# Artificial Alterations of Fluidity of Pea Thylakoid Membranes and Its Effect on Energy Distribution between Both Photosystems

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Two different membrane perturbing agents – cholesterol and benzyl alcohol were applied to modify the fluidity of pea thylakoid membranes. Well pronounced decrease of membrane fluidity was observed upon increasing of cholesterol added. Rigification of thylakoid membranes was accompanied by reduction of cation-induced increase of light-scattering intensity and results in lower extent of increase of spillover upon cation induced stack-unstack transition. Thylakoid membranes, treated with local anesthetic benzyl alcohol, showed an increase of membrane fluidity, but approximately the same behavior as untreated thylakoids in respect to the light scattering intensity dependence on cation concentration. Upon stack-unstack transition the observed changes of energy distribution for benzyl alcohol treated thylakoids are similar to those for controls. The data are discussed in terms of influence of membrane fluidity on lateral reorganization of pigment-protein complexes.

## Introduction

The components involved in the electron transport processes of photosynthesis are associated with proteins, which form macroscopic complexes within and on the surface of the membrane. The intrinsic complexes involved in energy capture and electron transport processes are PSII, LHC II, PSI and Cyt.$b_6/f$ complex (Anderson and Andersson, 1982). There are evidences that these complexes are laterally separated from each other (Barber, 1980; Anderson, 1981). PSII and LHCII are mainly located in the appressed membranes of the grana, whilst PSI complexes are preferentially situated in the exposed thylakoid membranes – stromal and end-granal lamellae. This model raises questions about long-range diffusion processes both at the energy transfer and electron-transport levels. The lateral separation of PSII and PSI requires long-range diffusing species such as plastocyanin to allow electron transport from $H_2O$ to NADP. On the other hand, the regulation of energy distribution between PSI and PSII involves the physical movement of the light harvesting chlorophyll a-b complex between appressed and non-appressed membranes (Barber, 1982; Haworth *et al.,* 1982; Horton, 1983). These concepts emphasize the role of the thylakoid membrane lipid matrix and in particular its fluidity.

The fluidity of a biological membrane is determined by its composition (lipid classes, degree of saturation of the fatty acid chains, protein level and sterol content) and by temperature. At room temperature the thylakoid membranes are relatively fluid (Ford and Barber, 1980). This is mainly due to the high level of unsaturated fatty acid chains and the absence of sterols (Leech and Murphy, 1976; Webb and Green, 1991).

The membrane fluidity can be altered by varying the growth temperature or the temperature of chloroplast suspension and by changing the lipid composition. It was shown that thylakoid fluidity decreased as the temperature was lowered towards $0^0$C (Barber *et al.,* 1984) and that at temperature above $0^0$C the granal and stromal lamellae had different degrees of fluidity with the for-

*Abbreviations:* PS, photosystem; LHCII, light-harvesting chlorophyll *a-b* complex II; ESR, electron spin resonance; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DPH, 1,6 diphenyl-1,3.5 hexatriene; Chl, chlorophyll.

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mer being more rigid, which agree with the data obtained by spin labels and ESR techniques (Ford *et al.*, 1982).

The influence of membrane fluidity on electron transport, proton gradient and others has been also investigated. The incorporation of cholesterol into pea thylakoid membranes results in a decrease of membrane fluidity accompanied by a decrease of the rate of uncoupled linear electron flow (Ford and Barber, 1983). After incorporation of cholesteryl hemicuccinate a decrease of the rate of $Fe(CN)_6$ photoreduction by 30% and about 10% inhibition of the rate of 2,6-dichlorophenol indophenol photoreduction have been obtained (Yamamoto *et al.*, 1981). Addition of cholesteryl hemissuccinate decreased the initial rate of $H^+$ uptake coupled to electron transport and the rate constant of $H^+$ release was also decreased. Cholesteryl hemissuccinate had also a marked effect on the $Mg^{2+}$-induced increase in fluorescence yield (Yamamoto *et al.*, 1981). The cholesterol incorporation decreased the relative size of cation-induced fluorescence increase and the initial rate of this fluorescence change and the benzyl alcohol treatment increased the initial rate of fluorescence rise (Pedersen and Cox, 1984). In the cholesterol treated thylakoids the $Mg^{2+}$-induced increase of PSII fluorescence was blocked and cation-induced particle segregation no longer occurred, although appression of adjacent membranes was still evident and corresponded to an increase of turbidity (Briantias *et al.*, 1984).

The objective of the present work was to investigate the influence of fluidity changes of thylakoid membranes on energy distribution between both photosystems upon cation-induced stack- unstack transition. The extent of rigification and fluidization of thylakoid membranes has been controlled by incorporation of different amounts of cholesterol and benzyl alcohol. The data presented clearly show the role of the fluidity of thylakoid membranes in the cation induced processes of stacking of thylakoids and redistribution of pigment – protein complexes in the membranes, and resulted in well – pronounced changes of energy distribution between both photosystems.

## Materials and Methods

Thylakoid membranes from 12–14 days old peas *(Pisum sativum L, Ran 1)* were isolated according to the method of Goetze and Carpentier, (1990). The final pellet was resuspended in buffer containing 10 mM Tricine – NaOH (pH 8.0), 0.33 M sorbitol and 10 mM NaCl. 5 mM $MgCl_2$ was present when indicated. Cholesterol incorporation was carried out as described by Ford and Barber (1980). The fluidization of thylakoid membranes was completed by incubation with different concentration of benzyl alcohol according to Pedersen and Cox (1984). The chlorophyll concentration was determined by the method of Lichtenthaler (1987).

The relative fluidity of the isolated membranes was estimated by measuring the steady-state fluorescence polarization of the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) according to Ford and Barber (1983). For DPH incorporation the membranes (100 mg chl/ml) were incubated for 40 min at room temperature with 10 μM DPH, added directly from a stock solution (3 mM DPH in tetrahydrofuran) and then washed. The fluorescence polarization of DPH was measured by a spectrofluorimeter Jobin Yvon JY3 with excitation at 360 nm and monitored at 460 nm. The degree of polarization ($P$) was calculated using the following expression:

$$P = \frac{I_{vv} - I_{vh}Z}{I_{vv} + I_{vh}Z}$$

where $I$ is the intensity of the fluorescence, $Z = I_{hv}/I_{hh}$ and the first and second subscript represent the position of the excitation and emission polarizers (vertical and horizontal). Cholesterol and benzyl alcohol were added to the DPH – treated thylakoid membranes.

Light scattering was measured at 540 nm at 90° with respect to the optical axis at a chlorophyll concentration 10 μg/ml.

Low temperature fluorescence spectra were registered by a Jobin Ivon JY3 spectrofluorimeter equipped with a low temperature device and a red sensitive photomultiplier (Hamamatsu R928). The data were digitized by an in-build A/D converter and transferred to an on-line IBM compatible computer for further retrieval and analyses.

## Results

Three different concentrations of cholesterol and benzyl alcohol were applied for rigification

and fluidization of pea thylakoid membranes. The values of the degree of polarization ($P$) of DPH fluorescence are presented in Table I. The incubation of thylakoid membranes with different concentrations of cholesterol and benzyl alcohol were carried out at a chlorophyll concentration of 50 μg /ml. The value for control thylakoids – 0.249 is in agreement with the data usually observed (Ford and Barber, 1983; Barber *et al.*, 1984). The increasing of cholesterol concentration leads to well – pronounced increase of the $P$ up to 0.377 for 500 μM cholesterol added. It must be noted that even at 200 μM cholesterol added, the change of $P$ is about 40%. The opposite effect was observed after incubation with different levels of benzyl alcohol. The decrease of $P$ (the increase of membrane fluidity) for benzyl alcohol concentration tested (100 mM) was about 21%.

To examine the effect of these membrane fluidity perturbing agents on the ability of thylakoid membranes to undergo restacking, the changes of light scattering upon addition of increasing concentrations of $MgCl_2$ were investigated. It was shown that light-scatter measurements were at least a qualitative indicator of the extent of membrane stacking (Wollman and Diner, 1980). Ratios S(C)/S(0), where S(C) is the light-scattering intensity upon addition of concentration C of $MgCl_2$ and S(0) is the light-scattering intensity without $MgCl_2$, are presented in Table II. For control thylakoids S(C)/S(0) increases with increasing of $Mg^{2+}$ concentration up to 5 mM and remains practically unchanged at higher cation concentration. The values of benzyl alcohol treated thylakoids are very close to those of the control, but increasing tendency is still observed at 15 mM $MgCl_2$. For cholesterol – treated thylakoid membranes an increase of this ratio is also observed, but it remains lower than that for control thylakoids – at 15 mM $MgCl_2$ S(c)/S(o) for cholesterol treated samples the value is about 77% of the value for control thylakoids. It must be noted that in our experiments the absolute values of the light scattering intensity at 0 and higher $MgCl_2$ concentrations are greater than the values for untreated thylakoids.

Low temperature fluorescence emission spectra of control, cholesterol – treated and benzyl alcohol – treated pea thylakoid membranes were registered upon low – salt condition and after incubation in the presence of 5 mM $MgCl_2$. The fluorescence emission spectra for control thylakoid membranes in low and high cation concentration medium are presented in Fig. 1A. Upon stack-unstack transition the ratio F735/F685 usually used as a measure of spillover increases from 1.2 up to 1.65. The typical fluorescence emission spectra in the region 660–760 nm with excitation 436 nm for cholesterol- and benzyl alcohol- treated membranes are presented at Fig. 1B and Fig. 1C, respectively. The change of the shape of the maximum in the region 685–695 nm are observed in treated membranes.

Table I. The steady-state fluorescence polarization values (P) of DPH incorporated into untreated and treated with cholesterol and benzyl alcohol thylakoid membranes. Chlorophyll concentration – 10 μg/ml. Treatments and measurements were carried out as described in Materials and Methods. Mean values ± S.E were calculated from 4 independent experiments.

| Samples | Fluorescence polarization ($P$) |
|---|---|
| Untreated thylakoid membranes | 0.249±0.013 |
| Thylakoid membranes treated with | |
| 200 μM cholesterol, | 0.350±0.008 |
| 400 μM cholesterol | 0.359±0.011 |
| 500 μM cholesterol | 0.377±0.008 |
| Thylakoid membranes treated with | |
| 20 mM benzyl alcohol, | 0.235±0.016 |
| 40 mM benzyl alcohol, | 0.224±0.013 |
| 100 mM benzyl alcohol, | 0.213±0.007 |

Table II. The ratio S(C)/S(0) (S(C) – light – scattering intensity in presence of $Mg^{2+}$ and S(0) – light – scattering intensity without of $Mg^{2+}$) for control, cholesterol – treated (500 μM) and benzyl alcohol – treated (100 mM) thylakoid membranes. Chlorophyll concentration 10 μg/ml. Mean values ± S.E were calculated from 3 independent experiments.

| Sample | Concentration ($c$) of $MgCl_2$ | | | | | |
|---|---|---|---|---|---|---|
| | 0 mM | 3 mM | 5 mM | 7 mM | 10 mM | 15 mM |
| Control | 1.0 | 1.47 ± 0.07 | 1.60 ± 0.01 | 1.50 ± 0.05 | 1.45 ± 0.06 | 1.59 ± 0.05 |
| Cholesterol – treated | 1.0 | 1.14 ± 0.04 | 1.16 ± 0.02 | 1.19 ± 0.03 | 1.19 ± 0.03 | 1.23 ± 0.01 |
| Benzyl alcohol – treated | 1.0 | 1.37 ± 0.05 | 1.48 ± 0.09 | 1.46 ± 0.05 | 1.57 ± 0.05 | 1.65 ± 0.02 |

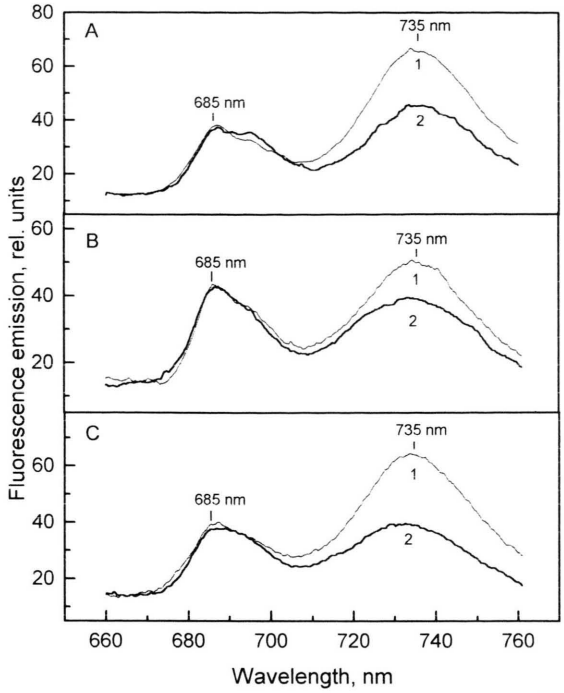


Fig. 1. 77K fluorescence emission spectra of thylakoid membranes. A – control; B – cholesterol – treated (500 μM); C – benzyl alcohol – treated (100 mM) thylakoid membranes. 1 – in unstack medium; 2 – in stack medium in the presence of 5 mM $MgCl_2$. The resuspending medium contains 10 mM Tricine-NaOH (pH 8.0), 330 mM sucrose and 10 mM NaCl. Chlorophyll concentration was 10 μg Chl/ml. Excitation wavelength: 436 nm.

The values for relative increase of the ratio F735/F685 upon unstacking for control, cholesterol- and benzyl alcohol- treated samples with excitation 436 nm and 472 nm are presented in Table III. The results for treated membranes concern the thylakoid membranes, treated with 500 μM cholesterol and 100 mM benzyl alcohol.

Table III. Relative changes (%) of the ratio F735/F685 with excitation 436 nm and 472 nm, due to the stack – unstack transition for control, cholesterol – treated (500 μM) and benzyl alcohol – treated (100 mM) thylakoid membranes. Ru=*F*735/*F*685 under low salt condition, Rs=*F*735/*F*685 under stack condition. Chlorophyll concentration – 10 μg/ml. Mean values ± S. E. were calculated from 4 independent experiments.

| Samples | Ru/Rs excitation 436 nm | Ru/Rs excitation 472 nm |
|---|---|---|
| Control | 49.00 ± 3.09 | 81.43 ± 9.22 |
| Cholesterol – treated | 27.63 ± 2.30 | 48.00 ± 2.68 |
| Benzyl alcohol – treated | 44.20 ± 7.11 | 61.00 ± 5.03 |

The values for the ratio F735/F685 upon excitation with 436 nm for benzyl alcohol- treated membranes are close to these for control thylakoids. The difference between control and benzyl alcohol treated thylakoids is observed upon excitation with 472 nm. Cholesterol treated membranes showed lower value for the increase of F735/F685 upon unstacking for both excitation wavelength. It must be noted that upon stack conditions the ratio F735/F685 for cholesterol-treated membranes is below or near to 1, while for untreated thylakoids this value is usually higher.

## Discussion

The changes of the thylakoid membrane fluidity affects most of the photochemical processes of photosynthesis – the rate of PSII – mediated electron transport, the rate of oxidation of primary electron acceptor of PSII and the initial rate of $H^+$ uptake (Yamamoto *et al.*, 1981), and the kinetics and relative size of cation-induced chlorophyll fluorescence increase (Pedersen and Cox, 1984). As the regulation of energy distribution between PSI and PSII includes lateral reorganization of pigment-protein complexes, it could be expected that any changes of the thylakoid membrane fluidity will affect the processes of energy redistribution during the stack-unstack transition.

In the present work the influence of two membrane fluidity perturbants on the possibility for stack-unstack transition and on the sequential changes of the energy distribution between both photosystems is investigated. Cholesterol is the most used agent for rigification of biological membranes and especially for thylakoid membranes (Ford and Barber, 1983; Yamamoto *et al.*, 1981).

The data shown in Table I relate to cholesterol concentrations corresponding to the incubation level of a cholesterol:chlorophyll ratio from 1 to 4 (w/w). The values for *P* increase remarkably at 200 μM cholesterol added. The increasing of cholesterol concentrations higher than 500 μM does not result in further pronounced change of *P* values. Our data for the fluidity of cholesterol-treated membranes are in agreement with those, obtained by use of DPH (Ford and Barber, 1980; Ford and Barber, 1983, Yamamoto *et al.*, 1981) and are consistent with the data about apparent correlation time, obtained by use of spin-labels (Pedersen and

Cox, 1984). Using the method for cholesterol treatment, described by Yamamoto *et al.*, 1981 the similar effects on membrane fluidity were observed.

The fluidity changes induced by incubation with benzyl alcohol are not so pronounced as compared to the control. In this case the values for *P* change considerably at first concentration applied and decrease slowly at higher benzyl alcohol concentrations. The decrease of values for *P* after treatment with benzyl alcohol are in agreement with the data about decreased values of apparent correlation time of spin labels reported by Pedersen and Cox (1984).

Using these methods to manipulate the membrane fluidity, we investigate the ability of destacked membranes, pretreated with cholesterol or benzyl alcohol to restack upon addition of different concentration of $Mg^{2+}$. The data presented in Table II showed similarity of control and benzyl alcohol treated samples in respect to the cation concentration requirements as well as the percentage of increase of light scattering intensity. It seems that fluidization of membrane does not affect the cation- dependence of light-scattering intensity.

The increase of turbidity of cholesterol treated thylakoids follows the same pattern as in control ones, but with lower amplitude. These values are lower than those reported by Briatias *et al.* (1984), that more than 90% of turbidity increase subsist in the cholesterol- pretreated chloroplasts compared to the control. The absolute values for light-scattering intensity are higher for cholesterol-pretreated thylakoids, what probably can be due to the swelling of cholesterol-treated thylakoids, as was reported by Scoufflaire *et al.* (1981). The other reason for lower increase of the ratio S(C)/S(0) in the case of cholesterol treatment can be any changes of the membrane surface properties if a part of the cholesterol molecules simply associated with the surface as it was proposed (Yamamoto *et al.*, 1981).

The membrane fluidity affects the lateral segregation of chlorophyll – protein complexes of PSI and PSII upon cation-induced stack – unstack transition. This influence can be followed by cation-induced change of the low temperature fluorescence ratio $F735/F685$. Under excitation with $\lambda = 436$ nm, the control and benzyl alcohol treated membranes showed the similar increase of this ratio upon unstacking. There is more pronounced difference of the ratio $F735/F685$ between control and benzyl alcohol treated samples under excitation with $\lambda = 472$ nm. As it was mentioned above, even at low benzyl alcohol concentration, the change of the shape of the fluorescence maximum at 685–695 nm is observed, so an influence of this agent on the coupling between LHCII and PSII core complex and their energy interaction can be conjectured. It seems that the increase of membrane fluidity does not alter remarkably the final lateral reorganization of pigment – protein complexes of PSI and PSII and after fluidization the same increase of spillover upon unstacking is observed.

The lower values for cholesterol – treated membranes (Table III) are probably due to the hindered lateral movements of chlorophyll–protein complexes as a result of decreased membrane fluidity and complete randomization of PSI and PSII does not occur. These data are in agreement with those reported by Scoufflaire *et al.* (1981), concerning the changes of spillover for cholesterol-treated thylakoids in the presence and absence of $MgCl_2$. Using freeze- fracture electron microscopy, it was shown that in cholesterol- pretreated thylakoids the reorganization of intramembrane particles has been initiated, so an increase of spillover upon unstacking can be expected (Briantias *et al.*, 1984).

The present data demonstrate the importance of fluidity of thylakoid membranes for their capability to undergo reversible lateral reorganization upon changes of the cation concentrations. Further investigations on the behavior of the thylakoid membranes with changed fluidity after stress treatments and on the kinetics properties of cation-induced fluorescence rise are in progress.

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Anderson J. (1981), Consequence of spatial separation of photosystem 1 and 2 in thylakoid membranes of higher plant chloroplasts. FEBS Lett. **124**, 1–10.

Anderson J. M. and Andersson B. (1982), The architecture of photosynthetic: lateral and transverse organization. Trends Biochem. **7**, 288–292.

Barber J. (1980), An explanation for the relationship between salt-induced thylakoid stacking and the chlorophyll fluorescence changes associated with changes in spillover of energy from photosystem II to photosystem I. FEBS Lett. **118**, 1–10.

Barber J. (1982), Influence of surface charges on thylakoid structure and function. Annu. Rev. Plant Physiol. **33**, 261–295.

Barber J., Ford R. C., Mitchell., R. A. C. and Millner P. A. (1984), Chloroplast thylakoid membrane fluidity and its sensitivity to temperature. Planta **161**, 375–380.

Briantias J. M., Vernotte C., Olivee J. and Wollman (1984), Kinetics of cation-induced changes of photosystem II fluorescence and of lateral distribution of the two photosystems in the thylakoid membranes of pea chloroplasts. Biochim. Biophys. Acta **766**, 1–8.

Ford R. C. and Barber J. (1980), The use of diphenylhexatriene to monitor the fluidity of the thylakoid membrane. Photobiochem. Photobiophys. **1**, 263–270.

Ford R. C. and Barber J. (1983), Incorporation of sterol into chloroplast thylakoid membranes and its effect on fluidity and function. Planta **158**, 35–41.

Ford R. C., Chapman D. J., Barber J., Pedersen J. Z. and Cox R. P. (1982), Fluorescence polarization and spin label studies of the fluidity of stromal and granal chloroplast membranes. Biochim. Biophys. Acta **681**, 145–151.

Goetze D. Ch. and Carpentier R. (1990), Oxygen mediated photosystem I activity in thylakoid membranes monitored by a photoelectrochemical cell. Photochem. Photobiol. **52**, 1057–1060.

Haworth P., Kyle D. J., Horton P. and Arntzen C. J. (1982), Chloroplast membrane protein phosphorylation. Photochem. Photobiol. **36**, 743–747.

Horton P. (1983), Control of chloroplast electron transport by phosphorylation of thylakoid proteins. FEBS Lett. **152**, 47–51.

Leech R. M. and Murphy D. J. (1976), The cooperative function of chloroplasts in the biosynthesis of small molecules. In: Topics in Photosynthesis (J. Barber, ed.), vol. **1**., 365–401, Elsevier, Amsterdam.

Lichtenthaler H. K. (1987), Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymol. **148**, 350–382.

Pedersen J. Z. and Cox R. P. (1984), Relationship between thylakoid membrane fluidity and the kinetics of salt-induced fluorescence changes: A spin label study. In: Adv. Photosynth. Res. (C. Sybesma ed.) Vol. **3**, 51–54. Martinus Nijhoff/Dr. Junk, W. Publishers, The Hague.

Scoufflaire C., Chow W. S., Barber J. and Lannoye R. (1981), Further studies of the relationship between thylakoid stacking and salt-induced chlorophyll fluorescence changes. In: Photosynthesis (G. Akoyunoglou, ed.) vol. **1**, pp. 605–615, Balaban Int. Science Services, Philadelphia.

Webb M. S. and Green B. R. (1991) Biochemical and biophysical properties of thylakoid acyl lipids. Biochim. Biophys. Acta **1060**, 133–158.

Wollman F.-A. and Diner B. A. (1980), Cation control of fluorescence emission, light scatter and membrane stacking in pigment mutants of *Chlamydomonas reinhardi*. Arch. Biochem. Biophys. **201**, 646–659.

Yamamoto Y., Ford R. C. and Barber J. (1981), Relationship between thylakoid membrane fluidity and the functioning of pea chloroplasts. Effect of cholesteryl hemisuccinate. Plant Physiol. **67**, 1069–1072.