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THE RELATIONSHIP BETWEEN THYLAKOID STACKING AND SALT INDUCED CHLOROPHYLL FLUORESCENCE CHANGES

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Summary

Salt induced chlorophyll fluorescence increase and thylakoid stacking have been measured under various conditions.

1. Aging of pea chloroplasts led to a loss of salt induced chlorophyll fluorescence increase and thylakoid stacking which is suggested to be due to a decrease in membrane fluidity as measured by 1,6-diphenylhextriene fluorescence polarization.

2. The aging treatment was accompanied by a decreased in surface charge density as indicated by chloroplast electrophoretic mobility measurements.

3. Lowering of the temperature to about 0°C retarded the time courses of salt induced stacking and chlorophyll fluorescence increase.

4. Like aging, addition of linolenic acid led to an inhibition of the salt induced fluorescence and stacking phenomena but in this case there was a concomitant increase in electrophoretic mobility without any detectable change in the polarization of 1,6-diphenylhextriene fluorescence.

5. Maximum stacking occurred in both aged and fresh chloroplasts in a low salt medium at about pH 4.3 and the time course for the pH induced process was rapid and relatively temperature insensitive when compared with salt induced stacking.

6. The chlorophyll *a*/chlorophyll *b* ratio was lower for salt induced 'grana' than for pH induced 'grana'.

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Abbreviations: Chl, chlorophyll; DCMU, 3(3',4'-dichlorophenyl)-1,1-dimethyl urea; DPH, 1,6-diphenyl-1,3,5-hexatriene; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid; Mes, 2-(*N*-morpholino)-ethanesulphonic acid; PS, Photosystem.

7. The results are discussed in terms of the hypothesis that changes in the lateral interaction of membrane pigment-protein complexes underlie the salt induced chlorophyll fluorescence increase and thylakoid stacking. It is argued that electrostatic screening by cations leads to the formation of domains of low-charge, fluorescent pigment-protein complexes, seggregated from domains of high-charge, quenching complexes, resulting in a increase in chlorophyll fluorescence yield and stacking at low-charge regions on adjacent membranes. In contrast to this, it is argued that the pH induced stacking occurs because of electrostatic neutralization, a mechanism which would not be expected to induce domain formation and associated chlorophyll fluorescence changes.

Introduction

In other publications [1,2] it has been suggested that the close correlation between salt induced thylakoid stacking and chlorophyll fluorescence yield changes [3—5] can be explained in terms of alterations in the balance of forces acting between different types of chlorophyll-protein complexes sited in the membrane. In short, it was argued that under low salt conditions when electrostatic screening of surface charges is poor and coulombic respulsion is at a maximum, the various pigment-protein complexes are homogeneously distributed in the membrane. Thus energy transfer between them is high such that chlorophyll fluorescence is at a minimum (assuming the existence of complexes having high and low fluorescence yields) and no thylakoid stacking occurs [2,6]. However, it was postulated that the addition of screening cations might give rise to the lateral diffusion of the various protein complexes in such a way as to create domains having either low or high net surface charge densities. Membrane stacking would now occur via attractive interactions (van der Waals forces) between the less charged or electroneutral regions on the adjacent membranes while the highly charged regions would not allow close membrane-membrane appression to occur [2,6].

The above ideas have been discussed in more detail elsewhere [1,2] and although at this stage are speculative, they do form the framework for new experiments and already have some support from freeze-fracture studies [3,7—9]. In this paper the model is explored experimentally by comparing the differences between electrostatic screening and neutralization of surfaces charges in controlling thylakoid stacking and chlorophyll fluorescence changes.

Materials and Methods

Chloroplasts were isolated from pea leaves using the procedure reported in Ref. 10, with the modification that the chloroplasts were subjected to a 10 s osmotic shock in distilled water followed by the addition of an equal volume of double-strength medium to give 0.1 M sorbitol, 1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes) and 1 mM KOH (pH 7.5, HCl). The chloroplasts were collected by centrifugation and resuspended in a basic medium of the same composition as above.

For experiments on the effects of aging, chloroplasts were diluted into the

basic medium described above and the resulting suspension was kept in the dark at room temperature. Chlorophyll concentration was 0.1 mg/ml. After various times of aging, aliquots were taken from the stock with or without further dilution depending on the type of measurement (see below).

Chlorophyll fluorescence measurements were made using chloroplast suspension consisting of 10 mM KCl and 10 μM DCMU added to the basic medium and containing 10 μg chlorophyll/ml. Chlorophyll fluorescence was excited with a beam transmitted by a filter combination consisting of Schott BG 18 (2 mm) and BG 38 (2 mM), giving an incident intensity of 30 W/m^2. The fluorescence emission was detected by an EMI 9558B S20 photomultiplier shielded by a Blazer B-40 693 nm interference filter and a Schott RG 695 cut-off filter.

For measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence polarization, 7.7 μM DPH was included in the incubation mixture during aging. After 40 min or more, an aliquot was diluted 5-fold with a 3 mM Hepes solution (pH 7.6 KOH). DPH fluorescence was excited at 360 nm and measured at 460 nm using a Perlin-Elmer Model MPF 44A fluorescence spectrophotometer. Polarized light for excitation was transmitted by a polarizer followed by a Schott UG1 bandpass filter which helped to minimize fluorescence from the polarizer itself. Polarization of DPH fluorescence emission was monitored by transmission through an analyser placed immediately after the sample. The polarization P was calculated according to

$$P = \frac{I_{vv} - I_{vh}(I_{hv}/I_{hh})}{I_{vv} + I_{vh}(I_{hv}/I_{hh})}$$

where I is the intensity of fluorescence measured with the polarizer (first subscript) and analyser (second subscript) in all four vertical (v) and horizontal (h) filter combinations [11,12]. Samples which lacked DPH gave negligibly small values of I. The temperature was maintained at 25°C.

The degree of thylakoid stacking was monitored qualitatively by the effectiveness of digitonin to disrupt the membrane system (cf. Refs. 13–15). The reliability and short comings of this approach compared with electron microscopy have been discussed elsewhere [15]. KCl (10 mM) was added to each 1 ml aliquot of thylakoid suspension with or without further addition of 120 mM KCl (added to induce stacking). The mixture was allowed to equilibrate for 10 min at room temperature before 0.1 ml of 4% digitonin solution was introduced. Upon incubation with digitonin for 4 min the mixture was diluted 20-fold with ice-cold basic medium (supplemented with KCl such that the final concentration was 10 mM) and then centrifuged at 10 000 × g and 0°C for 30 min. The percentage of chlorophyll appearing in the pellet was determined, and gives a relative measure of stacking/unstacking. When stacking was to be monitored at various pH values, the basic medium was supplemented by 1 mM 2-(N-morpholino)ethansulphonic acid (Mes) and 0.5 mM succinate, while the dilution medium included 10 mM Hepes (pH 7.5, KOH).

To monitor the time course of stacking, fresh samples of chloroplasts were first unstacked for 5 min at 23°C in 1 ml of basic medium supplemented by 10 mM KCl (0.1 mg chlorophyll/ml). The samples were either used at 23°C or 0°C. The chloroplasts were stacked by the addition of KCl (final con-

centration, 130 mM) or HCl in the presence of 0.5 mM succinate (final pH, 4.3). After various time periods of stacking, 0.1 ml of 4% digitonin was added. Treatment with digitonin was for 2 min, followed by 20-fold dilution as described above. The effectiveness of digitonin to disrupt thylakoid membranes was found to depend on temperature. In order to obtain comparable digitonin action at 23°C and 0°C, the cold samples were placed in a water bath at 23°C during the 2 min digitonin treatment, whereas the room-temperature samples were first cooled on ice/water to 11°C in 35 s and then placed in a water bath at 23°C for the remaining period of digitonin treatment. This procedure was found to give comparable values for fully stacked membranes incubated at the two temperatures.

Measurements of electrophoretic mobility were made according to the procedure reported in Ref. 16.

Digitonin and 1,6-diphenylhexatriene were purchased from Fisher Scientific Co. and Aldrich Chemical Co. respectively.

Results

(i) Aging and decrease in membrane fluidity

According to the concepts presented in the introduction and in previous discussion [1,2], the idea that protein complexes can diffuse in the plane of the membrane in response to changes in the balance between van der Waals and coulombic forces [17] requires that the lipid phase is relatively fluid. A reduction in the membrane fluidity would therefore be expected to inhibit both the thylakoid stacking process and the salt induced chlorophyll fluorescence changes. Although not previously studied in any depth, it has been noted for many years that the ability to induce an increase in chlorophyll fluorescence on adding various cations is variable and sensitive to 'aging' of the preparation (Barber, unpublished). We have used this aging process (defined as the loss in the ability of cations to increase chlorophyll fluorescence) to correlate the salt induced emission process with stacking and to test whether fluidity changes have occurred in the membranes.

As shown in Fig. 1 it can be seen that the aging process, as defined above and induced in the way described in Materials and Methods, resulted in not only an inhibition of the salt induced fluorescence increase but in the ability of the membrane to stack as monitored by the digitonin method.

According to our model, the aging process could involve a decrease in membrane fluidity so as to impede the lateral diffusion of chlorophyll-protein complexes even though electrostatic screening is high. Certainly it is well known that, amongst other things, the aging of the thylakoid membrane involves chemical modifications of its lipids [18—21]. To investigate the possibility of a decrease in membrane fluidity during the aging process we have carried out fluorescence polarization studies using the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene (DPH). This compound was introduced by Shinitzky and Barenholz in 1974 as a probe of microviscosity of the lipid matrix of biological membranes [22] and has been used extensively on a wide range of systems [12]. The molecule is rod-shaped with a length of 1.3 nm and exists

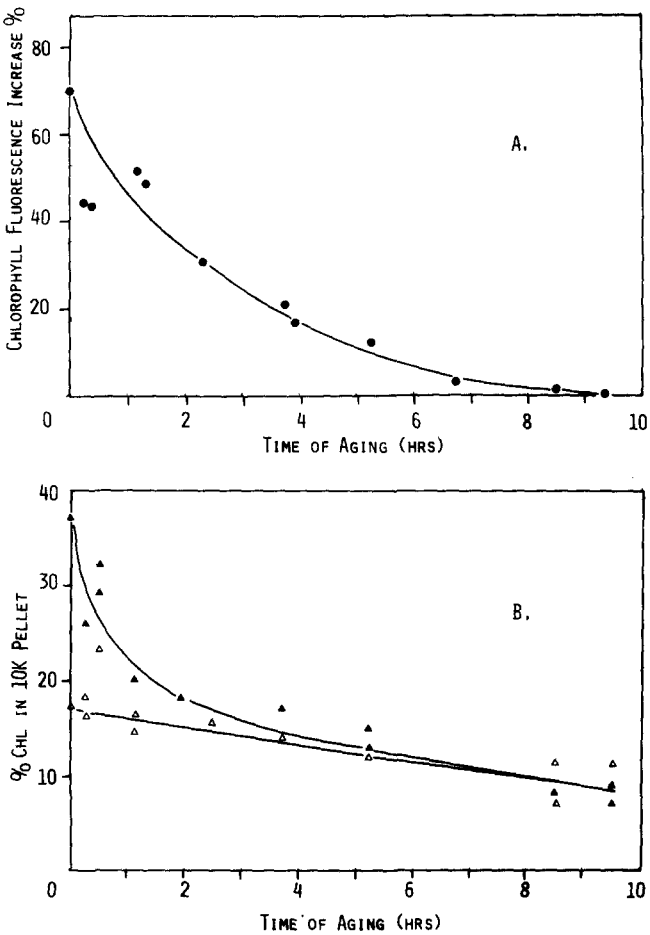

Fig. 1. (A) KCl induced chlorophyll fluorescence increase during aging of chloroplasts. The suspension contained 0.1 M sorbitol, 1 mM KOH, 1 mM Hepes (pH 7.5, HCl), 10 mM KCl and 10 μg chlorophyll/ml. KCl (120 mM) was added to give the fluorescence increase indicated. (B) The capacity for thylakoid stacking during aging of isolated chloroplasts, as monitored by a digitonin method. Chloroplasts were incubated in 0.1 M sorbitol, 1 mM KOH, 1 mM Hepes (pH 7.5, HCl), 10 mM KCl in the presence (▲) or absence (△) of 120 mM KCl, and then treated with digitonin. 10 K pellet, 10 000 × *g* pellet.

in the all-trans configuration and both the absorbance and fluorescence transition moments lie along its major axis.

As can be seen in Fig. 2, the polarization of DPH fluorescence, as calculated using the equation given in the Methods, increased as the thylakoid membranes aged and the cation induced fluorescence change was lost. This result gives support to the idea that there was a significant decrease in the membrane fluidity during the aging process although other interpretations are possible. The range of *P* values found (0.25 to 0.36) are typical of biological membrane systems [23] although highly fluid membranes can give a value of about 0.1 while a highly viscous system has a limiting value of about 0.46 [23].

(ii) The effect of linolenic acid

The aging of chloroplast membranes is also known to be accompanied by

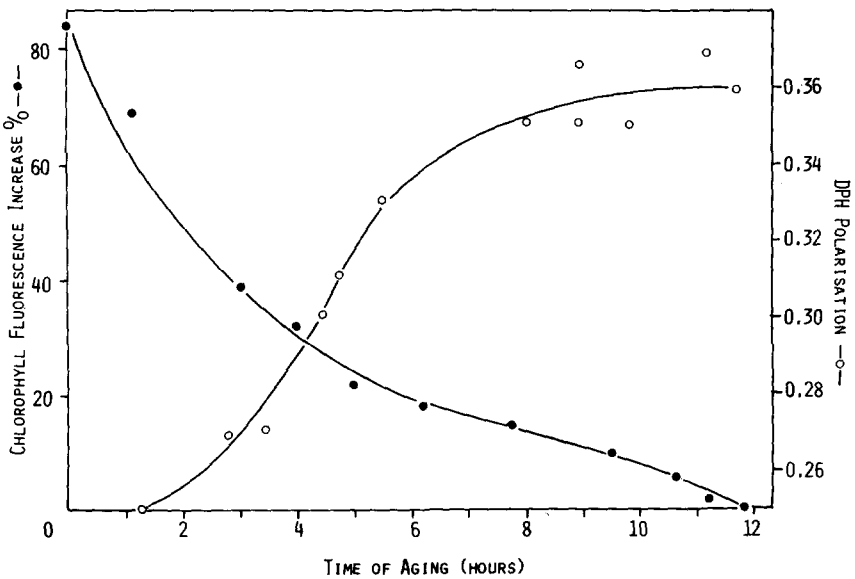

Fig. 2. Variation of $MgCl_2$-induced chlorophyll fluorescence increase and of polarization of DPH fluorescence during chloroplast aging. Chlorophyll fluorescence increase was measured in the same way as for Fig. 1A. For measurement of DPH polarization, see Materials and Methods.

the release of free fatty acids from endogenous lipids [18,20,24–26]. This effect could lead to an overall increase in the surface charge density of thylakoid membranes. If such an effect was to occur, especially in the proximity of the electroneutral components, then the proposed domain formation induced on adding salts would probably not occur. As a result then, the membrane would be expected to lose the ability to show salt induced stacking and associated chlorophyll fluorescence changes. In order to test the possibility that production of free fatty acids is the factor controlling aging of the two associated phenomena (stacking and fluorescence) we have treated fresh chloroplasts with linolenic acid.

Fig. 3 gives the anticipated result that by treating thylakoids with linolenic acid there is a concomitant loss of salt induced stacking and fluorescence. The ability of free fatty acid to inhibit stacking was previously reported by Shaw et al. [27]. However, Fig. 3 also shows that there is no change in membrane fluidity on adding increasing levels of the fatty acid assuming the DPH technique is a true measure of membrane viscosity. Only at a higher concentration of linolenic acid did the polarization decrease significantly. (At 0.48 mM linolenic acid, P was 0.19.)

To check whether the linolenic acid does indeed increase the surface charge density on the membrane, particle electrophoresis studies were undertaken. In Table I it can be clearly seen that linolenic acid treatment could be detected as an enhancement in electrophoretic mobility indicative of an increase in the negativity of the membrane surface. In contrast to this, Table I shows that for aged chloroplasts there is an overall decrease in the surface charge density.

Thus the DPH and electrophoresis studies indicate that the loss in the ability

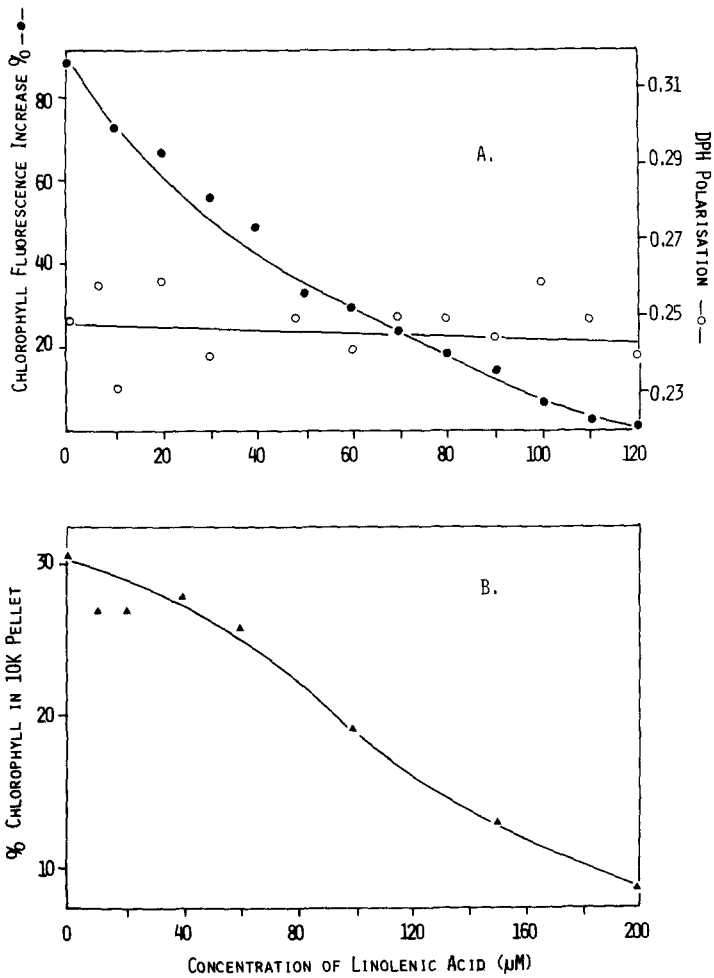

Fig. 3. (A) Effect of linolenic acid on $MgCl_2$-induced chlorophyll fluorescence increase and on DPH fluorescence polarization. (B) Effect of linolenic acid on KCl induced thylakoid stacking. The stacking medium contained 130 mM KCl, 0.1 M sorbitol, 1 mM KOH, 1 mM Hepes (pH 7.5, HCl), 10 K pellet, 10 000 × *g* pellet.

TABLE I

ELECTROPHORETIC MOBILITIES OF THYLAKOID MEMBRANES

Electrophoretic mobilities were determined according to the procedure in Ref. 16. The suspension medium consisted of 0.33 M sorbitol, 20 mM KCl and 5 mM Tris brought to pH 7.6 with HCl. The aging and linolenic acid treatments of the membranes were carried out as explained in Materials and Methods.

	$cm^2/V/s$
Control	$-1.40 \cdot 10^{-4}$
Aged (10 h)	$-1.23 \cdot 10^{-4}$
Control	$-1.32 \cdot 10^{-4}$
+Linolenic acid (120 μM)	$-1.51 \cdot 10^{-4}$

of cations to induce stacking and chlorophyll fluorescence changes during aging could be due to the overall decrease in the membrane fluidity and not brought about by changes in the density of electrical charge on the membrane surface.

(iii) Effect of aging on pH induced stacking

In principle neutralization of membrane surface charge should also bring about stacking without the need for lateral protein diffusion as suggested for the salt induced phenomenon. Surface charge neutralization can be done by lowering the pH to about 4.5 [16,28]. For this reason pH induced membrane stacking was studied both on fresh and aged membranes and the results shown in Fig. 4. As can be seen, both types of material had maximum stacking at about pH 4.3 in accordance with the isoelectric point of this membrane [16,28,29]. Unlike the salt induced stacking process, this type of stacking was not inhibited by aging the membranes. The inability of pH values above and below 4.3 to induce stacking in the low salt conditions used would reflect coulombic repulsion between the net negative charged (above pH 4.3) and net positive charge (below pH 4.3) on the membrane surface [16].

(iv) The effect of temperature on stacking induced either by charge screening or by charge neutralization

If the electrostatic screening mechanism for stacking involves the movement

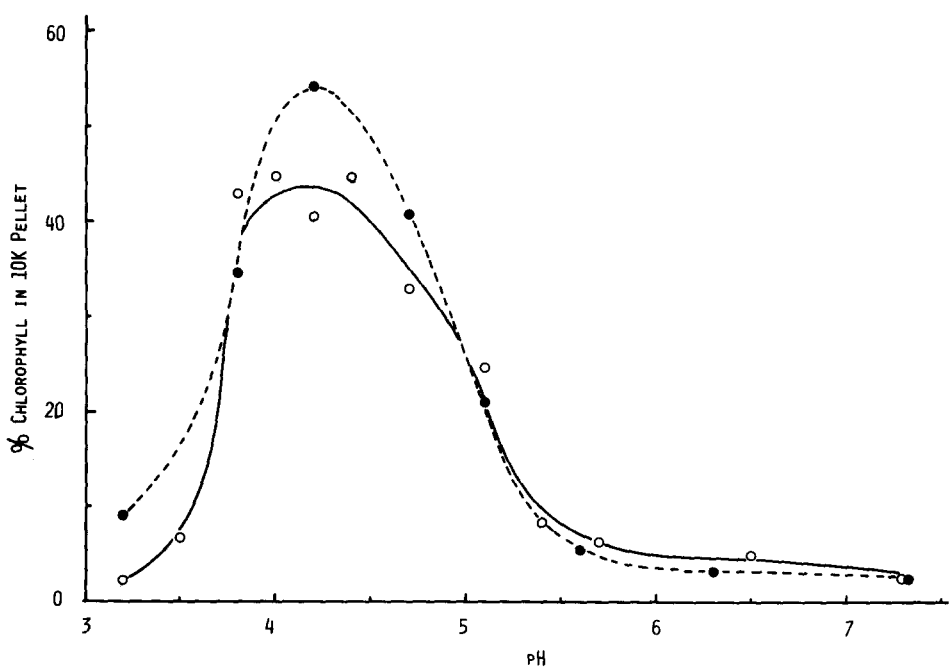

Fig. 4. The degree of stacking of chloroplasts suspended in a low salt medium of varied pH. The medium contained 0.1 M sorbitol, 1 mM Hepes, 1 mM Mes, 0.5 mM succinate, 3 mM KOH and 10 mM KCl, adjusted to required pH with HCl ○, fresh chloroplasts; ●, chloroplasts aged at room temperature for 7 h. 10 K pellet, 10 000 × *g* pellet.

of large protein complexes through the lipid matrix it would be expected that this process would be significantly sensitive to temperature. Indeed, the kinetics of the salt induced chlorophyll fluorescence rise is greatly reduced when the temperature is dropped from around 20°C to a few degrees above zero [1,30]. Barber found a linear response to temperature based on the Arrhenius plot (between 4 to 25°C, pea chloroplasts) and calculated that the activation energy of the salt induced fluorescence changes to about 12 kcal/mol [1]. On the other hand the aging experiment reported above (Fig. 4) gives some support to the idea that the stacking due to protonation (electrostatic neutralization) does not involve extensive diffusion of protein complexes through the lipid phase. Thus stacking due to electrostatic neutralization should be far less temperature sensitive.

Fig. 5 shows that the time courses for the salt and pH induced stacking processes are quite different, especially at low temperatures. Stacking induced by adding KCl was slower and temperature sensitive, taking several minutes to reach the maximum of around 40% stacking. The same chloroplasts also showed a significant temperature sensitivity in the KCl induced fluorescence. In contrast the pH induced stacking was much more rapid taking about one minute to reach the maximum. It was also less temperature sensitive as well as being more effective in terms of total amount of membrane stacked (70% with pH 4.3 and 40% with KCl as monitored by the 10 000 × *g* pellet).

If the stacking due to salt addition and to low pH occurs for the reasons

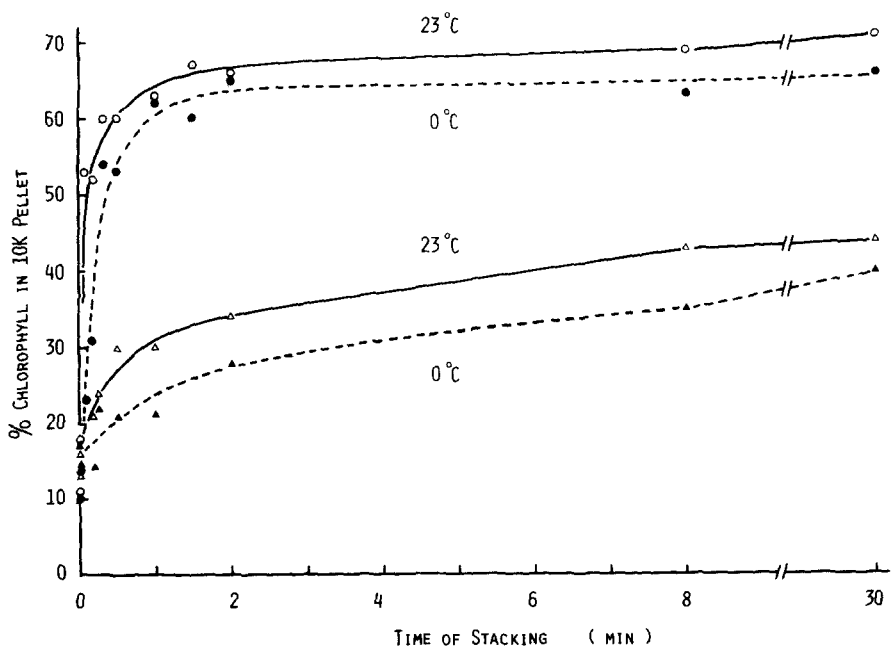

Fig. 5. The time courses of acid- and salt-induced stacking at two temperatures. The method is described in the text. ○ and ●, stacking on lowering pH to 4.3; △ and ▲, stacking on adding KCl (final 130 mM), pH 7.5. Time = 0 corresponds to addition of digitonin to unstacked chloroplasts. The medium for pH-induced stacking was the same as in Fig. 4, and that for KCl-induced stacking as in Fig. 1B. 10 K pellet, 10 000 × *g* pellet.

TABLE II

CHLOROPHYLL COMPOSITION OF 10000 × *g* PELLETS AFTER DIGITONIN TREATMENT OF STACKED CHLOROPLASTS

Chloroplasts were unstacked for 5 min at 23°C before restacking for 30 min in the conditions shown.

Stacking conditions	Chl *a*/Chl *b*
130 mM KCl 0°C	2.18
130 mM KCl 23°C	2.17
pH 4.3 0°C	2.57
pH 4.3 23°C	2.57
Untreated membranes	2.98

indicated above, that is, 'screening' versus 'neutralization', then it may be expected that the Chl *a*/Chl *b* ratios of the two types of 'grana' would be different. Indeed it was found that this ratio was lower for membranes stacked with KCl as compared with the ratio for membranes stacked at pH 4.3 (see Table II).

Discussion

Taken at their face value the results and arguments presented in this paper give some indirect support to the concept that salt induced stacking and chlorophyll fluorescence changes involve the lateral displacement and reorganisation of protein complexes in the lipid matrix of the thylakoid membrane in such a way as to bring about heterogeneity in surface charge distribution. Although evidence for rearrangement of thylakoid protein complexes on changing the salt regime of the suspending medium does exist from freeze-fracture studies [7—9] it would be inappropriate at this time to precisely identify the membrane particles observed by this technique with those postulated to give rise to domain formation. However, it would not be unreasonable to relate the changes in chlorophyll fluorescence with changes in distance between the chlorophyll *a*/chlorophyll *b* complex of PS II and the chlorophyll *a* complex of PS I (CP1, Ref. 31). Since most would agree that the Chl *a*/Chl *b* complex is associated with the appressed regions of the grana [3,32] then it seems likely that this increase in distance is brought about by displacement of some of the PS1 complexes into the stroma lamellae. In agreement with this is the well established fact that the stromal lamellae predominantly have PS I activity [33] but it should also be said that other charged protein complexes (e.g. coupling factor, ribulose-1,5-biphosphate carboxylase) would also be expected to be localized in the stromal region after inducing stacking by adding salts [3,34]. It is clearly wrong to devise a model in which the PS I complexes are entirely restricted to the stromal lamellae since PS I activity is also found in the stacked region. It could be that in the grana, the PSI complexes (because they carry surface charge) are located only on the exposed portions of this membrane system. However, it also seems likely that PS I complexes are located in the appressed portions of the grana [35,36]. If this is the case, according to our model, these complexes must have exposed

surfaces which are near to being electrically neutral if they are to occur in reasonable concentrations in the partition gap, or if they carry net negative charge, then there must be a substantial increase in attractive forces in these regions to overcome coulombic repulsion. Clearly the above ideas are only the starting point for explaining the inter-relationship between chlorophyll fluorescence and membrane stacking. For example, the extent and relative contributions of the various forces acting between adjacent complexes and membrane surfaces needs further clarification making allowances for such effects as lipid fluidity, lipid protein interactions, changes in water structure at surfaces, ionic adsorption, etc. One important consequences of the model is that it distinguishes between electrostatic screening and electrostatic neutralization. The former does not reduce the surface charge density and only weakens coulombic repulsion. It is because of this that the temperature and apparent 'fluidity' sensitive domain formation could occur giving rise to the chlorophyll fluorescence increase, membrane stacking and a decrease in the Chl *a*/Chl *b* ratio for the 'grana'. On the other hand, neutralization of surface charges reduces coulombic repulsion without the need for charge migration. In practice, complete neutralization of a complex electrically charged surface, like the thylakoid membrane, is not possible. Even at its isoelectric point (about 4.3; Refs. 16, 28, 29) positive and negative charge will still exist on the surface. As a consequence of this, localized membrane repulsion may well exist even at pH 4.3, accounting for the inability to observe 100% stacking at this pH (see Fig. 4). In addition to protonation, the surface charge density will be modified if ionic binding occurs, and indeed cations like poly(lysine) [37] and La^{3+} (Chow and Barber, unpublished) do induce thylakoid stacking. However, it would be anticipated that neutralization of surface charges by protonation or ion binding will not favour formation of the type induced by screening and would explain why this mechanism of stacking is not associated with an increase in fluorescence yield [38,39] and why the Chl *a*/Chl *b* ratio changes less for this type of 'grana'. Hopefully further support for these concepts will be obtained by the electron microscopists using the freeze-fracture technique.

Our results indicate that the cation induced increase in chlorophyll fluorescence can be used as a useful intrinsic probe of changes in the fluidity of the thylakoid membrane as already suggested by Murata and Fork [30]. Direct experimental evidence for this comes from the correlation found between this fluorescence phenomenon and the polarization of DPH fluorescence. This relationship needs further investigation since it is likely that DPH distribution in the membrane is not homogeneous and may tend to reflect fluidity changes in localized regions of the lipid matrix [23]. Also since no thorough study of DPH polarization in the thylakoids has been reported it is possible that the polarization values obtained reflect other phenomena unrelated to lipid fluidity. For this reason there is a need to carry out more detailed studied with this fluorescence probe and compare them with other techniques suitable for membrane fluidity measurements, such as EPR.

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