



# Thylakoid membrane unstacking increases LHCII thermal stability and lipid phase fluidity

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## Abstract

Thylakoids are highly protein-enriched membranes that harbor a number of multicomponent photosynthetic complexes. Similarly to other biological membranes the protein constituents are heterogeneously distributed laterally in the plane of the membrane, however the specific segregation into stacked (grana patches) and unstacked (stroma lamellae) membrane layers is a unique feature of the thylakoid. Both the lateral and the vertical arrangements of the integral membrane proteins within the three-dimensional thylakoid ultrastructure are thought to have important physiological function. In this work we explore the role of membrane stacking for the thermal stability of the photosynthetic complexes in thylakoid membranes. By means of circular dichroism and differential scanning calorimetry we demonstrate that the thermal stability of the monomeric and trimeric forms of the major light harvesting complex of photosystem II (LHCII) increases upon unstacking. This effect was suggested to be due to the detachment of LHCII from photosystem II and consequent attachment to photosystem I subunits and/or the fluidization of the lipid matrix upon unstacking. The changes in the physical properties of the protein and lipid membrane components upon unstacking result in strongly reduced photosystem II excitation energy utilization.

**Keywords** Thylakoid membrane · Thermal stability · Photosystem II · LHCII · Membrane stacking

## Introduction

Biological membranes are characterized by inhomogeneous protein distribution that is closely related to proteins functionality. In fact, membrane segregation into different domains appears as a general property for all biological membranes. So far our knowledge about the constitution of the thylakoid membranes of higher plants reveals strict protein segregation of their main functional components - photosystem I (PSI), photosystem II (PSII), the major light harvesting complex of PSII (LHCII) and the ATPase complex. Two types of organization can be distinguished – a lateral arrangement that is due to segregation of the protein complexes within the membrane plane and vertical arrangement due to proteins facing (and possibly interacting with) each other in the adjacent highly

invaginated membrane regions – the grana (Pribil et al. 2014; Kirchhoff 2018). The functionality of the photosynthetic apparatus is strongly dependent on the lateral and vertical order within the thylakoid membrane. The formation of grana stacks appears as ubiquitous property of all higher plants thylakoids. It is a product of the action of multiple attractive and repulsive forces with variable strength (Chow et al. 2005; Puthiyaveetil et al. 2017) and is suggested to be entropy-assisted (Chow 1999, 2005; Kim et al. 2005; Jia et al. 2014). Membrane unstacking induced by cation depletion leads to loss of the lateral order and protein intermixing; upon these conditions part of LHCII detaches from PSII and binds to PSI resulting in energy spillover from PSII towards PSI (Butler and Kitajima 1975; Briantais et al. 1984; van der Wij de Wit et al. 2007; Kirchhoff et al. 2007). This process also takes place in vivo (known as state transitions) and is an important component of the dynamic regulation of the excitation energy distribution between the photosystems (Boichenko 1998; Allen and Forsberg 2001; Kaftan et al. 2002; van der Wij de Wit et al. 2007).

The thermal stability of membrane proteins and supramolecular protein complexes is related to their structure/conformation, cofactor/ligand binding, lipid and ionic environment, intermolecular interactions, all of these factors being crucial for proteins functionality. In a number of works it was also shown

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that the temperature of protein denaturation is tightly related to molecule's flexibility (reviewed in Karshikoff et al. 2015). Translated to higher plants thylakoid membranes, the studies of the thermal stability of the photosynthetic apparatus components can give valuable information about the effects of different factors on the membrane structural organization and functionality. Differential scanning calorimetry (DSC) is the method of choice for the studies of proteins thermal stability since it directly evaluates the energetics of protein unfolding. For correct assignment of the observed heat-induced transitions, however, further spectroscopic and proteomic analyzes are needed. Employing DSC, circular dichroism (CD) and native gel electrophoresis in our earlier works we have identified a number of heat-induced events in thylakoid membranes and submembrane fragments. The first one, occurring below 50 °C, was found to be associated with the thermally induced impairment of the three dimensional structure of the thylakoid system leading to membrane unstacking and disassembly of the chirally ordered domains of photosynthetic complexes. This process was also shown to possess thermo-optical nature and to be sensitive to the ionic strength (Dobrikova et al. 2003). Data obtained for thylakoid membranes, as well as submembrane fractions and isolated complexes showed that the heat-induced transitions occurring at higher temperatures reflect the monomerization of the LHCII trimeric complexes (at around 60 °C) and the denaturation of different photosynthetic complexes, i.e. PSII core complexes denature at about 53–57 °C, the major transition at about 70 °C can be assigned to LHCII denaturation, while PSI core components degradation occurs at about 90 °C (reviewed in Krumova et al. 2014). In addition we have shown that LHCII stability is strongly affected by its protonation state (Stoichev et al. 2015) and the protein:lipid ratio (Holm et al. 2005). It can also be suggested that there is a correlation between LHCII flexibility and stability since isolated flexible LHCII assemblies (able to respond to illumination by changes in their conformation) are ca. 5 °C less stable than “rigid” LHCII microcrystals that are light insensitive (Simidjiev et al. 1997; Holm et al. 2005).

In this work we study how the thermal stability of the photosynthetic complexes is affected by the membrane stacking and also whether the lipid phase plays a role for the thermal stability of the proteins in the stacked and unstacked membrane states. Finally, we probe how the physical properties of the protein and lipid membrane components in those two configurations affect the photosystem II photochemical activity.

## Materials and methods

### Thylakoid membrane preparation

*Pisum sativum* cv. RAN 1 (garden pea) plants were grown hydroponically at 150  $\mu\text{mol}/\text{m}^2\cdot\text{sec}$  light intensity and

8 h day/ 16 h night photoperiod. Pea leaves were harvested in the end of the dark phase and were kept in the fridge for 1 h before thylakoids isolation (Harrison and Melis 1992). All isolation procedures were performed in dim light and on ice. Intact (stacked) thylakoid membranes were isolated as in Dobrikova et al. (2003) and resuspended in 20 mM tricine, 250 mM sorbitol, 5 mM  $\text{MgCl}_2$ , pH 7.6 (stacking buffer) to a final chlorophyll (chl) concentration of 2 mg chl/ml (determined according Arnon 1949), supplemented with 30% glycerol and stored at  $-20$  °C until further use. Before measurements the isolated stacked membranes were washed twice in the stacking buffer and diluted to desired concentration. Stacked membranes also served as a starting material for the preparation of unstacked and  $\beta$ -dodecyl maltoside ( $\beta$ -DM, Sigma-Aldrich) treated samples.

To trigger  $\text{Mg}^{2+}$  depletion induced membrane unstacking, the isolated thylakoids were resuspended in medium containing 15 mM HEPES, 5 mM KCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.6 (unstacking buffer) up to a concentration of 20  $\mu\text{g}$  chl/ml and centrifuged for 10 min at 5000 x rpm. The pellet was resuspended in 10 ml of the same buffer and incubated for 15 min. The samples were centrifuged once more (as above) and the pellet was resuspended and incubated for 45 min in the unstacking buffer. After a final washing procedure the pellet was resuspended in the unstacking buffer to the required concentration (see below) and used for experiments.

The mild non-ionic detergent  $\beta$ -DM was added to a final concentration of 0.1% to unstacked thylakoids with concentration of 1 mg chl/ml and incubated for 5 min prior calorimetric and spectroscopic measurements. For circular dichroism and fluorescence measurements the  $\beta$ -DM treated thylakoids were further diluted to 15  $\mu\text{g}$  chl/ml.

Five independent preparations of stacked, unstacked and  $\beta$ -DM treated thylakoids were used for each of the experiments.

### Circular dichroism

CD spectra of stacked, unstacked and  $\beta$ -DM treated thylakoid membranes were recorded by means of Jobin Yvon CD6 dichrograph in the 400–750 nm spectral range applying the following instrument settings: increment 1 nm, integration time 0.5 s, 2 nm bandpass. The chl concentration of the samples was 15  $\mu\text{g}/\text{ml}$ . Origin 8.0 software package was used to smooth the CD spectra and to subtract the CD signal of the corresponding buffer solution from each CD spectrum recorded for thylakoids.

For the temperature series the samples were heated sequentially for 5 min at defined temperatures in the range 20–90 °C (increasing at an increment of 5 °C) in water bath ( $\pm 0.5$  °C accuracy) prior to recording the CD spectra. The temperature at which the intensity of the respective CD band decreased by

50% in the series of CD spectra of heat-treated samples was regarded as a transition temperature ( $T_m^{CD}$ ). The intensity of the CD bands was determined by subtracting the intensities at defined pairs of wavelengths, that is (+)694 nm and (+)750 nm ( $CD_{694-750}$ ), (-)679 nm and (+)750 nm ( $CD_{750-679}$ ), (+)483 nm and (-)473 nm ( $CD_{483-473}$ ), (+)444 nm and (-)436 nm ( $CD_{444-436}$ ), (+)506 nm and (+)620 nm ( $CD_{506-620}$ ), (-)657 nm and 630 nm ( $CD_{630-657}$ ).

### Differential scanning calorimetry

The thermal stability of stacked, unstacked and  $\beta$ -DM treated thylakoid membranes was probed by means of DSC measurements using DASM-4 microcalorimeter (Privalov, Pushchino). Samples with a concentration of 1.0–1.3 mg chl/ml were scanned with a heating rate of 0.5 °C/min in the 30 °C - 110 °C temperature range. After a correction for a buffer-buffer baseline, a sigmoidal baseline was subtracted from the thermograms and they were normalized to the total chl content of the samples. Origin 8.0 software was used to determine the temperature of denaturation ( $T_m$ , the temperature at the peak of the thermal transition) and excess heat capacity ( $c_p^{ex}$ , the amplitude of the thermal transition) of the sequential thermal transitions.

### Fluorescence spectroscopy

Stacked and unstacked thylakoid membranes with a concentration of 15  $\mu$ g chl/ml were incubated with 0.2  $\mu$ M merocyanine 540 (MC540, using 1 mM stock solution dissolved in ethanol) or 30  $\mu$ M laurdan (using 1 mM stock solution dissolved in dimethyl sulfoxide) for 30 min at 20 °C. Control measurements were performed with fluorescent probes suspended in stacking and unstacking buffers. The emission and excitation spectra were recorded with 10 nm emission/excitation slits on LS-50B Perkin Elmer luminescence spectrometer and corrected for autoemission of untreated thylakoids or buffer solutions. The general polarization (GP) of laurdan was determined as  $GP = (I_{460} - I_{516}) / (I_{460} + I_{516})$ , where  $I_{460}$  is the fluorescence intensity at 460 nm (characteristic for tightly packed lipids) and  $I_{516}$  is the fluorescence intensity at 516 nm (characteristic for less tightly packed membranes), respectively (Szilágyi et al. 2008).

77 K fluorescence emission spectra of stacked and unstacked thylakoid membranes were recorded on Fluorolog spectrofluorometer (Jobin Yvon Horiba) upon excitation at 472 nm, utilizing 7 and 3 nm excitation and emission slits, respectively, and integration time of 1 s. Prior to measurements the thylakoid suspensions with a concentration of 200  $\mu$ g chl/cm<sup>2</sup> were first deposited onto filter paper disks and further frozen in liquid nitrogen.

### Fluorescence induction measurements by OJIP test

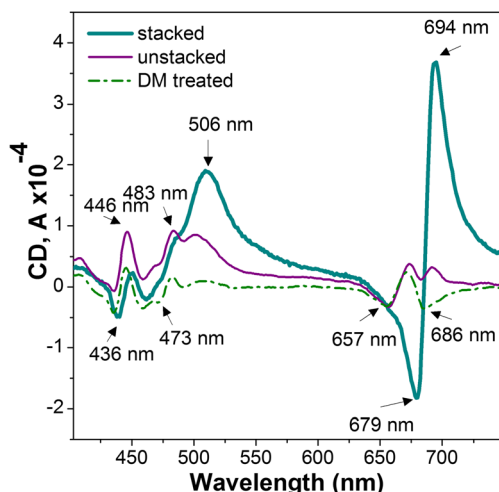
Fluorescence induction curves of isolated stacked and unstacked thylakoid membranes (1 mg chl/ml) were obtained by Multifunctional Plant Efficiency Analyzer (M-PEA), developed by Hansatech Instruments Ltd. (King's Lynn, UK). Prompt fluorescence was recorded for 120 s at light intensity of 4000  $\mu$ mol photons/m<sup>2</sup> s. For determination of the relative amount of PSII $\alpha$ , PSII $\beta$  and PSII $\gamma$  centers the thylakoids were incubated for 30 min with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (final concentration  $3.5 \times 10^{-5}$  mM). Prompt fluorescence transients of DCMU treated thylakoids were recorded for 1 s.

### Results

In this work we explored the role of membrane stacking for the thermal stability of the photosynthetic complexes in higher plants thylakoid membranes by means of CD and DSC. We compared stacked thylakoids with intact 3D structure and unstacked (Mg<sup>2+</sup> depleted membranes) with dismantled architecture that represent two limiting cases of ordered and disordered membranes with matching protein and lipid constituents but differing in their organization within the membrane. To check whether the observed variations in the thermal stability of LHCII are affected not only by the protein rearrangements upon membrane unstacking but also by the association of LHCII with PSI, we treated the unstacked membranes with the mild detergent  $\beta$ -DM that is known to impair the PSI-LHCII association (Wientjes et al. 2013).

### Changes in the thermal stability of thylakoid membranes upon unstacking

The CD spectra of intact stacked thylakoids (Fig. 1) exhibit spectral features similar to those already reported for a variety of plant species (Tóth et al. 2016). The high intensity psi-type CD bands  $CD_{694-750}$  and  $CD_{750-679}$  were demonstrated to originate from ordered chiral domains of PSII supercomplexes in stacked thylakoid configuration (Garab et al. 1988; Dobrikova et al. 2003; Tóth et al. 2016); the  $CD_{506-620}$  band was recently shown to be due to  $\beta$ -carotene located within PSII cores found in a long-range ordered structure (Tóth et al. 2016). In addition some of the excitonic CD bands can be attributed to specific pigment-pigment interactions within and between the pigment-protein complexes: the  $CD_{483-473}$  band reflects LHCII protein-protein interactions within the trimers affecting the peripheral chl *b* and neoxanthin molecules (Gradinaru et al. 2003; Dobrikova et al. 2003; Georgakopoulou et al. 2007; Lambrev et al. 2007); the  $CD_{444-436}$  band involves chl *a* and carotenoids (Garab et al. 1991; Georgakopoulou et al. 2007) and is present in unstacked thylakoids and isolated LHCII aggregates but is



**Fig. 1** CD spectra of stacked (thick line), unstacked (thin line) and  $\beta$ -DM treated (thin dash-dotted line) thylakoid membranes recorded at 20 °C. Peak positions are indicated by arrows. The chlorophyll concentration was 15  $\mu$ g chl/ml

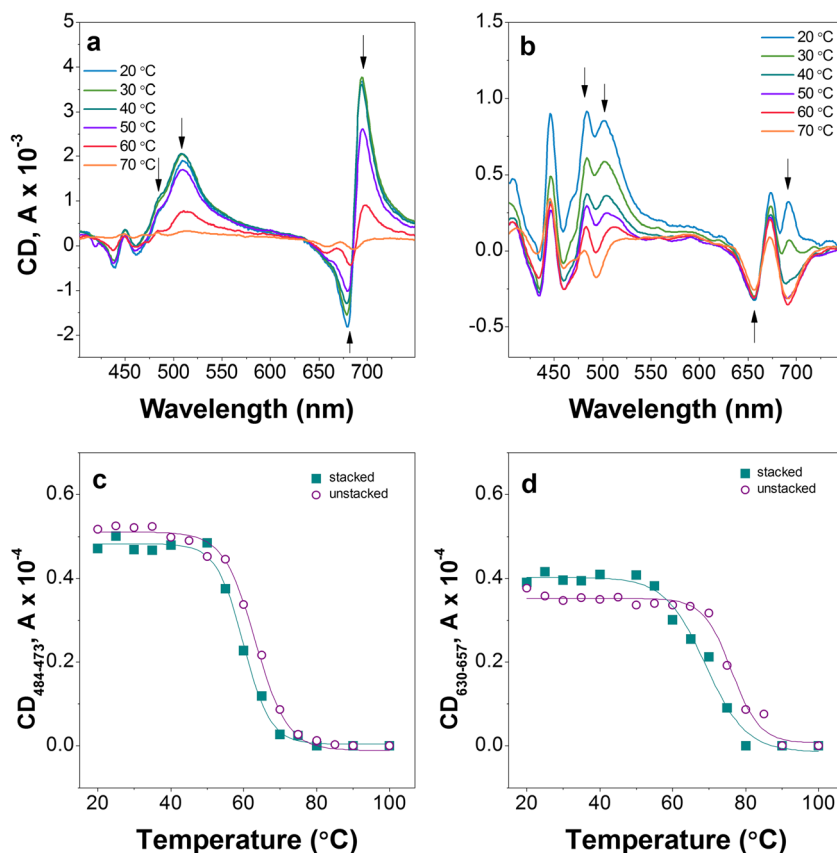
reduced in disaggregated LHCII (Lambrev et al. 2007; Akhtar et al. 2015);  $CD_{444-436}$  is also present in solubilized PSI-LHCI supercomplexes and PSI cores (Krumova et al. 2014) and the  $CD_{630-657}$  band involves chl *b* interactions within LHCII monomers (Garab et al. 1991; Dobrikova et al. 2003). Thus,  $CD_{694-750}$ ,  $CD_{750-679}$  and  $CD_{506-620}$  bands might serve as spectroscopic fingerprints of the lateral organization of the

PSII-LHCII supercomplexes within intact thylakoid membranes, while  $CD_{483-473}$  and  $CD_{630-657}$  bands reflect the stability of trimeric and monomeric LHCII complexes, respectively.

The unstacking procedure, as expected, led to dramatic reduction of  $CD_{694-750}$  and  $CD_{506-620}$  bands, and diminishment of the  $CD_{750-679}$  band (Fig. 1) indicating disassembly of the ordered PSII-LHCII domains and randomization of the complexes, similarly to earlier reports (Dobrikova et al. 2003; Lambrev et al. 2007). The amplitude of the  $CD_{446-436}$  band was higher in the unstacked than in the stacked sample. To probe the spectral properties of unstacked thylakoids when the LHCII-PSI association and/or LHCII aggregation is impaired we utilized 0.1%  $\beta$ -DM treatment. It led to complete diminishment of the (+)694 nm, (-)679 nm and (+)506 nm CD signals, without dramatic effects on the other excitonic CD bands (Fig. 1).

The CD spectra of heat treated stacked and unstacked thylakoids are presented in Fig. 2 and the transition temperatures derived for the different CD bands are compared in Table 1. In the stacked membranes the  $CD_{694-750}$ ,  $CD_{750-679}$  and  $CD_{506-620}$  bands were the least stable, followed by  $CD_{446-436}$ ,  $CD_{483-473}$  and  $CD_{630-657}$  bands (Fig. 2a, Table 1). For unstacked thylakoids the  $CD_{506-620}$  band was by more than 20 °C less stable than the excitonic  $CD_{630-657}$ ,  $CD_{483-473}$  and  $CD_{444-436}$  bands (Fig. 2b, Table 1).

**Fig. 2** Series of CD spectra of heat treated stacked (a) and unstacked (b) thylakoid membranes. The samples were incubated for 5 min at desired temperatures (denoted on each panel) and subsequently measured at 20 °C. For clarity the arrows indicate the direction of the reduction in amplitude of CD bands. Temperature dependences of  $CD_{484-473}$  and  $CD_{630-657}$  bands are presented in panels (c) and (d), respectively, for stacked (solid squares) and unstacked (open circles) samples



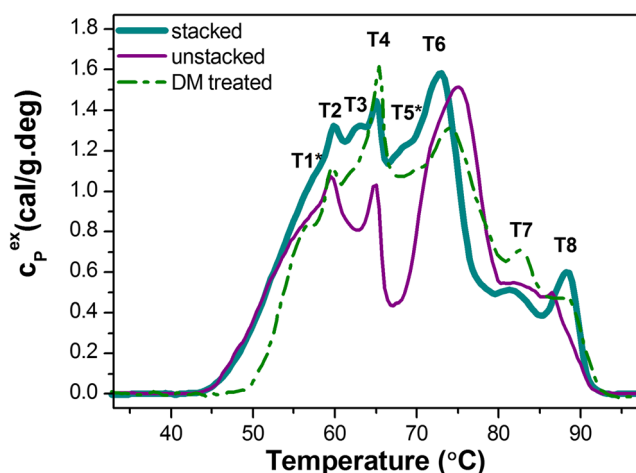


**Table 1** Transition temperatures ( $T_m^{CD}$ ) of characteristic CD bands at which the amplitude of the CD bands reduces by 50% of its value at 20 °C. Mean values  $\pm$  SD,  $n = 5$ 

| CD band               | $T_m^{CD}$ (°C) |                |
|-----------------------|-----------------|----------------|
|                       | stacked         | unstacked      |
| CD <sub>694–750</sub> | 53.5 $\pm$ 0.07 | –              |
| CD <sub>750–679</sub> | 54.8 $\pm$ 1.20 | –              |
| CD <sub>506–620</sub> | 56.5 $\pm$ 0.40 | 40.2 $\pm$ 3.1 |
| CD <sub>483–473</sub> | 59.5 $\pm$ 1.60 | 63.4 $\pm$ 0.3 |
| CD <sub>446–436</sub> | 62.2 $\pm$ 3.10 | 68.9 $\pm$ 1.7 |
| CD <sub>630–657</sub> | 69.3 $\pm$ 1.50 | 74.8 $\pm$ 1.6 |

The heat stability of LHCII monomers and trimers, corresponding to the thermal stability of the CD<sub>630–657</sub> and CD<sub>483–473</sub> bands, respectively, increased by 4–6 °C upon unstacking; in addition, the CD<sub>444–436</sub> band is also stabilized by 7 °C in the unstacked state (Table 1). On the other hand, the CD bands reflecting the macroorganization of the PSII-LHCII supercomplexes (CD<sub>506–620</sub>, CD<sub>694–750</sub> and CD<sub>750–679</sub>) were diminished or dramatically destabilized upon unstacking of the membranes (Table 1). These data demonstrate that the impairment of the lateral organization of the photosynthetic complexes upon unstacking is associated with significantly altered LHCII thermal stability that can be attributed to modified protein-protein and/or protein-lipid interactions.

The calorimetric profiles (thermograms) of stacked and unstacked pea thylakoids are compared in Fig. 3 and the denaturation temperatures and excess heat capacities of the resolved thermal transitions are summarized in Table 2. Eight endothermic events were resolved in the thermograms of stacked thylakoids with transition temperatures at 57, 59, 63,



**Fig. 3** Thermograms of stacked (thick line), unstacked (thin line) and  $\beta$ -DM treated (thin dash-dotted line) thylakoid membranes. Scanning rate 0.5 °C/min, sample concentration 1–1.3 mg chl/ml. For clarity the individual transitions are labeled T1–T8, \* indicates not well resolved transitions (shoulders)

65, 69, 73, 82 and 88 °C, denoted as T1\* – T8; the transitions at 57 °C and 69 °C are only observed as shoulders due to the overlap with neighboring transitions and therefore are marked with \*(T1\* and T5\*, respectively). Although the heating protocol for CD and DSC measurements are not identical, the temperature dependence of the CD bands (that specifically reflects the thermal stability of the pigment binding proteins), as well as previous calorimetric data on thylakoid membranes and submembrane fragments, can be used to gain insight on the nature of the calorimetric transitions in the ordered and disordered thylakoids under our experimental conditions. As mentioned above the CD bands related to the disassembly of the domains of ordered PSII supercomplexes upon unstacking (CD<sub>694–750</sub>, CD<sub>750–679</sub> and CD<sub>506–620</sub>) had very close transition temperatures in the range 53 °C – 56 °C. Therefore, it can be assumed that this process contributes to the first thermally-induced event, T1\* (57 °C), in stacked thylakoids as previously demonstrated (Dobrikova et al. 2003). The melting of PSII cores is expected to occur close to this temperature as well. The T2 (59 °C) transition had transition temperature identical to that of the CD<sub>483–473</sub> band (59 °C) and therefore it can be regarded that LHCII-LHCII interactions (within LHCII trimers) contribute to this thermal event. The sharp transition at 65 °C (T4) most probably originates from the denaturation of the thylakoid ATPase (Nolan et al. 1992). The transition temperature of the main calorimetric transition (T6, 73 °C) is close to that of the CD<sub>630–657</sub> band in the stacked membranes and therefore it can be regarded that this transition is dominated by the denaturation of LHCII, in line with previous reports (Dobrikova et al. 2003; Krumova et al. 2010a).

The unstacking procedure resulted in two main effects on the thylakoids DSC profiles: (i) reduced amplitude (excess heat capacity,  $c_p^{ex}$ ) of the transitions below 65 °C, an effect that cannot unambiguously be interpreted due to the overlap of multiple transitions in the 45–65 °C temperature range and (ii) clear shift of the 73 °C transition (and the shoulder at 69 °C) towards higher temperatures, that indicates higher resistance of LHCII monomers to thermal denaturation (Fig. 3, Table 2). The treatment of unstacked thylakoid membranes with 0.1%  $\beta$ -DM resulted in a shift of the T6 transition towards lower temperatures as compared to the unstacked thylakoids and significant increase of the amplitudes of T5\* and T3 transitions, thus leading to partial restoration of the calorimetric curve of stacked thylakoids (Fig. 3).

### Lipid phase behavior of stacked and unstacked thylakoid membranes

To determine whether the changes in the lipid phase interfere with the observed variations in the thermal stability of the components of the thylakoid membranes upon unstacking we probed the interaction of thylakoids with two polarity-

**Table 2** Transition temperatures ( $T_m$ ) and excess heat capacities ( $c_p^{ex}$ ) of the successive thermal transitions (T1\* - T8 as defined in Fig. 1 and in text), derived from the thermograms of stacked and unstacked pea thylakoids. The  $T_m$  values were determined at the peak of the calorimetric transitions. Mean values  $\pm$  SD,  $n = 5$

| DSC transition | stacked        |                        | unstacked      |                        |
|----------------|----------------|------------------------|----------------|------------------------|
|                | $T_m$ (°C)     | $c_p^{ex}$ (cal/g.deg) | $T_m$ (°C)     | $c_p^{ex}$ (cal/g.deg) |
| T1*            | 56.9 $\pm$ 1.1 | 1.16 $\pm$ 0.03        | 56.0 $\pm$ 0.1 | 0.83 $\pm$ 0.08        |
| T2             | 59.9 $\pm$ 0.2 | 1.33 $\pm$ 0.16        | 59.5 $\pm$ 0.0 | 1.07 $\pm$ 0.04        |
| T3             | 63.0 $\pm$ 0.2 | 1.32 $\pm$ 0.07        |                |                        |
| T4             | 65.0 $\pm$ 0.0 | 1.44 $\pm$ 0.08        | 64.9 $\pm$ 0.2 | 1.05 $\pm$ 0.01        |
| T5*            | 68.9 $\pm$ 0.5 | 1.23 $\pm$ 0.07        | 71.6 $\pm$ 1.0 | 1.30 $\pm$ 0.18        |
| T6             | 72.9 $\pm$ 0.2 | 1.58 $\pm$ 0.17        | 75.3 $\pm$ 0.8 | 1.53 $\pm$ 0.18        |
| T7             | 81.8 $\pm$ 0.2 | 0.51 $\pm$ 0.02        | 82.7 $\pm$ 0.5 | 0.54 $\pm$ 0.06        |
| T8             | 88.4 $\pm$ 0.2 | 0.61 $\pm$ 0.06        | 86.8 $\pm$ 0.9 | 0.50 $\pm$ 0.04        |

sensitive lipophilic markers - MC540 and laurdan. The fluorescence spectra of those markers were shown to be sensitive to the packing of the lipid molecules for a number of artificial and biological membranes, including thylakoids (Stillwell et al. 1993; Wilson-Ashworth et al. 2006; Szilágyi et al. 2008; Krumova et al. 2008, 2010a, 2010b; Garab et al. 2017).

As a first step we compared the fluorescence characteristics of MC540 and laurdan when suspended in the buffer media used to measure the stacked and unstacked membranes, and found no buffer related changes (data not shown).

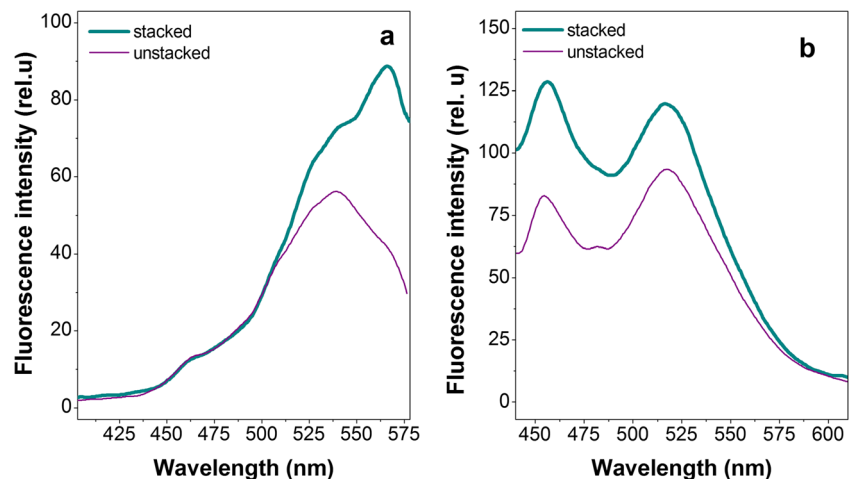
Stacked thylakoid membranes with added MC540 exhibited excitation spectra similar to previously reported (Krumova et al. 2008) with a typical maximum at 563 nm due to MC540 monomers buried deep into the membrane bilayer and a shoulder at 536 nm due to surface associated MC540 dimers (Fig. 4a). For stacked membranes the ratio of the fluorescence intensities at 563 and 536 nm,  $F_{563/536}$ , that reflects the extent of membrane incorporation of MC540, was found to be  $F_{563/536} = 1.30 \pm 0.09$ .

The incorporation of MC540 into unstacked thylakoids resulted in a significantly different excitation spectrum than the one recorded for stacked samples. The 563 nm band was essentially missing after unstacking and  $F_{563/536}$  ratio was

nearly two times lower than in stacked membranes ( $0.79 \pm 0.1$ ) (Fig. 4a), which clearly indicates that MC540 incorporation in unstacked is far lower than in stacked membranes.

The incorporation of laurdan in the two types of thylakoid membranes was confirmed by the significant change in the fluorescence intensity as well as the dramatic red shift of its emission spectra upon interaction with thylakoid membranes (Fig. 4b), while no difference was observed for laurdan suspended in stacking and unstacking buffer. When embedded into both stacked and unstacked thylakoid membranes laurdan exhibited two distinct emission maxima at 457 nm and 513 nm (Fig. 4b). This indicates that the probe distinguishes two types of microenvironments with more or less tightly packed lipids, respectively. The amplitude of the two emission maxima differed significantly in the two types of membranes (Fig. 4b) that in turn is reflected in the laurdan's GP values. The GP values varied in a broad range (0.03–0.10 for stacked and  $-0.06 - 0.03$  for unstacked thylakoids) but for each individual preparation the GP value for stacked was always higher than that for unstacked thylakoids ( $\Delta GP = 0.08 \pm 0.01$ ) revealing that the lipid matrix in the unstacked thylakoids is more disordered than in the stacked ones.

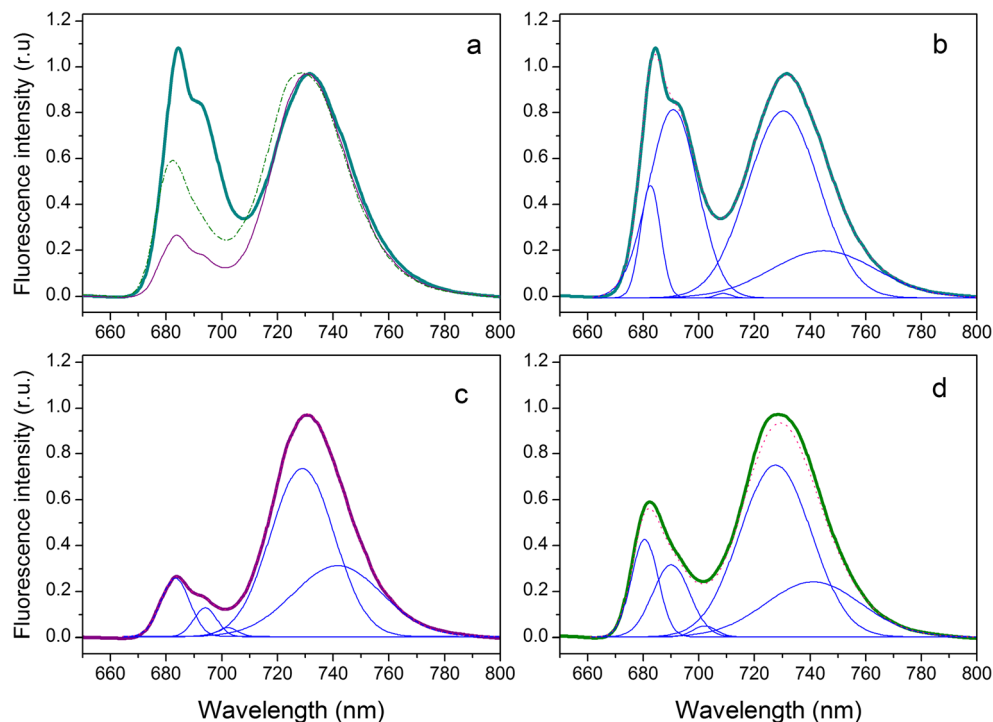
**Fig. 4** Fluorescence characteristics of MC540 (a) and laurdan (b) after 30 min incubation with stacked (thick lines) and unstacked (thin lines) thylakoid membranes at 20 °C. The excitation spectra of MC540 are recorded at 595 nm (a). The emission spectra of laurdan are recorded upon excitation at 390 nm (b). Excitation and emission slits 10 nm



## Effect of unstacking on the excitation energy distribution and photosystem II activity

The distribution of the excitation energy between the two photosystems in stacked and unstacked thylakoids was studied by 77 K steady state fluorescence. This technique allows to discriminate emission bands characteristic of free (detached from photosystems) LHCII (680 nm), CP43 and CP47 complexes of PSII (685 and 695 nm), LHCII aggregates (700 nm) and PSI-LHCI complex (730 nm) (van Grondelle et al. 1994; Andreeva et al. 2003; Miloslavina et al. 2008). As already reported by van der Wij de Witt et al. (2007) and Kirchoff et al. (2007) a significant energy spillover from PSII to PSI takes place upon membrane unstacking, evidenced by the increased PSI fluorescence (at 730 nm) at the expense of PSII emission (below 700 nm) visible from the ratio of the intensities of the emission maxima at 684 nm and 730 nm,  $F_{684}/F_{730}$  (Fig. 5, Table 3). The  $\beta$ -DM treatment resulted in an increase of the  $F_{684}/F_{730}$  ratio, due to lowering of the PSI fluorescence upon LHCII detachment from PSI complex. The PSII emission below 700 nm, however, did not fully recover and the emission peak at 684 nm shifted to 682 nm suggesting that part of LHCII did not attach back to PSII and remained free in the membrane (Fig. 5a). To check if the different treatments are associated with the formation of LHCII aggregates (emitting at ca. 700 nm) we fitted the emission spectra with 5 Gauss components (Fig. 5b-d). In all three cases (stacked, unstacked and  $\beta$ -DM treated membranes) the resolved 700 nm component contributed  $\leq 1\%$  to the total emission, therefore no extensive LHCII aggregation took place.

**Fig. 5** 77 K emission spectra of stacked (thick line), unstacked (thin line) and  $\beta$ -DM treated (thin dash-dotted line) thylakoid membranes upon excitation of 472 nm (a). For clarity the spectra are normalized to the emission maximum at 730 nm. (b-d) Mathematical fit of the emission spectra of stacked (b), unstacked (c) and  $\beta$ -DM treated (d) thylakoid membranes (thick lines) employing 5 Gauss components (thin lines), and their sum (thin dotted lines)

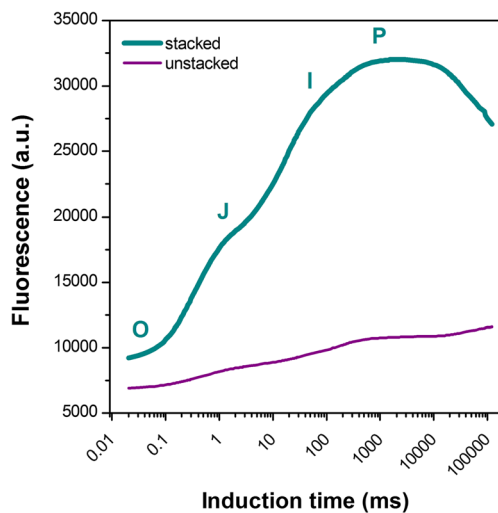


**Table 3** Ratio,  $F_{684}/F_{730}$ , of the intensities of the emission maxima at 684 nm (PSII emission) and 730 nm (PSI emission) for stacked, unstacked and  $\beta$ -DM treated thylakoid membranes. Mean values  $\pm$  SD,  $n = 5$

| sample                         | $F_{684}/F_{730}$ |
|--------------------------------|-------------------|
| stacked thylakoids             | $1.11 \pm 0.06$   |
| unstacked thylakoids           | $0.29 \pm 0.02$   |
| $\beta$ -DM treated thylakoids | $0.61 \pm 0.08$   |

To analyze the photochemical activity of PSII supercomplexes in stacked and unstacked thylakoids we applied OJIP test (Strasser et al. 2004). The recorded fluorescence induction curves of intact and unstacked thylakoids are shown in Fig. 6. The fast chl *a* fluorescence transient possessed four characteristic points: O, J, I and P which occur at 20  $\mu$ s,  $\sim 2$  ms,  $\sim 30$  ms and  $\sim 300$  ms, respectively. The fluorescence intensity values at these points (denoted as  $F_0$ ,  $F_J$ ,  $F_I$ ,  $F_M \equiv F_P$ ) were used for calculation of the following parameters:  $F_0$  (minimal fluorescence recorded at the beginning of the fluorescent induction curve);  $F_M$  (maximal fluorescence recorded at the end of the fluorescent induction curve); their ratio  $F_M/F_0$ ;  $F_V/F_0 = (F_M - F_0)/F_0$  (effectiveness of the primary photochemical reaction) and  $M_0 = 4(F_{300\mu s} - F_0)/F_V$  (the maximum rate of  $Q_A$  reduction, expressed as the initial slope of the fluorescence curve at the beginning of the illumination).

The initial slope of the induction curve was lower and the  $M_0$  parameter was consequently reduced for the unstacked membranes, strongly suggesting slower initial photochemistry rate (maximal  $Q_A$  reduction rate) in the unstacked than in the



**Fig. 6** Fluorescence induction curves of stacked (thick line) and unstacked (thin line) thylakoids

stacked membranes (Table 4). The  $F_M/F_0$  was found to be about 2 times higher for the unstacked than for the stacked membranes indicating large differences in the utilization of the excitation light energy in the two samples (Table 4). The fluorescence quantum yield in the unstacked samples was strongly decreased (both at  $F_0$  and  $F_M$  points) as compared to the stacked ones demonstrating significant quenching of fluorescence in unstacked condition. The four times reduced  $F_V/F_0$  parameter in unstacked membranes further showed dramatic change in the balance between photochemically and non-photochemically utilized excitation energy.

These data were further complemented by analysis of the fluorescence transients of thylakoids treated with DCMU. It was already reported that the complementary area growth curve (i.e. the area between the curve of fluorescence induction and the horizontal line determining the maximal fluorescence level,  $F_M$ ) can be described as a sum of three exponential phases. These three exponential curves correspond to PSII supercomplexes with different antenna size – PSII $\alpha$  (with the largest light harvesting antenna), PSII $\beta$  (with 2.5 times

**Table 4** OJIP parameters derived for stacked and unstacked thylakoids. Amount of PSII $\alpha$ , PSII $\beta$  and PSII $\gamma$  centers presented as a percentage of the total number of PSII centers. Mean  $\pm$  SEM,  $n = 5$

| parameter         | stacked          | unstacked        |
|-------------------|------------------|------------------|
| $F_0$             | 9238 $\pm$ 597   | 7427 $\pm$ 316   |
| $F_M$             | 31,744 $\pm$ 715 | 12,162 $\pm$ 854 |
| $F_V/F_0$         | 2.46 $\pm$ 0.15  | 0.64 $\pm$ 0.07  |
| $F_M/F_0$         | 3.46 $\pm$ 0.15  | 1.64 $\pm$ 0.07  |
| $M_0$             | 0.77 $\pm$ 0.02  | 0.48 $\pm$ 0.10  |
| PSII $\alpha$ (%) | 86.3% $\pm$ 5.6  | 74.7% $\pm$ 2.8  |
| PSII $\beta$ (%)  | 11.8% $\pm$ 5.6  | 22.6% $\pm$ 3.1  |
| PSII $\gamma$ (%) | 1.9% $\pm$ 0.42  | 2.6% $\pm$ 0.4   |

smaller antenna than PSII $\alpha$ ) and PSII $\gamma$  (with the smallest antenna), only the former two being photochemically active (Mehta et al. 2010 and the references therein). Our data show a decline by ca. 10% in the relative number of PSII $\alpha$  centers and increase in the PSII $\beta$  and PSII $\gamma$  supercomplexes in the unstacked compared to the stacked membranes (Table 4) – an effect that is similar but not identical to that observed for lettuce (Kaftan et al. 2002) and spinach (Kirchhoff et al. 2007) thylakoids, probably due to differences in the experimental conditions.

## Discussion

In this work we compared the thermal stability of the major antennae complex of photosystem II, which is also the most abundant thylakoid membrane protein, in stacked and unstacked thylakoid state. Although the chemically unstacked thylakoid membranes do not represent a native membrane state, they can be regarded as a system that mimics the plants' response to high light conditions which leads to significant enlargement of the unstacked membrane domains in vivo (Herbstová et al. 2012; Khatoon et al. 2009; Yamamoto et al. 2013). Therefore, unstacked membranes are a suitable model system to gain insight into the light adaptation mechanisms of plants and the role of membrane stacking for the optimization of the photosynthetic processes.

We demonstrated that thylakoid membrane unstacking (induced by  $Mg^{2+}$  depletion) results in significant thermal stabilization of LHCII complexes and change in the fluidity of the lipid matrix. The protein intermixing upon unstacking led to substantial energy spillover towards PSI and lower PSII photochemical activity.

Our data reveal that the thermal stability of LHCII monomers (CD<sub>630–657</sub> band, T5 calorimetric transition) and trimers (CD<sub>483–473</sub> band, T3 calorimetric transition) is significantly lower in stacked than in unstacked thylakoid membranes. Several factors might be responsible for the different thermal stability of LHCII in the two membrane states: (i) different ionic strength of the suspension medium, (ii) modified protein-protein associations and (iii) altered protein-lipid interactions and/or lipid phase fluidity. The first factor (presence or lack of  $Mg^{2+}$  in the suspension medium) apparently is not the major determinant of LHCII stability in the unstacked state since measurements on unstacked and  $\beta$ -DM treated thylakoids, both of which are suspended in cation depleted medium, resulted in different LHCII thermal stabilities (Fig. 3). The second factor seems more probable since there are number of evidences that LHCII changes its association partners upon unstacking. In the stacked condition LHCII serves as antenna for both PSII and PSI (Wientjes et al. 2013) but upon unstacking a large fraction of the LHCII complexes migrated from PSII to PSI that resulted in 4 times decrease in the



F684/F730 parameter in our study. Therefore, it can be suggested that the thermal stability of LHCII increases upon association with PSI. This is confirmed by the  $\beta$ -DM treatment experiment revealing that detergent induced dissociation of LHCII from PSI results in decrease in LHCII's thermal stability. In the stacked membrane state the stroma exposed N terminus of LHCII is highly disordered (Liguori et al. 2015), while in the unstacked state the N terminus of LHCII interacts with PSI (Pan et al. 2018) and hence becomes immobilized, which might exert stabilizing effect on LHCII thermal stability. However, it is to be noted that the change in the thermal stability of the subpopulation of LHCII that shuffles between the two photosystems might not be the sole contributor to the observed large changes in the CD and DSC features occurring upon unstacking.

LHCII stability is strongly dependent on the lipid-LHCII interactions as well as the physical state of the bulk lipid phase. The two fluorescent probes used in this study to probe the membrane fluidity (MC540 and laurdan) exhibited different behavior with respect to thylakoid membrane incorporation in the stacked and unstacked state. While MC540 was not able to penetrate into unstacked membranes and thus could not report on the physical properties of the lipid matrix, laurdan was well incorporated and provided solid evidence for membrane fluidization upon unstacking. The association of MC540 with lipidic membranes is known to be hampered by the presence of surface exposed negative charges (Matešik et al. 2002) and therefore MC540 repulsion from unstacked membranes is most probably electrostatically driven. Indeed,  $Mg^{2+}$  depletion exposes the protein negative charges on the membrane surface (Barber 1982). Furthermore, Sheng et al. (2018) have recently shown that the anionic lipid molecules are enriched on the stromal side of the membrane, thus forming a membrane domain with a high concentration of negative charges, which are screened by  $Mg^{2+}$  ions in stacked thylakoids but become surface exposed in unstacked membranes.

The decrease in the GP values of laurdan in unstacked as compared to stacked conditions clearly indicates increase in membrane fluidity upon unstacking. The decreased GP values of laurdan emission determined for unstacked thylakoids in our work might either be a result of more fluid bulk lipid phase (providing the relative proportion of tightly and loosely packed lipid areas is not changed) or of extended “fluid-like” regions (at the expense of shrunk tightly packed lipid domains). Either way the more fluid lipid phase in the unstacked than in the stacked membranes might facilitate LHCII diffusion towards PSI and the protein intermixing that is known to occur in unstacked thylakoids (Stahelin 1976; Kirchhoff et al. 2007). Contrary to our observations Rumak et al. (2010) showed that the lipid disorder and the protein:lipid ratio do not differ between  $Mg^{2+}$  supplemented and  $Mg^{2+}$

depleted thylakoid samples. However, it should be taken into account that in our case a more extensive cation-washing procedure was used, resulting in higher membrane unstacking than that achieved by Rumak et al. (2010). This is also evidenced by the larger difference in the ratio of the PSII and PSI emission peaks in our stacked and unstacked preparations than those of Rumak et al. (2010). Higher membrane fluidity was recently reported for partial unstacking of thylakoids as a response to illumination with moderately high light intensity (Yamamoto et al. 2013), indicating that the physical state of the membrane lipid phase is intricate part of the regulatory mechanisms of plants coping with different light intensities.

The lower thermal stability of LHCII in stacked than in unstacked membrane state might be associated with the higher LHCII flexibility that facilitates its regulatory functions and/or the recognition of interaction partners in stacked conditions. There are a number of studies demonstrating that the high LHCII flexibility is important for plants photoprotection - LHCII is able to exert reversible light-induced structural changes both in vivo and in vitro, and to switch between conformations with different capacity to quench the excess of excitation energy (reviewed in Garab 2014).

In functional terms our data confirm previous reports that unstacking induces excitation energy leakage to PSI (Butler and Kitajima 1975; Briantais et al. 1984; van der Weij-de Wit et al. 2007; Kirchhoff et al. 2007) that in our work is reflected by the overall decrease in fluorescence yield in unstacked membranes at room temperature, the increase in the relative fluorescence at 730 nm at 77 K and the significantly hampered PSII excitation energy utilization.

## Conclusions

The presented data demonstrate that the thylakoid membrane unstacking strongly affects the thermal stability of LHCII complexes and the fluidity of the lipid matrix. The stabilization of LHCII monomers and trimers in the unstacked state can be attributed to both increased membrane fluidity and LHCII-PSI association. The physical properties of the protein and lipid membrane components in the unstacked state result in strongly reduced photosystem II photochemical activity.

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