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Importance of trimer–trimer interactions for the native state of the plant light-harvesting complex II

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Abstract

Aggregates and solubilized trimers of LHCII were characterized by circular dichroism (CD), linear dichroism and time-resolved fluorescence spectroscopy and compared with thylakoid membranes in order to evaluate the native state of LHCII *in vivo*. It was found that the CD spectra of lamellar aggregates closely resemble those of unstacked thylakoid membranes whereas the spectra of trimers solubilized in n-dodecyl-β,D-maltoside, n-octyl-β,D-glucopyranoside, or Triton X-100 were drastically different in the Soret region. Thylakoid membranes or LHCII aggregates solubilized with detergent exhibited CD spectra similar to the isolated trimers. Solubilization of LHCII was accompanied by profound changes in the linear dichroism and increase in fluorescence lifetime. These data support the notion that lamellar aggregates of LHCII retain the native organization of LHCII in the thylakoid membranes. The results indicate that the supramolecular organization of LHCII, most likely due to specific trimer–trimer contacts, has significant impact on the pigment interactions in the complexes.

Keywords: Thylakoid membrane; Light-harvesting complex; Circular dichroism; Linear dichroism; Time-resolved fluorescence

1. Introduction

In green plants, half of the chlorophyll is bound by the most plentiful pigment–protein complex of the thylakoid membrane, the light-harvesting complex II (LHCII). LHCII has been extensively studied in the past two decades by biochemical, biophysical and molecular genetics approaches, validating not only its light-harvesting but also structural and regulatory roles. The light-harvesting complex II comprises a group of nuclear-encoded proteins of the *Lhcb* gene family [1] with six genes — *Lhcb*1–6. In this work, by the term LHCII we refer exclusively to the peripheral or "major" antenna of Photosystem II (LHCIIb), which is encoded by the genes *Lhcb*1–3. The X-ray crystal structure of the major LHCII was resolved independently from spinach [2] and from pea [3]. Each monomer subunit has a polypeptide chain of ~232 amino acids with three membrane-spanning α-helices and binds eight molecules of chlorophyll *a*, six Chl *b* and four carotenoids, identified in the structure as two luteins, one neoxanthin and one violaxanthin. The tight packing of the pigments in the protein bed effects strong chlorophyll–chlorophyll and chlorophyll–carotenoid interactions ensuring rapid and effective excitation energy exchange [4,5].

LHCII, assembled in trimers [6], is found in the apressed regions of the grana [7] where it serves its principal light-harvesting role. LHCII mediates lateral separation and stacking and stabilizes the grana [8–11] and is involved in various mechanisms for regulation of photosynthesis, e.g. state transitions [12] and nonphotochemical quenching [13]. These pro-

Abbreviations: Chl, chlorophyll; CD, circular dichroism; CMC, critical micelle concentration; DM, n-dodecyl-β-D-maltoside; LD, linear dichroism; LHCI-PSI, light-harvesting complex I-Photosystem I; LHCII, major light-harvesting chlorophyll *a*/*b* complex II; OG, n-octyl-β-D-glucopyranoside

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cesses are likely associated with changes in the LHCII structure and macroorganization [14–17] and therefore understanding them requires detailed knowledge of the native state of LHCII in the membrane.

There are several existing models for the supramolecular organization of LHCII in the thylakoid membrane [18,19]. LHCII readily forms 2D and 3D aggregates *in vitro* [10,20,21] and it has been postulated that *in vivo* it may exist in some form of an aggregated state higher than the trimer. One possible arrangement is the PSII–LHCII supercomplexes where 2–4 LHCII trimers are bound to a PSII dimer [19]. However this stoichiometry cannot account for all LHCII present in the membrane [22]. There is substantial evidence that LHCII can also form ordered arrays of trimers, or LHCII-only domains [18,19,22,23], in which trimers form close contacts between each other and can transfer excitation energy over a long distance [24]. It is very unlikely that isolated trimers, unconnected to the Photosystem II core, occur in the native membrane containing 70–80% protein [25]. Still, a large number of the *in vitro* spectroscopic studies on LHCII are done using isolated LHCII trimers solubilized in detergent micelles as a model for the native LHCII. For this purpose usually non-ionic detergents, such as Triton X-100, n-octyl-β,D-glucopyranoside (OG) [21,26,27], or n-dodecyl-β,D-maltoside (DM) [28–32], are used. In the solubilized state LHCII might possess different properties than in its native environment, on the one hand due to the lack of protein–protein contacts, and on the other hand due to protein–detergent interactions, which can rarely be neglected even with mild detergents, such as OG and DM [33,34]. In this respect, aggregated preparations of LHCII in a state as close to the native one as possible might serve as a more suitable model for studying LHCII. There are indications that loosely stacked lamellar aggregates of LHCII can mimic some of the properties of the native thylakoid membranes, like long-range chiral order of the chromophores [35,36], and light-induced reversible structural reorganizations [14,37,38].

In the present paper we analyze linear and circular dichroism and fluorescence lifetime data for lipid–protein macroaggregates and solubilized trimers of LHCII in comparison with thylakoid membranes and show that macroaggregates may represent one of the native states of LHCII better than solubilized trimers. We also show that different detergents perturb the pigment coordination in LHCII to different extents and in a different manner.

2. Materials and methods

2.1. Plant material

Market spinach or leaves of 2 weeks old pea plants grown on soil in the greenhouse were used. Thylakoid membranes were isolated by the following procedure. Leaves were homogenized with ice-cold buffer medium containing 20 mM Tricine (pH 7.5), 0.4 M Sorbitol, 5 mM $MgCl_2$, 5 mM KCl. The homogenate was filtered through four-layer cheesecloth and the remaining debris was centrifuged down at 200×*g* for 2 min. The supernatant was then centrifuged at 4000×*g* for 5 min. The pellet was resuspended in osmotic-shock medium (20 mM Tricine, 5 mM $MgCl_2$, 5 mM KCl, pH 7.5) and centrifuged at 7000×*g* for 5 min. Unstacked thylakoids were obtained after washing three times with buffer medium containing only 20 mM Tricine (pH 7.5) and centrifuging at 7000×*g* for 5 min.

Aggregates of LHCII were isolated by the method of Krupa et al. [39] with modifications described by Simidjiev et al. [40]. In brief, thylakoid membranes were suspended in 20 mM Tricine buffer (pH 7.8) at [Chl] 0.8 mg/ml and Triton X-100 was added to a final concentration of 0.5–0.7%. The suspension was incubated for 1 h and centrifuged at 30,000×*g* for 40 min. The supernatant was treated with 30 mM $MgCl_2$ and 100 mM KCl for 15 min and centrifuged on 0.5 M sucrose cushion for 10 min at 10,000×*g*. The pellet was resolubilized with 0.5% Triton and the LHCII was precipitated as before. The final preparation was washed with 20 mM Tricine (pH 7.8), centrifuged at 10,000×*g*, resuspended in the same buffer at [Chl] of ca. 1 mg/ml and stored at 4 °C. The LHCII aggregates isolated by this procedure are organized in lamellar structures with diameter of several μm, Chl a/b ratio 1.3 and ~300 μg lipids/mg Chl. PSII/PSI cores could not be detected by immunoblotting against D1 protein [40] or low-temperature fluorescence.

Trimers of LHCII were isolated by isoelectric focusing from BBY particles solubilized in DM as described in [41] and stored as suspension in HEPES buffer (pH 8.0) at −80 °C.

For spectroscopy measurements, samples were diluted in 20 mM Tricine buffer (pH 7.8) to a Chl concentration of 10 μg/ml (20 μg/ml for the detergent-solubilization experiments). 0.01% DM was added to the samples containing solubilized trimers.

2.2. Detergent solubilization

The detergents Triton X-100 (Sigma), n-dodecyl-β,D-maltoside (Sigma) and n-octyl-β,D-glucopyranoside (Sigma) were used to solubilize LHCII aggregates or thylakoid membranes. The detergents were added from 10% stock solutions to the sample diluted at 20 μg/ml Chl concentration in 20 mM Tricine buffer medium (pH 7.8); the sample was vigorously stirred (or vortexed) and incubated for 5 min before measuring. Titration was done by repetitively adding detergent aliquots and measuring the CD so that the total detergent concentration increased at each step.

2.3. Circular and linear dichroism measurements

Circular dichroism (CD) and linear dichroism (LD) spectra were registered with a modified CD6 dichrograph (Jobin-Yvon, France). The optical path length was 1 cm. The CD and LD was recorded in absorbance units but for easier comparison the data are plotted normalized to amplitude of the negative band at 650 nm (for CD) or to the maximal amplitude in the red region (for LD). For LD measurements the samples were fixed in polyacrylamide gel, containing 5% acrylamide:bis-acrylamide (30:1) polymerized with 0.2% ammonium persulfate and 0.2% *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), and oriented by two-dimensional squeezing [42]. CD and LD were measured at room temperature.

2.4. Time-resolved fluorescence

Time-resolved fluorescence measurements were performed as described in [43]. The fluorescence decays were recorded using a single-photon-counting apparatus with a resolution of 1–2 ps. The excitation beam was tuned at 645–650 nm and fluorescence was detected at different wavelengths between 660 and 740 nm. The fluorescence decays were analyzed by global lifetime analysis [44].

3. Results

3.1. CD spectra of thylakoids and isolated LHCII

The CD spectra of thylakoid membranes, aggregates of LHCII and solubilized LHCII trimers are shown in Fig. 1A. In order to minimize the contribution of the large psi-type CD [45] arising from the long-range chromophore order in the grana,

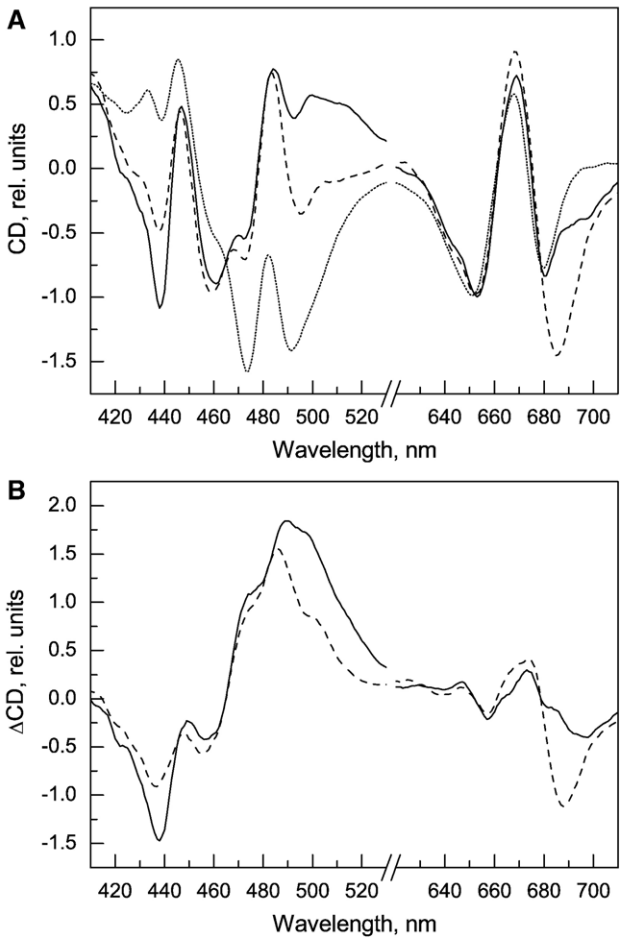

Fig. 1. (A) Circular dichroism spectra of thylakoid membranes unstacked in low-salt hypotonic buffer solution (solid line), aggregates of LHCII isolated according to Simidjiev et al. [40] (dashed line), and solubilized LHCII trimers, isolated by isoelectric focussing according to Ruban and Horton [41] (dotted line). The samples were diluted in 20 mM Tricine buffer, pH 7.8, to a Chl concentration of 10 μg/ml. CD was measured at room temperature in a cuvette of 1 cm optical pathlength. The spectra are normalized to the negative maximum at 650 nm. (B) CD difference spectra: solid line, thylakoid membranes minus LHCII trimers; dashed line, aggregates minus trimers.

thylakoids were washed and suspended in hypotonic, low-salt medium [46]. This enables the comparison of the excitonic features of the thylakoid membrane components with the isolated LHCII. The CD spectra of the thylakoids and solubilized trimers were essentially the same as those published previously [27,47–49]. The aggregate CD spectra were similar to the published spectra of LHCII isolated by the same procedure [40,50] but were not identical to those of aggregates prepared from solubilized trimers by removal of the detergent (cf. Fig. 6A in ref. [49]).

The CD of thylakoids in the blue region (Fig. 1A, solid line) revealed main excitonic bands at (−)438 nm, (+)446 nm, (−)460 nm, (−)473 nm, (+)483 nm and a broad composite band peaking at (+)502 nm. In the red region the main bands are found at (−)652 nm, (+)668 nm, and (−)680 nm. The CD of LHCII aggregates (dashed line) showed remarkable similarities with the thylakoids. The same bands were present in both spectra to a comparable extent except for the weaker (−)438 nm band in the aggregates, the missing (+)502 nm band, and an additional negative CD at 686 nm. The CD of solubilized trimers in the red region was similar to that of thylakoids and aggregates but it had drastically different excitonic band structure in the blue region. The (−)438 nm band was negligible, (−)460 nm was present as a shoulder, and the CD between 460 and 500 nm was strongly negative compared to the thylakoids and aggregates with two characteristic peaks at (−)472 nm and (−)492 nm.

Fig. 1B shows the CD difference spectra of thylakoids minus LHCII trimers and aggregates minus trimers. The two curves largely resemble each other in shape with negative difference bands at 438 nm and 456–457 nm and positive difference bands at 475 nm and 486 nm. In addition, the thylakoids-minus-trimers spectrum has a positive band at 500 nm.

3.2. Solubilization of thylakoid membranes and LHCII aggregates

Thylakoid membranes and LHCII aggregates were titrated with increasing amounts of n-dodecyl-β,D-maltoside (DM) measuring the CD spectrum at each step (see Materials and methods). Above the critical micelle concentration (~0.01% DM) there was a prominent change in the CD spectrum (inset in Fig. 2). Both in thylakoids and LHCII aggregates this change occurred in parallel with a 6–8-fold increase of the fluorescence yield, measured with a Walz PAM fluorometer (data not shown). However, complete solubilization, judging from the change in CD, was usually not achieved at DM concentrations below 0.1%. After addition of 0.1% DM (detergent:chlorophyll molar ratio 286:1) the CD spectra remained unchanged for 1 h in darkness and there was no apparent change in the absorption spectra (not shown). Moreover, addition of 0.1% DM to the already solubilized trimers did not induce further changes in the CD spectrum.

The CD spectra of thylakoid membranes, LHCII aggregates and LHCII trimers, registered in the presence of 0.1% DM are presented in Fig. 2A. There was no detectable change in the trimer spectra after addition of the detergent, whereas the spectra of the thylakoids and aggregates were converted in a way that they were virtually indistinguishable from the LHCII trimers. A few minor differences could be found after closer inspection: more positive CD around 500 nm in the thylakoids (Fig. 2A, solid line), and stronger negative and slightly red-shifted (−)680 nm band. These two differences can be explained with contribution from Photosystem I as the CD spectrum of solubilized PSI has positive peak at 499 nm and negative peak at 688 nm (data not shown).

3.3. Comparison of the effect of different detergents

In addition to DM, we titrated LHCII aggregates with two other detergents, commonly used in the literature — Triton X-100 and n-octyl-β,D-glucopyranoside (OG). All are non-ionic and considered as mild and non-denaturing solubilizing

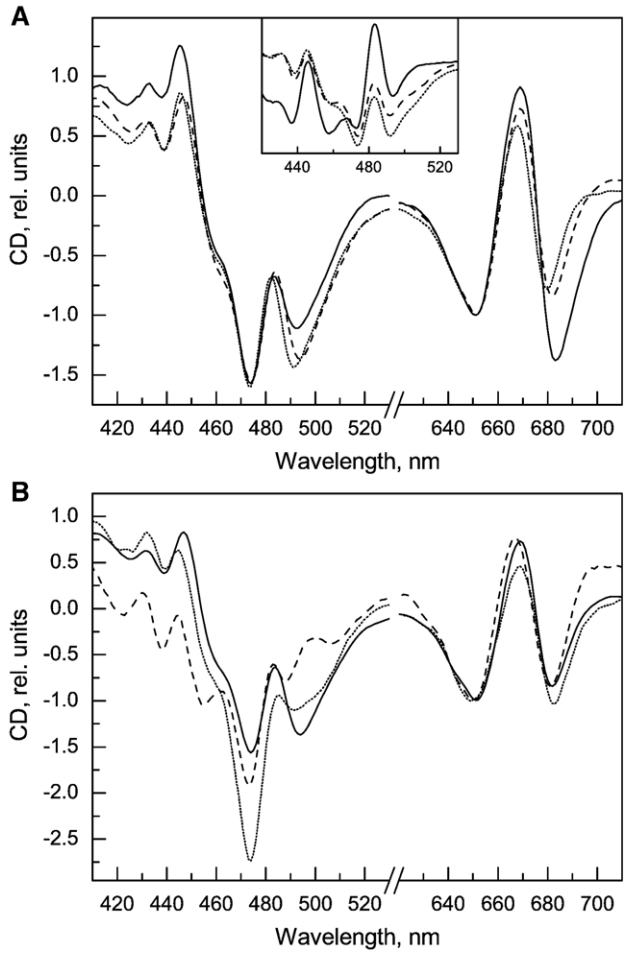

Fig. 2. (A) Circular dichroism spectra of thylakoid membranes (solid line), LHCII aggregates (dashed line), and LHCII trimers (dotted line), solubilized in 0.1% n-dodecyl-β,D-maltoside. The detergent was added to the diluted samples (20 μg/ml chlorophyll concentration) 5 min prior to the CD measurement. CD was measured at room temperature in a cuvette of 1 cm optical pathlength. The spectra are normalized to the negative maximum at 650 nm. Inset: CD of LHCII aggregates in the presence of 0.01% DM (solid line), 0.03% DM (dashed line) and 0.06% DM (dotted line). (B) CD spectra of LHCII solubilized in 0.1% n-dodecyl-β,D-maltoside (solid line), 0.01% Triton X-100 (dashed line), or 1% n-octyl-β,D-glucopyranoside (dotted line).

agents. We found that solubilization of the aggregates occurred at different concentrations, depending on the CMC of the given detergent. The CD spectra of LHCII aggregates, solubilized in 0.1% DM, 0.01% Triton (detergent:chlorophyll molar ratio 46:1) and 1% OG (detergent:chlorophyll 3000:1) are shown in Fig. 2B. When solubilized in Triton or OG, unlike DM, the LHCII complexes were unstable and showed gradual loss of the CD signal with time. The spectra shown were registered 5 min after addition of the detergent. Solubilization of LHCII with any of these three detergents brought about significant changes in the CD spectrum mainly in the Soret-carotenoid region and the changes depended on the type of detergent. In OG-solubilized LHCII, the (−)473 nm CD band was much stronger and the (−) 492 nm band was decreased. In Triton, the (−)492 nm band was absent and other, weaker, excitonic features could be seen in the same region.

3.4. LD spectra

The effect of solubilization of lamellar aggregates of LHCII with 0.1% DM on the visible LD spectrum is shown in Fig. 3. Due to their better alignment, the aggregates showed a six-fold stronger LD signal than the trimers at the same OD but changes in the shape of the normalized LD spectra were also found. The aggregates had two distinct positive maxima in the blue region — at 481 and 498 nm, and a red maximum at 688 nm. After solubilization the main maximum in the blue region was found at 482 nm and the maximum in the red region was at 685 nm. It must be noted that the LD spectra did not differ between aggregates of different size and macroaggregates with long-range order, which were characterized with strong psi-type CD showed the same LD spectrum as the one displayed in the figure.

3.5. Fluorescence lifetimes

It is well known that aggregation of LHCII leads to a strong, up to ten-fold decrease in the fluorescence yield and comparable decrease in the fluorescence lifetime at 77 K [26,41,51]. The decrease in lifetime has been demonstrated for aggregates prepared from trimers by removal of the detergent [26,51]. In this work we used aggregates that were directly isolated from the membrane containing LHCII in the aggregated form [39,40]. They display significantly different CD and LD properties than *in vitro* aggregated LHCII forms, which might influence the fluorescence relaxation kinetics. The fluorescence lifetimes of aggregates and trimers were determined at room temperature from fluorescence decays detected at 680 nm emission wavelength by single-photon counting. The results are presented in Table 1. Two lifetime components were sufficient to describe the relaxation kinetics of the trimers and three components were needed for the aggregates, as in the work of Vasil'ev et al. [51]. The main decay component in the solubilized trimers, with 90% relative amplitude, had lifetime

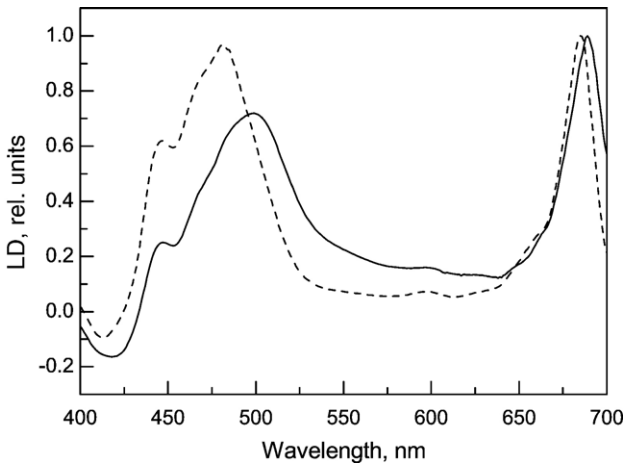

Fig. 3. Linear dichroism spectra of LHCII aggregates (solid line) and aggregates, solubilized with 0.1% DM (dashed line). The samples, at ~10 μg/ml chlorophyll concentration, were fixed in 5% polyacrylamide gel and oriented by squeezing. The spectra are normalized to the maximum in the red region.

Table 1
Fluorescence lifetime components of LHCII aggregates and trimers, obtained from global lifetime analysis of fluorescence decays at 680 nm, detected by single-photon timing at room temperature

	τ_1	a_1	τ_2	a_2	τ_3	a_3	τ_{av}
Aggregates	762 ps	0.78	246 ps	0.18	29 ps	0.03	644 ps
Trimers	3.89 ns	0.90	1.1 ns	0.10	–	–	3.52 ns

τ_1–τ_3—lifetimes, a_1–a_3—relative amplitudes, τ_{av}—average lifetime.

of 4 ns. The second, much less pronounced, component decayed with 1.1 ns lifetime. The average lifetime was 3.5 ns, in good agreement with all published data [21,26,51]. In contrast, the average lifetime of the aggregates was found to be ~600 ps and, contrary to previous studies, we could not detect any lifetimes significantly longer than 1 ns.

4. Discussion

In this work, we compared the CD spectra of isolated LHCII in aggregated and solubilized form with the CD of unstacked thylakoid membranes in order to get an insight into the native state of LHCII. Since LHCII represents a major part of the thylakoid membrane content, we expected to find features of LHCII in the CD of the membranes. The spectra showed strong similarity between LHCII aggregates and unstacked thylakoid membranes, on the one hand, and strong differences between thylakoid membranes and detergent-solubilized LHCII trimers, on the other hand. This result can be rationalized if we assume, first, that the CD signal of unstacked thylakoids originates mostly from LHCII aggregates and, second, that LHCII in isolated lamellar aggregates and in unstacked thylakoids is found in a similar state. In contrast, solubilization leads to changes in the inter-protein organization and the pigment–pigment interactions. In perfect agreement with this hypothesis, the solubilization of either thylakoid membranes or LHCII aggregates resulted in CD spectra that were virtually identical to the spectrum of solubilized trimers.

Based on comparison of the CD of DM-solubilized LHCII and thylakoid membranes in the chlorophyll Q region only, Hemelrijk et al. [28] concluded that CD of the thylakoids is dominated by the LHCII contribution. Furthermore, as there were no drastic differences in the red spectral region, it was concluded that detergent solubilization does not induce sizeable changes in the pigment–pigment interactions in LHCII. In accordance with this, we did not observe major alterations in the red region of the CD; however, we did observe changes in the blue region, which indicates that solubilization affects mostly carotenoids or Soret transitions of chlorophylls.

The CD and LD data give evidence that the supramolecular organization of LHCII has significant impact on the pigment–pigment interactions and the orientation of pigments in the complexes. These data are consistent with our earlier results showing that detergent solubilization of LHCII leads to well discernible absorbance changes both in the red and the blue spectral regions and – as suggested by triplet-minus-singlet absorbance transients – perturbs the carotenoid:chlorophyll interactions and leads to an increase in both the Chl *a* triplet and the Chl *a* fluorescence yields [52,53]. There are several possible reasons for the solubilization-induced changes: loss of interactions between peripheral pigments of neighbouring complexes; change in pigment–pigment interactions within the trimers due to the loss of trimer–trimer contacts; perturbation of the coordination of pigments due to conformational changes induced by the detergent molecules. Based on the CD data only, it is difficult to assign the specific pigments responsible for the observed effects. Ide et al. [21] attempted to assign excitonic features to chlorophyll *a* or *b* inferring from the central position of the (putative) excitonic splitting. They addressed three excitonic band pairs: (+)445/(−)495 nm, assigned to Chl *b*, (+)433/(−)477 nm, assigned to Chl *a*–*b*, and (+)487/(−)461 nm, assigned to Chl *b* (B_y). In our spectra these would correspond to (+)445/(−)492 nm, (+)433/(−)473 nm, and (+)483/(−)461 nm. We found a significant decrease in the amplitude of the latter excitonic band pair upon solubilization of thylakoid membranes with DM or LHCII aggregates with any of the three detergents used. This result is in accordance with the data of Ide et al. who used OG-solubilized LHCII. In contrast, the other two band pairs showed an increase or no change in the solubilized samples.

A different approach, namely resonance Raman spectroscopy, has been used by Ruban et al. [54] to characterize conformational changes in the pigments of LHCII induced by oligomerization. A specific change in the configuration of one carotenoid molecule, neoxanthin, has been found to occur when trimers are assembled into oligomers. Neoxanthin is bound in the periphery of the LHCII trimer and, as seen in the crystal structure, a large part of the molecule is protruding out the complex [2,3]. It is thus reasonable to assume that neoxanthin is sensitive to the environment around the complex. Neoxanthin is in close proximity with the chlorophyll *b* cluster in the corresponding LHCII monomer, with nearest contacts to Chl's *b*11[1] (608[2]), *b*12 (609), *b*13 (606), and Chl *a*6 (604). Modelling based on the LHCII structure has shown strong excitonic interactions between Chl's *a*6 (604) and *b*13 (606) and between *b*11 (608) and *b*12 (609) [5]. Both Chl's *a*6 (604) and *b*13 (606) form excitons also with Chl *b*10 (607). It may be speculated that distortion of the neoxanthin molecule changes the conformation of the neoxanthin binding pocket and thus affects also the excitonic features of the mentioned chlorophylls. The largest differences between the CD of aggregates/thylakoids and the solubilized trimers were around 437 nm and 486 nm (Fig. 2), which concurs with the absorption maxima of neoxanthin [54]. Furthermore, the CD spectrum of OG- and Triton-solubilized LHCII had common features with the spectrum of recombinant LHCII reconstituted in the absence of neoxanthin [55]. We can infer that OG and Triton have severe impact on the neoxanthin binding site or lead to loosening of the neoxanthin molecule.

The comparison of the three detergents (OG, DM and Triton) indicates that at least in some occasions the detergent has a profound effect on the state of the trimers. However, the CD features found in the aggregates used in this work could not be

[1] According to the nomenclature of Standfuss et al. [3].
[2] According to the nomenclature of Liu et al. [2].

restored in trimers simply by diluting the detergent below its CMC [49]. It is possible that specific trimer–trimer contacts occur depending on the conditions of aggregation. Alternatively, such specific trimer–trimer interactions may be mediated by lipids. This hypothesis could well explain the differences between our aggregates and aggregates prepared from solubilized trimers, since in our experiments the sample contained significant amounts of bound lipids [40], thus having the LHCII trimers interacting with a lipid matrix, as they do within the thylakoid membranes. Support for this suggestion can be found also in the recent publication of Yang et al. [56], which demonstrates that LHCII proteoliposomes have CD features similar to the CD of LHCII aggregates presented here.

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