Towards in-cell structural study of light-harvesting complexes : an investigation with MAS-NMR

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CHAPTER 6

General discussion and future prospects

Photosynthetic light harvesting complexes from plant and algae have the crucial role to capture the sunlight and transfer it to reaction centers for biochemical productions. Fluctuation in sunlight intensity is a challenge that plants face every day. To cope with this challenge, they develop complex protective mechanisms to protect themselves from photo-damage. The PhD research described in this thesis aimed to understand how the structural flexibility of photosynthetic light harvesting complex II effects on its function and how this can be controlled by membrane environments. In this light, obtaining atomic-level structures of these complexes under physiological conditions and in native environments is an essential step towards understanding the molecular mechanisms that regulate the excitation energy flow.

NMR spectroscopy has the advantage over crystallography that it is capable of studying conformational dynamics of membrane proteins in their native environments. *In-situ* and *in-cell* NMR spectroscopy allow to the researcher to simultaneously have an overview of membrane or cell chemical compositions and conformations (lipid isomers, degree of unsaturation). While *in-cell* or *in-situ* NMR spectroscopy for structure characterization is very challenging, several studies have been reported, for example solid-state NMR of recombinant-expressed PagL in *E. coli* whole cells and cell envelopes, *in-situ* NMR of the Chl-binding CsmA protein and Dynamic Nuclear Polarization NMR of proteins in native cellular membranes [1-3]. The *in-situ* NMR experiments reported in this thesis may serve as a steppingstone toward structural characterization of LHC pigment-protein complexes in their native environments.

Dynamics of LHCII and the role of the membrane environment: from protein and lipids to cell

In-vitro: LHCII

The conformational dynamics of LHCII is mainly studied via the spectroscopic characteristics of the pigments that are taken as reporters, resulting in lack of information on the dynamics of the protein helixes and loops. However, with our NMR approach we obtained simultaneously a direct view of the protein, pigment, associated intrinsic lipids of LHCII and their dynamics.

Combining the NMR results together, we could detect flexible sites of LHCII that have not been resolved by crystallography and cryo-EM, including residues of the N-terminus and 2 Chl tails (**Chapter 3**). Our findings in **Chapter 3** of a flexible N terminus are in agreement with a reported MD study on membrane-

embedded LHCII and with an EPR-spin label study [4]. Interestingly, the suggested flexible sites in our study are in close proximity to carotenoid and Chl sites that have been proposed to be involved in excitation quenching [5-9]. The NMR signals of different Lhcbm polypeptides could be distinguished in our NMR study, which could not be distinguished in X-ray or cryo-EM studies (chapter 3). Selective Lhcbm mutants may be used in future to identify chemical shift contributions of the different polypeptide types [10].

Moreover, we present the first study of the effect of Zea binding on the conformation and dynamics of LHCII (**Chapter 4**). Our NMR results on Zea binding LHCII from the *npq2* mutant reconstituted in a lipid bilayer reveal that Zea-LHCII binds many lipids that are immobilized and that protein dynamics is reduced compared to wildtype LHCII. In addition, evidences for structural change have been observed revealing that some parts of the Zea-containing LHCII folds into strands. Our novel results of reduced dynamics and strong lipid binding of Zea LHCII might be correlated with the reduced fluidity of Zea-accumulating thylakoid membranes that was observed in **Chapter 2**. Although our approach, where we did not perform a sequence-specific NMR assignment revealed interesting results on the conformational dynamics of LHCII in lipid environments, a sequential assignment will help to reveal site-specific structure and dynamics. This would require selective labeling of native LHCII that are extracted from *Cr.* or selective labeling of recombinant LHCII that is overexpressed in *E. coli.* However, for both approaches there are number of challenges. For *E. coli*, selective labeling methods are well established, however for *Cr.* selective labeling strategies would first have to be established. A challenge that recombinant expression of LHCII faces is the refolding of recombinant LHCII. The LHCII proteins expressed from *E. coli* come as inclusion bodies and should be refolded with pigments, and further purified to separate the refolded proteins from free pigments and unfolded proteins. Hence, obtaining NMR quantities (milligrams) of refolded LHCII may lead to long sample preparation time.

The conformational dynamics studies in **Chapter 3** and **4** were performed on proteoliposomes in conditions where the LHCII proteins were strongly fluorescence quenched due to the high protein to lipid ratio. An interesting study would be to investigate the conformational dynamics of LHCII under mild quenched conditions. Fluorescence quenching of LHCII in our preparations is induced by protein self-aggregation in liposomes [11]. Therefore dilution of the LHCII complexes in proteoliposomes would reduce the quenched state. This requires at least 10 times dilutions of protein content compared to the condition described in the **Chapter 3** and **4**. Therefore, the concentration might not be enough for the 2D ^{13}C-^{13}C NMR measurements. However 1D CP and INEPT or

^{1}H-^{13}C HETCOR still could provide information on the effect of the protein to lipid ratio on the conformational dynamics of LHCII.

In-situ: thylakoid membrane

We show that polarization transfer solid state NMR and biosynthetic isotope labeling methods have the ability for assessing protein and lipid molecular dynamics in native heterogeneous photosynthetic membranes, providing a microscopic picture on molecular components of thylakoid membranes (**Chapter 2**). Separation of the rigid and mobile components is possible by combining CP and INEPT based experiments. Our method has provided detailed information on molecular dynamics of protein, lipid and xanthophyll molecules in thylakoid membranes as a function of temperature and comparing wildtype and Zea accumulating membranes. Moreover, we demonstrate that the NMR signals of LHCII can be detected within native thylakoid membranes, which allowed us to explore the role of the native environment on the conformational dynamics of LHCII by comparing LHCII in native thylakoid membranes and in liposome membranes (**Chapter 3**). Our NMR results reveal that the dynamics of LHCII flexible sites are significantly suppressed in native thylakoid membranes. Current models for excitation migration assume that individual antenna proteins fluctuate between quenched and unquenched states. Our observation that the intrinsic dynamics of LHCII is constrained in native membranes raises questions by this view and suggests that environmental changes are necessary to enable their conformational switching.

For the purpose of studying active membranes on which only rapid, 1D experiments can be performed in order to maintain their states, a limitation is the lack of sensitivity for detection of specific proteins. In that respect, FRAP is more selective for study of light-harvesting protein mobility, but has limited spatial resolution since lateral diffusion is measured over long distances, while NMR detects rotational diffusion of molecules with atomistic resolution. Our *in-situ* NMR approach can be further exploited by integration with selective mutation or labeling strategies. *In-situ* NMR studies that are aimed at structural characterization of a target protein generally rely on genetic manipulation to reduce the background signals of other cellular or membrane components. Recently, a chloroplast biosynthesis induction/repression system was reported to create minimal cells with stripped thylakoid membranes containing LHCII as the only Chl-binding protein [12]. Such a system could be used to more selectively probe the dynamic behavior of LHCII in a membrane or cellular environment. Selective ^{13}C *in-vivo* labeling of Chls could be achieved by addition of δ–aminolevulinic acid to the cell growth medium [13], which would amplify the Chl signals with respect to the lipid and membrane background. Another interesting direction would be to perform *in-situ* NMR on LHCII,

comparing thylakoid membranes of cells grown under different light conditions to study the effect of environmental conditions at the protein level.

Furthermore, our NMR method can be combined with other spectroscopic techniques such as fluorescence, EPR, electron microscopy and Raman as complementary methods for quantitative analysis of protein and lipids structure and dynamics.

In-cell: thylakoid membrane dynamics in intact *Cr.* algae

Polarization transfer NMR was successfully extended to whole cells (**Chapter 5**) providing a molecular picture of cell components in intact *Cr.* cells. Intact *Cr.* cells and isolated thylakoid membranes share very similar NMR lipid spectral profiles, demonstrating that the lipid NMR profiles in the spectra of *Cr* cells are dominated by thylakoid lipids. This suggests that their molecular conformation and dynamics can be determined inside intact cells. The intrinsic dynamics of protein and lipid constituents was measured over a physiological temperature range. An overall increase of mobility was observed for the cell components with increasing temperature up to 13 ^{o}C Celsius. However, at higher temperatures, the dynamics of the lipids decreased or stabilized, which may suggest the existence of a protective mechanism in the membrane that prevents the membrane from extreme fluidity. In addition to the temperature dependence NMR analysis, simulated INEPT and CP NMR intensities provided quantitative information on values or ranges of order parameters and rotational correlation times of the lipid components, which may provide input for coarse-grain and molecular dynamics simulations.

In this thesis, cells were grown on a moderate light using acetic acid as carbon source, however different growth conditions such as high light, different CO_2 concentrations or salt stress could be tested in future to investigate the effect of various environmental conditions on dynamics of cell components.

For the *in cell* NMR experiments, the stability and physiological state of cells during the measurements has to be controlled. Controlling the physiological states of *Cr.* cells during NMR experiments could be a problem since without light and oxygen, the cells can switch on fermentation [14]. Further research is required to test and verify under which conditions *in-cell* NMR can best be performed.

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