Project closing (final) report

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"Structural and functional plasticity of thylakoid membranes"

1. Major aims and questions of the reserach

In this project we addressed key questions of the multilevel regulatory mechanisms of oxygenic photosynthetic organisms. These mechanisms fine-tune the structure and function of the photosynthetic machineries at different levels of complexity, the pigment-protein complexes, their macro-assemblies and the entire thylakoid membrane system and intact organisms (algal cells and whole leaves). The physical and molecular background of most of these different regulatory mechanisms are not understood satisfactorily, and very often important details are missing from our knowldedge both regarding the corresponding structural-functional units and the nature and mechanism and forms of plasticity that are involved in the regultarory mechanisms.

In our work, we focused our attention to the following areas and main questions:

1.1. Light-harvesting antenna complexes

The main function of LHCII is to absorb the incident photons and to supply the excitation energy for the photochemical reaction centers (RC). LHCII is the major light-harvesting antenna complex of green plants, the most abundant membrane protein in the Biosphere. LHCII is capable of performing the light-harvesting function with nearly 100% quantum efficiency, i.e. the pigment-protein complexes are capable of transferring the excitation energy toward the RCs without quantum losses. This requires a highly organized molecular architecture. While the near-atomic resolution cystal structure of LHCII is known (Liu et al. 2004), the pigment-pigment interactions that are involved in the energy migration have not or have only partially been identified; the same holds true for the ultrafast energy transfer steps in the trimeric complex containing 42 chlorophyll (Chl) and a number of different carotenoid molecules. These, and their association with each other and the RC can be explored primarily with the aid of circular dichroism (CD) spectroscopy, including, for the first time in photosynthesis research and excitonic CD, the technique of anisotropic CD (ACD), as well as by using advanced fs time-resolved spectroscopic techniques.

Another important question is the participation of these complexes in photoprotection. In this process LHCII switches to a regime to safely dissipate the excess excitation energy (which is not utilized for energy conversion and would otherwise damage the photosynthetic apparatus) (Demmig-Adams et al. 2014). This function, opposite to its main, light-harvesting function, evidently cannot be performed without adjustment in the molecular architecture; this demands structural flexibility – which we aimed to reveal by using mainly chlorophyll-fluorescence

lifetime measurements and CD spectroscopy, and by exposing the complexes to different molecular environments.

1.2. Photosystem II reaction center

Our earlier investigations have suggested that the RC of Photosystem II (PSII) is undergoing light-induced reorganizations that affect their chlorophyll-a fluorescencence (Schansker et al. 2011, 2014). The nature and significance of these changes, however, have not been revealed. The chlorophyll-a fluorescence transients were thus further investigated. The additional aim of these studies were to shed light on the origin of variable fluorescence (Fv) of PSII. Fv/Fm (where Fm is the maximal fluorescence yield of PSII) is probably the most widely used parameter of photosysnthesis research. Accordding to the widely accepted model, to reach Fm it is necessary and sufficient to reduce Q_A the first quinone acceptor of PSII (Stirbet and Govindjee 2012). We reinvestgated this 'dogma'.

1.3. Role of non-bilayer lipids and lipid phases in the assembly and flexibility of thylakoid membranes

The functional state of thylakoid membranes is a bilayer, which warrants the build-up of the electrochemical potential gradient for protons and its utilization for ATP synthesis. In spite of this strong restriction on the functional state, the non-bilayer lipid monogalactosyl-diacylglycerol (MGDG) comprises about half of their lipid content. It is also notheworthy that non-bilayer lipids dominate the lipid composition in all energy-converting biological membranes - their roles are still poorly understood.

It has been proposed that non-bilayer lipids, via their segregation capability, regulate the protein-to-lipid ratio of the membranes, and contribute to the structural flexibility of thylakoid membranes (Garab et al. 2000). Accordingly, a thylakoid membrane model (tagged later as DEM, dynamic exchange model) has been hypothesized, suggesting the co-existence of the bilayer membrane with a non-bilayer lipid phase.

Earlier, by employing ^{31}P-NMR we have demonstrated the co-existence of an isotropic, non-bilayer lipid phase and the bilayer phase of thylakoid membranes (Krumova et al. 2008a); the heterogeneity of lipid phases has also been indicated by steady-state and time-resolved fluorescence measurements using the lipid-label fluorescence dye, merocyanine 540 (MC540) (Krumova et al. 2008b). However, the identity of this non-bilayer lipid phase in terms of structural entity and its role in the plasticity of the membranes have not been revealed – these questions were addressed in the present proposal.

1.4. Changes in the macro-organization of the protein complexes and remodeling of the membrane system

In the thylakoid membranes the protein complexes are packed in ordered arrays and the membranes are assembled into highly organized multilamellar systems, an organization warranting a substantial degree of stability (Garab 2014). At the same time, the photosynthetic

machinery possesses the ability for reorganizations in response to rapidly changing environmental conditions. It has been shown earlier, by using respectively CD spectroscopy (Garab and van Amerongen 2009) and small-angle neutron scattering (Nagy et al. 2013, Ünnep et al. 2014), that the chirally ordered arrays of pigment-protein complexes and the periodicty of the multilamellar membrane system are capable of undergoing reorganizations. These structural changes were proposed to be associated with different regulatory mechanisms in different organisms. These studies are still in exploratory phase – and thus our aim was to carry out systematic investigations on this area.

2. Results - highlights

In the report, we describe the main results, which were in the focal points of our investigations. For the results from several additional studies, which were carried out either for their methodological relevance or linked to the main questions indirectly, see the yearly reports.

2.1. Light-harvesting antenna complexes

The structural-functional plasticity was shown by using CD spectrocopy and chlorophyll-a fluorescence lifetime measurements of LHCII in diifferent molecular environments (Akhtar et al. 2015). These investigations revealed distinct structural effects of protein-protein, lipid-protein, and detergent interactions. It was concluded that "the native state of complexes is perturbed by detergents and best retained in lipid:LHCII assemblies". (The purchese of key components of the fluorescence lifetime apparatus was financed by the project.)

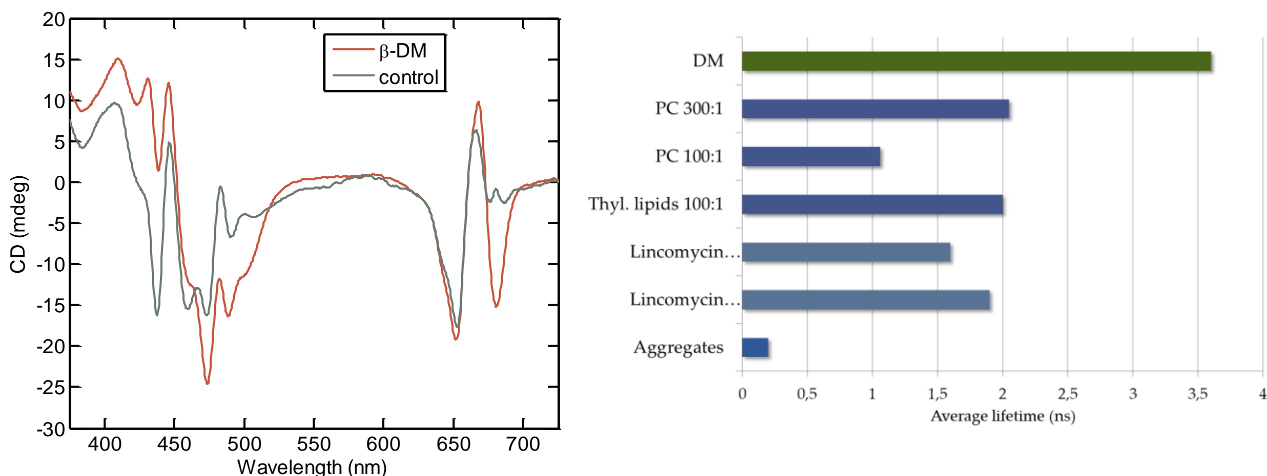

Fig. 1. Perturbation of the molecular architecture of LHCII by the detergent ß-DM (left) and Chl fluorescence lifetimes of LHCII in different molecular environments (right) , with different lipid (PC and thylakoid lipid) to protein ratios, in lincomycin-treated native membranes, and in aggregates.

Based on these data, we carried out experiments on proteoliposomes, using isolated thylakoid lipids and LHCII, and have demonstrated efficient energy transfer between LHCII and PSI in

reconstituted membranes (Akhtar et al. 2016). The embedding of complexes in the lipid vesicles was verified by freeze-fracture electron microscopy; the molecular architecture and pigment interactions were tested by CD spectroscopy; energy transfer between the respective units was measured by steady-state and time-resolved fluorescence spectroscopy.

With regard to the dramatically different behavior of solubilized and aggregated forms of LHCII, reflected, among others, in their excited-state lifetimes (cf. Fig. 1), we investigated the energy transfer dynamics in LHCII trimers and aggregates using the femtosecond 2D electronic spectroscopy (2DES) (Enriquez et al. 2015). "2DES allows direct correlation of excitation and emission energies of coupled states over population time delays, hence enabling mapping of the energy flow between Chls." It was concluded that "owing to slow energy equilibration processes, long-lived intermediate Chl-a states are present in solubilized trimers, while in aggregates, the population decay of these excited states is significantly accelerated, suggesting that, overall, the energy transfer within the LHCII complexes is faster in the aggregated state" (Fig. 2).

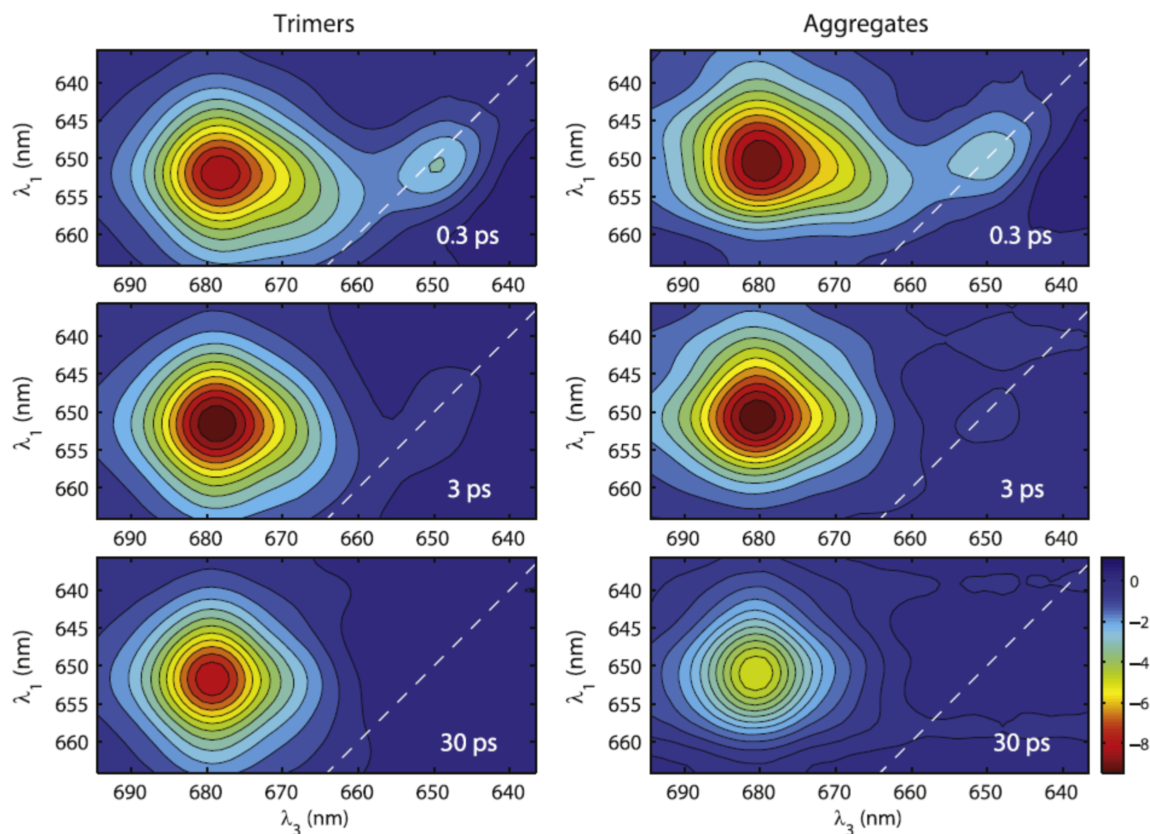

Fig. 2. Purely absorptive 2D spectra of LHCII trimers (left column) and aggregates (right column) recorded in the Chl Qy region at 300 fs, 3 ps, and 30 ps waiting times. The 2D spectra are plotted in terms of excitation wavelength λ_1 and detection wavelength λ_3.

2DES also allowed us to directly observe, for the first time, the bidirectional (uphill and downhill) energy transfer of the equilibration process between excited states (Akhtar et al. 2017). The multistep excitation-energy transfer processes in LHCII were also monitored using the novel technique of ultrafast fifth-order three-dimensional electronic spectroscopy (3DES), with the aid of which we measured cross peaks that directly indicate energy transfer from excitons in the Chl-b manifold to the low-energy level chlorophyll a Chl-a via mid-level Chl-a energy states (Zhang et al. 2015).

In order to apply ACD to LHCII and other constituents of the photosynhetic apparati, with solid experimental and theoretical background,we searched for a simpler system, where we could test the validity of the basic assumptions and where the strength of ACD could be demonstrated. This was achieved by applying this technique to the baseplate of carotenosomes, a chlorosome mutant lacking the BChl c antenna but containing the fully functional baseplate – containing complexes with dimeric Bchl-a; on these we could, for the first time, experimentally separate the two orthogonal components

2.2. Photosystem II reaction center

PSII is a large multi-subunit homodimeric protein complex embedded in the thylakoid membranes of cyanobacteria, algae and vascular plants. Our knowledge about the structure and function of PSII and the PSII reaction center (RC), in particular, is very well advanced: the ultrafast primary and slower secondary processes, charge separation and stabilization, electron and proton transfer and recombination pathways occurring in the RC have been determined (Cardona et al. 2012) and these processes can now be interpreted using the atomic resolution crystallographic structures (Suga et al. 2015). Nevertheless, several important questions remain to be answered. Among these: (i) is the structure of RC static or possesses significant structural dynamics - essential for or associated with its function; (ii) are there conformational changes in the PSII RC that are similar to those reported in the literature on purple bacterial RC; (iii) is the variable chlorophyll uorescence, that is widely used for testing photosynthetic performance, reflects solely the reduction of Q_A?

In the project, "we examined the nature of gradual fluorescence rise of PSII elicited by trains of single-turnover saturating flashes (STSFs) in the presence of DCMU, a PSII inhibitor, permitting only one stable charge separation. We show that a substantial part of the fluorescence rise originates from light-induced processes that occur after the stabilisation of charge separation, induced by the first STSF; the temperature-dependent relaxation characteristics suggest the involvement of conformational changes in the additional rise. In experiments using double flashes with variable waiting times ($\Delta\tau$) between them, we found that no rise could be induced with zero or short $\Delta\tau$, the value of which depended on the temperature - revealing a previously unknown rate-limiting step in PSII" (Fig. 3) (Magyar et al. 2018). These investigations have opened a new research area – which are being conducted, in large part via international collaborations, after the closing of the present project.

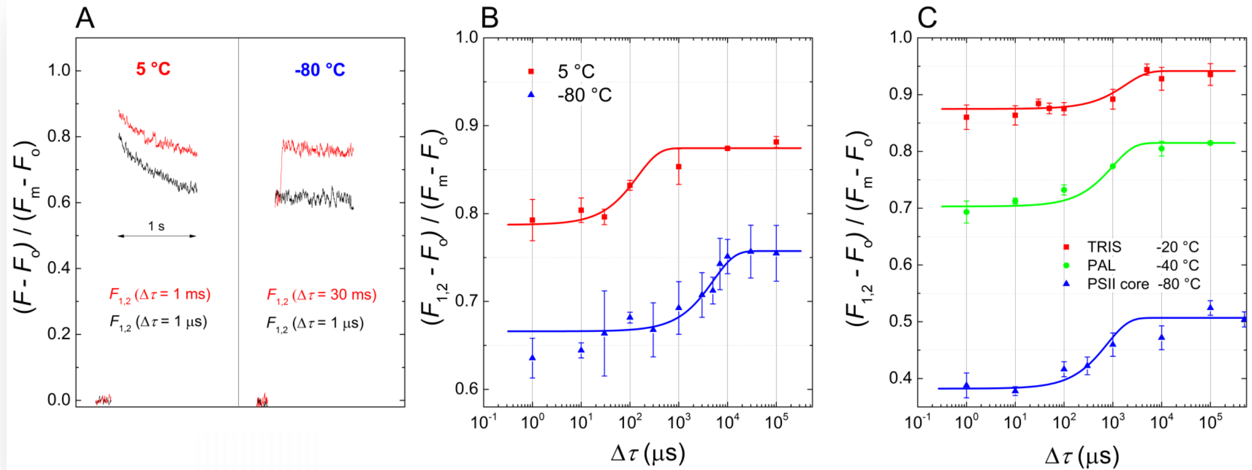

Fig. 3. Chl-*a* fluorescence induced by double flashes. Kinetic traces of the F_1-to-F_2 increment with two different dark times (Δτ) between the first and second flashes, at two different temperatures in isolated spinach thylakoid membranes (A) and dependences of the F_1-to-F_2 increments on the waiting time (Δτ) between the flashes in thylakoid membranes (**B**), and in TRIS-washed thylakoid membranes, intact cells of the PAL mutant of *Synechocystis* PCC6803 and in the PSII core particles (C) at different temperatures as indicated. $F_{1,2}$ denotes the fluorescence intensity level following the first double STSF.

2.3. Role of non-bilayer lipids and lipid phases in the assembly and flexibility of thylakoid membranes

Our recent ^{31}P-NMR experiments, using higher resolution spectrometers, have clearly shown the presence of two isotropic phases, in addition to the bilayer phase, in freshly isolated, fully functional spinach thylakoid membranes; an inverted hexagonal (H_{II}) phase was also observed, which is accounted for by the extruded excess amounts of lipid molecules (Fig. 4 - left) (Garab et al. 2017). We have shown that the phase behavior of isolated thylakoid membranes (i) is sensitive to the osmolarity and ionic strength of the medium, (ii) can be modulated by rigidifying the membranes, and (iii) exhibits a marked, largely reversible increase of one of the isotropic phases upon lowering the pH of the medium; (iv) the heterogeneity of lipid phases has also been confirmed by time-resolved MC540 fluorescence spectroscopy (Garab et al. 2017 Sci Rep). In a series of experiments, we also applied co-solute (2 M sucrose) treatment of isolated thylakoid membranes, which had earlier been shown to lead to the extrusion of lipids from the bilayer membrane and thermal stabilization of photosystem II (PSII). We have found that the changes in PSII stability, and in the chiral macro-organization of the complexes, as well as the associated changes in the lipid phases, as detected by time-resolved MC540 fluorescence spectroscopy, are almost fully reversible - suggesting that most of the extruded lipids remain closely associated with the bilayer membrane, in good accordance with the DEM (Kotakis et al. 2018).

We propose a chloroplast thylakoid membrane model in which fusion channels at the granum-stroma junctions, and lipocalins and lipocalin-like molecules, such as VDE, ZE (the zeaxanthin epoxidase) and CHL (the chloroplastic lipocalin) - water soluble proteins capable of binding lipid molecules, account for the presence of the two isotropic phases (Fig. 4 - right).

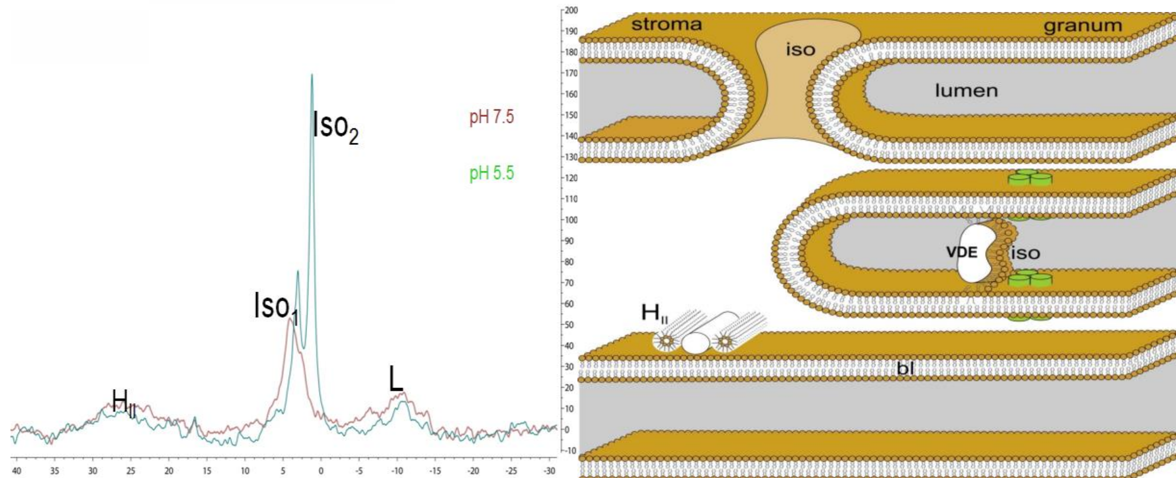

Fig. 4. ^{31}P-NMR spectra of isolated thylakoid membranes at neutral and acidic pH (left) – the low-pH induced changes are largely reversible, and show the structural flexibility of lipid phases, specific reorganizations which appear to be associated with the (lumenal) low-pH associated NPQ; and the dynamic excange model of thylakoid membranes – proposed by us (Garab et al. 2017) based on the results from the present OTKA project.

2.4. Changes in the macro-organization of the protein complexes and remodeling of the membrane system

With regard to the chiral macro-organization of the protein complexes in the thylakoid membranes of different oxygenic photosynthetic organisms, we have fingerprinted isolated plant thylakoid membranes and intact algal cells using their psi-type CD spectra, and found that (i) the chiral macrodomains disassemble upon mild detergent treatments, but not after crosslinking the protein complexes; (ii) in different wild-type leaves of dicotyledonous and monocotyledonous angiosperms the CD features are quite robust, displaying very similar excitonic and psi-type bands, suggesting similar protein composition and (macro-) organisation of PSII supercomplexes in the grana; (iii) the main positive psi-type bands depend [on the LHCII] contents of the membranes; (iv) the (+)506 and appears only in the presence of PSII–LHCII supercomplexes and does not depend on the xanthophyll composition of the membranes (Tóth et al. 2016).

The periodic organization of thylakoid membranes have been studied under different experimental conditions – using SANS, in most cases in combination with CD spectrosocopy, including desert crust cyanobacteria during desiccation and rehydration (Eyal et al. 2017) and the presence and absence of different ion channels (Herdean et al. 2016a,b).

We studied the effect of low pH. “Energization of thylakoid membranes brings about the acidification of the lumenal aqueous phase, which activates important regulatory mechanisms. Earlier Jajoo et al. (2014) have shown that low pH in isolated plant thylakoid membranes induces

changes in the excitation energy distribution between the two photosystems. In order to elucidate the structural background of these changes, we [SANS] on thylakoid membranes exposed to low p^2H (pD) and show that gradually lowering the p^2H from 8.0 to 5.0 causes small but well discernible reversible diminishment of the periodic order and the lamellar repeat distance [which can also be seen on the 2D scattering profile of the membranes (Fig. 5)] and an increased mosaicity – similar to the effects elicited by light-induced acidification of the lumen. Our data strongly suggest that thylakoids dynamically respond to the membrane energization and actively participate in different regulatory mechanisms." (Ünnep et al. 2017)

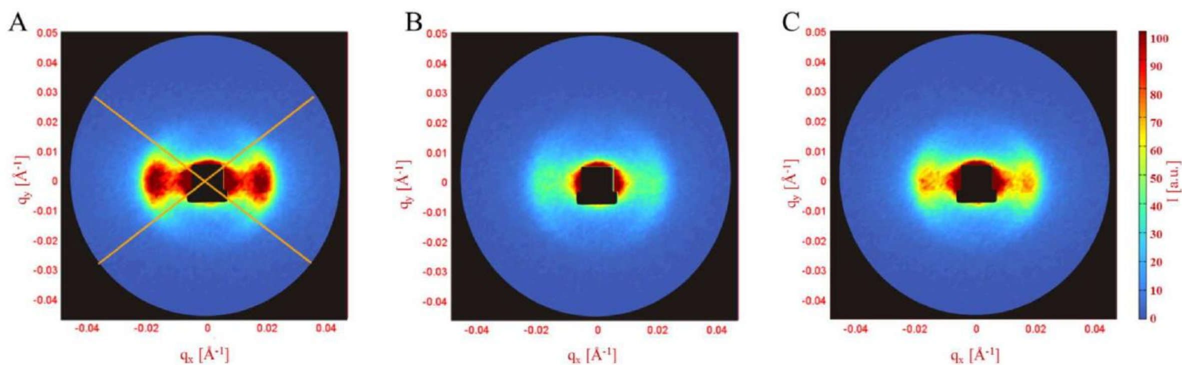

Fig. 5. 2D small-angle neutron scattering profiles of magnetically oriented thylakoid membranes isolated from pea leaves and suspended in p^2H 8.0 reaction medium (A), resuspended in the same medium adjusted to p^2H 5.0 (B), and returned to the p^2H 8.0 medium (C).

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