Answers to Prof. Béla Böddi

General comments

I accept the criticism that the structure of the dissertation is perhaps too complex and requires significant effort to navigate. I wish to clarify that all figures in the expeirmental sections represent original results obtained with my indvidual contribution. In some cases I have created original figures specifically for the dissertation that are not found in other publications.

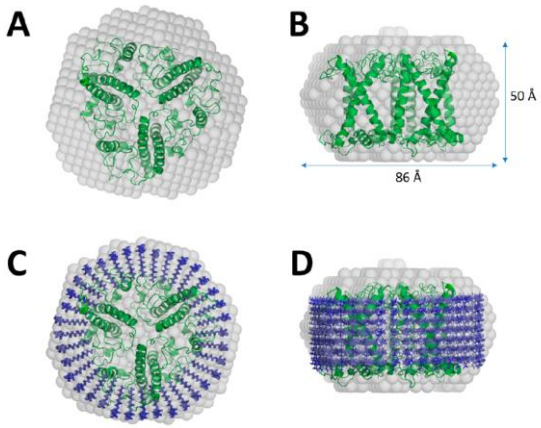
Answers to specific questions

1) *Comments and questions about the research materials containing detergents: In the Materials and Methods, we can find that the Candidate used α-DDM and β-DDM detergents for the isolation of LHCII: 0.7 % α-DDM or β-DDM and 15 min incubation on ice was used at extraction and then, after centrifugation the supernatant was transferred into a 0.06 % detergent containing 5mM Tricine buffer and 0.4 M sucrose. (Page 48) Among the results, 0.03 % detergent concentration is written in the 5.2 figure legend, 0.1 % β-DDM in 5.3 figure legend.* ***What was the reason for using various detergent concentrations?***

   This is an important methodological question that probably deserved separate clarification in the dissertation. The optimal detergent concentration depends on many factors, among others the ionic composition of the medium, the protein concentration, the detergent to protein ratio, etc. In my experience, it has been often necessary to optimize the detergent concentration empirically for specific conditions. For example, 0.03% dodecyl maltoside was found sufficient to support the soluble state of already isolated LHCII, as in figure 5.2; however, higher concentration was necessary to extract the complexes from the native membrane as in figure 5.3.

2) *According to Protein Science (1994) 3:1975-1983, and Methods in Enzymology Volume 182, 1990, Pages 239-253, the critical micellar concentration of DDM is 0.01 % in water at room temperature. The great concentration values during isolation and then their dilution brings up the possibility of preparing first reversed micellar systems (with hydrophilic phase inside) and then phase transition into usual (with hydrophilic phase outside) micellar systems at dilution.* ***In which phase are the LHCII units localized?***

   I would expect the formation of inverted micelles primarily in non-polar solvents, whereas in water LHCII would be surrounded by a monolayer belt of detergent molecules such that the polar head faces the aqueous phase and the acyl chain is toward the hydrophobic residues and pigments in the membrane-intrinsic section of the protein. This arrangement has been confirmed for several membrane protein complexes, including an LHCII-octylglucoside complex, by small-angle X-ray and neutron scattering (Cardoso et al., 2009, J. Phys. Chem. B. 113:16377).



Structural reconstruction of the LHCII-OG complex from SAXS data (Golub et al., 2022, J. Phys. Chem. Lett. 13:1258)

3) ***Can we get information about the structures of these colloid systems? Are there results in this research field about the DDM concentration dependence of the LHCII solubilization and the parallel changes of the spectral properties?***

Yes, solubilization of LHCII occurs around the critical micelle concentration of DDM (~0.01%), whereas aggregates are formed below that concentration. Increasing the DDM concentration beyond the CMC usually has little to no effect on the absorption, CD, or fluorescence spectra, as shown for example by Voigt et al. (2008, Photosynth. Res. 95:317-325). Curiously, these authors found marked changes in the nonlinear polarization spectra of LHCII that only disappear at DDM concentrations much higher than the CMC. They interpret these observations in terms of residual aggregation of LHCII that persists until DDM concentrations around 0.06% but is not detectable by conventional spectroscopy. A more recent study interestingly shows that LHCII can adopt an oligomeric structure of three trimers in DDM micelles but not in OG micelles (Golub et al., 2022, J. Phys. Chem. Lett. 13:1258). This new finding, if confirmed, may partly explain the differential effect of detergents on the CD spectra of LHCII.

4) ***What are the particle sizes in the samples with various DDM concentrations; what is the DDM micelle aggregation number?***

The aggregation number for beta dodecyl maltoside is 70–140 with micelle sizes of 60–70 kDa (Stetsenko & Guskov, 2017, Crystals 7:197). Interestingly, a recent X-ray/neutron scattering study by Golub et al. (2022, J. Phys. Chem. Lett. 13:1258) has revealed unexpectedly large sizes of LHCII-DDM particles with diameters of around 128 Å, which were modelled as trimers of trimers. On the other hand, gel filtration and native PAGE have shown that LHCII is stable as a trimer in DDM, so there must be a specific condition under which the oligomeric complex is formed.

5) *Comments on the appearance of the (-) 491 nm CD band: These experiments are logically planned and the interpretations are correct: Varying the components of the different systems, the (-) 491 nm CD band was proven as an effect of interactions of LHCII units with detergent or lipid structures. I fully agree with the Candidates' comment that the detergent micelles are only flawed representations*

of the native state and the model systems must be interpreted properly. ***Is it possible to identify the electronic transition corresponding to this signal?***

This would indeed be very useful to know. I am familiar with at least one article (Georgakopoulou 2007) where the authors attribute the transition to a combination of excitonic interactions between the S2 state of carotenoids and chlorophyll *b*. However, the level of theory used is too simple for an accurate analysis. We are currently starting a collaboration project with the Institute of Theoretical Physics in Linz, with one of the aims being precisely the identification of the origin of the CD bands in the blue region.

6) *I found in chapter 5.1. variability of the CD band ratios in spectra of the aggregates shown in figures 5.1B, 5.2B.* ***What is the reason for these band amplitude variations?***

Figure 5.1B shows the CD spectrum of LHCII aggregates, whereas Figure 5.2B shows the difference between the CD spectrum of aggregates minus the spectrum of LHCII in detergent-free gel. The CD difference spectrum shows only the spectral changes that can be associated with the aggregation of LHCII.

7) *Comments on the Far-red-emitting states associated with quenching: 5.2.2.: These measurements were done with samples frozen at 77 K. At this temperature, the molecular environment and the CT distances, therefore the energy migration efficiency and the vibrational freedom of the complexes differ from those at 293 K.* ***What is the opinion of the Candidate, how can we interpret these results on TM-s in their physiological states?***

The association between the fluorescence quenching in LHCII and far-red emission was first proposed by Ruban and Horton based on the 77 K fluorescence spectra of aggregates. At this temperature the far-red emission is the most prominent and easily identifiable. However, the responsible low-energy states exist at physiological temperatures as well and we have shown that they are responsible for at least part of the photoprotective quenching in the thylakoid membranes.

8) *I could not find enough explanation to the Figure 5.10. (page 65.). In panel A, curves are shown in different colours, without the meaning of these colours, we can find out that they correspond to the colours of block frames in panel B. An explanation of the figure labels is missing. In the cited paper (Ostroumov et al. 2020) I found data about measurements with samples cooled to 5, 30, 50, 100 and 170 K in the referred publication but no data with 77 K.* ***Why were the 77 K results presented here, where were these results (shown in Figure 5.10.) published?***

I apologize, there is a mistake in the legend of figure 5.10. The legend erroneously states that the measurements were done at 77 K, while in fact the results presented are for 100 K.

9) *The chapter on the two-dimensional electronic spectroscopy results is significant and it is an attractive part of the dissertation. This method and the analyses of the results provide us with important details of the energy distribution within the LHCII trimers and aggregates. A very important part of the dissertation is the comparison of the results measured on LHCII trimers to those of LHCII aggregates.* ***It would be interesting to know the aggregation number of the studied LHCII macrodomains.***

Unfortunately, I do not have data on the aggregation number. LHCII aggregates can vastly vary in size depending on the conditions. It was shown that in the presence of Mg ions, aggregates have average sizes of several hundred nanometers and can extend to tens of micrometers but without Mg they are smaller (Barzda et al. 1996). My best guess based on

dynamic light scattering data, is that the particles have diameter of 200−300 nm. A single-layer patch with diameter of 200 nm can pack about 250 LHCII trimers, which is more than the estimated functional domain size.

10) *The native thylakoid membrane components must influence the charge distribution in the whole photosynthetic unit.* ***Are or can be the two-dimensional spectroscopy measurements extended to the thylakoid membrane units?***

2DES measurements on thylakoid membranes should be possible, although technically challenging because of the high degree of light scattering, pulse depolarization and dephasing. Perhaps a bigger problem obtaining meaningful interpretation of such measurements. We are currently working on at an intermediate step – consolidating the knowledge obtained from isolated complexes and collecting data from various membrane supercomplexes containing LHCII as well as photosystem core complexes to map the dynamics of energy transfer in a larger system that is closer to the intact photosynthetic unit (Do et al. 2022, J. Phys. Chem. Lett. 13:4263).

Comments to the chapter "Summary"

11) *I expected a list of conclusions showing the results that the Candidate considers as the main conclusions of his scientific work. Instead, a 3.5-page long description is here, containing 7 titles printed in bold (and a little shorter text with inserted figures is in the thesis booklet). My opinion is, that not all of these emphasized prints are specific. I won't repeat these titles, instead, I summarize my opinion about the contents of the chapters. I consider new scientific results the statements printed in bold and italic in the next 7 points:*

1, This paragraph states first the importance of the molecular surroundings of the LHCII complexes. To my knowledge, this is basic and general information in biochemistry. My opinion is that the main result of this subject is that the Candidate could create different models which have native absorption and fluorescence properties but their structures can be different. However, the structural and functional plasticity of the complex needs to be considered when inferring physiological function from in vitro experiments. This statement is important because both, the DDM micellar solutions, the proteoliposomes, and the reconstituted membranes are generally used in projects studying the LHCII complexes.

2, The Candidate experimentally proved that the LCHII complexes may have distinct charge transfer states at excitation which is important in the self-regulation of light harvesting and in the directional

energy migration. With sophisticated spectroscopy methods, fluorescence lifetimes, and kinetic components of non-photochemical quenching were analyzed.

3, Structure-based exciton models were identified and validated; the experimental results were compared with calculated (predicted) ACD spectra.

4, Important details of the energy transfer between the Chl-s were identified, which contributed to creating the structural model.

5, Further and theoretically important details of the LHCII properties were given in works with the alga Bryopsis corticulans having special pigment composition which proves that the LHCII is a spectrally tunable light-harvesting antenna.

6, Determined the maximal aggregation number of LHCII trimeric subunits to be limited to about 25, fitting to the general idea about the "photosynthetic unit".

7, ***The delivery of the excitation energy from LHCII directly to PSI was shown in model membranes which may not have the native structure of thylakoids****. This result can be important in constructing efficient artificial structures but* ***its inference to the general plant physiology is uncertain.***

I agree with the seven points that the reviewer listed as main scientific results.

Regarding point 7, namely that the inference of the result from reconstituted membranes to the general plant physiology is uncertain: Recently several groups have published new reports confirming that LHCII acts as a PSI antenna in the native stromal membranes (Bos et al. 2017, BBA 1858:371-378; Bressan et al. 2018, Photosynth. Res. 135:251-261; Chukhutsina et al. 2020, Nat. Plants 6:860-868; Schiphorst et al. 2022, Plant Physiol. 188:2241-2252). Their time-resolved fluorescence data and findings on the binding of LHCII and the efficiency and dynamics of energy transfer are in agreement with our study on model membranes.

Minor comments

12) *The* ***Title*** *seems to show a kind of uncertainty. Why "On the role" is used to formulate it? A direct statement would be more expressive.*

I agree with the critical remark. Perhaps a more appropriate direct statement summarizing the main results of the dissertation could be constructed.

13) *The last paragraph of the Introduction is a description of the "Table of contents", it is redundancy.*

I respect the reviewer's opinion.

14) *Page.9 : The subscription of Figure 2.1 contains a mistake: "the methyl group (-CHO) at position C7 is replaced by a formyl group (-CHO)". The figure is correct, but in the legend, a real methyl (–CH_3) group is needed.*

Indeed, the methyl group is mistyped in the legend.

15) *Page, Q, and B absorption maxima of Chl-a dissolved in acetone are indicated. It's very difficult to remove water from acetone thus in addition to the Chl-a-acetone monosolvates different Chl-water species are present. The details of the spectral properties for Chls were studied in a water-free diethyl ether solution which contains exclusively mono-solvate Chl-a-ether molecules (see for example*

Houssier, C. and Sauer, K. BBA 172, 476-491 (1969), BBA 172, 492-502 (1969), J. Am. Chem. Soc. 92, 779-791 (1970)).

I agree. On the other hand, spectroscopic determination of Chls in acetone or 80% acetone in water is standard in plant biology and the spectra are well known.

16) *Page 58.: Figure 5.3 B. The figure legend contains a mistype: The difference spectrum "unsolubilized minus solubilized" must be "solubilized minus unsolubilized"*

The legend to Fig. 5.3B ("unsolubilized minus solubilized") is correct.

17) *What is the reason of the few nm differences (shifts) in the spectra? Are all spectra shown in figures means of several recordings or are the band position differences due to baseline correction difficulties or data collection frequency problems? (My experience is that to calculate the difference spectrum, at least 0.1 nm data collection frequency is needed.)*

Technical issues such as imperfect instrument calibration or insufficient sampling can cause small errors in the peak positions. Most CD spectra were recorded with spectral resolution of 3 nm and fluorescence spectra with 5 nm. However, in the CD difference spectra, peak positions can be shifted because the environment affects only some excitonic transitions contributing to a given CD band.

18) *Some of the results are presented on wave number basis in the original publications, however, the dissertation shows spectra and calculations in wavelength function. What is the reason for this conversion?*

I chose to present all spectra throughout the dissertation on a wavelength scale in nanometers for consistency and easier comparison, regardless of the units used in the original publication.

Szeged, 28 February 2023

(Petar Lambrev)