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KINETICS OF CATION-INDUCED CHANGES OF PHOTOSYSTEM II FLUORESCENCE AND OF LATERAL DISTRIBUTION OF THE TWO PHOTOSYSTEMS IN THE THYLAKOID MEMBRANES OF PEA CHLOROPLASTS

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Time-courses of increase in Photosystem (PS) II fluorescence and of stacking on addition of 5 mM $MgCl_2$ were studied under the same experimental conditions. It was shown that PS II–PS I segregation as well as the decrease of PS II–PS I exciton transfer were completed within the same time. Furthermore, in cholesterol-pretreated thylakoids, where Mg^{2+}-induced increase of PS II fluorescence is blocked, cation-induced particle segregation no longer occurred, although appression of adjacent membranes was still evident and corresponded to an increase of turbidity. Cholesterol rigidification did not suppress the Mg^{2+}-induced increase of exoplasmic fracture face particles size nor the stimulation of the rise time of PS II fluorescence induction in the presence of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.

Introduction

Lutz [1] and Markwell et al. [2] gave evidence that all chlorophylls are localized in chromoprotein complexes. Thus, no exciton transfer between photosynthetic units can occur via free chlorophylls embedded within the lipid matrix of the thylakoid membrane.

At low monovalent cation concentration, thylakoids are unstacked [3] and the protein complexes are homogeneously distributed within the membrane [4]. Screening the surface-negative charges by cations induces stacking [3] which corresponds to a lateral segregation of PS I and PS II [5]. This segregation is, as suggested by Barber and Chow [6] and by Anderson [5], the simplest explanation for the decrease in PS-II-to-PS-I exciton transfer. However, there is no direct correlation between stacking or partitioning of PS I and PS II markers and PS II fluorescence enhancement obtained by addition of various cation concentrations [7,8]. In addition, the kinetics of fluorescence enhancement and of increase of scattering are different [8]. Although the cation induced increase of PS II fluorescence can be explained by an increase of the average distance between PS I and PS II units [9], the fluorescence enhancement observed in this work [9] is very much faster than the segregation of PS I and PS II markers measured by Staehelin et al. [10] using electron microscopy. The same kind of discrepancy is also observed between rates of quenching of PS II fluorescence (Ref. 11, see also Briantais, J.-M., and Vernotte, C., unpublished data) and PS I and PS II randomization [10] upon removal of cations.

On the basis of these large differences between the kinetics of the two phenomena it has been

Abbreviations: PS I, Photosystem I; PS II, Photosystem II; Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

proposed [9] that a more discrete separation of the two photosystems than the large-scale segregation of PS I and PS II into stroma and grana, respectively, is responsible for the decrease of PS-II-to-PS-I exciton transfer. Among fast structural changes induced by cations are appression and deappression of adjacent membranes [10]. Arntzen [12] has suggested that appression may induce transfer between PS II units of contiguous membranes.

In order to clarify these problems, a reinvestigation of the kinetics of Mg^{2+}-induced stacking and increase of PS II fluorescence of the same thylakoid preparation have been done.

In an attempt to distinguish between the effects of appression and of lateral segregation, cation-induced changes of fluorescence and structure have been measured in cholesterol-supplemented thylakoids. Cholesterol addition will not increase the density of negative charges of the thylakoid surface but, as shown by Scoufflaire et al. [13], it decreases membrane fluidity and inhibits the Mg^{2+}-induced increase of PS II fluorescence.

The data presented here show that the decrease of PS-II-to-PS-I exciton transfer measured by a PS II fluorescence increase, and PS-II–PS-I segregation are both completed within the same time (3 min) in our experimental conditions. It is also shown that the lateral segregation of the two photosystems, but not the simple appression, is correlated with a decrease of exciton transfer from PS II to PS I.

Material and Methods

Broken chloroplasts were isolated at 0–4°C from pea leaves grown in a green house. Leaves were ground in 0.4 M Sorbitol, 0.1 M tricine (pH 7.8) and 0.25% bovine serum albumin; after filtration through four, then eight layers of cheese cloth, the chloroplasts were centrifuged and washed in 0.01 M NaCl and resuspended at 1–3 mg chlorophyll/ml in 0.4 M Sorbitol. 0.01 M tricine (pH 7.8) and 0.01 M NaCl (called 'resuspending medium') plus $1 \cdot 10^{-6}$ M Gramicidine D. 5 mM $MgCl_2$ was added when necessary to the different media as indicated in the text. All the experiments, unless otherwise stated, are performed in the resuspending medium.

Chlorophyll fluorescence was measured using 2.5 ml samples of chloroplast suspension at a concentration of 1 μg chlorophyll per ml in a 1×1 cm stirred cuvette. The excitation beam was passed through Corning 4-96 and 3-69 filters; the fluorescence emitted at 90° to the exciting beam was selected using red filters (Corning 2-64 plus Wratten 92). The excitation intensity was about 30–50 photons per center per s. The shutter opening time was less than 1 ms. The signal was recorded utilizing a multichannel analyser (SEIN).

Turbibidity of chloroplasts (5 μg chlorophyll per ml) was measured at 540 nm using a Cary 14 spectrophotometer, in a 1×1 cm stirred cuvette.

Digitonin fractionation was performed following the procedure of Chow and Barber [14], modified by Gerola et al. [15]: chloroplasts were resuspended at 300 μg Chl/ml in the resuspending medium with or without 5 mM $MgCl_2$ added. Digitonin (2% w/v) was added to 0.4% final concentration. Treatment with digitonin was done with agitation at room temperature for 2 min. Then the chloroplast membrane-detergent mixture was diluted 20-fold with a cold (2°C) medium, containing 0.1 M Sorbitol/1 mM NaCl/1 mM Hepes (pH 7.5). Separation of the heavy and light membrane fractions was achieved by centrifugation at $10000 \times g$ for 30 min at 2°C. Chlorophyll was extracted from the 10 k pellet with 80% acetone and chlorophylls a and b determined.

Cholesterol (10 mg/ml Ethanol) was added at a final concentration of 40 μg/ml to chloroplast suspension at 5 μg Chl/ml. After 3 or 5 min incubation under stirring, 5 or 10 mM $MgCl_2$ were added. After 20 min, samples were quickly pelleted for electron microscopic studies. Fluorescence levels were recorded during the cholesterol incubation and subsequent $MgCl_2$ addition. For the turbidity measurements, the chloroplast suspension was centrifuged (3 min at $3000 \times g$) 5 min after cholesterol addition, rediluted to 5 μg Chl/ml in the resuspending medium and finally 5 mM $MgCl_2$ was added (fluorescence changes have also been measured in these conditions).

For the freeze-fracturing studies, the thylakoid membranes were rapidly frozen by a propane jet in a cryojet Balzers and stored in liquid nitrogen. Freeze-fracturing and platinum shadowing were carried out with a Balzers apparatus at −150°C.

Thin sections have been obtained from the thylakoïd membranes according to a method described by Martin and Goodenough [16]. Replicas and thin sections were examined in an EM 400 Philips electron microscope.

Results

Kinetics of PS II and PS I segregation upon $MgCl_2$ addition to destacked thylakoids

Fig. 1 shows variations of the amount of chlorophyll in the 10 k pellet (a) and its chlorophyll *a/b* ratio (b) after digitonin treatment of thylakoids which have been restacked at 20°C during various times. Before $MgCl_2$ addition, thylakoids were resuspended in the medium without $MgCl_2$ and kept several hours at 2°C in this destacking condition. The amount of membrane in the 10 k pellet is indicative of the degree of membrane appression and the chlorophyll *a/b* ratio a measure of PS I and PS II segregation. We observed that at 20°C

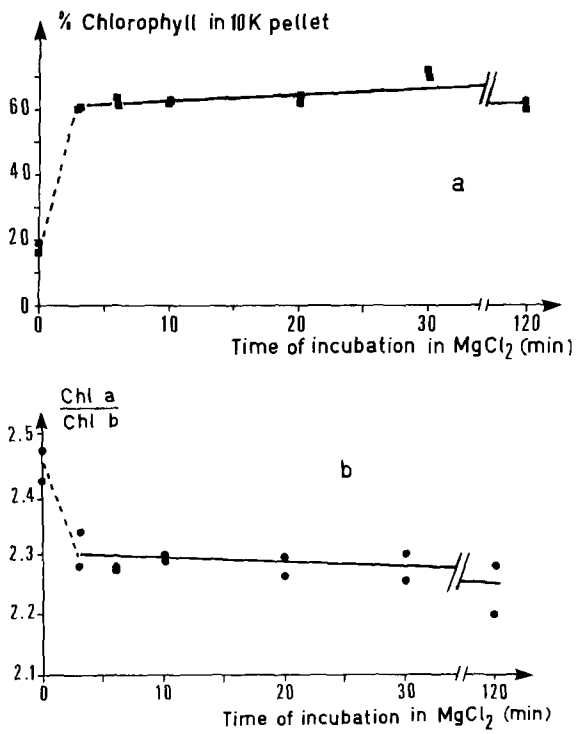

Fig. 1. Effects of digitonin treatment of thylakoids, incubated various times in resuspending medium + 5 mM $MgCl_2$; for details see the Material and Methods section and the Results section.

the formation of grana appears to be complete within 3 min after $MgCl_2$ addition. The half-time of PS II fluorescence increase, in the same conditions and for the same preparation, is 9 s, and also complete within 3 min. Thus it emerges that cation-induced segregation of the two photosystems and PS II fluorescence enhancement are fast phenomena and occur in the same time range.

As shown in Fig. 2a, thin section of the Mg^{2+}-untreated thylakoid membranes indicate that the membranes are completely unstacked. As expected for this unstacked pattern, the intramembranous particles observed on the freeze-fracture surfaces are randomly distributed on both protoplasmic and exoplasmic fracture faces (Fig. 2b). Conversely, a large proportion of the membranes incubated 3 min or 20 min with $MgCl_2$, are appressed as shown in the thin sections (Figs. 3a and 4a). Freeze-fracturing of the Mg^{2+}-treated thylakoids showed the known differences between stacked and unstacked membranes (Figs. 3b and 4b). The protoplasmic fracture faces are characterized by a higher proportion of particles larger than 100 Å in the unstacked (u) than in the stacked (s) membranes, while on the exoplasmic fracture faces the particle density is lower on the unstacked exoplasmic fracture faces than on the stacked ones, and the large particles (greater than 150 Å) are restricted to the stacked membranes.

The most important information given by these electron micrographs is that the 3 min and the 20 min $MgCl_2$-treated membranes cannot be distinguished either by thin section or by freeze fracture. As an example, there is no significant difference of the stacked exoplasmic fracture face particle densities in the two cases: 1420 and 1395/μm^2 for the 3 min and 20 min $MgCl_2$-treated samples, respectively. The stacking and segregation processes seem to be completed within 3 min after $MgCl_2$ addition. The electron microscope studies are, therefore, consistent with the digitonin fractionation results.

We also measured the fluorescence induction kinetics, in the presence of DCMU, of thylakoids incubated for various times in $MgCl_2$. Table I and Fig. 5 show that there is no difference between the samples incubated 5 min or 30 min in 5 mM $MgCl_2$. This indicates that the changes of the PS II–PS II transfer (sigmoidicity, relationship of flu-

orescence versus complementary area), the changes of PS II antenna size (half-time of variable fluorescence increase) and the changes of PS II → PS I transfer (F_m and F_o levels) are all completed within 5 min, and no long time range effects can be detected.

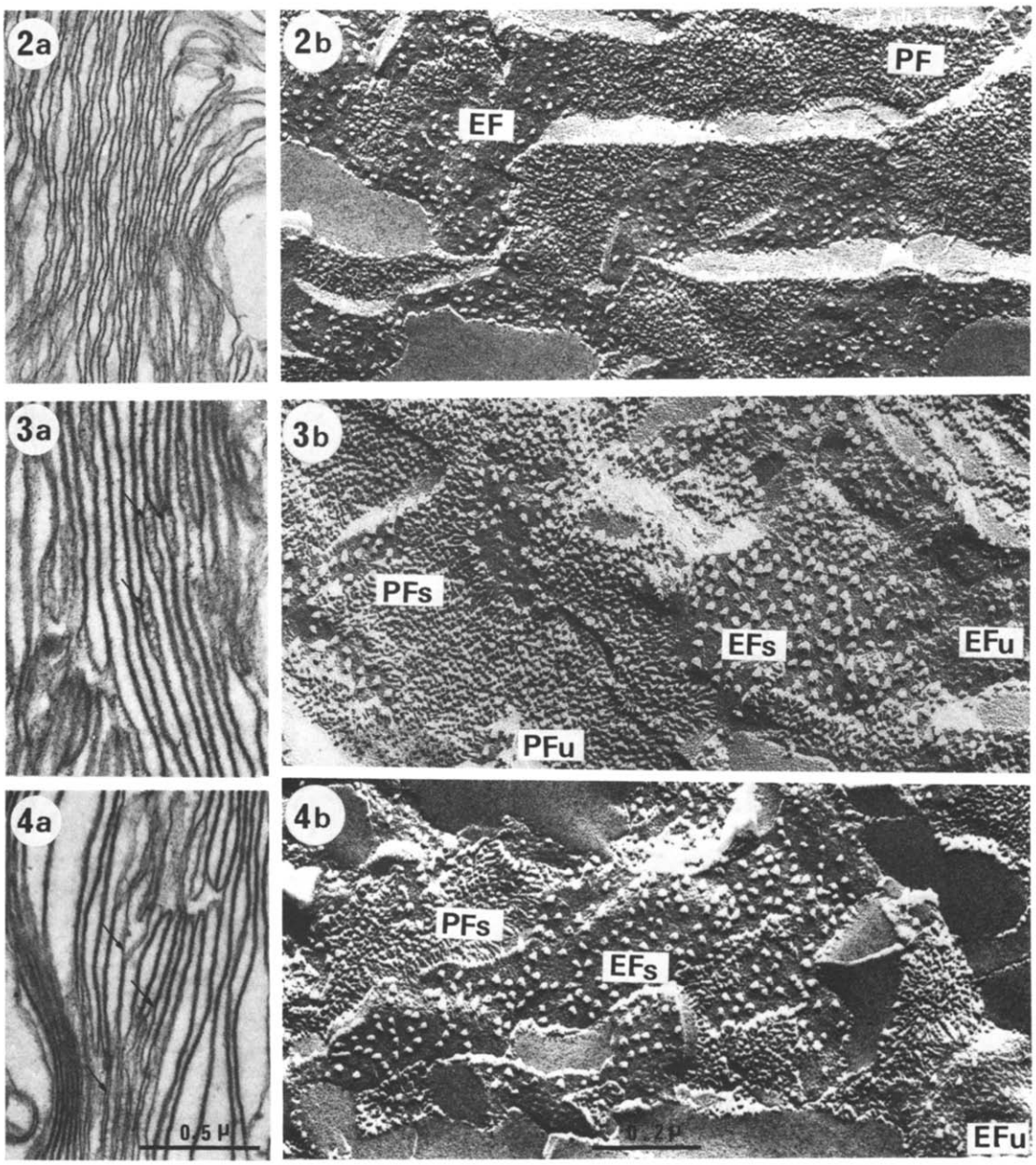

Fig. 2. The $MgCl_2$-untreated thylakoid membranes are completely unstacked, as shown on thin sections (a) and on the freeze-fractured surfaces (b), where the particles are randomly distributed on both protoplasmic (PF) and exoplasmic (EF) fraction face.
Figs. 3 and 4. Thylakoid membranes treated 3 min (Fig. 3) and 20 min (Fig. 4) with $MgCl_2$. In both conditions, a large proportion of membranes are stacked, as shown on this sections (Figs. 3a and 4a). The arrows indicate regions where the membranes are not appressed. On freeze-fractured surfaces (Figs. 3b and 4b), the segregation of particles into stacked (s) and unstacked (u) protoplasmic and exoplasmic fraction face domains is visible.

TABLE I

CHARACTERISTICS OF FLUORESCENCE INDUCTION IN THE PRESENCE OF DCMU AS A FUNCTION OF TIME IN RESUSPENDING MEDIUM PLUS $MgCl_2$ (STACKING CONDITIONS)

Thylakoids have been incubated more than 1 h in the medium without $MgCl_2$ at 4°C. Then they are diluted to 2.5 μg Chl/ml at 20°C; 5 mM $MgCl_2$ is added and the induction measured 5 and 30 min later. F_m = maximum fluorescence, F_0 = initial fluorescence, F_v = variable fluorescence.

Time in resuspending medium + $MgCl_2$ (min)	F_0	F_m	F_v/F_0	$t_{1/2}F_v$ (ms)	Sigmoidicity [a]
0	0.78	1.67	1.14	46	≈ 0
5	1.00	3.66	2.66	31	+
30	0.95	3.31	2.48	31	+

[a] Sigmoidicity has been visualized through automatic plot of F_v versus the complementary area of F_v.

Effect of cholesterol supplementation of thylakoids on cation-induced increase of fluorescence and structural changes

We measure the amplitudes of PS II fluorescence and turbidity increases after adding $MgCl_2$ to destacked thylakoids which have or have not been pretreated by cholesterol. It is observed that cholesterol blocks PS II fluorescence enhancement (5% only in cholesterol-pretreated thylakoids instead of 75–100% in the control) but does not inhibit the increase of turbidity (more than 90% of turbidity increase subsist in the cholesterol-pretreated chloroplasts compared to the control). It should be noticed that the two phases, fast and slow, of turbidity increase still exist in the cholesterol-treated sample, their relative amplitudes are the same as in the control (not shown).

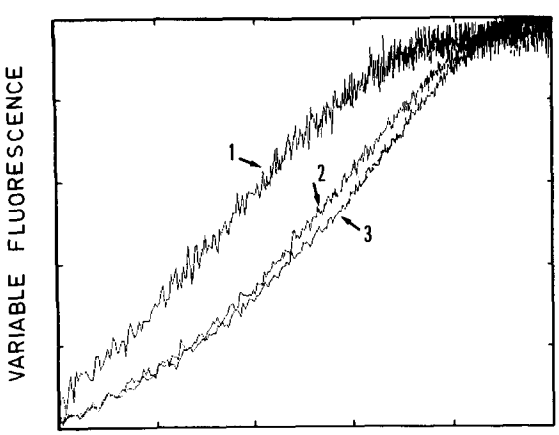

Fig. 5. Variable fluorescence versus complementary area over the fluorescence curve during induction in the presence of DCMU $1 \cdot 10^{-5}$ M, in thylakoids which have been preincubated various times in plus $MgCl_2$ medium (see Table I). 1, No $MgCl_2$; 2, 5 min in $MgCl_2$ medium; 3, 30 min in $MgCl_2$ medium.

In Table II are presented the characteristics of fluorescence induction in the presence of DCMU, of untreated and cholesterol-treated thylakoids. In the absence of $MgCl_2$, control and cholesterol-treated thylakoids present the same characteristics. When $MgCl_2$ is added to the cholesterol-treated thylakoids, no major changes occur except for a decrease in the half-time of the variable fluorescence induction. This diminution is comparable to that obtained in the control thylakoids by addition of $MgCl_2$, but it is not accompanied by a significant variation of the variable fluorescence amplitude.

In the absence of $MgCl_2$, cholesterol-pretreated thylakoid membranes, like the control membranes, show the same unstacked pattern, both in thin sections and freeze fracturing (Fig. 6a and b).

The same cholesterol-pretreated membranes, incubated with $MgCl_2$ for 20 min, displayed a large proportion of appressed regions as shown in thin sections (Fig. 7a); the same result has been obtained by Gerola, P.D. (personal communication). But, in contrast to the control membranes, particle segregation does not occur and, in freeze-fracturing, the protoplasmic and exoplasmic fracture surfaces are characterized by a random particle distribution (Fig. 7b). However, some limited movement of the intramembranous particles can be detected on the exoplasmic fracture faces to

TABLE II

CHARACTERISTICS OF FLUORESCENCE INDUCTION IN THE PRESENCE OF DCMU OF CONTROL AND CHOLESTEROL-TREATED CHLOROPLASTS WITH AND WITHOUT $MgCl_2$

In all cases thylakoids (5 μg Chl/ml) were incubated 9 min in the dark before addition of $1 \cdot 10^{-5}$ M DCMU. 30 s later, the fluorescence induction curve was recorded. When present, cholesterol 940 μg/ml) was added after 3 min of incubation in the dark, and $MgCl_2$ (10 mM) was added after 6 min.

		F_0	F_m	F_v/F_0	$t_{1/2}F_v$ (ms)	Sigmoidicity
Control	no $MgCl_2$	0.78	1.44	0.84	48	0
	10 mM $MgCl_2$	1.00	2.88	1.88	28	+
Cholesterol-treated	no $MgCl_2$	0.72	1.28	0.78	44	0
	10 mM $MgCl_2$	0.81	1.42	0.76	28	0

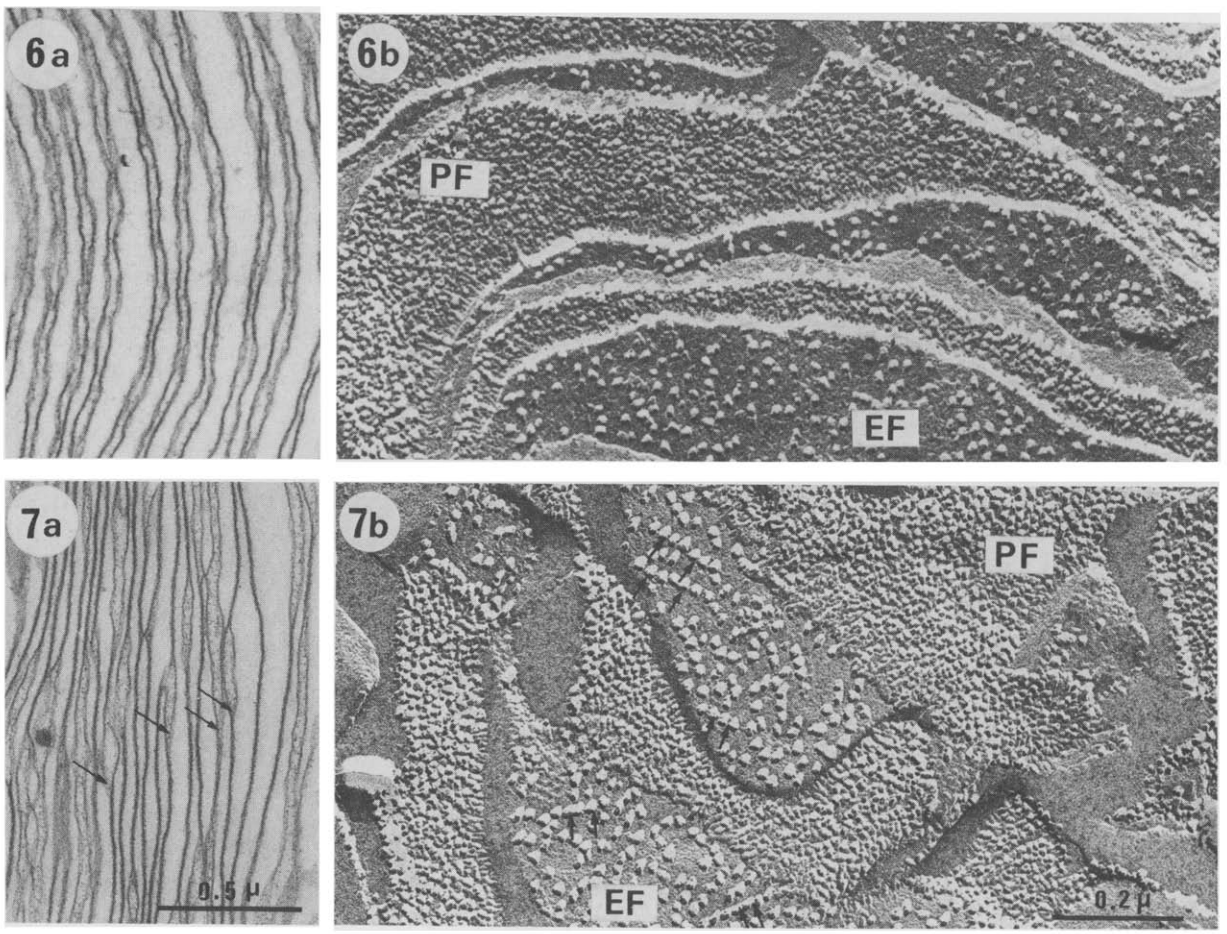

Fig. 6. Cholesterol-treated thylakoid membranes show, both in thin sections (a) and in freeze-fracturing (b), the same unstacked pattern observed in control membranes. EF and PF, exoplasmic and protoplasmic fraction face, respectively.

Fig. 7. Cholesterol-pretreated membranes incubated 20 min with 10 mM $MgCl_2$. Thin sections (a) show that the membranes are appressed in a large proportion. Conversely, in freeze-fractured surfaces (b), the segregation of particles corresponding to stacked and unstacked protoplasmic (PF) and exoplasmic (EF) fraction face regions does not occur. Alignment of some particles (arrows) indicate that a reorganization of the intramembranous particles has been initiated but probably limited by the rigidification of the membrane due to the cholesterol.

give a linear organization of small numbers of particles (Fig. 7b, arrows); further particle reorganization is probably prevented by the rigidification of the membrane by cholesterol.

The main ultrastructural change observed on the freeze-fracture faces upon addition of $MgCl_2$ to cholesterol-treated membranes is an increase of the exoplasmic fracture face particle size (Fig. 8). $MgCl_2$ addition does not induce a major change of the particle densities on either face.

Discussion

The data presented in this paper complete at the functional level Staehelin's structural studies [10], determining both kinetics of PS II fluorescence enhancement and of PS I and PS II segregation in the same experimental conditions.

Staehelin [10] showed that the movements of the intramembraneous particles (seen by freeze fracture) are paralleled by changes in the distribution of chlorophyll molecules between stacked and unstacked membrane regions (isolated after French-press disruption of chloroplasts). We also obtain the same duration for segregation of PS I and PS II particles utilizing electron microscope observations and digitonin fractionation.

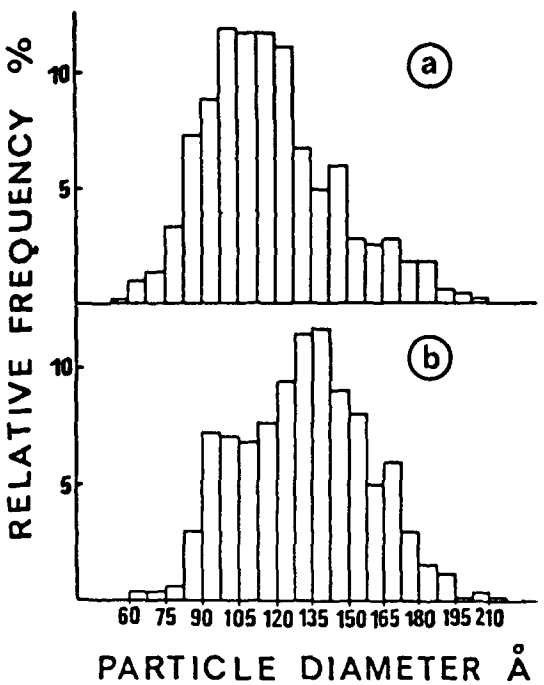

Fig. 8. Particle size histogram from freeze-fractured external faces of cholesterol-treated membranes, in absence (a) or in presence during 20 min (b) of $MgCl_2$. In (a), the majority of the particle diameters is around 120 Å. The distribution is shifted toward the larger sizes (140 Å) upon addition of $MgCl_2$ (b).

In our conditions, it is also observed that both segregation of the two photosystems and PS II fluorescence enhancement are completed within 3 min after cation addition. Thus, as hypothetized by Barber [17] cation-induced spatial separation of the two photosystems into two distinct zones of thylakoid membrane is enough to explain the decrease of PS-II-to-PS-I exciton transfer.

This conclusion is further reinforced by our results with cholesterol-treated thylakoids where cations induce an appression of membranes which is not followed, either by particle segregation or by an increase of PS II fluorescence. It emerges that simple appression, to which the turbidity increase is correlated, does not induce changes in exciton transfer between photosystems. This conclusion is in agreement with the results of Wollman and Diner [8]: they showed, comparing the effects of mono and divalent cations, that the increase of fluorescence is not directly related to the increase of scattering or to the extent of membrane appression.

Several parameters can influence the rate of cations-induced modifications. In particular, membrane fluidity influences the rate of cation-induced changes of fluorescence. Chapman et al. [18] pointed out that the higher the growth temperature of peas, the less fluid is the thylakoid membrane and the slower the $MgCl_2$-induced fluorescence increase. From growth temperature ranges of 7–13°C to 20–23°C the $MgCl_2$-induced enhancement of PS II fluorescence is decreased 4-fold. Variations of diphenyl hexatriene fluorescence polarization show that the fluidity of thylakoids is also very much decreased during aging of the chloroplasts [19]. This must also slow down the rate of PS-II–PS-I segregation. The sum of such differences affecting membrane fluidity may explain why the structural changes reported in this paper are much faster than those previously observed by Staehelin [10].

It is known that variation of the cation concentration can induce various effects on the distri-

bution of light energy. Several authors have suggested that, in addition to decreasing PS-II-to-PS-I exciton transfer, $MgCl_2$ increases the coupling between light harvesting complex (LHC) and PS II [20–22] and also increases the rate constant for exciton trapping by open PS II centers [23]. Olive et al. [24] showed that the $MgCl_2$-induced decrease of the half-times of variable fluorescence in the presence of DCMU and of O_2 evolution activation were, in some cases, accompanied by a shift to larger sizes of the exoplasmic fracture face particles and by a decrease of particle density on the protoplasmic fracture face. $MgCl_2$ produces, on the cholesterol-pretreated chloroplasts, some of these structural and functional changes without producing PS-I–PS-II particle segregation. Then, in cholesterol rigidified membrane, modifications of protein-protein interactions by screening of negative charges of the membrane can still occur, leading to a larger size of exoplasmic fracture face particles and a decrease of the half-time of variable fluorescence.

There is, however, no accompanying changes either in the maximal fluorescence level at room temperature or in the variable to initial fluorescence ratio as would be expected if an increase in LHC-PS II coupling was still occurring in these Mg^{2+}-treated sample. Then an alternative effect of Mg^{2+} such as an increase in the efficiency of charge separation in PS II centers will have to be looked for in the cholesterol treated membranes.

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