The role of lipids in the global organization of thylakoid membranes of higher plants

Promotor:
Prof.dr. Herbert van Amerongen
Hoogleraar Biofysica
Wageningen Universiteit, Nederland

Co-promotor:
Dr. Győző Garab
Group leader, Institute of Plant Biology
Biological Research Center, Szeged, Hungary

Samenstelling promotiecommissie:
Prof.dr. Sacco C. de Vries (Wageningen Universiteit, Nederland)
Prof.dr. Roberta Croce (Rijksuniversiteit Groningen, Nederland)
Prof.dr. Egbert J. Boekema (Rijksuniversiteit Groningen, Nederland)
Dr. Zoltan Gombos (Institute of Plant Biology, Szeged, Hungary)

Dit onderzoek is uitgevoerd binnen de onderzoeksschool EPS.

The role of lipids in the global organization of thylakoid membranes of higher plants

Sashka Krumova

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof.dr. M.J. Kropff,
in het openbaar te verdedigen
op vrijdag 16 januari 2009
de namiddags te half twee in de Aula

Sashka Krumova, 2009
The role of lipids in the global organization of thylakoid membranes of higher plants
PhD thesis, Wageningen Universiteit, Nederland
met een samenvatting in het Nederlands

ISBN: 978-90-8585-292-6

"Tiger got to hunt, bird got to fly;
Man got to sit and wonder why, why, why;
Tiger got to sleep, bird got to land;
Man got to tell himself he understand."

Kurt Vonnegut, “Cat’s cradle”

The first steps of photosynthesis (capturing of light and conversion of light energy into chemical energy) occur in the thylakoid membrane of the chloroplasts. It consists for 75% of membrane proteins and 25% of lipid molecules. In this thesis the global properties of the lipids in the thylakoid membrane are studied with a variety of spectroscopic techniques. Special attention is paid to the lipid packing, phase behavior and membrane permeability, as well as the role of the lipids in structural rearrangements and the overall organization of the membrane. The presented results reveal heterogeneity in the packing of the bulk lipid molecules and the participation of the lipids in both bilayer and non-bilayer structures. It is demonstrated that the lipid mixture has an active role in the lateral arrangement of the protein complexes, the formation of macrodomains and their thermal stability and indirectly in the excitation energy trapping by the photosynthetic complexes. This work provides a basis for further future investigations of the properties of the thylakoid lipid matrix and of the lipid-protein interactions in thylakoid membranes and their relevance for different functions of the membrane.

Table of Contents

Chapter 1

General Introduction

Chapter 1

Macroorganization of the thylakoid membrane

Oxygenic photosynthesis is a process which is highly relevant for human kind. In higher plants the first steps of photosynthesis (capturing of light and the conversion of solar energy into chemical energy) occur in the internal membranes of chloroplasts – the thylakoid membranes. These constitute a complex ensemble of pigments, proteins and lipid molecules, forming a continuous 3D membrane network that encloses a single interior aqueous phase (lumen). It has long been recognized that in higher plants the thylakoid is highly folded and forms membrane domains with strikingly different protein composition (see below) - grana stacks and unstacked (stromal) lamellae (Fig. 1, cf [1,2]). According to the most recent 3D models the stroma membranes are arranged in a circular fashion around the granum and ensure the connections between neighboring grana stacks [3] or alternatively the granum layers are formed by bifurcations of stromal sheets that fuse within the granum body, the adjacent granum layers being connected via membrane bridges [4]. Grana formation leads to lateral segregation of the main photosynthetic complexes - photosystem II (PSII) and photosystem I (PSI). The function of grana has long been discussed (see for example [5,6]). It appears that grana are not indispensable for plant survival but are needed for better adaptation to land conditions via regulation of light harvesting and controlling the balance of energy flow between PSII and PSI [6]. The spatial separation of PSII and PSI in the stacked and unstacked membrane regions, respectively, as well as the macromolecular organization of PSII in stacked grana thylakoids is a self-organizing process, which most likely is an important feature for the maintenance of the functional integrity of the photosynthetic machinery [7].

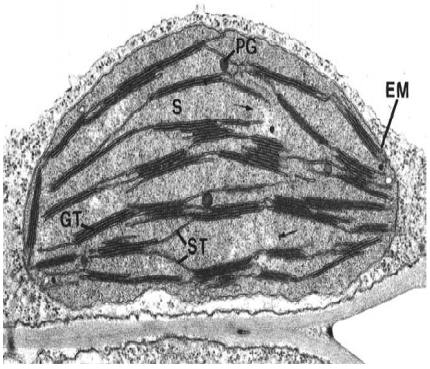


Figure 1. Thin section micrograph of a young tobacco chloroplast [2]. Two envelope membranes (EM) surround the chloroplast in which stacked grana thylakoids (GT) and unstacked stroma thylakoids (ST) can be recognized. Plastoglobules (PG, lipoprotein particles) are also visible in the stromal space (S).

Assembly of the pigment-protein complexes

The thylakoid membrane is highly enriched in proteins (about 70% of its area, [8] and in the last decade most of the crystal structures of the membrane-embedded pigment-protein complexes have become available. Moreover, it was established that they are able to form higher order assemblies.

The most abundant protein in thylakoids is the major light-harvesting complex of PSII (LHCII). It was established to play a structural role: (i) it has been shown to stabilize the granum ultrastructure, and to participate in the cation-mediated stacking of the membranes [9-11]; (ii) it is involved, via electrostatic and osmotic forces, in the lateral organization of the membranes [12]. Furthermore, LHCII participates in the regulation of the energy distribution between the two photosystems and the harmless dissipation of excess excitation energy into heat under conditions of light stress [13]. It was proposed that LHCII-only domains in one layer of the thylakoid membrane are able to transfer excitation energy to the PSII complexes in the opposing layer [14,15].

The two main pigment-protein complexes in thylakoids – PSII and PSI consist of core (where charge separation occurs) and antenna (where light harvesting occurs) complexes. PSII contains more than 20 different proteins [16]. The core complex of PSII forms a dimeric supercomplex together with light-harvesting antenna complexes in different architectures and stoichiometries (Fig. 2, see also [17,18]). The type of assembly presented in Fig. 2 was found only in the stacked grana regions, whereas PSII core dimers

lacking some of the light-harvesting units and PSII core monomers were distributed over all regions of the thylakoid membrane, but mostly over the grana and stroma lamellae, respectively [19].

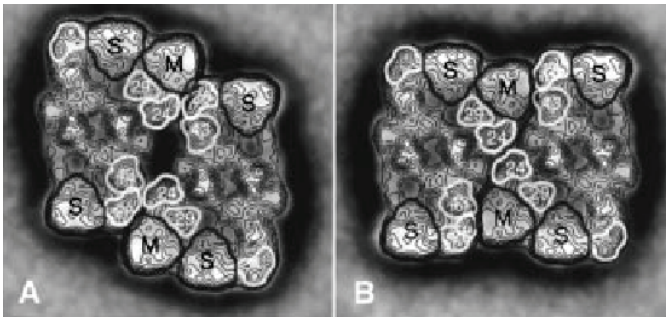


Figure 2. Arrangements of the subunits participating in the formation of supercomplexes of PSII and LHCII. Trimeric LHCII complexes are denoted as S (strongly bound) and M (moderately bound); the numbers 24, 26, 29 and 43 indicate the positions of the complexes CP24, CP26, CP29 and CP43, respectively. The unlabeled central density represents the dimeric core complexes [17].

Two types of semi-crystalline macrodomains of PSII supercomplexes were observed [20] and it was proposed that they might face domains formed by the major light-harvesting complex of PSII, LHCII, in the opposite layer of the granum [20]. Pairs of PSII-LHCII supercomplexes, interacting with their stromal sides were also identified [21]. The presence of large ordered protein domains has been revealed earlier by means of circular dichroism spectroscopy (CD) and the appearance of very intense and "anomalously shaped" CD bands, Ψ-type CD bands. It was established that these bands correlate with the macro-organization of LHCII in LHCII-only domains (e.g. [12,22]). Recent data show that the arrays of PSII-supercomplexes might also contribute to the Ψ-type CD signal. [23].

This intrinsic variability at the macroorganizational level was suggested to result in different light-harvesting efficiencies and thus to provide the system with a way to optimize its function under various light or stress conditions that green plants are constantly exposed to [20]. For example it has been demonstrated that, upon illumination with moderate and

high light intensities, thylakoid membranes undergo reversible structural changes, which, as revealed by CD spectroscopy, affect the long-range chiral order of the chromophores, whereas prolonged illumination with intense light generally leads to similar, but irreversible changes [24]. Moreover, the physiological role of the PSII reaction center oligomerization might be to minimize the contact between PSII and PSI in a crowded membrane and, thus, to regulate the light energy channeled to the two photosystems [25].

Whereas PSII is predominantly located in the stacked regions of grana membranes, PSI is found in the stroma lamellae and the stroma-exposed membranes of the grana stacks, the so called end and marginal membranes [26].

In higher plants it is monomeric and the core complex is associated with four asymmetrically bound light-harvesting complexes (Fig. 3). Higher order spatial macroorganization of PSI was not observed so far. The core complex is composed of 12 subunits [27]. The proteins constituting the light-harvesting antenna possess high sequence homology with LHCII, however they are organized in heterodimers (Lhca1/4 and Lhca2/3). Two other Lhca genes, Lhca5 and Lhca6, have been identified in *Arabidopsis thaliana* as well as in other plant species [28]. The corresponding complexes seem to be only minor components of the antenna, which however could play a role under specific environmental conditions. The interaction between Lhca subunits and PSI core is strongly cooperative and when one subunit is missing, the whole LHCI system is destabilized [29].

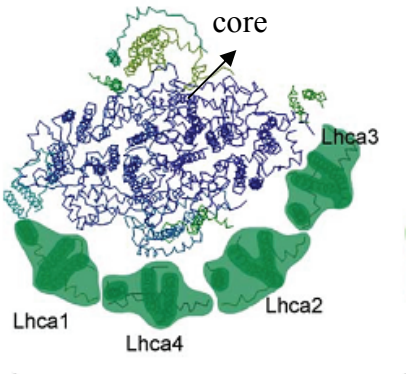
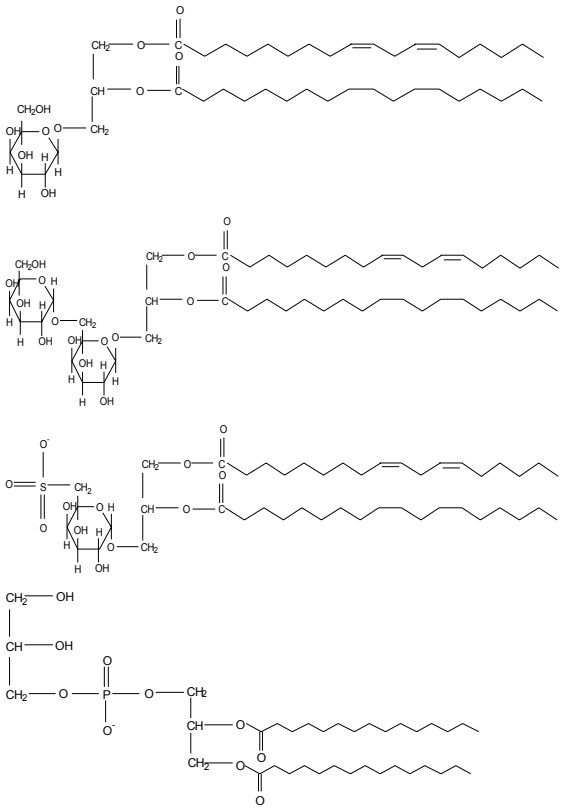


Figure 3. Structural model of Photosystem I complex, obtained at 4.4 Å [27]. The core complex is surrounded asymmetrically by the light-harvesting antenna complexes denoted as Lhca1, Lhca2, Lhca3 and Lhca4.

Lipid matrix

The lipid matrix of thylakoids constitutes about 30% of the membrane area [8] and consists of four lipid classes: monogalactosyl diacylglycerol (MGDG, ~50%), digalactosyl diacylglycerol (DGDG, ~30%), sulfoquinovosyl diacylglycerol (SQDG, ~5–12%) and phosphatidylglycerol (PG, ~5–12%) [30] (Fig. 4). In contrast to the large lateral heterogeneity in the distribution of proteins, the relative distribution of the four lipid classes as well as the relative amount of the different lipid species was found to be identical in intact thylakoid membranes and in isolated granal, stromal and marginal membrane fragments [31].



Monogalactosyl diacylglycerol (MGDG)

Digalactosyl diacylglycerol (DGDG)

Sulfoquinovosyl diacylglycerol (SQDG)

Phosphatidyl glycerol (PG)

Figure 4. Structural formulas of the thylakoid membrane lipids. Each lipid species can bind different fatty acids – 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3.

Different lipid molecules were resolved in the crystal structures of photosynthetic pigment-protein complexes and they were also shown to be important for the assembly of higher-order complexes in the plane of the membrane [32-36].

The boundary lipids (motion-restricted lipids, close to membrane proteins) have been shown to be important for the spacing of the proteins and the supercomplexes in such a way that they ensure fast diffusion of plastoquinol needed for efficient electron transfer [8,37]. Bulk lipids (not associated with proteins) were shown to be important for membrane flexibility – isolated lamellar aggregates of LHCII enriched in different thylakoid lipids exhibited light-induced reversible structural rearrangements, very similar to the ones observed in native thylakoid membranes [38], with the largest enhancement obtained upon addition of MGDG [39].

Depending on their molecular structure, the lipid molecules, when isolated, can form bilayers (bilayer-prone lipids) such as DGDG, SQDG and PG, whereas MGDG prefers to assemble in non-bilayer structures (non-bilayer forming lipid, NBL). The high abundance of MGDG makes the thylakoid membrane comparable to other energy converting membranes (such as mitochondria, (bacterio)rhodopsin-containing membrane) where the relative ratio of non-bilayer lipids is likewise high.

The structural organization and phase properties of the total lipid extract of thylakoids are highly dependent on the ionic strength and pH of the suspending medium due to the high abundance of NBL on the one hand and the presence of negatively charged lipids (PG and SQDG) on the other. In the presence of ions at concentrations typical of those found *in vivo* and/or standard chloroplast isolation media, the structural organization of the total polar lipid extracts of thylakoid membranes is predominantly non-bilayer [40]. Nevertheless, the assembled functional thylakoid is believed to adopt a bilayer configuration [41]. Apparently, the presence of proteins plays a major part in maintaining the bilayer configuration in the native membranes and demonstrates the importance of lipid-protein interactions for the organization of the membrane. For example it was established that LHCII can force purified MGDG to form bilayer structures [42].

Non-bilayer lipids in general are hypothesised to have a high physiological significance and to maintain a high lateral packing pressure on proteins, thus keeping them

in their functional state [43]. It was proposed that in thylakoids they are required for the maintenance of curvature stress in the bilayer needed for the optimal operation of the enzyme violaxanthin de-epoxidase (located in the thylakoid lumen) which converts violaxanthin to zeaxanthin during the xanthophyll cycle [44]. This is one of the main protection mechanisms by which the plants dissipate the excess light energy which otherwise would be harmful. According to the model of Szilagyi et al. [44] zeaxanthin with its longer hydrophobic stretch promotes lamellar arrangements of the membrane and as a result relieves the curvature elastic stress, which in turn leads to inactivation of violaxanthin de-epoxidase.

Thylakoid membrane as a dynamic system

The thylakoid membrane is a dynamic system which is able to adapt to the constantly changing environmental conditions, including variations in temperature, light intensity and quality. For example during the so called state-transitions (light adaptation) LHCII has been shown to detach from PSII and migrate towards PSI [45]. Recent reports reveal that this process indeed encompasses major structural changes at the interface of the grana and stroma membrane domains resulting in macroscopic rearrangements of the entire membrane network [46] and moreover it is accompanied by movement of PSI-LHCI complexes towards the grana and phosphorylated LHCII-PSII towards the stroma lamellae [47]. Damaged PSII complexes have also been shown to migrate from the grana to the stroma lamellae where their repair takes place [48].

Significant structural rearrangements can also be induced by high temperatures – monomerization of both LHCII and the PSII core [49], increased phosphorylation of LHCII and migration to the PSI complexes [50-53]. Growing of plants at low temperatures was shown to result in monomerization of LHCII [54,55], increased phosphorylation levels of CP29 [56], higher amounts of xanthophylls and early-light-inducible-protein complexes [55].

The overall ultrastructure of chloroplasts is dynamically changing - in darkness the chloroplasts have been shown to be enlarged and round, the thylakoids are swollen and

grana stacks to a large extent disintegrated, whereas in light the chloroplasts have an ellipsoid shape and the typical differentiation of grana and stroma membranes is observed [57]. High temperatures result in destacking of the thylakoid membranes and lipid segregation [58]. Cold stress also results in major changes of the chloroplast ultrastructure (cf [59]), but detailed structural information is not available.

Most of the above mentioned structural changes are realized via rearrangements of the protein complexes in the thylakoid membrane. The significance of the bulk lipid mixture for the above mentioned processes is not yet well studied, although the lipid molecules provide the matrix where the protein complexes are embedded and assembled. So far it has been established that the thylakoid membrane has the ability to adjust its fluidity to variations in the temperature by controlling the fatty acid composition and the relative amount of the different lipid species [60-62]. However, it is not known yet how the changes in the lipid matrix affect the lipid-protein interactions and *vice versa*, how the membrane macroorganization ensures efficient diffusion of protein complexes, whether the role of non-bilayer lipids is confined to maintenance of a certain packing stress or also whether it should induce the formation of non-bilayer structures and finally how it is related to response to stress and adaptation of plants.

This thesis is focused mainly on the global properties of the thylakoid lipid matrix - with special attention to phase behavior and lipid packing - and their correlation with the macroorganization of the pigment-protein complexes.

Main Experimental Methods

Circular dichroism

(probing the molecular architecture and assembly of pigment-protein complexes)

Circular dichroism (CD) is defined as the differential absorption of left and right circularly polarized light:

$$\mathbf{CD} = (\mathbf{A_L} - \mathbf{A_R})$$

$\mathbf{A_L}$ – absorption of left circularly polarized light

$\mathbf{A_R}$ – absorption of right circularly polarized light

CD is exhibited by chiral molecules [63] and in molecular complexes (e.g. pigment-protein complexes) it originates from short-range excitonic interactions between the chromophores [64-66]. In large systems, such as the thylakoid membrane and isolated LHCII lamellar aggregates, also high-intensity CD bands are observed (Ψ-type CD), reflecting the long-range order of the chromophores [66-71].

^{31}P-Nuclear magnetic resonance

(probing the lipid phase behavior)

^{31}P-Nuclear magnetic resonance (^{31}P-NMR) is a powerful technique for the study of lipid phase behavior in model and biological membranes. The interaction of an unpaired proton with the applied magnetic field results in well defined spectra, whose shape is determined by the local environment of ^{31}P. The NMR spectra are expressed in terms of chemical shift. The chemical shift of a nucleus (δ, in ppm), relative to the standard, is the difference between the resonance frequency of the nucleus (ν) and the standard (ν_{ref}):

$$\delta = (\nu - \nu_{ref}) \times 10^6 / \nu_{ref}$$

In phosphorus-containing lipid systems three types of ^{31}P-NMR spectra can be observed depending on the lipid phase (Fig. 5). Relatively narrow ^{31}P-NMR spectra (Fig. 5B) may be obtained from small vesicles, where their fast isotropic tumbling is the dominant line narrowing mechanism [72]. Micelles, cubic and rhombic phases give the same averaged signal due to the fact that the motion of phospholipids is allowed in all directions. Much broader and asymmetric ^{31}P-NMR lineshapes (Fig. 5A) reflecting the restricted anisotropic motion of the phospholipid molecules are observed for liposomes (model for lamellar phase) with sizes of about and above 250 nm [73]. In these systems isotropic averaging mechanisms due to tumbling and lateral diffusion of the phospholipid in the plane of the membrane are not effective, and the obtained spectra only reflect the local anisotropic motions in the phosphate region of the polar head-group, restricted to the 2D plane of the membrane.

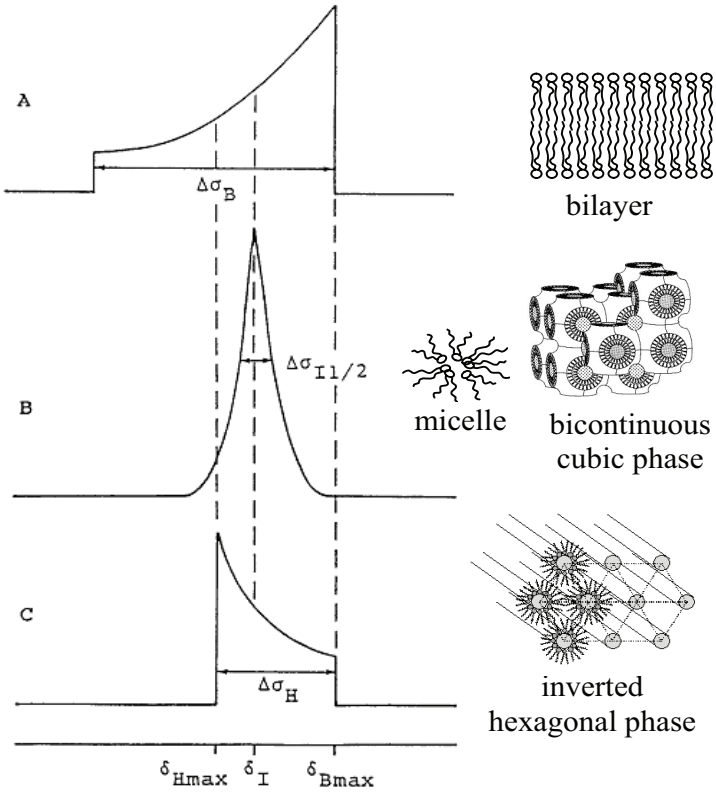


Figure 5. Simulated [76] ^{31}P-NMR spectra of phospholipids organized in different phases (examples for the arrangement of the lipids in the corresponding phases are given on the right): (A) lamellar/bilayer phase; (B) phases where isotropic motion occurs; (C) inverted hexagonal phase. For ease of presentation, the intensities of spectra B and C are reduced by a factor of two. The field is increasing from left to right; the chemical shift (δ) is increasing from right to left. The effective chemical shift anisotropy for the bilayer phase ($\Delta\sigma_B$) is defined as the chemical shift difference between the high-field peak and the low-field shoulder and for the inverted hexagonal phase ($\Delta\sigma_H$) – as the chemical shift difference between the low-field peak and the high-field shoulder, respectively. For the isotropic phases a symmetrical peak is observed whose linewidth at half height ($\Delta\sigma_{I1/2}$) is about 5 ppm.

The corresponding ^{31}P-NMR signal is asymmetric, with a high-field peak and a low-field shoulder (Fig. 5A). These signals are insensitive to the fatty acid composition [74], however below the hydrocarbon phase transition the motion of the lipid head-group is progressively restricted, which results in the increase of the ^{31}P-NMR line-width and changes in the lineshape [72,74,75].

For the inverted hexagonal phase (H_{II}) the phospholipids are allowed to move both along and around the cylinders and the ^{31}P-NMR spectrum has reversed symmetry as compared to the one for bilayers, and the width of the spectrum is reduced to half compared to that of the lamellar phase (Fig. 5C).

Time-correlated single photon counting

(probing the assembly and functionality of pigment-protein complexes and the local environment of fluorescent probes)

Time-Correlated Single Photon Counting (TCSPC) is based on the detection of single fluorescence photons after pulsed-light excitation with picosecond time resolution. The time between the excitation pulse and the observed photon is measured and stored in a histogram. Deconvolution of the data allows the determination of the excited-state lifetimes. Fluorescence intensity as a function of time (*I(t)*) is typically fitted to a multi-exponential model:

$$I(t) = N\sum_i \alpha_i \exp(-t/\tau_i) \otimes Irf(t)$$

N – scaling factor, α_i - pre-exponential factors proportional to the fractional population, with their sum normalized to unity, τ_i – fluorescence lifetimes, *Irf (t)* – instrumental response function, and t – time

Outline of the thesis

Chapter 2: *Structural rearrangements in chloroplast thylakoid membranes revealed by differential scanning calorimetry and circular dichroism spectroscopy. Thermo-optic effect*

In this chapter the thermally induced rearrangements of the pigment-protein complexes in thylakoid membranes in the range 30 - 100 °C are explored. The detected transitions are largely dominated by LHCII, due to the high abundance of this protein. The presented data suggest the following sequence of events upon raising the temperature: (i) unstacking of the thylakoid membranes and lateral disassembly of the chiral protein macrodomains occurring at about 43 °C, being largely influenced by the ionic strength of the medium and by illumination with photoinhibitory light, (ii) monomerization of LHCII trimers at around 60 °C and (iii) degradation of LHCII and other pigment-protein complexes above 70 °C.

Chapter 3: *Phase behavior of phosphatidylglycerol in spinach thylakoid membranes as revealed by ^{31}P-NMR*

In this chapter the lipid phases present in intact functional thylakoids are studied by means of ^{31}P-NMR. This is a non-invasive method, well elaborated for studies of the lipid phase behavior in model and biological systems. In thylakoids the sole phospholipid is phosphatidylglycerol (PG) and it accounts for about 10 % of the total lipid content. Since PG is distributed over all parts of the thylakoid membrane, it can be regarded as a reporter of the phase behavior of the bulk lipid mixture. In intact thylakoid membranes the characteristic lamellar signal is observed only below 20 °C. Moreover, it is superimposed on an isotropic signal, which becomes even dominant between 14 and 28 °C despite the presence of fully functional large membrane sheets that are capable of generating and maintaining a trans-membrane electric field. These data demonstrate the co-existence of lamellar and non-lamellar, isotropic phases. Tris-washed thylakoid membranes exhibit similar behavior but the lamellar phase is observed up to higher temperatures.

Chapter 4: *Temperature dependence of the lipid packing in thylakoid membranes studied by time- and spectrally resolved fluorescence of Merocyanine 540*

The lipid packing and fluidity of thylakoid membranes have been shown to play a role in a variety of photosynthetic functions. This chapter provides further information on the nature of lipid microenvironments in thylakoids and their temperature dependence. A systematic study of the steady-state and time-resolved fluorescence properties of the polarity probe Merocyanine 540 (MC540) incorporated in isolated spinach thylakoid membranes is carried out. For comparison, also model lipid systems (dipalmitoyl phosphatidylcholine and dioleoyl phosphatidylethanolamine) that adopt different phases are investigated. The presented data demonstrate a marked heterogeneity in the lipid packing of thylakoid membranes at all temperatures. Above 25 °C a rearrangement in the bulk lipid phase occurs, leading to the extrusion of MC540 from the membrane.

Chapter 5. *The role of DGDG in the overall organization and thermal behavior of thylakoid membranes*

In this chapter the functional role of one of the major thylakoid lipids, DGDG is studied employing a mutant of *Arabidopsis* (*dgd1*) in which the amount of DGDG is reduced by about 90%, and consequently the non-bilayer:bilayer lipids ratio is increased. It is demonstrated that the DGDG deficiency substantially influences both the overall organization and functioning of the thylakoid membrane and its thermal stability. At room temperature (25 °C) the arrangement of the pigment-protein complexes in large macrodomains of *dgd1* is different from that in the wild type and this is accompanied by a longer excitation trapping time in the mutant.

The data also reveal a different thermal behavior of the lipid and protein moieties for *dgd1* and wild type. In the case of *dgd1* the thylakoid membranes are more susceptible to heat-induced changes; they become "leaky" already at 35 °C, whereas for the wild type this is observed at 40 °C. The fluorescent probe Merocyanine 540 gradually becomes extruded from the *dgd1* membrane with the increase of temperature, whereas for the wild type this is not prominent even at 45 °C. Moreover, the temperature dependence of the average lifetime

of lipid-bound Merocyanine 540 is substantially shifted towards lower temperatures. The mutant thylakoids also demonstrate a lower thermal stability of the protein macrodomains (by ~5 °C) and the Chl *a*-binding proteins (by ~10 °C), meaning that the lipid matrix of *dgd1* is not able to maintain the functional state of the protein molecules at moderately elevated temperatures.

Reference List

[1]Boardman N.K. and J. M. Anderson, Isolation from spinach chloroplasts of particles containing different proportions of chlorophyll *a* and chlorophyll *b* and their possible role in the light reactions of photosynthesis, *Nature* 206 (1964) 166-167.
[2]Staehelin L.A., Chloroplast structure and supramolecular organization of photosynthetic membranes, In: *Photosynthesis III: Photosynthetic Membranes and Light-Harvesting Systems*, Staehelin L.A., Arntzen C.J. (Eds.), Springer Verlag (1986) 1-84.
[3]Mustardy L. and G. Garab, Granum revisited. A three-dimensional model – where things fall into place, *Trens Plant Sci*. 8 (2003) 117-122.
[4]Shimoni E., O. Rav-Hon, I. Ohad, V. Brumfeld and Z. Reich, Three-dimensional organization of higher-plant chloroplast thylakoid membranes revealed by electron tomography, *The Plant Cell* 17 (2005) 2580-2586.
[5]Trissl H.W. and C. Wilhelm, Why do thylakoid membranes from higher plants form grana stacks? *Trends Biochem. Sci*. 18 (1993) 415-419.
[6]Mullineaux C.W., Function and evolution of grana, *Trends Plant Sci*. 10 (2005) 521-525.
[7]Kirchhoff H., W. Haase, S. Haferkamp, T. Schott, M. Borinski, U. Kubitscheck and M. Rögner, Structural and functional self-organization of photosystem II in grana thylakoids, *Biochim. Biophys. Acta* 1767 (2007) 1180 -1188 .
[8]Kirchhoff H., U. Mukherjee and H.-J. Galla, Molecular architecture of the thylakoid membrane: Lipid diffusion space for plastoquinone, *Biochemistry* 41 (2002) 4872-4882.
[9]Arntzen C.J., Dynamic structural features of chloroplast lamellae, *Curr. Top. Bioenerg*. 8 (1978) 111-160.
[10]Duniec J.T., J.N. Israelachvili, B.W. Ninham, R.M. Pashley and S.W. Thorne, An ion-exchange model for thylakoid stacking in chloroplasts. *FEBS Lett*. 129 (1981) 193-196.
[11]Barber J., Influence of surface charges on thylakoid structure and function, *Annu. Rev. Plant Physiol.* 33 (1982) 261-295.
[12]Garab G., J. Kieleczawa, J. C. Sutherland, C. Bustamante and G. Hind, Organization of pigment-protein complexes into macrodomains in the thylakoid membranes of wild type and chorophyll *b*-less mutant of barley as revealed by circular dichroism, *Photochem. Photobiol.* 54 (1991) 273-281.
[13]Bassi R., D. Sandona and R. Croce, Novel aspects of chlorophyll *a/b* proteins, *Plant Physiol.* 100 (1997) 769-779.
[14]Trissl H.W., J. Breton, J. Deprez and W. Leibl, Primary electrogenic reactions of photosystem II as probed by the light-gradient method, *Biochim. Biophys. Acta* 893 (1987) 305-319.
[15]Leibl W., J. Breton, J. Deprez and H.W. Trissl, Photoelectric study on the kinetics of trapping and charge stabilization in oriented PS-II membranes, *Photosynth. Res.* 22 (1989) 257-275.
[16]Hankamer B., J. Barber and E. Boekema, Structure and membrane organization of photosystem II in green plants, *Annu. Rev. Plant Physiol. Plant Mol. Biol*. 48 (1997) 641-671.
[17]Boekema E.J., H. van Roon, F. Calkoen, R. Bassi and J.P. Dekker, Multiple types of association of photosystem II and its light-harvesting antenna in partially solubilized photosystem II membranes, *Biochemistry* 38 (1999) 2233-2239.

[18]Boekema E.J., H. van Roon, J.F.L. van Breemen and J.P. Dekker, Supramolecular organization of photosystem II and its light-harvesting antenna in partially solubilized photosystem II membranes, *Eur. J. Biochem*. 266 (1999) 444-452.
[19]Danielsson R., M. Suorsa, V. Paakkarinen, P.-A. Albertsson, S. Styring, E.-M. Aro and F. Mamedov, Dimeric and monomeric organization of photosystemII. Distribution of five distinct complexes in the different domains of the thylakoid membrane, *J. Biol. Chem.* 281 (2006) 14241-14249.
[20]Boekema E.J., J. F. L. van Breemen, H. van Roon and J. P. Dekker, Arrangement of photosystem II supercomplexes in crystalline macrodomains within the thylakoid membrane of green plant chloroplasts, *J. Mol. Biol*. 301 (2000) 1123-1133.
[21]Bumba L., M. Husak and F. Vacha, Interaction of photosystem 2-LHC2 supercomplexes in adjacent layers of stacked chloroplast thylakoid membranes, *Photosynthetica* 42 (2004) 193-199.
[22]Garab G. and L. Mustárdy, Role of LHCII-containing macrodomains in the structure, function and dynamics of grana. *Aust. J. Plant Physiol*. 26 (1999) 649-658.
[23]Kovács L., J. Damkjar, S. Kereiche, C. Ilioaia, A. V. Ruban, E. J. Boekema, S. Jansson and P. Horton, Lack of the light-harvesting complex CP24 affects the structure and function of the grana membranes of higher plant chloroplasts, *The Plant Cell* 18 (2006) 3106-3120.
[24]Cseh Z., S. Rajagopal, T. Tsonev, M. Busheva, E. Papp and G. Garab, Thermooptic effect in chloroplast thylakoid membranes. Thermal and light stability of pigment arrays with different levels of structural complexity, *Biochemistry* 39 (2000) 15250-15257.
[25]Mullineaux C.W., The thylakoid membranes of Cyanobacteria: Structure, dynamics and function. *Aust. J. Plant Physiol*. 26 (1999) 671-677.
[26]Andersson B. and J.M. Anderson, Lateral heterogeneity in the distribution of chlorophyll–protein complexes of the thylakoid membranes of spinach chloroplasts, *Biochim. Biophys. Acta* 593 (1980) 427-440.
[27]Ben-Shem A., F. Frolow and N. Nelson, Crystal structure of plant photosystem I, *Nature* 426 (2003) 630-635.
[28]Jansson P., A Guide to the Lhc genes and their relatives in *Arabidopsis*, *Trends Plant Sci.* 4 (1999) 236-240.
[29]Morosinotto T., M. Ballottari, F. Klimmek and S. Jansson, The association of the antenna system to photosystem I in higher plants. Cooperative interactions stabilize the supramolecular complex and enhance red-shifted spectral forms, *J. Biol. Chem*. 280 (2005) 31050-31058.
[30]Douce R. and Joyard J., Biosynthesis of thylakoid membrane lipids, In: *Advances in Photosynthesis/Oxygenic Photosynthesis: The Light Reactions*, Ort D.R.; Yacum C.F. (Eds.) Kluwer Academic Publishers (1996) 69-101.
[31]Duchene S. and P. Siegenthaler, Do glycerolipids display lateral heterogeneity in the thylakoid membrane?, *Lipids* 35 (2000) 739-744.
[32]Nuβberger S., K. Dorr, D.N. Wang and W. Kühlbrandt, Lipid-protein interactions in crystals of plant light-harvesting complex, *J. Mol. Biol*. 234 (1993) 347-356.
[33]Liu Z.F., H.C. Yan, K.B. Wang, T.Y. Kuang, J.P. Zhang, L.L. Gui, X.M. An and W.R. Chang, Crystal structure of spinach major light-harvesting complex at 2.72 angstrom resolution, *Nature* 428 (2004) 287-292.

[34]Loll B., J. Kern, W. Sänger, A. Zouni and J. Biesiadka, Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II, *Nature* 438 (2005) 1040-1044.
[35]Loll B., J. Kern, W. Sänger, A. Zouni and J. Biesiadka, Lipids in photosystem II: Interactions with protein and cofactors, *Biochim. Biophys. Acta* 1767 (2007) 509-519.
[36]Yan H., P. Zhang, C. Wang, Zh. Liu and W. Chnag, Two lutein molecules in LHCII have different conformations and functions: Insights into the molecular mechanism of thermal dissipation in plants, *Biochem. Biophys. Res. Comm*. 355 (2007) 457-463.
[37]Kirchhoff H., I. Tremmel, W. Haase and U. Kubitscheck, Supramolecular photosystem II organization in grana thylakoid membranes: Evidence for a structured arrangement, *Biochemistry* 43 (2004) 9204-9213.
[38]Barzda V., A. Istokovics, I. Simidjiev and G. Garab, Structural flexibility of chiral macroaggregates of light-harvesting chlorophyll *a/b* pigment-protein complexes. Light-induced reversible structural changes associated with energy dissipation, *Biochemistry* 35 (1996) 8981-8985.
[39]Simidjiev I., V. Barzda, L. Mustardy and G. Garab, Role of thylakoid lipids in the structural flexibility of lamellar aggregates of the isolated light-harvesting chlorophyll *a/b* complex of photosystem II, *Biochemistry* 37 (1998) 4169-4173.
[40]Gounaris K., A. Sen, A.P.R. Brain, P.J. Quinn and W.P. Williams, The formation of non-bilayer structures in total polar lipid extracts of chloroplast membranes, *Biochim. Biophys. Acta* 728 (1983) 129-139.
[41]Williams W.P., The physical properties of thylakoid membrane lipids and their relation to photosynthesis, In: *Advances in Photosynthesis. Lipids in Photosynthesis,* Siegenthaler, P.-A. and Murata, N. (Eds.), Kluwer Academic Publishers (1998) 103-118.
[42]Simidjiev I., S. Stoylova, H. Amenitsch, T. Jávorfi, L. Mustárdy, P. Laggner, A. Holzenburg and G. Garab, Self-assembly of large, ordered lamellae from non-bilayer lipids and integral membrane proteins in vitro, *Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 1473-1476.
[43]de Kruijff B., Lipids beyond the bilayer, *Nature* 386 (1997) 129-130.
[44]Szilagyi A.M., M. Sommarin and H.-E. Akerlund, Membrane curvature stress controls the maximal conversion of violaxanthin to zeaxanthin in the violaxanthin cycle-influence of α-tocopherol, cetylethers, linolenic acid, and temperature, *Biochim. Biophys. Acta* 1768 (2007) 2310-2318.
[45]Allen J.F. and J. Forsberg, Molecular recognition in thylakoid structure and function, *Trends Plant Sci*. 6 (2001) 317-326.
[46]Chuartzman S.G., R. Nevo, E. Shimoni, D. Charuvi, V. Kiss, I. Ohad, V. Brumfeld and Z. Reich, Thylakoid membrane remodeling during state transitions in *Arabidopsis*, *The Plant Cell* 20 (2008) 1029-1039.
[47]Tikkanen M., M. Nurmi, M. Suorsa, R. Danielsson, F. Mamedov, S. Styring and E.M. Aro, Phosphorylation-dependent regulation of excitation energy distribution between the two photosystems in higher plants, *Biochim. Biophys. Acta* 1777 (2008) 425-432.
[48]van Wijk K.J., B. Andersson and E.-M. Aro, Kinetic resolution of the incorporation of the D1 protein into photosystem II and localization of assembly intermediates in thylakoid membranes of spinach chloroplasts, *J. Biol. Chem.* 271 (1996) 9627-9636.
[49]Takeuchi T.S. and J.P. Thornber, Heat-induced alterations in thylakoid membrane-protein composition in barley, *Aust. J. Plant Physiol*. 21 (1994) 759-770.

[50]Sundby C., P. Maenpaa and B. Andersson, Temperature dependent changes in the antenna size of photosystem II, *Biochim. Biophys. Acta* 851 (1986) 475-483.
[51]Anderson J.M. and B. Andersson, The dynamic photosynthetic membrane and regulation of solar energy conservation. *Trends Biochem. Sci*. 593 (1988) 351-355.
[52]Timmerhaus M. and E. Weis, Regulation of photosynthesis: α- to β-convertion of photosystem II and thylakoid protein phosphorylation, In: *Current Research in Photosynthesis*, M. Baltscheffsky (Ed.), Kluwer Academic Publishers (1990) 771-774.
[53]Mohanty P. and J.S.S. Prakash, Elevated temperature treatment induced alteration in thylakoid membrane organization and energy distribution between the two photosystems in *Pisum Sativum*, *Z. Naturforsch*. 57c (2002) 836-842.
[54]Huner N.P.A., M. Krol, J.P. Williams, E. Maissan, P.S. Low, D. Roberts and J.E. Thompson, Low temperature development induces a specific decrease in trans-Δ3-hexadecenoic acid content which influences LHCII organization, *Plant Physiol*. 84 (1987) 12-18.
[55]Montanem H., B. Petzold and K. Kloppstech, Formation of early-light-inducible-protein complexes and status of xanthophyll levels under high light and cold stress in barley (*Hordeum Vulgare L.*), *Planta* 208 (1999) 519-527.
[56]Bergantino E., P. Dainese, Z. Cerovic, S. Sechi and R. Bassi, A post-translational modification of the photosystem II subunit CP29 protects maize from cold stress, *J. Biol. Chem.* 270 (1995) 8474-8481.
[57]Pfeiffer S. and K. Krupinska, New insights in thylakoid membrane organization, *Plant Cell Physiol*. 46 (2005) 1443-1451.
[58]Gounaris K., A.P.R. Brain, P.J. Quinn and W.P. Williams, Structural and functional changes associated with heat-induced phase-separations of non-bilayer lipids in chloroplast thylakoid membranes, *FEBS Lett.* 153 (1983) 47-52.
[59]Trunova T.I, N.V. Astakhova, A.N. Deryabin and E.P. Sabełnikova, Ultrastructural organization of chloroplasts of the leaves of potato plants transformed with the yeast invertase gene at normal and low temperature, *Dokl. Biol. Sci*. 389 (2003) 176-179.
[60]Vigh L., I. Horváth, P.R. van Hasselt and P.J.C. Kuiper, Effect of frost hardening on lipid and fatty-acid composition of chloroplast thylakoid membranes in two wheat-varieties of contrasting hardiness, *Plant Physiol*. 79 (1985) 756-759.
[61]Vigh L., I. Horváth, J. Woltjes, T. Farkas, P. R. van Hasselt and P. J. C. Kuiper, Combined electron-spin-resonance, X-ray-diffraction studies on phospholipid-vesicles obtained from cold-hardened wheats. 1. An attempt to correlate electron-spin-resonance spectral characteristics with frost-resistance. *Planta* 170 (1987) 14-19.
[62]Harwood J.L., Involvement of chloroplast lipids in the reaction of plants submitted to stress, In: *Advances in Photosynthesis*, Siegenthaler P-A., Murata N. (Eds.), Kluwer Academic Publishers (1998) 287-302.
[63]Woody R.W., Circular dichroism of peptides, In: *The Peptides*, Hraby,V.J. (Ed), Academic Press, New York (1985) 15-114.
[64]Tinoco I.J., Theoretical aspects of optical activity. 2. Polymers, *Adv. Chem. Phys*. 4 (1962) 113-160.
[65]De Voe H., Optical properties of molecular aggregates. II. Classical theory of the refraction, absorption, and optical activity of solutions and crystals, *J. Chem. Phys*. 43 (1965) 3199-3208.

[66]Garab G., Linear and circular Dichroism, In: *Biophysical Techniques in Photosynthesis*, Amesz, J, Hoff, A.J. (Eds.), Kluwer Academic Publishers (1996) 11-40.
[67]Keller D. and C. Bustamante, Theory of the interaction of light with large inhomogeneous molecular aggregates. I. Absorption, *J. Chem. Phys*. 84 (1986) 2961-2971.
[68]Keller D. and C. Bustamante, Theory of the interaction of light with large inhomogeneous molecular aggregates. II. Psi-type circular dichroism, *J. Chem. Phys*. 84 (1986) 2972-2980.
[69]Tinoco I., W. Mickols, M.F. Maestre and C. Bustamante, Absorption, scattering, and imaging of biomolecular structures with polarized-light, *Ann. Rev. Biophys. Biophys. Chem.* 16 (1987) 319-349.
[70]Garab G., A. Faludi-Daniel, J.C. Sutherland and G. Hind, Macroorganization of chlorophyll *a/b* light-harvesting complex in thylakoids and aggregates: Information from circular differential scattering, *Biochemistry* 27 (1988) 2425-2430.
[71]Finzi L., C. Bustamante, G. Garab and C.B. Juang, Direct observation of large chiral domains in chloroplast thylakoid membranes by differential polarization microscopy, *Proc. Natl. Acad. Sci. U. S. A.* 86 (1989) 8748-8752.
[72]McLaughlin A.C., P.R. Cullis, J.A. Berden and R.E. Richards, ^{31}P NMR of phospholipid membranes: Effects of chemical shift anisotropy at high magnetic field strengths , *J. Magn. Res*. 20 (1975) 146-165.
[73]Burnell E.E., P. R. Cullis and B. de Kruijff, Effects of tumbling and lateral diffusion on phosphatidylcholine model membrane ^{31}P-NMR lineshapes, *Biochim. Biophys. Acta* 603 (1980) 63-69.
[74]Cullis PR, B. de Kruiyff and R.E. Richards, Factors affecting the motion of the polar headgrouop in phospholipid bilayers. A ^{31}P NMR study of unsonicated phosphatidylcholine liposomes, *Biochim. Biophys. Acta* 426(1976) 433-446.
[75]McLaughlin A.C., P.R. Cullis, M.A. Hemminga, D.I. Hoult, G.K. Radda, G.A. Ritchie, P.J. Seeley and R.E. Richards, Application of ^{31}P-NMR to model and biological membrane systems , *FEBS Lett*. 57 (1975) 213-218.
[76]Schanck A., A method for determining the proportions of different phases in hydrated phospholipids by ^{31}P Nuclear Magnetic Resonance (NMR) spectroscopy , *Appl. Spectr*. 46 (1992) 1435-1437.

Chapter 2

Structural Rearrangements in Chloroplast Thylakoid Membranes, Revealed by Differential Scanning Calorimetry and Circular Dichroism Spectroscopy. Thermo-optic Effect

A. G. Dobrikova, Z. Várkonyi, S. B. Krumova, L. Kovács, G. K. Kostov, S. J. Todinova, M. C. Busheva, S. G. Taneva, G. Garab

Published in Biochemistry 42 (2003) 11272-11280

Structural rearrangements in chloroplast thylakoid membranes, revealed by differential scanning calorimetry and circular dichroism spectroscopy. Thermo-optic effect

Thermo-optic mechanism in thylakoid membranes was earlier identified by measuring the thermal and light stabilities of pigment arrays with different levels of structural complexity [Cseh, Z., et al. (2000) *Biochemistry 39,* 15250-15257]. The same mechanism was found to be responsible for the light-induced trimer-to-monomer transition in LHCII, the main chlorophyll *a/b* light-harvesting antenna of photosystem II (PSII) [Garab, G., et al. (2002) *Biochemistry 41,* 15121-15129]. In this paper, differential scanning calorimetry (DSC) and circular dichroism (CD) spectroscopy on thylakoid membranes of barley and pea are used to correlate the thermo-optically inducible structural changes with well-discernible calorimetric transitions. The thylakoid membranes exhibited six major DSC bands, with maxima between about 43 ºC and 87 ºC. The heat sorption curves were analyzed both by mathematical deconvolution of the overall endotherm and by a successive annealing procedure; these yielded similar thermodynamic parameters, transition temperature and calorimetric enthalpy. A systematic comparison of the DSC and CD data on samples with different levels of complexity revealed that the heat-induced disassembly of chirally organized macrodomains contributes profoundly to the first endothermic event, a weak and broad DSC band between 43 and 48 °C. Similarly to the main macrodomain-associated CD signals, this low enthalpy band could be diminished by prolonged photoinhibitory preillumination, the extent of which depended on the temperature of preillumination. By means of CD fingerprinting, it is shown that the second main endotherm, at around 60 ºC, originates to a large extent from the monomerization of LHCII trimers. The main DSC band, at around 70 ºC, which exhibits the highest enthalpy change, and another band at around 75-77 ºC relate to the dismantling of LHCII and other pigment-protein complexes, which under physiologically relevant conditions cannot be induced by light. The currently available data suggest the following sequence of events of thermo-optically inducible changes: (i) unstacking of membranes, followed by (ii) lateral disassembly of the chiral macrodomains, and (iii) monomerization of LHCII trimers.

1. Introduction

The photosynthetic system of higher plants possesses the ability to be regulated by short-term variations in the external environmental conditions such as temperature and illumination [1,2]. Most components of the multilevel regulatory mechanisms are known to involve structural changes in the thylakoid membranes. *Vice versa*, significant variations in the environmental conditions often induce structural changes, which in turn affect the photosynthetic functions.

The relative light-harvesting capability of the two photosystems can be regulated by redox-regulated reversible phosphorylation of LHCII [1]; during this process, part of LHCII is transferred from the stacked to the unstacked region of the thylakoid membranes [3-5]. In strong light, i.e. when plants are exposed to excess radiation that cannot be used in photosynthesis, different protective mechanisms are induced, which down-regulate the energy utilization and thus prevent the excess excitation energy from causing sustained damage to the plant. For instance, the short-term (1-2 min) component of the regulated energy dissipation in the antenna system, qE, the energy-dependent non-photochemical quenching, which depends on ΔpH, also involves conformational changes that modulate the aggregation state of LHCII [6]. The non-photochemical quenching of the excess excitation energy also depends on the presence of zeaxanthin and the PsbS protein of PSII [7,8].

There is ample evidence for structural changes induced directly by environmental factors. Significant structural rearrangements, involving the dissociation and movement of LHCII, can be induced by the heat treatment of thylakoid membranes [9]. It has been demonstrated that, upon illumination with moderate and high light intensities, thylakoid membranes, undergo reversible structural changes, which, as revealed by CD spectroscopy, affect the long-range chiral order of the chromophores [10]; prolonged illumination with intense light generally leads to similar, but irreversible changes [11]. Although they display some similarities to the conformational changes accompanying qE [12], these structural rearrangements are largely independent of the photochemical activity of the membranes and of the transmembrane ΔpH. Further, lamellar aggregates of isolated LHCII have been shown to be capable of undergoing very similar reversible structural reorganizations [13,14], and light induces a significant degree of monomerization of LHCII in vivo and in vitro, which is in contrast with the preferentially trimeric organization of the complexes in

the dark [15]. These rearrangements are accompanied by Chl fluorescence quenching [13,16]. Light-induced reorganizations, probably of a similar nature, have been found to be directly involved, at the substrate level, in the regulation of the phosphorylation of LHCII in vivo and in vitro [17-19].

Light-induced reorganizations in the isolated light-harvesting complexes and in the native membrane have been observed to be driven by a thermo-optic effect. In other terms, they are ascribed to fast, local thermal transients, T-jumps, due to the dissipation of the excess excitation energy in the antenna system, which in turn lead to elementary structural changes in the vicinity of the dissipating centers [15,20]. This conclusion has been reached mainly from analyses of the thermal and light stabilities of macroarrays of different levels of structural complexity in thylakoid membranes and isolated complexes. The thermo-optic mechanism depends on the 'built-in' thermal instability of molecular assemblies, which ensures the susceptibility of these structures to fast local thermal transients. It is usually assumed, and verified experimentally, that the thermal instability of macroassemblies does not affect the molecular structure of the constituents. Hence, the arrays can undergo thermo-optically driven reorganizations without irreversible damage to their constituents. Indeed, the presence of such thermally unstable assemblies, LHCII-containing macrodomains and trimeric structures of LHCII, have been identified mainly via CD spectroscopy and biochemical tools [15,20]. However, no calorimetric analysis has been performed and accordingly the thermodynamic parameters of the thermo-optically inducible changes remain unknown.

Earlier calorimetric analyses on thylakoids revealed the band structure of the thermograms and identified the origin of several bands with respect to the stroma and granum membranes and their membrane-associated enzymes and complexes [21-29]. However, they have not been analyzed with respect to their sensitivity toward preillumination and other treatments affecting the long-range organization of the complexes. Furthermore, CD spectroscopy, which is a particularly sensitive tool for the detection of structural reorganizations both at the macro-organization level and in the individual complexes and particles [30,31] can be applied to establish correlations between the most prominent structural changes in the pigment system and some DSC bands.

In the present work, by using DSC and CD spectroscopy, we have identified the broad but relatively weak DSC band at around 43 °C as being associated with the LHCII-containing chiral macrodomains. The calorimetric data presented are fully consistent with the notion that the structural changes in the chiral macrodomains are of thermo-optic origin. Among the additional thermal transients, a band at around 60 °C is found to be involved in the thermo-optically driven trimer-to-monomer transition of LHCII.

2. Materials and methods

Isolation of thylakoid membranes; preillumination. Thylakoid membranes were isolated from 10-day-old barley seedlings (Hordeum vulgare L.) or 2-week-old pea (Pisum sativum L.), grown in the greenhouse, by a slight modification of a procedure described earlier [32]. No significant difference was observed between the two plant materials. The membranes of 1-2 mg Chl ml^{-1} were suspended in a medium containing 250 mM sorbitol, 5 mM $MgCl_2$ and 10 mM Tricine (pH 7.6), and stored on ice in the dark until use within 4-6 hrs after the isolation. Aliquots from this stock were suspended in the same medium, or washed twice in Tricine buffer (10 mM, pH 7.6) in the presence of 50 μM EDTA, or in 10 mM Tricine buffer supplemented with 250 mM sorbitol.

The chlorophyll concentration was determined spectrophotometrically [33].

Preillumination of the thylakoid membranes (in 10 mM Tricine, pH 7.6, 250 mM sorbitol), on ice or thermostated at 25 °C, was performed with heat-filtered white light (30 or 60 min, 1600 μE m^{-2} s^{-1}) at 2 mg Chl ml^{-1}, in a thin layer, for DSC measurements, and at 20 μg Chl ml^{-1}, in a cell of 1 cm pathlength, for CD.

DSC measurements. Calorimetric measurements were performed with a DASM4 (Biopribor, Pushchino, Russia) high-sensitivity scanning microcalorimeter [34]. Runs were routinely made in the temperature range between 20 °C and 100 °C, at a heating rate of 1 °C min^{-1}. A second heating of the sample in the calorimetric cell, immediately after cooling to 20 °C following the first run, checked the reversibility of the thermally induced transitions. The transition temperature (T_m) was defined as the temperature at the maximum of the excess heat capacity curve. The calorimetric enthalpy (ΔHcal) of the transition was determined as the area under the excess heat capacity curve.

CD measurements, temperature dependence. CD was measured between 400 and 800 nm in a Jobin-Yvon CD6 dichrograph equipped with a side-illumination attachment and a thermostatable sample holder. The Chl content of the samples was adjusted to 20 μg ml^{-1}, the optical pathlength of the cell was 1 cm, and the distance of the sample from the photomultiplier was 5 cm. The spectra were recorded in 0.5 nm steps with an integration time of 0.2 s and a bandpass of 2 nm.

The temperature dependence of the CD spectra of EDTA-washed thylakoid membranes was measured either in the thermostated sample holder after a 5 min incubation at the given temperature, or at room temperature following the heat treatment of the sample in a block-heater unit. In the range of interest, between 40 °C and 80 °C, only minor differences were observed between the two types of treatments.

Each experiment was repeated on at least three independent batches, with identical tendencies and similar results. However, the thermal and light stabilities of the preparations from different batches of thylakoids differed somewhat from each other. In the paper, typical thermograms and CD spectra and temperature- and light-induced changes are presented.

3. Results and Discussion

Mathematical deconvolution and experimental dissection of thylakoid endotherms. Figure 1 presents the DSC profile of barley thylakoids, measured in medium of low ionic strength. The thermogram is characterized by 6 main endothermic transitions, centered at 43 °C, 62 °C, 69.6 °C, 76.5 °C, 80 °C and 87 °C, denoted as bands A, B, C, D, E and F, respectively [21]. The overall endotherm was deconvoluted mathematically, using theoretical Gaussian fitting (Figure 1). With this, band B was split into bands B1 and B2, with peak positions at 59 °C and 61.8 °C, respectively. In our deconvolution, the sharp and relatively weak band A1, which has been shown to be associated with the oxygen-evolving complex [22-24], remained unresolved in the broad and rather asymmetric and weak band A.

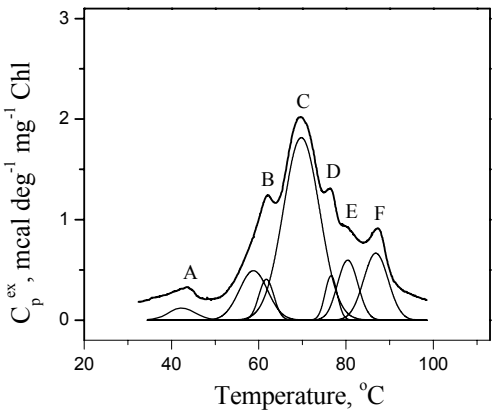


Figure 1. DSC thermogram of isolated thylakoid membranes (after baseline correction) suspended, at a concentration of 2 mg Chl ml^{-1}, in a reaction medium containing 10 mM Tricine (pH 7.6), 250 mM sorbitol; and mathematical deconvolution of the DSC curve by Gaussian fitting. The scanning rate was 1 ºC min^{-1}. The endothermic transitions are denoted as A, B, C, D, E and F following [21]. C_p^{ex}, excess heat capacity.

As shown in Figure 2, the heat-sorption curves can be dissected into their constitutive bands by applying a successive annealing procedure [35,36]. This experimental dissection procedure can be applied because the successive endothermic events are largely irreversible under our experimental conditions. This was confirmed by recording the second scans in barley and pea thylakoids (data not shown). Similarly to the endotherm, annealing yielded 6 DSC transitions. On the same thylakoid preparation, the peak positions were found at 43 °C, 60 °C, 69.2 °C, 77.6 °C, 81.5 °C and 85.3 °C (Table 1), in good agreement with the fitted data. It is to be noted, however, that, whereas the peak positions and bandwidths did not depend noticeably on the heating protocol, the amplitudes were somewhat sensitive to it. In particular, the amplitudes of the main bands, B and C, decreased upon application of the annealing procedure. It was also observed that the amplitudes of these bands were reduced when the thylakoids were preincubated for several minutes between 40 and 48 °C. This effect was largely independent of the peak position of band A, which could be shifted by varying the composition of the suspending medium, as shown in Table 1. This suggests that slower structural changes induced by heat, or other processes, e.g. lipid peroxidation, phase transitions in the membrane, activation of proteases etc., are involved in the destabilization of the structure(s) associated with the high-temperature bands. The identification of these processes, which modify the relative intensities only slightly, is beyond the scope of the present work. However, comparison of

these two protocols is important mainly because this permits us to apply variations in the experimental conditions. In particular, the fact that continuous heating, annealing, and preincubation at a given temperature followed by resumed heating, yielded very similar endotherms, and hence similar thermodynamic parameters (Table 1), is important when the experimental protocol of DSC, i.e. continuous, slow heating, is difficult to apply, e.g. in CD measurements.

Table 1. Thermodynamic parameters of the heat sorption curves of barley thylakoid membranes in the absence (control) and presence of 5 mM $MgCl_2$, and after 5 min preincubation of the sample at 48 °C, followed by DSC measurements between 20 ºC and 100 °C. The parameters were derived from the band parameters obtained either from mathematical deconvolution of the endotherm or from the experimentally dissected curves (using successive annealing). Thylakoid membranes were suspended, at a Chl concentration of 2 mg ml^{-1}, in 10 mM Tricine, 250 mM sorbitol (pH 7.6); the scanning rate was 1 ºC min^{-1}. Tm (ºC) is the temperature at maximum heat capacity and ΔHcal (mcal mg^{-1} Chl) is the calorimetric enthalpy of the transitions. Mean values and standard errors were obtained from three to five independent experiments, except in the last row, where two essentially identical DSC curves were recorded. The standard error for T_m is less than 0.5 ºC in all cases.

	Peak	A	B1	B2	C	D	E	F
Control deconvoluted	T_m	43	59	61.8	69.8	76.6	80.4	86.6
	ΔH_{cal}	2±0.4	3.6±0.3	2.7±0.7	19.0±0.5	1.8±0.2	3±0.5	4.9 ±0.7
Control annealing	T_m	43	-	60	69.2	77.6	81.5	85.3
	ΔH_{cal}	4.6±0.4		6.8±0.6	14.8±0.7	1.5±0.1	0.8±0.1	2.7 ±0.2
+ Mg^{2+} deconvoluted	T_m	46.6	-	62	69	76.3	80.5	85.3
	ΔH_{cal}	3.7±0.5		25±0.4	9.2±0.9	2.2±0.4	1.5±0.3	2.3±0.5
+ Mg^{2+} annealing	T_m	48	-	61	66.9	74.7	80.4	84.4
	ΔH_{cal}	3.9±0.3		17.7±0.1	13.2±0.4	4.3±0.5	1.3±0.3	0.4±0.1
Preincubated at 48 °C (+Mg^{2+}) deconvoluted	T_m	-	57	62.2	69	76	81	86
	ΔH_{cal}		4.5	10.4	6.2	4.6	0.4	3.1

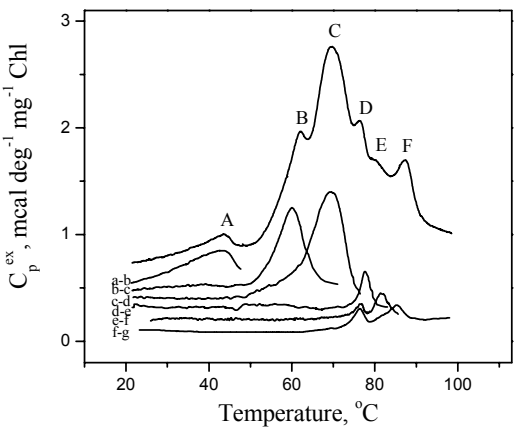


Figure 2. DSC profile of barley thylakoid membranes, from the same batch and under the same experimental conditions as in Figure 1; and dissection of the heat capacity curve by successive annealing procedure. Profiles of the difference curves of the successive annealings (a-b, b-c, c-d, d-e, e-f, f-g) are obtained by subtracting each scan from the previous one. C_P^{ex}, excess heat capacity.

Thermal and heat instability of LHCII-containing chiral macrodomains, first endothermic event (band A). It was earlier shown that the thylakoid membranes of higher plant chloroplasts exhibit thermal instability, which originates from the disassembly of chirally organized LHCII-containing macrodomains. The fact that this transition occurs at relatively low temperatures (below 55 °C) suggests the involvement of macrodomains in the first DSC band. It has also been demonstrated that the transition temperature, determined from the temperature dependence of the main, psi-type CD bands, depends on the suspending medium: the removal of sorbitol or Mg^{2+} (i.e. decreasing the osmolarity or the ionic strength, respectively) has been found to destabilize the macrodomains [20,32]. It is therefore of interest that the position of the first endothermic event (band A) is very sensitive to the presence of millimolar concentrations of $MgCl_2$, which shift the peak position to higher temperatures (Figure 3, Table 1). This observation is in good agreement with the notion that the first DSC band involves a significant contribution from the heat-induced disassembly of the chiral macrodomains. Table 1 further reveals that the enthalpy changes associated with this disassembly are considerably smaller than those associated with the higher-temperature bands. These latter bands evidently involve thermal transitions associated with further disassembly of the particles and the unfolding and denaturation of the main pigment-protein complexes (see also below).

While DSC and CD data demonstrate that the correlation between band A and the disassembly of the macrodomains is satisfactory (and further support is given below), it must be noted that the two measurements are unlikely to yield exactly the same transition temperatures, even if the two measurements could be run under identical conditions. Analysis of the thermal instability of the main psi-type bands in intact thylakoids revealed two components: the diminishment of the (-) 676 nm psi-type band, associated with the stacking, preceded that of the (+) 689 nm band [20]. As pointed out earlier, the disassembly of macrodomains begins with the unstacking of membranes and then proceeds with a lateral disorganization of the chiral macrodomains [37]. These two steps are not resolved in band A. Further, psi-type CD is lost below a certain size of the domain, when the diameter falls below one-quarter of the wavelength of the corresponding band [38]. On the other hand, the heat sorption can continue as long as the disassembly proceeds, albeit its magnitude may depend on the actual size of the domain. Additionally, DSC may contain other transitions, such as from the oxygen-evolving complex [22,24], which in part may be related to the macroorganization of LHCII [29,39]; further independent or partly independent transitions may also be present, e.g. rearrangements in the lipid bilayer [40-43]. Nonetheless, the thermodynamic parameters associated with the macrodomain organization of the complexes set the upper limits of the corresponding values, and serve for comparison with other, more intense DSC transitions.

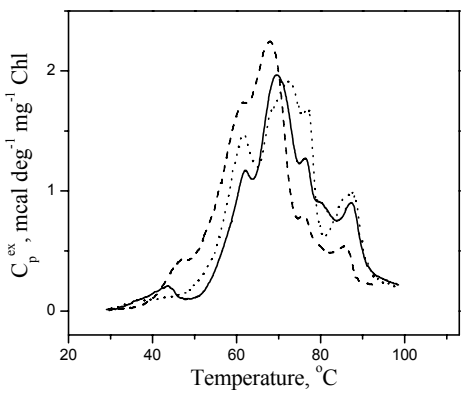


Figure 3. Effects of the ionic strength and osmolarity on the calorimetric transitions of barley thylakoid membranes at a concentration of 2 mg Chl ml^{-1}, suspended in 10 mM Tricine (pH 7.6), supplemented with 250 mM sorbitol (as in Figure 1, full line), 250 mM sorbitol and 5 mM $MgCl_2$ (dashed line), and 50 μM EDTA (dotted line). The scanning rate was 1 ºC min^{-1}; for other conditions, see Materials and Methods. C_P^{ex}, excess heat capacity.

In order to support the above notion concerning the origin of band A, and to reduce the contributions from independent factors, we performed experiments in the absence of chiral macrodomains. In hypotonic low-salt media in the presence of EDTA, thylakoid membranes do not contain sizeable macrodomains [11,32]. As can be seen in Figure 3 and Table 2, transition A is suppressed in ion-depleted thylakoids suspended in Tricine. Cations also affected transitions D and E and changed the relative intensities of the DSC peaks. This is in agreement with earlier data [27] and not analyzed further in the present work.

It is known that the chiral macrodomains can be disassembled by preilluminating the thylakoid membranes with intense, photoinhibitory light [11]. In accordance with the thermo-optic mechanism, this depends on the temperature of preillumination [20]. In good agreement with expectations, band A could be eliminated by preillumination at 25 °C (Figure 4), whereas the same light treatment at 0 °C led only to a shift toward lower temperatures (Figure 4; Table 2). It is interesting to observe in the DSC curves that, fully consistent with the thermo-optic mechanism, preillumination at 25 °C exerted an effect very similar to that of preincubation of the sample at 48 °C; it also diminished the amplitudes of bands B and C (data not shown) without affecting the peak positions.

Table 2. Thermodynamic parameters of the heat sorption curves of barley thylakoid membranes: transition temperatures (T_m) and calorimetric enthalpies (ΔHcal), mean values and standard errors calculated using the band parameters from mathematical deconvolution (from three to five independent experiments). The standard error for Tm is less than 0.5 ºC in all cases. The membranes, at 2 mg Chl ml^{-1}, were suspended in 10 mM Tricine (pH 7.6) and 250 mM sorbitol (control) or washed with EDTA-containing media (see Methods). The control thylakoid samples were preilluminated (PI) with white light of 1600 μE m^{-2} s^{-1} at 0 °C for 30 or 60 min and at 25 °C for 30 min, as indicated. The scanning rate was 1 °C min^{-1}.

	Peak	A	B1	B2	C	D	E	F
Control	T_m	43.0	59.0	61.8	69.8	76.6	80.4	86.6
	ΔH_{cal}	2.0±0.4	3. 6±0.3	2.8±0.7	18.9±0.5	1.8±0.2	3.0±0.5	5.0±0.7
EDTA- washed	T_m	-	58.8	61.3	69	73.2	76.8	85.5
	ΔH_{cal}		6.1±0.5	5.3±0.8	10 ±0.9	4.6±0.4	5.5±0.5	7.3±0.3
PI 30 min at 0 ºC	T_m	35.0	59.0	61.0	70.9	76.7	81.4	87.4
	ΔH_{cal}	3.1±0.5	4.5±0.6	2.1±0.2	22 ±0.6	1.8 ±0.5	2.7 ±0.5	4.0±0.4
PI 60 min at 0 ºC	T_m	26.0	58.8	61.3	70.9	76.4	80.4	86.8
	ΔH_{cal}	4.1±0.5	4.3±0.3	1.9±0.1	22.0±0.2	1.1 ±0.4	1.5±0.2	5.7±0.2
PI 30 min at 25 ºC	T_m	-	57.9	60.9	69.8	76.8	81.5	87
	ΔH_{cal}		6.0±0.6	1.7±0.1	14.2±0.2	2.5±0.1	3.5±0.2	3.6±0.4

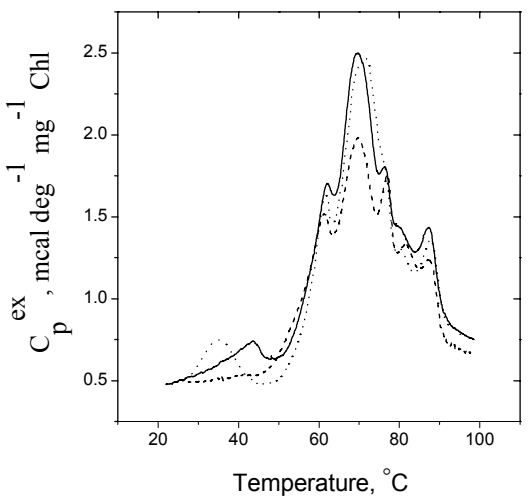


Figure 4. Effect of preillumination on the DSC profiles of barley thylakoid membranes suspended in 10 mM Tricine (pH 7.6), 250 mM sorbitol at a concentration of 2 mg Chl ml^{-1}. The thylakoids were illuminated at 0 ºC (dotted line) and 25 ºC (dashed line) for 30 min with white light of 1600 μE m^{-2} s^{-1} (dashed lines), or incubated in the dark (continuous line). C_P^{ex}, excess heat capacity.

As expected, destabilization of the macrodomains by preillumination led to a similar shift in CD as that seen in DSC (Figure 5). In the dark, as indicated by the main, psi-type CD bands, the macrodomains were quite stable up to 40 ºC, and even at 50 ºC the signal was strong. In contrast, upon preillumination at 0 ºC and 25 ºC, these bands were significantly reduced and almost fully eliminated, respectively (Figure 5). As shown in Figure 6, in the dark, the transition temperature (the temperature at which the intensity of the psi-type CD bands decreases to 50% of the original value) was to be found at around 45 °C. After a 30 min preillumination at 0 °C (Figure 6), the residual psi-type bands shifted their transition from 47 °C to 39 °C, whereas longer preilluminations (≥ 60 min) at the same temperature (not shown) or the same preillumination at 25 °C led to essentially complete loss of the psi-type bands (Figures 5C and 6). This finding is fully consistent with the thermo-optic origin of these changes. Moreover, it can be seen that, as observed earlier, photoinhibitory preillumination diminished mainly the array of complexes, i.e. the main, psi-type bands (cf. Figures 5A-C), without eliminating the excitonic band structure. In other terms, the preillumination did not destroy the structure of the individual complexes, as can be seen from the main excitonic bands, e.g. at around 440 nm and 650 nm. It must be noted, however, that some of the excitonic bands also exhibited a heat sensitivity, as evident from a closer inspection of the CD spectra. For instance, the excitonic band centered at around 476 nm displayed high susceptibility to heating (Figure 5). This will be further analyzed in the following section. At this point we conclude that the currently available DSC data and CD data indicate that the first DSC band includes a significant contribution from the disassembly of the chiral macrodomains. These data are consistent with the earlier conclusion that these macrodomains can be disassembled thermo-optically. Further we demonstrate that this disassembly consumes relatively low thermal energies as compared with the main endothermic events in the thylakoid membranes. This explains why the thermal energy available locally and transiently from the dissipation of the unused excitation energy of the absorbed photons does not lead to the destruction of the complexes, at least under physiologically relevant conditions.

Trimer-to-monomer transition in LHCII, second endothermic event (band B). As pointed out above, besides causing the loss of the chiral macrodomains, the preillumination of the thylakoids with strong light resulted in weakening of the excitonic couplet centered

at around 476 nm. The same band-pair, (+) 483 nm (-) 470 nm, dominated the trimer-minus-monomer difference spectra, after both phospholipase A and light treatments [15]. Further, monomerization of LHCII has also been ascribed to a thermo-optic effect. The (+) 483 nm (-) 470 nm excitonic bands most likely originate from Chl *b* and/or neoxanthin molecules [44]. The disruption of interactions between pigments on different monomeric subunits upon monomerization might be responsible for such an effect although the structural model of LHCII provides no likely candidates for such a change in interaction. Alternatively, monomerization might lead to a (small) structural change of monomeric subunits that causes a change in pigment-pigment interactions. Nevertheless, this band-pair can be used as a fingerprint of the trimer-to-monomer transition [15]. Hence, it is of interest to investigate whether or not the same 'spectral transition' can be induced by heat in thylakoid membranes in the dark. Complementary data, relating to the heat-induced conversion of trimers into monomers in isolated LHCII at around 55 °C [15] and the onset of monomerization, following heat treatment (45-47 °C, 1 hr) of leaves were published earlier [9]. Here, we inspected the thermal stability of the (+) 483 nm (-) 470 nm excitonic CD band pair in the thylakoid membranes. In all samples, we found a clear indication of a thermal transition between 55 °C and 65 °C. However, in intact membranes the large CD variations associated with the gradual loss of the macrodomains hindered the resolution of this band-pair (data not shown, see Figure 5A). This could be achieved more readily in samples that contained no macrodomains.

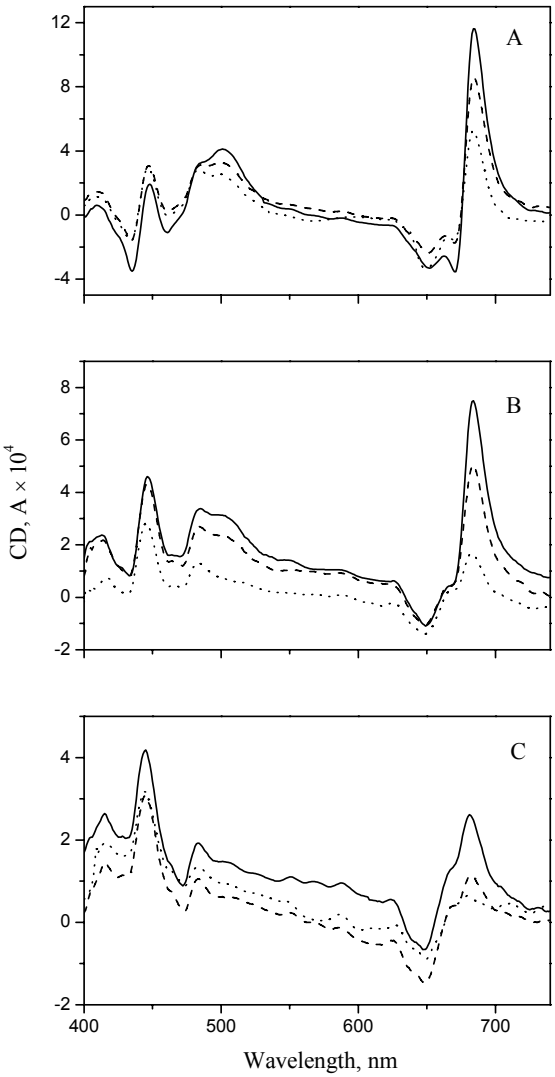


Figure 5. Thermal stability of barley thylakoid membranes suspended in 10 mM Tricine (pH 7.6), 250 mM sorbitol at a concentration of 20 μg Chl ml^{-1} in the dark (A) and following a preillumination (30 min, white light of 1600 μE m^{-2} s^{-1}) at 0 ºC (B) and 25 ºC (C). Spectra were recorded at 25 ºC (continuous line), 40 ºC (dashed line) and 50 ºC (dotted line). The light treatment and the CD measurements were carried out as described in Materials and Methods.

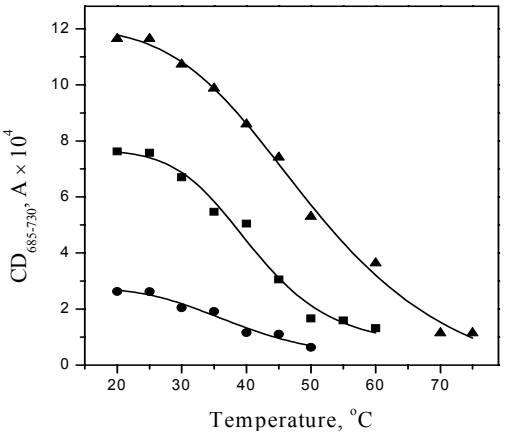


Figure 6. Temperature dependence of the intensity of the (+) 685 nm psi-type CD band, with reference to 730 nm, in barley thylakoids in the dark (▲) and preilluminated with white light of 1600 μE m^{-2} s^{-1} for 30 min at 0 ºC (■) and 25 ºC (●). Other conditions as in Figure 5.

The spectra in Figure 7 clearly show that the intensity of this band at 62 °C is largely diminished as compared with the control (at 45 °C) and the heat-treated sample (incubated for 5 min at 53 °C). At 45 °C, the residual macrodomain signals are eliminated in most samples, but minor contributions are often found, e.g. at 680 nm and 503 nm, even in EDTA-washed membranes (Figure 7).

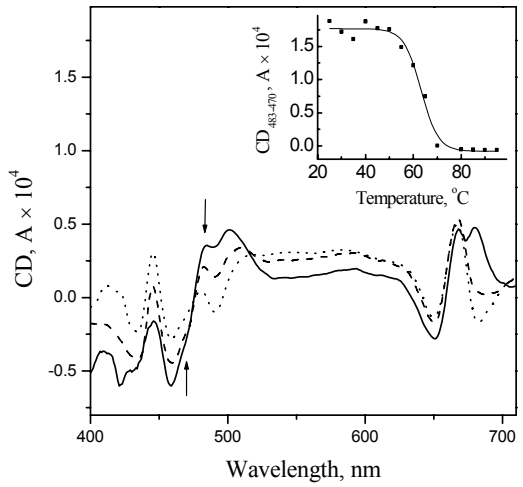


Figure 7. CD spectra of EDTA-washed barley thylakoid membranes incubated for 5 min at 45 ºC (solid line), 53 ºC (dashed line) and 62 ºC (dotted line). Inset, thermal stability of the (+) 483 nm/(-) 470 nm CD band pair, indicated by arrows. (For other conditions, see Materials and Methods.)

To further substantiate the findings that elevated temperatures and light can induce monomerization of the trimeric LHCII in thylakoid membranes, we analyzed the oligomerization state of LHCII in the membrane by using a combination of green gel electrophoresis and CD spectroscopy. As shown in Figure 8, short (5 min) treatment of isolated thylakoid membranes at 65 °C leads to a substantial monomerization of LHCII; these data are in good agreement with those obtained after prolonged (1 h) heat stress at 45 and 60 °C on barley leaves [9]. However, under our experimental conditions, i.e., following a much shorter (5 min) incubation on isolated thylakoids, no noticeable change occurred in the oligomerization (trimer/monomer) state of the complexes at 45 and 55 °C. This was also testified by CD spectroscopy, which revealed no substantial variation between the 45 °C (Figure 8B) and the 55 °C trimeric bands of the green gel, compared to the control also excised from the green gel, and only minor alterations at 65 °C (data not shown). On the other hand, the bands produced by heat treatment or preillumination with strong light exhibited virtually identical CD spectra which were essentially also identical with those obtained in isolated trimeric LHCII upon monomerization by phospholipase treatment or by preillumination [15]. Further, similarly to our earlier report on isolated LHCII [15], the difference spectra between the trimeric and monomeric spectra in Figure 8B were also dominated by the CD band pair (+) 483 nm, (-) 470 nm (data not shown).

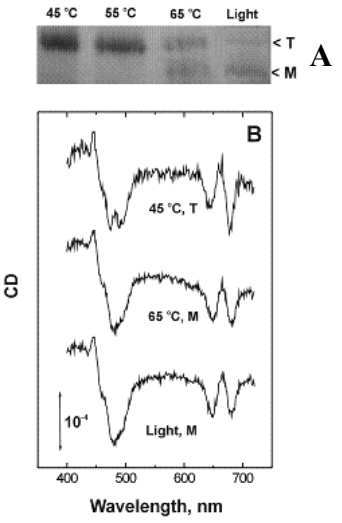


Figure 8. LHCII-containing bands from non-denaturing green gel electrophoresis (A) on EDTA-washed barley thylakoid membranes and CD spectra of excised bands (B). Thylakoid membranes were incubated for 5 min at 45, 55, and 65 °C, as indicated, or preilluminated at 25 °C for 1 h with white light of 1600 μE m^{-2} s^{-1}. The CD spectra, measured on the excised bands, were normalized for their (-) 650 nm band intensities, $CD_{650\text{-}625nm}$. T and M, respectively, denote bands containing predominantly trimeric and monomeric forms of LHCII.

It can be seen in both Figures 7 and 8B that the bands around (-) 650 nm and (+) 446 nm, which originate from excitonic interactions involving Chl *b* (of LHCII) and Chl *a* respectively [32], retain their intensities even at 62 °C. (Their thermal destabilization between 65 and 70 °C will be addressed in the following section.) These data reveal that, as expected for the trimer-to-monomer transition, the changes around 60 °C do not lead to the disorganization of the main pigment protein complex constituents of the membranes. Hence, it can be concluded that, as far as the main pigment protein complexes are concerned, the second main endothermic event, band B, involves the monomerization of LHCII trimers but not the denaturation of the main complexes. These data are consistent with the assignment of this transition as of thermo-optic nature.

Disassembly of LHCII, the main DSC band (band C). As revealed by the loss of its characteristic CD fingerprint at 625-650 nm, the disorganization of LHCII occurs in a narrow temperature range, between 65 °C and 70 °C (Figure 9). This is in reasonable agreement with earlier published data, though these are at variance with each other as concerns the denaturation temperature (around 66 °C and 74 °C reported in [24] and [27], respectively). This variation in the transition temperature is probably due to inherent thermal stability differences between different LHCII preparations [15,46]. Variations are somewhat smaller in the thylakoid membranes, where the transition temperature was always found between 68 °C and 72 °C (Figure 8, inset). Similarly, the variations in the position of band C in different samples were confined to a relatively narrow range, between 67 °C and 71 °C. Hence, these data indicate, that band C contains a contribution from the disassembly of the LHCII complexes, which can be inferred, to lead to unfolding and denaturation of the polypeptide [47-49]. Although contributions from other complexes cannot be ruled out, it seems very likely that LHCII furnishes the main contribution to band C: LHCII is the most abundant protein complex in the thylakoid membranes. Interestingly, however, even at 70 °C some CD bands are retained, albeit with reduced amplitudes, some of which, e.g. at 680 nm, are also present in LHCII. This suggests that the disassembly of LHCII is a multistep event, as revealed by recent light-sensitivity data suggesting the existence of internal pigment clusters in LHCII [49,50], and/or some other complexes which exhibit higher heat resistance than LHCII. These bands were eliminated between 72

°C and 80 °C, which suggests that this additional denaturing of the complexes contributes to band D.

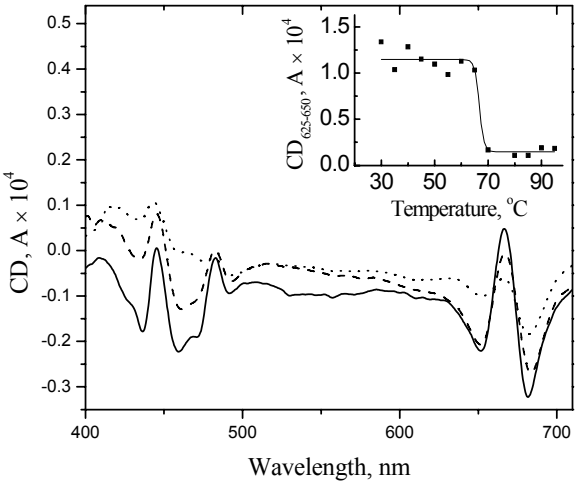


Figure 9. CD spectra of EDTA-washed barley thylakoids at 50 ºC (full line), 60 ºC (dashed line) and 70 ºC (dotted line). Inset, thermal stability of the (+) 650 nm band, with reference to 625 nm, $CD_{625\text{-}650}$.

At present, we have no indication as to whether these transitions, relating to bands C and D are inducible thermo-optically under physiologically relevant conditions (i.e. in the temperature range below 45 °C and with moderate preillumination). A challenging question, far beyond the scope of the present paper, however, is that of what molecular organization ensures the high heat stability of these complexes, and the safe 'handling' of the heat packages from the dissipative processes.

4. Conclusions

Systematic comparison of CD data and the endothermic transitions in the thylakoid membranes revealed that characteristic structural transitions of the pigment-protein complexes, at different levels of structural complexity, contribute significantly to different calorimetric events, i.e. DSC bands. In particular, the first endothermic event, at around 43 °C, could be assigned to the LHCII-containing chiral macrodomains, i.e. a well-organized macroarray of complexes and/or PSII particles in the granum. The second endothermic reaction, band B, involves a trimer-to-monomer transition in LHCII in the native thylakoid membrane. These structural transitions can also be induced thermo-optically, i.e. by fast local thermal transients in the close vicinity of the dissipating centers

[15,20]. On the other hand, the thermodynamic parameters associated with the molecular disorganization of the main light-harvesting pigment–protein complexes do not appear to favor the thermo-optic mechanism. Hence, the available data suggest the sequence of events in thermo-optically driven transitions. The first target appears to be the disassembly of the macrodomains. As shown earlier and pointed out above, this begins with the unstacking of the membranes [20,37]. This event is followed closely by a lateral disassembly of the macroarray; evidently, disassembly cannot occur without at least a partial unstacking. Following these steps, which might be essential in ensuring the mobility of certain protein complexes, including LHCII [3-5], the trimers of LHCII are transformed into monomers [15]. This lends further structural flexibility to the membrane, which might be needed for different enzymatic activities [17,51]. It is important to emphasize that, when these changes are driven thermo-optically, i.e. the alterations are confined in space and time to the dissipation of the unused excitation energy, structural flexibility can be ensured, locally and temporarily, without attacking the overall organization of the membrane. In other terms, the main significance of the thermo-optical structural reorganizations might be that they ensure the dual requirement of structural stability, required to preserve the integrity of the membranes and the entire organelle, and flexibility to ensure the adaptability of the system, and evidently required in various steps of the multilevel regulatory system.

Acknowledgment

We are indebted to Prof. H. van Amerongen for advice concerning the origin of the (+) 483 nm (-) 470 nm excitonic CD bands, and for critically reading the manuscript.

Rerefences

[1]Anderson J. M., and B. Andersson, The dynamic photosynthetic membrane and regulation of solar-energy convertion, *Trends Biochem. Sci.* 13 (1988) 351-355.
[2]Horton P., A.V. Ruban and R.G. Walters, Regulation of light harvesting in green plants, *Annu. Rev. Plant Physiol. Plant Mol. Biol*. 47 (1996) 655-684.
[3]Allen J. F. and J. Forsberg, Molecular recognition in thylakoid structure and function, *Trends Plant Sci*. 6 (2001) 317-326.
[4]Barber J., Influence of surface-charges on thylakoid structure and function, Annu. Rev. *Plant Physiol.* 33 (1982) 261-295.
[5]Arntzen C. J., Dynamic structural features of chloroplast lamellae, *Curr. Top. Bioenerg*. 8 (1978) 111-160.
[6]Ruban A. V., D. Rees, A.A. Pascal and P. Horton, Mechanism of delta-pH-dependent dissipation of absorbed excitation energy by photosynthetic membranes. 2. The relationship between LHCII aggregation in vitro and qE in isolated thylakoids, *Biochim. Biophys. Acta* 1102 (1992) 39-44.
[7]Li X. P., O. Bjorkman, C. Shih, A.R. Grossman, M. Rosenquist, S. Jansson and K.K. Niyogi, A pigment binding protein essential for regulation of photosynthetic light harvesting, *Nature* 403 (2000) 391-395.
[8]Aspinall-O'Dea M., M. Wentworth, A. Pascal, B. Robert, A. Ruban and P. Horton, In vitro reconstitution of the activated zeaxanthin state associated with energy dissipation in plants, *Proc. Natl. Acad. Sci. U.S.A*. 99 (2002) 16331-16335.
[9]Takeuchi, T. S. and J.P. Thornber, Heat-induced alterations in thylakoid membrane protein composition in barley, *Austr. J. Plant Physiol*. 21 (1994) 759-770.
[10]Garab G., A. Faludi-Dániel, J.C. Sutherland and G. Hind, Macroorganization of chlorophyll a/b light-harvesting complex in thylakoids and aggregates: Information from circular differential scattering, *Biochemistry* 27 (1988) 2425-2430.
[11]Gussakovsky E. E., V. Barzda, Y. Shahak, and G. Garab, Irreversible disassembly of chiral macrodomains in thylakoids due to photoinhibition, *Photosynth. Res*. 51 (1997) 119-126.
[12]Istokovics A., I. Simidjiev, F. Lajkó and G. Garab, Characterization of the light induced reversible changes in the chiral macroorganization of the chromophores in chloroplast thylakoid membranes. Temperature dependence and effect of inhibitors, *Photosynth. Res.* 54 (1997) 45-53.
[13]Barzda V., A. Istokovics, I. Simidjiev and G. Garab, Structural flexibility of chiral macroaggregates of light-harvesting chlorophyll a/b pigment-protein complexes. Light-induced reversible structural changes associated with energy dissipation, *Biochemistry* 35 (1996) 8981-8985.
[14]Simidjiev I., V. Barzda, L. Mustárdy and G. Garab, Role of thylakoid lipids in the structural flexibility of lamellar aggregates of the isolated light-harvesting chlorophyll a/b complex of photosystem II, *Biochemistry* 37 (1998) 4169-4173.
[15]Garab G., Z. Cseh, L. Kovács, S. Rajagopal, Zs. Várkonyi, M. Wentworth, L. Mustárdy, A. Der, A.V. Ruban, E. Papp, A. Holzenburg and P. Horton, Light-induced trimer to monomer transition in the main light-harvesting antenna complex of plants: Thermo-optic mechanism, *Biochemistry* 41 (2002) 15121-15129.

[16]Barzda V., R.C. Jennings, G. Zucchelli and G. Garab, Kinetic analysis of the light-induced fluorescence quenching in light-harvesting chlorophyll a/b pigment-protein complex of photosystem II, *Photochem. Photobiol*. 70 (1999) 751-759.
[17]Zer H., M. Vink, S. Shochat, R.G. Herrmann, B. Andersson, and I. Ohad, Light affects the accessibility of the thylakoid light harvesting complex II (LHCII) phosphorylation site to the membrane protein kinase(s), *Biochemistry* 42 (2003) 728-738.
[18]Zer H., M. Vink, N. Keren, H.G. Dilly-Hartwig, H. Paulsen, R.G. Herrmann, B. Andersson and I. Ohad, Regulation of thylakoid protein phosphorylation at the substrate level: Reversible light-induced conformational changes expose the phosphorylation site of the light-harvesting complex II, *Proc. Natl. Acad. Sci. U. S. A*. 96 (1999) 8277-8282.
[19]Aro E. M. and I. Ohad, Redox regulation of thylakoid protein phosphorylation, *Antioxidants & Redox Signaling* 5 (2003) 55-67.
[20]Cseh Z., S. Rajagopal, T. Tsonev, M. Busheva, E. Papp and G. Garab, Thermooptic effect in chloroplast thylakoid membranes. Thermal and light stability of pigment arrays with different levels of structural complexity, *Biochemistry* 39 (2000) 15250-15257.
[21]Cramer W. A., J. Whitmarsh, and P.S. Low, Differential scanning calorimetry of chloroplast membranes – identification of an endothermic transition associated with the water-splitting complex of photosystem II, *Biochemistry* 20 (1981) 157-162.
[22]Thompson L. K., J.M. Sturtevant and G.W. Brudvig, Differential scanning calorimetry studies of photosystem II – evidence for a structural role for cytochrome b559 in the oxygen-evolving complex, *Biochemistry* 25 (1986) 6161-6169.
[23]Thompson L.K., J.M. Sturtevant and G.W. Brudvig, In: *Progress in Photosynthesis Research*, Biggins, J. (Ed.), Martinus Nijhoff Publishers, Dordrecht (1987) pp 609
[24]Thompson L.K., R. Blaylock, J.M. Sturtevant and G.W. Brudvig, Characterization of the multiple forms of cytochrome b599 in photosystem II, *Biochemistry* 28 (1989) 6686-6695.
[25]Smith K.A., Ardelt, B. K. and Low, P. S., Identification of the soluble coupling factor transition in calorimetric scans of chloroplast membranes, *Biochemistry* 25 (1986) 7099-7105.
[26]Smith K.A. and P.S. Low, Identification and partial characterization of the denaturation transition of thephotosystem II reaction center of spinach chloroplast membranes, *Plant Physiol.* 90 (1989) 575-581.
[27]Smith K.A., B.K. Ardelt, N.P.A. Huner, M. Krol, E. Myscich and P.S. Low, Identification and partial characterization of the denaturation transition of the light harvesting complex II of spinach chloroplast membranes, *Plant Physiol*. 90 (1989) 492-499.
[28]Nolan W.G., H.P. Hopkins and S.A.M. Kalini, Differential scanning calorimetric investigation of pea chloroplast thylakoids and thylakoid fractions, *Arch. Biochem. Biophys.* 297 (1992) 19-27.
[29]Shutilova N., G. Semenova, V. Klimov and V. Shnyrov, Temperature-induced functional and structural transformations of the photosystem II oxygen-evolving complex in spinach subchloroplast preparations, *Biochem. Mol. Biol. Internatl*. 35 (1995) 1233-1243.
[30]Garab G., Linear and circular dichroism, In: *Biophysical Techniques in Photosynthesis*, Amesz, J. and Hoff, A. J. (Eds.), Kluwer Academic Publishers, Dordrecht (1996) pp 11-40

[31]van Amerongen H., L. Valkunas and R. van Grondelle, *Photosynthetic Excitons*, World Scientific Publishing Co., Singapore (2000)
[32]Garab G., J. Kieleczawa, J.C. Sutherland, C. Bustamante and G. Hind, Organization of pigment protein complexes into macrodomains in the thylakoid membranes of wild-type and chlorophyll-b-less mutant of barley as revealed by circular dichroism, *Photochem. Photobiol.* 54 (1991) 273-281.
[33]Lichtenthaler H.K., *Meth. Enzymol*. 148 (1987) 350-382.
[34]Privalov P.L., V.V. Plotnikov and V.V. Filmonov, Precision scanning microcalorimetry for study of liquids*, J. Chem. Thermodyn*. 7 (1975) 41-47.
[35]Lyubarev A.E. and B.I. Kurganov Rec. Res. Devel. Biophys. Chem. 2 (2001) 141-165.
[36]Shnyrov V.L. and P.L. Mateo, Thermal transitions in the purple membrane from Halobacterium halobium, *FEBS Lett.* 324 (1993) 237-240.
[37]Garab G. and L. Mustárdy, Role of LHCII-containing macrodomains in the structure, function and dynamics of grana, *Aust. J. Plant Physiol*. 26 (1999) 649-658.
[38]Keller D. and C. Bustamante, Theory of the interaction of light with large inhomogeneous molecular aggregates. 2. Psi-type circular dichroism, *J. Chem. Phys*. 84 (1986) 2972-2979.
[39]Havaux M. and F. Tardy, Thermo-stability and photo-stability of photosystem II in leaves of the Chlorina-f2 barley mutant deficient in light-harvesting chlorophyll a/b protein complexes, *Plant Physiol*. 113 (1997) 913-923.
[40]Berry, J. and O. Bjornman, Photosynthetic response and adaptation to temperature in higher plants, *Annu. Rev. Plant Physiol.* 31 (1980) 491-543.
[41]Gounaris K., A.R.R. Brain, P.J. Quinn and W.P. Williams, Structural reorganization of chloroplast thylakoid membranes in response to heat-stress, *Biochim. Biophys. Acta* 766 (1984) 198-208.
[42]Quinn P. and W.P. Williams, In: *Photosynthetic Mechanisms and the Environment*, Barber, J. and Baker, N. R. (Eds.), Elsevier, Amsterdam (1985) pp 1-47.
[43]Kóta Z., L.I. Horváth, M. Droppa, G. Horváth, T. Farkas and T. Páli, Protein assembly and heat stability in developing thylakoid membranes during greening, *Proc. Natl. Acad. Sci. U. S .A*. 99 (2002) 12149-12154.
[44]Gradinaru C.C., van R. Grondelle and H. van Amerongen, Selective interaction between xanthophylls and chlorophylls in LHCII probed by femtosecond transient absorption spectroscopy, *J. Phys. Chem*. B 107 (2003) 3938-3943.
[45]Taneva S.G., I. Simidjiev, G. Garab and A. Muga, In: *Photosynthesis: Mechanisms and Effects*, Garab, G. (Ed.), Kluwer Academic Publishers, Dordrecht (1998) pp 325-328
[46]Horn, R. and H. Paulsen, Folding in vitro of light-harvesting chlorophyll a/b protein is coupled with pigment binding, *J. Mol. Biol.* 318 (2002) 547-556.
[47]Paulsen H., B. Finkenzeller, and N. Kuhlein, Pigments induce folding of light-harvesting chlorophyll a/b-binding protein, *Eur. J. Biochem*. 215 (1993) 809-816.
[48]Meyer M., C. Wilhelm and G. Garab, Pigment-pigment interactions and secondary structure of reconstituted algal chlorophyll a/b-binding light-harvesting complexes of Chlorella fusca with different pigment compositions and pigment-protein stoichiometries, *Photosynth. Res*. 49 (1996) 71-81.
[49]Olszowka D., W. Maksymiec, Z. Krupa and S. Krawczyk, Spectral analysis of pigment photobleaching in photosynthetic antenna complex LHCIIb, *J. Photochem. Photobiol*. B 70 (2003) 21-30.

[50]Yang D.H., H. Paulsen and B. Andersson, The N-terminal domain of the light-harvesting chlorophyll a/b-binding protein complex (LHCII) is essential for its acclimative proteolysis, *FEBS Lett.* 466 (2000) 385-388.

Chapter 3

Phase Behavior of Phosphatidylglycerol in Spinach Thylakoid Membranes as Revealed by ^{31}P-NMR

S. B. Krumova, C. Dijkema, P. de Waard, H. V. As, G. Garab, H. van Amerongen

Published in Biochim. Biophys. Acta 1778 (2008) 997-1003

Phase Behavior of Phosphatidylglycerol in Spinach Thylakoid Membranes as Revealed by ^{31}P-NMR

Non-bilayer lipids account for about half of the total lipid content in chloroplast thylakoid membranes. This lends high propensity of the thylakoid lipid mixture to participate in different phases which might be functionally required. It is for instance known that the chloroplast enzyme violaxanthin de-epoxidase (VDE) requires a non-bilayer phase for proper functioning in vitro but direct evidence for the presence of non-bilayer lipid structures in thylakoid membranes under physiological conditions is still missing.

In this work, we used phosphatidylglycerol (PG) as an intrinsic bulk lipid label for ^{31}P-NMR studies to monitor lipid phases of thylakoid membranes. We show that in intact thylakoid membranes the characteristic lamellar signal is observed only below 20 °C. But at the same time an isotropic phase is present, which becomes even dominant between 14 and 28 °C despite the presence of fully functional large membrane sheets that are capable of generating and maintaining a transmembrane electric field. Tris-washed membranes show a similar behavior but the lamellar phase is present up to higher temperatures. Thus, our data show that the location of the phospholipids is not restricted to the bilayer phase and that the lamellar phase co-exists with a non-bilayer isotropic phase.

1. Introduction

In the thylakoid membranes, lipids constitute ~20% of the dry mass and the protein content of thylakoids is likewise high (~80%); the non-bilayer lipid monogalactosyl diacylglycerol (MGDG) is the major lipid constituent, accounting for about half of the total lipid content of the thylakoid membrane [1]. The rest of the thylakoid lipids are bilayer-prone lipids; in higher plant chloroplasts, digalactosyl diacylglycerol DGDG (30%), sulfoquinovosyl diacylglycerol SQDG (5-12%) and phosphatidylglycerol PG (5-12%). The high content of non-bilayer lipids lends high non-bilayer propensity to the whole lipid mixture, which thus can readily participate in different lipid phases [1].

The relative amounts of non-bilayer lipids change readily in response to variations in environmental conditions [2] but it is generally believed that in biological membranes

non-lamellar phases are not present in sizeable quantities and for substantial time periods [1,3]. However, it has been shown that total lipid extracts from thylakoid membranes and inner membrane chloroplast envelope form non-lamellar structures in dilute salt solutions [1,4]. It has also been established that non-bilayer phases can be formed in the thylakoid membrane at low pH [5], in the presence of high concentrations of metal cations [6], phospholipase A2 [7], at high temperature [8] and in the presence of co-solutes [9].

In addition to the well established functional roles in the activities of various membrane proteins [3,10,11] non-bilayer lipids have been hypothesized to play important roles in the bulk phase. It has been proposed that they exert high lateral packing pressure on intrinsic membrane proteins, thereby influencing their functional activity [12]. The ability of the lipid mixture to segregate into bilayer and non-bilayer phases has been proposed to regulate the protein content of the membranes, and it was suggested that the non-lamellar phase might be closely associated with the membrane and might exist in a dynamic equilibrium with the bilayer phase, thus contributing to the structural flexibility of the membranes [13]. It was shown that the major thylakoid protein, the main light-harvesting complex of photosystem II, LHCII, can force purified MGDG to form bilayer structures [14]. LHCII–lipid macroassemblies were also obtained from the association of freshly prepared lamellar aggregates of LHCII and different purified thylakoid lipids. They exhibited a remarkable structural flexibility, being capable of undergoing light-induced reversible structural rearrangements, which closely resembled the analogous transients in the native membranes [15]. These reorganizations were enhanced by the addition of lipids, with the largest enhancement obtained with MGDG [16].

It was shown that MGDG [17] and non-bilayer phases in general [18] are needed for the operation of VDE, an enzyme that is involved in the photoprotection of plants [19]. It was proposed that in the thylakoid membrane MGDG serves to solubilize the xanthophyll cycle pigments and furthermore provides inverted hexagonal phase (H_{II}) structures associated with the membrane bilayer, which are essential for efficient xanthophyll de-epoxidase activity [20]. Recently, Szilágyi et al. [21] proposed that the operation of VDE requires negative curvature elastic stress in the thylakoid lipid bilayer. These data show that non-bilayer lipids and non-lamellar phases play important roles in the operation of VDE in thylakoid membranes. Their role, however, is evidently not confined to this particular

enzymatic function of the thylakoid membrane: e.g. cyanobacteria lack VDE but the lipid composition of their thylakoid membranes is essentially the same as in higher plants. Also, all energy converting membranes contain high amounts of non-bilayer lipids, which has been suggested to play a role in safe-guarding their high protein content and enhancing their structural flexibility [13].

Despite the relatively low concentration of PG in thylakoid membranes (about 10% of the total lipid content) and association of a number of PG molecules with pigment-protein complexes this lipid species is present in the bulk; one can estimate that the PG which is not associated with proteins represents nearly 90% of the total PG content [22]. As estimated from electron paramagnetic resonance measurements, about 30% of the PG is part of the solvation shell of membrane proteins and hence motionally restricted and 70% of PG can be rapidly exchanged by laterally diffusing membrane lipids [23]. In ^{31}P-NMR experiments all PG molecules with the exception of the insufficiently mobile ones (e.g. protein-bound) do contribute to the signal. Furthermore, the inorganic phosphorus content of isolated thylakoid membranes is low; with the exception of a possible inclusion of some inorganic phosphate in the lumen, it can be washed out together with the stroma liquid. Thus, PG is part of the bulk lipid mixture and hence can be used as a reporter of variations in the bulk lipid phase behavior of thylakoid membranes. Moreover, although PG is a bilayer forming lipid, it can also participate in non-bilayer structures, which were detected by ^{31}P-NMR, upon neutralization of its negative surface charge [24] and also in total lipid extract of thylakoid membranes [25] and chloroplast envelope membranes [4].

^{31}P-NMR studies of thylakoid membranes have been performed before. However, the membranes were washed in 0.8 M Tris (pH 8.0) in order to remove the loosely bound Mn, which might broaden the ^{31}P-NMR signal [26], and were used either as a suspension or were freeze-dried and subsequently rehydrated with D_2O [26,27] in order to ensure better proton decoupling. These treatments, however, perturb the functional activity of membranes: they loose the oxygen evolving capability [28] and lead to changes in the macroorganization of the membrane proteins [29], also observed as a loss of the psi-type circular dichroism signal originating from LHCII-containing chiral macrodomains (Krumova, S.B. and Garab, G., unpublished data). Hence, these treatments might affect the hydration shell of the lipids, and thus the physical state of the lipid phase. To our

knowledge, intact thylakoid membranes (capable of oxygen evolution and retaining the macroorganization of the complexes) have not been studied with ^{31}P-NMR. In our experiments freshly isolated thylakoid membranes were suspended in isotonic medium with an ionic strength optimum for their functions, and the measurements were performed within a few hrs during which time the membranes retained their structural integrity and functional activity, including their electrical impermeability. Our results revealed isotropic motion of the phospholipids in the temperature range 7 – 40 °C, in both intact and Tris-washed thylakoids, thus suggesting the presence of non-bilayer structures in these membrane preparations.

2. Materials and methods

2.1. Isolation and treatment of thylakoid membranes

Dark adapted spinach leaves were homogenized in a medium containing 50 mM Tricine (pH 7.5), 400 mM sorbitol, 5 mM $MgCl_2$ and 5 mM KCl; the suspension was filtered through 4 layers of cheese cloth and centrifuged for 4 min at 4000 x g. The chloroplasts were osmotically shocked in a hypotonic medium containing 50 mM Tricine (pH 7.5), 5 mM $MgCl_2$ and 5 mM KCl, and centrifuged for 5 min at 6000xg. After washing in the same medium supplemented with 400 mM sorbitol, the pellet was resuspended in this isotonic medium.

Electrochromic absorbance changes, ΔA515, induced by saturating single turnover flashes, were measured at 515 nm, the maximum of the transients in thylakoid membranes, in a set-up described earlier [30]. The samples were thermostated; the time constant was set to 100 μs; 32 kinetic traces were collected with a repetition rate of 1 s^{-1} and averaged.

Oxygen evolving activity of thylakoid membranes upon illumination was measured polarographycally using a Clark-type oxygen electrode (Hansatech Instruments, King's Lynn, UK) in the presence of 5 mM potassium ferricyanide. The samples were illuminated with saturating white light provided by a KL1500 lamp source (Schott, Germany). The temperature was controlled by a circulating water bath during the measurements.

Thermoluminescence measurements were carried out in a home-built apparatus described by Demeter et al. [31]. The samples were excited at -40 ^{o}C by two saturating single-turnover flashes and the glow curves were recorded at a heating rate of 20 °C/min.

For ΔA515, oxygen evolution and thermoluminescence experiments, the thylakoid membranes were pre-incubated at 7, 20 or 30 °C at a concentration of 5 mg chl/ml. They were subsequently diluted to 60 and 200 μg chl/ml for ΔA515 and oxygen evolution, and thermoluminescence measurements, respectively.

For Tris-washing, the thylakoid membranes were isolated as described above and treated according to [26] with some modifications – the Tris-treatment was only 1 h and after washing with the same buffer, the sediment was resuspended in non-deuterated buffer 0.8 M Tris (pH 8.0).

In all our experiments, the leaves used for isolation of thylakoid membranes were dark adapted for prolonged periods (1-8 h), which results in dephosphorylation of the membrane proteins [32], hence significant contribution of protein-bound phosphate can be neglected under our experimental conditions.

2.2. ^{31}P-NMR Measurements

^{31}P-NMR spectra were recorded with an AMX300 wide-bore spectrometer (Bruker, Germany) tuned at the resonance frequency of the ^{31}P nucleus (121.500 MHz, 7 T). 20 mm outer diameter tubes were used containing 15 ml of concentrated thylakoid suspension (~5 mg chl/ml). The temperature was controlled within 0.1 °C; spectra were recorded using a 40° pulse, an interpulse time of 0.5 s and no ^{1}H-decoupling was applied. Chemical shifts were scaled by referencing against a capillary containing methylene diphosphonic acid pH 8.9 (0.2 M) providing a resonance at 16.92 ppm.

For saturation transfer experiments, 0.3 s saturation irradiation (power 40 dB and frequency -1800 Hz) was applied before switching on the measuring pulse (acquisition time 0.2 s).

Since we wanted to record the ^{31}P-NMR spectra within the shortest possible time, all spectra were collected for only 1 h. In this way the signal to noise ratio remained low, but the characteristic features for the different phases were still clearly discernible. All experiments were repeated at least 3 times and the observed tendencies were always the same. Stirring of the sample had no noticeable effect on the spectra, indicating that no significant magnetic orientation of membranes occurred. For the determination of the chemical shift position of isotropic di-myristoyl PG and inorganic phosphate, PG micelles formed with excess amounts of SDS in 20 mM tricine buffer were used; inorganic

phosphorus was added to the suspension at a concentration of 1 mM. It was established that isotropic di-myristoyl PG resonates at +1 ppm and the inorganic phosphate at 2.6 ppm at pH 7.5.

3. Results and discussion

3.1. Line shape analysis of ^{31}P-NMR spectra of isolated intact thylakoid membranes at 7 °C

The ^{31}P-NMR spectrum of isolated thylakoid membranes (Fig. 1) detected at 7 °C shows that the signal is originating from several different chemical environments of the phosphorous nucleus. The inorganic phosphate content of washed thylakoid membranes, isolated from dark-adapted leaves, is largely reduced because their phosphoproteins are dephosophorylated and the stroma liquid is washed out (cf. Materials and methods). Thus, the observed resonances originate from PG and possibly from inorganic phosphate in the lumen.

As expected, the contribution of the lamellar phase could clearly be seen – with the high intensity peak at high field at about -10 ppm and the low intensity shoulder at low field around 30 ppm. This lamellar signal is broader than found in model systems [33], which is probably due to a larger heterogeneity in the dimensions of the lamellae. Besides this signal, we also observed a pronounced resonance at 4 ppm and weaker resonances at 2.6 ppm and 0 ppm (Fig. 1). It was established that the resonance at 2.6 ppm, which appears only as a shoulder on the 4 ppm signal, originates from inorganic phosphate (probably in the lumen), since it increased after the addition of potassium phosphate (data not shown). This resonance also dominates the ^{31}P-NMR spectrum of unbroken chloroplasts (data not shown), due to the high amount of phosphate in the stroma.

In order to determine the isotropic resonance position of the PG molecules constituting the thylakoid, the membranes were solubilized with SDS. After this treatment the spectra are dominated by the isotropic peak (Fig. 2), centered at about 0 ppm, coinciding with the low intensity resonance in unsolubilized membranes around 0 ppm (Fig. 1) and similar to the resonance frequency of di-myristoyl PG in SDS micelles (1 ppm, see Materials and methods). At small line-broadening the 4 ppm resonance can be seen as a shoulder (data not shown).

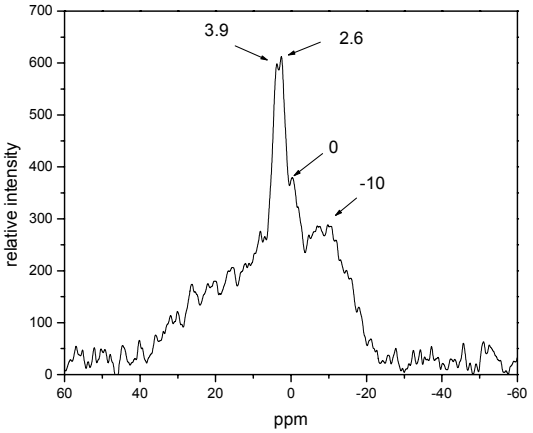


Figure 1. ^{31}P-NMR spectrum of thylakoid membranes at 7 °C. 6700 free induction decays were accumulated, the line broadening was 100 Hz. The positions (in ppm) of the different resonances are denoted by arrows.

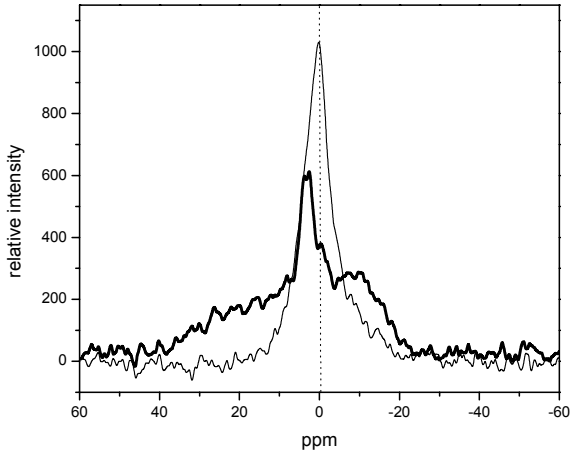


Figure 2. ^{31}P-NMR spectra of thylakoid membranes before (thick line) and after (thin line) solubilization with 3 % SDS. For each spectrum 6700 free induction decays were accumulated, the line broadening was 100 Hz. The spectrum of non-solubilized membranes was recorded at 7 °C and the one of solubilized membranes at 14 °C. The dashed line denotes the isotropic position of PG determined after solubilization of the thylakoid membranes by SDS.

Since the resonance at around 4 ppm appears close to the position where the high intensity edge of the H_{II} phase is expected, we checked whether this resonance possesses the asymmetrical shape characteristic for the H_{II} phase [33]. The spectral shape of this 4 ppm resonance can be obscured by its overlap with the resonance of the lamellar phase. To probe its spectral shape, we performed saturation transfer experiments (Fig. 3), where the irradiation frequency was set at -10 ppm, i.e., at the high intensity peak of the lamellar phase. This resulted in sufficient elimination of the lamellar resonance and revealed that the remaining 4 ppm peak is isotropic in nature. Moreover, its intensity was not decreased, suggesting that under these experimental conditions, at 7 °C, there is no considerable magnetization transfer between the lipids experiencing isotropic motion and the ones motionally restricted in the bilayer. This indicates that at 7 °C there is little or no exchange (on the ^{31}P-NMR timescale) between the phospholipids giving rise to these two resonances. In order to avoid irradiation of the high field shoulder of the potentially present H_{II} phase, we also performed saturation transfer experiments applying strong pulses at frequencies between 2500 and 800 Hz (10-30 ppm), where no contribution of the H_{II} signal can be expected. The lamellar signal was eliminated upon irradiation at 800 Hz, applying 30 dB pulse power. The intensity of the 4 ppm peak was also strongly reduced but its shape was rather symmetric (data not shown), thus giving no indication that it originated from H_{II} phase. An isotropic peak at this position was also observed in total lipid extracts of wheat leaves [25] and was attributed to cubic phase. However, we have shown that the isotropic position of thylakoid PG is at about 0 ppm, and hence the cubic phase should emerge at this resonance position. Thus the nature of the peak at 4 ppm remains unclear. Due to the low intensity of the 0 ppm peak in this preparation it is difficult to judge whether the saturation of the lamellar signal affects the isotropic peak at 0 ppm. However, this is clearly demonstrated in Tris-washed membranes (see section 3.3 and Fig. 5).

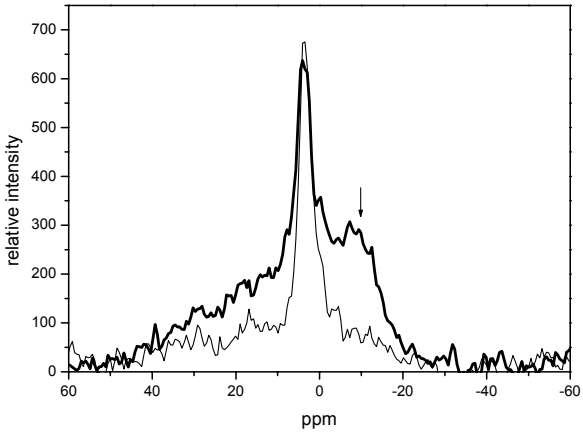


Figure 3. ^{31}P-NMR spectra of thylakoid membranes without (thick line) and with (thin line) saturation at 7 °C. For the saturation transfer, irradiation with 0.3 s pulses of power 40 dB at -1800 Hz (-10 ppm, designated by arrow) was applied. For each spectrum 6700 free induction decays were accumulated, the line broadening was 100 Hz.

3.2. Thermally induced changes in the lipid phase behavior of intact thylakoid membranes

The heat-induced changes in the ^{31}P-NMR spectra are presented in Fig. 4A. With the increase of the temperature from 7 °C to 21 °C the lamellar signal gradually disappears. The intensities of the 4 and 0 ppm peaks increase, both resonances start to broaden and the weak resonance at 2.6 ppm is no longer detectable. At 35 °C only one isotropic peak (0 ppm) remains, whereas the intensity of the 4 ppm resonance is obscured. At higher temperatures also the 0 ppm peak is decreasing in intensity, probably due to an increased T1 relaxation time (longer than the interpulse time of 0.5 s used in our experiments). Upon cooling of the samples (Fig. 4B) the contribution of the lamellar phase could not be recovered, however, two high intensity peaks at 0 ppm and 2.9 ppm were detected.

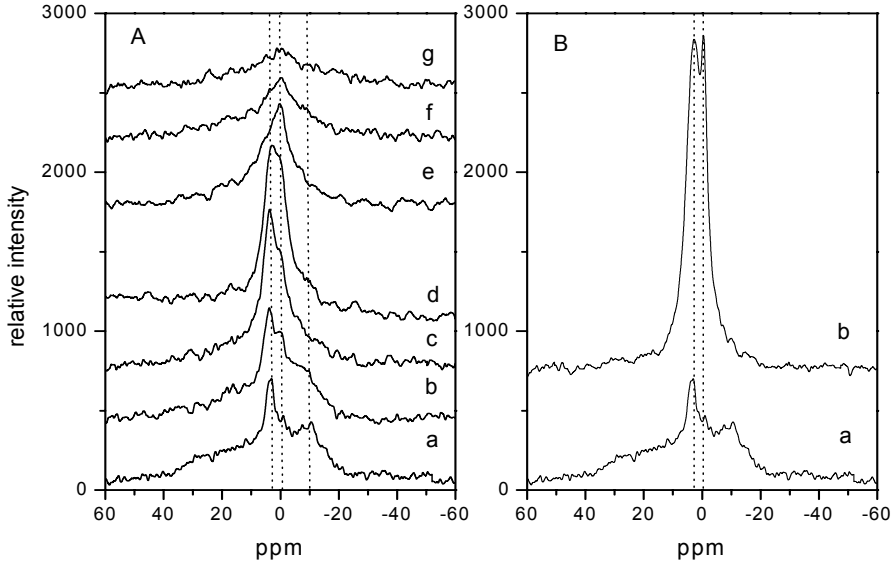


Figure 4. Temperature dependence (A) of the ^{31}P-NMR spectra of thylakoid membranes: (a) 7 °C, (b) 14 °C, (c) 21 °C, (d) 28 °C, (e) 35 °C, (f) 42 °C, (g) 49 °C and comparison (B) of the spectra at 7 °C obtained before (a) and after (b) heating of the sample to 49 °C. For each spectrum 6700 free induction decays were accumulated, the line broadening was 100 Hz. The dashed lines indicate the positions of the different resonances.

Since isotropic signals originate from regions which allow the motion of phopsholipid molecules in all directions, one might think that the marginal regions of the thylakoids could give rise to such resonances, since the margins are strongly curved, protein free areas ([34] and references therein). However, the marginal regions are much smaller in size as compared to the granal and stromal surfaces (at most 5% of the total volume of thylakoid mebranes [34]) and moreover their contribution is largely reduced in unstacked (Tris-washed and heat-treated) thylakoids. Thus, the isotropic peaks can at most only partly be due to the margin regions.

Alternatively, the isotropic signals might be generated upon destruction of the membrane and formation of small micelles. In order to test the integrity of the membranes, we recorded electrochromic absorbance changes at 515 nm (ΔA515) induced by single turnover flashes. The amplitude of this absorbance transient measures the capability of

membranes to generate a transmembrane electric potential gradient and its decay kinetics probes the permeability of the membranes [35]. Our results showed that 1 h incubations of the membranes at 7, 20 and 30 °C did not affect the magnitude of transmembrane electrical field; the membrane permeability was also retained at 7 and 20 °C, with a temperature dependence similar to that of freshly isolated intact chloroplasts [36]. Substantial deteriorations of these membrane functions and the overall ultrastructure, revealed by electron microscopy, were observed only in samples incubated at and above 30 °C (data not shown). We have also determined that the oxygen evolving capability, and the charge separation and stabilization in photosystem II (as measured by thermoluminescence) were preserved up to 30 °C; moreover, there was no substantial lipid peroxidation as indicated by the very low intensity (data not shown) of the high-temperature thermoluminescence band at around 75 °C [37] (data not shown). All these complementary experiments show that the integrity and functionality of the thylakoid membranes are retained between 7 and 28 °C, hence under conditions where isotropic peaks in the ^{31}P-NMR spectrum were observed. Thus, these resonances can not be attributed to degradation and vesicularization of the membrane.

This apparent contradiction (loss of the lamellar signal but presence of intact bilayer) can be explained only if the motion of PG molecules is not restricted to the bilayer, i.e., they are either released from the existing bilayer and form small vesicles or micelles or they are allowed to exchange with other non-bilayer structures, which results in averaging of the ^{31}P-NMR signal. The first possibility is highly unlikely taking into account that under our experimental conditions the large sheets of granal thylakoids and stromal lamellae are preserved and they do contain PG - it was shown [38] that the relative distribution of the four lipid classes, as well as the relative amount of the different PG molecular species was identical in intact thylakoids and isolated granal, stromal and marginal membrane fragments. It seems more likely that with the increase of temperature fast exchange of lipids between the bilayer and non-bilayer structures occurs which leads to averaging of the ^{31}P-NMR signal. These non-bilayer structures which, in isolated thylakoids are probably located in the lumen, must be rather small and without long-range order since their presence cannot be detected with small angle X-ray scattering (Holm, J.K., Kovács L., Garab G. and Posselt D., unpublished data).

3.3. Thermally induced changes in the lipid phase behavior of Tris-washed thylakoid membranes

Tris is known to remove the proteins of the oxygen-evolving complex from the thylakoid membrane [28]. Moreover, it was shown that Tris treatment results in changes in the macroorganization of the membrane proteins [29]; it eliminates for instance the psi-type circular dichroism signal originating from the LHCII-containing chiral macrodomains (Krumova, S.B. and Garab, G., unpublished data, cf. [39]). Previously ^{31}P-NMR spectra of Tris-washed wheat thylakoid membranes, accumulated at different temperatures, were reported [26]. Here we show the ^{31}P-NMR spectra of Tris-washed spinach thylakoids and compare them with intact (non-Tris-washed) membranes of the same plant species. The ^{31}P-NMR spectrum of Tris-washed spinach thylakoid membranes at 7 °C (Fig. 5) is very similar to the one obtained for non-washed membranes (cf. Fig. 1). A clear lamellar signal (with a high intensity shoulder at -10 ppm) is present and two isotropic peaks at about 0 and 4 ppm are superimposed on it. Saturation-transfer experiments (applying 35 dB at -1800 Hz) resulted in elimination of the lamellar signal and showed that similarly to non-washed membranes, the isotropic peaks at 4 ppm and 0 ppm are magnetically decoupled from the bilayer lipids giving rise to the lamellar ^{31}P-NMR signal (Fig. 5).

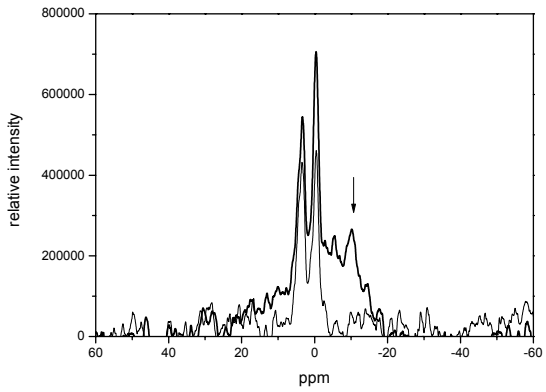


Figure 5. ^{31}P-NMR spectra of Tris-washed thylakoid membranes without (thick line) and with (thin line) saturation at 7 °C. For the saturation transfer, irradiation with 0.3 s pulses of power 35 dB at -1800 Hz (-10 ppm, designated by arrow) was applied. For each spectrum 6700 free induction decays were accumulated, the line broadening was 100 Hz.

The heat induced changes in Tris-washed thylakoids (Fig. 6A) followed a similar trend as the ones observed for similar preparations (see Materials and methods) from wheat [26]. The differences between the data of Harańczyk et al [26] and our experiments are

possibly due to the different plant species used and the slightly modified preparation protocol (see Materials and methods). Our data show that the trend of the events observed for Tris-washed membranes is similar to the one for non-washed samples (Fig. 4A), however, the lamellar signal was preserved until 42 °C. Similarly to non-washed membranes only a broad isotropic signal at 0 ppm could be detected at 49 °C. However, its intensity was much higher as compared to the one in non-washed samples. Cooling of the Tris-washed membranes from 49 °C back to 7 °C resulted in a recovery of the lamellar phase observed in the spectrum of non-heated samples. However, the intensity of the isotropic peaks was higher in comparison with non-heated membranes (Fig. 6B). These data clearly show that the lamellar-to-isotropic phase transition could be reversed under certain conditions, but only in Tris-washed thylakoids. The exact reason for the differences in the phase behavior of PG in non-washed and Tris-washed thylakoids is not clear; possibly it is due to the specificity of the lipid-protein interactions in these two preparations.

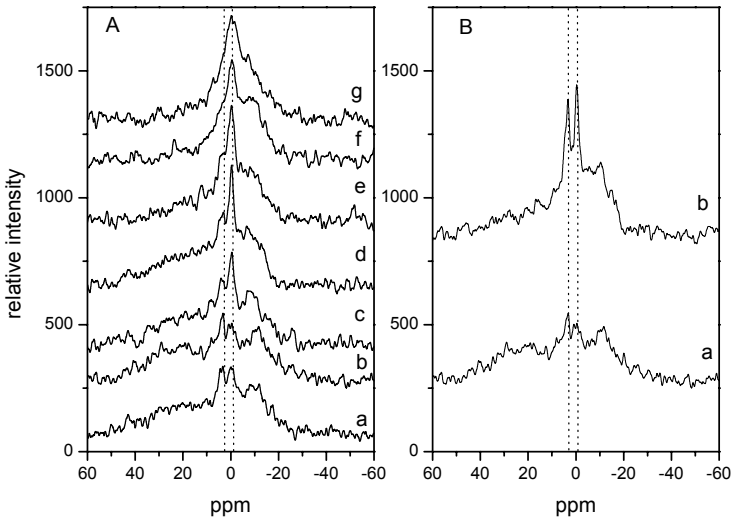


Figure 6. Temperature dependence (A) of the ^{31}P-NMR spectra of Tris-washed thylakoid membranes: (a) 7 °C, (b) 14 °C, (c) 21 °C, (d) 28 °C, (e) 35 °C, (f) 42 °C, (g) 49 °C and comparison (B) of the spectra at 7 °C obtained before (a) and after (b) heating of the sample to 49 °C. For each spectrum 6700 free induction decays were accumulated, the line broadening was 150 Hz.

4. Conclusions

In this work we have used the non-invasive ^{31}P-NMR technique, in which the phosphate nucleus of the membrane phospholipids serves as an internal label and hence reporter of the lipid phase behavior. Both the intact and Tris-washed thylakoid membranes show complex lipid phase behavior – the ^{31}P-NMR lineshapes reveal the co-existence of lamellar and isotropic signals. Similar isotropic resonances were detected also in other native [40] and model membranes [41,42], however their nature is still obscure. Our data show that isotropic motion (isotropic peak at 0 ppm) of the PG molecules occurred at all temperatures below 40 °C, where only the lamellar phase was expected. We attribute this isotropic signal to a separate pool of PG molecules involved in non-bilayer structures. These non-bilayer structures remain in contact with the membrane and the exchange of lipids between them and the lamellae is temperature-dependent; it is increased at temperatures higher than 14 °C which results in disappearance of the lamellar spectrum and averaging of the ^{31}P-NMR signal while the integrity of the membrane is preserved.

Acknowledgments

The authors are indebted to Profs. B. de Kruijff and L. Vigh for helpful discussions. The authors benefited from critical comments by Dr. Z. Gombos. We wish to thank to Dr. L. Mustárdy for performing electron microscopy on our samples. This work has been supported in part by the WNMRC, an EU FP5 grant (HPRI-CT-2001-00178 (2003-2004)) to H.V.A. and grants by the Hungarian Fund for Basic Research (OTKA K 63252 and T42696) and by EU FP6 MC RTN (INTRO2) to G.G.; S.K. received Sandwich-Programme fellowship from Wageningen University, The Netherlands.

References

[1] Williams W.P., The physical properties of thylakoid membrane lipids and their relation to photosynthesis, in: P.-A. Siegenthaler, N. Murata (Eds.), *Advances in Photosynthesis. Lipids in Photosynthesis*, Kluwer Academic Publishers, Dordrecht, 1998, pp. 103-118.
[2] Harwood J.L., Involvement of chloroplast lipids in the reaction of plants submitted to stress, in: P.-A. Siegenthaler, N. Murata (Eds.), *Advances in Photosynthesis. Lipids in Photosynthesis*, Kluwer Academic Publishers, Dordrecht, 1998, pp. 287-302.
[3]Epand R.M., Lipid polymorphism and protein-lipid interactions, *Biochim. Biophys. Acta* 1376 (1998) 353-368.
[4]Steponkus P.L., M. Uemura, R.A. Joseph, S.J. Gilmour, M.F. Thomashow, Mode of action of the COR15a gene on the freezing tolerance of Arabidopsis thaliana, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 14570-14575.

[5]Semenova G.A., The thylakoid membrane in wide pH range, *J. Plant Physiol*. 159 (2002) 613-625.
[6]Gounaris K., A. Sen, A.P.R. Brain, P.J. Quinn, W.P. Williams, The formation of non-bilayer structures in total polar lipid extracts of chloroplast membranes, *Biochim. Biophys. Acta* 728 (1983) 129-139.
[7]Thomas P.G., A.P.R. Brain, P.J. Quinn, W.P. Williams, Low pH and phospholipase A2 treatment induce the phase-separation of non-bilayer lipids within pea chloroplast membranes, *FEBS Lett.* 183 (1985) 151-166.
[8]Gounaris K., A.P.R. Brain, P.J. Quinn, W.P. Williams, Structural reorganization of chloroplast thylakoid membranes in response to heat-stress, *Biochim. Biophys. Acta* 766 (1984) 198-208.
[9]Williams W.P., A.P.R. Brain, P.J. Dominy, Induction of non-bilayer lipid phase separations in chloroplast thylakoid membranes by compatible co-solutes and its relation to the thermal stability of Photosystem II, *Biochim. Biophys. Acta* 1099 (1992) 137-144.
[10]Lee A.G., Lipid-protein interactions in biological membranes: a structural perspective, *Biochim. Biophys. Acta* 1612 (2003) 1-40.
[11]Lee A.G., How lipids affect the activities of integral membrane proteins, *Biochim. Bioph. Acta* 1666 (2004) 62-87.
[12]de Kruijff B., Lipid polymorphism and biomembrane function, *Curr. Opin. Chem. Biol*. 1 (1997) 564-569.
[13]Garab G., K. Lohner, P. Laggner, T. Farkas, Self-regulation of the lipid content of membranes by non-bilayer lipids: a hypothesis, *Trends Plant Sci*. 5 (2000) 489-494.
[14]Simidjiev I., S. Stoylova, H. Amenitsch, T. Jávorfi, L. Mustárdy, P. Laggner, A. Holzenburg, G. Garab, Self-assembly of large, ordered lamellae from non-bilayer lipids and integral membrane proteins in vitro*, Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 1473-1476.
[15]Barzda V., A. Istokovics, I. Simidjiev, G. Garab, Structural flexibility of chiral macroaggregates of light-harvesting chlorophyll a/b pigment-protein complexes. Light-induced reversible structural changes associated with energy dissipation, *Biochemistry* 35 (1996) 8981-8985.
[16]Simidjiev I., V. Barzda, L. Mustárdy, G. Garab, Role of thylakoid lipids in the structural flexibility of lamellar aggregates of the isolated light-harvesting chlorophyll a/b complex of photosystem II, *Biochemistry* 37 (1998) 4169-4173.
[17]Yamamoto H.Y., R.M. Higashi, Violaxanthin de-epoxidase. Lipid composition and substrate specifity, *Arch. Bioch. Biophys* 190 (1978) 514-522.
[18]Latowski D., J. Kruk, K. Burda, M. Skrzynecka-Jaskier, A. Kostecka-Gugała, K. Strzałka, Kinetics of violaxanthin de-epoxidation by violaxanthin de-epoxidase, a xanthophyll cycle enzyme, is regulated by membrane fluidity in model lipid bilayers, *Eur. J. Biochem.* 269 (2002) 4656-4665.
[19]Yamamoto H.Y., R.C. Bugos, A.D. Hieber, Biochemistry and molecular biology of the xanthophyll cycle, in: H.A. Frank, A.J. Young, G. Britton, R.J. Cogdell (Eds), *The Photochemistry of Carotenoids*, Kluwer Academic Publishers, Dordrecht, 1999, pp. 305-325.
[20]Goss R., M. Lohr, D. Latowski, J. Grzyb, A. Vieler, C. Wilhelm, K. Strzałka, Role of hexagonal structure-forming lipids in diadinoxanthin and violaxanthin solubilization and de-epoxidation, *Biochemistry* 44 (2005) 4028-4036.

[21]Szilágyi A., M. Sommarin, H.-E. Åkerlund, Membrane curvature stress controls the maximal conversion of violaxanthin to zeaxanthin in the violaxanthin cycle—influence of α-tocopherol, cetylethers, linolenic acid, and temperature, *Biochim. Biophys. Acta*. 1768 (2007) 2310-2318.
[22]Douce R., J. Joyard, Biosynthesis of thylakoid membrane lipids, in: D.R. Ort, C.F. Yacum (Eds), *Advances in Photosynthesis/Oxygenic Photosynthesis: The Light Reactions*, Kluwer Academic Publishers, Dordrecht, 1996, pp. 69-101.
[23]Ivancich A., L. Horváth, M. Droppa, G. Horváth, T. Farkas, Spin label EPR study of lipid solvation of supramolecular photosynthetic protein complexes in thylakoids, *Biochim. Biophys. Acta* 1196 (1994) 51-56.
[24]Lewis R.N.A.H., R.N. McElhaney, Surface charge markedly attenuates the nonlamellar phase-forming propensities of lipid bilayer membranes: calorimetric and ^{31}P-Nuclear Magnetic Resonance studies of mixtures of cationic, anionic, and zwitterionic lipids, *Biophys. J*. 79 (2000) 1455-1464.
[25]Selstam E., I. Brentel,G. Lindblom, Phase structure of lipids of the photosynthetic membrane, in: P.J. Quinn, J.L. Harwood, (Eds.), *Plant Lipid Biochemistry, Structure and Utilization*, Portland Press Limited, London, 1990, pp. 39-46.
[26]Harańczyk H., K. Strzałka, W. Dietrich, J.S. Blicharski, P-31-NMR observation of the temperature and glycerol induced non-lamellar phase-formation in wheat thylakoid membranes, *J. Biol. Phys*. 21 (1995) 125-139.
[27]Harańczyk H., K. Strzałka, T. Bayerl, G. Klose, J.S. Blicharski, ^{31}P-NMR measurements in photosynthetic membranes of wheat, *Photosynthetica* 19 (1985) 414-416.
[28]Seibert M., M. de Wit, L.A. Staehelin, Structural localization of the O_2-evolving apparatus to multimeric (tetrameric) particles on the lumenal surface of freeze-etched photosynthetic membranes, *J. Cell Biol*. 105 (1987) 2257-2265.
[29]Dekker J.P., H. van Roon, E. Boekema, Heptameric association of light-harvesting complex II trimers in partially solubilized photosystem II membranes, *FEBS Lett*. 449 (1999) 211-214.
[30]Büchel C., G. Garab, Electrochromic absorbance changes in the chlorophyll-c-containing alga *Pleurochloris meiringensis* (Xanthophyceae), *Photosynth. Res*. 43 (1995) 49-56.
[31]Demeter S., I. Vass, G. Horváth, A. Läufer, Charge accumulation and recombination in photosystem II studied by thermoluminescence. II. Oscillation of the C band induced by flash excitation, *Biochim. Biophys. Acta* 764 (1984) 33–39.
[32]Bennett J., Chloroplast phosphoproteins. Evidence for a thylakoid bound phosphoprotein phopshatase, *Eur. J. Biochem*. 104 (1980) 85-89.
[33]Cullis P.R., B. Kruijff, Polymorphic phase behaviour of lipid mixtures as detected by 31P NMR. Evidence that cholesterol may destabilize bilayer structure in membrane systems containing phosphatidylethanolamine, *Biochim. Biophys. Acta* 507 (1978) 207-218.
[34]Dekker J.P., E.J. Boekema, Supramolecular organization of thylakoid membrane proteins in green plants, *Biochim. Biophys. Acta* 1706 (2005) 12-39.
[35]W. Junge, Membrane-potentials in photosynthesis, *Ann. Rev. Plant Physiol*. 28 (2007) 503-536.

[36]Farineau J., G. Garab, G. Horváth, A. Faludi-Daniel, Proton translocation in the slow rise of the flash-induced 515 nm absorbance change of intact chloroplasts, *FEBS Lett*. 118 (1980) 119-121.
[37]Hideg E., I. Vass, The 75 ^{o}C thermoluminescence band of green tissues: chemiluminescence from membranechlorophyll interaction, *Photochem. Photobiol*. 58 (1993) 280-283.
[38]Duchene S., P. Siegenthaler Do glycerolipids display lateral heterogeneity in the thylakoid membrane?, *Lipids* 35 (2000) 739 744.
[39]Garab G., Linear and circular dichroism, in: J. Amesz, A.J. Hoff, (Eds.), *Biophysical Techniques in Photosynthesis. Advances in Photosynthesis and Respiration*, Springer, Dordrecht, 1996, pp. 41-61.
[40]Cullis P.R., B. de Kruijff, M.J. Hope, A. Rietveld, A.J. Verkleij, Structural properties of phospholipids in the rat liver inner mitochondrial membrane. A ^{31}P-NMR study, *Biochim. Biophys. Acta* 600 (1980) 625-635.
[41]de Kruijff B., A.J. Verkley, C.J.A. van Echteld, W.J. Gerritsen, C. Mombers, P.C. Noordam, J. de Gier, The occurrence of lipidic particles in lipid bilayers as seen by ^{31}P NMR and freeze-fracture electron-microscopy, *Biochim. Biophys. Acta* 555 (1979) 200-209.
[42]Killian J.A., I. Salemink, M.R.R. de Planque, G. Lindblom, R.E. Koeppe, D.V. Greathouse, Induction of nonbilayer structures in diacylphosphatidylcholine model membranes by transmembrane r-helical peptides: Importance of hydrophobic mismatch and proposed role of tryptophans, *Biochemistry* 35 (1996) 1037-1045.

Chapter 4

Temperature Dependence of the Lipid Packing in Thylakoid Membranes Studied by Time- and Spectrally Resolved Fluorescence of Merocyanine 540

S. B. Krumova, R. B. M. Koehorst, A. Bóta, T. Páli, A. van Hoek, G. Garab, H. van Amerongen

Published in Biochim. Biophys. Acta, 1778 (2008) 2823-2833

Temperature dependence of the lipid packing in thylakoid membranes studied by time- and spectrally resolved fluorescence of Merocyanine 540

The lipid packing of thylakoid membranes is an important factor for photosynthetic performance. However, surprisingly little is known about it and it is generally accepted that the bulk thylakoid lipids adopt the liquid-crystalline phase above -30 °C and that a phase transition occurs only above 45 °C. In order to obtain information on the nature of the lipid microenvironment and its temperature dependence, steady-state and time-resolved fluorescence measurements were performed on the fluorescence probe Merocyanine 540 (MC540) incorporated in isolated spinach thylakoids and in model lipid systems (dipalmitoyl phosphatidylcholine and dioleoyl phosphatidylethanolamine) adopting different phases. It is demonstrated that the degree and way of incorporation differs for most lipid phases – upon selective excitation at 570 nm, the amplitude of the fluorescence component that corresponds to membrane-incorporated MC540 is about 20% in gel-, 60% in rippled gel-, and 90% in liquid-crystalline and inverted hexagonal phase, respectively. For thylakoids, the data reveal hindered incorporation of MC540 (amplitude about 30% at 7 °C) and marked spectral heterogeneity at all temperatures. The incorporation of MC540 in thylakoids strongly depends on temperature. Remarkably, above 25 °C MC540 becomes almost completely extruded from the lipid environment, indicating major rearrangements in the membrane.

1. Introduction

Many physiological processes that take place in the photosynthetic thylakoid membrane as well as a variety of its properties have been demonstrated to depend on temperature. These include oxygen evolution [1], CO_2 assimilation, non-photochemical quenching, aggregation of the major light-harvesting complex LHCII [2], photophosphorylation [2,3], expression of proteins [4], lipid-protein ratio [5] and lipid-protein interactions ([6] and references therein), membrane permeability [7-9], electron transport activity [10-14], proton efflux [15] and the rate of NADP-reduction [16]. The

temperature dependences of some of these processes and properties were correlated with (or related to) the mobility of the chloroplast-membrane lipids as measured by EPR spin-label spectroscopy [17-19]. Abrupt changes in the membrane fluidity were also reported at 10-12 °C [20].

It is known that the lipid packing and phase behavior are essential for the operation of violaxanthin de-epoxidase [21-23] and that they are modulated by the xanthophyll cycle pigments solubilized in the bilayer [24]. They might also play a significant role in providing the optimal conditions for diffusion of hydrophobic molecules such as plastoquinol [25,26] or integral protein complexes (e.g. during state transitions [27,28]).

It is generally accepted that the bulk lipid mixture in the thylakoid lamellae exists in the liquid crystalline phase (Lα) above -30 °C [29] and no phase transition is expected to occur up to about 45 °C; above this temperature the thylakoid lipids segregate from the membrane and form non-bilayer structures [30,31]. This, however, does not exclude the possibility of changes in the lipid packing and/or formation of membrane domains with distinct physical properties at lower temperatures. Since the thylakoid membranes contain about 70-80% proteins, and only 20-30% lipids, the lipid packing might also be influenced by changes in the macro-organization of the protein complexes, as indeed indicated by temperature- and light-induced reorganizations in the LHCII-containing chiral macrodomains (see e.g. [32]). The packing of biological membranes in general, as well as the nature of lipid-protein interactions, are also largely influenced by the presence of high concentrations of non-bilayer lipids [33,34].

The thylakoid membrane is constituted of four lipid classes – monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol, phosphatidylglycerol and sulfoquinovosyl. It is known that half of the thylakoid membrane lipids (MGDG) are non-bilayer forming lipids (NBL), which are nevertheless believed to be organized predominantly in bilayers assuming the liquid-crystalline phase in the intact membrane [29,35]. Their significance for the macroorganization and function of the membrane is still unknown. It was hypothesized that the presence of NBL is needed for the maintenance of a certain lipid:protein ratio, essential for the optimal functioning of the membrane [36]. The adjustment of the MGDG:DGDG ratio was found to be a way for adaptation to cold stress [37,38]. Phase segregation of thylakoid lipids into the inverted hexagonal phase (H_{II}) phase

was shown to occur in spinach plants grown under low-light conditions [39] and also under various other stress conditions like high temperatures (above 45 °C) [30,31,40], low pH [41] and high concentrations of metal cations [42]. These data show that under certain conditions a marked heterogeneity can be induced in the lipid phase of thylakoid membranes. Our recent ^{31}P-NMR data have shown that heterogeneity is also present in freshly isolated intact thylakoid membranes. It has been shown that the location of the phospholipids is not restricted to the bilayer phase and the lamellar phase co-exists with a non-bilayer isotropic phase, with a strong temperature dependence between 14 and 20 °C [43].

In general, modulations in the lipid packing and co-existence of different lipid phases and domains are rather difficult to detect in complex biological membranes such as thylakoids due to limitations of the available techniques. Here we apply a new approach, the combination of steady-state and time-resolved fluorescence spectroscopy of the lipophylic fluorescent dye Merocyanine 540 (MC540). The spectroscopic properties of this probe have been shown to be sensitive to the phase behavior and lipid packing/spacing in model membranes [44-51], which has been used for identifying domains with different packing in different biological membranes [52-56]. MC540 is incorporated into lipid membranes as monomers [57,58] or forms dimers and higher aggregates on the surface [46,49,59,60].

In this work, we have determined the steady-state and time-resolved fluorescence properties of MC540 in model lipid systems that can adopt different phases, depending on the temperature of the suspension, and have compared them to those in thylakoid membranes. The combination of steady-state and time-resolved fluorescence spectroscopy led to the unequivocal identification of three forms of MC540, with characteristic spectral properties and fluorescence lifetimes, in model lipid systems as well as in thylakoid membranes, which can be assigned to dyes in different microenvironments. Our results reveal that incorporation of MC540 in thylakoids (whose lipids are believed to adopt liquid-crystalline phase, see above) is far less pronounced than in model liquid-crystalline phase and strongly depends on the temperature. Above 25 °C the dye molecules are progressively extruded from their hydrophobic lipid environment, indicating the onset of structural rearrangements in the thylakoid membrane.

2. Materials and Methods:

2.1. Sample preparation

Liposomes of dipalmitoyl phosphatidylcholine (DPPC) and dioleoyl phosphatidylethanolamine (DOPE) were prepared in 20 mM Tricine (pH 7.8) using the procedure described by Csiszar et al. [61]. MC540 (Fig. 1) was purchased from Sigma-Aldridge Co. The lipids were purchased from Avanti Polar Lipids, USA.

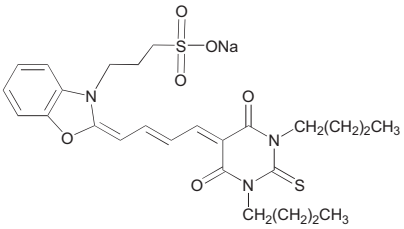


Figure 1. Structural formula of Merocyanine 540.

Dark-adapted leaves of market spinach were homogenized in a medium containing 20 mM Tricine (pH 7.5), 400 mM sorbitol, 5 mM $MgCl_2$ and 5 mM KCl; the suspension was filtered through 4 layers of cheese cloth and centrifuged for 4 min at 4000 x g. The chloroplasts were osmotically shocked in a hypotonic medium containing 20 mM Tricine (pH 7.5), 5 mM $MgCl_2$ and 5 mM KCl, and centrifuged for 5 min at 6000 x g. The pellet was finally resuspended in the same medium supplemented with 400 mM sorbitol (resuspension buffer); the chlorophyll (Chl) content was adjusted to 20 μg Chl/ml and quantified according to Arnon [62].

Before performing the measurements the liposomes and the isolated thylakoid membranes were kept on ice and MC540 was added from 1 mM ethanol stock solution; the samples were gently stirred in the dark for 30 min. The final concentration of MC540 was 0.6 μM for the lipid suspensions and 0.2 μM for the thylakoid membrane preparations, respectively. During the measurements the samples were thermostated for 15 min at each temperature. For each experiment five independent repetitions were performed.

2.2. Fluorescence spectroscopy

Steady-state fluorescence spectra were recorded with a Jobin Yvon Fluorolog FL3-22 spectrofluorimeter and corrected for the detection sensitivity. In order to correct for the contribution of Chl fluorescence, excitation and emission spectra of thylakoid membranes were recorded in the absence of MC540 and subtracted from the corresponding spectra

obtained after the addition of the dye. The emission wavelength for the excitation spectra was set at 580 nm, unless stated otherwise. For the model lipid systems the bandwidth for both excitation and emission was 0.75 nm, and for thylakoid membranes it was 3 nm; the step size was 0.2 nm. The emission spectra were detected using 2 nm bandwidth both for the excitation and the emission monochromators for the lipid systems and 3 nm for thylakoid membranes, the step size was 0.5 nm.

2.3. Fluorescence lifetime measurements

Time-correlated single photon counting technique was used to perform time-resolved fluorescence measurements. A CW diode-pumped, frequency-doubled Nd:YVO_4 laser (Coherent Inc., Santa Clara, CA, model Verdi V10) was used to pump a titanium:sapphire laser (Coherent Inc., Santa Clara, CA, model Mira 900-D in fs mode) that was passively mode-locked and tuned to either 540 or 570 nm. The channel spacing was 5 ps. The excitation intensity was reduced with neutral density filters to obtain a count-rate of 30000 s^{-1}. The emission filters were two 3 mm thick Schott RG 610 nm cut-off filters for the lipid systems and a 3 mm thick Schott RG 610 nm cut-off filter and an additional Balzers broadband interference filter model K60 for the thylakoid suspensions, thus collecting the fluorescence between 610 and 630 nm in chlorophyll containing (thylakoid membranes) samples. For the fitting procedure, the dynamic instrumental response of the experimental setup was recorded using the fast and single-exponential fluorescence decay (6 ps) of the reference compound pinacyanol in methanol [63]. Data analysis was performed using the computer program described earlier [64,65]. During the deconvolution procedure the lifetimes for different preparations of the same kind (measured upon excitation at 540 and 570 nm) and certain temperature were linked and their relative amplitudes were free. For example the lifetimes were linked for five samples of MC540 added to DPPC vesicles at 7 °C measured at 540 nm excitation and five samples but measured at 570 nm excitation. In this way the variation of the relative amplitudes corresponding to certain decay component could be followed in the different samples and at different temperatures. The fit quality was evaluated from χ^2, and from plots of the weighted residuals and the autocorrelation thereof (see e.g. [66]). Typical values of χ^2 were 1.1-1.2.

3. Results

Before studying the lipid packing in a system as complex as the thylakoid membrane, we first address the fluorescence properties of MC540 in model systems: DPPC and DOPE, which adopt different phases. It has been shown that the fluorescence yield and spectra of MC540 depend on the lipid phase and packing [46-50]. Here we further extend this approach by combining the steady-state properties of MC540 with its fluorescence lifetime characteristics and apply this information for thylakoid membranes.

Time-resolved fluorescence measurements of MC540 incorporated in lipid systems adopting different lipid phases, to our knowledge, have not been reported previously. The combination of the steady-state and time-resolved fluorescence spectroscopy techniques is particularly informative in cases where different microenvironments co-exist and when the fluorescence yields vary in a broad interval. With steady-state spectroscopy, heterogeneity can be revealed by varying either the excitation or the detection wavelength. However, molecules that have a short excited-state lifetime contribute considerably less to the excitation/emission spectra than the molecules with a longer excited-state lifetime, which makes their identification and determination of their contribution difficult. These can readily be performed with the aid of time-resolved fluorescence measurements, where the amplitude of a decay component is linearly proportional to the number of MC540 molecules in the corresponding environment.

In the following sections, steady-state and time-resolved fluorescence properties of different model lipid systems and of isolated thylakoid membranes will be presented at different temperatures. The temperature dependences will be confined for a range between 5 and 45 °C, the physiological range for thylakoid membranes. Above this temperature, as pointed out in the Introduction, thylakoid lipids segregate in large quantities from the membrane and form extended non-bilayer structures.

3.1. Steady-state fluorescence

MC540 in aqueous environment

In the aqueous phase (resuspension buffer, see Materials and Methods) the excitation spectrum of MC540 is characterized by bands at around 500 and 540 nm (Fig. 2, see also [67]). The spectrum is not changing significantly upon varying the detection wavelength.

The same holds true also for the emission spectrum – it peaks at 575 nm and the shape is only weakly dependent on the excitation wavelength.

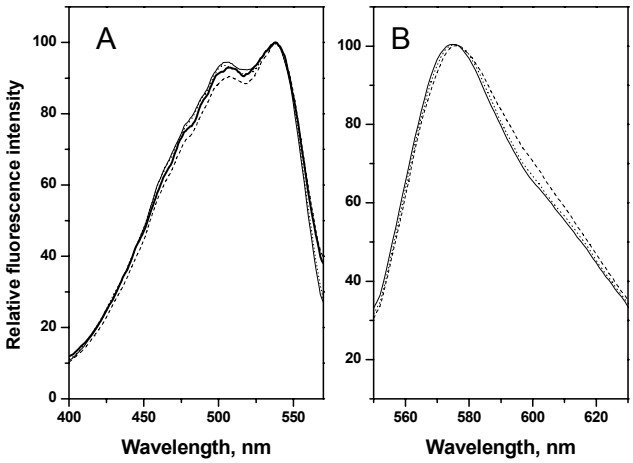


Figure 2. (A) Excitation spectra of MC540 in resuspension buffer at 7 °C, recorded with the fluorescence emission collected at 572 nm (thick line), 580 nm (thin line), 595 nm (dashed line) and 620 nm (dotted line). (B) Emission spectra of MC540 in resuspension buffer at 7 ºC, excited at 480 nm (solid line), 505 nm (dashed line), and 540 nm (dotted line). The spectra are normalized to their maxima.

MC540 in DPPC

The main phase transition for DPPC (L_{β}' to L_{α} phase) occurs at about 41.5 °C with a pre-transition (rippled gel phase, P_{β}') at about 33 °C [68]. When MC540 is added to vesicles in L_{β}' phase (temperature below 33 °C) the most striking difference between its excitation spectrum and that of MC540 in an aqueous environment (cf. Fig. 2) is the reduction of the 500 nm band and the presence of an additional band at 566 nm (Fig. 3A). This latter band has been shown to originate from MC540 monomers incorporated in the lipid bilayer [69].

As reported previously the fluorescence intensity is increasing dramatically upon the gel to liquid crystalline phase transition (Fig. 3B, C see also [44,47,58]) and the fluorescence ratio between the 566 nm and 536 nm bands in the excitation spectra recorded at 580 nm, F_{566}/F_{536} increases from 1.20±0.11 (at 7 °C) – 1.95±0.05 (at 25 °C) in the L_{β}'

phase to 2.83±0.08 in the P_{β}' and 2.94±0.04 in the L_{α} phase. Subtraction of the normalized excitation spectrum of MC540 in the L_{α} phase (recorded at 45 °C) from the spectrum in the L_{β}' phase (recorded at 7 °C) results in a difference spectrum very similar to the one of MC540 in buffer (Fig. 2A). The shape of the emission spectrum strongly depends on the excitation wavelength for the L_{β}' phase (Fig. 3C), but remains unchanged for the P_{β}' and L_{α} phases (Fig. 3C). These latter two phases cannot be distinguished from each other by comparing the steady-state properties of MC540 – the excitation (Fig. 3B) and emission (Fig. 3C) spectra have identical shapes.

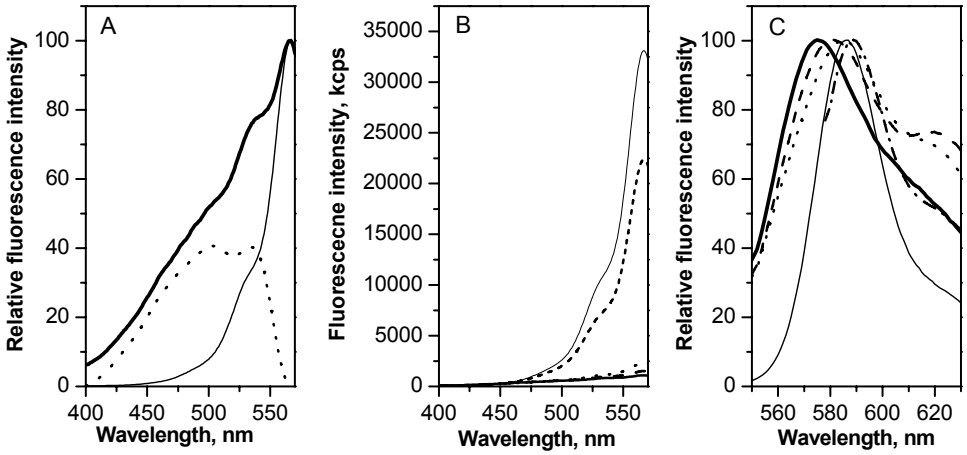


Figure 3. (A) Excitation spectra of MC540 added to dipalmitoyl phosphatidylcholine (DPPC) vesicles adopting different phases [68]: the gel (L_{β}') phase at 7 ºC (thick line), and the liquid crystalline (L_{α}) phase at 45 ºC (thin line). The spectra are normalized to their emission maxima. Their difference spectrum is represented by the dotted line. The emission was collected at 580 nm. (B) Temperature dependence of the excitation spectra of MC540, recorded at 580 nm: 7 ºC (thick line), 14 ºC (dashed line), 25 ºC (dotted line), 35 ºC (short dashed line) and 45 ºC (thin line). (C) Emission spectra of MC540 added to DPPC vesicles in the L_{β}' phase at 7 ºC, using different excitation wavelengths: 480 nm (thick line), 517 nm (dashed line), 540 nm (dotted line) and 570 nm (dash-dotted line). The emission spectrum of DPPC in L_{α} phase at 45 ºC upon excitation 540 nm is presented with thin line. The spectra are normalized to their emission maxima.

MC540 in DOPE

The shape of the excitation and emission spectra of MC540 in the H_{II} phase of DOPE (DOPE adopts the H_{II} phase above 3 °C [70] at 7 °C (Fig. 4A) is almost identical to the ones of DPPC in the L_α phase at 45 °C ($F_{566/536}$ is 2.81±0.01, as compared to 2.94±0.04 for DPPC at 45 °C, see also Fig. 3B). The fluorescence intensity is linearly decreasing with the increase, as was observed for MC540 in ethanol [47], and the spectral shape remains unchanged. As observed for the L_α phase of DPPC, the shape of the emission spectrum does not depend on the excitation wavelength (Fig. 4B).

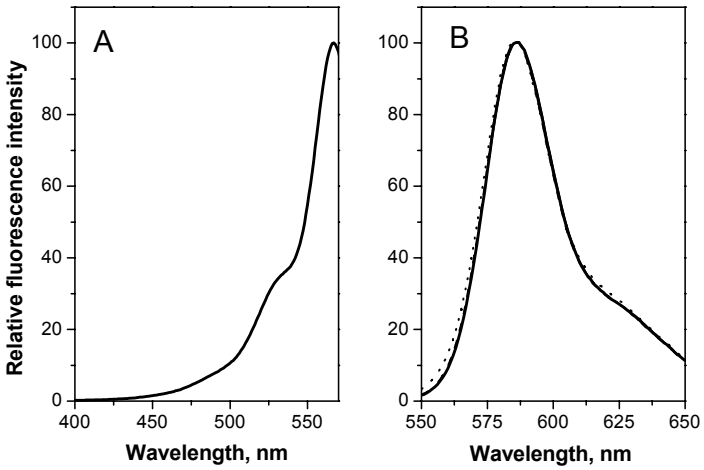


Figure 4. (A) Excitation spectra of MC540 added to dioleoyl phosphatidylethanolamine (DOPE) adopting the inverted hexagonal phase (H_{II}, [70]), at 7 ºC. The emission was collected at 580 nm. (B) Emission spectra of MC540 added to DOPE adopting the H_{II} phase at 7 ºC. The excitation wavelengths are: 480 nm (thick line), 517 nm (dashed line), 540 nm (dotted line), 570 nm (thin line). The spectra are normalized to their emission maxima.

MC540 in thylakoid membranes

The excitation spectrum of MC540 in the presence of thylakoid membranes at 7 °C (Fig. 5A) is dominated by a band at 566 nm with a shoulder at 536 nm, whereas no pronounced 500 nm band is observed. The ratio F_{566}/F_{536} depends on the concentration of MC540. Over the whole concentration range studied – between 0.04 and 1.2 μM MC540

(at a fixed chlorophyll concentration of 20 μg Chl/ml) - the shoulder at 536 nm is more pronounced than for MC540 in the $H_{II}/P_{\beta}'/L_{\alpha}$ lipid systems. The highest value for F_{566}/F_{536} (2.21±0.09) is obtained at concentrations below 0.2 μM but it is significantly lower than the one observed for the L_{α} phase of DPPC ($F_{566/536}$ = 2.94±0.04). Above this concentration, the shoulder at 536 nm becomes even more prominent. In the rest of our experiments we used 0.2 μM MC540, i.e. the highest concentration of MC540 at which $F_{566/536}$ is still 2.21. When the excitation spectrum of MC540 in the L_{α} phase (DPPC at 45 ºC) is subtracted from the excitation spectrum of MC540 in the presence of thylakoid membranes, the resulting difference spectrum (Fig. 5A) resembles but is not identical to the one of MC540 in buffer (Fig. 2A). In addition, the shape of the emission spectrum depends on the wavelength of excitation (Fig. 5B).

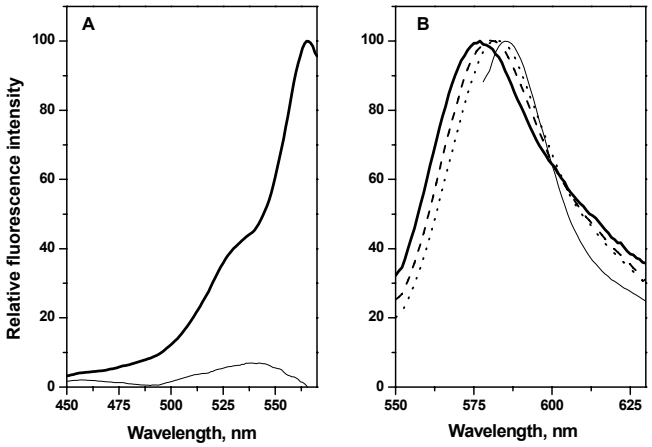


Figure 5. (A) Excitation spectra of MC540 in thylakoid membranes. The spectra were recorded at 7 °C and the emission was collected at 580 nm. The difference between the spectra of MC540 added to thylakoids and to dipalmitoyl phosphatidylcholine (DPPC) in the liquid crystalline phase (L_{α}) at 45 °C, collected at 580 nm, is represented by the thin line. (B) Emission spectra of MC540 added to thylakoid membranes at 7 ºC. The excitation wavelengths are – 480 nm (thick line), 517 nm (dashed line), 540 nm (dotted line) and 570 nm (thin line) The spectra are normalized to their emission maxima.

With the increase of temperature, significant changes are observed both in the intensity and the shape of the excitation spectrum (Fig. 6A). The relative intensity of the

band at 566 nm decreases significantly and at 45 °C $F_{566/536}$ equals 1.27±0.12. The intensity of the emission spectrum of MC540 is decreasing with the increase of temperature from 7 °C to 45 °C, but no major spectral change is observed (Fig. 6B, C).

The thylakoid membrane is enriched in proteins and hence it cannot be ruled out that MC540 is not only incorporated in the bulk lipid phase but also in the solvation shell of the proteins. In order to study this in more detail, we have checked whether there is excitation energy transfer between MC540 and the Chl molecules in the pigment-protein complexes of the thylakoids. Emission spectra were recorded both in the absence and presence of MC540 (selective excitation at 570 nm). It was established (data not shown) that there is absolutely no change/increase in the intensity of Chl *a* fluorescence (684 nm), upon excitation in the MC540 absorption region, demonstrating that no detectable energy transfer is taking place and the fluorescence lifetimes of MC540 are not governed by excitation energy transfer but by their direct environment, which is either the water or the lipid phase. However, it should be noted that the possibility of association of part of the MC540 molecules to proteins which do not contain pigments can not be ruled out.

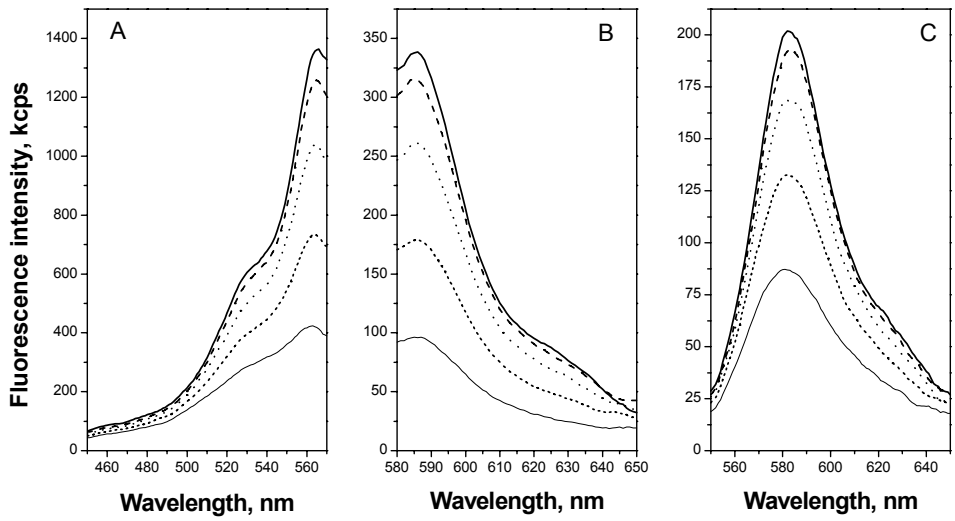


Figure 6. Temperature dependence of the excitation (A) and emission (B, C) spectra of MC540 added to thylakoid membranes: 7 ºC (thick line), 14 ºC (dashed line), 25 ºC (dotted line), 35 ºC (short dashed line) and 45 ºC (thin line). The emission was collected at 580 nm. The emission spectra were recorded upon 570 nm (B) and 540 nm (C) excitation.

3.2. Time-resolved fluorescence

MC540 in aqueous environment

Time-resolved fluorescence measurements revealed a mono-exponential decay upon exciting at 540 nm with a lifetime (somewhat) dependent on the temperature (Fig. 7). At 25 °C it is 110 ps, equal to the one obtained by Mandal et al. [71]. Identical lifetimes were found upon excitation at 570 nm, although a small fraction of an additional longer-lived fluorescence (~1 ns) was also present (data not shown), which is probably due to a small amount of aggregated dye molecules.

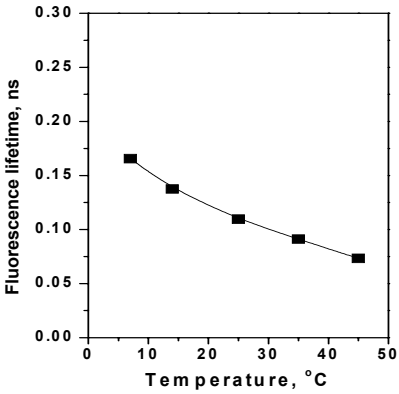


Figure 7. Fluorescence lifetime (τ) obtained after analysis of the fluorescence decay traces of MC540 in the aqueous phase (resuspension buffer, see Materials and Methods) at different temperatures. For the global fitting procedure the lifetimes for the different measurements at certain temperature were linked, thus resulting in only a single value and the respective amplitudes were free; see also Materials and methods. The fluorescence decay curves were recorded upon excitation at 540 nm. The samples were thermostated for 10 min at each temperature.

MC540 in DPPC

Fitting of the time-resolved fluorescence decay curves revealed three decay times in most cases, and the relative amplitudes were different for 540 nm and 570 nm excitation. The lifetimes depended strongly on the lipid phase – at temperatures below 33 °C, where the L_{β}' phase is adopted, they were about 2 ns, 0.8 ns and 150 ps (Fig. 8A). Under these conditions the relative contribution of the shortest component (about 150 ps) was

particularly pronounced upon 540 nm excitation (Fig. 8B) and it could be ascribed to MC540 in the aqueous phase. The two long components (2 ns and 0.8 ns) are ascribed to MC540 associated with the lipids since their relative contributions are enhanced significantly upon 570 nm excitation (Fig. 8B). The relative amplitudes of these two components were equal in the $L_{\beta'}$ phase and upon the transition to $P_{\beta'}$ and L_{α} phases the relative amplitude of the 2 ns became significantly higher. The phase transition from $L_{\beta'}$ to $P_{\beta'}$ also resulted in a significant decrease of the relative amplitude of the 150 ps component and its disappearance in the L_{α} phase, demonstrating that no MC540 remains in the water phase. Apparently the dye molecules were easily dissolved in the hydrophobic lipid environment in both the $P_{\beta'}$ and L_{α} phases. In order to check whether the relative amplitudes of the two slow components are dependent on the excitation wavelength, we compared the normalized relative amplitudes (Fig. 8C). For the $L_{\beta'}$ phase (7 – 25 °C) the relative amplitude of the 0.8 ns component is somewhat increased upon 540 nm excitation, as compared to 570 nm excitation, respectively. For the $P_{\beta'}$ and L_{α} phases no change in the relative amplitudes of the 0.8 and 2 ns components was observed upon variation of the excitation wavelength.

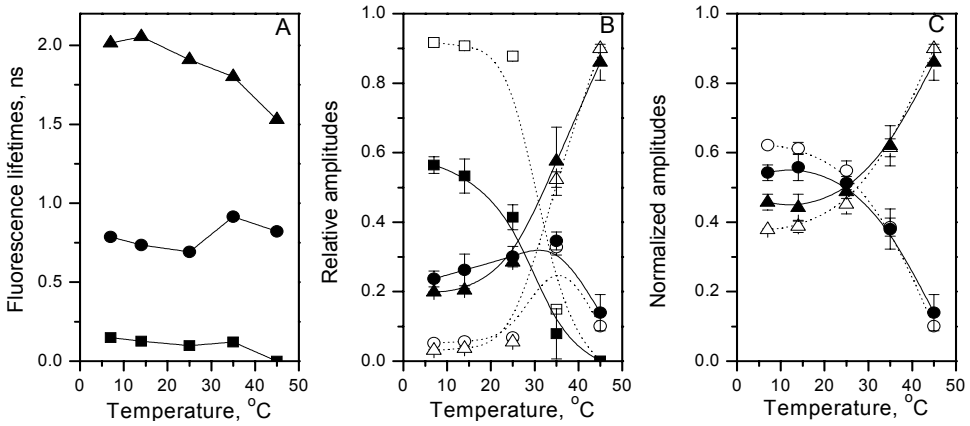


Figure 8. Parameters obtained from the analysis of the fluorescence decay traces of MC540 added to dipalmitoyl phosphatidylcholine (DPPC) adopting different phases at different temperatures (gel phase (L_{β}') below 33 °C, "rippled" gel phase (P_{β}') at 33 °C and liquid crystalline (L_{α}) above 41.5 °C, [68]): (A) fluorescence lifetimes (For the global fitting procedure the lifetimes for the different measurements at certain temperature were linked, thus resulting in only a single value, and the respective amplitudes were free; see also Materials and methods); (B) their relative amplitudes upon 570 nm (full symbols) and 540 nm (open symbols) excitation and (C) normalized amplitudes of the two long components (2 ns (▲, Δ) and 1 ns (●, ○) resolved upon 540 nm (open symbols) and 570 nm (full symbols) excitations). The error bars represent the standard error. Solid (for 570 nm excitation) and dashed (for 540 nm excitation) lines serve as a guide to the eye to follow the changes in the amplitudes of the fast, intermediate and slow components as a function of temperature. The samples were thermostated for 10 min at each temperature.

MC540 in DOPE

The fluorescence decay curves of MC540 added to DOPE at different temperatures were fitted with 3 components. The 200 ps component, indicative of MC540 in the water phase, was clearly resolved upon excitation at 540 nm (amplitude, 20%) but was absent upon 570 nm excitation (Fig. 9). Again, the longest component (about 2 ns) had by far the largest amplitude as was observed for the L_{α} phase for DPPC at 45 °C, and normalization

of the relative amplitudes of the two long components revealed no dependence on the excitation wavelength (Fig. 9C).

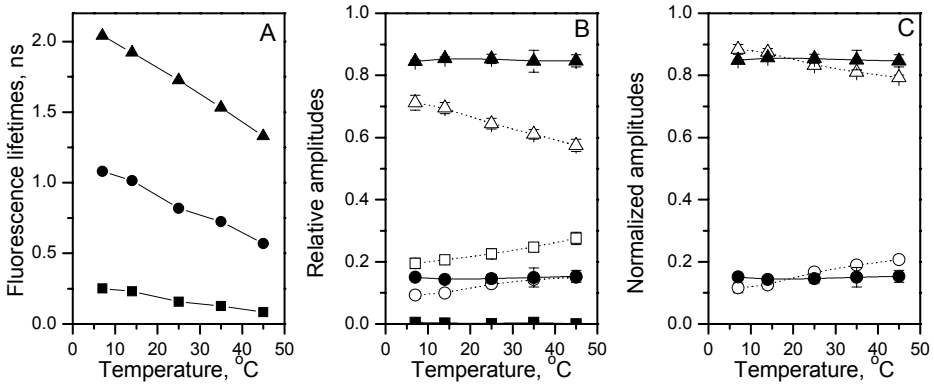


Figure 9. Parameters obtained from the analysis of the fluorescence decay traces of MC540 added to dioleoyl phosphatidylethanolamine (DOPE) adopting inverted hexagonal phase (H_{II}) at different temperatures [70]: (A) fluorescence lifetimes (For the global fitting procedure the lifetimes for the different measurements at certain temperature were linked, thus resulting in only a single value, and the respective amplitudes were free; see also Materials and methods); (B) their relative amplitudes upon 570 nm (full symbols) and 540 nm (open symbols) excitation and (C) normalized amplitudes of the two long components (2 ns (▲, Δ) and 1 ns (●, ○) resolved upon 540 nm (open symbols) and 570 nm (full symbols) excitations). The error bars represent the standard error. Solid (for 570 nm excitation) and dashed (for 540 nm excitation) lines serve as a guide to the eye to follow the changes in the amplitudes of the fast, intermediate and slow components as a function of temperature. The samples were thermostated for 10 min at each temperature.

MC540 in thylakoid membranes

Three lifetime components were found in the time-resolved measurements - at 7 °C they are approximately 2.0 ns, 1.0 ns and 170 ps (Fig. 10A), very similar to the values determined in DOPE and DPPC at this temperature. The 170 ps component had a very high relative contribution, about 50% even upon 570 nm excitation and upon 540 nm excitation it reaches a value of about 85% (Fig. 10B). Moreover, the relative amplitude of the 1 ns component was comparable to the one of the 2 ns component (Fig. 10C).

Upon increasing the temperature the relative contribution of the 170 ps component increased substantially and its relative amplitude became larger than 70% even upon 570 nm excitation (Fig. 10B), indicating the extrusion of the dye molecules from the thylakoid membrane. The relative amplitude of the 1 ns component was somewhat enhanced upon 540 nm excitation (Fig. 10C). The amplitudes of the two long-lived components showed a clear temperature dependence - the relative amplitude of the 2 ns component decreased with the increase of temperature, most prominently above 25 °C.

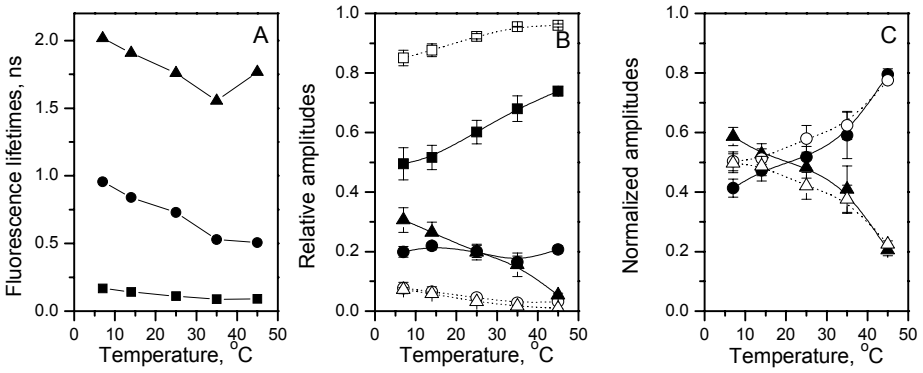


Figure 10. Parameters obtained from the analysis of the fluorescence decay traces of MC540 added to suspension of thylakoid membranes: (A) fluorescence lifetimes (For the global fitting procedure the lifetimes for the different measurements at certain temperature were linked, thus resulting in only a single value, and the respective amplitudes were free; see also Materials and methods); (B) their relative amplitudes upon 570 nm (full symbols) and 540 nm (open symbols) excitation and (C) normalized amplitudes of the two long components (2 ns (▲, Δ) and 1 ns (●, ○) resolved upon 540 nm (open symbols) and 570 nm (full symbols)). The error bars represent the standard error. Solid (for 570 nm excitation) and dashed (for 540 nm excitation) lines serve as a guide to the eye to follow the changes in the amplitudes of the fast, intermediate and slow components as a function of temperature. The samples were thermostated for 10 min at each temperature.

4. Discussion

In this work, in order to obtain information on the lipid packing in thylakoid membranes, we have performed time-resolved and steady-state spectroscopy measurements on thylakoids and model membranes. This combination of fluorescence measurements, which to our knowledge is applied for the first time in this systematic manner, has revealed the existence of different microenvironments in different model systems. These data, in turn, can help us to assess the lipidic microenvironments in the thylakoid membranes. These types of measurements may thus complement the information for the lipid phase of the thylakoid membranes at physiological temperatures, i.e. before the break-down of the membrane impermeability and the segregation of the lipids from the membrane, and the denaturation of proteins. It is believed that the bulk lipid mixture in thylakoid membranes exists in liquid crystalline phase [29] and, although marked temperature dependences were reported (see Introduction), phase transitions were detected only above 45 °C [30,31]. Also labeling of thylakoid membranes with the fluorescent probe DPH [72] and the EPR probe TEMPO [40] showed a monotonous increase in the fluidity of the membrane upon increasing the temperature from 10 °C to 45 °C. In contrast, our previous work pointed towards a more complex behavior and organization/packing of the lipid molecules; ^{31}P-NMR measurements showed a change in the bulk phospholipid behavior between 7 °C and 20 °C and revealed the presence of small non-bilayer structures in thylakoids co-existing with the lipid bilayer at all temperatures [43]. Here we show that steady-state and time-resolved MC540 measurements also reveal different microenvironments for the dye in model lipid systems and in intact thylakoids in the physiological range of temperatures; these will be discussed below.

4.1. MC540 in different lipid systems

The fluorescence spectra of MC540 have already been shown to be sensitive to the lipid packing and phase behavior of different model lipid systems [46,47,49,50]. In short, it was established that the fluorescence yield is substantially increased upon relocation of MC540 from aqueous to lipidic environment and there is a red-shift in the absorption and fluorescence spectra [44,58,73]; moreover the transition from gel to liquid crystalline and from liquid crystalline to inverted hexagonal phases were also correlated with higher

fluorescence yields [47,50]. Here we further extend this knowledge by exploring the excitation wavelength dependence of the spectral shapes of MC540 in different environments.

In buffer solution the excitation spectrum is very similar to the absorption spectrum of MC540 in water [49,58,73-75] and does not change substantially upon detection at different wavelengths (Fig. 2A). Correspondingly the emission spectrum also changes only slightly with the variation of the excitation wavelength (Fig. 2B), reflecting a rather homogeneous dye solution. It was reported that the spectral properties of MC540 depend on the presence of salts [76]. Under our experimental conditions no change in the spectral properties of MC540 was observed when solved in the buffer used for the preparation of the model lipid systems (20 mM Tricine, pH 7.5) or the one for the thylakoid membranes (20 mM Tricine, pH 7.5, 400 mM sorbitol, 5 mM $MgCl_2$, 5 mM KCl).

In contrast to MC540 free in the water phase, for MC540 in the L_{β}' phase a strong wavelength-dependence was observed - the emission maximum varies between 575 nm and 589 nm (Fig. 3C), revealing a large heterogeneity in the MC540 population, i.e. the existence of different microenvironments for the dye. Moreover, in accordance with previous studies [46,49] we also observe MC540 dimer formation in the L_{β}' phase of DPPC, reflected by a distinct fluorescent band at 620 nm (Fig. 3C). The transition to the P_{β}' and L_{α} phases leads to a pronounced change in the shape of both the excitation and emission spectra – the 566 nm band becomes the dominating one in the excitation spectra (Fig. 3A, B) and no 620 nm band is observed in the emission spectra (Fig. 3C). The same holds true for the H_{II} phase (Fig. 4). For the P_{β}', L_{α} and H_{II} phases no heterogeneity can be detected upon varying the excitation and detection wavelength, indicating that the spectra are largely dominated by one spectral form of MC540, which is attributed to MC540 monomers incorporated into the hydrophobic lipid environment [57,58]. The excitation spectra for P_{β}', L_{α} and H_{II} phases showed no evidence for the presence of MC540 molecules free in the buffer, as shown by the absence of a well discernible band at 500 nm; however those could be detected by time-resolved measurements (see below).

Furthermore, our work correlates the steady-state characteristics of MC540 with its time-resolved fluorescence properties and assigns the different fluorescence lifetime components to distinct microenvironments of the dye.

In the lipid systems studied here, MC540 exhibits three different fluorescence lifetime components, which in most cases decrease with the increase of the temperature, similarly to MC540 in buffer solution (Fig. 7) due to enhanced photoisomerization [67]. However, their relative amplitudes depend exclusively on the local environments of MC540 and on the physical state of the lipid system (Figs. 8B, 9B and 10B). The different lifetime components will briefly be discussed.

The short-lifetime component - MC540 in the aqueous phase

The short-lifetime component (113 ps - 214 ps) is found with different relative amplitudes in the various lipid-containing systems (Figs. 8, 9 and 10) but it is by far the dominating component for MC540 in buffer (Fig. 7). In the aqueous buffer phase at 25 °C a value of 110 ps is observed, identical to the one determined by Mandal et al. for MC540 in water [71]. In the studied lipid systems its relative contribution was always higher upon 540 nm than upon 570 nm excitation (Figs. 8B, 9B and 10B). This is in accordance with the expectations (as judged by the absorption spectrum of MC540 in aqueous environment, where the absorption at 570 nm is far less than at 540 nm [49,58,73-75], see also Fig. 2), and the short lifetime component thus represents MC540 molecules which remain free in the aqueous phase. Therefore, it can also be used as an indicator of the accessibility of the lipid membranes for MC540. We have established that the L_{β}' phase is particularly inaccessible for the dye molecules (Fig. 8), presumably due to the tight packing of the lipids. In all other model lipid phases studied, the short component was not present or had small amplitude (Figs. 8 and 9).

The long-lifetime components – MC540 interacting with lipids

The 1 ns and 2 ns components are originating from MC540 in hydrophobic environments (in the lipid phase) and hence provide information about the lipid packing and phase behavior. These lifetimes might be assigned to two discrete populations of the molecules, reflecting two different microenvironments. It is also possible that there is a broad distribution of lifetimes due to incorporation of MC540 in a variety of environments with only small differences in their physical properties (dielectric constant, lipid packing).

Discrete vs. broad lifetime distributions

According to the discrete lifetime distribution model, the two components should be assigned to MC540 residing in different environments. The 1 and 2 ns lifetimes are

somewhat similar to the ones obtained by Aramendia et al. [77] for dimyristoyl phosphatidylcholine vesicles in the L_α phase at 20 °C – 1.87 ns and 410 ps. It should be pointed out that these authors did not resolve the 200 ps component. Therefore, the 410 ps might be a weighted average of the values of 200 ps and 1 ns, which were resolved in the present study. Similarly to Aramendia et al. [77] we assign the 2 ns component to MC540 incorporated deep in the bilayer with parallel orientation with respect to the lipid molecules and the 1 ns component to surface-associated MC540 oriented perpendicular to the membrane. This is also substantiated by the fact that red-shifted absorption and fluorescence originate from MC540 located in an environment with a lower dielectric constant [58]. Moreover, a higher relative amplitude is observed for the 1 ns component upon 540 nm excitation in the case of L_β' and thylakoids. This is not detected for the P_β', L_α and H_{II} phases, probably due to the pronounced dominance of the 2 ns component. Thus, it can be concluded that the red-most emission upon 570 nm excitation originates mainly from the 2 ns component (MC540 in hydrophobic lipid environment) and the blue shifted emission obtained after excitation at 540 nm from the 1 ns component (more hydrophilic environment), respectively.

As mentioned above the relative amplitudes of the two slow components are strongly dependent on the lipid phase – the 2 ns component is substantially more pronounced in the P_β', L_α (Fig. 8) and H_{II} (Fig. 9) phases. This reveals that the majority of the MC540 molecules are incorporated completely between the lipid molecules. In the case of the L_β' phase on the other hand, the 2 ns and 1 ns components have almost equal amplitudes (Fig. 8). In accordance with this, the fluorescence intensity ratio $F_{566/536}$ is significantly higher for the P_β', L_α and H_{II} phases (2.81 – 2.94) than for the L_β' phase (1.20 – 1.95), thus indicating that a large fraction of the MC540 molecules penetrates less well into the bilayer and remains closer to the more polar environment of the lipid headgroups.

In the case of a broad distribution of lifetimes, the weighted average lifetime of the two long components discussed above should be considered. The average lifetime estimated in this way is not dependent on the excitation wavelength (Fig. 11) and the obtained values are of the order of the ones obtained by Onganer et al. [67] by phase modulation fluorometry. It is longer for the H_{II} and L_α phases than for the L_β' phase. Mandal et al. [71] have shown that the fluorescence lifetime and quantum yield are

significantly lower in aqueous than in polar environment and this was attributed to the increased rate of photoizomerization in polar solvents. This is in line with our observations - for the $L_{\beta'}$ phase the majority of the MC540 molecules remains on the surface of the vesicles and are thus exposed to the aqueous phase or remain free in the buffer phase, allowing fast photoisomerization, causing a shortening of the average fluorescence lifetime (Fig. 11). On the other hand the MC540 molecules that are buried in the hydrophobic lipid environment of H_{II} and L_{α} phases exhibit longer average fluorescence lifetimes due to the suppressed non-radiative processes (Fig. 11).

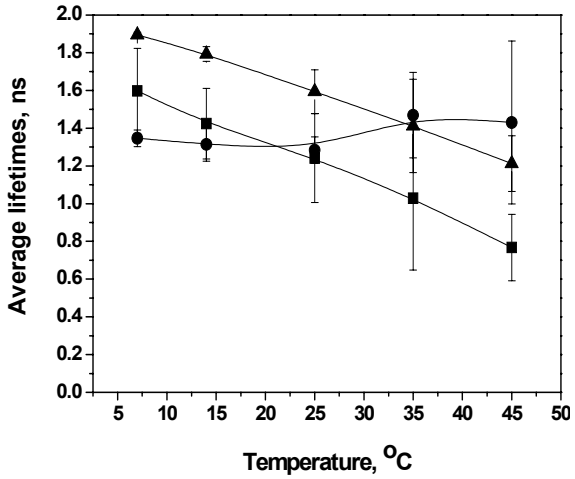


Figure 11. Average lifetimes obtained from the weighted lifetimes of the two longest decay components (~2 ns and ~1 ns) for MC540 in dipalmitoyl phosphatidylcholine (DPPC, ●), dioleoyl phosphatidylethanolamine (DOPE, ▲) and thylakoids (■) upon 570 nm excitation at different temperatures. The error bars represent the standard error. Excitation at 540 nm resulted in essentially the same average lifetimes.

4.2. MC540 in thylakoid membranes

Accessibility

The partitioning of MC540 in the thylakoid membrane at different temperatures can in principle be followed by the ratio of the 570 nm and 530 nm absorption peaks ($A_{570/540}$), as suggested by Bakalcheva et al. [78]. However, this approach could not be applied here – due to the low concentration of MC540 and its strong absorption overlap with Chl no good quality difference absorption spectra could be obtained. Instead, the fingerprint (566 nm

band in the excitation spectra) for monomeric MC540, and the fluorescence intensity ratio $F_{566/536}$ was used in order to probe the microenvironments of MC540 when incorporated in the thylakoid membranes. A concentration of MC540 was used that results in the highest $F_{566/536}$ ratio (and thus the highest amount of MC540 monomers located in the thylakoids) without the formation of sizeable amounts of MC540 dimers. The incorporation of MC540 appeared to be far less pronounced in thylakoids than in the model L_α phase. For comparison $F_{566/536}$ for thylakoids at 7 °C is 2.21±0.09, whereas for DPPC in the L_α phase it is 2.94±0.04. The combined steady-state and time-resolved fluorescence spectroscopy measurements reveal that a relatively large fraction of the MC540 molecules remains close to or free in the aqueous phase and are not incorporated in the thylakoid membrane. This could not reliably be inferred from the steady-state spectra due to the low fluorescence quantum yield of the short-lived component (Figs. 5 and 10). The lifetime measurements, showed however that even upon 570 nm excitation the relative amplitude of the shortest lifetime component (100-200 ps), and thus the fraction of water-exposed MC540 molecules is very high - it is 50% upon 570 nm and 85% upon 540 nm excitation, respectively (Fig. 10B). The thylakoid membrane thus appear to be less accessible for MC540 than the L_α, P_β' (Fig. 8) and H_{II} (Fig. 9) phases in model systems, while the amplitude of the short component is close to the one observed for the L_β' phase (Fig. 8). This might either be due to the tight lipid packing in the thylakoid membrane or to the presence of large amount of proteins whose surface charges might prevent the incorporation of MC540 in their vicinity and thus decrease the lipid area available for MC540.

Heterogeneity

Variation of the excitation wavelength in the steady-state spectra of MC540 added to thylakoids reveal heterogeneity in its spectral properties (Fig. 5C) and thus the presence of more than one type of local environment for MC540. Moreover, the difference spectrum presented in Fig. 5A also indicates that a fraction of MC540 is not well incorporated in the membrane. It also shows that these MC540 molecules are located in a rather hydrophilic environment since the difference spectrum is blue-shifted in comparison with the spectrum of monomeric MC540 in a lipid environment. This, in principle, might be due to the presence of MC540 molecules free in the aqueous phase. However, they have a very short lifetime and thus do not contribute substantially to the steady-state spectra. Furthermore, as

mentioned above the ratio F_{566}/F_{536} is significantly lower than the one observed for the L_α phase. These observations show that the bulk lipid phase behavior in thylakoids can not be described satisfactorily with the properties of the L_α phase and suggests that either the lipid packing is different from the one in the L_α phase or different lipid domains with distinct properties co-exist in the thylakoid. This is in agreement with our recent ^{31}P-NMR data, obtained on spinach thylakoid membranes, which revealed a substantial degree of heterogeneity in the lipid phases of the thylakoid membranes at temperatures below 45 °C [43].

Lipid packing

When using the discrete lifetime distribution model, it can be seen that up to 25 °C the relative amplitudes of the 1 ns and 2 ns components are almost equal (Fig. 10C), resembling very much the situation for the $L_{\beta'}$ phase (Fig. 8C). Above 25 °C, the 1 ns component becomes the dominant one (Fig. 10C). This was not observed for any of the model lipid phases studied, and it indicates the onset of a rearrangement of the bulk lipid matrix of the thylakoids – the MC540 which was buried inside the membrane becomes extruded from this hydrophobic environment and hence localized in an environment with higher water content. This conclusion is corroborated with the changes in the steady-state spectral properties between 25 ºC and 45 ºC – a strong reduction of the intensity of the 566 nm peak (hydrophobic environment), as compared to the 536 nm band (Fig. 6).

The temperature dependence of the average lifetime of the two long components (Fig. 11) does not correspond to the ones determined for model lipid systems in different phases. The nature of this effect is still unclear; the high protein content might influence the MC540 fluorescence lifetime to some extent. It is also worth mentioning that the temperature dependence of the average lifetime for thylakoid membranes is linear and thus does not reveal any major phase transition. However, it does reveal rather complex behavior of the bulk lipid matrix in the thylakoid membrane, since it differs from the mono-phasic model lipid systems. It has been shown that the decrease in $A_{570/540}$ is proportional to the increase of the dielectric constant [78]. In the present experiments the significant decrease in $F_{566/536}$ for thylakoids between 7 and 45 °C should be regarded as a decrease in the $A_{570/540}$, which thus indicates relocation of MC540 to an environment with higher dielectric constant. Taking into account also the strong reduction of the fluorescence

intensity upon an increase in temperature (Fig. 6), it can be concluded that MC540 is extruded from the hydrophobic lipid membrane at high temperatures. The observed changes in the excitation spectra of MC540 in the suspension of thylakoid membranes with the increase of temperature strongly resemble the absorption changes observed by Mateasik et al. induced by increasing the negative surface charge of model lipid membranes [79]. Their data show that electrostatic repulsion prevents the penetration of MC540 into the lipid membrane. The surface charge of thylakoids is also negative and it has been shown to arise mainly from the exposed charged amino acids of the membrane proteins [80,81]. Thus a rearrangement of the protein complexes and/or the lipid molecules upon the increase of temperature might lead to more surface-exposed charges and consequently lead to extrusion of MC540 from the membrane. This hypothesis is further supported by the data of Dobrikova et al. [82] showing that in the temperature range 20-30 °C changes in the permanent dipole moment (transversal charge asymmetry) and the electric polarizability (motion of charges in the diffuse electric double layer) of pea thylakoid membrane occur, which have also been shown to strongly depend on the supramolecular organization of LHCII [83].

5. Conclusion

The steady-state and time-resolved spectroscopic data shown here demonstrate that in a system as complex as the thylakoid membrane the lipophylic fluorescent probe MC540 is exposed to different microenvironments and thus the thylakoid membrane can not be described satisfactorily with the properties of simple, mono-phasic model lipid systems. This conclusion is in line with our earlier results, based on ^{31}P-NMR studies, indicating heterogeneity in the bulk lipid phases of thylakoid membranes [43]. The results presented here also reveal a strong temperature dependence of the spectral properties of MC540 in the thylakoid membranes. At temperatures between 25 and 45 °C rearrangements in the bulk lipid phase occur leading to relocation of MC540 into more hydrophilic environment and its extrusion from the membrane. The formation of inverted hexagonal lipid phase (H_{II}), i.e., the segregation of lipids from the membrane, and the degradation of the pigment-protein complexes occur at higher temperatures, at about 45 °C [30,31,40] and 65-70 °C [84], respectively, thus the changes detected by MC540 can not be correlated with the

above mentioned events. Most probably they are due to internal protein rearrangements or changes in the lipid-protein interactions in the otherwise intact and functional membrane. For example they might be associated with the destacking of the membranes at about 30 °C, observed by negative staining electron microscopy (data not shown) and consequent changes in the macroorganization of the thylakoid membranes.

Acknowledgements

This work has been supported in part by grants by the Hungarian Fund for Basic Research (OTKA K 63252) and by EU FP6 MC RTN (INTRO2) to G.G.; S.K. received Sandwich-Programme fellowship from Wageningen University, The Netherlands. The authors wish to thank to Sergey Laptenok for the help with the analysis of the time-resolved fluorescence measurements.

References

[1]Nash D., M. Miyao, N. Murata, Heat inactivation of oxygen evolution in Photosystem II particles and its acceleration by chloride depletion and exogenous manganese, *Biochim. Biophys. Acta* 807 (1985) 127-133.
[2]Tang Y., X. Wen, Q. Lu, Z. Yang, Z. Cheng, C. Lu, Heat stress induces an aggregation of the light-harvesting complex of Photosystem II in spinach plants, *Plant Physiol*. 143 (2007) 629-638.
[3]Mohanty P., J.S.S. Prakash, Elevated temperature treatment induced alteration in thylakoid membrane organization and energy distribution between the two photosystems in *Pisum sativum*, *Z. Naturforsch*. 57c (2002) 836-842.
[4]Caffarri S. , S. Frigerio, P.G. Righetti, Differential accumulation of Lhcb gene products inthylakoid membranes of *Zea mays* plants grown under contrasting light and temperature conditions, *Proteomics* 5 (2005) 758-768.
[5]Chapman D.J., J. De-Felice, J. Barber, Growth temperature effects on thylakoid membrane lipid and protein content of pea chloroplasts, *Plant Physiol*. 72 (1983) 225-228.
[6]Páli T., G. Garab, L.I. Horváth, Z. Kóta, Functional significance of the lipid-protein interface in photosynthetic membranes, *Cell. Mol. Life Sci.* 60 (2003) 1591-1606.
[7]Gräber P., H.T. Witt, M. Avron, The effect of temperature on flash induced transmembrane currents in choroplasts of spinach, in: *Proccedings of the Third International Congress on Photosynthesis*, Avron, M. (Ed.) Rehovot, Elsevier, Amsterdam (1974) pp. 951-956.
[8]Yamamoto Y., M. Nishimura, Characteristics of light-induced 515-nm absorbance change in spinach chloroplasts at lower temperatures I. Participation of structural changes of thylakoid membranes in light-induced 515-nm absorbance change, *Plant Cell Physiol*. 18 (1977) 55-66.
[9]Havaux M., F. Tardy, J. Ravenel, D. Chanu, P. Parot, Thylakoid membrane stability to heat stress studied by flash spectroscopic measurements of the electrochromic shift in intact potato leaves: influence of the xanthophyll content, *Plant Cell & Env*. 19 (1996) 1359-1368.
[10]McEvoy F.A., W.S. Lynn, The effects of temperature on photophosphorylation and on the two forms of cytochrome 559 in subchloroplast particles, *Arch. Biochem. Biophys*. 150 (1972) 632-635.
[11]Nolan W.G., R.M. Smillie, Temperature-induced changes in Hill activity of chloroplasts isolated from chilling-sensitive and chilling-resistant plants, *Plant Physiol*. 59 (1977) 1141-1145.
[12]Inoue H., Break points in Arrhenius plots of Hill reaction of spinach chloroplast fragments in the temperature range from -25 to 25 °C, *Plant Cell Physiol*. 19 (1978) 355-363.
[13]Nolan W.G., The effect of temperature on electron transport activities of isolated chloroplasts, *Plant Physiol*. 66 (1980) 234-237.
[14]Nolan W.G., Effect of temperature on proton efflux from isolated chloroplast thylakoids, *Plant Physiol.* 67 (1981) 1259-1263.
[15]Yamamoto Y., Y.M. Nishimura, Characteristics of light-induced H^+ transport in spinach chloroplast at lower temperatures. I. Relationship between H^+ transport and

physical changes of the microenvironment in chloroplast membranes, *Plant Cell Physiol*. 17 (1976) 11-16.
[16]Shneyour A., J.K. Raison, R.M. Smillie, The effect of temperature of the rate of photosynthetic electron transfer in chloroplasts of chilling-sensitive and chilling-resistant plants, *Biochim. Biophys. Acta* 292 (1973) 152-161.
[17]Raison J.K, The influence of temperature-induced phase changes on the kinetics of respiratory and other membrane-associated enzyme systems. *J. Bioenerg*. 4 (1973) 285-309.
[18]Torres-Pereira J., R. Mehlhorn, A.D. Keith, L. Packer, Changes in membrane lipid structure of illuminated chloroplasts: studies with spin-labeled and freeze-fractured membranes, *Arch. Biochem. Biophys*. 160 (1974) 90-99.
[19]Jursinic P., Govindjee, Temperature dependence of delayed light emission in the 6 to 340 microsecond range after a single flash in chloroplasts, *Photochem. Photobiol*. 26 (1977) 617-628.
[20]Carlberg I., S. Bingsmark, F. Vennigerholz, U.K. Larsson, B. Andersson, Low temperature effects on thylakoid protein phosphorylation and membrane dynamics, *Biochim. Biophys. Acta* 1099 (1992) 111-117.
[21]Goss R., M. Lohr, D. Latowski, J. Grzyb, A. Vieler, C. Wilhelm, K. Strzałka, Role of hexagonal structure-forming lipids in diadinoxanthin and violaxanthin solubilization and de-epoxidation, *Biochemistry* 44 (2005) 4028-4036.
[22]Szilágyi A., M. Sommarin, H.-E. Åkerlund, Membrane curvature stress controls the maximal conversion of violaxanthin to zeaxanthin in the violaxanthin cycle - influence of α-tocopherol, cetylethers, linolenic acid, and temperature, *Biochim. Biophys. Acta* 1768 (2007) 2310-2318.
[23]Vieler A., H.A. Scheidt, P. Schmidt, C. Montag, J.F. Nowoisky, M. Lohr, C. Wilhelm, D. Huster, R. Goss, The influence of phase transitions in phosphatidylethanolamine models on the activity of violaxanthin de-epoxidase, *Biochim. Biophys. Acta* 1778 (2008) 1027-1034.
[24]Szilágyi A., E. Selstam, H.-E. Åkerlund, Laurdan fluorescence spectroscopy in the thylakoid bilayer: The effect of violaxanthin to zeaxanthin conversion on the galactolipid dominated lipid environment, *Biochim. Biophys. Acta* 1778 (2008) 348-355.
[25]Kirchhoff H., S. Horstmann, E. Weis, Control of the photosynthetic electron transport by PQ diffusion microdomains in thylakoids of higher plants, *Biochim. Biophys. Acta* 1459 (2000) 148-168
[26]Kirchhoff H., U. Mukherjee, H.-J. Galla, Molecular architecture of the thylakoid membrane: Lipid diffusion space for plastoquinone, *Biochemistry* 41 (2002) 4872-4882.
[27]Allen J.F., J. Forsberg, Molecular recognition in thylakoid structure and function, *Trends Plant Sci*. 6 (2001) 317-326.
[28]Tikkanen M., M. Nurmi, M. Suorsa, R. Danielsson, F. Mamedov, S. Styring , E.M. Aro, Phosphorylation-dependent regulation of excitation energy distribution between the two photosystems in higher plants, *Biochim. Biophys. Acta* 1777 (2008) 425-432.
[29]Williams W.P., P.J. Quinn, The phase behavior of lipids in photosynthetic membranes, *J. Bioenerg. Biomembr*. 19 (1987) 605-624.
[30]Gounaris K., A.P.R. Brain, P.J. Quinn, W.P. Williams, Structural and functional changes associated with heat-induced phase-separations of non-bilayer lipids in chloroplast thylakoid membranes, *FEBS Lett*. 153 (1983) 47-52.

[31]Kóta Z., B. Szalontai, M. Droppa, G. Horváth, T. Páli, Fourier transform infrared and electron paramagnetic resonance spectroscopic studies of thylakoid membranes, *J. Mol. Str*. 480-481 (1999) 395-400.
[32]Cseh Z., S. Rajagopal, T. Tsonev, M. Busheva, E. Papp, G. Garab, Thermooptic effect in chloroplast thylakoid membranes. Thermal and light stability of pigment arrays with different levels of structural complexity, *Biochemistry* 39 (2000) 15250-15257.
[33]Epand R.M., Functional roles of non-lamellar forming lipids, *Chem. Phys. Lipids* 81 (1996) 101-104.
[34]de Kruijff B., Lipids beyond the bilayer, *Nature* 386 (1997) 129-130.
[35]W.P. Williams, The physical properties of thylakoid membrane lipids and their relation to photosynthesis, in: Advances in *Photosynthesis. Lipids in Photosynthesis*, Siegenthaler, P-A., Murata, N. (Eds), Kluwer Academic Publishers (1998) 103-118.
[36]Garab G., K. Lohner, P. Laggner, T. Farkas, Self-regulation of the lipid content of membranes by non-bilayer lipids: a hypothesis, *Trends Plant Sci*. 5 (2000) 489-494.
[37]Vigh L., I. Horváth, P.R. van Hasselt, P.J.C. Kuiper, Effect of frost hardening on lipid and fatty-acid composition of chloroplast thylakoid membranes in two wheat-varieties of contrasting hardiness, *Plant Physiol*. 79 (1985) 756-759.
[38]J.L. Harwood, Involvement of chloroplast lipids in the reaction of plants submitted to stress, in: *Lipids in Photosynthesis: Structure, Function and Genetics*, Siegenthaler, P.-A., Murata N. (Eds), Kluwer Academic Publisher (1998) pp. 287-302.
[39]Kirchhoff K., W. Haase, S. Wegner, R. Danielsson, R. Ackermann, P.-A. Albertsson, Low-light-induced formation of semicrystalline Photosystem II arrays in higher plant chloroplasts, *Biochemistry* 46 (2007) 11169-11176.
[40]Kóta Z., B. Szalontai, M. Droppa, G. Horváth, T. Páli, The formation of an inverted hexagonal phase from thylakoid membranes upon heating, *Cell. Mol. Biol. Lett*. 7 (2002) 126-128.
[41]G.A. Semenova, The relationship between the transformation of thylakoid acyl lipids and the formation of tubular lipid aggregates visible on fracture faces, *J. Plant Physiol*. 155 (1999) 669-677.
[42]Gounaris K., A. Sen, A.P.R. Brain, P.J. Quinn, W.P. Williams, The formation of non-bilayer structures in total polar lipid extracts of chloroplast membranes, *Biochim. Biophys. Acta* 728 (1983) 129-139.
[43]Krumova S.B., C. Dijkema, P. de Waard, H.V. As, G. Garab, H.van Amerongen, Phase behavior of phosphatidylglycerol in spinach thylakoid membranes as revealed by ^{31}P-NMR, *Biochim. Biophys. Acta* 1778 (2008) 997-1003.
[44]Williamson P., K. Mattocks, R. Schlegel, Merocyanine 540, a fluorescent probe sensitive to lipid packing, *Biochim. Biophys. Acta* 732 (1983) 387-393.
[45]Yu H., S.-W. Hui, Merocyanine 540 as a probe to monitor the molecular packing of phopshatidylcholine: a monolayer epifluorescence microscopy and spectroscopy study, *Biochim. Biophys. Acta* 1107 (1992) 245-154.
[46]Bernik D.L., E.A. Disalvo, Gel state surface-properties of phosphatidylcholine liposomes as measured with merocyanine 540, *Biochim. Biophys. Acta* 1146 (1993) 169-177.
[47]Langner M., S.W. Hui, Merocyanine interaction with phosphatidylcholine bilayers, *Biochim. Biophys. Acta* 1149 (1993) 175-179.

[48]Stillwell W., S.R. Wassail, A.C. Dumaual, W.D. Ehringer, C.W. Browning, L.J. Jenski, Use of merocyanine (MC540) in quantifying lipid domains and packing in phospholipid vesicles and tumor cells, *Biochim. Biophys. Acta* 1146 (1993) 136-144.
[49]Bernik D., E. Tymczyszyn, M.E. Daraio, R.M. Negri, Fluorescent dimers of merocyanine 540 (MC540) in the gel phase of phosphatidylcholine liposomes, *Photochem. Photobiol*. 70 (1999) 40-48.
[50]Langner M., S.W. Hui, Merocyanine 540 as a fluorescence indicator for molecular packing stress at the onset of lamellar-hexagonal transition of phosphatidylethanolamine bilayers, *Biochim. Biophys. Acta* 1415 (1999) 323-330.
[51]Wilson-Ashworth H.A., Q. Bahm, J. Erickson, A. Shinkle, M.P. Vu, D. Woodbury, J.D. Bell, Differential detection of phospholipid fluidity, order, and spacing by fluorescence spectroscopy of bis-pyrene, prodan, nystatin, and merocyanine 540, *Biophys. J*. 91 (2006) 4091-4101.
[52]Valinsky J.E., T.G Easton, E. Reich, Merocyanine 540 as a fluorescent probe of membranes: selective staining of leukemic and immature hemopoietic cells, *Cell* 13 (1978) 487-499.
[53]Schlegel R.A., B.M. Phelps, Binding of Merocyanine 540 to normal and leukemic erythroid cells , *Cell* 20 (1980) 321-328.
[54]Williamson P.L., W.A. Massey, B.M. Phelps, R.A. Schlegel, Membrane phase state and the rearrangement of hematopoietic cell surface receptors, *Mol. Cell Biol*. 1 (1981) 128-135.
[55]Phelps B.M., P. Williamson, R.A. Schlegel, Lecitin induced rearrangement of an immature hematopoietic cell surface marker, *J. Cell Physiol*. 110 (1982) 245-248.
[56]Schlegel R.A., B.M. Phelps, G.P. Cofer, P. Williamson, Enucleation eliminates a differentiation-specific marker from normal and leukemic murine erythroid cells, *Exp. Cell Res*. 139 (1982) 321-328.
[57]Waggoner A.S., A. Grinvald, Mechanisms of rapid optical changes of potential sensitive dyes, *Ann. N. Y. Acad. Sci*. 303 (1978) 217-241.
[58]Lelkes P.I., I.R. Miller, Perturbations of membrane structure by optical probes: I. Location and structural sensitivity of merocyanine 540 bound to phospholipid membranes, *J. Membr. Biol*. 52 (1980) 1-15.
[59]Verkman A.S., M. Frosch, Temperature-jump studies of merocyanine-540 relaxation kinetics in lipid bilayer membranes, *Biochemistry* 24 (1985) 7117-7122.
[60]Sikurova L., R. Frankova, Temperature induced changes in monomer-dimer distribution of merocyanine 540 in dimyristoyl lecithin liposomes, *Studia Biophys*. 140 (1991) 21-28.
[61]Csiszar A., E. Klumpp, A. Bota, K. Szegedi, Effect of 2,4-dichlorophenol on DPPC/water liposomes studied by X-ray and freeze-fracture electron microscopy, *Chem. Phys. Lipids* 126 (2003) 155-166.
[62]D. Arnon, Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris, *Plant Physiol*. 24 (1949) 1-15.
[63]van Oort B., A. Amunts, J.W. Borst, A. van Hoek, N. Nelson, H. van Amerongen, R. Croce, Picosecond fluorescence of intact and dissolved PSI-LHCI crystals, *Biophys. J.* (2008) *in press*
[64]Novikov E.G., A. van Hoek, A.J.W.G. Visser, J.W. Hofstraat, Linear algorithms for stretched exponential decay analysis, *Opt. Commun*. 166 (1999) 189-198.

[65]Digris A.V., V.V. Skakun, E.G. Novikov, A.van Hoek, A. Claiborne, A.J.W.G. Visser, Thermal stability of a flavoprotein assessed from associative analysis of polarized time-resolved fluorescence spectroscopy, *Eur. Biophys. J.* 28 (1999) 526-531.
[66]Visser N.V., A.H. Westphal, A. van Hoek, C.P.M. van Mierlo, A.J.W.G. Visser, H. van Amerongen, Tryptophan-tryptophan energy migration as a tool to follow apoflavodoxin folding , *Biophys. J.* 95 (2008) 2462-2469.
[67]Onganer Y., E.L. Quitevis, Dynamics of Merocyanine-540 in model biomembranes – photoisomerization studies in small unilamellar vesicles, *Biochim. Biophys. Acta* 1192 (1994) 27-34.
[68]Bursing H., S. Kundu, P. Vohringer, Solvation dynamics at aqueous lipid membrane interfaces explored by temperature-dependent 3-pulse-echo peak shifts: Influence of the lipid polymorphism, *J. Phys. Chem*. B 107 (2003) 2404-2414.
[69]Garcia D.A., M.A. Perillo, Effects of flunitrazepam on the L-H_{II} phase transition of phosphatidylethanolamine using merocyanine 540 as a fluorescent indicator, *Coll. Surf*. B 37 (2004) 61-69.
[70]Toombes G.E.S., A.C. Finnefrock, M.W. Tate, S.M. Gruner, Determination of L–H_{II} phase transition temperature for 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine, *Biophys. J*. 82 (2002) 2504-2510.
[71]Mandal D., S.K. Pal, D. Sukul, K. Bhattacharyya, Photophysical processes of merocyanine 540 in solutions and in organized media, *J. Phys. Chem*. A 103 (1999) 8156-8159.
[72]Barber J., R.C. Ford, R.A.C. Mitchell, P.A. Millner, Chloroplast thylakoid membrane fluidity and its sensitivity to temperature, *Planta* 161 (1984) 375-380.
[73]Sato C., J. Nakamura, Y. Nakamaru, A chemometric approach to the estimation of the absorption spectra of dye probe merocyanine 540 in aqueous and phopsholipid environments, *J. Biochem*. 127 (2000) 603-610.
[74]Kaschny P., F.M. Goñi, The components of Merocyanine-540 absorption spectra in aqueous, micellar and bilayer environments, *Eur. J. Biochem*. 207 (1992) 1085-1091.
[75]Ehrenberg B., E. Pevzner, Spectroscopic properties of the potentiometric probe merocyanine-540 in solutions and in liposomes, *Photochem. Photobiol*. 57 (1993) 228-234.
[76]Sikurova L., B. Cunderlikova, J. Turisova, I. Waczulikova, Interaction of merocyanine-540 with cations of physiological solutions, *Anal. Chim. Acta* 303 (1995) 79-83.
[77]Aramendia P.F., M. Krieg, C. Nitsch, E. Bittersmann, S.E. Braslavsky, The photophysics of merocyanine 540. A comparative study in ethanol and in liposomes, *Photochem. Photobiol*. 48 (1988) 187-194.
[78]Bakaltcheva I., W.P. Williams, J.M. Schmitt, D.K. Hincha, The solute permeability of thylakoid membranes is reduced by low concentrations of trehalose as a co-solute, *Biochim. Biophys. Acta* 1189 (1994) 38-44.
[79]Mateasik A., L. Sikurova, D. Chorvat, Interaction of merocyanine 540 with charged membranes, *Bioelectrochemistry* 55 (2002) 173-175.
[80]Barber J., Membrane surface charges and potentials in relation to photosynthesis, *Biochim. Biophys. Acta* 594 (1980) 253–308.
[81]Nakatani H.Y., J. Barber, Further studies of the thylakoid membrane surface charges by particle electrophoresis, *Biochim. Biophys. Acta* 591 (1980) 82–91.

[82]Dobrikova A.G., I. Petkanchin, S.G. Taneva, Temperature-induced changes in the surface electric properties of thylakoids and photosystem II membrane fragments, *Coll. Surf*. A 209 (2002) 185-192.
[83]Dobrikova A.G., R.M. Morgan, A.G. Ivanov, E. Apostolova, I. Petkanchin, N.P.A. Huner, S.G. Taneva, Electric properties of thylakoid membranes from pea mutants with modified carotenoid and chlorophyll–protein complex composition, *Photosynth. Res*. 65 (2000) 165-174.
[84]Dobrikova A.G., Z. Várkonyi, S.B. Krumova, L. Kovács, G.K. Kostov, S.J. Todinova, M.C. Busheva, S.G. Taneva, G. Garab, Structural Rearrangements in chloroplast thylakoid membranes revealed by differential scanning calorimetry and circular dichroism spectroscopy. Thermo-optic effect, *Biochemistry* 42 (2003) 11272-11280.

Chapter 5

The Role of DGDG in the Overall Organization and Thermal Behavior of Thylakoid Membranes

S. B. Krumova, S. Laptenok, L. Kovács, T. Tóth, A. van Hoek, G. Garab, H. van Amerongen

submitted for publication

The role of DGDG in the overall organization and thermal behavior of thylakoid membranes

We investigated the effects of digalactosyl-diacylglycerol (DGDG) on the organization of thylakoid membranes, using wild-type *Arabidopsis* and the DGDG-deficient mutant, *dgd1*. Circular-dichroism and picosecond (micro)spectroscopy measurements reveal that DGDG-deficiency hampers the formation of the chirally organized macrodomains and slows down the excitation energy trapping by the reaction centers, and it also alters the lipid packing, as shown by time-resolved measurements using the lipophylic fluorescence probe, Merocyanine 540. Electrochromic absorbance transients reveal that *dgd1* thylakoids are more susceptible to heat-induced injuries. In general, *dgd1* thylakoids possess 5-10 °C lower thermal stability than the wild-type, as revealed by the disassembly of the chiral macrodomains and chlorophyll *a*-containing complexes (presumably the cores of the photosystems) at elevated temperatures, the temperature dependence of the fluorescence lifetimes and the amount of Merocyanine 540 found in the membrane bilayer. It is concluded that DGDG plays important roles in the overall organization of thylakoid membranes especially at elevated temperatures.

1. Introduction

Digalactosyl-diacylglycerol (DGDG) is one of the main bilayer-prone lipid species in thylakoid membranes of higher plants, constituting about 30 % of the total lipid content [1]. Its structural importance is well established for several (super)complexes of the photosynthetic machinery. It has been shown to be bound to photosystem II (PSII) [2,3], it forms hydrogen bonds with tyrosine in PSII [4] and it is important for the binding of extrinsic proteins required for the stabilization of the oxygen-evolving complex [5]. DGDG was resolved in the crystal structure of the major light-harvesting complex of PSII (LHCII) – the head groups of two DGDG molecules are simultaneously hydrogen bonded to the lumenal-surface amino acids from two adjacent LHCII trimers, functioning as a bridge [6,7]. DGDG appears to be required for the formation of 2D and 3D crystals of LHCII [8]. The functional significance of this lipid was studied employing a genetic approach – a

mutant of *Arabidopsis* (*dgd1*) was generated which lacks more than 90% of the DGDG content of the membranes [9]. This results in a changed chloroplast ultrastructure – the thylakoid membranes are highly curved and displaced from the central stroma area towards the envelope, the length of both grana and stroma membranes and the total length of the thylakoid membrane are increased in the mutant [9]. This is followed by a decrease of the total chlorophyll (Chl) content on a fresh weight basis of about 25 %, in the Chl *a*/*b* ratio by about 20 % and a 1.7 times higher xanthophyll content [10]; however the amount of metabolic intermediates (products of the dark reactions of photosynthesis) were found to be indistinguishable from those of the wild type [11]. Ivanov et al. [12] have established that the DGDG deficiency has a larger effect on photosystem I (PSI) structure than on PSII: the relative abundance of the reaction center protein of PSII (PsbA) and the light-harvesting proteins associated with PSII (Lhcb1, Lhcb2, Lhcb3 and Lhcb5) are not changed in the mutant, whereas the reaction center proteins of PSI (PsaA and PsaB) are significantly reduced (by about 50 %) and the abundance of the PsaC, PsaL, PsaH subunits is also substantially decreased compared to the wild type (WT) [12]. Moreover, compared to the WT, in *dgd1* PSI has been shown to be less stable against treatment with chaotropic salts and the light-harvesting antenna complexes of PSI (LHCI) could more easily be detached from the core complex [13].

The modified protein content in *dgd1* is accompanied by differences in various functional parameters. For example, the amount of non-photochemical quenching in *dgd1* is increased at the expense of PSII photochemistry [11]; the linear electron transport between the two photosystems is restricted, the plastoquinol pool is more reduced, PSI has an increased capacity for cyclic electron transfer [12] and the capacity for state transitions is reduced by 50 % as compared to the WT [12]. Another lipid mutant of *Arabidopsis* (*mgd1-1*, lacking about 42 % of the total MGDG content) was characterized with even lower chlorophyll content (only about 50 % of the normal amount of chlorophyll) and disrupted plastid ultrastructure [14]. Although being less well characterized with respect to its photosynthetic protein content and function, recent results have shown that it exhibits inefficient operation of the xanthophyll cycle and increased conductivity of the thylakoid membrane at high light intensities [15].

Hendrickson et al. [16] have proposed that the effects of the reduced DGDG content in thylakoids are due to the global physical properties of the thylakoid membrane. Indeed, the ratio of non-bilayer/bilayer lipids is significantly higher in the mutant (4.5; for comparison, in the WT this ratio is 1.9) [12]. This might affect the physical state of the lipid membrane, which in turn might influence the membrane permeability, protein content and macro-organization of the membranes as well as their structural flexibility [17,18]. In this work we study the effect of the significantly reduced DGDG content in the *dgd1* mutant on some of the global properties of the thylakoid membranes – protein organization and thermal stability, membrane packing and permeability. Our results reveal a different organization and decreased thermal stability of the protein macroaggregates. Chlorophyll *a*-binding proteins also degrade faster in the mutant than in the wild type (WT). This is accompanied by modified excitation energy trapping. Furthermore the lipid packing in the thylakoid membrane appears to be different for the WT and *dgd1*, and the membrane permeability is more sensitive to elevated temperatures in *dgd1* than in the WT.

2. Materials and methods

Plant material

Both the WT *Arabidopsis thaliana* ecotype *Columbia* and the *dgd1* mutant were grown at 20/18 °C (day/night), at light intensity of 200-250 W/m^2, about 70 % humidity and a day/night cycle of 16/8h.

Isolation of thylakoid membranes

Dark-adapted leaves were homogenized in a medium containing 50 mM Tricine (pH 7.5), 400 mM sorbitol, 5 mM $MgCl_2$ and 5 mM KCl; the suspension was filtered through 4 layers of cheese cloth and centrifuged for 4 min at 4000 x g. The chloroplasts were osmotically shocked in a hypotonic medium containing 50 mM Tricine (pH 7.5), 5 mM $MgCl_2$ and 5 mM KCl, and centrifuged for 5 min at 6000 x g. After washing in the same medium supplemented with 400 mM sorbitol, the pellet was resuspended in this isotonic medium and used for the fluorescence and circular-dichroism measurements.

Circular-dichroism measurements

Circular dichroism (CD) was measured on isolated thylakoid membranes between 400 and 800 nm with a Jasco J-715 spectropolarimeter. The Chl content of the samples was

adjusted to 15 μg/ml, the optical pathlength of the cell was 1 cm. The spectra were recorded in 1 nm steps with an integration time of 2 s, a band-pass of 2 nm, and scanning speed of 100 nm/min. The samples were sequentially thermostated for 10 min at each temperature starting from 3 °C up to 80 °C. Each experiment was repeated five times with freshly isolated thylakoids.

Fluorescence lifetime imaging microscopy

Fluorescence lifetime imaging microscopy (FLIM) was performed *in vivo* on detached leaves of WT and *dgd1*, with the setup described previously [19]. In short, two-photon excitation pulses (860 nm, 150 fs pulse duration, 76 MHz repetition rate) were focused into the sample with a 60x water immersion objective lens (CFI Plan Apochromat, numerical aperture 1.2). Fluorescence was detected via non-descanned single photon counting detection, through two band-pass filters of 700 nm (75 nm width) (HQ700/75 Chroma, Vermont, USA). Images of 64x64 pixels were obtained, with 1024 time channels of 12 ps. The fluorescence was collected for 30 min; low excitation power (of the order of 600 μW average power) was used in order to keep the reaction centers open and to minimize photodamage. The fluorescence decays were analyzed by software provided by Becker & Hickl (SPCImage). All measurements were performed at 22 °C. The plants were dark-adapted at 20 °C for 30 min before the measurements.

Time-correlated single photon counting

Time-correlated single photon counting (TCSPC) technique was used to perform time-resolved fluorescence measurements with a setup described earlier [19]. For the fitting procedure, the dynamic instrumental response of the experimental setup was recorded using the fast and single-exponential fluorescence decay (6 ps) of the reference compound pinacyanol in methanol [20]. Data analysis was performed using the computer program described earlier [21,22]. The fit quality was evaluated from χ^2, and from plots of the weighted residuals and the autocorrelation thereof [23]. Typical values of χ^2 were 1.0-1.1

For Chl *a* fluorescence measurements the samples were excited at 470 nm and the emission was collected using a Balzers 688 nm interference filter with a bandwidth of 10 nm. The samples were sequentially thermostated for 10 min at each temperature starting from 7 °C up to 70 °C. The decay curves were analyzed by a four-exponential model and for each decay trace the average lifetime (τ_{ave}) was calculated by the formula:

$$\tau_{ave} = \sum_{i=1}^{n} \alpha_i . \tau_i$$

τ being the fluorescence lifetime and α – pre-exponential factor proportional to the fractional population, with $\sum_{i=1}^{n} \alpha_i = 1$. For the τ_{ave} calculation the minor contribution (typically about 1-2 %) of a component with a lifetime above 1 ns, originating from closed reaction centers, was not taken into account. The mean value of τ_{ave} and its standard error presented in this paper were determined from five different decay curves measured on different samples.

For Merocyanine 540 (MC540, purchased from Sigma-Aldrich) fluorescence measurements the excitation wavelength was set to 570 nm and the emission was collected between 610 and 630 nm using a Schott OG 610 nm (3 mm) cut-off filter and a Balzers K60 interference filter. MC540 was added from a 1 mM ethanol stock solution (to a final concentration of 0.2 μM) to a suspension of thylakoid membranes (containing 20 μg Chl/ml) and incubated for 30 min before the experiments. During this time the sample was gently stirred on ice and kept in the dark. During the measurements the samples were sequentially thermostated at increasing discrete temperatures, between 7 °C and 45 °C, for 15 min at each temperature. For the analysis of the fluorescence decay a three-exponential model was used – MC540 can be located in three types of environments (water, on the surface of the thylakoid membrane or incorporated deep in the hydrophobic lipid bilayer), each characterized with a specific fluorescence lifetime [24,25]. Since these three types of environments are the same for WT and *dgd1*, the MC540 fluorescence lifetimes for the different WT or *dgd1* samples were linked during the fitting procedure (resulting, at a given temperature, in equal lifetime values for all samples) whereas their relative amplitudes were left free. In this way the changes in the distribution of MC540 over the different environments can be followed for WT and *dgd1*.

Electrochromic absorbance transients

Electrochromic absorbance changes (ΔA515) induced by saturating single turnover flashes, were measured at 515 nm, in a setup described earlier [26]. The plants used for the measurements were dark-adapted at 20 °C for 30 min and detached leaves of WT and *dgd1* were infiltrated with water and incubated for 10 min at different temperatures and then

measured at 25 °C; 64 kinetic traces were collected with a repetition rate of 1 s^{-1} and averaged; the duration of the flashes was about 5 μs; the time constant of the measurements was adjusted to 100 μs. The measurements were repeated five times with leaves from different plants.

3. Results and discussion

Pigment-protein complexes – (macro-)organization, excitation energy migration and trapping

Circular dichroism

CD spectroscopy in the visible range is a valuable tool for probing the molecular architecture of the complexes and supercomplexes and their macro-organization in the membrane system [27]. The CD spectra of thylakoid membranes isolated from WT and *dgd1* are presented in Fig. 1a.

Two types of CD bands are relevant for the study of thylakoid membranes:

(i) excitonic bands which originate from short-range (nanometer) excitonic interactions between pigments within a pigment-protein complex or on adjacent complexes [28,29,30], and can be used for testing the intactness of individual complexes or supercomplexes. Such interactions give rise to a conservative band structure – i.e., the positive and negative bands of the split spectrum have equal areas. In a system as complex as the thylakoid membrane a variety of excitonic bands is superimposed on top of each other. These are difficult to discriminate and here we shall use only two characteristic bands, at around 650 and 440 nm. It has been established that the (-)650 nm band originates from Chl *b* and is regarded as a fingerprint of the LHCII complexes [31,32], while the CD bands that appear between 400 and 450 nm mainly originate from Chl *a* [33].

(ii) Ψ-type CD bands - high-intensity bands, originating from long-range order (hundreds of nanometers) of the chromophores in chirally-organized macroarrays. They are very intense and "anomalously shaped" [27]; in thylakoids and isolated LHCII-aggregates they appear at around (+)685, (-)671 nm and (+)505 nm [34,35]; these bands are also associated with long tails outside the principal absorbance bands, which originate from differential scattering of the left and right circularly polarized light [27]. Ψ-type bands correlate with the macro-organization of the main Chl *a/b* light harvesting complexes, e.g.

in LHCII-only domains, as indicated by correlations between the intensity of these bands and the LHCII-content of the sample (e.g. [33,36]). The arrays of PSII-supercomplexes might also contribute to the Ψ-type CD signal. For example, in a mutant lacking one of the minor light-harvesting complexes, namely CP24, the macro-organization of the PSII-supercomplexes is modified as compared to WT. This results in the loss of the main Ψ-type band in the red at around (+)690 nm [37].

As can be seen from Fig. 1a at 25 °C the amplitudes of the excitonic signal of Chl *b*, at around (-)650 nm, are approximately identical for both WT and *dgd1*. Also the CD signals between 400 and 450 nm are not affected significantly by the deficiency of DGDG. In contrast, the intensities of the main Ψ-type CD bands between 660 and 700 nm and around 505 nm are substantially smaller for *dgd1* (Fig. 1a). This might either be due to a smaller size of the chiral macrodomains or to a different organization of the complexes. It should be noted that DGDG has been found to be required for the formation of ordered 3D crystals of LHCII [8]; the present data strongly suggest that also in the thylakoid membranes DGDG modulates the macroorganization of the main light-harvesting complexes of PSII.

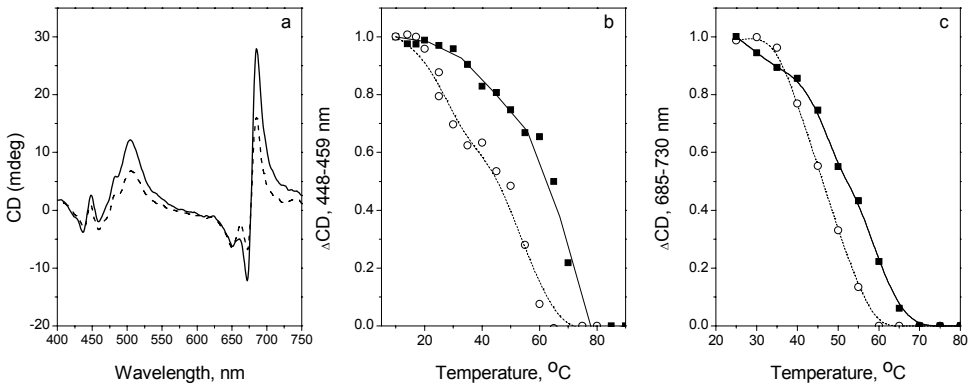


Figure 1. (a) CD spectra of thylakoid membranes isolated from WT (solid line) and *dgd1* (dashed line) leaves. The spectra are measured at 25 °C at identical Chl concentrations (15 μg/ml). Temperature dependence of the 448-459 nm (b) and 685-730 nm (c) CD signals for the WT (■) and *dgd1* (○). The lines (solid for the WT and dashed for *dgd1*) serve as a guide to the eye.

The amplitudes of the different excitonic and Ψ-type CD bands are determined by using reference wavelengths, e.g. by the subtraction of the maximum intensity of the positive signal at a specified wavelength and the corresponding minimum of the negative signal (for example the amplitude of the 448-459 nm band is obtained by subtracting the CD at 459 nm from the signal at 448 nm). For strongly overlapping CD bands, such as the Ψ-type CD band at 685 nm and the excitonic band at 650 nm, the amplitude is estimated by subtracting a reference zero-value CD signal ($CD_{(685-730)}$ and $CD_{(610-650)}$). The thermal destabilization of different protein complexes is monitored via the amplitudes of their corresponding CD bands. The (-)650 nm Chl *b* excitonic band exhibits the same temperature dependence for WT and *dgd1* with a transition temperature (T_m) of ~60 °C (Table 1, T_m is defined as the temperature at which the intensity of the CD band is decreased by 50 % of its value at 25 °C, similarly to Cseh et al. [38]). This shows that the molecular architecture of LHCII is not significantly affected by the mutation. On the other hand, the mutation significantly affected thermal stability of the Chl *a* excitonic bands at around 450 nm (determined either as $CD_{(448-438)}$ or $CD_{(448-459)}$) – the T_m values (Table 1) are lower by ~6 °C in the mutant (54±1 °C for 448-459 nm and 50±1 °C for 448-438 nm bands) than in the WT (59±2 °C and 57±1 °C, respectively). The thermal behavior for the $CD_{(448-459)}$ band is presented in Fig. 1b. The $CD_{(448-438)}$ and $CD_{(448-459)}$ excitonic bands most probably originate from the core complexes of PSII and/or PSI which bind only Chl *a* [39,40,41] and thus their thermal behavior indicates a lower stability of these complexes in the mutant than in the WT. Studies of the thermal stability of isolated PSI and PSII core complexes would be advantageous in clarifying this point.

The Ψ-type signal ($CD_{(685-730)}$) also exhibits a different temperature dependence for WT and *dgd1* (Fig. 1c). The transition temperature for this band is 54±2 °C for the WT, whereas for *dgd1* it is 48±1 °C (Table 1), showing that the chiral macro-organization in *dgd1* is characterized by a lower thermal stability.

Table 1. Transition temperatures (T_m) of selected CD bands or band pairs for WT and *dgd1* thylakoid membranes. The membranes were thermostated for 10 min at different temperatures in the range between 5 °C and 80 °C before recording the CD spectra at the given temperature; the amplitudes for the individual bands were calculated from the difference in the intensity at specific wavelengths (see also the text). T_m is defined as the temperature at which the intensity of the CD band is decreased by 50 % of its value at 25 °C.

CD signal	assignment	T_m, °C (WT)	T_m, °C (*dgd1*)
685-730 nm	Ψ-type	54±2	48±1
685-671 nm	Ψ-type	54±1	49±1
505-550 nm	Ψ-type	56±1	51±1
610-650 nm	Excitonic (Chl *b,* LHCII)	61±2	58±2
448-459 nm	Excitonic (Chl *a*)	59±2	54±1
448-438 nm	Excitonic (Chl *a*)	57±1	50±1

Chlorophyll a fluorescence

The functional activity of the photosystems was studied with the aid of Chl *a* fluorescence lifetime measurements. The assignment of the fluorescence lifetimes to particular protein complexes or macroassemblies is a rather complicated task for intact chloroplasts and isolated thylakoids where a large variety of complexes and supercomplexes co-exist. For example, most studies on whole chloroplasts and intact thylakoid membranes suggested average values for the trapping time in PSII between ~300 to ~500 ps (see e.g. [42-44]). For isolated BBY particles two major lifetimes of about 74-80 ps and 175-212 ps were observed [45,46], whereas three major decay times are found for PSI: 5-20 ps, 20-60 ps, 80-130 ps [20,47-51]. In the present paper only the average lifetimes (τ_{ave}) measured for WT and *dgd1* thylakoid membranes and intact leaves are compared.

The Chl *a* fluorescence decay curves were measured using two techniques: (i) *in vivo* FLIM measurements on detached leaves at room temperature (22 °C) and (ii) TCSPC measurements on isolated thylakoid membranes at different temperatures.

The FLIM images are plotted in Figs. 2a and b (WT), and c and d (*dgd1*). The bright spots in the intensity images (Figs. 2a and c) originate from distinct chloroplasts.

Their brightness is proportional to the intensity of the emission, the brightest chloroplasts being located in the focal plane, whereas the lower intensity pixels probably represent somewhat out-of-focus chloroplasts. The fluorescence decay traces recorded for each pixel were analyzed by a three-exponential model and their average lifetimes are plotted in Figs. 2b and d for the WT and *dgd1*, respectively. The sum of the decays recorded for all pixels in the image of WT and *dgd1* leaves is presented in Fig. 2e. The distribution histogram of the average lifetime is presented in Fig. 2f and clearly shows that it is longer in the mutant – the average fluorescence lifetime in the majority of the pixels of the WT-image is 180-220 ps, whereas for the *dgd1*-image it is about 250-300 ps.

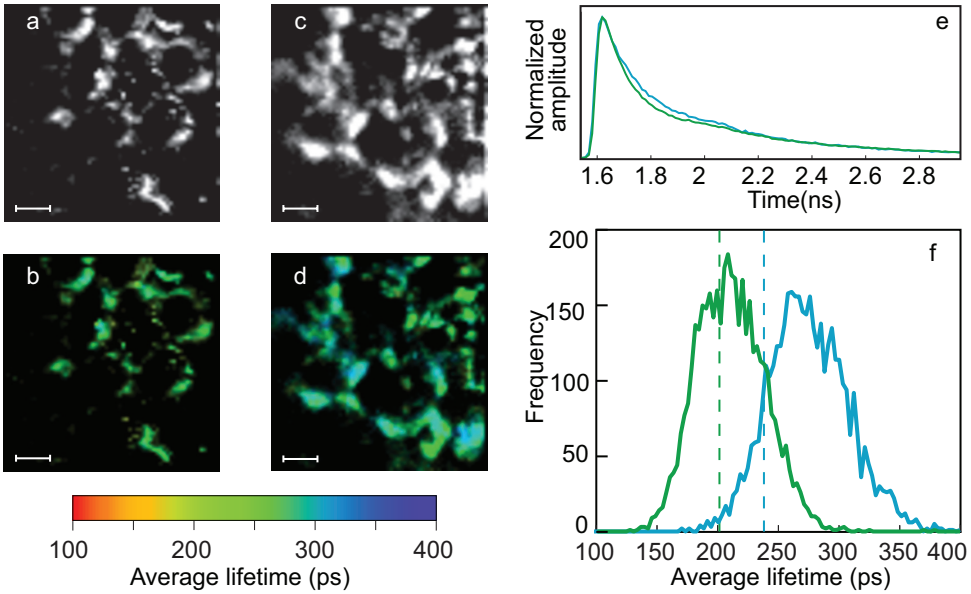


Figure 2. FLIM results on dark-adapted detached WT and *dgd1* leaves. The fluorescence images are shown in panel (a) for the WT and panel (c) for *dgd1*. The color-coded average fluorescence lifetime images are presented in panel (b) for the WT and panel (d) for *dgd1*. The scale bars are 20 μm. The decay traces, recorded for each pixel in the images were added and their sums are presented in panel (e) for the WT (green trace) and *dgd1* (blue trace). The distribution histograms of the average lifetimes, obtained from a total of 4096 pixels for each sample, plotted with 3 ps steps, are given in panel (f) (green curve for the WT and blue for *dgd1*). The dashed lines represent the average lifetime values for WT and *dgd1*, obtained for isolated thylakoid membranes by TCSPC at 25 °C.

The FLIM setup used can only be applied for measurements at 22 °C. In order to check the temperature dependence of the average Chl *a* fluorescence lifetime (τ_{ave}), it was determined for isolated intact thylakoid membranes using the TCSPC technique. The fluorescence decay curves for WT and *dgd1,* as determined by TCSPC, are shown in Fig. 3a and the parameters obtained from the fit are plotted as a table in the figure. At 25 °C the fitting analysis results in longer fluorescence lifetimes for *dgd1* than for WT, τ_{ave} = 202±5 ps for WT and 236±13 ps for *dgd1* (Fig. 3b); these values are similar to the ones determined with the FLIM technique (Fig. 2e). It must be noted that the temperature dependence of the average fluorescence lifetime is characterized by different phases (Fig. 3b); interestingly, these variations were less marked for *dgd1* than for the WT. Interpretation of these results is beyond the scope of the present study. The only aspect of the temperature dependence that we want to point out is the strong decrease of the average lifetime above 50 °C (reaching 83 ps at 65 °C). For *dgd1* the same sharp drop in τ_{ave} occurs at lower temperatures and begins at around 45 °C. The decrease of the average lifetimes at high temperatures is most probably related to the degradation of membrane proteins, as suggested by the observation that the CD bands at around 450 nm exhibit similar temperature dependences (cf. Table 1).

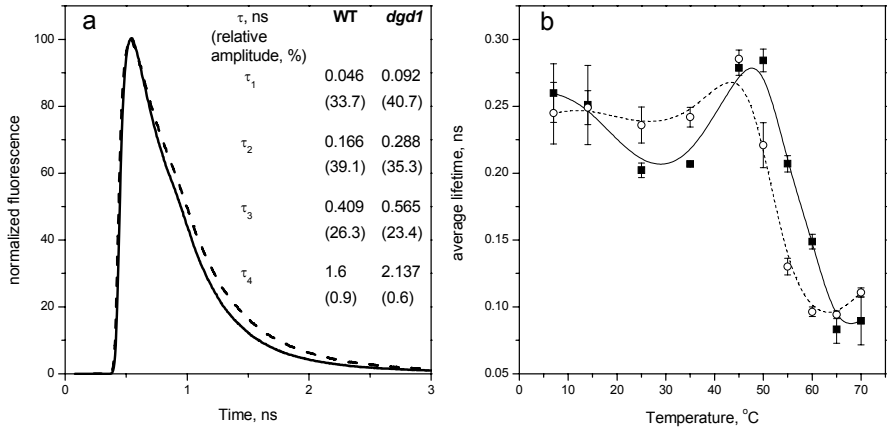


Figure 3. (a) Chlorophyll *a* fluorescence decay traces for isolated thylakoid membranes from WT (thick line) and *dgd1* (dashed line), recorded by TCSPC. The presented curves are the sums of five independent measurements on different preparations. The excitation wavelength is 430 nm, and the emission is recorded at 688 nm at 25 °C. The corresponding fitting results (fluorescence lifetimes (τ) and relative amplitudes, given in brackets) are also presented. (b) Temperature dependence of the average fluorescence lifetime for the WT (■) and *dgd1* (○). The details about the fitting procedure are described in Materials and methods. The lines (solid for WT and dashed for *dgd1*) serve as a guide to the eye.

Lipid matrix – lipid packing and membrane permeability

In order to study the global physical properties of the lipid matrix of thylakoids two methods were applied: (i) time-resolved fluorescence of MC540 in thylakoid membranes, which reports on the packing of the lipid molecules (see Chapter 4 and references therein), and (ii) electrochromic absorbance transients on whole leaves, which probe the membrane permeability (see e.g. [52,53]).

Incorporation of MC540 into thylakoid membranes

MC540 is a lipophilic fluorescence probe, the spectroscopic properties of which are determined by the dielectric constant of its local environment [54]. Thus, it exhibits different fluorescent lifetimes when present in different environments (interacting with lipids or solubilized in the aqueous phase). A three-exponential model was used for the analysis of the fluorescence decay of MC540 (see also Materials and methods) and

lifetimes of the order of 0.19-0.23 ns, 0.66-1.08 ns and 1.71-2.15 ns were obtained (Fig. 4a). Hereafter, in this paper they are referred to as 200 ps, 1 ns and 2 ns components, respectively. These values shorten with the increase of temperature (Fig. 4a) due to enhanced izomerization [55].

As shown in Figs. 4b and c, the relative amplitudes of the different lifetime components of MC540 differ for WT and *dgd1*. The 200 ps component is due to MC540 free in the aqueous buffer phase, not interacting with the membrane [24,56]. The relative amplitude of this component remains constant up to 45 °C for the WT (Fig. 4b), whereas for *dgd1* it increases with the increase of temperature, most prominently above 25 °C (Fig. 4d). As discussed in Chapter 4 the two long-lived components (1 ns and 2 ns) might either reflect two discrete populations of MC540 molecules, corresponding to two different microenvironments, or originate from a broad distribution of lifetimes due to incorporation of MC540 in a variety of environments with (small) differences in their physical properties (dielectric constant, lipid packing). Within the framework of the discrete population model, the 2 ns component reflects MC540 incorporated deep in the lipid bilayer, whereas the 1 ns component corresponds to surface-associated MC540. Comparison of the relative amplitudes of these two components (Figs. 4b and c) reveals that for WT the relative amplitude of the 1 ns component is slightly larger than the one of the 2 ns component, indicating that the amount of MC540 incorporated into the bilayer and MC540 located on the surface are almost equal (Fig. 4b). In contrast, for *dgd1* the relative amplitude of the 1 ns component is significantly larger than the one of the 2 ns component, indicating that in *dgd1* the MC540 molecules are preferentially surface-associated (Fig. 4c). The lower extent of incorporation of MC540 in the thylakoid membranes isolated from *dgd1* in comparison with the ones from WT might be due to two factors: (i) tighter lipid packing in *dgd1*, (ii) modified surface charge of the membrane (due to conformational changes in the protein complexes or to different lipid-protein interactions).

If the two slow components originate from a broad distribution of lifetimes, then their weighted average lifetime is a more appropriate parameter to consider. As can be seen from the comparison of Fig. 4b and c insets, at 7 °C this average lifetime is shorter for *dgd1* (1.35±0.1 ns) than for WT (1.52±0.01 ns). This shows that the MC540 molecules are facing a more hydrophilic environment in *dgd1* than in WT. These data indicate differences in the

lipid packing. The average lifetime for both WT and *dgd1* is decreasing with the increase in temperature (due to enhanced isomerization, [55] (see also Fig. 4a)) but the difference, shorter average lifetime in *dgd1*, is retained at all temperatures between 7 and 35 °C; at 45 °C the two lifetimes become almost identical, about 1.1 ns.

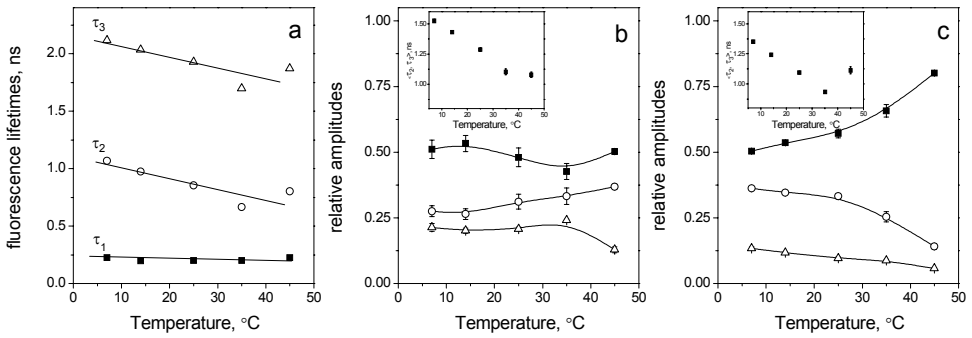


Figure 4. Parameters obtained from the global analysis of the fluorescence decay traces recorded for MC540 added to thylakoid membrane suspensions of WT and *dgd1* (details for the used fitting model are described in Materials and methods, see also text) - fluorescence lifetimes (a) and their corresponding relative amplitudes for WT (b) and *dgd1* (d). The symbols used for the relative amplitudes correspond to the symbols used for the lifetime components (~200 ps (■),~1 ns (○) and ~2 ns (Δ)). The weighted average lifetimes of the two long-lived components for WT (■) and *dgd1* (□) are plotted as insets in (b) and (c), respectively. The samples were thermostated for 10 min at each temperature before starting the measurements.

Electrochromic absorbance changes (ΔA515) in WT and dgd1

In order to test the membrane permeability, ΔA515 measurements were performed. Saturating single turnover flashes were applied to detached leaves. Light-induced primary charge separation in the reaction centers, followed by vectorial transport of charges, generates a trans-membrane electrochemical potential difference, which consists of a pH gradient and an electric potential difference. By a reverse flow of protons, the electrochemically stored energy is used for ATP synthesis [57]. The potential gradient can also be dissipated by the basal ion efflux, which depends on the electrical permeability

of the membranes. The rise and decay of the transmembrane electrical difference can be followed by the electrochromic absorbance changes (ΔA515) of the pigments embedded in the membrane, which correlates with the trans-membrane electric field [52,53].

On the time scale of the experiment, the rise of ΔA515, due to primary charge separations, is instantaneous. The initial amplitude of ΔA515 differs for WT and *dgd1*, as can be seen in Fig. 5a, b, which is most probably due to the decreased Chl and PSI content in the mutant [12]. At 25 °C the decay time of ΔA515 for the mutant ($t_{1/2}$ = 226±15 ms) is essentially the same as for the WT ($t_{1/2}$ = 227±19 ms). These decay times are comparable with those observed for barley under similar conditions [58]. Our data indicate that despite the altered lipid composition (increased non-bilayer:bilayer lipid ratio), the membranes are perfectly adjusted to generate and maintain the trans-membrane electrochemical potential difference at 25 °C. This is in line with the data of Härtel et al. [10] showing that *dgd1* is capable of maintaining a low lumenal pH, needed for the xanthophyll cycle operation. Although our MC540 measurements reveal that the lipid packing at 25 °C is different for *dgd1* and the WT (Fig. 4), this apparently does not influence the permeability. This demonstrates that the behavior of MC540 (with regard to its incorporation in the membrane and its fluorescence lifetime) is determined by the packing of the lipid molecules and not by the membrane permeability.

At 35 °C, the decay of ΔA515 is significantly faster for the *dgd1* mutant (Fig. 5b); the corresponding halftimes are 237±16 ms for WT and 154±19 ms for *dgd1*, indicating increased membrane permeability for *dgd1*. No change in the decay rate is observed for the WT leaves at this temperature; only at 40 °C the decay becomes faster ($t_{1/2}$ = 36±12 ms) for the WT; at this latter temperature no ΔA515 signal can be discerned for *dgd1*. Dependence of the membrane permeability on the lipid content of thylakoids was also demonstrated for a mutant of *Arabidopsis* (*mgd1-1*) with decreased amount of MGDG – the thylakoid membranes of *mgd1-1* were shown to exhibit increased conductivity at high light intensities, which resulted in inefficient operation of the xanthophyll cycle [15] and which further demonstrates the importance of the lipid matrix content for the physical properties of the membrane.

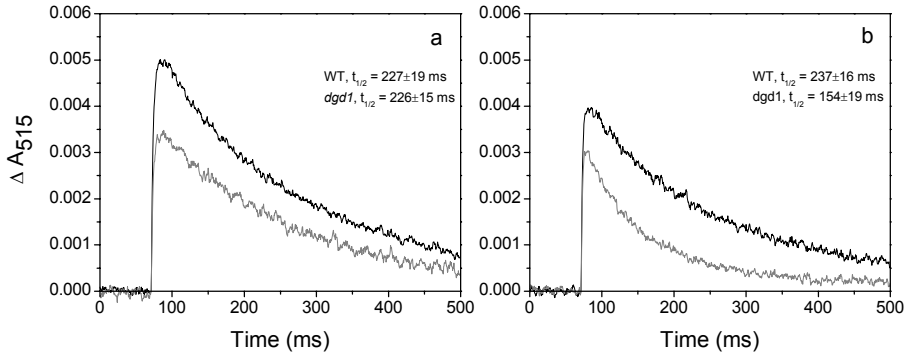


Figure 5. Typical electrochromic absorbance transients recorded at 515 nm (ΔA515), induced by saturating single-turnover flashes on detached WT (black trace) and *dgd1* mutant (gray trace) leaves incubated in the dark for 10 min at 25 °C (a) and 35 °C (b) and subsequently measured at 25 °C. The kinetic traces are obtained by averaging 64 transients with a repetition rate of 1 s^{-1}. The corresponding decay halftimes for WT and *dgd1* (average from 5 independent experiments) are also plotted in the figure.

4. Conclusions

It has become clear in this study that the DGDG deficiency substantially influences both the overall organization and functioning of the thylakoid membrane and its thermal stability. At room temperature (25 °C) the arrangement of the pigment-protein complexes in *dgd1* is different than for the WT: the Ψ-type CD bands, originating from large macrodomains of pigment-protein complexes, have significantly lower intensity for *dgd1*, and probably because of this inferior macroorganization of the complexes, the excitation trapping time in the mutant is substantially longer than in the WT (measured both by FLIM on leaves and by TCSPC on isolated thylakoids). Experiments using the fluorescent lipid probe MC540 revealed differences in the packing of the lipid molecules, indicating a tighter packing or a modified surface charge density in the mutant thylakoid membranes.

Our data also reveal differences in the thermal behavior of the lipid and protein moieties in *dgd1* and WT thylakoid membranes. While at room temperature, which is close to the growth temperature, the thylakoid membranes both in the WT and mutant leaves exhibit the same low basal ion flux, with the increase of temperature *dgd1* becomes “leaky”

at lower temperature, at 35 °C, than the WT. As revealed by time-resolved fluorescence measurements on MC540, with the gradual increase of temperature, this fluorescent lipid probe becomes gradually extruded from the *dgd1* thylakoid membranes, whereas for WT this is not prominent even at 45 °C. Also, the temperature dependence of the average lifetime of lipid-bound MC540 is substantially shifted towards lower temperatures. The mutant thylakoids also possess a lower thermal stability (by ~6 °C) of the protein macrodomains and the Chl *a*-binding proteins, suggesting that the lipid matrix of *dgd1* is not able to maintain the functional state of the protein molecules at moderately elevated temperatures.

Acknowledgements

The authors wish to thank Dr. Eva Selstam for providing the *dgd1* seeds and for fruitful discussions and Mr. Milán Szabó for help with the electrochromic absorbance change measurements. This work was supported by grants from the Hungarian Fund for Basic Research (OTKA K 63252) to G.G., the Sandwich-Programme of Wageningen Uniiversity, The Netherlands to S.B.K. and the EU 6th Framework Programme Grant MRTN - CT - 2005 – 019481 to HvA and SBK.

References

[1]Douce R. and J. Joyard, Biosynthesis of Thylakoid Membrane Lipids, In: *Advances in Photosynthesis/Oxygenic Photosynthesis: The Light Reactions*, Ort D.R.; Yacum C.F. (Eds.) Kluwer Academic Publishers (1996) 69-101.
[2]Loll B., J. Kern, W. Saenger, A. Zouni and J. Biesiadka, Towards Complete Cofactor Arrangement in the 3.0 E Resolution Structure of Photosystem II, *Nature* 438 (2005) 1040-1044.
[3]Loll B., J. Kern, W. Saenger, A. Zouni and J. Biesiadka, Lipids in Photosystem II: Interactions With Protein and Cofactors, *Biochim. Biophys. Acta* 1767 (2007) 509-519.
[4]Gabashvili, I.S., A. Menikh, J. Segui and M. Fragata, Protein structure of photosystem II studied by FTIR spectroscopy. Effect of digalactosyldiacylglycerol on the tyrosine side chain residues, *J. Mol. Str.* 444 (1998) 123-133.
[5]Sakurai, I., N. Mizusawa, H. Wada and N. Sato, Digalactosyldiacylglycerol is required for stabilization of the oxygen-evolving complex in photosystem II, *Plant Physiol*. 145 (2007) 1361-1370.
[6]Liu, Z.F., H.C. Yan, K.B. Wang, T.Y. Kuang, J.P. Zhang, L.L. Gui, X.M. An and W.R. Chang, Crystal structure of spinach major light-harvesting complex at 2.72 angstrom resolution, *Nature* 428 (2004) 287-292.

[7]Yan H., P. Zhang, C. Wang, Zh. Liu and W. Chang, Two lutein molecules in LHCII have different conformations and functions: Insights into the molecular mechanism of thermal dissipation in plants, *Biochem. Biophys. Res. Commun*. 355 (2007) 457-463.
[8]Nuβberger S., K. Dorr, D.N. Wang and W. Kühlbrandt, Lipid-protein interactions in crystals of plant light-harvesting complex, *J. Mol. Biol.* 234 (1993) 347-356.
[9]Dörmann P., S. Hoffmann-Benning, I. Balbo and C. Benning, Isolation and characterization of an *Arabidopsis* mutant deficient in the thylakoid lipid digalactosyl diacylglycerol , *The Plant Cell* 7 (1995) 1801-1810.
[10]Härtel H., H. Lokstein, P. Dörmann, B. Grimm and C. Benning, Changes in the composition of the photosynthetic apparatus in the galactolipid-defficient *dgd1* mutant of *Arabidopsis thaliana* , *Plant Physiol*. 115 (1997) 1175-1184.
[11]Härtel H., H. Lokstein, P. Dörmann, R. N. Trethewey and C. Benning, Photosynthetic light utilization and xanthophyll cycle activity in the galactolipid deficient *dgd1* mutant of *Arabidopsis Thaliana*, *Plant Physiol. Biochem*. 36 (1998) 407-417.
[12]Ivanov A.G., L. Hendrickson, M. Krol, E. Selstam, G. Öquist, V. Hurry and N.P.A. Huner, Digalactosyl-diacylglycerol deficiency impairs the capacity for photosynthetic intersystem electron transport and state transitions in *Arabidopsis thaliana* due to photosystem I acceptor-side limitations, *Plant Cell Physiol*. 47 (2006) 1146-1157.
[13]Guo J., Zh. Zhang, Y. Bi, W. Yang, Y. Xu and L. Zhang, Decreased stability of photosystem I in *dgd1* Mutant of *Arabidopsis thaliana*, *FEBS Lett*. 579 (2005) 3619-3624.
[14]Jarvis P., P. Dörmann, C.A. Peto, J. Lutes, C. Benning and J. Chory, Galactolipid deficiency and abnormal chloroplast development in the *Arabidopsis* MGD synthase 1 mutant, *Proc. Natl. Acad. Sci. U. S. A*. 97 (2000) 8175-8179.
[15]Aronsson H., M.A. Schottler, A.A. Kelly, C. Sundqvist, P. Dörmann, S. Karim and P. Jarvis, Monogalactosyldiacylglycerol deficiency in *Arabidopsis* affects pigment composition in the prolamellar body and impairs thylakoid membrane energization and photoprotection in leaves, *Plant Physiol*. 148 (2008) 580-592.
[16]Hendrickson L., A. Vlckova, E. Selstam, N. Huner, G. Öquist and V. Hurry, Cold acclimation of the *Arabidopsis dgd1* mutant results in recovery from photosystem I-limited photosynthesis, *FEBS Lett*. 580 (2006) 4959-4968.
[17]Garab G., K. Lohner, P. Laggner and T. Farkas, Self-regulation of the lipid content of membranes by non-bilayer lipids: a hypothesis, *Trends Plant Sci*. 5 (2000) 489-494.
[18]Simidjiev I., V. Barzda, L. Mustardy and G. Garab, Role of thylakoid lipids in the structural flexibility of lamellar aggregates of the isolated light-harvesting chlorophyll *a/b* complex of photosystem II, *Biochemistry* 37 (1998) 4169-4173.
[19]Borst J.W., M.A. Hink, A. van Hoek and A.J.W.G. Visser, Effects of refractive index and viscosity on fluorescence and anisotropy decays of enhanced cyan and yellow fluorescent proteins, *J. Fluores*. 15 (2005) 153-160.
[20]van Oort B., A. Amunts, J.W. Borst, A. van Hoek, N. Nelson, H. van Amerongen and R. Croce, Picosecond fluorescence of intact and dissolved PSI-LHCI crystals, *Biophys. J.*, in press
[21]Novikov E.G., A. van Hoek, A.J.W.G. Visser and J.W. Hofstraat, Linear algorithms for stretched exponential decay analysis, *Opt. Commun*. 166 (1999) 189-198.
[22]Digris A.V., V.V. Skakun, E.G. Novikov, A. van Hoek, A. Claiborne and A.J.W.G. Visser, Thermal stability of a flavoprotein assessed from associative analysis of polarized time-resolved fluorescence spectroscopy, *Eur. Biophys. J*. 28 (1999) 526-531.

[23]Visser N.V., A.H. Westphal, A. van Hoek, C.P.M. van Mierlo, A.J.W.G. Visser and H. van Amerongen, Tryptophan-tryptophan energy migration as a tool to follow apoflavodoxin folding, *Biophys. J.* 95 (2008) 2462-2469.
[24]Mandal D., S.K. Pal, D. Sukul and K. Bhattacharyya, Photophysical processes of Merocyanine 540 in solutions and in organized media, *J. Phys. Chem*. A 103 (1999) 8156-8159.
[25]Aramendia P.F., M. Krieg, C. Nitsch, E. Bittersmann and S.E. Braslavsky, The photophysics of Merocyanine 540. A comparative study in ethanol and in liposomes, *Photochem. Photobiol*. 48 (1988) 187-194.
[26]Büchel C and G. Garab, Electrochromic absorbance changes in the chlorophyll-*c*-containing alga *Pleurochloris Meiringensis* (Xanthophyceae), *Photosynth. Res*. 43 (1995) 49-56.
[27]Garab G., Linear and Circular Dichroism, In: *Biophysical Techniques in Photosynthesis*, Amesz, J; Hoff, A.J. (Eds.), Kluwer Academic Publishers (1996) 11-40.
[28]Tinoco I.J., Theoretical aspects of optical activity. 2. Polymers, *Adv. Chem. Phys*. 4 (1962) 113-160.
[29]De Voe H., Optical properties of molecular aggregates. II. Classical theory of the refraction, absorption, and optical activity of solutions and crystalls, *J. Chem. Phys*. 43 (1965) 3199-3208.
[30]Somsen O.J.G., R. van Grondelle and H. van Amerongen, Spectral broadening of interacting pigments: Polarized absorption by photosynthetic proteins, *Biophys. J.* 71 (1996) 1934-1951.
[31]van Metter R.L., Excitation energy transfer in the light-harvesting chlorophyll *a/b* protein, *Biochim. Biophys. Acta* 462 (1977) 642-658.
[32]Georgakopoulou S., G. van der Zwan, R. Bassi, R. van Grondelle, H. van Amerongen and R. Croce, Understanding the changes in the circular dichroism of light harvesting complex II upon varying its pigment composition and organization, *Biochemistry* 46 (2007) 4745-4754.
[33]Garab G., J. Kieleczawa, J.C. Sutherland, C. Bustamante and G. Hind, Organization of pigment-protein complexes into macrodomains in the thylakoid membranes of wild type and chorophyll *b*-less mutant of barley as revealed by circular dichroism, *Photochem. Photobiol*. 54 (1991) 273-281.
[34]Barzda V., L.A. Mustardy and G. Garab, Size dependency of circular dichroism in macroaggregates of photosynthetic pigment-protein complexes, *Biochemistry* 33 (1994) 10837-10841.
[35]Dobrikova A.G., Z. Várkonyi, S.B. Krumova, L. Kovács, G.K. Kostov, S.J. Todinova, M.C. Busheva, S.G. Taneva and G. Garab, Structural rearrangements in chloroplast thylakoid membranes revealed by differential scanning calorimetry and circular dichroism spectroscopy. Thermo-optic effect, *Biochemistry* 42 (2003) 11272-11280.
[36]Garab G. and L. Mustardy, Role of LHCII-containing macrodomains in the structure, function and dynamics of grana., *Aust. J. Plant Physiol*. 26 (1999) 649-658.
[37]Kovács L., J. Damkjar, S. Kereiche, C. Ilioaia, A. V. Ruban, E.J. Boekema, S. Jansson and P. Horton, Lack of the light-harvesting complex CP24 affects the structure and function of the grana membranes of higher plant chloroplasts, *The Plant Cell* 18 (2006) 3106-3120.

[38]Cseh Z., S. Rajagopal, T. Tsonev, M. Busheva, E. Papp and G. Garab, Thermooptic effect in chloroplast thylakoid membranes. Thermal and light stability of pigment arrays with different levels of structural complexity, *Biochemistry* 39 (2000) 15250-15257.
[39]Chitnis P.R., Photosystem: function and physiology, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52 (2001) 593-626.
[40]Ben-Shem A., F. Frolow and N. Nelson, Crystal structure of plant photosystem I, *Nature* 426 (2003) 630-635.
[41]Smith P.J., S. Peterson, V.M. Masters, T. Wydrzynski, S. Styring, E. Krausz and R.J. Pace, Magneto-optical measurements of the pigments in fully active photosystem II core complexes from plants, *Biochemistry* 41 (2002) 1981-1989.
[42]Roelofs T.A., C.-I. Lee and A.R. Holzwarth, Global target analysis of picosecond chlorophyll fluorescence kinetics from pea chloroplasts: a new approach to the characterization of the primary processes in photosystem II α-units and β-units, *Biophys. J.* 61 (1992) 1147-1163.
[43]Gilmore A.M., T.L. Hazlett, P.G. Debrunner and Govindjee, Photosystem II chlorophyll *a* fluorescence lifetimes and intensity are independent of the antenna size differences between barley wild-type and *Chlorina* mutants: Photochemical quenching and xanthophyll cycle dependent non-photochemical quenching of fluorescence, *Photosynth. Res.* 48 (1996) 171-187.
[44]Vasilev S., S. Wiebe and D. Bruce, Non-photochemical quenching of chlorophyll fluorescence in photosynthesis. 5-hydroxy-1,4-naphthoquinone in spinach thylakoids as a model for antenna based quenching mechanisms. *Biochim. Biophys. Acta* 1363 (1998) 147-156.
[45]Broess K., G. Trinkunas, C.D. van der Weij-de Wit, J.P. Dekker, A. van Hoek, and H. van Amerongen, Excitation energy transfer and charge separation in photosystem II membranes revisited, *Biophys. J.* 91 (2006) 3776-3786.
[46]Broess K., G. Trinkunas, A. van Hoek, R. Croce and H. van Amerongen, Determination of the excitation migration time in photosystem II. Consequences for the membrane organization and charge separation parameters, *Biochim. Biophys. Acta* 1777 (2008) 404-409.
[47]Turconi S., N. Weber, G. Schweitzer, H. Strotmann and A.R. Holzwarth, Energy transfer and charge separation kinetics in photosystem I. 2. Picosecond fluorescence study of various PS I particles and light-harvesting complex isolated from higher plants. *Biochim. Biophys. Acta*. 1187 (1994) 324-334.
[48]Ihalainen J.A., P.E. Jensen, A. Haldrup, I.H.M. van Stokkum, R. van Grondelle, H.V. Scheller and J.P. Dekker, Pigment organization and energy transfer dynamics in isolated photosystem I (PSI) complexes from *Arabidopsis thaliana* depleted of the PSI-G, PSI-K, PSI-L, or PSI-N subunit, *Biophys. J.* 83 (2002) 2190-2201.
[49]Ihalainen J.A., I.H.M. van Stokkum, K. Gibasiewicz, M. Germano, R. van Grondelle, and J.P. Dekker, Kinetics of excitation trapping in intact photosystem I of *Chlamydomonas reinhardtii* and *Arabidopsis thaliana*, *Biochim. Biophys. Acta*. 1706 (2005) 267-275.
[50]Croce R., D. Dorra, A.R. Holzwarth and R.C. Jennings, Fluorescence decay and spectral evolution in intact photosystem I of higher plants, *Biochemistry* 39 (2000) 6341-6348.

[51]Slavov C., M. Ballottari, T. Morosinotto, R. Bassi and A.R. Holzwarth, Trap-limited charge separation kinetics of photosystem I complexes from higher plant, *Biophys. J.* 94 (2008) 3601-3612.
[52]Junge W., Membrane potentials in photosynthesis, *Ann. Rev. Plant Physiol*. 128 (1977) 503-536.
[53]Witt H.T., Energy conversion in the functional membrane of photosynthesis. Analysis by light pulse and electric pulse methods. The central role of the electric field, *Biochim. Biophys. Acta* 505 (1979) 355-427.
[54]Lelkes P.I. and I.R. Miller, Perturbations of membrane structure by optical probes: I. Location and structural sensitivity of Merocyanine 540 bound to phospholipid membranes, *J. Membr. Biol*. 52 (1980) 1-15.
[55]Onganer, Y. and E.L. Quitevis, Dynamics of Merocyanine-540 in model biomembranes - photoisomerization studies in small unilamellar vesicles, *Biochim. Biophys. Acta* 1192 (1994) 27-34.
[56]Krumova S.B., C. Dijkema, P. de Waard, H.V. As, G. Garab and H. van Amerongen, Phase behavior of phosphatidylglycerol in spinach thylakoid membranes as revealed by ^{31}P-NMR, *Biochim. Biophys. Acta* 1778 (2008) 997-1003.
[57]Mitchell P., Chemiosmotic coupling in oxidative and photosynthetic phosphorylation, *Biol. Rev. Cambridge Phil Soc*. 41 (1966) 445-502.
[58]Garab G., A.A. Sanchez Bargos, L. Zimanyi and A. Faludi-Daniel, Effect of CO_2 on the organization of thylakoids in leaves of higher plants, *FEBS Lett*. 154 (1983) 323-327.

Chapter 6

Summary and Discussion

Summary and Discussion

Life on earth, as we know it, relies on photosynthesis – the process in which light energy is converted into chemical energy. A large variety of organisms (higher plants, algae, cyanobacteria), adapted to different biotopes, are able to perform oxygen evolution and CO_2 fixation, and they all possess a similar, although not identical, photosynthetic machinery. In higher plants all the light-harvesting and energy transduction functions are performed by protein complexes (photosystems) embedded in a lipid matrix - the thylakoid membrane.

In the past two decades, after the crystallization and the near-atomic resolution structure of the bacterial photosynthetic reaction center [1], high resolution crystal structures of the most important photosynthetic pigment-protein complexes and of other protein constituents have become available (see e.g. [2-7]). This has advanced tremendously our understanding of the functioning of the photosynthetic apparatus. Many of the bound lipids, which are associated with the membrane proteins, have also been identified. Advanced electron microscopy studies revealed that the protein complexes are assembled in large, highly-organised macrostructures of dynamic nature, providing the cell with possibilities for regulation and optimization of the light energy utilization. This structural flexibility however has scarcely been correlated with the properties of the lipid constituents of the thylakoid membranes. In fact, our knowledge of the effects of the bulk lipids on the overall organization and functions of the thylakoid membranes is far less advanced than the studies of the protein complexes, and the bulk lipids are generally regarded as a rather passive matrix accommodating the protein complexes. On the other hand, numerous data show that in many organisms the fluidity of the lipid membrane and its composition (type of lipids and their relative ratios) vary over a broad interval upon changes in the environmental conditions; also, the protein structures and their macroassemblies can depend on the lipid environments. Hence, a the better understanding of the structure and functions of the thylakoid membranes requires a deeper understanding of the impact of the bulk lipids on the organization and physical properties of the thylakoid membranes.

Four lipid classes constitute the thylakoid membrane: the non-bilayer monogalactosyl diacylglycerol (MGDG, ~50 %) and the bilayer-prone digalactosyl diacylglycerol (DGDG, ~30 %), sulphoquinovosyl diacylglycerol (SQDG, ~10 %) and phosphatidylglycerol (PG, ~10 %) [8]. When isolated under conditions close to the ones found in thylakoid membranes these lipid molecules adopt predominantly non-bilayer phases [9]. However, when embedded in the intact thylakoid membrane, they are believed to be organized in a bilayer, which is also the functional state of the thylakoid membrane [10,11]. Thus isolated lipids can not be regarded as a proper model system for the studies of the phase behavior of lipids that are incorporated into the thylakoid membrane. The significance of the lipid-protein interactions was demonstrated for a system containing the major lipid species in thylakoids (the non-bilayer lipid, MGDG) and the most abundant photosynthetic protein (the major light-harvesting complex of photosystem II, LHCII), where the incorporation of these two components resulted in destruction of the inverted hexagonal phase formed by MGDG and the consequent formation of large, ordered lamellar structures involving both the protein and the lipid molecules [12]. The presence of a large variety of different proteins, four distinct lipid classes and specific lipid/protein interactions, makes the thylakoid membrane a very complex system to study. Nevertheless, a variety of specific roles for lipids has been found which will be summarized bellow:

- There is ample evidence that lipid molecules are important for the protein structures and formation of higher-order protein complexes [7,13-15].
- Lipids prevent non-specific protein aggregation and provide a matrix for diffusion of molecules with indispensable physiological significance for photosynthesis (such as plastoquinones, [16]) and whole protein complexes (for example during state-transitions or protein repair of PSII core, [17-19]).
- The membrane curvature stress, determined by the thylakoid lipids, controls the optimal functioning of violaxanthin de-epoxidase (VDE, an enzyme located in the thylakoid lumen which plays important role in the photoprotection in plants) [20]. In addition, the curvature stress might be important for the binding of peripheral proteins to the membrane (such as some subunits involved in the formation of the photosystems) or for the operation of heat-shock proteins (which were shown to bind to model and

cyanobacterial thylakoid membranes, regulate their fluidity and inhibit inverted hexagonal phase formation [21-23]).

- Recently it was shown that the lipid:protein ratio is of functional importance - lipid dilution of BBY membranes (isolated granal membranes) results in lowering of the photosynthetic efficiency due to detachment of LHCII from the PSII core complex [24]. It is yet not clear how the lipid:protein ratio is regulated in the intact thylakoid membrane.
- Under stress conditions such as temperatures above 45 °C [25,26], high concentration of metal cations [9] and co-solutes [27], low pH [28] and low light [29] the lipids segregate from the thylakoid membrane and form extended inverted hexagonal phase structures.

Aim of the thesis

The major goal of this thesis is to contribute to a better understanding of the global properties of the thylakoid lipid matrix of higher plants regarding the lipid packing, phase behavior and permeability, and their relevance for the structural rearrangements observed for protein complexes and the overall macroorganization of the membrane. We confine our study to the physiological range of temperatures - before the onset of pronounced lipid segregation from the membrane.

Lipid packing at physiological temperatures

In order to determine the temperature at which major structural rearrangements and protein degradation in the thylakoid membrane occur, differential scanning calorimetry (DSC) and circular-dichroism (CD) spectroscopy are applied (Chapter 2). It is established that the first endothermic event, observed at 43-48 °C correlates with the disassembly of the protein macrodomains, whereas the transitions occurring at higher temperatures are associated with further disassembly and denaturation of the main pigment-protein complexes. It has previously been shown that at this temperature also a large-scale segregation of the lipid molecules into inverted hexagonal phase occurs [25,26], however the thermograms of thylakoid membranes are largely dominated by the endothermic events

involving protein complexes and no lipid-related transition could be detected via DSC. Thus we apply alternative approaches to study the lipid matrix in thylakoids.

In Chapter 3 we use ^{31}P-NMR, a method well established for studying the lipid packing and phase behavior (cf. [30]). Here it is applied for the first time to intact functional thylakoid membranes and demonstrates heterogeneity in the lipid packing due to the co-existence of different lipid phases at temperatures lower than 45 °C (thus before the onset of lipid segregation and protein degradation). These are namely the lamellar and the isotropic phases, which are found in the membrane bilayer and in small non-bilayer structures, respectively. Moreover, it is shown that these two phases are in close contact with each other since exchange of lipids between them is allowed, whereas its rate is characterized by a strong temperature-dependence. The segregation of lipids in non-bilayer structures was observed so far only under extreme stress and had not been shown before for physiological conditions.

Heterogeneity in the lipid matrix is furthermore detected via the specific fluorescence properties of the lipophilic polarity probe Merocyanine 540 (MC540, Chapter 4). Our data reveal that there is more than one type of environment for the MC540 molecules interacting with isolated thylakoid membranes. Comparison with model lipid systems indicates that, similarly as concluded from the ^{31}P-NMR measurements, the bulk lipid phase behavior in thylakoids can not be described satisfactorily with the properties of a mono-phasic liquid crystalline phase. This suggests that either the lipid packing is different from the one in the model liquid crystalline phase or different lipid domains with distinct properties co-exist in the thylakoid membrane. Furthermore, this experimental approach proved to be useful for comparative studies of different plant species and/or mutants with modified lipid content (Chapter 5). In addition, we apply MC540 for the study of an *Arabidopsis* mutant (*dgd1*) with modified lipid content (strongly reduced amount of one of the major thylakoid (bilayer) lipids, DGDG [31]). This approach shows that thylakoids isolated from *dgd1* exhibit modified lipid packing which might account for the observed differences in the macroorganization of the protein complexes and the prolonged excitation trapping time in the mutant.

Temperature dependences

Previous studies have reported a monotonous increase of the fluidity of thylakoid membranes with the increase of temperature from 7 °C up to 45 °C [32,33], as would be expected for a fluid membrane. On the other hand, in the same range break-points have been observed in the temperature dependence of several functional parameters: at 10-14 °C for the electron transport [34]; 16-18 °C for cytb$_{559}$ oxidation [35]; 15 °C for the electric field formation across the thylakoid membrane and related structural or conformational changes [36]; 9-12 °C for the Hill reaction for chilling-sensitive plants [37-40], and 10-12 °C for the increase in the lateral mobility of phosphorylated LHCII [41].

The results presented in this thesis also reveal changes occurring in the lipid matrix below 45 °C. The ^{31}P-NMR data show that between 14 and 21 °C there is a significant increase in the rate of lipid exchange between the bilayer and the isotropic lipid structures. Labeling with MC540 reveals the onset of rearrangement in the bulk lipid matrix of the spinach thylakoids above 25 °C – the MC540 which is buried inside the membrane at low temperatures becomes extruded from this hydrophobic environment to an environment with higher water content. In contrast to the ^{31}P-NMR measurements, the MC540 data do not allow the discrimination of different lipid phases in thylakoids. It is possible that MC540 is distributed over both the bilayer (lamellar phase) and the small non-bilayer phases (isotropic phase), but if the hydration level of the lipid molecules involved in these two phases is not significantly different or alternatively if the distribution of MC540 in the lamellar phase is much more pronounced than in the isotropic phase, they will remain unresolved by this method. Nevertheless, these two approaches reveal that the complex thylakoid system can not be regarded as a simple fluid bilayer, but requires a more sophisticated description. The fact that the observed changes in the lipid phase/packing occur at physiological temperatures (14-25 °C) suggests that they might have a regulatory role in the adaptation of plants to variations in the temperature. For example they might be correlated with destacking of the membranes, disassembly of the protein macrodomains, and changes in the macroorganization of the PSII-supercomplexes. These processes are important for the regulation/optimization of light-harvesting or they might be required for the diffusion of protein complexes over large distances. The correlation between the physical properties of the lipid matrix and the macroorganization and thermal stability of

thylakoid membranes is demonstrated in chapter 5, where the DGDG-deficient mutant of *Arabidopsis* (*dgd1*) is studied. It is shown that protein macrodomain formation, excitation energy trapping and thermal stability are indeed dependent on the bulk lipid properties. Apparently a thylakoid membrane with a higher non-bilayer propensity (higher ratio non-bilayer:bilayer lipids) is less resistant to thermal challenge; it loses its barrier properties earlier than the wild type and is not able to maintain the functional state of the protein molecules at moderately elevated temperatures - as judged from membrane permeability measurements, the thermal stability of the protein macrodomains and the denaturation of proteins.

Conclusion

Results of this thesis reveal heterogeneity in the packing of the bulk lipid molecules and their participation in both bilayer and non-bilayer structures. Furthermore, the composition of the lipid mixture appears to have an active role in the lateral arrangement of the protein complexes, formation of macrodomains and their thermal stability and indirectly in the excitation energy trapping by the photosynthetic complexes. This work might provide a basis for further investigation of the properties of the lipid matrix and of the lipid-protein interactions in thylakoid membranes and their relevance for different functions of the membrane.

Reference List

[1]Deisenhofer J. and H. Michel, Nobel Lecture. The photosynthetic reaction centre from the purple bacterium *Rhodopseudomonas viridis*., *EMBO J.* 8 (1989) 2149-2170.
[2]Hankamer B., J. Barber and E. Boekema, Structure and membrane organization of Photosystem II in green plants, *Annu. Rev. Plant Physiol. Plant Mol. Biol*. 48 (1997) 641-671.
[3]Boekema E. J., H. van Roon, J.F.L. van Breemen and J.P. Dekker, Supramolecular organization of Photosystem II and its light-harvesting antenna in partially solubilized Photosystem II membranes, *Eur. J. Biochem*. 266 (1999) 444-452.
[4] Groth G. and E. Pohl, The structure of the chloroplast F1-ATPase at 3.2 Å resolution, *J. Biol. Chem* 276 (2001) 1345-1352.
[5]Ben-Shem A., F. Frolow and N. Nelson, Crystal Structure of Plant Photosystem I, *Nature* 426 (2003) 630-635.
[6]Kurisu G., H. Zhang, J.L. Smith and W.A. Cramer, Structure of the cytochrome b(6)f complex of oxygenic photosynthesis: Tuning the cavity, *Science* 302 (2003) 1009-1014.
[7]Liu Z.F., H.C. Yan, K.B. Wang, T.Y. Kuang, J.P. Zhang, L.L. Gui, X.M. An and W.R. Chang, Crystal structure of spinach major light-harvesting complex at 2.72 angstrom resolution, *Nature* 428 (2004) 287-292.
[8]Douce R. and J. Joyard, Biosynthesis of thylakoid membrane lipids, In: *Advances in Photosynthesis/Oxygenic Photosynthesis: The Light Reactions*, Ort D.R.; Yacum C.F. (Eds.) Kluwer Academic Publishers (1996) 69-101.
[9]Gounaris K., A. Sen, A.P.R. Brain, P.J. Quinn and W.P. Williams, The formation of non-bilayer structures in total polar lipid extracts of chloroplast membranes, *Biochim. Biophys. Acta* 728 (1983) 129-139.
[10]Williams W.P. and P.J. Quinn, The phase behavior of lipids in photosynthetic membranes, *J. Bioenerg. Biomembr*. 19 (1987) 605-624.
[11]Williams W.P., The physical properties of thylakoid membrane lipids and their relation to photosynthesis, *In: Advances in Photosynthesis. Lipids in Photosynthesis*, Siegenthaler P.A. and Murata N. (Eds), Kluwer Academic Publishers (1998) 103-118.
[12]Simidjiev I., S. Stoylova, H. Amenitsch, T. Jávorfi, L. Mustárdy, P. Laggner, A. Holzenburg and G. Garab, Self-assembly of large, ordered lamellae from non-bilayer lipids and integral membrane proteins in vitro, *Proc. Natl. Acad. Sci. U. S. A*. 97 (2000) 1473-1476.
[13]Nuβberger S., K. Dorr, D.N. Wang and W. Kühlbrandt, Lipid-protein interactions in crystals of plant light-harvesting complex, *J. Mol. Biol*. 234 (1993) 347-356.
[14]Loll B., J. Kern, W. Sänger, A. Zouni and J. Biesiadka, Lipids in photosystem II: interactions with proteins and cofactors, *Biochim. Biophys. Acta* 1767 (2007) 509-519.
[15]Yan H., P. Zhang, C. Wang, Zh. Liu and W. Chnag, Two lutein molecules in LHCII have different conformations and functions: Insights into the molecular mechanism of thermal dissipation in plants, *Biochem. Biophys. Res. Commun*. 355 (2007) 457-463.
[16]Kirchhoff H., U. Mukherjee and H.-J. Galla, Molecular architechture of the thylakoid membrane: Lipid diffusion space for plastoquinone, *Biochemistry* 41 (2002) 4872-4882.
[17]van Wijk K.J., B. Andersson and E.-M. Aro, Kinetic resolution of the incorporation of the D1 protein into photosystem II and localization of assembly intermediates in thylakoid membranes of spinach chloroplasts, *J. Biol. Chem*. 271 (1996) 9627-9636.

[18]Allen J.F. and J. Forsberg, Molecular recognition in thylakoid structure and function, *Trends Plant Sci*. 6 (2001) 317-326.
[19]Tikkanen M., M. Nurmi, M. Suorsa, R. Danielsson, F. Mamedov, S. Styring and E.-M. Aro, Phosphorylation-dependent regulation of excitation energy distribution between the two photosystems in higher plants, *Biochim. Biophys. Acta* 1777 (2008) 425-432.
[20]Szilagyi A.M., M. Sommarin and H.-E. Akerlund, Membrane curvature stress controls the maximal conversion of violaxanthin to zeaxanthin in the violaxanthin cycle-influence of α tocopherol, cetylethers, linolenic acid, and temperature, *Biochim. Biophys. Acta* 1768 (2007) 2310-2318.
[21]Horváth I., A. Glatz, V. Varvasovszki, Z. Török, T. Páli, G. Balogh, E. Kovács, L. Nadasdi, S. Benko, F. Joo and L. Vigh, Membrane physical state controls the signaling mechanism of the heat shock response in *Synechocystis* PCC 6803: Identification of Hsp17 as a "fluidity gene", *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 3513-3518.
[22]Tsvetkova N.M., I. Horváth, Z. Török, W.F. Wolkers, Zs. Balogi, N. Shigapova, L.M. Crowe, F. Tablin, E. Vierling, J.H. Crowe and L. Vigh, Small heat-shock proteins regulate membrane lipid polymorphism, *Proc. Natl. Acad. Sci. U. S. A*. 99 (2002) 13504-13509.
[23]Horváth I., G. Multhoff, A. Sonnleitner and L. Vigh, Membrane-associated stress proteins: more than simply chaperones, *Biochim. Biphys. Acta* 1778 (2008) 1653-1664.
[24]Haferkamp S. and H. Kirchhoff, Significance of molecular crowding in grana membranes of higher plants for light harvesting by photosystem II, *Photosynth. Res*. 95 (2008) 129-134.
[25]Gounaris K., A.P.R. Brain, P.J. Quinn and W.P. Williams, Structural and functional changes associated with heat-induced phase-separations of non-bilayer lipids in chloroplast thylakoid membranes, *FEBS Lett*. 153 (1983) 47-52.
[26]Gounaris K., A.R.R. Brain, P.J. Quinn and W.P. Williams, Structural reorganization of chloroplast thylakoid membranes in response to heat-stress, *Biochim. Biophys. Acta* 766 (1984) 198-208.
[27]W.P. Williams, A.P.R. Brain and P.J. Dominy, Induction of non-bilayer lipid phase separations in chloroplast thylakoid membranes by compatible co-solutes and its relation to the thermal stability of Photosystem II, *Biochim. Biophys. Acta* 1099 (1992) 137-144.
[28]Semenova G., The relationship between the transformation of thylakoid acyl lipids and the formation of tubular lipid aggregates visible on fracture faces, *J. Plant Physiol*. 55 (1999) 669-677.
[29]Kirchhoff H., H. Haase, S. Wegner, R. Danielsson, R. Ackermann and P.-A. Albertsson, Low-light-induced formation of semicrystalline photosystem II arrays in higher plant chloroplasts, *Biochemistry* 46 (2007) 11169-11176.
[30]Cullis P.R. and B. de Kruijff, Lipid polymorphism and the functional roles of lipids in biological membranes, *Biochim. Biophys. Acta* 559 (1979) 399-420.
[31]Dörmann P., S. Hoffmann-Benning, I. Balbo and C. Benning, Isolation and characterization of an *Arabidopsis* mutant deficient in the thylakoid lipid digalactosyl diacylglycerol, *The Plant Cell* 7 (1995) 1801-1810.
[32]Barber J., R.C. Ford, R.A.C. Mitchell and P.A. Millner, Chloroplast thylakoid membrane fluidity and its sensitivity to temperature, *Planta* 161 (1984) 375-380.
[33]Kota Z., B. Szalontai, M. Droppa, G. Horvath and T. Pali, The formation of an inverted hexagonal phase from thylakoid membranes upon heating, *Cell. Mol. Biol. Lett.* 7 (2002) 126-128.

[34]Nolan W.G., Effect of temperature on proton efflux from isolated chloroplast thylakoids, *Plant Physiol*. 67 (1981) 1259-1263.
[35]Yamamoto Y. and K.E. Bacon, A temperature-dependent conformational change in photosystem II thylakoid membrane, *FEBS Lett*. 107 (1979) 137-140.
[36]Yamamoto Y. and M. Nishimura, Characteristics of light-induced 515-nm absorbance change in spinach chloroplasts at lower temperatures I. Participation of structural changes of thylakoid membranes in light-induced 515-nm absorbance change, *Plant Cell Physiol*. 18 (1977) 55-66.
[37]Raison J.K., The influence of temperature-induced phase changes on the kinetics of respiratory and other membrane-associated enzyme systems, *J. Bioenerg*. 4 (1973) 285-309.
[38]Shneyour A., J.K. Raison and R.M. Smillie, The effect of temperature on the rate of photosynthetic electron transfer in chloroplasts of chilling-sensitive and chilling-resistant plants, *Biochim. Biophys. Acta* 292 (1973) 152-161.
[39]Nolan W.G. and R.M. Smillie, Temperature-induced changes in Hill activity of chloroplasts isolated from chilling-sensitive and chilling-resistant plants, *Plant Physiol*. 59 (1977) 1141-1145.
[40]Inoue H., Break points in Arrhenius plots of Hill reaction of spinach chloroplast fragments in the temperature range from -25 to 25 °C, *Plant Cell Physiol*. 19 (1978) 355-363.
[41]Carlberg I., S. Bingsmark, F. Vennigerholz, U.K. Larsson and B. Andersson, Low temperature effects on thylakoid protein phosphorylation and membrane dynamics, *Biochim. Biophys. Acta* 1099 (1992) 111-117.

Nederlandse samenvatting

De eerste stappen in de fotosynthese (lichtinvangst en conversie van lichtenergie in chemische energie) vinden plaats in het thylakoïde membraan van bladgroenkorrels. Het bestaat voor 75 % uit membraaneiwitten en voor 25 % uit lipiden. In dit proefschrift worden de globale eigenschappen van de lipiden in het thylakoïde membraan van hogere planten bestudeerd met een verscheidenheid aan technieken, met speciale aandacht voor lipide-pakking, fasegedrag en membraan permeabiliteit. Tevens wordt de rol van de lipiden bestudeerd voor waargenomen structurele herrangschikkingen van eiwitcomplexen en de algehele organisatie van het membraan. De beschreven resultaten laten heterogeniteit in de pakking van het merendeel van de lipide-moleculen zien en hun deelname aan zowel bilaag als non-bilaag lipide-structuren. Aangetoond wordt dat het lipiden-mengsel een actieve rol speelt in de laterale organisatie van de eiwitcomplexen, in de vorming van macrodomeinen en hun thermische stabiliteit en indirect in het vastleggen van de excitatie-energie door de fotosynthetische complexen. Dit proefschrift vormt een basis voor toekomstige studies van de eigenschappen van de lipide-matrix in thylakoïde membranen en de relevantie ervan voor verschillende membraanfuncties.

Acknowledgements

My training in science started when I was working on my diploma work in the Bulgarian Academy of Sciences, Sofia. I would like to express my greatest gratitude to my supervisor Dr. Stefka Taneva who was the first to give me confidence and strength to go further into research.

I am very grateful that I had the chance to work on this challenging subject in Wageningen and in Szeged and I thank to my supervisors Prof.dr. Herbert van Amerongen and Dr. Győző Garab for enrolling me in this topic, supporting me during my research and giving me a lot of opportunities to develop. I would like to thank also Cor Dijkema and Peter de Waard for helping me complete probably the most difficult part of my PhD work – the ^{31}P-NMR project. Rob Koehorst became involved in my work in the second part of my studies and I profited and enjoyed a lot working and discussing with him. Thank you! Special thanks to Dr. Bart van Oort and Sergey Laptenok for helping me incalculably with data analysis, keeping me safe from viruses and all other troubles, Prof.dr. Dane Bicanic for the slavic solidarity and all group-members in Szeged and Wageningen who helped me with my work in various respects.

During this project I have met a lot of impressive people in and out of science, who have broadened my visions and ideas, which I appreciate a lot!

I thank all my friends for staying close to me even when we were worlds apart! Special thanks to Miguel Oliveira and Miroslava Zhiponova for always keeping my spirit high.

Thanks to my parents and my sister for the great support and patience!

Curriculum Vitae

Sashka Boychova Krumova was born on the 11th December of 1977 in Kyustendil, Bulgaria. After completing her studies in a High school for foreign languages (German and English) in her home-town, she became enrolled in Sofia University "St. Kliment Ohridski", Faculty of Biology in the period 1996-2001. She received a M.Sc. degree in Biotechnological Processes, specialization Molecular Biotechnology, and the qualification Biotechnologist. In the last year of her university studies she has completed her diploma work in the Institute of Biophysics, Bulgarian Academy of Sciences, Sofia, Bulgaria, entitled: "Fluorescence and electric characteristics of purple membranes in presence of 1,4-anthraquinone and substituted 1,4-anthraquinones" under the supervison of Dr. Stefka Taneva. In the year 2002-2003 she participated for 11 months in an International Training Course on Selected Topics in Modern Biology, organized by the Biological Research Center, Szeged, Hungary. During this time she was trained in the Thylakoid Membrane Energization Group, headed by Dr. Győző Garab. In January 2004 she started her studies at the Laboratory of Biophysics, Wageningen University, as a sandwich PhD-fellow, under the supervision of Prof.dr. Herbert van Amerongen and Dr. Győző Garab. Her research was focused on the role of lipids for the overall organization of thylakoid membranes. During her doctoral study she has attended a variety of international meetings on photosynthesis and seminar series of the Laboratory of Biophysics, WUR and Institute of Plant Biology, BRC, Szeged, Hungary. She has followed in-depth courses on advanced spectroscopy techniques, the role of lipids in photosynthesis, biochemical and computational methods. She completed her PhD project in the end of 2008.

List of publications

E. Apostolova, **S. B. Krumova**, N. Tuparev, M. T. Molina, Ts. Filipova, I. Petkanchin and S. G. Taneva, Interaction of biological membranes with substituted 1,4-anthraquinones, *Coll. Surf. B* 29 (2003) 1-12

A. Dobrikova, Z. Várkonyi, **S. B. Krumova**, L. Kovács, G. Kostov, S. Todinova, M. Busheva, S. Taneva and G. Garab, Structural rearrangements in chloroplast thylakoid membranes revealed by differential scanning calorimetry and circular dichroism spectroscopy. Thermo-optic effect, *Biochemistry* 42 (2003) 11272-11280

E. Apostolova, **S. B. Krumova**, Tz. Markova, Tz. Filipova, M. T. Molina, I. Petlanchin and S.G. Taneva, Role of LHCII organization in the interaction of substituted 1,4-anthraquinones with thylakoid membranes, *J. Photochem. Photobiol. B* 78 (2005) 115–123

S. B. Krumova, S. J. Todinova, M.C. Busheva and S.G. Taneva, Kinetic nature of the thermal destabilization of LHCII macroaggregates, *J. Photochem. Photobiol. B*. 78 (2005) 165-170

S. B. Krumova, C. Dijkema, G. Garab, H. van Amerongen and A. van Hoek, Polymorphyc phase behaviour of phosphatidylglycerol in spinach thylakoid membranes, In: *Photosynthesis: Fundamental Aspects to Global Perspectives*, A. van der Est and D. Bruce Eds (2004) pp. 791-793

J.K. Holm, **S. B. Krumova,** Z. Várkonyi, S.J. Todinova, S. Rajagopal, D. Posselt, S.G. Taneva and G. Garab, Thermo-optically driven reversible reorganizations in LHCII. Significance of thermal instability, role of LHCII-only domains in thylakoids, In: *Photosynthesis: Fundamental Aspects to Global Perspectives*, A. van der Est and D. Bruce Eds (2004) pp. 579-581

Z. Cseh, A. Vianelly, S. Rajagopal, **S. B. Krumova,** L. Kovács, E. Papp, V. Barzda, R. Jennings and G. Garab, Thermo-optically induced reorganizations in the main light-harvesting antenna of plants. I. Non-Arrhenius type of temperature dependence and linear light-intensity dependencies, *Photosynth. Res.* 86 (2005) 1-2, 263-273

P.H. Lambrev, Z. Várkonyi, **S. B. Krumova**, L. Kovács, Y. Miloslavina, A.R. Holzwarth and G. Garab, Importance of trimer-trimer interactions for the native state of the plant light-harvesting complex II, *Biochim. Biophys. Acta* 1767 (2007) 847-53

S. B. Krumova, C. Dijkema, P. de Waard, H.V. As, G. Garab and H. van Amerongen, Phase behavior of phosphatidylglycerol in spinach thylakoid membranes as revealed by ^{31}P-NMR, *Biochim. Biophys. Acta* 1778 (2008) 997-1003

S. B. Krumova, R.B.M. Koehorst, A. Bóta, T. Páli, A. van Hoek, G. Garab and H. van Amerongen, Temperature dependence of the lipid packing in thylakoid membranes studied by time- and spectrally resolved fluorescence of Merocyanine 540, *Biochim. Biophys. Acta*, 1778 (2008) 2823-2833

S. B. Krumova, S. Laptenok, L. Kovács, T. Tóth, A. van Hoek, G. Garab and H. van Amerongen, The role of DGDG in the overall organization and thermal behavior of thylakoid membranes, *submitted for publication*

Education Statement of the Graduate Schoo l
Experimental Plant Science s

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Sashka Boychova Krumova
Date: 16 January 2009
Group: Laboratory of Biophysics, Wageningen Universit y

1) Start-up phase	date
► **First presentation of your project**	
Oral Presentation at Laboratory of Biophysics "Role of non-bilayer lipids in chloroplast thylakoid membranes"	January 2004
► **Writing or rewriting a project proposal**	
Application for a research project at the Wageningen NMR Centre (granted)	2003
Application for a research project HMI, Berlin (granted)	2005
Application for a continuation of a research project at the Wageningen NMR Centre (granted)	2006
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*4.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Wageningen University	13 September 2008
► **EPS theme symposia**	
► **NWO Lunteren days and other National Platforms**	
EPW Lunteren meeting	07-08 April 2008
Annual dutch meeting on molecular and cellular biophysics, Veldhoven	29-30 September 2008
► **Seminars (series), workshops and symposia**	
Biophysica seminar series, WUR, Lab. of Biophysics	2004
Seminar series Institute of Plant Biology, Szeged	2005
Workshop INTRO2: Proteomics, Metabolomics and Transcriptomics, Umea, Sweden	08-12 June 2005
Seminar Series Institute of Plant Biology, Szeged	2006
Caput Series: Structure and dynamics of biomolecules in vitro and in vivo	October-December 2006
Biophysica seminar series, WUR, Lab. of Biophysics	2007
User meeting NMR Centre, Wageningen University	29-30 November 2007
Biophysica seminar series, WUR, Lab. of Biophysics	2008
Farewell Symposium Antonie J.W.G. Visser	19 September 2008
► **Seminar plus**	
► **International symposia and congresses**	
13th International Congress of Photosynthesis, Montreal, Canada	29 August-03 September 2004
30th FEBS Congress Budapest, Hungary	02-07 July 2005
INTRO2 network meeting, Umea, Sweden	13-15 June 2005
XII Congress of the Hungarian Biophysical Society, Debrecen, Hungary	28-30 June 2005
Central-Europea n Conferenc e on Biophysica l and Biochemica l Methods in Photosynthesi s Research ("Photosynthesi s and Stress"), Brno, Czech Republic	15-16 September 2005
Photosynthesis in the post-genomic era, Pushchino, Russia	20-26 August 2006
47th International Conference on the Bioscience of Lipids, Pecs, Hungary	05-10 September 2006
INTRO2 network meeting, Rójtökmuzsaj, Hungary	13-16 May 2007
14th Congress on Photosynthesis, Glasgow, 2007	22-27 July 2007
INTRO2 network meeting, Lanzarote, Spain	07-11 February 2008
► **Presentations**	
Poster at 13th International Congress of Photosynthesis, Montreal, Canada	29 August-03 September 2004
Oral presentation at INTRO2 network meeting, Umea, Sweden	13-15 June 2005
Oral presentation at XII Congress of the Hungarian Biophysical Society, Debrecen, Hungary	28-30 June 2005
Poster at 30th FEBS Congress Budapest, Hungary	02-07 July 2005
Poster at "Photosynthesis and Stress"	August 2005
Poster at Photosynthesis in the post-genomic era, Pushchino, Russia	20-26 August 2006
Poster in ICBL Pecs, Hungary	05-10 September 2006
Oral presentation at Seminar series, Institute of Plant Biology, BRC, Szeged, Hungary	2005-2006
Oral presentation at INTRO2 network meeting, Rójtökmuzsaj, Hungary	13-16 May 2007
Poster on the EPS PhD student days	13 September 2007
Oral presentation at User meeting NMR Centre, Wageningen University	29-30 November 2007
Oral presentation at Biophysics Day, WUR	19 December 2007
Oral presentation at Seminar series, Laboratory of Biophysics, WUR	2007-2008
Oral presentation at INTRO2 network meeting, Lanzarote, Spain	07-11 February 2008
Poster in EPW Lunteren meeting	07-08 April 2008
Poster in Annual dutch meeting on molecular and cellular biophysics, Veldhoven	30-31 September 2008
► **IAB interview**	
► **Excursions**	
Subtotal Scientific Exposure	*27.7 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
EPR Course Advanced ESR Spectroscopy in Membrane Biophysics	29 March - 01 April 2004
Structural role of lipids in photosynthetic membranes, PhD Course at BRC, Szeged, Hungary	12 January-28 April 2005
INTRO2 network course: Proteomics, Metabolomics and Transcriptomics, Umea, Sweden	08-12 June 2005
Modern Methods in Computational Chemistry, Synthetic Organic Chemistry and Fluorescence Spectroscopy	13-16 February 2008
Time-resolved microspectroscopy in the life sciences, Wageningen University	30 June-03 July 2008
► **Journal club**	
Institute of Plant Biology, BRC, Szeged, Hungary	2005-2006
► **Individual research training**	
QENS measurements at Hahn-Meitner Institute, Berlin	5 days, July 2005
QENS measurements at Hahn-Meitner Institute, Berlin	5 days, July 2006
Training at the laboratory of Peter Horton, Sheffield, UK	14 days, June 2005
Subtotal In-Depth Studies	*12.1 credits**

4) Personal development	date
► **Skill training courses**	
Personal Competence, Assertiveness, Time Management & Project Planning	15-18 March 2004
Spanish language	June-August, 2004
► **Organisation of PhD students day, course or conference**	
Time-resolved microspectroscopy in the life sciences	June-July 2008
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.3 credits**

TOTAL NUMBER OF CREDIT POINTS*	**47.6**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

Cover desigh: Sergey Laptenok