimers with respect to the water environment and the hydrophobic core of the detergent micelle. In this experiment, the hyperfine interaction of spin labels with deuterium nuclei of heavy water was measured for the 12 N-terminal residues, and the water accessibility parameter II(D₂O) was calculated as described previously (Fig. 5, gray dots and black triangles, respectively) (18).

Early experiments showed that residues near the LHCl core, which reside inside the hydrophobic micelle, exhibit a very low water accessibility parameter II(D₂O) (0.075–0.12), whereas the free label in detergent-containing buffer exhibits II(D₂O) = 0.35 (18). Uncertainty of the experimental values is ΔII(D₂O) = ±0.02. Overall, N-terminal positions have enhanced access to the hydrophilic environment, but in some cases differences between monomers and trimers appear. Residues (34, 14, 12, 10, 7, and 3) in monomers and trimers exhibit identical water accessibility, so they reside in similar environments. Monomeric samples 59, 22, 11, 9, and 4 show slightly but significantly enhanced water accessibility as compared with their trimeric equivalents, whereas the environment of residue 29 is slightly but significantly more hydrophobic in monomers than in trimers. Although the change in sample 59 can be due to the labeling position close to the interface between two monomers in a trimer, the other positions can hardly be affected by oligomerization and thus show conformational change of the N-terminal protein domain in response to the state of oligomerization.

In the trimer, water accessibility is roughly the same in samples 14, 12, 11, 9, 7, 4, and 3. This indicates that the localization of these residues with respect to detergent and lipid headgroups remains approximately the same throughout this section. The mean conformation of this section thus runs nearly parallel to the headgroup layer.

**DISCUSSION**

Core Structure of LHCl in Solution—The x-ray crystal structures of LHCl from spinach and pea (2, 3) can be superimposed to yield an almost perfect overlap of the main chain polypeptide atoms of residues 14–231. Additionally, the three trans-membrane helices of LHCl superimpose almost perfectly with the polypeptide backbones of LHCl apoproteins, corroborating the assumption of a conserved core structure of LHCs with little flexibility between individual α helices (7).

In our EPR study, the core structure of monomeric and trimeric LHCl in detergent solution was analyzed to scan the LHCl interior for flexible protein domains. Recently, DEER distance mapping was shown to be an excellent technique to analyze structural dynamics of proteins in solution (20, 25, 26). Therefore, interspin distances were measured between PROXYL labels, attached to each of the trans-membrane helices of LHCl, and these distances were compared with distances determined in a simulation based on label rotamers in the structure of trimeric LHCl crystals (28).

The DEER distance map of LHCl in aqueous solution displays a core structure identical to that seen in crystals with regard to both the sizes and the arrangement of membrane-spanning helices. Measured distance distributions between positions near the LHCl core are even narrower than the ones predicted from the rotamer library, indicating that spin labels have less rotational freedom than expected and verifying the tight packing of the complex in detergent solution. As pointed out by Barros and Kühlbrandt (7), a rigid scaffold fixing the pigments in their optimum position for both anisotropic light absorption and efficient energy transfer is a prerequisite for proper light harvesting.

The side peaks at 5.1 nm in the distance distributions 90/196 and 123/196 (Fig. 2) are puzzling. In both cases, the maximum peak of the EPR distance distribution coincides with the maximum peak of distances predicted by the rotamer simulation, but the 5.1 nm side peaks exceed these maxima by more than 1 nm and therefore cannot be due to the spin mobility simply being larger than expected from the molecular model. A distance change of this size in a subpopulation of LHCl would require either an altered tilt or partial unfolding at the luminal end in at least one of the trans-membrane helices. This would jeopardize proper binding of the Chls situated between the trans-membrane helices or bound to ligands near the luminal surface, respectively. Either appears highly unlikely in a functional LHCl structure.

An alternative explanation for the existence of side peaks is the accidental formation of LHCl trimers or aggregates due to the high LHCl concentration in the EPR sample (300 μM), which is approximately the concentration of LHCl trimers in...
the mother liquor of typical crystallization experiments (35). Highly concentrated LHCII monomers can form trimers in the presence of phosphatidylglycerol (PG) (36), of which trace amounts are contained in the pigments used for reconstituting LHCII. However, in CD and PAGE analyses of these LHCII solutions, no significant amounts of trimerized complexes were detectable (supplemental Figs. S2–S4). In the CD spectra and in partially denaturing electrophoresis, LHCII trimers would have shown up as a trimer signal at 473 nm (37) and as a distinct band partially denaturing electrophoresis, LHCII trimers would have detectable (supplemental Figs. S2–S4). In the CD spectra and in partially denaturing electrophoresis, LHCII trimers would have shown up as a trimer signal at 473 nm (37) and as a distinct band partially denaturing electrophoresis, LHCII trimers would have detectable (supplemental Figs. S2–S4). In the CD spectra and in partially denaturing electrophoresis, LHCII trimers would have shown up as a trimer signal at 473 nm (37) and as a distinct band partially denaturing electrophoresis, LHCII trimers would have detectable (supplemental Figs. S2–S4). In the CD spectra and in partially denaturing electrophoresis, LHCII trimers would have shown up as a trimer signal at 473 nm (37) and as a distinct band partially denaturing electrophoresis, LHCII trimers would have detectable (supplemental Figs. S2–S4). In the CD spectra and in partially denaturing electrophoresis, LHCII trimers would have shown up as a trimer signal at 473 nm (37) and as a distinct band.

Hence, we cannot exclude that the 5.1-nm peak is the remainder of the main peak, is narrow. Furthermore, contributions from longer distances are suppressed by background correction. Hence, we cannot exclude that the 5.1 nm peak is the remainder of a broad aggregate peak.

Structure and Flexibility of Loop Domains—By contrast to the rigid core domains, loop regions revealed more flexibility. As noted before, part of the broad distance distributions found in monomeric sample 106/160 and trimeric sample 106 + 106 + 106 may stem from a larger mobility of the spin labels than the one seen in α-helical labeling positions, as deduced from the broader modeled distance distribution. However, the DEER distance distributions measured were even broader than the predicted ones. Consistently, a certain degree of flexibility in LHCII loop regions has been deduced from comparing the two crystal structures and from inspecting crystallographic B factors (7). No bimodal distance distributions were seen in these EPR measurements, so these data do not support the notion of a co-existence of preferred conformers. Conformational changes in the luminal loop and core structures of LHCII, triggered by lumen acidification and leading to nonphotochemical quenching, have been proposed by some (2, 6) and doubted by others (7). The EPR data presented here indicate a considerable flexibility in an aqueous environment of the apoprotein in its loop domains but not in the hydrophobic core where the chlorophylls are bound.

Structure and Flexibility of the N-terminal Domain of LHCII in Solution—To discuss the structure and flexibility of the N terminus of monomeric and trimeric LHCII in solution, the domain will be divided into three individual sections, proceeding from the N-proximal end of the first trans-membrane helix toward the N terminus, and each section will be compared with the structural data published in the latest x-ray studies (2, 3).

The flexibility of the first section, covering the polypeptide backbone from residue 59 to 26, is low in LHCII crystals and in solubilized trimers. Interestingly, only this section of the N terminus could be traced out in the first LHCII structure in two-dimensional crystals at 3.4 Å resolution (38), a first evidence for a low flexibility of the section in trimeric LHCII. According to the crystal structure, residues 51 to 42 form a short loop that submerges into the lipophilic membrane environment. In this loop, the polar headgroup of the lipid PG is in contact with Tyr-44, and one of its fatty acid chains expands deeply into the core of the trimer along the interface of two monomers, probably undergoing hydrophobic interactions (36). Thus, PG may anchor the first N-terminal section to the rigid core of LHCII, which may explain the rigid structure of this part of the N-terminal protein domain in trimeric LHCII.

The second section, covering amino acid residues 26 to 14, was more difficult to resolve in crystallography and was defined only by the newer x-ray studies by Liu et al. (2) (PDB code 1RWT) and by Standfuss et al. (3) (PDB entry 2BH7). EPR measurements of trimeric LHCII in solution confirm the low flexibility expected from the crystal data, whereas the broadened distance distribution in sample 14/29 indicates a less defined and more flexible structure for this section in monomeric LHCII. The main peak in the distance distribution of the same sample is shifted in LHCII monomers compared with trimers, and the water accessibility parameter in positions 29 and 22 show significant differences, both indicating this protein domain to adopt a slightly different structure in monomers compared with that in trimers. This is not unexpected because according to the crystal structure of LHCII trimers, amino acid Tyr-24 is the protein ligand for the central Mg$^{2+}$ of Chl 9. The pigments Chl 9 and violaxanthin and the lipid PG, which run parallel to each other, are positioned in monomer–monomer interfaces in the trimer and are only weakly, if at all, bound to monomers. Therefore, in monomeric LHCII, these contact sites may be missing Chl 9 and thus the middle section in the
N-terminal domain, rendering it more flexible in comparison with its situation in LHCII trimers.

The last section of the N-terminal domain covers residues 14 to 1 of which the first nine residues have not been resolved in the x-ray studies. Thus, for these positions, we cannot compare the EPR data to the x-ray structure. Residues 14 to 10 were resolved in structure 2BHW only, featuring strongly enhanced B factors, and at least for residues 11 and 10 it is safe to state that their mean location with respect to the C$_5$ symmetry axis of the trimer in solution differs strongly from the one observed in crystals. We have also tested whether the width of the distance distributions for sites 11, 10, 9, 7, 4, and 3 can be explained by any well defined conformation of the N-terminal domain and conformation distribution of the spin label. The full width at half-height of the distance distributions simulated for completely unrestricted label conformation varies slightly with the distance between the labeled residues and their relative orientation. However, this width is typically 1.5–1.7 nm and in no case exceeds 2 nm, whereas all experimental distance distributions in this section are at least as broad as 3 nm. We therefore conclude that in aqueous solution the backbone of the N-terminal domain in this section exhibits a spatial distribution between 0.5 and 1.5 nm. A flexible N terminus in LHCII has been proposed to explain the low resolution in this segment of the LHCII crystal structure (3), but it was never confirmed experimentally for LHCII. The DEER distance mapping in our study provides evidence for a highly disordered domain-terminal protein section in both monomeric and trimeric LHCII. This is the protein section that carries the phosphorylation site in LHCII (Thr-5 and -6). LHCII phosphorylation triggers the so-called state transition, a re-distribution of LHCII to balance the excitation energy flow between PSI and PSII (39). A different EPR structural study on the N-terminal domain of the CP29, one of the monomeric minor light-harvesting complexes of PSII, also indicated that the phosphorylation site is located in a mobile protein domain (40). This local flexibility of the substrate protein may be a prerequisite for its interaction with its cognate kinase.

In the distance distributions in the trimeric complexes labeled in positions 9, 7, 4, and 3, two maxima can be noted. These do not immediately suggest a bimodal distance distribution because there is no base-line separation between the two peaks. However, some of the primary EPR data show a kink in the time trace that does indicate bimodality. There is a trend when going from 9 to 7 and 4 to 3 for this kink to become more pronounced. This may indicate two families of preferred conformations, with the difference between the families increasing toward the N terminus, confirming our earlier notion of two preferred conformations of the N-terminal LHCl domain (24). However, as long as such conformations have not been correlated with functional states such as the phosphorylated and nonphosphorylated state or the attachment to PSI or PSII, their significance remains unclear.

The structural information on LHCl presented here refers to the complex in aqueous detergent solution. It has been shown that detergent micelles are able to mimic the membrane environment quite well for at least some membrane proteins (41). LHCl like many other membrane proteins is able to adopt its functional structure in aqueous detergent solution, taking efficient intra-molecular energy transfer from Chl b to Chl a as a criterion for functionality. It is difficult to decide whether the LHClII environment in crystals used for x-ray analyses is a better approximation to the one provided by the thylakoid membrane. On the other hand, the stacked two-dimensional crystals containing lipids of pea LHClII (3) or the icosahedral proteoliposomes of spinach LHClII (2) contain a more or less continuous hydrophobic phase in which the complexes are inserted rather than individual micelles enclosing the hydrophobic section of each LHClII. On the other hand, the non-natural up/down orientation of neighboring LHClII trimers as well as crystal packing effects may distort parts of the protein, particularly hydrophilic domains. In the detergent situation, those hydrophilic domains protrude into a truly aqueous environment. Consequently, differences seen between the LHClII crystal structures and structural data derived from EPR measurements should be taken as a reminder that the native structure of membrane-embedded LHClII may still be different from what we have seen so far. Because of the improved sensitivity of Q-band EPR, it may soon be possible to perform structural studies on LHClII embedded in thylakoid membranes.

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