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Increased thermal stability of photosystem II and the macro-organization of thylakoid membranes, induced by co-solutes, associated with changes in the lipid-phase behaviour of thylakoid membranes

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Abstract

The principal function of the thylakoid membrane depends on the integrity of the lipid bilayer, yet almost half of the thylakoid lipids are of non-bilayer-forming type, whose exact functions are not fully understood. Non-bilayer lipids can be extruded from the membrane in the presence of high concentrations of co-solutes. We applied 2 M sucrose to induce lipid phase separation in isolated thylakoid membranes, following consequent structural and physiological effects. Circular dichroism spectroscopy indicated significant changes in the chiral macro-arrangement of the pigment–protein complexes, which were reversed after washing out the co-solute. Similarly, merocyanine-540 fluorescence suggested reversible changes in the lipid phases. The PSII function, as tested by chlorophyll fluorescence induction transients and time-resolved fluorescence, was almost unaffected. However, the presence of sucrose dramatically increased the PSII thermostability, which can partly be explained by a direct osmolyte effect and partly by the lipid phase separation stabilizing the stacked membrane.

Additional key words: circular dichroism; merocyanine-540; non-bilayer lipids; osmolyte; time-resolved fluorescence spectroscopy.

Introduction

The thylakoid membranes of oxygenic photosynthetic organisms embed virtually all components, which actively participate in the light reactions of photosynthesis. The thylakoids are flattened lipid vesicles, which separate the inner (lumenal) and outer (stromal side in chloroplasts, cytoplasmatic side in cyanobacteria) aqueous phases. The primary charge separations in the two photosystems, PSII and PSI, and the associated electron and proton transport generate a transmembrane electrochemical potential gradient, $\Delta\mu_H^+$, consisting of electrical potential ($\Delta\Psi$) and proton gradient (ΔpH), which is utilized for the synthesis of ATP (Mitchell 1966). The build-up of $\Delta\mu_H^+$ and its utilization is warranted by the organization of the thylakoid membrane as a bilayer, which is largely impermeable to water and most water-soluble molecules and ions.

The strong restriction on the functional state of the thylakoid membrane as a bilayer is not easy to reconcile with the fact that only about half of the thylakoid lipids are capable to self-assemble into bilayers (Douce and Joyard 1996). These bilayer lipids are the digalactosyl-diacylglycerol (DGDG), sulfoquinosyl-diacylglycerol (SQDG), and phosphatidylglycerol (PG). In contrast, the major lipid species of thylakoids, constituting about the other half (45–55%) of the total lipid content, is the non-bilayer lipid monogalactosyl-diacylglycerol (MGDG). In aqueous media under physiologically relevant conditions, non-

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Abbreviations: CD – circular dichroism; Chl – chlorophyll; DAES – decay-associated emission spectra; DGDG – digalactosyl-diacylglycerol; IRF – instrument response function; MC540 – merocyanine-540; MGDG – monogalactosyl-diacylglycerol; PG – phosphatidylglycerol; psi – polymer and salt induced; SQDG – sulfoquinosyl-diacylglycerol; TCSPC – time-correlated single-photon counting
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bilayer lipids are not capable to form bilayers but self-assemble into different non-bilayer lipid phases, such as the inverted hexagonal (H_{II}), isotropic, and cubic phases (Williams 1998). MGDG, along with the bilayer lipid species might play important roles in the molecular architecture of different protein complexes and super-complexes (Garab *et al.* 2016). This, however, does not explain their large abundance in the bulk phase: about 60% of the total thylakoid lipids are found in a fluid-like phase (Páli *et al.* 2003).

As shown by mixing together isolated MGDG and light-harvesting complex II (LHCII), these non-bilayer lipids can be forced into bilayer membrane *via* lipid–protein interactions, during which LHCII destroys the H_{II} phase and forms a bilayer phase (Simidjiev *et al.* 2000). These membranes, however, appear to be in a frustrated state with high structural flexibility (Simidjiev *et al.* 1998). In good accordance with this, *dgd1*-mutant thylakoid membranes with an increased MGDG content have been shown to exhibit higher thermal susceptibility (*i.e.* lower thermal stability) compared to the wild type (Krumova *et al.* 2010).

Numerous data have shown that *in vitro*, in etioplasts, and *in situ*, in plants exposed to stress conditions, thylakoid lipids are capable of forming different non-bilayer structures (Williams 1998, Kirchhoff *et al.* 2007, Janik *et al.* 2013, Demé *et al.* 2014, van Eerden *et al.* 2015, Kowalewska *et al.* 2016). MGDG, and non-bilayer lipid phases in general, have been shown to play key role in the functioning of the violaxanthin de-epoxidase enzyme, responsible for the transformation of violaxanthin to zeaxanthin (Yamamoto and Higashi 1978, Latowski *et al.* 2004, Goss *et al.* 2005, 2007; Jahns *et al.* 2009). The coexistence of the bilayer and non-bilayer lipid phases and dynamic exchange between the phases has been hypothesized by Garab *et al.* (2000) and indicated by ^{31}P-NMR data on freshly isolated, intact plant thylakoid membranes (Krumova *et al.* 2008a). Further, marked heterogeneity of lipid phases of thylakoid membranes has also been shown by fluorescence spectroscopic experiments and lifetime measurements using the lipophilic fluorescent dye MC540. Most recently, by employing ^{31}P-NMR spectroscopy on isolated thylakoid membranes, we have shown the presence of three non-bilayer lipid phases in addition to the bilayer phase; time-resolved MC540 fluorescence spectroscopic measurements, by detecting four components in the decay-associated spectra, confirmed that lipids in the thylakoid membranes cannot be portrayed by a single, bilayer phase, but should rather be characterized by a significant degree of heterogeneity (Garab *et al.* 2017). Nevertheless, the nature and physiological significance of different lipid phases remain to be elucidated.

It has been shown that high concentrations of co-solutes, such as sucrose, glycerol, and glycine-betaine, induce the formation of H_{II} phase (Williams *et al.* 1992), also leading to the appearance of large quasi-crystalline protein arrays in the thylakoid membrane (Tsvetkova *et al.* 1995). In association or parallel with the extrusion of lipids from the membrane, high concentrations of co-solutes have been shown to stabilize PSII and the oxygen-evolving complex, in particular – significantly elevating its thermal stability (Williams *et al.* 1992, Williams and Gounaris 1992). In this work, by employing chlorophyll (Chl) *a* fluorescence transients, circular dichroism, and time-resolved MC540 fluorescence spectroscopy, we analysed the effects of the co-solute 2 M sucrose on the activity of PSII, on the macro-organization of protein complexes in the thylakoid membrane systém, and on the lipid phase behaviour of membranes. We show that, in agreement with Williams *et al.* (1992), 2 M sucrose significantly enhances the thermal stability of PSII. Further, we report on the stabilization of the chiral macrodomains of thylakoid membranes at elevated temperatures. As expected, 2 M sucrose also induces prominent changes in the lipid phase behaviour of thylakoid membranes. The fact that all these changes are largely reversible strongly suggests that the co-solute-induced non-bilayer lipid phase(s) remain(s) in close contact with the bilayer membrane.

Materials and methods

Isolation of thylakoid membranes: Thylakoid membranes were prepared from pea or spinach leaves, according to Krumova *et al.* (2008b), with minor modifications. Briefly, 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 and the supernatant was centrifuged for 10 min at 6,000 × *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 for 10 s followed by the immediate addition of the same medium supplemented with 800 mM sorbitol before centrifugation for 10 min at 6,500 × *g*. The pellet was finally resuspended in a medium containing 50 mM Tricine, 5 mM $MgCl_2$, and 5 mM KCl supplemented with 400 mM sorbitol (called control resuspension buffer below).

Sucrose treatment was performed according to Williams *et al.* (1992). Membrane suspension of 40 μg(total Chl) in a volume of ~20 μl was centrifuged for 5 min at 3,000 × *g*. The obtained pellet was resuspended in equal volume of resuspension buffer containing additionally 2M sucrose.

For the recovery, sucrose-treated sample was centrifuged in 100× volume of control resuspension buffer for 15 min at 30,000 × *g* to remove the sucrose. Finally, the sample was resuspended in control resuspension buffer.

Merocyanin-540 (MC540) treatment: Suspension of thylakoid membranes containing 60 μg(Chl) in 50 μl was mixed with 10 mM MC540 and incubated for 30 min at room temperature. The mixture was diluted with 1 ml of the resuspension buffer and centrifuged for 15 min at

30,000 × *g*. The pellet was resuspended in 1 ml of resuspension buffer (control) or buffer supplemented with 2 M sucrose. For recovery, the pellet from the above step was resuspended with 0.5 ml of 2 M sucrose buffer and then diluted to 2 ml with control buffer, centrifuged for 15 min at 30,000 × *g* in order to wash the sucrose, and finally resuspended again in control buffer.

Absorption and circular dichroism spectroscopy: Room temperature absorption and circular dichroism (CD) spectra were recorded with a *Nicolet Evolution 500* dual-beam spectrophotometer (*Thermo Scientific*) and a *J-815* spectropolarimeter (*Jasco*), in the range of 350 and 750 nm with 3-nm spectral resolution. The samples were diluted to absorbance of 1.0 at the red maximum in 1-cm optical path length cell. Temperature dependence of the CD spectra were measured between 25–60°C in steps of 5°C, as by Krumova *et al.* (2010); the samples were thermostated for 5 min before each measurement.

Chl fluorescence transients: Fluorescence induction transients were recorded from thylakoid membrane suspensions diluted to 25 μg(Chl) ml^{-1} and pre-incubated for 5 min in the dark at temperatures from 25–60°C. Fluorescence was excited by a 5-s flash of red light [light intensity of 3,500 μmol(photon) m^{-2} s^{-1}] using a *Handy-PEA* fluorimeter (*Hansatech Instruments,* UK).

Time-resolved fluorescence: Excited-state decay kinetics were measured with picoseconds resolution using a *FluoTime 200* spectrometer (*PicoQuant*, Germany) equipped with a microchannel plate detector (*Hamamatsu*) and a *PicoHarp 300* TCSPC set-up (*PicoQuant*), as previously described (Akhtar *et al.* 2016). A *WhiteLase Micro* supercontinuum laser (*NKT Photonics*, UK) was used as an excitation source. Excitation wavelengths of 632 nm or 540 nm were selected by a monochromator to excite Chl or MC540, respectively. Fluorescence emission was detected through a second monochromator at wavelengths between 670–740 nm for Chl and between 560–620 nm for MC540. Samples were diluted to OD 0.03 at the excitation wavelength and continuously circulated through a 1.5-mm path length flow cell. All measurements were performed at room temperature. Fluorescence decays were analysed by a global lifetime fitting routine using a kinetic model and convolution with the measured IRF using homebuilt *MATLAB* routines.

Results

Absorption and CD spectra: High concentrations of co-solutes, such as sucrose, are known to induce phase separation of non-bilayer thylakoid membrane lipids (Williams *et al.* 1992). Significant changes in the overall granal membrane organization are to be expected as a consequence of the lipid phase separation, even though the stacked granal structures are shown to be largely preserved. We employed circular dichroism (CD) spectroscopy in the visible range to monitor for changes in the supramolecular structure of the thylakoid membranes. The CD signals in this region originate from Chls and carotenoids bound to the photosynthetic pigment–protein complexes (Garab and van Amerongen 2009). The CD spectra of granal thylakoid membranes are characterized by the presence of intense anomalously shaped "psi-type" CD bands, which are associated with the long-range chiral order of the pigment molecules and are highly sensitive to the three-dimensional supramolecular organization of the membrane (Barzda *et al.* 1996, Garab *et al.* 1988b).

Typical absorption and CD spectra of pea thylakoid membranes resuspended in control buffer, in buffer containing 2 M sucrose, or in control buffer after washing out the sucrose, are presented in Fig. 1. The absorption spectra of the control and sucrose-treated samples showed almost no differences. In contrast, significant changes occurred in the CD spectra, especially in the psi-type bands, at (+)506, (−)672, and (+)688 nm (peak positions in control buffer). The psi-type CD band at 506 nm, which is associated with carotenes in the photosystem core complexes (Kovács *et al.* 2006), completely disappeared

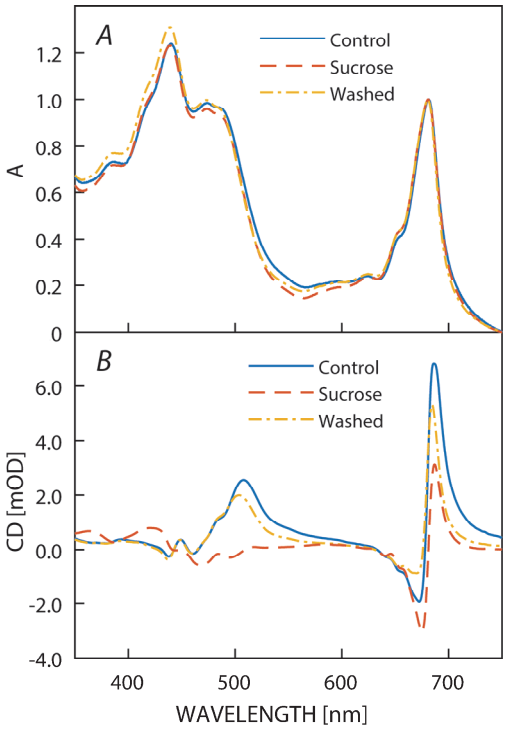

Fig. 1. Absorption (*A*) and CD (*B*) spectra of thylakoid membranes suspended in control buffer (*blue solid line*), in buffer with additional 2 M sucrose (*red dashed line*) and re-suspended in control buffer after incubation in 2 M sucrose buffer (*yellow dot-dashed line*). Spectra are normalized to the Q_y absorbance maximum.

in 2 M sucrose, exposing the excitonic CD bands characteristic for unstacked thylakoid membranes and isolated pigment–protein complexes. In the Chl Q_y region (650–700 nm), the amplitude of the (+)688 nm band was less than half in 2 M sucrose buffer compared with control buffer. The CD band at (−)672 nm shifted to 677 nm gaining negative amplitude, apparently as a consequence of the diminished (+)688 nm band, which partially overlaps with it. Such drastic changes in the CD spectra cannot be attributed to optical artefacts, as the absorption spectra of the same samples were virtually unaffected. Rather, they are evidence for changes in the macro-organization of the thylakoid membranes, leading to a significant reduction in the lamellar distances (Posselt *et al.* 2012), and at least partially caused by lipid phase separation affecting the protein density in the membrane and thus the long-range chiral order of the chromophores. In addition, the CD spectra of sucrose-treated samples exhibited changes outside the psi-type bands, most noticeably the appearance of new CD bands around 360 and 430 nm. This intriguing phenomenon warrants further inquiry into the nature of these signals.

Interestingly, after washing out the sucrose and resuspending the thylakoid membranes in control buffer, the control CD spectrum was essentially restored. The positive psi-type CD bands almost fully recovered and the additional sucrose-induced CD signals vanished. This shows that the sucrose-induced lipid phase separation and consequential macrostructural changes are almost fully reversible. The remaining differences with the original control-buffer CD spectrum were mostly in the differential scattering tails of the positive psi-type bands, which may be related to the different particle size (Garab *et al.* 1988a), and the suppressed 672 nm band, probably due to repeated mechanical stress (discussed below).

Temperature dependence of the CD spectra: A key characteristic of compatible co-solutes is their potency to protect proteins and protein complexes from thermal denaturation. Accordingly, treatment of chloroplast membranes with high concentrations of sucrose has been shown to significantly widen the temperature interval of PSII function (Williams *et al.* 1992). To evaluate the thermal stability of the thylakoid membrane assembly in the presence of sucrose, thylakoid membranes were subjected to gradual heating and CD spectra were recorded at every 5°C (Fig. 2). The maximal amplitude of the CD signal in the red region, which is equal to the 688 nm psi-type CD band intensity, decreased gradually above 40°C for thylakoids in control buffer, losing 80% at 60°C. In contrast, for thylakoids resuspended in buffer with 2 M sucrose, although the CD amplitude was much smaller to begin with, it was maintained to higher temperatures. A noticeable reduction of the psi-type CD in the Q_y region was observed only at 60°C; apart from this reduction, the spectra were virtually unaffected by temperature. After treating with 2 M sucrose and resuspending back in the control buffer, the temperature curve of the CD amplitude was fully restored (data not shown).

The differential response of the three psi-type CD bands to external factors was manifested also in their temperature dependence, in a similar fashion as reported by Cseh *et al.* (2000). The CD signal at (−)672 nm was the most temperature-sensitive, losing 50% amplitude at 48°C, whereas the equivalent transition temperatures for the 688 and 506 nm bands were 52 and 56°C, respectively.

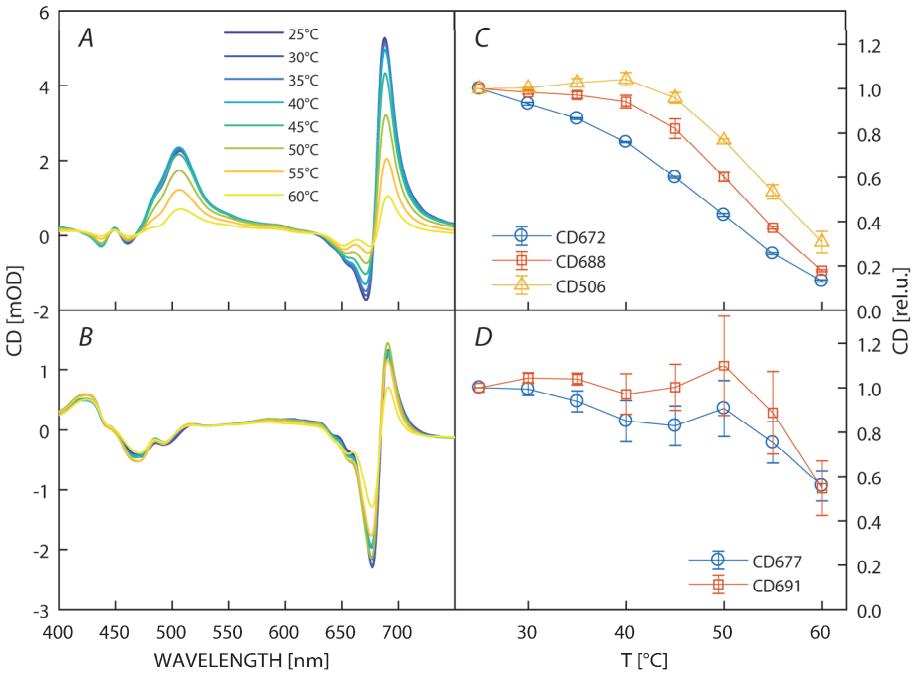

Fig. 2. Temperature dependence of the CD spectra of thylakoid membranes: (*A*) CD spectra in control buffer, (*B*) in buffer with additional 2 M sucrose, (*C*) temperature dependence of the intensity of the three psi-type CD bands of thylakoid membranes resuspended in control buffer, and (*D*) intensity of the psi-type CD bands in buffer with 2 M sucrose. The data points in (*C*) and (*D*) are normalized to the respective values at 25°C. Vertical bars indicate standard errors from three measurements.

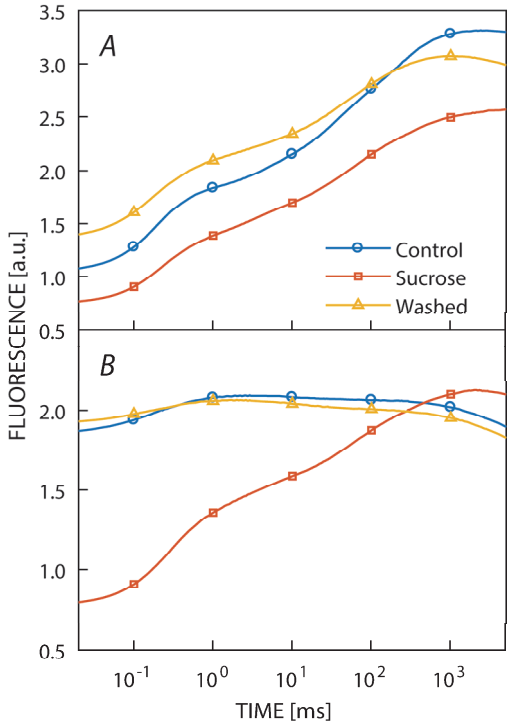

Fig. 3. Effect of 2 M sucrose on the fast chlorophyll fluorescence induction transients of thylakoid membranes in control buffer, buffer with additional 2 M sucrose and control buffer after washing out the sucrose measured after 5-min incubation at room temperature (*A*) or 45°C (*B*).

The temperature dependence of the Q_y-region psi-type bands remaining in 2 M sucrose buffer had a very different shape compared with control buffer, with both bands losing 45% amplitude at 60°C. The distinct behaviour suggests that the sequence of events in the thermal disassembly of the thylakoid structure is different in control and sucrose buffer.

Chl fluorescence: The presence of high sucrose concentrations in the resuspension medium not only stabilizes the thylakoid structure against thermal disassembly but also extends the temperature interval for photochemistry (*vide supra*). To examine this effect further, we measured fast Chl fluorescence induction transients after heating the thylakoid membranes up to 60°C in control and in 2 M sucrose medium (Fig. 3; Fig. 1S, *supplement available online*). It is well known that temperatures of 45°C and above inactivate the oxygen-evolving complex of PSII as well as the acceptor-side electron transfer (Goltsev *et al.* 1994, Kouřil *et al.* 2004). This is accompanied by a significant increase in the initial fluorescence intensity F_0 and a drop in the variable fluorescence $F_v = F_m - F_0$. After temperature treatment, the fluorescence transients of thylakoids in control medium not only showed strongly reduced variable fluorescence but almost no rise after ~2 ms (the J induction peak), which can be attributed to inhibition of the electron flow beyond Q_A. Addition of 2 M sucrose exerted a very minor effect on the fluorescence transients at 25°C and largely preserved the PSII activity after temperature treatment. The transient of thylakoids (Fig. 3*B*) preheated to 45°C showed no increase in F_0 and clearly distinguished O–J (0–2 ms) and J–P (2 ms – 2 s) phases, although the latter was reduced in amplitude compared to the room-temperature control.

The addition of sucrose led to a decrease in the initial slope of the normalized relative variable fluorescence curve after incubation at room or elevated temperature (Fig. 4). In the presence of active RCs, the initial slope of the relative variable fluorescence curve normalized at 2 ms, $W(t) = (F_{(t)} - F_0)/(F_{2ms} - F_0)$, depends on the rate of reduction of Q_A, which in turn depends on the effective absorption cross section of the PSII antenna (Strasser *et al.* 2004). The parameter J^{ABS}/RC, as described in Stirbet and Govindjee (2011), which reflects the effective number of antenna Chls per actively working RC, was reduced by ~20% in sucrose-treated thylakoids. This effect was fully reversed after washing out the sucrose.

Fig. 5 reports two key fluorescence induction parameters for thylakoids incubated at different temperatures in control medium, 2 M sucrose, and resuspended in control medium after washing out the sucrose. The maximal variable fluorescence F_v/F_m is a measure of the maximal photochemical efficiency of PSII and the second parameter, $1 - V_j$ [V_j is the relative variable fluorescence at 2 ms, $V_j = (F_{2ms} - F_0)/(F_m - F_0)$], measures the efficiency of acceptor-side electron transfer (Strasser *et al.* 2004),

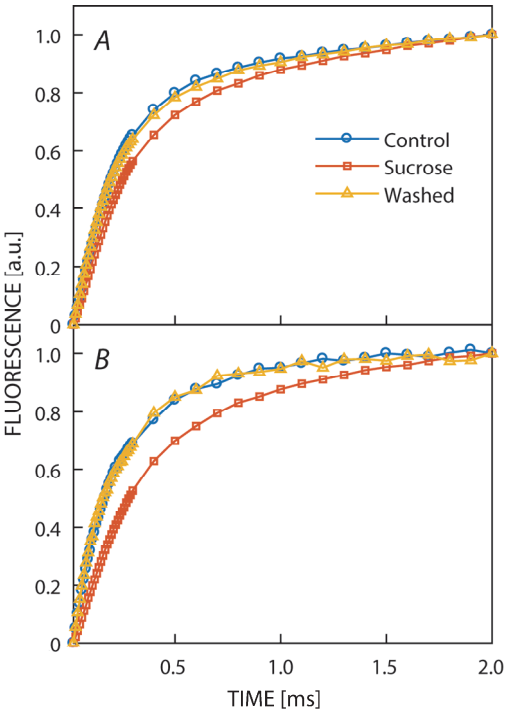

Fig. 4. Effect of 2 M sucrose on the initial rise of the relative variable fluorescence of thylakoid membranes incubated for 5 min at 25°C (*A*) or 45°C (*B*). Fluorescence is expressed as $W(t) = (F_{(t)} - F_0)/(F_{2ms} - F_0)$.

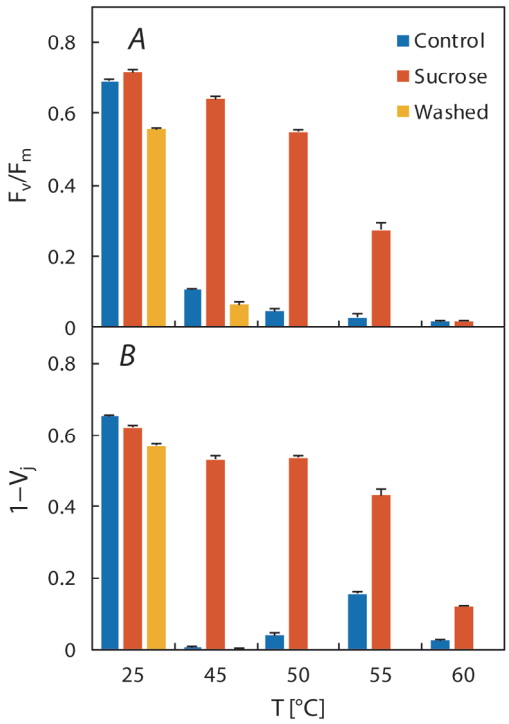

Fig. 5. Fluorescence parameters calculated from the induction transients of thylakoid membranes in control buffer, buffer with 2 M sucrose or in control buffer after washing out the sucrose, measured after incubation at different temperatures. (*A*) Maximal variable fluorescence, F_v/F_m; (*B*) $1 - V_j = (F_m - F_j)/F_v$.

For thylakoids in control medium, both parameters showed severely impaired PSII function already at 45°C and almost zero PSII activity at higher temperatures. When the medium was supplemented with 2 M sucrose, the photochemical and electron transport activity was retained to higher temperatures with only 11 and 14% drop in F_v/F_m and $1 - V_j$, respectively, at 45°C. The half-inhibition temperature for F_v/F_m was 52°C and somewhat higher, 57°C, for the electron transport parameter $1 - V_j$. Thus, the temperature interval of overall PSII activity was extended by more than 10°C. Moreover, sucrose appeared to exhibit a stronger protective effect on the PSII acceptor side at temperatures above 50°C, at which the activity was evidently limited by inhibition of the donor side. The enhanced thermal stability was entirely lost once the co-solute was removed and the thylakoid membranes were resuspended back in control buffer.

Time-resolved Chl fluorescence: In order to further analyse the functional effects of co-solute treatment on the photosynthetic apparatus, we recorded the picosecond Chl fluorescence decay kinetics by TCSPC. One specific aim of these measurements was to test the hypothesis raised by Williams *et al.* (1992) that the separation of non-bilayer lipids in medium with high sugar concentration causes detachment of LHCII from PSII. For a good fit of the fluorescence kinetics at wavelengths in the 670–740 nm, a minimum of four exponential decay components were needed – in control buffer the lifetimes were 79 ps, 0.28 ns, 0.69 ns, and 2.2 ns. The decay-associated emission spectra (DAES) are plotted in Fig. 6*A*. The 79-ps component mainly reflects the energy trapping in PSI, which has a strong emission in the far-red region. All other lifetimes are associated exclusively with the kinetics of PSII, hence the DAES have similar shape and only differ in magnitude. The 0.3 and 0.7-ns lifetime components are typical for functional, open PSII RCs and the 2.2 ns lifetime reflects closed RCs (Broess *et al.* 2008, Roelofs *et al.* 1992). The average lifetime at 688 nm was 0.33 ns, which is also typical for dark-adapted plants (F_0). There were minor differences in the lifetimes, DAES, and their relative contributions between sucrose-treated (Fig. 6*B*) and control thylakoids. If LHCII get detached from the PSII core, the PSII fluorescence lifetimes are expected to change and new decay components originating from uncoupled LHCIIs should appear. We consistently determined a slightly longer ns-decay lifetime and hence average fluorescence lifetime in 2 M sucrose but the differences were not significant. Moreover, the relative amplitude of the longest lifetime component was not significantly larger in sucrose compared to the control values. From these results it can be concluded that sucrose induces detachment of a minor fraction, if any, of PSII antenna. The effects of sucrose treatment were reversed upon washing the thylakoids with control medium (not shown). Generally, we can say that the light-harvesting and photochemical functions of PSII and PSI are mostly retained in the presence of 2 M sucrose, despite the apparently disturbed membrane macro-organization.

Fig. 6 also shows DAES of chloroplasts after incubation for 5 min at 45°C in control medium or 2 M sucrose (panels *C, D*). Heat treatment of control thylakoids resulted in discernible changes in the lifetimes, shape of the DAES, and their relative magnitudes. The 230-ps DAES could no longer be attributed purely to PSII emission due to its altered shape (notice the shoulder at 720–730 nm). Most noticeably, the relative amplitude of the 2-ns component increased 4-fold, resulting in a longer average fluorescence lifetime (0.51 ns). The overall longer PSII lifetime testifies for the inhibited photochemical energy conversion in PSII. None of these changes were incurred by heat treatment of thylakoids suspended in 2 M sucrose medium. Therefore, the time-resolved fluorescence data reaffirm that sucrose strongly enhances the thermal stability of PSII.

Time-resolved fluorescence of MC540: The fluorescence emission of the lipophilic dye MC540 is sensitive to the local dielectric constant and the structural rigidity of the molecular surroundings. Earlier MC540 has been used as a probe for different lipid phases and changes in the packing of lipid bilayers (Krumova *et al.* 2008b, Langner and Hui 1999). Here we tested the effect of 2 M sucrose, as a factor inducing phase separation of non-bilayer lipids,

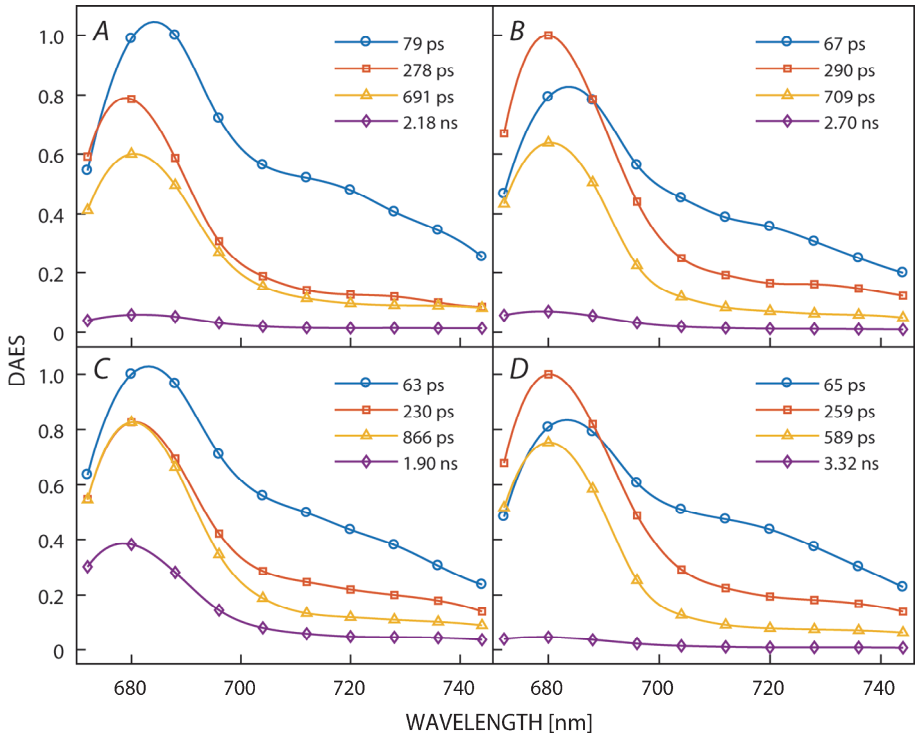

Fig. 6. Decay-associated fluorescence emission spectra (DAES) obtained from global analysis of the fluorescence kinetics of spinach chloroplasts in control buffer or supplemented with 2 M sucrose and incubated for 5 min at 25 or 45°C. (*A*) Control buffer, 25°C; (*B*) 2 M sucrose, 25°C; (*C*) control buffer, 45°C; (*D*) 2 M sucrose, 45°C.

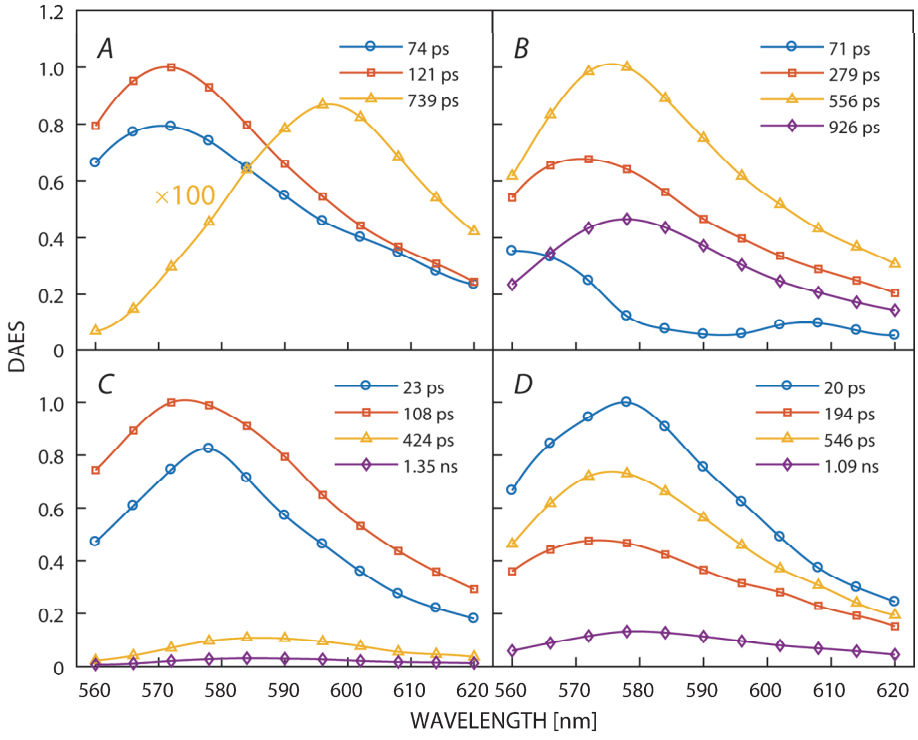

Fig. 7. Decay-associated fluorescence emission spectra (DAES) of MC540. (*A*) 10 μM MC540 in control buffer; (*B*) 10 μM MC540 in buffer with additional 2 M sucrose; (*C*) thylakoid membranes resuspended in control buffer + 10 μM MC540; (*D*) thylakoid membranes in buffer with 2 M sucrose + 10 μM MC540. The DAES were obtained by global lifetime analysis of the fluorescence decay traces recorded with 540-nm excitation.

on the time-resolved fluorescence characteristics of MC540 bound to thylakoid membranes. In order to confirm that MC540 was stably bound to the membrane and in order to wash out the excess dye from the aqueous phase, the thylakoid membrane suspension was repeatedly centrifuged and washed with fresh buffer, making sure that no dye remained in the supernatant. Thus, we can assume that fluorescence is emitted exclusively from membrane-bound MC540. In agreement with Krumova *et al.* (2008b), we did not observe direct or indirect energy transfer from MC540 to Chl, which could otherwise affect the MC540 fluorescence lifetimes. Because sucrose itself alters the dielectric and solvation properties of the medium, fluorescence measurements were performed also with

MC540 dissolved in control buffer and in buffer with 2 M sucrose, without thylakoid membranes.

The results of global multiexponential analysis of the fluorescence kinetics – lifetimes and DAES – are plotted in Fig. 7. Dissolved in control buffer, MC540 exhibited biexponential decay with lifetimes of 70 ps and 120 ps and maximal amplitudes at 572 nm (Fig. 7*A*). A third longer decay lifetime of a very low (1%) amplitude peaking at 590 nm could be associated with a small amount of MC540 dimers (Bernik *et al.* 1999). In buffer with 2 M sucrose (Fig. 7*B*), the fluorescence decay was markedly slower resulting in a 3-fold increase of the average fluorescence lifetime. The major decay lifetimes were in the range of 280–930 ps and the fast, 70-ps decay component had a very small contribution. There are several reasons for the change of the fluorescence yield/lifetime in sucrose medium: the lower dielectric constant and higher refractive index of the sucrose solution, the higher viscosity and the reduced motion and collisions of the dye and solvent molecules (Krishna and Periasamy 1998). In addition, in sucrose medium, the peak emission wavelength gradually red-shifted for longer lifetime components, possibly related to slower solvation dynamics and consequently time-dependent Stokes shift of the dye (Pal *et al.* 2000, Stratt and Maroncelli 1996). Dynamic Stokes shift is probably also the reason for the observed trough in the 70-ps component around 590 nm.

The fluorescence of MC540 bound to thylakoid membranes (Fig. 7*C,D*) exhibited similar behaviour with respect to the presence of sucrose, but there were notable differences between the membrane-bound and free dye. In thylakoid membranes resuspended in control buffer, new decay components appeared – a 20-ps component with a maximum at 578 nm and longer lifetime components (0.4 and 1.4 ns) peaking at 585 nm. Neither of the slower decay components could be attributed to dye oligomers, as the latter emit at longer wavelengths. The observed lifetime heterogeneity of MC540 is understood in terms of the lipid phase polymorphism in the thylakoid membranes (Garab *et al.* 2017). When thylakoid membranes were resuspended in medium with 2 M sucrose, the fluorescence intensity and average lifetime increased owing to the enhanced amplitude of long-lifetime components in the range of 0.5–1 ns. Interestingly, whereas sucrose caused a red-shift of the fluorescence emission of MC540 micelles in buffer, the emission of the membrane-bound dye was blue-shifted in sucrose medium, as evident from the DAES as well as the stationary spectra (Fig. 2S, *supplement available online*). These differential effects are an indication that the changes in the fluorescent properties of membrane-bound MC540 are not only a direct consequence of the interaction with the solvent, but also reflect the altered lipid environment of the dye molecules, partitioned between the different lipid phases, separated in the presence of the co-solute.

The effect of sucrose on the fluorescent properties of MC540 in thylakoid membranes was fully reversible – after washing out the sucrose, the lifetimes and the DAES were virtually the same as in thylakoid membranes originally resuspended in control buffer (Fig. 3S, *supplement available online*).

Discussion

Co-solutes are known to stabilize the tertiary and quaternary structure of proteins (Yancey 1994), which is ascribed to entropic reasons, *i.e*. replacing and expelling the water molecules from the protein surface and driving the proteins (or any macromolecular assemblies) to attain a conformation minimizing their solvent-exposed surface. For example, it could be envisioned that sucrose stabilizes the oxygen-evolving complex preventing the dissociation of the membrane-extrinsic subunits and the loss of the Mn–Ca–O cluster, which are normally observed at elevated temperatures (Pueyo *et al.* 2002). However, the direct entropy effect, while well recognized and likely having a significant contribution to the observed stabilizing effects, cannot fully explain the observed results, including the changes in the fluorescence of the lipid-bound MC540. In the case of thylakoid membranes, co-solutes also exert an indirect effect on the proteins by altering the macro-organization of the lipid-protein membrane and inducing non-bilayer lipid phase separation (Williams *et al.* 1992). Earlier it was shown by small-angle neutron scattering that osmotically-active substances, such as sucrose, sorbitol or inorganic salts, can alter the membrane repeat distances in thylakoid membranes with pronounced shrinkage at high osmolyte concentrations (Nagy *et al.* 2012, 2014; Posselt *et al.* 2012, Ünnep *et al.* 2014). Here we show unambiguously by CD spectroscopy that the thylakoid membrane undergoes sizeable structural rearrangements in medium with 2 M sucrose.

The three main psi-type CD bands all responded differently to the presence of the co-solute, 2 M sucrose – one disappeared completely, the second only partially, and the third one did not decrease at all. These results may seem counterintuitive, because the psi-type CD of chloroplasts is associated with the three-dimensional structure of stacked granal thylakoids, which is supposedly stabilized by the co-solute. However, it has been shown earlier that the psi-type bands originate from distinct structural entities in the thylakoid membranes. For instance, Cseh *et al.* (2000) found that the 672–676 nm band, which had earlier been preferentially associated with stacking (Garab *et al.* 1991), was more sensitive to temperature and light treatment than the 686–690 nm band. Moreover, the 672–676 nm band tends to be the most sensitive to structural destabilization of the stacked membranes by various means, including low detergent concentrations, chaotropic agents (Tóth *et al.* 2016), enzymatic degradation or

mechanical manipulation (unpublished). Here, we report the opposite effect – the (−)672 nm band was apparently unaffected by the sucrose treatment. On the other hand, the positive 506 and 688 nm bands, which do not absolutely depend on the granal stacking, but on the lateral supramolecular organization of PSII and PSII–LHCII supercomplexes (Kovács *et al.* 2006), were significantly diminished. This can be explained if we assume that stacking *per se* is maintained in high-sucrose medium, in agreement with earlier electron microscopy observations (Williams *et al.* 1992), but the supramolecular arrangement of the pigment–protein complexes in the membrane is perturbed, leading to the loss of the 506 and 688 nm CD bands. We can speculate that structural re-arrangements in the membrane occur as a consequence of the lipid phase separation.

The results presented here confirm that co-solutes, in this case sucrose, are extremely effective in protecting PSII from thermal deactivation, extending its functional window to temperatures as high as 60°C. At physiological temperatures, the presence of 2 M sucrose did not significantly disturb the energy and electron transfer dynamics in PSII, as confirmed by both the fast fluorescence induction transients and the picosecond time-resolved fluorescence kinetics. In particular, we tested whether the sucrose-induced lipid phase separation led to a disturbed connectivity between the PSII antenna and the RC, which is known to occur with the temperature-induced separation of non-bilayer phases. Detachment of PSII antenna would be detectable by an increased dark-adapted fluorescence yield and lifetime and the appearance of unconnected lifetime components in the PSII fluorescence kinetics (Holzwarth *et al.* 2009). We could clearly detect all these effects in heat-treated thylakoids in control medium – the F_0 level in the fluorescence induction transients was higher, as was the average fluorescence lifetime. The DAES showed the appearance of both long (2–3 ns) and short (200–300 ps) lifetime components that seemed to be unconnected to the functional PSII. There are two possible interpretations for the appearance of the shorter lifetime component. It could be associated with detached LHCII-forming quenched aggregates, which have a characteristic far-red emission shoulder (Miloslavina *et al.* 2008). The far-red emission may also reflect spillover or energy transfer between LHCII and PSI after disorganization of the membrane and loss of lateral segregation (Akhtar *et al.* 2016, van der Weij-de Wit *et al.* 2007). Neither of signs were found in sucrose-treated chloroplasts. Therefore, we can conclude that gross changes in the supramolecular organization of PSII and loss of peripheral LHCII do not occur despite the overall rearrangements of the thylakoid membrane detected by CD. A minor reduction in the PSII antenna size could be the reason for the slightly slower rise in the normalized fluorescence induction curves, which may be correlated to the relatively longer PSII lifetimes in 2 M sucrose, but we found these effects of small, if any, significance. Moreover, sucrose effectively counteracts against the antenna detachment and loss of PSII function that is associated with lipid phase separation at high temperatures.

An important, albeit somewhat surprising, result from the experiments reported here was that all observed structural and functional effects of the co-solute treatment were essentially reversible once the co-solute was washed out of the thylakoid membrane suspension. Not only the temperature stability of PSII function was lost but also, interestingly, the membrane macro-organization, chlorophyll and merocyanine fluorescence lifetimes and spectra were all restored. Hence, we must conclude that the non-bilayer lipid phases are transiently and reversibly formed in the presence of co-solute. Moreover, the structural and functional changes (CD, Chl fluorescence) reported here concern specifically the pigment–protein assemblies, which are embedded in the bilayer and not in the expulsed non-bilayer phases. Therefore, the reversible nature of these changes necessitates the conclusion that upon removal of the co-solute the extruded lipids must be re-incorporated into the bulk membrane so that the membrane macro-organization and function is restored. This is only possible if the extruded lipids are always in contact with the membrane and never fully dissociate but remain as an available pool, much in line with the hypothesis by Garab *et al.* (2000, 2016). This would be a remarkable feat of the thylakoid membrane, as it shows its extraodinary flexibility and capability to self-regulate its structure and composition.

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