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Kinetic relationships between structural changes in the thylakoid membranes and photosystems organization

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Events involved in cation-induced aggregation of thylakoid membranes and grana formation were studied in broken chloroplasts. Membrane stacking was evaluated through changes in light scattering while lateral interactions and movements of pigment-protein complexes were monitored through changes in steady-state, maximum and minimum chlorophyll fluorescence (F_s, F_m and F_o resp.). Changes in the imbalance between the photoactivities of PS I and PS II were derived from these fluorescence parameters and the rates of these changes were compared at room temperature and at 5 ° C. Particularly, a distinction was made between altered protein–protein interactions within the membrane (microstructural changes) and altered membrane–membrane interactions (macrostructural changes). Following addition of $MgCl_2$ (5 mM), the increase in F_s and in the photosystems' imbalance lagged behind the increase in F_m, all of which lagged behind the increase in light scattering. This effect was mostly pronounced at the low temperature. One may conclude that membrane stacking (reflected mainly by the scattering increase) does not require and is not simultaneously coordinated with the lateral movements of pigment complexes (reflected by an increase in the imbalance) and the lateral organization of the photosystems (reflected by an increase in F_m) but is rather a prerequisite for it. Such photosystem organization occurs in later stages of the cation-induced overall process. On the other hand, in the reverse processes, which were followed by depleting Mg^{2+} with EDTA, membrane destacking and grana disintegration lagged behind the decrease in light excitation imbalance in favor of PS II and thus are probably limited by lateral rearrangements of pigment complexes. The decrease in the imbalance was much faster than the decrease in F_m, the last one presumably reflecting an increased exciton interaction between PS I and PS II (i.e., quenching of PS II excitation by energy transfer to PS I – 'spillover' – when the two photosystems are sufficiently close, as the diffusional movement progresses). The difference between the kinetics of F_m and the imbalance when they increase upon cation addition or decrease upon cation depletion implies that the changes in the imbalance of the photosystems and spillover are largely independent processes.

Introduction

Granal structures of thylakoid membranes in suspension are stabilized by a 'high-salt' medium (e.g., 100 mM NaCl or 5 mM $MgCl_2$) [1,2]. Such a state is also characterized by a high level of the variable chlorophyll *a* fluorescence relative to the non-variable fluorescence, resembling the in vivo situation [3–5]. In a 'low-salt' medium (e.g., 10 mM NaCl), the granal structures of the photosynthetic membranes disintegrate and the membranes become completely unstacked with a lower variable chlorophyll *a* fluorescence [2,5]. An additional effect of the 'high-salt' medium which has been found [6] and reconfirmed recently in detail [7], is that it induces an imbalance in light excitation distribution in favor of PS II for light of short wavelengths, (in particular those favorably absorbed by chlorophyll *b*). This effect is most pronounced when an uncoupler is present at neutral pH or below [7].

The strong dependence of the variable fluorescence

Abbreviations: LHC II, light-harvesting chlorophyll *a*/*b*-protein complex of Photosystem II; PS, Photosystem; MeV, methylviologen; GD, gramicidin-d.

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increase on the cation charge has suggested that the cation effect is due to the screening of the negatively charged surfaces of the thylakoid membranes [8]. This results in less electrostatic and hydration repulsive forces and helps in bringing together adjacent membranes to form finally the grana stacks by attractive Van der Waals forces [8,9]. A specific suggestion was made that the adhesion of thylakoid membranes is facilitated by a coupled simultaneous lateral separation between PS II and PS I complexes, such that finally PS II resides in the grana stackes and PS I in the unstacked membranes [8]. The separation between the photosystems could by itself lead to the appearance of the imbalance of excitation, which is not apparent in the 'low-salt' unstacked membranes, presumably due to the possibility of excitation transfer ('spillover') from PS II to PS I [10,11]. Such energy transfer was invoked in order to explain the low level of the maximum fluorescence of PS II (F_m) in 'low-salt', relative to 'high-salt', while the basal fluorescence (F_0) is only marginally different [3–5]. It is possible, however, that the appearance of the imbalance at the 'high-salt' medium is due to an additional process of pigment redistribution. Also, the question whether membrane–membrane interaction (stacking and grana formation) is a prerequisite for the lateral separation between PS II and PS I or whether membrane stacking requires a pre-segregation of the photosystems is still unresolved. In artificial model systems other than thylakoids (e.g., phospholipid vesicles), membrane stacking also occurs as a result of negative surface charge screening by cations [12]. In this case, there is no specificity in different regions in the aggregating membranes, provided the negative charges are randomly laterally distributed. On the other hand, in thylakoid stacking there are indications for a more specific role of mediators like the light-harvesting complex of PS II (LHC II). Indeed, it has been shown in a model phosphatidylcholine vesicle system that surface residues of reconstituted LHC II are required in order to obtain cation-induced aggregation and stacking of the vesicles [13,14].

Light- and cation-induced changes in light scattering from energized chloroplasts have been shown to be correlated with ultrastructural changes (15,16]. Addition of cations to broken chloroplasts, initially suspended in a 'low-salt' medium, resulted in an increase in light scattering which has been attributed to membrane stacking and grana formation [16]. An almost linear relationship between the degree of stacking of grana membranes and the cation-induced light-scattering increase at 540 nm was demonstrated by comparing light-scattering data and electron micrographs at different monovalent and divalent cation concentrations [16,17]. At a given level of light scattering, the same degree of stacking was obtained, regardless of the type of cation used to reach such a level of light scattering [16]. This supports the reliability of light scattering as a monitor of membrane stacking, implying only a marginal contribution of lateral and conformational changes in membranal complexes to cation-induced changes in light scattering. On the other hand, if lateral interactions and conformational changes of membranal proteins affected cation-induced changes in light scattering substantially, one would expect that such lateral interactions between proteins in the membrane and their conformational changes would be proportional and would occur in parallel to membrane stacking [16]. In this context, the cation-induced increase in chlorophyll fluorescence has been claimed to be a monitor of membrane stacking and of a simultaneous lateral segregation of the photosystems [10,11]. However, no direct strict correlation between stacking and the segregation of the photosystems could be found by freeze-fracture electron microscopy [18]. Also, the cation-induced changes in chlorophyll fluorescence and light scattering [16] had different kinetics. In the present study we confirm that indeed the macrostructural changes (e.g., membrane stacking and grana formation) and the microstructural changes (lateral segregation of the photosystems and pigment distribution between them) do not depend on each other. In the forward direction (induced by cation addition), the second lags behind the first. In the reverse direction (induced by cation depletion), grana disintegration is preceded by a lateral reorganization of the pigment complexes between the two photosystems. Another outcome of this study is the demonstration that the cation-induced changes in the photosystems' imbalance do not necessarily depend on 'spillover' changes.

Materials and Methods

Materials. Hepes, gramicidin-d (GD), methylviologen (MeV) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were purchased from Sigma.

Chloroplast preparation. Broken chloroplasts from market lettuce or from greenhouse-grown spinach were prepared and stored in a liquid-nitrogen container as described in Ref. 19. The storage buffer contained 0.3 M sorbitol, 20 mM Hepes, 10 mM NaCl, 5 mM $MgCl_2$ and 30% (v/v) ethylene glycol (pH 7.3). Total chlorophyll concentration was determined according to Ref. 20.

Reaction mixtures. Chloroplasts were diluted in a regular spectrophotometric cuvette to less than 10 μg/ml and kept in the dark for 2 min prior to the experiment. The standard reaction buffer (2 ml) contained 20 mM Hepes (pH 7.3), 10 mM NaCl, 1 μM GD and 200 μM MeV as an electron acceptor. $MgCl_2$ or EDTA were added by a fast mixing of 80 μl of concentrated solution to a final concentration of 5 mM. At such EDTA concentration, the chelation of Mg^{2+} was very effective and the resulting rates of change in fluorescence and light scattering were saturated. Some experiments were performed in the light in presence of 10 μM DCMU, which effectively causes the closure of PS II reaction centers, thus yielding the fluorescence to reach its maximum level, F_m. Measurements were done at 24 °C and 5 °C. Temperature was controlled by water flow from a thermostatted cooler.

Chlorophyll a fluorescence measurements. Measurements of modulated chlorophyll *a* fluorescence emission at 683 nm and the experimental definitions of the fluorescence parameters, F_o, F_s and F_m, were as previously described [7]. The photoactivity distribution coefficient to PS II, β, was calculated as described before [7]:

$$\beta = (F_m - F_o)/(2F_m - F_s - F_o)$$

This relation is derived by assuming that $\alpha + \beta = 1$ where α is the activity distribution coefficient of PS I. Another parameter, the imbalance term, defined conveniently as $(\beta/\alpha) - 1$, was calculated directly from the fluorescence parameters without any need for such an assumption [7,21]:

$$(\beta/\alpha) - 1 = (F_m - F_o)/(F_m - F_s) - 1.$$

It should be noted that the above relations are correct to the extent that PS II has more or equal photoactivity than PS I. In such a case, the imbalance term may vary between zero (when the photosystems are balanced) to infinity (when there is a complete imbalance in favor of PS II).

Light-scattering measurements. Light scattering was measured at an angle of 90 ° using the same set-up.

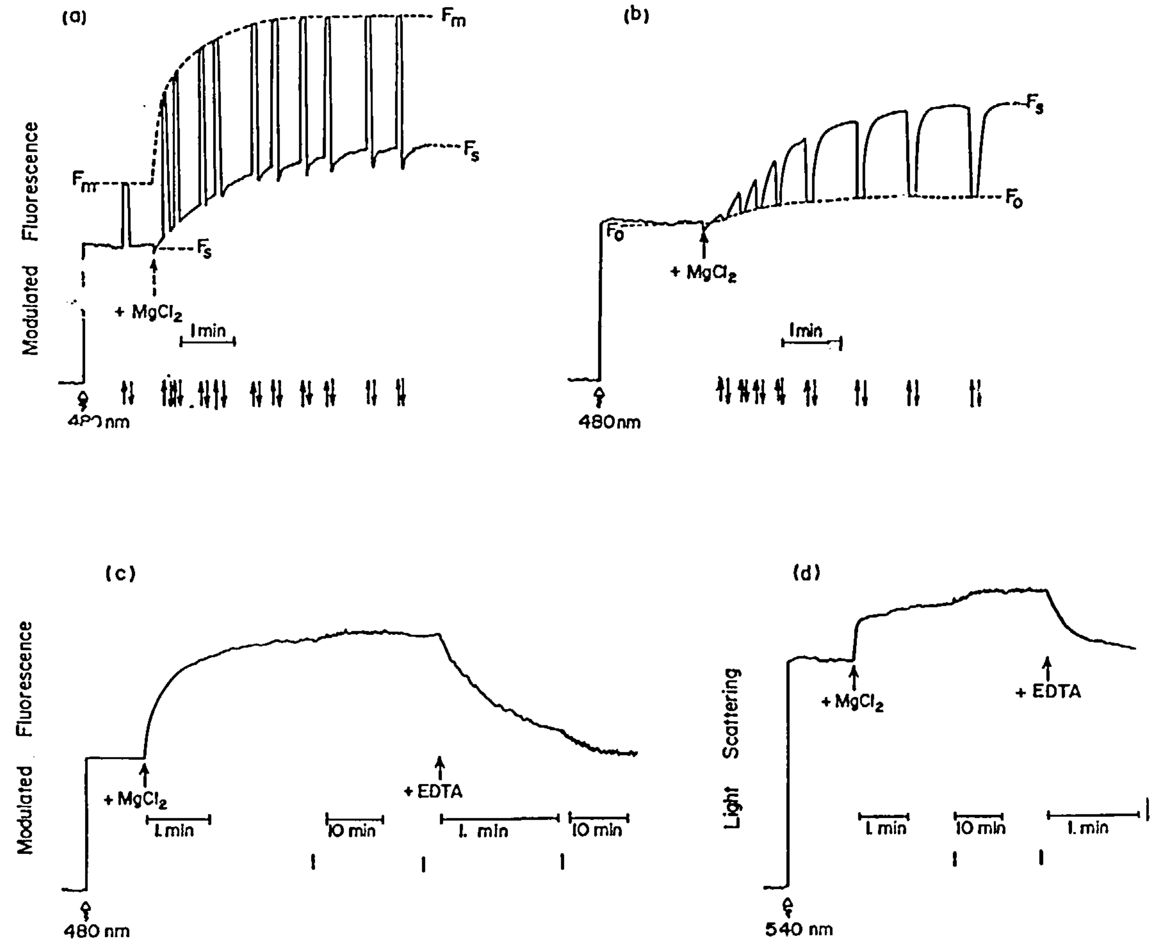

Fig. 1. The effect of $MgCl_2$ addition and depletion on fluorescence parameters and light scattering. Steady-state fluorescence, F_s, was monitored continuously by a weak modulated 480 nm excitation (1 nE cm^{-2} s^{-1}) while the fluorescence parameters F_m (a) and F_o (b) were determined intervally by superimposing saturating blue or far-red light (120 and 30 nE, cm^{-2} s^{-1}, respectively). A continuous monitor of F_m with the modulated light alone was obtained in the presence of 10 μM DCMU (c). Light scattering (d) was monitored in the presence or absence of 10 μM DCMU. These measurements were done at 24 °C. The reaction mixture contained the chloroplasts suspended in the standard reaction buffer. $MgCl_2$ and EDTA were added to final concentrations of 5 mM. The upward and downward arrows in (a) and (b) denote turning the saturating lights on and off, respectively. The upward wavy arrow denotes turning on the modulated light. Points of change in the time scale in (c) and (b) are marked by short vertical lines below the scales.

Modulated incident 540 nm beam was obtained using a Schott interference filter (about 0.5 nE cm^{-2} s^{-1}, an intensity which hardly introduced any actinic effects). Scattered light was collected close to the reaction cuvette by a light guide positioned 90 ° relative to the incident beam. The modulated scattering signal was obtained by the photodiode which was protected by a 540 nm interference filter (Balzers), amplified and processed by the lock-in amplifier. Actinic illumination was obtained by a non-modulated light source with a 480 nm interference filter (Baird atomic). The intensity of the actinic light in the scattering measurements was set to approximately equal the modulated 480 nm light of the fluorescence measurements, neglecting the negligible actinic contribution of the weaker modulated 540 nm light.

Results

In order to examine the kinetics of the cation-induced increase in chlorophyll fluorescence, chloroplasts were first suspended in a 'low-salt' medium in the presence of the uncoupler GD with or without DCMU. The reason for the addition of GD was to prevent energization effects which affect the fluorescence parameters and the imbalance [7] and also to avoid energization-induced effects on light scattering [9]. Addition of 5 mM $MgCl_2$ (i.e., enough to stack the grana membranes and cause an increase in the photosystem imbalance [7]), resulted in a considerable increase in both F_s and of F_m. Fig. 1a illustrates an experiment in which the Mg^{2+}-induced increase with time of F_s and F_m was monitored. In this experiment, the steady-state fluorescence was followed continuously while F_m was determined in intervals, by briefly illuminating the chloroplasts with the saturating blue light. In a parallel similar experiment (Fig. 1b), changes in F_o were also monitored in intervals using brief periodic illumination with saturating far-red light. It was also possible to monitor F_m continuously by including DCMU in the reaction mixture and illuminating the chloroplasts with the modulated light alone. Similar kinetics in F_m increase were obtained as in the previous experiment. For experimental convenience and for consistency, we further included DCMU in all samples where F_m alone was monitored.

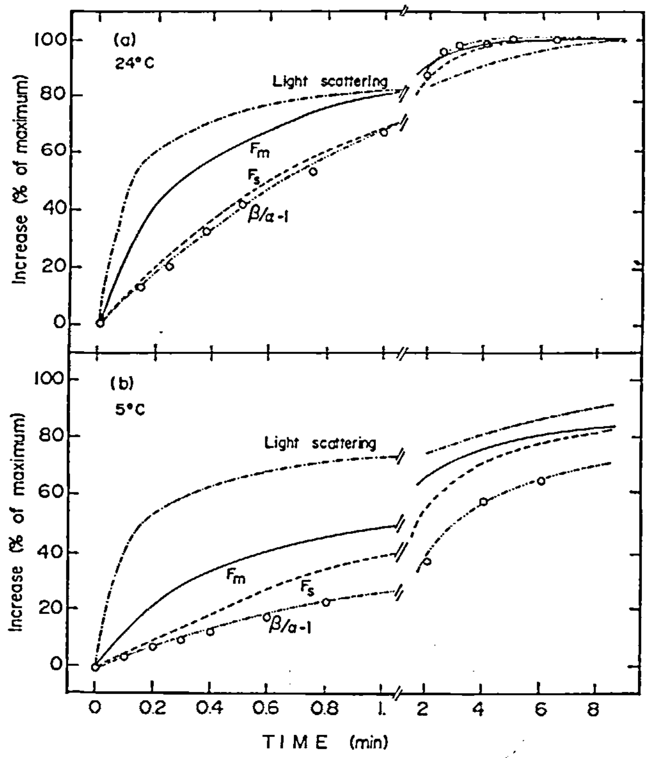

Fig. 2. A comparison between the kinetics of the $MgCl_2$-induced increase in F_m, F_s, the imbalance term and in light scattering at two temperatures. Fluorescence parameters, F_s (dashed curve), F_m (continuous curve), the calculated imbalance term $(\beta/\alpha)-1$, (open circles, dotted curve) and light scattering (dotted curve) were monitored continuously at 24 °C (a) and at 5 °C (b). The reaction mixtures contained the chloroplasts suspended in the standard reaction buffer. For the experiments where F_m and light scattering were monitored, 10 μM DCMU was included. (It was checked separately that changes in light scattering were not affected by the presence of DCMU.) $MgCl_2$ was added to a final concentration of 5 mM. The kinetics were each normalized to the maximum increase obtained in each case.

Parallel continuous changes in F_m and in light scattering due to cation addition and removal are presented in Fig. 1c and d. Addition of Mg^{2+} caused an increase in both F_m and light scattering. Chelation of Mg^{2+} by EDTA in a following experiment resulted in the reverse process where F_m and light scattering decreased to their former values. Raw data similar to those in Fig. 1 were used to compare the kinetics of the changes in the fluorescence parameters and the light scattering, normalizing the full extent to 100%. Such a comparison is presented both for the increase (Fig. 2) and the decrease (Fig. 3) in these parameters. The kinetics are complex and are probably made up of at least two different phases. Nevertheless, the comparison of the overall kinetics is quite illustrative. The cation-induced light scattering increases at a considerably higher rate than F_s but only slightly faster than F_m (Fig. 2a). The kinetics of the increase in F_s and in the derived imbalance term $(\beta/\alpha)-1$, are quite similar, both reflecting the change in the imbalance of excitation, while the kinetics of F_m are faster. Since the fluorescence changes are believed to represent lateral diffusion-controlled processes, which depend largely on the membrane fluidity, lowering the temperature is expected to result in a decrease in their rate [22]. Indeed, a change of the temperature from 24 °C to 5 °C caused a dramatic decrease in the rate of the cation-induced increase in F_s, F_m and the imbalance, with the rate of increase in the imbalance becoming slower than the change in F_s. The scattering change,

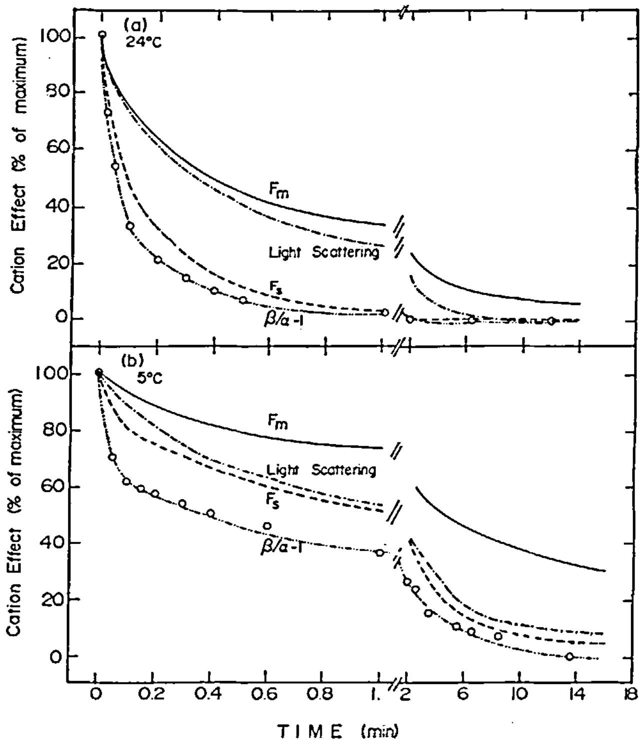

Fig. 3. A comparison between the kinetics of the EDTA-induced decrease in F_m, F_s, the imbalance term and in light scattering at two temperatures. Fluorescence parameters, F_s (dashed curve), F_m (continuous curve), the imbalance term, $(\beta/\alpha)-1$, (open circles, dotted curve) and light scattering (dotted curve) were monitored continuously at 24 °C (a) and at 5 °C (b). The reaction mixtures contained the chloroplasts suspended in the standard reaction buffer. The procedure described in Figs. 1c and d was followed: 5 mM $MgCl_2$ was added to each sample and when a steady state was reached, 5 mM EDTA was added and the signal was monitored continuously. In the experiments where F_m and light scattering were monitored, 10 μM DCMU was included. The changes in light scattering, however, were not affected by the presence of DCMU. The kinetics were each normalized to the previous maximum effect of 5 mM $MgCl_2$ addition.

on the other hand, was only little affected by temperature (Fig. 2a and b), with the second phase of increase at a longer time being only slightly slower at the lower temperature.

Reversing the cation-induced stacking by EDTA was expected to occur at an even higher rate than the cation-induced stacking, according to what is known of aggregation and deaggregation interactions between colloidal surfaces [12,23]. Comparing the scattering curve in Fig. 2a to that presented in Fig. 3a, it is evident that this is not the case here. EDTA induced a decrease in light scattering rather at a somewhat slower rate. There is no first fast phase such as that which occurred in the cation-induced increase response. At room temperature, the EDTA-induced decrease in light scattering and the parallel decrease in F_m were comparable, both quite slower than F_s decrease (Fig. 3a). The imbalance term, however, decreased only slightly faster than F_s. At a lower temperature (Fig. 3b), all processes became slower with the biggest effect on the fluorescence. Nevertheless, the light scattering change, too, became slower and its kinetics approached that of F_s, with F_m still the slowest. At the lower temperature, the imbalance term decreased significantly faster than F_s (Fig. 3b). Comparing Figs. 2 and 3, it is evident that, unlike F_m, F_s and the imbalance term decrease upon cation depletion at room temperature in a much faster rate than their increase upon cation addition. This difference in the rates tends, however, to disappear at a lower temperature.

Discussion

Two processes most probably take place as a result of addition of cation to 'low-salt' chloroplasts. One is represented by a relatively fast increase in light scattering and involves macrostructural changes (i.e., altered membrane–membrane interactions) which lead to membrane stacking and grana formation. The second involves microstructural changes (i.e., altered protein–protein interactions within the membrane), represented by the relatively slower fluorescence increase attributed to the lateral segregation and reorganization of the photosystems and to enhanced imbalance in favor of PS II. Low temperature slowed down the rates of increase in fluorescence and the imbalance, which lagged considerably behind the scattering increase, whose rate remained almost unaffected. The stacking process thus does not require any lateral segregation of the photosystems per se. This is in line with the results on a mutant of *Chlamydomonas reinhardtii* lacking chlorophyll-protein complex I, where addition of cations still induced an increase in light scattering while chlorophyll fluorescence even decreased [16].

Aggregation and stacking of artificial membranes (e.g., phospholipid vesicles) was predicted and expected to depend only slightly on temperature [12,23]. Thus, a lack of a strong temperature effect on the cation-induced scattering increase is quite expected. De-aggregation in such model systems occurred much faster than the aggregation process, as also predicted [12]. It depended more strongly on the temperature, slowing down as the temperature decreased [23]. In contrast to model systems, however, the EDTA-induced destacking of thylakoid membranes was appreciably slower than the cation-induced stacking at room temperature and more so at 5 °C. This suggests that the thylakoid destacking does not obey a model deaggregation, probably as a result of an additional limitation which is even more pronounced at a low temperature. A decrease in temperature results in slower lateral diffusions in the membrane [22,24–26], particularly because of gross changes in its fluidity [24–27]. It is possible that a certain degree of a prior lateral

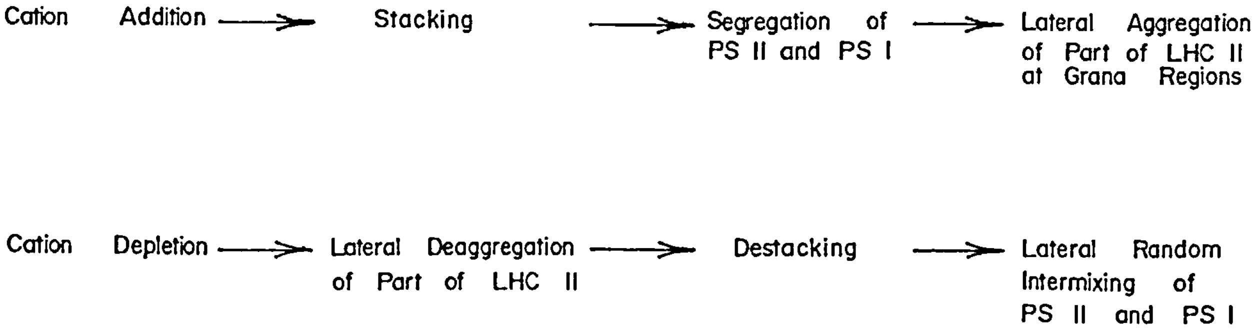

Fig. 4. A suggested order of events following addition of cations to, and their depletion from, thylakoid membranes.

diffusion-controlled rearrangement of the complexes, which otherwise stabilize the granal structure, is required for grana disintegration. Indeed, the fastest process following the depletion of Mg^{2+} is the decrease in the photoactivity imbalance between PS II and PS I, which presumably results from a lateral redistribution of at least a part of the pigment-protein complexes. This part could be related to LHC II, shown already [13,14] to mediate cation-induced stacking in a model system. Another evidence which links changes in the imbalance with redistribution of pigment complexes is obviously the wavelength dependence of the imbalance [7].

In this respect, one should note the difference between the kinetics of the change in F_m, and that of the imbalance. Cation-induced increase in F_m, which is attributed to a decrease in excitation energy transfer from PS II to PS I (spillover) [3–5,8,28], occurred faster than the parallel increase in the imbalance of the photosystems. The order of rates is turned around in the reverse process. Thus, spillover changes cannot be solely responsible for the changes in the photosystems' imbalance. These must be additionally accounted by movement of pigment complexes between the two photosystems, most likely accessory pigment complexes such as LHC II.

Following the above, a model for the events connected with stacking and destacking is featured and presented schematically in Fig. 4. It suggests that, following cation-induced stacking, there is a segregation of PS II and PS I, followed later by a lateral aggregation of LHC II units at the grana, stabilizing the grana regions at a tightly stacked conformation. The destacking occurs at a slower rate, requiring that the lateral deaggregation of LHC II occurs first. A recent indirect analysis [29] in a model system, consisting of peptides and phospholipid vesicles, has indicated that the peptides, which interact with the vesicles, undergo lateral deaggregation faster than aggregation. Here too, the faster rate of the imbalance decrease compared to its increase may thus reflect a faster random lateral intermixing of membrane complexes compared to their cation-induced segregation or lateral aggregation, in line with the above interpretation.

A closer inspection of Fig. 2 reveals that at least two kinetic phases exist in the cation-induced increase of light scattering, as also was shown previously [16]. The first fast phase most probably reflects indeed the fast gross changes as a result of an immediate stacking of adjacent membranes and is completely unaffected by temperature. The following slow increase is slightly slowed at low temperature. This phase could represent fine structural rearrangements which might involve some partial lateral movements of membrane complexes. The existence of two phases indicates that at least two stages are involved in the cation-induced structural change. The first is presumably an adhesion interaction between the thylakoid membranes which is not specific to a certain kind of surface and follows a mechanism of aggregation by surface negative charge screening. The second is a structural organization, in which the thylakoid membranes reorganize in a favorable conformation where the membranes form two distinct types of domain, the tightly stacked grana and the unstacked stroma lamellae domains. The above mechanism may serve as a general model for membrane–membrane interactions in biological systems.

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