Response of isolated thylakoid membranes with altered fluidity to short term heat stress

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ABSTRACT

The effect of alterations of lipid phase order of thylakoid membranes on the thermosensitivity of photosystem I (PS I) and photosystem II (PS II) was studied. Plant sterols stigmasterol and cholesterol were applied to decrease the fluidity in isolated membranes. After sterol treatment, a decrease of the temperature of 50 % inhibition of PSII activity was observed. Heat stress-induced stimulation of PSI-mediated electron transport rate was registered for control, but not for sterol-treated membranes. Effect of altered lipid order on oxygen evolving complex was evaluated by means of flash oxygen yields revealing changes in the stoichiometry of PSII_α and PSII_β centers. The effect of sterol incorporation on the changes in the thermotropic behavior of the main pigment-protein complexes was studied by differential scanning calorimetry (DSC). DSC traces of control thylakoids in the temperature range 20–98 °C exhibited several irreversible endothermic transitions. Incorporation of cholesterol and stigmasterol results in superimposition of the transitions and only two main bands could be resolved. While high temperature band peaks at the same temperature after treatment with both sterols, the band that combines low temperature transitions shows different melting temperature (T_m): 70 °C for stigmasterol- and 65 °C for cholesterol-treated membranes. The data presented here emphasise the crucial role of lipid order for the response of thylakoids to high temperatures, mediated not only by changes in the fluidity of bulk lipid phase as result of sterol incorporation but also by changes in the thermotropic properties of pigment-protein complexes. [*Physiol. Mol. Biol. Plants* 2009; 15(1) : 43–52] E-mail : mayav@bio21.bas.bg

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INTRODUCTION

The fluidity and permeability of membrane lipid phase play an important role in controlling light reactions of photosynthesis, for effective electron and energy transfer (Siegenthaler and Tremolieres, 1998). The lateral separation of the main pigment-protein complexes in thylakoid membranes, participation of mobile electron carriers in the electron transport reactions and regulation of energy distribution between PSI and PSII through physical movement of the light-harvesting chlorophyll *a/b* complex emphasize the role of lipid matrix and in particular, of its fluidity for the effectiveness of the photosynthetic process – linear electron transport, capture and transmitting of light energy. Several studies on this topic, including artificially manipulated thylakoids (incorporation of cholesterol or cholesteryl hemisuccinate, catalytic hydrogenation) or by using

membranes from lipid mutants (genetically modified membrane fluidity) discuss the effects of altered properties of lipid matrix on functional characteristics of photosynthetic apparatus (Siegenthaler and Tremolieres, 1998; Williams, 1998). The importance of membrane fluidity is clearly evident in respect to plant response to changes of environmental conditions. Changes of lipid saturation level inevitably reflect membrane fluidity, which is well-characterized phenomenon of plant and algal acclimation to temperature and light conditions (Raison *et al.*, 1982; Klyachko-Gurvich *et al.*, 1999). To date, a number of studies have been reported on the influence of the degree of fatty acid unsaturation on the extent of low temperature photoinhibition of some cyanobacteria (Kanavero *et al.*, 1997) and tobacco transgenic plants (Moon *et al.*, 1995).

Photosynthetic reactions exhibit different heat sensitivity. Exposure of isolated chloroplast or leaves of higher plants to elevated temperatures leads to considerable changes in structural organization of thylakoid membranes and their photosynthetic activities

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(Bukhov and Mohanty, 1999). PSII-catalyzed water oxidation is especially sensitive to heat, while PSI demonstrates much higher heat tolerance. Heat-induced changes of thylakoid stacking and rearrangement of lipids and of pigment-protein complexes as a result of heat stress are well documented (Gounaris *et al.*, 1983; Sundby and Andersson, 1985). The heterogeneity of PSII centers is also influenced by heat treatment (Bukhov and Carpentier, 2000). Alterations of grana stacking, partial dissociation of LHCII from the core complex of PSII and other heat-induced changes of mutual organization of thylakoid membranes and their constituents are dependent on the properties and composition of the lipid phase. It has been shown that the thermal stability of thylakoid membrane proteins in the process of development of thylakoid membrane during greening is related to the concomitant change of composition, dynamics and overall fluidity of the lipid component in greening seedlings (Kota *et al.*, 2002). All reported data indicate that the changes in the properties of lipid matrix affect the effectiveness of the photosynthetic process, but the mechanisms underlying are very complex and are far from completely understood.

The aim of the present study was to investigate the effect of changes in lipid phase properties on the response of isolated thylakoid membranes to high temperature stress. Artificial manipulation of membrane fluidity was achieved by treatment with cholesterol or stigmasterol. Although sterols are mainly found in plasma membranes of animals and higher plants and only in very low concentration in intracellular membranes, they could be used for artificial manipulation of thylakoid membrane fluidity (Ford and Barber, 1983). It is shown that incorporation of sterol molecules results in changes in the response of thylakoid membranes to short term heat stress and alters the thermotropic behavior of the main pigment-protein complexes.

MATERIALS AND METHODS

Isolation of thylakoid membranes

Thylakoid membranes from 14 day-old pea leaves (*Pisum sativum* L., Ran 1) were isolated by the procedure described by Popova *et al.* (2007). The final pellet was resuspended in a medium containing 0.33 M sucrose, 10 mM N-(2-hydroxy-1,1-bis(hydroxymethyl) ethyl) glycine (TRICINE) (pH 8.0), 5 mM MgCl₂ and 10 mM NaCl. Chlorophyll concentration was determined according to the procedure described in Lichtenthaler (1987).

Incorporation of sterols

For cholesterol and stigmasterol incorporation, a procedure described in Popova *et al.* (2007) was followed. Sterols were added to thylakoid membranes from a stock solution in ethanol and incubated in the dark at room temperature while stirring gently for 10 min. Ethanol concentration in the samples was kept as low as not to affect the measurements proven by control samples treated only with ethanol. After incubation, the membranes were washed two times in order to remove the non-incorporated sterol and resuspended in a medium appropriate for further measurements. Alternatively, the method of Yamamoto *et al.* (1981) to incorporate cholesterol by the use of polyvinyl pyrrolidone as a mediator was checked. Fluorescence polarization (P) of 1,6-diphenyl-1, 3, 5-hexatriene (DPH) indicates that with both methods, the level of cholesterol incorporation is approximately the same. The level of cholesterol incorporation depends on the concentration of sterol during incubation (Yamamoto *et al.*, 1981; Ford and Barber, 1983). Application of the first method - direct treatment with cholesterol (from ethanol solution) could be complicated by the presence of cholesterol micelles, which at high concentration form aggregates with thylakoid membranes. These complications were observed at cholesterol:chlorophyll ratio higher than 5 during incubation (Ford *et al.*, 1981). In our experiments, we used a lower incubation ratio of cholesterol:chlorophyll – 2.32, thus avoiding aggregation. At this incubation ratio, the cholesterol:chlorophyll value in the final pellet was about 0.26 (Ford *et al.*, 1981).

Estimation of membrane fluidity

The fluidity of isolated membranes was estimated by measuring the steady-state fluorescence polarization of DPH at room temperature, as described previously (Dobrikova *et al.*, 1997). Fluorescent probe is often used to determine the fluidity of the hydrophobic interior of biological membranes and especially for thylakoid membranes, as it tends to distribute reasonably evenly between all lipid domains and no energy transfer occurred between DPH and photosynthetic pigments (Ford and Barber, 1980). A P value of about 0.23 indicates a relatively fluid membrane, around zero for an isotropic solution and is higher - 0.49, for a totally rigid system (Kinosita *et al.*, 1981).

High temperature treatment

Short term heat treatment was performed in a water thermostat. Thylakoid membranes were incubated at

designated temperatures (± 0.2 °C) for 5 min in the dark and then cooled in a water bath to 20 °C.

Photochemical activity of PSI and PSII

Photochemical activity was polarographically determined by a Clark-type electrode (Model DW1, Hansatech Instruments Ltd., King's Lynn, Norfolk) in a temperature-controlled cuvette. The activity of PSII was determined by the rate of oxygen evolution with exogenous electron acceptor 1, 4-benzoquinone (BQ) at a concentration of 0.1 mM in a reaction medium containing 0.33 M sucrose, 5 mM MgCl₂, 10 mM NaCl and 20 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) (pH 6.5). PSI-mediated electron transport was determined by the rate of oxygen uptake in a medium containing 0.33 M sucrose, 5 mM MgCl₂, 10 mM NaCl, 20 mM Tricine (pH 7.5) and artificial electron donors and acceptors as follows: 0.1 mM 2,6-dichlorophenolindophenol (DCPIP), 4 mM Na ascorbate, 0.4 μ M 3-(3',4' dichlorophenyl)-1,1'-dimethylurea (DCMU), 0.5 mM NH₄Cl and 0.1 mM methyl viologen (MV). Both photochemical activities were measured at room temperature and thylakoid membranes equivalent to 20 mg chl/ml.

Flash oxygen yields and oxygen burst measurements

Determination of oxygen flash yields and initial oxygen burst was performed using home-built equipment, described in detail in Zeinalov (2002). Its main device is a fast oxygen rate electrode equipped with a system for flash, modulated and continuous modes of illumination. Thylakoid membranes were suspended in a medium without artificial electron acceptor containing: 0.33M sucrose, 10 mM NaCl, 5 mM MgCl₂ and 20 mM MES (pH 6.5). Chlorophyll concentration was 150 μ g chl/ml. The volume of the reaction compartment was 100 μ l in which the sample formed a layer of 2 mm height. Thylakoid membranes were pre-illuminated with ca. 25 flashes and then dark-adapted for 5 min before measurements. Oxygen flash yields were induced by saturating (4 J) and short ($t_{1/2}$ = 10 μ s) periodic flash sequences with 0.466 s dark spacing between the flashes. The initial oxygen burst was recorded after illumination with continuous white light (420 μ mol photons m⁻² s⁻¹). Data were digitized by a built-in A/D converter and transferred to an online IBM-compatible computer for further analysis. The induction curves after oxygen burst exhibit biphasic exponential decay. Decomposition of the oxygen burst decay was performed by fitting the curve with a function with two exponential components. The initial S_0 state distribution in dark, misses (α) and double hits (β) were calculated by a

software based on fitting of the experimentally obtained oxygen flash yields to the theoretically calculated yields according to the model of Kok *et al.* (1970).

Differential scanning calorimetry

Microcalorimetric measurements were performed on differential scanning microcalorimeter DASM4 (NPO "Biopribor", Pushchino, Russia). The stainless steel cell volume was of 0.466 ml and a constant pressure of 2 atm was maintained during all experiments. Data were digitized by an in-built A/D converter and transferred to an online IBM compatible computer for further retrieval and analysis. Scans were recorded in the temperature interval 20 °C - 100 °C at heating rate of 1 °C min⁻¹. After the first scan, the sample was cooled to 20 °C and a second scan was performed immediately in order to examine the reversibility of thermal transitions. The baseline was registered before sample recording with both cells filled with buffer used for membrane resuspension. Chlorophyll concentration of the samples was 2 mg chl/ml. Temperature of thermal transition (T_m) and calorimetric enthalpy of denaturation were estimated from DSC scans after subtraction of baseline and normalization of the data for chlorophyll concentration. T_m was determined as the temperature of the maximum of the excess heat capacity curve peaks and calorimetric enthalpy (ΔH) of the transition as the area under the excess heat capacity curve peaks.

RESULTS

Fluidity of thylakoid membranes was determined by the degree of polarization of fluorescence (P) of incorporated DPH. For control thylakoid membranes, the value for P was 0.249 ± 0.013 . After incorporation of cholesterol and stigmaterol, the measured values for P were 0.354 ± 0.008 ($p < 0.001$) and 0.314 ± 0.042 , respectively, thus indicating rigidification of the lipid phase as was earlier reported (Popova *et al.*, 2007).

The effect of incorporation of cholesterol and stigmaterol on the PSI- and PSII-mediated electron transport activities after short term heat stress is illustrated in Fig. 1. The temperature dependencies of oxygen evolution for control, cholesterol and stigmaterol are shown in Fig. 1A. Treatment with both sterols results in the lowering of temperature at which 50 % inhibition ($T_{50\%}$) of oxygen evolution occurs. For control thylakoids $T_{50\%}$ is determined at about 46 °C, while stigmaterol incorporation shifts $T_{50\%}$ to 40 °C. Cholesterol treatment decreases $T_{50\%}$ to a lesser extent, with only about 3 °C in comparison to the control.

Fig. 1. Dependence of photochemical activity of PSII (A) and PSI (B) on the temperature of short term heat stress (5 min at designated temperature) for control – (□), stigmasterol-treated – (Δ) and cholesterol-treated – (○) thylakoid membranes. Activities are represented as % of activities at 20°C. Experimental conditions are as described in Material and Methods.

The effect of sterol incorporation on PSI-mediated electron transport (Fig. 1B) is more significantly expressed. For control thylakoid membranes, a heat-induced stimulation of PSI electron transport is observed up to 50 °C, while after cholesterol and stigmasterol treatment, PSI activity is almost constant up to 50 °C and slows down thereafter.

For characterization of the activity of oxygen evolving complex in control and sterol-treated thylakoid membranes at elevated temperatures, the flash oxygen yields were investigated. This part of the electron transport machinery is very heat sensitive, evidenced by a considerable decrease in the flash yields with increase in temperature. Typical traces of flash oxygen yields at 20 and 40 °C for control and sterol-treated membranes are presented at Fig. 2 (panel I). For control thylakoids, oxygen yields are not significantly reduced at 40 °C while for sterol-treated membranes at this temperature, they are minimal and no longer registered

at 45 °C. Data regarding S_0 (centers in the most reduced and stable state), α (misses) and β (double hits) are shown in Table 1. In control thylakoid, S_0 is about 26–27 % and stable up to 40 °C but a noticeable decrease is observed at 45 °C. In membranes with incorporated sterols, the initial level of S_0 is lower than in controls but with increase in temperature its level increases, more pronounced in stigmasterol-treated membranes. This increase starts at 30 °C for stigmasterol- and at 35 °C for cholesterol-treated samples. The values for misses are higher for cholesterol-treated membranes and lowest for control. The probability for double hits is not influenced by sterol incorporation.

The pattern and parameters of initial oxygen burst under continuous illumination are significantly affected by sterol incorporation (Fig. 2, panel II) The typical oxygen induction curve under continuous illumination exhibits a second order exponential decay kinetics due to the functioning of two mechanisms of oxygen evolution by both types of PSII centers – PSII $_{\alpha}$ and PSII $_{\beta}$ – that differ in respect to their antenna size as well as in their location in the membranes. PSII $_{\beta}$ centers, situated mainly in the stroma thylakoids, evolve oxygen by cooperative mechanism, after cooperation of oxygen precursors (positive charges) obtained in different reaction centers, while PSII $_{\alpha}$ centers in grana thylakoids produce oxygen by non-cooperative mechanism (Zeinalov, 1982; Zeinalov, 2005). The contribution of PSII $_{\alpha}$ and PSII $_{\beta}$ in oxygen evolving process can be estimated by decomposition of oxygen burst decay kinetics into two kinetic components: the fast component being related to the fast PSII $_{\alpha}$ centers and the slow ones – to the slower PSII $_{\beta}$. The temperature dependence of kinetic parameters of both resulting components – amplitude A_1 and time constant t_1 (for fast component) and A_2 and t_2 (for slow component) for control, cholesterol- and stigmasterol-treated membranes are presented in Table 2. At 20 °C, the values for t_1 are much higher for sterol treated thylakoids than for control ones. For stigmasterol-treated membranes the fast component is more sensitive to elevated temperatures – the values for t_1 increase rapidly with the increase of temperature. At 40 °C, the oxygen burst is considerably reduced and no different kinetic components could be distinguished. The contribution of fast component to the oxygen burst is major in control thylakoid membranes at 20 °C and the ratio A_1/A_2 decreases with increasing temperature, mainly due to a decrease of A_1 . Incorporation of both sterols decreases the ratio A_1/A_2 , indicating greater contribution of stroma-situated slower PSII $_{\beta}$ centers. Elevated temperatures also lead to

Fig. 2. Typical traces of flash oxygen yields (panel I) and of initial oxygen burst (panel II) for control (A), stigmasterol-treated (B) and cholesterol-treated (C) thylakoid membranes after incubation for 5 min at 20 °C (solid lines) and 40 °C (dashed lines). Experimental conditions are as described in Materials and Methods.

increases in time constants of both types of centers, indicating that heat treatment slows down the turnover rate of oxygen evolving centers.

In order to relate the sterol-induced changes in the thermosensitivity of isolated thylakoids with changes in thermotropic properties of pigment-protein complexes, calorimetric measurements were performed. Figure 2 shows DSC scans of control (A) and stigmasterol- and cholesterol-treated (B and C, respectively) thylakoid membranes. In control thylakoids six endothermic transitions peaking at 46 °C, 64.8 °C, 69.8 °C, 74.6 °C, 82.3 °C, 89 °C and a shoulder at 56–60 °C are observed. Treatment with cholesterol and stigmasterol results in significant changes of the shape of endothermic curves, and superimposition of different transitions into two bands occurs – for stigmasterol at 70 °C and 84 °C (Fig. 2, B) and for cholesterol at 64.6 °C and 84 °C (Fig. 2, C).

The transitions in control and sterol-treated membranes are independent from each other, as revealed by successive heating to different temperatures (data not shown). This procedure was performed as follows: samples were heated to T_m of the first transition, then

cooled to 20 °C and reheated to T_m of the next transition. This procedure (cooling and heating) was repeated for the T_m of all observed transitions. The higher temperature transitions were not influenced by the occurrence of the previous one for all studied samples.

DISCUSSION

Alteration of the lipid composition and extent of unsaturation of fatty acid chains is a well documented phenomenon in plant response to high and low temperature (Harwood, 1999). In the present study, the thylakoid membrane fluidity was artificially manipulated with sterols and the sensitivity of different components of the electron transport chain towards short term heat stress was investigated. The data presented here indicate that PSI associated electron transport rate was affected by sterol incorporation. The heat-stress induced stimulation of PSI activity in non-treated thylakoids (Fig. 1B) is in agreement with literature data (Goumaris *et al.*, 1983, Thomas *et al.*, 1986, Sundby and Anderson, 1985, Ivanov and Velitchkova, 1990). This stimulation has been attributed to several reasons. It has been established

Fig. 3. DSC thermograms (after baseline correction) of isolated thylakoid membranes: Control- A; Stigmasterol-treated – B; Cholesterol-treated – C. Scanning rate – 1 °C min. Chlorophyll concentration - 2 mg/ml. Membranes were resuspended in a medium containing: 10 mM Tricine (pH 8.0), 0.33 M sucrose, 5 mM $MgCl_2$ and 10 mM NaCl.

that after heating to 55 °C a loss of grana stacking and dissociation of a part of LHCII complex from core complex of PSII occurred (Gounaris *et al.*, 1983; Sundby and Andersson 1985). These structural rearrangements were accompanied by an increase of PSI absorption cross-section and an increase in the energy delivered to PSI and subsequent increase of PSI efficiency (Ivanov and Velitchkova, 1990). In addition, an increased affinity for electron donor – reduced DCPIP in cytochrome *f/b₆* complex has been proposed (Thomas *et al.* 1986). After treatment with both sterols, stimulation of PSI-mediated electron transport was abolished. These results support the data reported by Vigh *et al.* (1989) that lipid saturation above 35 % (where an increase of lipid order parameter can be expected) totally prevents the appearance of any increase in PSI electron flow upon heat treatment. Our data show that PSI-mediated electron transport rate is affected by cholesterol and stigmasterol

treatment in the same manner. Therefore, it can be supposed that cholesterol and stigmasterol incorporate in a similar way in the membrane regions where PSI complexes are situated. DSC data support this assumption: - both sterols influence the last two transitions similarly, at 83 °C and 89 °C, they overlap into one band peaking at 85 °C. Although the membrane constituents responsible for these two transitions are not definitely identified, it has been proposed that the peak at 83 °C is predominantly or exclusively associated with the stroma membranes where PSI is situated (Nolan *et al.* 1992). Therefore, it is very likely that incorporation of sterol molecules in stroma regions could avoid structural and conformational changes responsible for heat induced stimulation of PSI activity.

An enhanced stability of thylakoid organization and of the thermal stability of PSII (estimated by Fv/Fm ratio) has been reported as a result of increased saturation level of fatty acids of membrane lipids obtained by using catalytic hydrogenation (Thomas *et al.* 1986; Horvath *et al.* 1987). It is questionable whether this effect of lipid saturation is related to the changes of membrane fluidity. Results reported here (Fig. 1A) show that the decrease of membrane fluidity, induced by incorporation of sterols, exhibits opposite effect – the inhibition of PSII-mediated electron transport from H_2O to artificial electron donor BQ was shifted to lower temperatures. Therefore, the preservation effect of fatty acid saturation on thermal stability of PSII complex is hardly mediated by increase of lipid ordering in the regions where PSII is situated. Results from flash-induced yields and initial oxygen burst permits us to estimate the extent of oxygen evolution inhibition and also the heat stress-induced changes in the kinetic characteristics of PSII centers and alteration of PSII heterogeneity. It should be noted that the inhibition of flash oxygen yields started at lower temperatures than that of oxygen burst. By analyzing the oxygen burst decay kinetics, we show that sterol incorporation leads to a decrease of PSII_α centers in favor to PSII_β centers. This is in line with earlier reported data about the effect of cholesterol incorporation on fluorescence induction kinetics of isolated thylakoids (Busheva *et al.* 1998). It is worth noting that an increase in the proportion of PSII_β in the membrane has also been observed after catalytic hydrogenation of lipids (Horvath *et al.* 1987). The data obtained from decay kinetics of oxygen burst show that heat treatment leads to a decrease in the ratio A_1/A_2 in control and sterol treated thylakoids, indicating heat-induced transformation of PSII_α centers into PSII_β (Table 2). Similar results about the effect of heat stress

Table 1. Changes in S_0 state, misses (α) and double hits (β) for control, stigmasterol- and cholesterol-incorporated thylakoid membrane as a result of short term (5 min) heat stress at different temperatures in the range 20–45 °C. Values for S_0 are presented as % from the value of the respective sample at 20 °C. Mean values and SE are calculated from 3 to 5 independent experiments.

T °C	Control			Cholesterol-incorporated			Stigmasterol-incorporated		
	S_0	α	β	S_0	α	β	S_0	α	β
20	27.6±2.5	0.18±0.03	0.04±0.009	22.2±4.8	0.27±0.03	0.04±0.006	21.8±1.4	0.23±0.02	0.04±0.004
30	26.0±0.8	0.20±0.02	0.04±0.003	19.4±4.7	0.27±0.03	0.03±0.006	24.3±5.7	0.23±0.02	0.04±0.003
35	28.6±4.3	0.21±0.04	0.05±0.006	21.3±6.2	0.29±0.04	0.03±0.007	28.5±6.5	0.23±0.04	0.05±0.013
40	29.2±3.2	0.21±0.03	0.05±0.004	27.4±4.4	0.23±0.03	0.04±0.004	34.3±9.3	0.18±0.04	0.06±0.004
45	20.4±10	0.14±0.04	0.07±0.002	—	—	—	—	—	—

on the heterogeneity of PSII has been reported based on the investigation of fluorescence induction in isolated spinach chloroplasts (Sundby *et al.* 1986). Sterol incorporation affects PSII thermostability - PSII complex is more sensitive than PSI to elevated temperatures with respect to electron transport from H_2O to artificial electron acceptor as well as to flash oxygen yields.

The effects of incorporation of sterols are studied mainly in respect to their influence on the properties of lipid component in biological membranes and model lipid membranes. In the case of isolated thylakoid membranes, it has been shown that alteration of lipid order parameter induced by the incorporation of cholesterol-influenced characteristics of primary photosynthetic reactions – electron transport, proton gradient and energy

distribution (Yamamoto *et al.* 1981; Dobrikova *et al.* 1997). These effects have been attributed mainly to changes in the physicochemical properties of the lipid bilayer and especially to the role of fluidity in the functioning of the mobile electron carriers. No attention was paid to the effect of incorporation of these membrane-perturbing agents on the properties of pigment-protein complexes. Here we show that besides fluidity alterations, changes in the thermotropic behavior of main pigment-protein complexes occur. For spinach thylakoid membranes, seven bands peaking at 42.5 °C, 60.6 °C, 64.9 °C, 69.6 °C, 75.8 °C, 84.3 °C and 88.9 °C have been resolved. A number of attempts have been made in order to assign the different bands to respective membrane constituents. Some of the peaks were identified to originate from LHCII (at 74 °C) (Smith *et al.*

Table 2. Effect of short term heat treatment (5 min) on parameters of initial oxygen burst of control, cholesterol- and stigmasterol-treated pea thylakoid membranes. A_1 and A_2 and t_1 and t_2 are amplitudes and time constants of fast (subscript 1) and slow (subscript 2) components. Mean values and SE are calculated from 3 to 5 independent experiments.

T °C	Control			Cholesterol-treated			Stigmasterol-treated		
	τ_1	τ_2	A_1/A_2	τ_1	τ_2	A_1/A_2	τ_1	τ_2	A_1/A_2
20	0.19±0.03	2.26±0.43	3.27	0.30±0.08	3.24±0.34	1.63	0.28±0.07	2.48±0.38	1.20
30	0.19±0.02	2.28±0.18	2.82	0.37±0.06	3.21±0.71	1.27	0.48±3.35	3.35±0.34	0.83
35	0.22±0.05	2.32±0.09	2.83	0.51±0.11	3.77±0.34	0.99	1.39±0.14	7.76±1.23	0.73
40	0.28±0.06	2.46±0.11	1.73	2.23±0.33	11.55±1.53	0.97	—	—	—
45	1.00±0.57	4.12±1.1	1.07	—	—	—	—	—	—

1989), from oxygen-evolving complex (at 42.4 °C) (Cramer *et al.* 1981; Thompson *et al.* 1986), reaction center of PSII (at 60.6 °C) (Smith and Low, 1989). Later on, Nolan *et al.* (1992) identified the peaks for pea thylakoid membranes and associated them with granal and stromal constituents of thylakoid membranes. In our control thylakoids, several endothermic transitions peaking at 46 °C, 64.8 °C, 69.8 °C, 74.6 °C, 82.3 °C and 89 °C and a shoulder at around 56–60 °C are observed (Fig. 2A). Treatment with cholesterol and stigmasterol resulted in significant changes of the shape of endothermic curves. After cholesterol incorporation, only two main peaks are resolved - at 64.6 °C and 82 °C representing superimpositions of transitions observed in control thylakoid membranes (Fig. 2C). Treatment with stigmasterol also results in the association of different transitions into two bands at 70 °C and 84 °C. However, the T_m of the low temperature band is higher than for cholesterol-treated samples (Fig. 2B). The T_m of the highest maximum is situated at 75 °C for control and at 70 °C and 65 °C for stigmasterol- and cholesterol-treated samples, respectively. Both sterols influence the last two transitions at 83 °C and 89 °C in a similar manner. In control thylakoids, the transition at 75 °C is considered to be associated with the most abundant chlorophyll-protein complex in thylakoid membranes (LHCII) and the band at approx. 60 °C is attributed to the PSII core complex (Smith *et al.* 1989; Smith and Low 1989).

The chemical structures of both sterols are similar - four fused rings (common for cholesterol and stigmasterol) form the rigid part of the molecule and an aliphatic side chain with slight differences - stigmasterol has an additional ethyl group attached to C-24 and trans double bond between C-22 and C-23. These structural discrepancies determine the different effects of plant sterol incorporation in lipid bilayers when compared with those of cholesterol. Cholesterol is known to partition preferentially into bilayers with saturated acyl chains while plant sterol stigmasterol - into membrane regions with a higher degree of unsaturated lipid acyl chains (Bernsdorff and Winter, 2003). Recently, it has been shown that incorporation of stigmasterol in phospholipid bilayers resulted in a decrease of the phase transition temperatures of both the sterol-poor and sterol-rich domains in DPPC bilayers (Halling and Slotte, 2004). In thylakoid membranes, stigmasterol incorporation induces a decrease of $T_{50\%}$ after short term heat stress. This influence could be regarded as the effect of stigmasterol on the properties of the bulk lipid phase, but also on bound lipid environment of protein complexes and on protein complexes themselves.

Difference in the action of both sterols could be due to the different partitioning of both sterols in different regions of the thylakoid membranes, which exhibit lateral heterogeneity with respect to lipid content. In addition, specific affinity of both sterols to different lipid classes could not be excluded.

The data presented here demonstrate that the decrease of membrane fluidity leads to an increase in thermal sensitivity of oxygen evolution and PSII electron transport rate. On the other hand, rigidification of the thylakoid membranes abolishes heat-induced stimulation of PSI activity. Sterol incorporation results in the alteration of thermotropic characteristics of pigment-protein complexes. It can be supposed that two high temperature transitions originate from components situated in the membrane regions readily accessible for both sterols. The effect of sterol incorporation on the T_m of the transition of the most abundant pigment-protein complex in thylakoid membranes - LHCII, could be regarded in the light of the recently published data about the effect of lipid classes and lipid/protein ratio on the dissociation temperature of trimeric LHCII incorporated into liposomes of thylakoid lipids (Yang *et al.* 2006).

Short term heat stress induces the formation of hexagonal inverted micelles by non-bilayer forming lipids in thylakoid membranes (Gounaris *et al.* 1983). It could be that insertion of cholesterol and stigmasterol molecules in the lipid phase modifies this process and different regions of the lipid component are affected in a different manner. The data presented demonstrate that the altered heat sensitivity of PSI and PSII photochemical activities is related not only to sterol-induced changes of lipid order but also to altered thermotropic behavior of the main pigment-protein complexes.

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REFERENCES

- Bernsdorff C and Winter R (2003). Differential properties of the sterols cholesterol, ergosterol, β -sitosterol, trans-7-dehydrocholesterol, stigmasterol and lanosterol on DPPC bilayer order. *J. Phys. Chem.* 107: 10658-10664.

- Bukhov NG and Carpentier R (2000). Heterogeneity of photosystem II reaction centers as influenced by heat treatment of barley leaves. *Physiol. Plant.* 110: 279-285.
- Bukhov NG and Mohanty P (1999). Elevated temperature stress effects on photosystems: Characterization and evaluation of the nature of heat induced impairments. In: *Concepts of Photobiology: Photosynthesis and Photomorphogenesis* (Eds. Singhal G.S., Renger G., Sopory S.K., Irgang K-D., Govindjee), Narosa Publishing House, New Delhi, India, pp. 617-648.
- Busheva M, Velitchkova M, Markova Tz and Zanev Y (1998). Effects of cholesterol and benzyl alcohol on fluorescence transients of thylakoids. *J. Photochem. Photobiol. B* 42: 240-244.
- Dobrikova A, Tuparev N, Krasteva I, Busheva M and Velitchkova M (1997). Artificial alteration of fluidity of pea thylakoid membranes and its effect on energy distribution between both photosystems. *Z. Naturforsch.* 52c: 475-480.
- Cramer WA, Whitmarsh J and Low PS (1981). Differential scanning calorimetry of chloroplast membranes: Identification of an endothermic transition associated with the water-splitting complex of photosystem II. *Biochemistry* 20: 157-162.
- Ford RC and Barber J (1980). The use of diphenylhexatriene to monitor the fluidity of the thylakoid membrane. *Photobiochem. Photobiophys.* 1: 263-270.
- Ford RC and Barber J (1983). Incorporation of sterols into chloroplast thylakoid membranes and its effect on fluidity and function. *Planta* 158: 35-41.
- Ford RC, Yamamoto Y and Barber J (1981). Lipid fluidity and photosynthetic electron flow: Effect of cholesterol. In: *Photosynthesis I. Photophysical Processes-Membrane Energization* (Ed. Akoyunoglou G), Balaban International Science Services, Philadelphia, Pa., vol. 1, pp. 617-626.
- Gounaris K, Brian APR, Quinn PJ and Williams WP (1983). Structural reorganization and functional changes associated with heat-induced phase separation of non-bilayer lipids in chloroplast thylakoid membranes. *FEBS Lett.* 153: 47-52.
- Halling KK and Slotte JP (2004). Membrane properties of plant sterols in phospholipid bilayers as determined by differential scanning calorimetry, resonance energy transfer and detergent-induced solubilization. *Biochim. Biophys. Acta* 1664: 161-171.
- Harwood J (1999). Involvement of chloroplast lipids in the reaction of plants submitted to stress. In: *Lipids in Photosynthesis: Structure, Function and Genetics* (Eds. Siegenthaler P-A, Murata N), Kluwer Publishers, Dordrecht, Boston, London, pp. 287-302.
- Horvath G, Melis A, Hideg E, Droppa M and Vigh L (1987). Role of lipids in the organization and function of photosystem II studied by homogeneous catalytic hydrogenation of thylakoid membranes in situ. *Biochim. Biophys. Acta* 891: 68-74.
- Ivanov A and Velitchkova M (1990). Heat-induced changes in the efficiency of P700 photo-oxidation in pea chloroplast membranes. *J. Photochem. Photobiol. B* 4: 307-320.
- Kanavero E, Tasaka Y, Murata N and Aro E-M (1997). Membrane lipid unsaturation modulates processing of the photosystem II reaction center protein D1 at low temperature. *Plant Physiol.* 114: 841-849.
- Kinosita K, Kataoka R, Kimura Y, Gotoh O and Ikegami A (1981). Dynamic structure of biological membranes as probed by DPH: a nanosecond depolarization study. *Biochemistry* 20: 4270-4277.
- Klyachko-Gurvich GL, Tzoglun LN, Doucha J, Kopetski J, Shebatina (Ryabych) IB and Semenenko VE (1999). Desaturation of fatty acids as an adaptive response to shifts in light intensity. *Physiol. Plant.* 74: 566-574.
- Kok B, Forbush B and McGloin M (1970). Co-operation of charges in photosynthetic O₂. I. A linear four step mechanism. *Photochem. Photobiol.* 11: 457-475.
- Kota Z, Horvath LI, Droppa M, Horvath G, Farkas T and Pali T (2002). Protein assembly and heat stability in developing thylakoid membranes during greening. *Proc. Natl. Acad. Sci. USA*, 99: 12149-12154.
- Lichtenthaler HK (1987). Chlorophylls and carotenoids: Pigments of photosynthetic membranes. *Methods Enzym.* 148:350-382.
- Loll B, Kern J, Saenger W, Zouni A and Biesiadka J (2005). Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II. *Nature* 438: 1040-1044.
- Moon BY, Higashi ST, Gombos Z and Murata N (1995). Unsaturation of the membrane lipids of chloroplasts stabilizes the photosynthetic machinery against low-temperature photoinhibition in transgenic tobacco plants. *Proc. Natl. Acad. Sci. USA* 92: 6219-6223.
- Nolan WG, Hopkins JrHP and Kalini SAM (1992). Differential scanning calorimetric investigation of pea chloroplast thylakoids and thylakoid fractions. *Arch. Biochem. Biophys.* 297: 19-27.
- Popova A, Velitchkova M and Zanev Y (2007). Effect of membrane fluidity on photosynthetic oxygen production reactions. *Z. Naturforsch.* 62c: 253-260.
- Raison JK, Roberts JKM and Berry JA (1982). Correlation between the thermal stability of chloroplast (thylakoid) membrane and the composition and fluidity of their polar lipids upon acclimation of the higher plant, *Nerium oleander*, to growth temperature. *Biochim. Biophys. Acta* 688: 218-228.
- Siegenthaler P-A and Tremolieres A (1998). Role of acyl lipids in the function of photosynthetic membranes in higher plants. In: *Lipids in Photosynthesis: Structure, Function and Genetics* (Eds. Siegenthaler P-A, Murata N), Kluwer Publishers, Dordrecht, Boston, London, pp. 145-173.
- Smith KA, Ardel BK, Huner NPA, Krol M, Myscich E and Low PS (1989). Identification and partial characterization of the denaturation transition of the light harvesting complex II of spinach chloroplast membrane. *Plant Physiol.* 90: 492-499.
- Smith KA and Low PS (1989). Identification and partial characterization of the denaturation transition of the

- photosystem II reaction center of spinach chloroplast membranes. *Plant Physiol.* 90: 575-581.
- Sundby C and Andersson B (1985). Temperature induced reversible migration along thylakoid membranes of photosystem II regulates its association with LHCII. *FEBS Lett.* 191: 24-28.
- Sundby S, Melis A, Maenpaa P and Andersson B (1986). Temperature-dependent changes in the antenna size of Photosystem II. Reversible conversion of Photosystem II_a to Photosystem II_b. *Biochim. Biophys. Acta* 851: 475-483.
- Thomas PG, Dominy PJ, Vigh L, Mansourian AR, Quinn PJ and Williams WP (1986). Increased thermal stability of pigment protein complexes of pea thylakoids following catalytic hydrogenation of membrane lipids. *Biochim. Biophys. Acta*, 849: 131-140.
- Thomas PG, Quinn PJ and Williams WP (1986). The origin of photosystem I-mediated electron transport stimulation in heat-stressed chloroplasts. *Planta* 167: 133-139.
- Thompson LK, Sturtevant JM and Brudvig GW (1986). Differential scanning calorimetric studies of photosystem II: Evidence for a structural role for cytochrome b559 in the oxygen-evolving complex. *Biochemistry* 25: 6161-6169.
- Velitchkova M, Popova A and Markova Tz (2001). Effect of membrane fluidity on photoinhibition of isolated thylakoid membranes at room and low temperature. *Z. Naturforsch.* 56c: 369-374.
- Vigh L, Gombos Z, Horvath I and Joo F (1989). Saturation of membrane lipids by hydrogenation induces thermal stability in chloroplast inhibiting the heat-dependent stimulation of photosystem I-mediated electron transport. *Biochim. Biophys. Acta*, 979: 361-364.
- Williams WP (1998). The physical properties of thylakoid membrane lipids and their relation to photosynthesis. In: *Lipids in Photosynthesis: Structure, Function and Genetics* (Eds. Siegenthaler P-A, Murata N), Kluwer Publishers, Dordrecht, Boston, London, pp. 103-118.
- Yamamoto Y, Ford RC and Barber J (1981). Relationship between thylakoid membrane fluidity and the functioning of pea chloroplasts. Effects of cholesteryl hemisuccinate. *Plant Physiol.* 67: 1069-1072.
- Yang C, Boggasch S, Haase W and Paulsen H (2006). Thermal stability of trimeric light-harvesting chlorophyll a/b complex (LHCIIb) in liposomes of thylakoid lipids. *Biochim. Biophys. Acta* 1757: 1642-1648.
- Zeinalov Y (1982). Existence of two different ways for oxygen evolution in photosynthesis and photosynthetic unit concept. *Photosynthetica* 16: 27-35.
- Zeinalov Y (2002). An equipment for investigation of photosynthetic oxygen production reactions. *Bulg. J. Plant Physiol.* 28: 57-67.
- Zeinalov Y (2005). Mechanisms of photosynthetic oxygen evolution and the fundamental hypothesis of photosynthesis. In: *Handbook of photosynthesis* (Ed. Pessarakly M.), 2nd ed. CRC Press, Taylor and Francis Group, Boca Raton, FL, USA, pp. 3-19.