Effect of Membrane Fluidity on Photosynthetic Oxygen Production Reactions

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The effect of changes of membrane fluidity on the oxygen evolving capability of isolated thylakoids was investigated. Alteration of the lipid phase fluidity was achieved by incorporation of the plant sterol stigmasterol. Incorporation of stigmasterol in the lipid bilayer of thylakoid membranes results in rigidization of the hydrophobic phase of thylakoid membranes and decreases the degree of packing of the lipid head groups. These changes of lipid order are accompanied by a reduction of oxygen evolution, measured with 1,4-benzoquinone as an electron acceptor, and by a more pronounced inhibition of PSI-mediated electron transport. By analysis of the parameters of oxygen flash yields and oxygen burst under continuous illumination it was shown that after treatment with stigmasterol: 1.) the number of active oxygen-evolving centres decreased; 2.) the remaining active oxygen-evolving centres were not affected in respect to the oscillation pattern; 3.) the contribution of the slow oxygen-evolving centres in oxygen burst yield was increased. The effect of stigmasterol was compared with the well-studied effect of cholesterol. Results were discussed in terms of determining the role of lipid order for the organization and functioning of the photosynthetic machinery.

Key words: Thylakoid Membrane Fluidity, Oxygen Evolution, Stigmasterol

Introduction

Lateral separation of main pigment-protein complexes in thylakoid membranes of higher plants, involvement of mobile electron carriers in the electron transport chain and physical movement of the light-harvesting chlorophyll *a/b* complex during state I-state II transitions emphasize on the role of the 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 that use thylakoid membranes with artificially manipulated lipid phase (incorporation of cholesterol or cholesteryl hemisuccinate) or lipid mutants (genetically altered membrane fluidity) discuss the importance of fluidity of the lipid matrix on functional characteristics of the photosynthetic apparatus, located in thylakoid membranes (Ford and Barber, 1983; Siegenthaler and Tremolieres, 1998; Yamamoto *et al.*, 1981).

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 alteration of thylakoid membrane fluidity. Sterols are essential constituents of eukaryotic membranes and play multiple roles in membrane organization, dynamics, function and sorting (Lindsey *et al.*, 2003). Intermolecular interactions between sterols and membrane lipids modulate the physical state of the bilayer via restricting the mobility of the fatty acyl chains and in turn regulate the membrane fluidity and permeability (Hartmann, 1998). Sterols modulate also the activity of membrane-bound proteins and enzymes by affecting either their conformation or protein activity by direct protein-sterol interactions (Cooke and Burden, 1990). It has been widely reviewed that cholesterol exhibits an ordering effect on the packing of phospholipids in their liquid-crystalline state and an disordering effect below the chain melting transition temperature – in the gel phase. Incorporation of cholesterol in pure bilayers of naturally occurring phospholipids induces the formation of liquid-ordered

Abbreviations: 1,4 BQ, 1,4-benzoquinone; chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; MES, 2-(morpholino)ethanesulfonic acid; MV, methyl viologen; PSI (II), photosystem I (II); TMA-DPH, trimethylammonium-diphenyl-DPH; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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phases (Ipsen *et al.*, 1987) and, at elevated concentrations, gradually abolishes the pre-transition and the main gel to liquid-crystalline phase transition temperature (McMullen *et al.*, 1993). However, the extent of these effects depends on the particular type of the lipid and also on the chemical structure of sterol incorporated. Intermolecular interactions between dipalmitoylphosphatidylcholine (DPPC) and cholesterol as well as different representatives of the plant sterols family (β-sitosterol, stigmasterol, etc.) depend on the structure of the side chain and the rigid ring nucleus of sterols. For example, comparing the effects of cholesterol and β-sitosterol, the additional ethyl group of the alkyl side chain of the latter markedly reduces its effectiveness on ordering of fluid DPPC bilayers (Bernsdorff and Winter, 2003). Previously, we have shown that alterations of the membrane fluidity by incorporation of cholesterol or benzyl alcohol influenced some of the membrane-related processes such as energy transfer between the main pigment protein complexes (Dobrikova *et al.*, 1997), fluorescence transitions (Busheva *et al.*, 1998) and kinetics of fluorescence decay (Zaharieva *et al.*, 1998a).

The complex nature of oxygen evolution and some observed rate phenomena, especially biphasic decay kinetics of initial oxygen burst in the absence of an artificial electron acceptor, led to the idea of existing of two different mechanisms of oxygen evolution. It has been proposed that in addition of a non-cooperative mechanism of oxygen evolution (Kok *et al.*, 1970) the cooperation between oxidizing intermediates from different oxygen-evolving centres can occur. This so-called cooperative mechanism of oxygen evolution takes place mainly in photosystem II (PSII) centres situated in non-appressed thylakoids (Diner, 1974; Lavorel, 1976; Maslenkova *et al.*, 1989; Zeinalov, 1982). According to a recently reported organization of thylakoid membranes, in stroma lamellae PSII complexes are present as monomers while in grana regions they are organized in dimers (Dekker and Boekema, 2005). Recently, structurally and functionally different populations of PSII centres have been isolated from stroma and grana regions of thylakoid membranes (Danielsson *et al.*, 2004 and references therein). PSII centres from grana regions contain the functional acceptor side and an active oxygen-evolving complex, while a large part of the stroma-situated PSII is inactive on either or both acceptor or donor side. According to Ford *et al.* (1982) stroma and grana membranes differ not only in respect to activity of PSII and photosystem I (PSI) population but also by the fluidity of the lipid phase, the stroma membranes being more fluid.

PSII complexes with different structural and functional properties are located in different parts of thylakoid membranes and work in different lipid environments and membrane fluidities. The question arises about the role of membrane fluidity in functioning of oxygen-evolving complexes, if any. The aim of the present study was to investigate the effect of alterations of thylakoid membrane fluidity on the oxygen evolution competence of isolated membranes using the plant sterol stigmasterol as lipid order-perturbing agent. Stigmasterol shares structural analogies with cholesterol (concerning the rigid ring structure) with slight modifications in the side chain – extra ethyl group at C24 position and a double bond between C22 and C23. Its plant origin makes stigmasterol very suitable for artificial alteration of fluidity of thylakoid membranes.

Materials and Methods

Isolation of thylakoid membranes

Thylakoid membranes were isolated from 14-day-old pea plants *(Pisum sativum* L. *Ran 1.)* as described by Goetze and Carpentier (1990). The final pellet was resuspended in a buffer containing 0.33 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl and 20 mM Tricine (pH 7.5). Concentration of chlorophyll was determined according to Lichtenthaler (1987).

Manipulation of the lipid phase order by stigmasterol and cholesterol

Increase of the degree of order of fatty acyl chains of thylakoid membrane lipids was performed by incorporation of either stigmasterol or cholesterol using the method described by Dobrikova *et al.* (1997). Increasing concentrations of stigmasterol (from a stock solution in ethanol) were added to the thylakoid membranes (50 μg chlorophyll/ml) and incubated at room temperature for 10 min in the dark while being gently stirred. For comparison, we incubated thylakoid membranes also with 300 μM cholesterol. After incubation, the samples were twice washed and the final pellet was resuspended to an appropriate chlorophyll concentration in a resuspending buffer containing

0.33 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl and 20 mM Tricine (pH 7.5). The amount of ethanol added did not exceed 1%. Preliminary experiments have shown that up to this ethanol content the rate of oxygen evolution and oxygen uptake of washed samples were not affected.

Steady-state fluorescence polarization measurements

Alteration of the fluidity of the lipid phase of thylakoid membranes after incorporation of different concentrations of stigmasterol or cholesterol was determined by measuring the degree of polarization of the steady-state fluorescence emitted from the probes 1,6-diphenyl-1,3,5-hexatriene (DPH) or trimethylammonium-diphenyl-DPH (TMA-DPH). Both probes were incorporated in different locations in the membrane (Lentz, 1993). DPH is a rod-like hydrophobic molecule and partitions into the hydrophobic interior of the bilayer, while TMA-DPH, a derivative of DPH with a positively charged trimethylammonium moiety attached to the *para* position of one of the phenyl rings, anchors in the head group region of the bilayer (Prendergast *et al.*, 1981). Both fluorescent probes are often used for determination of the fluidity of thylakoid membranes, as they do not participate in the energy transfer with chlorophylls (Ford and Barber, 1980). DPH and TMA-DPH were added to a final concentration of 2.5 μM from stock solutions. Measurements were performed at room temperature in the resuspending buffer (0.33 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl and 20 mM Tricine, pH 7.5) using a fluorimeter Jobin Ivon 3Y (Jobin Yvon, ISA, Longjumeau, France), equipped with polarization filters. Background fluorescence was measured by using identical samples without probes and was found to be negligibly small. Fluorescence was excited at 360 nm and registered at 450 nm. The slit widths were 10 nm. The degree of polarization (P) was estimated as described previously (Dobrikova *et al.*, 1997). Chlorophyll concentration was 5 μg/ml.

Photochemical activity

Photochemical activity was polarographically measured by a Clark-type electrode (Model DW1, Hansatech Instruments Ltd., King's Lynn, Norfolk) in a temperature-controlled cuvette and at saturating light intensity. Activity of PSII was determined by the rate of oxygen evolution with the exogenous electron acceptor 1,4-benzoquinone (1,4 BQ) (0.1 mM) in a reaction medium containing 0.33 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl, 20 mM MES (pH 6.5). PSI-mediated electron transport was determined by the degree of oxygen uptake in a medium containing 0.33 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl, 20 mM Tricine (pH 7.5), 0.4 μM DCMU, 0.5 mM NH_4Cl and artificial electron donors and acceptors as follows: 0.1 mM DCPIP, 4 mM Na ascorbate, and 0.1 mM MV. Both photochemical activities were measured at room temperature and at a chlorophyll concentration of 20 μg/ml.

Measurement of oxygen flash yields

Determination of oxygen flash yields and initial oxygen burst was performed using a home-constructed equipment, described in details in Zeinalov (2002). Its main device is a fast oxygen rate electrode equipped with a system for flash, modulated and continuous illumination permitting the estimation of oxygen production reactions. Each sample (100 μl) was pre-illuminated with 25 flashes followed by a 5 min dark adaptation. For measuring of flash oxygen yields thylakoid membranes were illuminated with short (10 μs) saturating (4 J) flashes with a dark period of 0.466 s between the flashes. For continuous illumination measurements, a cold light supplier (LED LXHL-LW3C, Luxeon, Philips Lumileds Lighting Company, San Jose, USA) providing irradiation on the surface of the sample (420 μmol m^{-2} s^{-1}) was used. Data were digitized by a built-in A/D converter and transferred to an online IBM-compatible computer for further analysis. The S_i state population, misses (α) and double hits (β) were calculated by a software based on fitting the theoretically calculated oxygen burst yields according to the non-cooperative Kok's model of oxygen evolution (Kok *et al.*, 1970) to the experimentally obtained values. For all measurements thylakoid membranes were resuspended in a buffer containing 0.33 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl and 20 mM MES (pH 6.5) at a chlorophyll concentration of 150 μg/ml, without addition of artificial electron acceptor.

Results

Here we present data about the effects of the plant sterol stigmasterol on the lipid order of isolated thylakoid membranes and consequently on

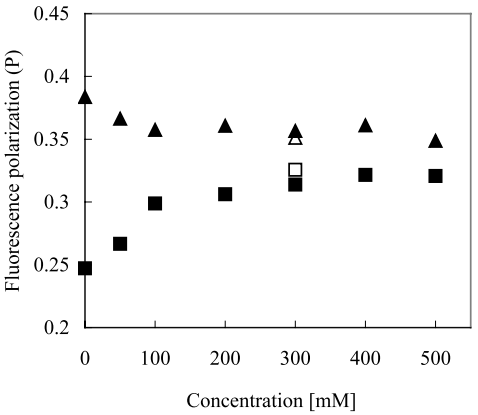

Fig. 1. Fluidity of isolated thylakoid membranes, artificially manipulated with increasing concentrations of the plant sterol stigmasterol at room temperature for 10 min, determined by the degree of polarization (*P*) of the fluorescent probes DPH (■) and TMA-DPH (▲). For comparison the values for thylakoid membranes treated with 300 μM cholesterol are also included (open symbols). Chlorophyll concentration was 5 μg chl/ml. Values are the mean of 5 independent experiments and SD did not exceed 8%. Fluorescence was excited at 360 nm and emission registered at 450 nm.

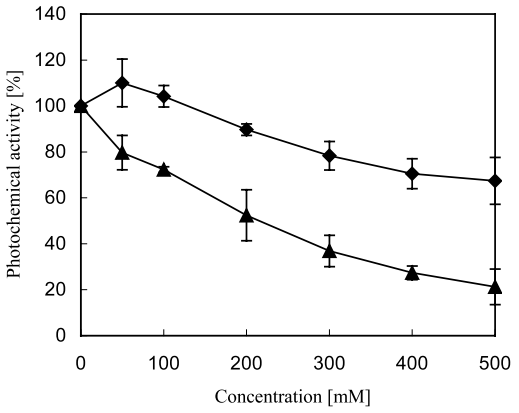

Fig. 2. Photochemical activity of PSI (▲) and PSII (◆) of thylakoid membranes manipulated with different concentrations of stigmasterol expressed as percent of control (non-treated thylakoid membranes). Photochemical activity of PSI was estimated by the rate of oxygen uptake and of PSII by the rate of oxygen evolution. 100% corresponds to: PSI = (324.89 ± 14.40) μmol O_2 mg^{-1} chl h^{-1}; PSII = (79.35 ± 4.33) μmol O_2 mg^{-1} chl h^{-1}. Values are means of 5 independent experiments.

the light-driven photosynthetic reactions. In Fig. 1 the dependence of the degree of polarization of the fluorescent probes DPH and TMA-DPH on the treatment with different concentrations of stigmasterol is presented. The changes reflect the alterations in the motional freedom of the thylakoid lipid phase in its hydrophobic (DPH) and interfacial region (TMA-DPH). Incubation of thylakoid membranes with increasing concentrations of stigmasterol leads to a well-pronounced increase in the degree of fluorescence polarization of DPH indicating rigidization of the hydrophobic phase of the lipid bilayer. Contrary, the degree of packing of the lipid head groups in the interface region, determined by TMA-DPH, is decreased. In both cases, *P* changes considerably up to 100 μM stigmasterol. Above 300 μM of stigmasterol, no further changes in anisotropy are observed. For comparison the degree of polarization of both fluorescent probes for thylakoid membranes treated with 300 μM cholesterol is also included. It is worth noting that the values of *P* for membranes treated with 300 μM stigmasterol or 300 μM cholesterol are close, indicating that the effect of both sterols, at least for concentration of 300 μM, are similar.

In order to determine the effect of stigmasterol on the photosynthetic apparatus we determined the photochemical activity of PSI and PSII of thylakoid membranes, treated with increased concentrations of the plant sterol (Fig. 2). With increase of the concentration of stigmasterol, the rate of electron transport through PSI decreases, reaching 20% of the control value at the highest concentration. The PSII-mediated electron transport, determined with the artificial electron acceptor 1,4 BQ is not affected up to 100 μM stigmasterol and decreases at higher concentrations but to a lower extent as compared with the decrease of PSI activity.

For characterization of the activity of the oxygen-evolving complex we investigated the flash-induced oxygen yields of control and sterol-treated membranes. Fig. 3 represents typical graphs based on the oxygen flash yields induced by short saturating flashes after 5 min dark incubation of the isolated pea thylakoids. The results show well-known oscillations of the amplitudes of the oxygen flash yields with maximum amplitudes at the third and the seventh flash. Although the flash yield amplitudes are reduced with increasing stigmasterol concentrations, the oscillation patterns are identical.

For further investigation of the effect of lipid chain order on the functioning of the oxygen-evolving complex of thylakoid membranes we cal-

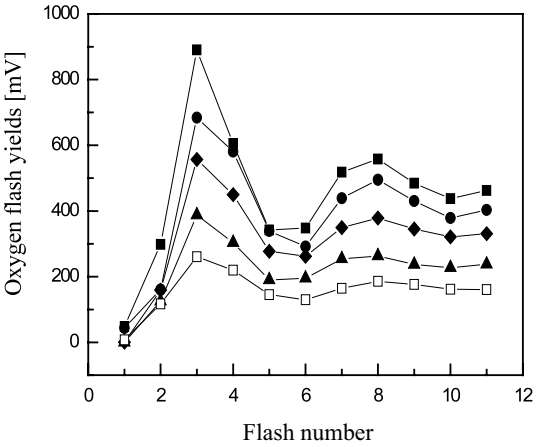

Fig. 3. Effect of incorporation of different concentrations of stigmasterol on flash-induced oxygen evolution of thylakoid membranes. For comparison, the flash oxygen yields patterns for thylakoid membranes treated with 300 μM cholesterol are also included. ■ – 0 μM; ● – 100 μM stigmasterol; ◆ – 400 μM stigmasterol; ▲ – 500 μM stigmasterol; □ – 300 μM cholesterol. Thylakoid membranes were resuspended in a buffer containing 0.33 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl and 20 mM MES (pH 6.5) at a chlorophyll concentration of 150 μg/ml.

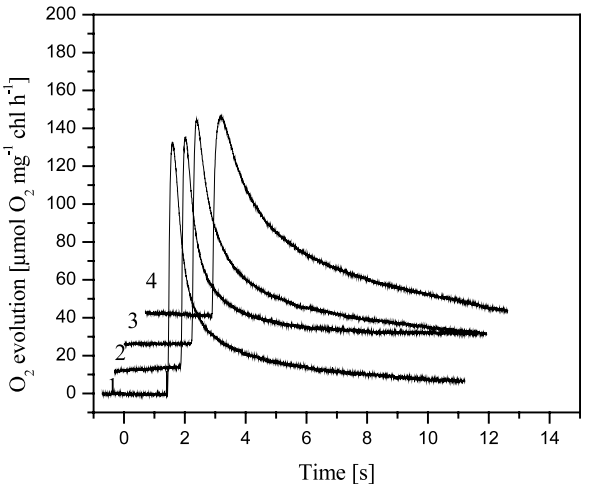

Fig. 4. Oxygen burst after continuous illumination of thylakoid membranes: control (1) and treated with 100 μM (2), 300 μM (3) and 500 μM (4) stigmasterol. Resuspending medium and chlorophyll concentration are as in Fig. 3. For better presentation, the curves are shifted vertically and horizontally.

culated the dark distribution of S_i states at different concentrations of stigmasterol incorporated. Data for 300 and 500 μM stigmasterol and for 300 μM cholesterol are summarized in Table I. Our calculations show a relatively low value of S_0 state population, which increases slightly with the increase of stigmasterol concentration up to 300 μM at the expense mainly of number of centres in S_1 state. Misses (α) (centres do not convert to higher state-zero-step advance) and the double hits (β) (double step advances) apparently are not affected by sterol treatment.

In Fig. 4 are arranged representative traces of oxygen burst under continuous illumination of thylakoid membranes, control and treated with different concentrations of stigmasterol. The typical oxygen induction curve under continuous irradiation exhibits a second order exponential decay kinetics due to the functioning of two mechanisms of oxygen evolution. It is believed that the population of $PSII_\beta$ centres, situated mainly in the stroma thylakoids, evolve oxygen by the cooperative mechanism, after cooperation of oxygen precursors (positive charges) obtained in different reaction centres, while $PSII_\alpha$ centres produce oxygen by non-cooperative mechanisms. Every reaction centre operates independently from the others and

Table I. Effect of incorporation of 300 or 500 μM stigmasterol (SS) or 300 μM cholesterol (Chol) on the S-state distribution and on the transition probabilities of flash-induced O_2 evolution. Numbers of oxygen-evolving centres in S_0 and S_1 states are presented as % of all centres. α and β are probabilities for misses and double hits, respectively. A_1 and A_2 and t_1 and t_2 represent amplitudes and time constants of fast and slow components of initial oxygen burst. Mathematical fit of the photosynthetic oxygen evolution amplitudes was performed using the computer-simulating program according to Kok's model.

Sample	S_0 (%)	S_1 (%)	α (%)	β (%)	t_1 [s]	t_2 [s]	A_1/A_2
0	4.04	87.8	21.6	4.9	0.27	2.84	2.06
300 μM SS	13.15	83.74	21.6	5.4	0.44	3.00	0.93
500 μM SS	6.63	89.94	20.6	4.4	0.54	3.30	0.58
300 μM Chol	14.94	73.42	20.3	4.4	0.50	3.65	0.87

evolves one molecule oxygen after four successive photoreactions (Zeinalov, 2005). The contribution of $PSII_\alpha$ and $PSII_\beta$ centres to the total amount of oxygen evolved can be estimated by decomposition of the oxygen burst decay curve into two kinetic components. The fast component is connected with $PSII_\alpha$ centres and the slow component is connected with $PSII_\beta$ centres. The kinetic parameters of both resulting components (amplitude – A_1 and A_2, time constants – t_1 and t_2) of the oxygen burst decay of control thylakoid membranes and of those treated with stigmasterol and cholesterol are arranged in Table I. The most pronounced effect of stigmasterol on the photosynthetic parameters is expressed in a decrease of the ratio A_1/A_2 – from 2.06 for control, untreated membranes to 0.58 for treated with 500 μM stigmasterol. This decrease indicates that the relative contribution of the slow components increases with an increase of the concentration of stigmasterol.

Discussion

Plant response to environmental stress conditions (temperature or light) includes changes of the lipid saturation level and consequently of membrane fluidity. Investigation of the condensing effect of different sterols on thyalkoid membranes *in vitro* is biologically relevant. We have shown that the controlled perturbation of membrane fluidity achieved by treatment with cholesterol (decreased fluidity) and benzyl alcohol (fluidizing effect) results in an alteration of the energy distribution between both photosystems upon cation-induced stack-unstack transitions (Dobrikova *et al.*, 1997), fluorescent transitions (Busheva *et al.*, 1998) and kinetics of fluorescence decay (Zaharieva *et al.*, 1998a). *In vitro* alteration of membrane fluidity modifies also the response of thylakoid membranes to short time heat stress (Zaharieva *et al.*, 1998b) or to photoinactivation of PSI and PSII (Velitchkova *et al.*, 2001) and energy distribution between the main pigment-protein complexes upon high light treatment at room and low temperatures (Velitchkova and Popova, 2005).

Treatment of thylakoid membranes with increasing concentrations of stigmasterol results in a decrease of fluidity of the hydrophobic interior of the lipid phase and a more loosely packing of the head groups in the interfacial region of the thylakoid membranes (Fig. 1). Both sterols demonstrate similar effects, at least at a concentration of 300 μM. The chemically related structure of stigmasterol and cholesterol and their similar effects on the lipid chain order allow us to suppose that both sterols incorporate in the lipid bilayer of thylakoid membranes in a similar manner and place – restricting the motional freedom of the fatty acyl chains and decreasing the degree of packing of the head groups.

For realizing an effective electron transport between both photosystems (PSII and LHCII, mainly located in the appressed membranes, and PSI complexes, situated preferentially in the stroma and end-grana lamellae), the plastoquinone pool acts as a mobile electron carrier (Andersson and Anderson, 1980). In this respect, the fluidity of the lipid phase plays an important role. The PSI-driven electron transport in thylakoid membranes is more sensitive towards a decrease of membrane fluidity than the photochemical activity of PSII (Fig. 2). This different effect of stigmasterol on the photochemical activity of PSII and PSI is comparable with our previous investigation of the rigidifying effect of cholesterol (Busheva *et al.*, 1998) and data reported by Yamamoto *et al.* (1981), indicating that the photochemical activity of PSII is less affected by treatment with cholesterol and cholesteryl hemisuccinate. The reason could be that the stroma-exposed thylakoid membranes, where the multiprotein complex of PSI is situated, are more accessible to insertion of the sterol molecules, and the lipids-surrounding PSI complex becomes more ordered. It has to be mentioned that under normal conditions PSI operates in a more fluid environment (Ford *et al.*, 1982).

The oxygen-evolving complex of thylakoid membranes is the most sensitive part of the photosynthetic apparatus towards different stress treatments. Our results show that the photoreducing ability of PSII is not dramatically affected after reduction of membrane fluidity but the O_2-evolving capacity is modified. Flash-induced oxygen evolution of photosynthetic membranes is associated mainly with the oxygen-evolving complex, located in the grana regions. Although stigmasterol incorporation results in a reduced amplitude of flash oxygen yields, the oscillations are retained. This means that even the number of operating oxygen-evolving complexes is reduced the remaining centres function "properly" with the characteristics of non-affected ones. Therefore, sterol incor-

poration does not modify the active oxygen-evolving complexes themselves.

The oxygen burst under continuous irradiation represents the amount of oxygen evolved by centres situated in the grana and stroma regions. It has been shown that at low light intensities a limited cooperativity between PSII units exists (Diner, 1974; Lavorel, 1976). It has been suggested that the non-cooperative oxygen-evolving mechanism ($PSII_\alpha$ centres) operates mainly in grana regions of thylakoid membranes while the cooperative mechanism ($PSII_\beta$ centres) takes place predominantly in stroma thylakoids (Maslenkova *et al.*, 1989). It is considered that the centres associated with $PSII_\beta$ (situated mainly in the stroma) operate slower than those from the grana-situated ($PSII_\alpha$) centres (Zeinalov, 2005). The two kinetic components existing at the oxygen burst under continuous illumination reflect the participation of the two different oxygen-evolving mechanisms – non-cooperative (Kok's) mechanism realized by $PSII_\alpha$ centres and cooperative one connected with $PSII_\beta$ (Maslenkova *et al.*, 1989; Zeinalov, 1982, 2005). The contribution of the slower ($PSII_\beta$) component increases after stigmasterol incorporation (Fig. 4). Previously, it has been shown that after cholesterol treatment the number of $PSII_\beta$ centres increases (Busheva *et al.*, 1998). It could be expected that stigmasterol incorporation results also in an increase of these types of PSII centres. In addition, according to the recently reported organization of thylakoid membranes, in stroma lamellae PSII complexes are present as monomers in contrast to dimers in grana regions (Dekker and Boekema, 2005). It cannot be excluded as a possibility that complexes in different oligomeric states could be influenced by different manner and extent of sterol incorporation.

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