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Summary of the PhD thesis

Excitonic States and Excitation Energy Transfer in Plant Light-Harvesting Complexes in Different Molecular Environments

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Background and aims

In chloroplast thylakoid membranes of plants, absorbed light energy is collected by the light-harvesting antenna complexes and delivered, by way of excitation energy transfer (EET), to the photochemical reaction centres (RCs) in Photosystem I (PSI) and Photosystem II (PSII). The antenna functions depend largely on the molecular architecture of the pigment–protein complexes and their organization in the thylakoid membranes, which is exploited by the photosynthetic machinery to dynamically tune light harvesting in response to the physiological and environmental conditions.

Light-harvesting complex II (LHCII) is the main light-harvesting antenna complex of plants, associated with PSII. It comprises about half of the photosynthetically active chlorophyll (Chl) pigments; it is the most abundant integral membrane protein in nature. The major LHCII exists as a trimer and binds 8 Chl *a*, 6 Chl *b* and 4 xanthophylls as pigment cofactors per monomeric subunit. The pigment molecules are at close distances such that their electronic transitions are coupled creating delocalized exciton states. The excitonic interactions are the basis for fast energy transfer between them. In addition to its light-harvesting function, LHCII plays regulatory roles. Under excess light conditions LHCII can switch from its light harvesting function to energy dissipating function, which is termed non-photochemical quenching (NPQ). Activation of NPQ is triggered by acidification of the thylakoid lumen and is associated with structural rearrangements of the thylakoid membrane and conformational changes in LHCII.

The functions of LHCII are based on and controlled by the innate structural flexibility of the complex and its intermolecular interactions in the thylakoid membrane. The general aim of this thesis work is to **clarify the changes in the molecular and excitonic structure of LHCII that are incurred by interactions with its environment, and how these changes affect the excitation energy transfer within the complex, and between LHCII and other pigment–protein complexes in the membrane.**

Integral membrane proteins can be studied in isolated forms solubilized with detergents or in reconstituted membranes. Although it is assumed that the protein conformation is generally retained, minute structural perturbations can alter the pigment–pigment excitonic interactions. Removal of the detergent from a solution of LHCII invariably leads to aggregation of the highly hydrophobic complexes. The visible-region of the CD spectra of LHCII aggregates, which are very sensitive to the pigment–pigment excitonic interactions, are markedly different from those of detergent-solubilized LHCII trimers. The origin of the differences

has not been clarified — whether the native conformation of the protein is perturbed by the detergent or by aggregation.

***Goal**: Identify the origin of the CD spectral changes observed when LHCII is placed in different molecular environments.*

The excitonic CD spectra of antenna complexes are hard to interpret in terms of specific molecular excitonic states because of the large number of spectrally overlapping transitions with positive and negative CD signals. The technique of anisotropic CD (ACD) can, in principle, alleviate this problem by separating the excitonic transitions based on their orientation with respect to the main axes of the complexes.

***Goal**: Determine the orientation dependence of the pigment excitonic transitions of isolated LHCII by recording and comparing isotropic and anisotrorpic CD spectra.*

Ultimately, structural changes in the antenna complexes can result in changes in their light-harvesting functions, altering the dynamics of EET and the excited-state lifetime, both of which are crucial for maintaining high photosynthetic quantum efficiency.

***Goal**: Extract qualitative and quantitative information on the fluorescence quenching in LHCII in different molecular environments.*

EET in LHCII has been studied by different time-resolved spectroscopy techniques and modelled by structure-based quantum computations. However, the different models still exist that only partially agree on the structural identities of the excitonic states and the time constants of EET between them.

***Goal**: Determine the kinetics of EET between different Chls in LHCII and test how it is affected by the molecular environment.*

One role of LHCII is balancing the excitation energy between the two photosystems by the process of state transitions, which shuttles mobile LHCIIs between PSII and PSI. There is substantial recent evidence that LHCII and PSI can interact in the non-appressed thylakoid membrane regions (stromal thylakoids and grana margins) even if state transitions are not activated. Temporary unstacking of the membranes can increase the contacts between LHCII and PSI.

***Goal**: Evaluate the capability of LHCII to function as a light-harvesting antenna of PSI.*

In PSI most of the Chls are located in the core antenna which is fused with the RC. Plant PSI additionally binds four peripheral antenna subunits, LHCIs. A characteristic feature of PSI is that the antenna contains Chls whose transition energy is lower than that of the RC. These low-energy forms, called "red Chls", are present in LHCI and give it a distinct spectral profile. For the absorbed energy to be utilized by photochemistry it has to be transferred against the energy gradient. Despite that, the photochemical yield of PSI is nearly unity. The complexity of the PSI antenna, the large number of pigments and spectral overlap makes it difficult to unambiguously interpret the spectroscopy data, and distinguish between different kinetic models.

***Goal**: Gain a more detailed understanding of the kinetics of EET and charge separation in isolated PSI.*

Methods

- Sample preparation
  - Isolation of LHCII
  - Isolation of PSI and LHCI
  - Preparation of liposomes
- Biochemical analysis
  - Lipid:protein estimation
  - Pigment composition
  - Freeze-fracture electron microscopy
  - Dynamic light scattering
  - Absorption and CD spectroscopy
  - Linear dichroism and ACD spectroscopy
- Spectroscopic analysis of energy transfer
  - Steady-state and time-resolved fluorescence spectroscopy
  - Two-dimensional electronic spectroscopy

Results

Variations in the excitonic interactions in LHCII detected by circular dichroism

Extraction of LHCII from the native membrane leads to significant changes in the CD spectrum that could reflect e.g. detergent–protein interactions in detergent micelles or protein–protein interactions upon aggregation. To separate these effects, we systematically analysed the differences in the CD spectra of LHCII upon changing the molecular environment: from detergent micelles to aqueous buffer (promoting aggregation), gel (preventing aggregation), reconstituted lipid bilayers, and crystals. We identified spectral changes specific to protein–protein interactions, at (−)437 nm and (+)484 nm, and changes specific to the interaction with the detergent n-dodecyl-β-D-maltoside (β-DM), at (−)494 nm. The latter was attributed to a conformational change of the LHCII-bound carotenoid neoxanthin, based on comparison with neoxanthin-deficient plant thylakoid membranes. The neoxanthin-specific band was not pronounced when LHCII was solubilized with the *α* isomer of DM but was present when LHCII was reconstituted in lipid membranes, indicating that the conformation of neoxanthin is sensitive to the molecular environment. Based on these results we concluded that the interactions of LHCII with the surrounding membrane or solvent alter the molecular and exciton structure of the complex.

Anisotropic circular dichroism of LHCII

We recorded ACD spectra of macroscopically-aligned LHCII with light directions parallel and perpendicular to the membrane plane. In line with theoretical considerations, the ACD spectra of oriented LHCII in face-aligned orientation exhibited only some of the bands present in the CD spectra of randomly oriented (isotropic) solution and the amplitudes of these bands were strongly amplified. For example, in LHCII membranes the CD bands at (+)445 nm and (+)483 nm were enhanced in the face-aligned ACD and were thus assigned to excitonic transitions oriented in the membrane plane. Conversely, the bands at (−)437 and (−)473 nm were absent from the face-aligned ACD and were assigned to excitonic transitions with polarization preferentially perpendicular to the membrane plane. Thus, ACD spectra provide direct structural constraints on the interpretation and assignment of the CD bands and, respectively, CD spectral changes.

Modulation of the fluorescence lifetime by the molecular environment

Intermolecular interactions have a profound effect on the excited-state lifetime of LHCII switching from light-harvesting to energy-dissipating mode. Confirming previous studies by other groups, our results showed that the lifetime of excited Chls in LHCII strongly depends on the environment (from 4 ns in detergent micelles to 2 ns in native membranes and 0.2 ns in aggregates) and that excitation quenching in LHCII aggregates is activated by the aqueous environment rather than protein–protein interactions. In the absence of detergent both in polymer gel or aggregates, the fluorescence was strongly quenched. From the fact that quenching could be induced without aggregation and, conversely, the aggregation CD signature could be observed without significant quenching (in LHCII-enriched native membranes), it was concluded that the Chl–carotenoid excitonic interactions responsible for the CD changes upon aggregation are not involved in the mechanism of quenching.

We employed a novel protocol for preparing reconstituted LHCII membranes with defined lipid:protein ratios. The fluorescence decays were markedly multiexponential, indicating structural and functional heterogeneity in the membranes. Evidently, protein–protein and lipid–protein interaction forces established an equilibrium of multiple structural states/phases of greatly different, structural properties, such as membrane diameters, protein densities, etc. The average fluorescence lifetimes of LHCII profoundly changed depending on the lipid:protein ratio — as the protein density in the membranes increased, fluorescence quenching was triggered. Concomitantly, we observed far-red fluorescence emission characteristic for quenched artificial aggregates, but without aggregation-specific CD signature, reaffirming the conclusion that the underlying Chl–carotenoid states are not associated with the quenching.

Excitation energy transfer in LHCII

We studied the EET pathways and dynamics in LHCII in different environments by using two-dimensional electronic spectroscopy (2DES) — an advanced spectroscopic technique that allows us to resolve and correlate the absorption frequencies of coupled donor and acceptor molecules. 2DES revealed the kinetics of EET between different Chl pools in LHCII at physiological temperature. EET in isolated LHCII complexes is a multi-phasic process with kinetics that range from hundreds of femtoseconds to several picoseconds. To study EET from Chl *b* to Chl *a*, 2D spectra were recorded with the pump pulses covering only the Chl *b*

absorption band. The time dependence of the 2D spectra was described with four exponential components. The 2D decay-associated spectra (DAS) revealed that the first two components (0.27 and 2.7 ps) were dominated by EET from strongly coupled Chl *b* to Chl *a* excitonic states, and slower energy transfer pathways between Chls, mediated by intermediate energy states, respectively. Decay cross-peaks in the 2D spectra showed the existence of two EET bottlenecks — intermediate states weakly coupled with the surrounding Chls.

The kinetics of exciton equilibration within the Chl *a* domain (intraband relaxation) was followed with excitation pulses covering the Chl *a* absorption band. We found two dominant EET timescales — 0.5 and 5 ps — reflecting strongly and weakly-coupled Chl *a*, respectively. From the position of diagonal and cross-peaks on the 2D DAS (Figure 1) we identified the corresponding Chl states. Moreover, we demonstrated that forward and reverse EET pathways can be resolved and quantified by the appearance of symmetric cross-peaks on the 2D DAS. From the dependence of the 2D spectra on excitation wavelength, we could unambiguously determine that all exciton states were thermally equilibrated in less than 10 ps.

By comparing the 2DES results obtained from solubilized and aggregated LHCII we found that aggregation alters the dynamics of EET, accelerating the transfer between certain Chls — further emphasizing the role of the molecular environment on the structure and function of LHCII.

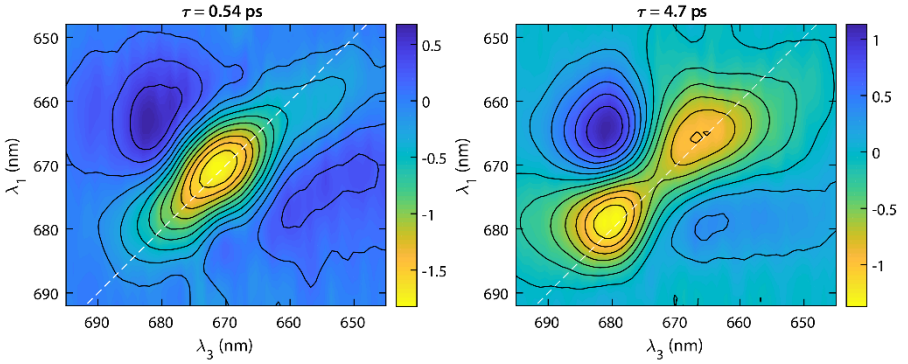

Figure 1. 2D decay-associated spectra of LHCII trimers, resulting from a three-exponential fit of the transient 2D signals from 150 fs to 64 ps with best-fit lifetimes of 0.54 ps, 4.7 ps, and 3.2 ns. The last 2D DAS is omitted from the plot

Energy transfer and charge separation in PSI

We studied the kinetics of plant PSI–LHCI supercomplexes compared with isolated PSI core complexes by time-resolved fluorescence spectroscopy and 2DES. We have revealed, for the first time, the two-dimensional spectral evolution of PSI in the time range from 100 fs to 1 ns. Global analysis of the 2DES data have shown that the dominant timescale of exciton equilibration in the PSI core antenna is about 0.5 ps. We observed the appearance of a bleaching signal at around 700 nm on a timescale of 3 ps, and assigned it to the formation of the oxidized RC Chl $P700^+$. The main photochemical trapping time in the core complex was about 20 ps, by which time the core antenna excitations were fully equilibrated. In the PSI–LHCI supercomplex trapping was slowed down by excitation equilibration with LHCI, which was observed by the population of red Chls decaying with an effective lifetime of 50–70 ps. These data show that trapping in the RC and equilibration with the peripheral antenna occur on similar timescales of about 20–30 ps. The kinetics of EET and charge separation in PSI determined by 2DES showed excellent agreement with TRF, while providing a wealth of complementary information.

Excitation energy transfer between complexes in artificial membranes

In contrast to LHCII aggregates, reconstituted LHCII:lipid membranes showed that excitations lived long enough (2 ns) to enable efficient light harvesting function. Moreover, excitation energy could migrate over several LHCII complexes in the membranes, providing degree of energetic connectivity similar to native thylakoid membranes. We took advantage of this characteristics to test another known function of thylakoid membranes, namely the ability of LHCII to donate excitations to PSI. To this end, we reconstituted membranes with LHCII and PSI at different stoichiometric ratios and monitored energy transfer between them by steady-state and time-resolved fluorescence spectroscopy. Employing kinetic modelling to fit the time-resolved data, we were able to estimate the rate constants and the efficiency of EET from LHCII to PSI. Different pools of LHCII were found to transfer energy to PSI on time scales from less than 10 ps to hundreds of ps, contributing significantly to the effective antenna size of PSI. The overall efficiency of transferring excitations from LHCII was up to 70%. Moreover, due to the remarkably efficient charge separation in PSI, the overall photochemical quantum yield remained very high, demonstrating the feasibility to construct artificial systems with desired functional antenna sizes without a significant loss in quantum efficiency.

Conclusions

The following list summarizes the main novel scientific results of this thesis work:

1. Structural changes induced in LHCII by changing its molecular surroundings — protein–protein, detergent–protein or lipid–protein — were identified by their specific CD signatures.
2. Excitonic CD bands were separated based on the polarization direction of the respective transition dipole moments by recording the ACD spectra of macroscopically oriented LHCII membranes.
3. Due to self-segregation, LHCII formed protein-dense domains in reconstituted membranes wherein fluorescence quenching occurred with a mechanism similar to NPQ *in vivo*.
4. Simultaneously observing uphill and downhill energy transfer pathways, 2DES revealed the dynamics of exciton equilibration in the Chl *a* domain in LHCII, which was found to occur with characteristic times up to 5 ps at physiological temperature.
5. The dynamics of EET in plant PSI–LHCI and isolated PSI core complexes were observed for the first time by 2DES. A refined kinetic model was proposed, according to which primary charge separation in the PSI RC occurs after full equilibration of the excitations in the core antenna, with an effective time constant of 3–4 ps.
6. In artificial reconstituted membranes, LHCII acts as efficient antenna of PSI increasing its functional antenna size by up to 50% with a minor loss of photochemical efficiency.

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List of publications

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Publications related to the PhD thesis

1. **Akhtar, P.,** Zhang, C., Tan, H., S., Lambrev, P.H. (2017) Excitation transfer and trapping kinetics in plant Photosystem I probed by two-dimensional electronic spectroscopy. ***Photosynthesis Research***, 135:239–250 (IF 3.864)
2. **Akhtar, P.,** Zhang, C., Do, T.N., Garab, G., Lambrev, P.H., Tan, H.-S. (2017) Two-dimensional spectroscopy of chlorophyll *a* excited-state equilibration in Light-harvesting complex II. ***Journal of Physical Chemistry Letters***, 8:257–263 (IF 9.353)
3. **Akhtar, P.,** Lingvay, M., Kiss, T., Deák, R., Bóta, A., Ughy, B., Garab, G., Lambrev, P.H. (2016) Excitation energy transfer between Light-harvesting complex II and Photosystem I in reconstituted membranes. ***BBA-Bioenergetics***, 1857:462–472 (IF 5.353)
4. Enriquez, M.G.M., **Akhtar, P.,** Zhang, C., Garab, G., Lambrev, P.H., Tan, H.-S. (2015) Energy transfer dynamics in trimers and aggregates of Light-harvesting complex II probed by 2D electronic spectroscopy. ***Journal of Chemical Physics***, 142:2124325 (IF 3.122)
5. **Akhtar, P.,** Dorogi, M., Pawlak, K., Kovács, L., Bóta, A., Kiss, T., Garab, G., Lambrev, P.H. (2015) Pigment interactions in Light-harvesting complex II in different molecular environments. ***Journal of Biological Chemistry***, 290:4877–4886 (IF 4.258)

Other publications

1. Kotakis, C., **Akhtar, P.,** Zsiros, O., Garab, G., Lambrev, P.H. (2018) Increased thermal stability of Photosystem II and the macro-organization of thylakoid membranes, induced by co-solutes, associated with changes in the lipid-phase behaviour of thylakoid membranes. ***Photosynthetica***, 56:254–264 (IF 1.507)
2. Garab, G., Ughy, B., de Waard, P., **Akhtar, P.,** Javornik, U., Kotakis, C., Šket, P., Karlický, V., Materová, Z., Vladimír S., Plavec, J., van Amerongen, H., Vígh, L., van As, H., Lambrev, P.H. (2017) Lipid polymorphism in chloroplast thylakoid membranes — as revealed by ^{31}P-NMR and time-resolved merocyanine fluorescence spectroscopy. ***Scientific Reports***, 7:13343 (IF 4.122)
3. Ghazaryan, A., **Akhtar, P.,** Garab, G., Lambrev, P.H., Büchel, C. (2016) Involvement of the Lhcx protein Fcp6 of the diatom *Cyclotella meneghiniana* in the macro-organization and structural flexibility of thylakoid membranes. ***BBA-Bioenergetics***, 1857:1373–1379 (IF 5.353)

Conference lectures

1. **Akhtar, P.**, Tutkus, M., Görföl, F., Trinkunas, G., Rutkauskas, D., Lambrev, P.H., Self-aggregation of Light-harvesting complex II in reconstituted membranes mimics non-photochemical quenching in plants. *8th Regional Biophysics Conference*, May 16–20, 2018, Zreče, Slovenia
2. **Akhtar, P.**, Tutkus, M., Trinkunas, G., Rutkauskas, D., Lambrev, P.H., Fluorescence quenching in transmembrane light-harvesting complexes measured in single proteoliposomes. *26th Congress of the Hungarian Biophysical Society*, Aug. 22–25, 2017, Szeged, Hungary
3. **Akhtar, P.**, Dorogi, M., Enriquez, M.M., Zhang, C., Tan, H.-S., Garab, G., Lambrev, P.H., Excitation energy transfer in model photosynthetic membranes. *PHOTOTECH 2015: Towards a photosynthesis-biobased economy*, Oct. 7–9, 2015, Rome, Italy
4. **Akhtar, P.**, Dorogi, M., Pawlak, K., Garab, G., Lambrev, P.H., Effects of detergents, lipids and trimer–trimer contacts on the pigment excitonic interactions in plant Light-harvesting complex II. *International Workshop on Ultrafast Processes in Photosynthesis. New Vistas at ELI-ALPS and COST PHOTOTECH Training School on "Advance Laser Spectroscopy in Green Photobiology"*, Oct. 18–21, 2014, Szeged, Hungary

Conference posters

1. **Akhtar, P.**, Biswas, A., Zakar, T., Petrova, N., Sipka, G., Gombos, Z., Lambrev, P.H., Excitation energy transfer from the phycobilisomes antenna to the photosystems in the cyanobacterium *Anabaena sp*. *The 1st European Congress on Photosynthesis Research ePS-1*, Jun. 25–28, 2018, Uppsala, Sweden
2. **Akhtar, P.**, Lingvay, M., Zsiros, O., Jávorfi, T., Siligardi, G., Garab, G., Lambrev, P.H., Anisotropic circular dichroism of macroscopically oriented photosynthetic light-harvesting complexes. *42nd Congress of the Brazilian Biophysics Society*, Oct. 27–29, 2017, Santos, Brazil
3. **Akhtar, P.**, Lingvay, M., Garab, G., Lambrev, P.H., Reconstituted membranes of Photosystem I and LHCII show efficient energetic connectivity and resistance to photodamage. *17th International Congress on Photosynthesis Research*, Aug. 7–12, 2016, Maastricht, The Netherlands
4. **Akhtar, P.**, Lingvay, M., Garab, G., Lambrev, P.H., Reconstituted membranes of Photosystem I and LHCII show efficient energetic connectivity and resistance to photodamage. *The 4th International Workshop on Solar Energy for Sustainability "Photosynthesis and Bioenergetics"*, Mar. 21–24, 2016, Singapore
5. **Akhtar, P.**, Dorogi, M., Enriquez, M.M., Pawlak, K., Zhang, C., Tan, H.-S., Garab, G., Lambrev, P.H., Exitonic interactions and energy transfer in Light-harvesting complex II in different environments. *10th European Biophysics Congress (EBSA 2015)*, Jul. 18–22, 2015, Dresden, Germany
6. **Akhtar, P.**, Enriquez, M.M., Zhang, C., Garab, G., Lambrev, P.H., Tan, H.-S., Environment-induced changes in the energy transfer dynamics of Light-Harvesting Complex II probed by 2D electronic spectroscopy. *The 3rd Workshop on Coherent Energy Transport and Optimization in Photosynthesis*, May 1–3, 2015, Singapore
7. **Akhtar, P.**, Dorogi, M., Pawlak, K., Garab, G., Lambrev, P.H., Effects of detergents, lipids and trimer-trimer contacts on the pigment excitonic interactions in plant Light-harvesting complex II. *Photosynthesis Research for Sustainability*, Jun. 2–7, 2014, Pushchino, Russia
8. **Akhtar, P.**, Pawlak, K., Dorogi, M., Garab, G., Lambrev, P.H., Detergent, lipid, and protein interaction effects on the CD spectrum of Light-harvesting complex II. *Eurosolar Fuel meeting*, Apr. 12–15, 2014, Passau, Germany
9. **Akhtar, P.**, Pawlak, K., Dorogi, M., Garab, G., Lambrev, P.H., Differentiation between detergent, lipid, and protein interactions in the CD spectrum of Light-harvesting complex II. *Biophysics of Photosynthesis*, Oct. 28–30, 2013, Rome, Italy

Declaration from the corresponding author

on the contribution of Parveen Akhtar to the scientific articles:

1. **Akhtar, P.,** Dorogi, M., Pawlak, K., Kovács, L., Bóta, A., Kiss, T., Garab, G., Lambrev, P.H. (2015) Pigment interactions in light-harvesting complex II in different molecular environments. Journal of Biological Chemistry, 290: 4877–4886.

2. **Akhtar, P.,** Lingvay, M., Kiss, T., Deák, R., Bóta, A., Ughy, B., Garab, G., Lambrev, P.H. (2016) Excitation energy transfer between light-harvesting complex II and photosystem I in reconstituted membranes. BBA-Bioenergetics, 1857: 462–472.

I declare that Parveen Akhtar has had the lead contribution to the preparation and implementation of the experiments described in the articles, the analysis of the experimental results and the writing of the articles.

The articles and the results published therein have not been referred to for the purpose of acquiring any other academic degrees or titles.

Dr. Petar Lambrev

Szeged, 19th June 2018.