Supporting Information

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SI Text

Site-Directed Spin Labeling (SDSL) of LHCII. The spin label used in SDSL was PROXYL, which we found advantageous compared to TEMPO used earlier (1). Besides the smaller size of PROXYL with lower internal dynamics, its chemical stability was higher, particularly toward low pH and reductants. TEMPO spin label reacted quite sensitive toward chemicals such as trichloroacetic acid, DTT, or β-mercaptoethanol (β-ME). The latter are thought to reduce the paramagnetic nitroxyl group to the nonparamagnetic hydroxylamine form (2–4). Reductants are an essential component in the reconstitution of Lhcb1; therefore, PROXYL, compared to TEMPO, yielded an acceptable signal-to-noise ratio at lower protein concentrations.

The efficiency of spin labeling was quantified by subsequent fluorescence labeling with a 50-fold molar excess of BODIPY 507/545 IA [N-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-2-yl)iodoacetamide] as described previously (1) and found to be higher than 90% for mutants containing a singular Cys, indicating that more than 80% of the mutants used for distance measurements carried two labels. An Lhcb1 mutant possessing no Cys did not give any EPR signal after the spin labeling reaction indicating that labels were specifically attached to sulfhydryl groups.

The labeled apoproteins were reconstituted with pigments according to the standard procedure as described in (5) with the exception that 10 mM β-ME was used as reductant. Purification of LHCII and preparation of EPR samples are described in (1). Purified complexes were compared to those reconstituted with the non-labeled WT protein. Neither CD spectra in the visible range nor the intramolecular energy transfer from chlorophyll (Chl) *b* to Chl *a* (6) showed any difference between the two, demonstrating that spin labeling did not affect the structure or function of LHCII.

Folding and Characterization of Recombinant Spin-Labeled LHCII Using a Modified Reconstitution Protocol for EPR Kinetic Measurements. For measuring the kinetics of Lhcb1 folding by time-resolved EPR measurements (mutants 106/160 and 90/196) we used the well-established reconstitution in vitro of LHCII from its protein and pigment compounds. However, to achieve the spin label concentration required for a good signal-to-noise ratio in EPR (7), the reconstitution procedure had to be modified substantially, raising protein and pigment concentrations by a factor of 20 as compared to earlier time-resolved fluorescence measurements of LHCII assembly (8). A ternary mixture of detergents was necessary to avoid Chl aggregation during the experiments.

Pigments were extracted as described in (9) except that the extraction step with diethyl ether was omitted. Freeze-dried pigments (3-fold molar excess of total Chl over protein) were dissolved in ethanol (20 mg/mL) and mixed by vortex with reconstitution buffer 1 containing 100 mM lithium borate (pH 8.1), 12.5% (wt/vol) sucrose, 6% (wt/vol) n-octyl-ß-D-glucoside (OG), 1% (vol/vol) Triton X-100 (Tx-100), 0.26% (wt/vol) 1,2-dipalmitoyl-sn-glycero-3-phosphatidylglycerol (PG), 0.02% (wt/vol) deoxycholic acid, and 10 mM β-ME. The protein solution contained 160 μM spin-labeled Lhcb1 and 1% (wt/vol) lithium dodecyl sulfate (LDS) in reconstitution buffer 2 [10 mM β-ME, 100 mM lithium borate (pH 8.1), and 12.5% (wt/vol) sucrose].

Reconstitution was initiated by manually mixing the protein and pigment solutions at a ratio of 1:1 in a 1.5-mL reaction tube by using a vortex mixer at room temperature ("reconstitution for EPR measurements"). After the reaction time the sample was mixed at a 1:1 volume ratio with 80% glycerol as a cryoprotectant and then immediately loaded into an EPR tube and flash-frozen in liquid nitrogen. This manual freeze-quench procedure took (10 ± 1) s. Each data point in the EPR-kinetic measurements represents an independently mixed protein sample exhibiting a final protein concentration of about 40 μM LHCII. To check that all labeled versions of the various Lhcb1 mutants had folded into a functional structure, 50 μL of the EPR sample of each mutant were diluted with 100 mM lithium borate (pH 8.1, 100 μL) lacking sucrose and concentrated at 3,000 × *g* in a 30 kDa Centricon filter unit (Millipore) at 4 °C. After repeating this step twice, samples were purified via ultracentrifugation as reported before (1) to compare them with complexes reconstituted by standard reconstitution procedure that have lower protein and pigment concentrations. Fluorescence emission spectroscopy (6) and CD spectroscopy in the visible domain (10) were performed as described elsewhere.

As shown for spin-labeled mutant 106/160 in Fig. S5, the complexes folded via the modified method exhibited the expected separation into free pigment (FP), folded protein-pigment complexes (LHCII) and aggregated Chls (a Chls). The isolated monomeric LHCII complexes were checked for their functionality and correct pigment assembly by measuring the intramolecular energy transfer from Chl *b* to Chl *a* and CD spectra in the visible range, respectively (Fig. S6, trace b). The CD showed the characteristic "fingerprint" signals of native LHCII or recombinant LHCII containing the WT protein sequence reconstituted according to the standard protocol (Fig. S6, trace a).

Spin-labeled LHCII was further characterized by comparing the DEER dipolar spectra (Fig. S7) of isolated monomeric complexes reconstituted by the standard method with those of non-isolated complexes folded by the modified method for EPR measurements. As shown for the mutants 106/160 (Fig. S7*A*) and 90/196 (Fig. S7*B*), the different reconstitution procedures yielded identical folding products.

Time-Resolved Fluorescence Measurements Using the Modified Reconstitution Protocol for EPR Measurements. Although the modified reconstitution procedure for EPR measurements yielded correctly assembled complexes, the kinetics of complex formation by the modified and the standard reconstitution procedure still had to be compared. In this experiment an acceptor fluorophore (DY731, Dyomics) was covalently attached to a single Cys in the stromal loop domain in LHCII (mutant 160, single Ser replaced by Cys) and time-resolved fluorescence measurements were performed according to Horn et al. (8) using excitation and emission wavelengths of 470 and 759 nm, respectively.

Purified dye-labeled LHCII exhibited a high Förster-type energy transfer efficiency showing that both Chl *a* and *b* are energy donors to the attached fluorophore. Measurements of the energy transfer during LHCII assembly resulted in an increase of the acceptor dye's fluorescence emission due to Chl binding in dependency on the folding time. The data revealed two apparent kinetic phases for the binding of the pigments (Table S1, standard reconstitution), a faster one ($\tau 1$) in the range of seconds to 1 min and a slower one ($\tau 2$) in the range of several minutes. This experiment was repeated (Fig. S4) using the reconstitution protocol for EPR measurements with high protein and pigment concentrations. The analysis of this kinetic

trace yielded two time constants of about 26 s ($\tau 1$) and 178 s ($\tau 2$) as shown in Table S1. Both apparent reaction phases are somewhat faster than seen in the earlier measurements, as is expected because of the higher concentrations of reactants (11). This shows that the modified reconstitution procedure does not substantially alter the kinetics of LHCII assembly as compared to the standard procedure.

Rapid Freeze-Quench of Samples for DEER Experiments. Samples were prepared by a two-step mixing of LDS-denatured apoprotein first with the pigment solution and then with 80% glycerol as a cryoprotectant. An Update Instruments System 1000 rapid mixer (Update Instruments Inc.) with four independent syringe shafts was used for this purpose. Three syringes were filled with apoprotein solution, pigment solution, and 80% glycerol as a cryoprotectant. The protein and pigment solutions were combined in a first grid mixer. After the reaction period, this mixture was combined with an equal volume of 80% glycerol in a second grid mixer. All tubes connecting the syringe outlets and grid mixers were kept as short as possible to avoid unnecessary dead volume. From the output of the second mixer the solution was sprayed onto a brass funnel cooled with liquid nitrogen. A dry nitrogen gas stream was directed to the funnel parallel to the mixture to stop recoiling sample droplets and lead them back to the funnel. This stream also minimized water condensation on the funnel and the sample particles. Sample particles were transferred to the sample tube by manually scraping them from the brass surface into an EPR tube attached to the lower end of the funnel.

In a control experiment, the protein/pigment mixture was extracted from the outlet of the first mixer before glycerol addition, was allowed to fold for 30 min in the dark, and the extent of folding was checked by denaturing LDS-PAGE (vide infra).

Check of Samples for Correct Protein Folding Using Partially Denaturing LDS-PAGE, ESE EPR, and T_2 Relaxation Measurements. Kinetic EPR experiments with shorter folding times yielded only partially assembled LHCII. To verify that all these samples had the same competence for folding and resulted in the same fractions of folded and unfolded protein, an aliquot was taken from each EPR sample at the end of the reaction time, before adding glycerol, and folding was completed for 30 min at room temperature. The aliquots were then run on a partially denaturing LDS-PAGE (12). Only samples that exhibited a strong green band of folded complex (Fig. S8*A*) and displayed around 50% of folded LHCII after Coomassie Brilliant Blue-Staining (Fig. S8*B*) were used for evaluating the EPR data. Hence, unfolded protein adds a constant offset to the high-frequency integral used in evaluating folding kinetics, so that the time-dependence is not influenced.

Samples that passed this first check were subjected to ESE EPR and T_2 relaxation measurements (13). EPR spectra were measured with field-swept echo detected EPR using a Hahn echo sequence $\pi/2$-τ-π-τ-*echo* and a 15-mT field sweep. The interpulse delay time τ was 200 ns and the pulse lengths were 16 ns for $\pi/2$ pulse and 32 ns for π pulse and the integration gate length was 200 ns.

The effect of short distances in unfolded or partially folded LHCII on transverse relaxation time T_2 was estimated by instantaneous diffusion measurements using a two-pulse ESE sequence. The flip angle β of the second pulse was varied by reducing microwave power with the main attenuator, and phase memory times $T_m(\beta)$ were obtained by fitting exponentials to the ESE decays measured as a function of the interpulse delay. The slope of linear fits to plots of $1/T_m$ as a function of $\sin^2(\beta/2)$ is a measure for local spin concentration. A significant increase of $1/T_m$ with $\sin^2(\beta/2)$ thus indicates either aggregation of spin-labeled molecules or a high prevalence of short intramolecular distances in a broad conformational ensemble of structures.

As shown in Fig. S3, aggregated Lhcb1 apoprotein (90/196) folded in mock reconstitutions lacking pigments (gray line), resulted in a characteristic ESE spectra showing only slight differences in the intensity of the major peak at 329 mT and the side peaks at 326 mT and at 332 mT. In comparison, LHCII folded by the reconstitution procedure for EPR measurements resulted in characteristic ESE spectra with a distinct major peak at 329 mT, indicating that no protein aggregates were formed (Fig. S3, black line). At the same time such samples displayed relaxation times T_2 longer than 2,000 ns also indicating the absence of protein aggregates. Only samples that passed these criteria were used for time-resolved DEER measurements.

Processing Parameters in DEER Data Analysis. For measurements with $\tau_2 = 1{,}500$ ns, data after a maximum evolution time of 1,400 ns were cut off. This cut-off time was set to 2,400 ns for $\tau_2 = 2{,}500$ ns and to 800 ns for the kinetic measurements with the unfolded protein in buffer 1.

A background function was fitted to the data at dipolar evolution times $t > t_b$. Starting time t_b was determined automatically as described in Jeschke et al. (14) and is given explicitly in the *Results* section. For measurements with unfolded and completely folded samples, experimental background functions were determined from data of mixtures of the two corresponding singly spin-labeled proteins by fitting a fifth-order polynomial. For measurements with partially folded samples, an exponential decay function corresponding to a 3-D homogeneous distribution of spins was used. After background correction of the data the distance distribution was determined by Tikhonov regularization with a regularization parameter $\alpha = 1{,}000$, except for the folded double mutant 90/196, where $\alpha = 1{,}000$ lead to overdamping and $\alpha = 100$ was used. Dipolar spectra were normalized to their integral.

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14. Jeschke G, et al. (2006) DeerAnalysis2006 - A comprehensive software package for analyzing pulsed ELDOR data. *Appl Magn Reson* 30:473–498.

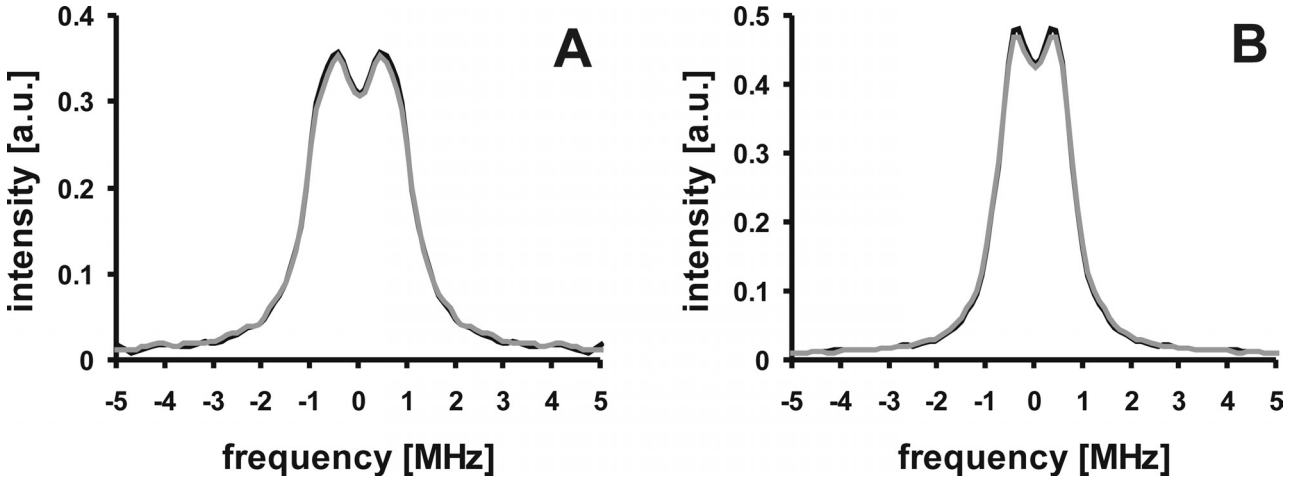

Fig. S1. Check of the reproducibility of DEER dipolar data of different LHCII double mutants. Monomeric LHCII samples were purified by ultracentrifugation and checked by CD measurements. DEER spectra were scanned with a dipolar evolution time of 2,500 ns, the background correction starting time for *A* and *B* was 224 and 320 ns, respectively. (*A*) comparison of two separately reconstituted LHCII samples of the double mutant 90/196 (gray and black trace) and in (*B*) double mutant 106/160 (gray and black trace).

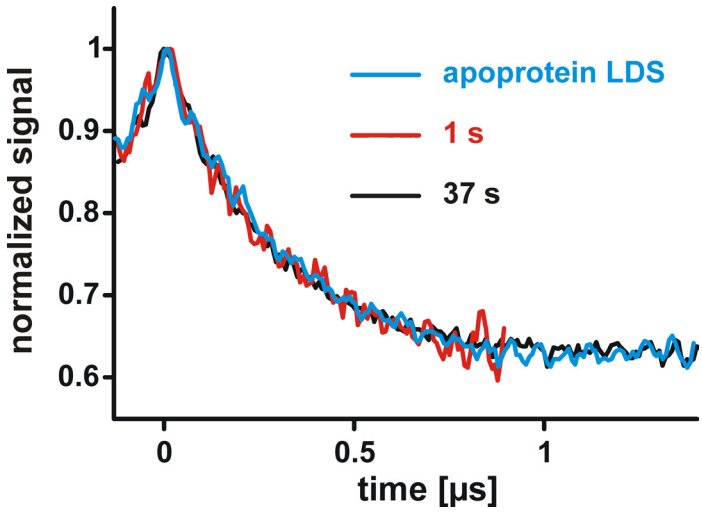

Fig. S2. Primary DEER EPR data of the LHCII mutant 90/196. The blue line represents the apoprotein in LDS micelles, the red line a sample shock frozen immediately after mixing the protein and pigment solutions (rapid freeze quench), and the black line a sample shock frozen 37 s after mixing (manual freeze quench). Data traces are normalized to the intensity at zero time and vertically scaled to match modulation depths. Modulation depth differences were smaller than 5% of the normalized maximum echo amplitude.

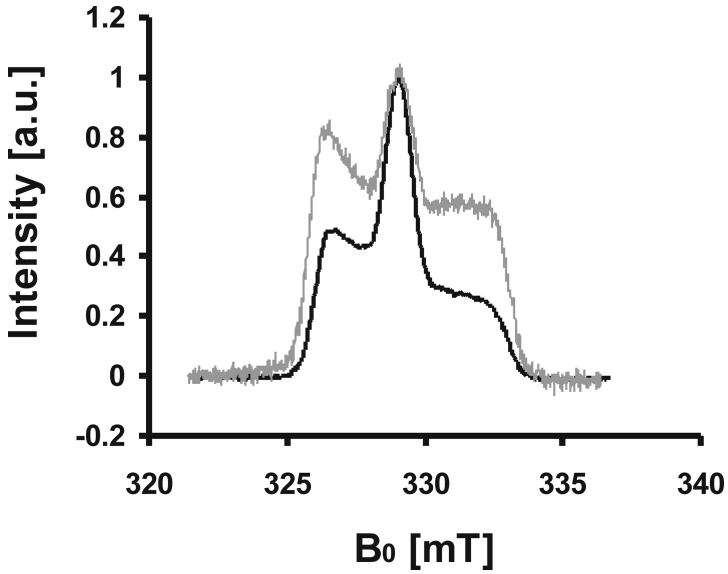

Fig. S3. ESE EPR spectra of two samples of the LHCII mutant 90/196. The black line represents folded complexes (reconstitution for EPR measurements); the gray line represents aggregated Lhcb1 apoprotein obtained by a mock reconstitution containing no pigments. Spectra are B_0 field-shifted and normalized on the intensity of the central peak.

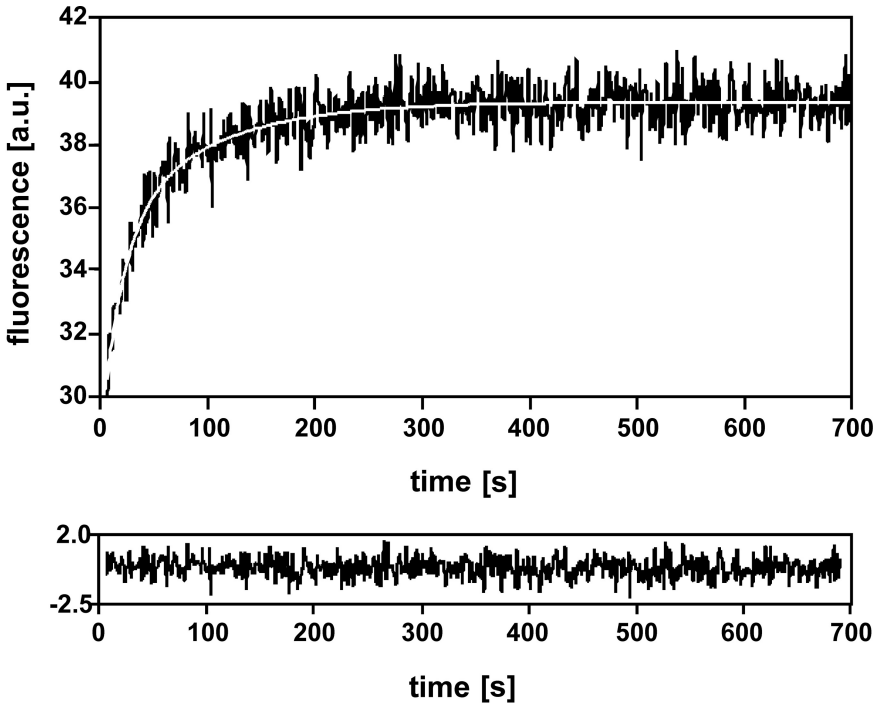

Fig. S4. Folding of LHCII as monitored by the rise of Chl-stimulated acceptor dye (DY731) fluorescence at 759 nm. Reconstitution was performed by using the EPR reconstitution procedure. The white line in the trace represents the biexponential fit in Table S1 (EPR reconstitution). Residuals, that is, kinetic trace minus fitted curve, are given in the lower panel.

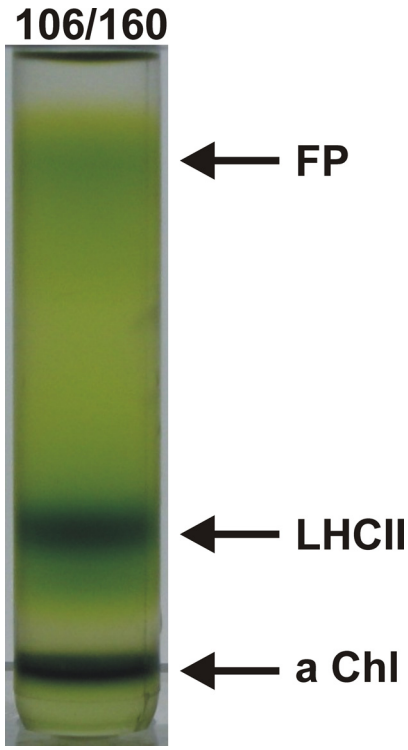

Fig. S5. Purification of reconstituted LHCII mutant 106/160 via sucrose gradient ultracentrifugation. After centrifugation in a Beckman SW 60 Ti rotor for 17 h at 230,000 × *g* and at 4 °C, three bands were distinguished. FP is free pigment, a Chl are aggregated Chls, and LHCII is reconstituted and spin-labeled LHCII.

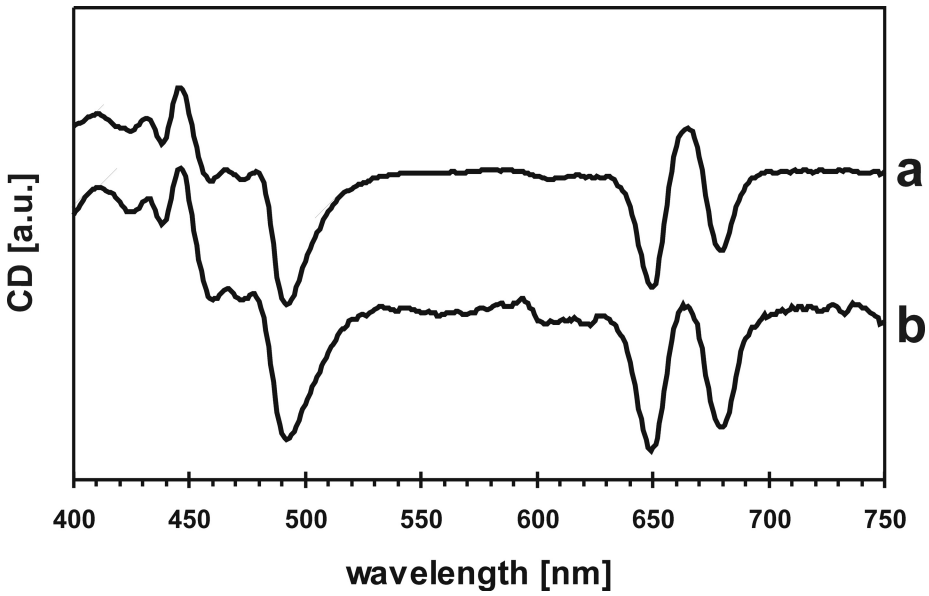

Fig. S6. CD spectra of LHCII after purification via sucrose gradient ultracentrifugation, (*A*) non-labeled LHCII apoprotein (WT protein sequence) reconstituted by standard reconstitution and (*B*) spin-labeled mutant 106/160 reconstituted for kinetic measurements (reconstitution for EPR measurements).

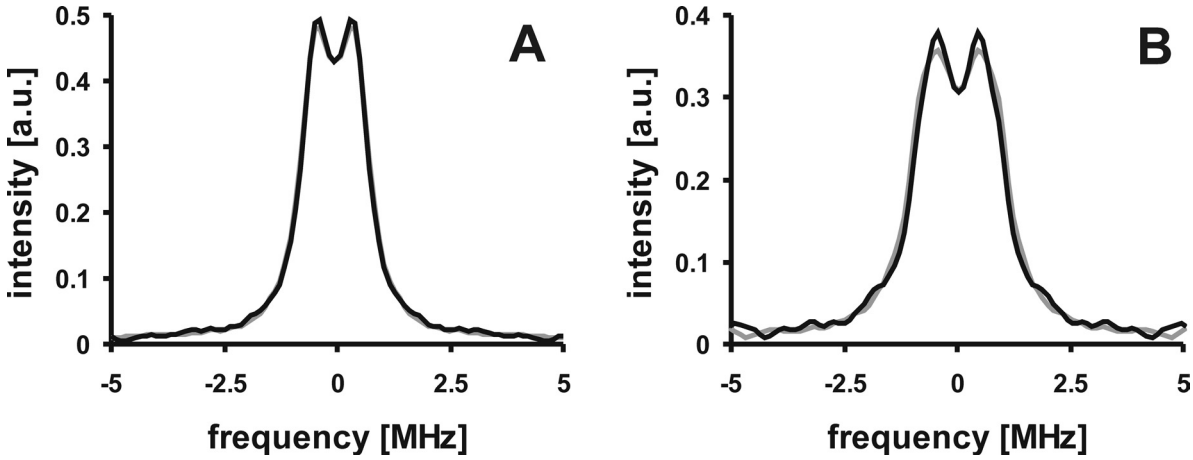

Fig. S7. Comparison of DEER dipolar spectra of the spin-labeled LHCII mutants 106/160 (*A*) and 90/196 (*B*) reconstituted by different protocols. Gray traces are monomeric LHCII samples reconstituted by the standard procedure and purified by ultracentrifugation, black traces are monomeric LHCII samples reconstituted by the protocol for EPR measurements without purification (≈930 s folding time). Spectra were scanned with a dipolar evolution time of 2,500 ns, the background correction time for *A* and *B* was 320 and 224 ns, respectively.

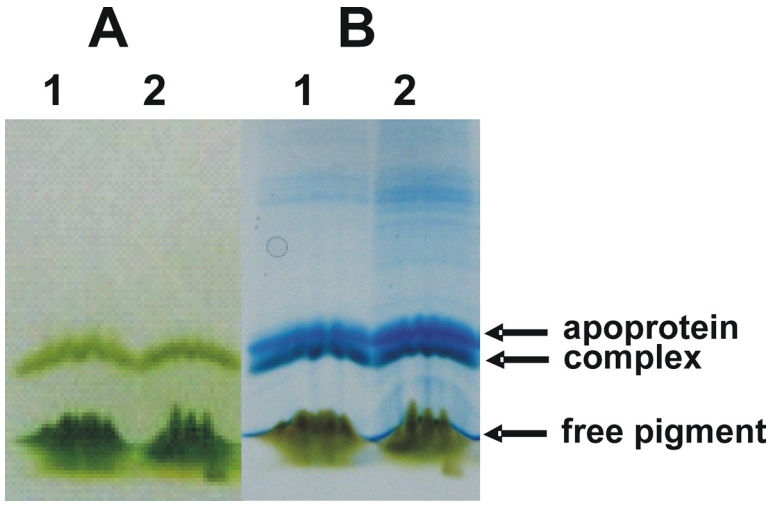

Fig. S8. PAGE analysis of reconstituted LHCII (reconstitution for EPR measurements). Partially denaturing LDS-PAGE on 10% polyacrylamide gel at 4 °C and 80 V, (*A*) unstained, (*B*) Coomassie Brilliant Blue-Stained. (1) Spin-labeled double mutant 106/160 and (2) spin-labeled double mutant 90/196

Table S1. Kinetic data extracted from time-resolved fluorescence measurements of LHCII assembly

Experiments	Conc.	τ1 (s) ± SD	τ2 (s) ± SD	*A1/A2* ± SD
standard reconstitution	3.9 μM	41 ± 9	260 ± 53	0.9 ± 0.08
EPR reconstitution	78 μM	26 ± 11	178 ± 114	3.1 ± 1.84

Standard reconstitution is described in Paulsen H, Rümler U, Rüdiger W (1990) Reconstitution of pigment-containing complexes from light-harvesting chlorophyll-*a*/*b*-binding protein overexpressed in *Escherichia coli*. *Planta* 181:204–211. Conc., Protein concentration after reconstitution. SD, standard deviation.