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Hypothesis

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

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1. Introduction

Following on from the initial work of Homann [1], Murata and colleagues established the basic properties of the salt-induced changes in chlorophyll fluorescence from isolated thylakoids and correlated this effect with changes in spillover of energy from photosystem two (PSII) to photosystem one (PSI) at the light-harvesting pigment level [2–4]. Many workers have confirmed Murata's findings and additional data has accumulated in support of the concept that the relative quantal delivery to the two photosystems is controlled by cation induced conformational changes within the thylakoid membrane [5–7]. Along side these fluorescence and electron-transport studies has been the observation that salt also controls the degree of thylakoid stacking [8–12] and from time-to-time these changes in grana formation have also been linked with the PSII to PSI transfer mechanism [5,6,13–17].

In this paper I present the known experimental facts regarding the salt-induced chlorophyll fluorescence and membrane stacking changes and put forward a model to explain their relationship. The model presented will hopefully give a framework for further experimental investigation.

2. Well-established experimental facts

To formulate a realistic model we must ask ourselves what are the basic properties shared by the salt-induced chlorophyll and thylakoid stacking changes:

(i) They are essentially independent of the anion used and cannot be explained in terms of changes in osmotic strength [5,12,18,19].

(ii) When the thylakoids are carefully isolated so as not to subject them to a media containing significant levels of salts and suspended in a cation free medium, the thylakoids remain stacked [11,19–21] and the chlorophyll fluorescence yield is at a maximum in the presence of DCMU [22,23].

(iii) On adding low levels of monovalent cations (1–5 mM) to the condition described in 2 (ii) the membranes unstack [11,12,20,21] and the chlorophyll fluorescence yield in the presence of DCMU drops to a lower value [19,20,22,23]. For this effect a wide range of inorganic and organic monovalent cations can be used [12,19]. The concentration of monovalent cation required to bring about these effects is dependent on the background level of divalent cation [20,24].

(iv) The restacking of the membranes and establishment of the high fluorescence yield can be accomplished by adding cations of various valencies [4,11, 12,21,25] to the medium described in 2 (iii) with the order of effectiveness being $C^{3+} > C^{2+} > C^{+}$. There is little or no specificity between cations in the same valency group as long as no strong binding occurs at the membrane surface [19]. The concentration of C^{3+} and C^{2+} required for this effect is dependent on the background level of monovalent cation [19,20,23].

(v) The relationship between stacking and chlorophyll fluorescence does not occur if added cations strongly bind to the membrane surface. In general neutralization of surface negative charges by cation binding, including protonation, induces membrane stacking but not the concomitant fluorescence rise as described in 2 (iv) [26,27].

3. An explanation for the valency sensitivity and the independence to chemical species within a valency group

As already emphasised in a number of publications from my laboratory the above properties indicate that the cation-induced chlorophyll fluorescence and thylakoid stacking changes must be controlled by a general electrostatic phenomenon rather than some specific chemical mechanism [12,18,28,29]. Attempting to find explanations for the cation-induced effects we, and Duniec et al. [30] have turned our attentions to the established electrostatic theory of Gouy and Chapman [31,32] and in particular the application of this theory to the concepts which underlie the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory of stability of lyophobic colloids [33]. The basis of this theory is that the ability of electrically charged macroscopic surfaces to approach each other is governed by two forces, van der Waals attraction and electrostatic repulsion. It is the electrostatic repulsive force which varies when the salt concentration is changed. In the case of the thylakoid membranes, repulsion is due to fixed negative charges and thus it is the addition of cations which brings about the variations in electrostatic screening. When electrostatic screening is high then charged surfaces are able to approach each other. We have shown that the sensitivity of chlorophyll fluorescence and thylakoid stacking to different cation levels, types and mixtures, can be correlated with changes in electrostatic screening as determined by calculation of the integrated space charge density adjacent to the membrane surface [28,29]. Duniec et al. were also able to predict the experimental findings for mixed electrolyte conditions using an extension of the DLVO approach [30] and we too have equated our calculations with a force parameter [29,34]. Sculley et al. have also presented force calculations applicable to thylakoid stacking [34a].

4. How can changes in electrostatic repulsion between negatively charged surfaces control chlorophyll fluorescence yield and membrane stacking?

In order to attempt to answer the above question and formulate a model the following properties must be considered:

(i) Both the cation-induced fluorescence and thylakoid stacking changes are very sensitive to temperature (E_a ~12 kcal/mol) [18,35] and to 'ageing' or 'chemical' treatment (e.g., trypsin) [27,36–40].

(ii) The cation-induced changes in chlorophyll fluorescence seems to reflect changes in spillover of energy from PSII to PSI at the pigment bed level [4–7,17].

(iii) That cation-induced stacking results in appressed and non-appressed regions (i.e., grana and stroma lamellae, respectively), with appressed distances in the grana of ~40 Å [41–43].

(iv) That stacking is enhanced when the chlorophyll *a*/chlorophyll *b* light-harvesting pigment–protein is present [13,17,38,42–45].

(v) That the stromal lamellae are significantly enriched in PSI while the grana contain both PSII and PSI [17,42,43,46].

(vi) The external thylakoid membrane surface carries a net negative charge at pH > 5, but is electroneutral at pH 4.3 [47–49].

(vii) That the surface electrical charge is associated with membrane proteins rather than with the lipid matrix [26,40,49].

(viii) That the thylakoid structural lipids are electrically neutral (mainly monogalactosyl diglyceride and digalactosyl diglyceride) and also have extensively unsaturated acyl chains (predominantly 18:3) which gives rise, at room temperature, to a fluid membrane [27,50,51].

(ix) That major components of the integral proteins of the thylakoid membrane are the light harvesting pigment–protein complexes [13,43,52,53].

(x) Cations which strongly bind to the membrane so as to neutralize the surface charges, e.g., La^{3+}, polylysine, Zn^{2+}, H^+ (at pH 4.3) do not cause the characteristic fluorescence changes indicative of spillover between PSII and PSI but do induce stacking [19,24–27,54,55].

5. The model

Recognising the validity of the fluid mosaic model as a general description of biological membrane structure [56], it would be naïve to attempt to explain the cation-induced fluorescence and thylakoid-stacking changes in terms of interactions between two fixed negatively-charged homogeneous membrane surfaces [12]. As already indicated in section 4, the thylakoids have a particularly fluid lipid matrix and of course contain a number of different types of integral and intrinsic proteins. It is the exposed surfaces of the protein which carry the majority of the electrical surface charge. There is every reason to predict that the density of charge on the various protein complexes will depend on their functional properties. In this article we will concern ourselves only with chlorophyll–protein complexes but recognise that other protein complexes associated with electron transport and photophosphorylation will exist in the real membrane. For simplicity I wish to define two types of chlorophyll–protein complexes:

1. One which contains the light-harvesting chlorophyll *a*/chlorophyll *b* complex (LHC) and is closely associated with PSII activity. It is established that it is this complex which gives rise to the major portion of room temperature chlorophyll fluorescence [57] and is known to be required in order to observe large cation-induced changes in chlorophyll fluorescence and stacking [13,17,43]. This com-

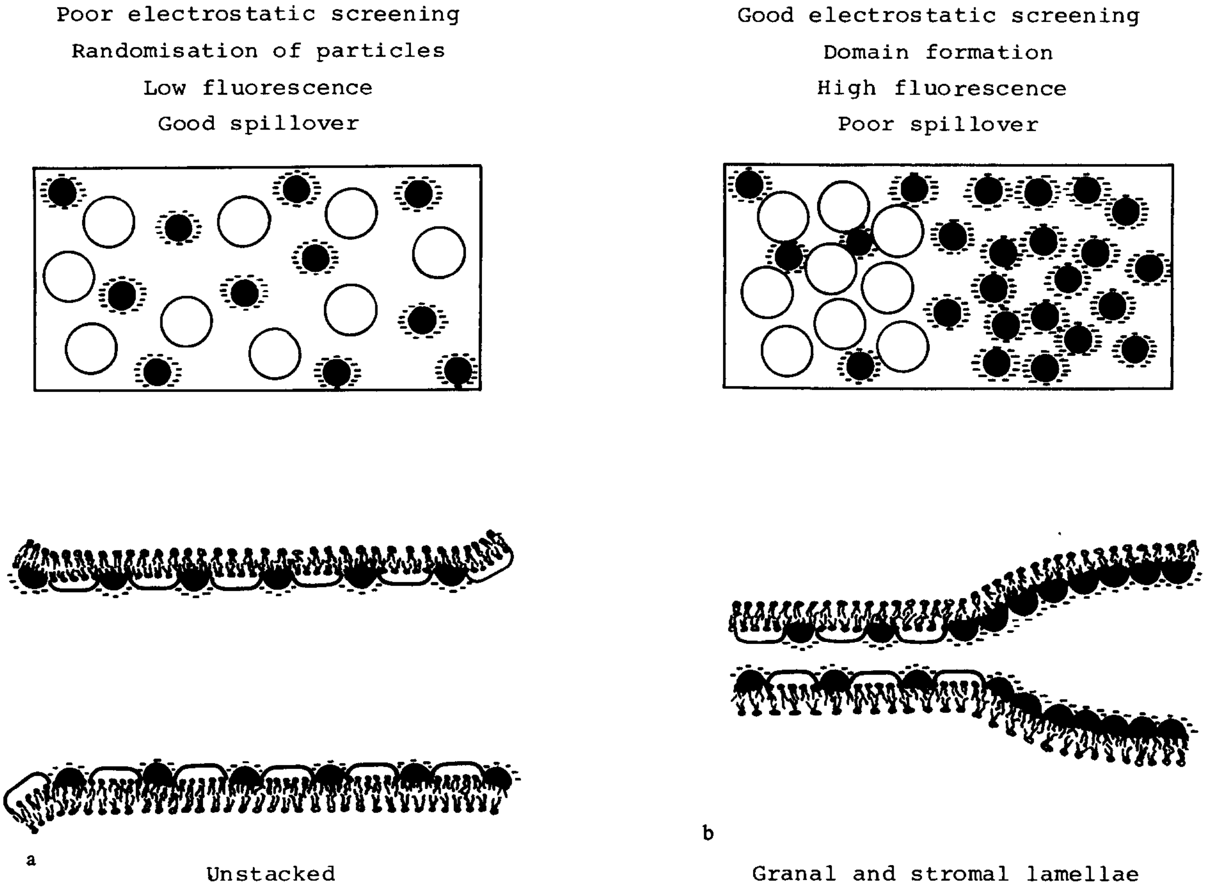

Fig.1. A simplified model of lateral protein diffusion and membrane stacking induced by changes in electrostatic screening. The white particles represent the exposed segments of a pigment protein complex which can be identified with the light harvesting chlorophyll *a*/*b*–PSII complex, while the black particles are the surfaces of the PSI–chlorophyll *a* light-harvesting protein complex. The white particles carry little or no negative charge on their surface (i.e., are electroneutral) and have a high chlorophyll fluorescence yield while the dark particles are negatively charged on their exposed surfaces and have a low chlorophyll fluorescence yield (i.e., are fluorescence quenchers).

plex is shown in fig.1 as white circles and in the model it is assumed that the segment of this protein exposed at the membrane surface carries no, or a low level of, net electrical charge. That is not to say that it is not charged, but simply that its exposed surface is close to electroneutrality. For convenience it can be given dimensions in the region of 150 Å and identified with the large EF freeze-fracture particle thought to contain the LHC and a PSII chlorophyll a light-harvesting core as advocated by Staehelin et al. [58] and Arntzen et al. [59].

2. The other complex, shown as black circles in fig.1, can be identified with a light-harvesting chlorophyll–protein complex associated with PSI activity. This complex contains only chlorophyll a, has a low fluorescence yield and is postulated to carry a net negative charge on its exposed surface. It is reasonable to assume that it has a diameter of $\sim$75 Å and can be identified with some of the smaller PF freeze-fracture particles [17,58,59].

When electrostatic screening is low, coulombic repulsion between the PSI complexes within the membrane plane and also between adjacent membrane surfaces, would be at a maximum. Thus, as indicated in fig.1a, the PSI complexes would be widely dispersed in the membrane and adjacent membranes would not stack. It is suggested that the dispersion of the PSI particles leads to a randomization such that there is a possibility for good energy transfer from the PSII–chlorophyll b containing complex to the PSI complex. This would explain the low level of the chlorophyll fluorescence yield observed under these conditions and the existence of significant exciton spillover from PSII to PSI. Manipulation of the cation levels in the bathing medium to induce good electrostatic

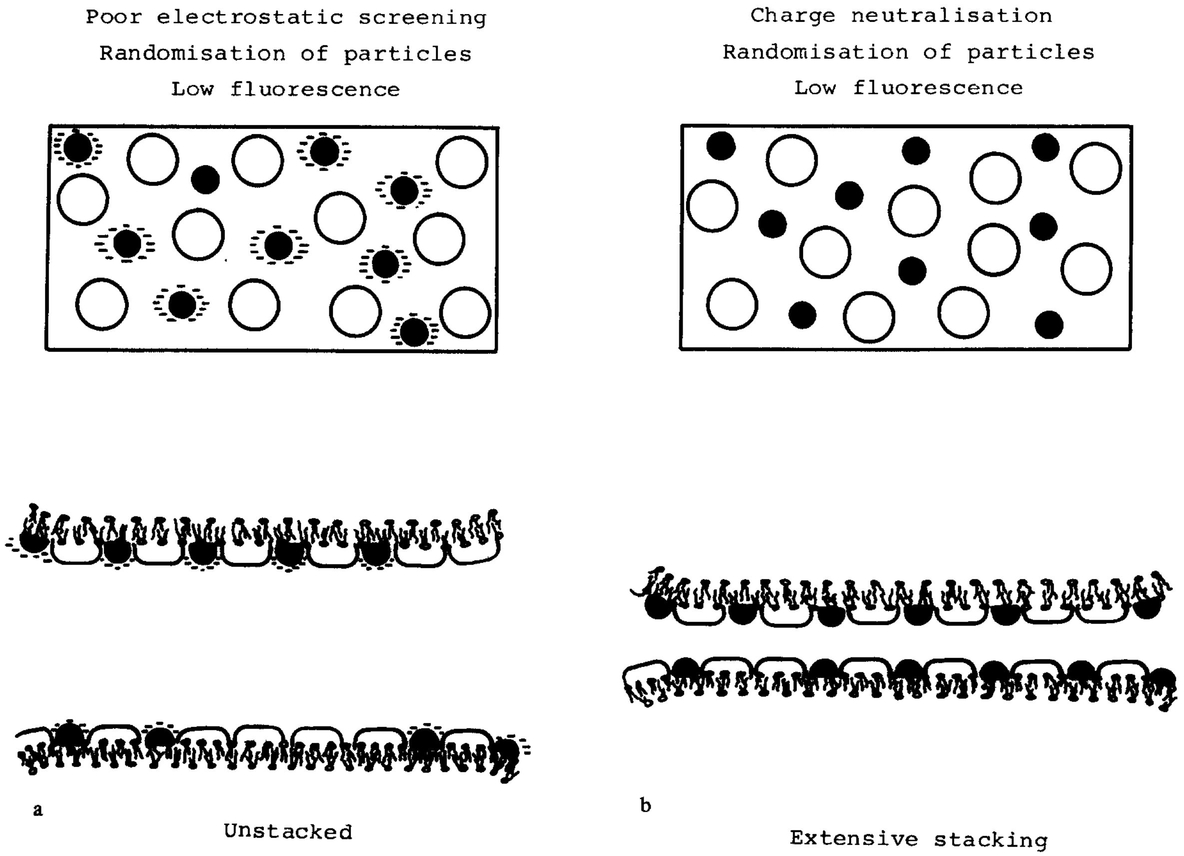

Fig.2. A simplified model of membrane stacking due to neutralization of surface charges on the pigment–protein complex of PSI. Description of particles the same as fig.1, except in this case the stacking/unstacking process is not associated with changes in chlorophyll fluorescence since no significant lateral protein diffusion occurs.

screening will allow reorganisation of the system to occur. It is proposed that the increase in electrostatic screening will allow the two membrane surfaces to come closer together and also encourage aggregation of complexes within the membrane plane itself. Thus at short distances between the two membrane surfaces each membrane will respond to the presence of the other by redistribution of its membrane components so as to relieve the energetically unfavourable repulsive forces in accordance with Le Chatelier's principle [60]. As a consequence, some of the charged components would be expected to migrate away from the area where appression is occurring while uncharged or hydrophobic proteins would preferentially be located in the stacked regions [61]. This is shown in fig.1b, where after stacking has occurred, the PSII–chlorophyll *b* complexes are located predominantly in the partition gap of the grana and a portion of PSI complexes are partitioned into the stromal lamellae. Since the final distribution of these two components is governed by what is energetically favourable, it would not be unreasonable to have some PSI complexes trapped in the appressed regions in the way indicated in fig.1b, consistent with experimental observations [62,63]. However, overall there will be a decrease in energy transfer from PSII to PSI and consequently an increase in chlorophyll fluorescence.

The model in fig.1 should be contrasted with that in fig.2. All the same conditions apply for fig.2, except in this case the surface charge on the PSI complexes has been neutralized by either cation binding or by protonation due to lowering the pH. As shown, electrostatic neutralization will also allow membrane stacking to occur because of the reduction of coulombic repulsion but now there is no significant domain formation and thus no change in chlorophyll fluorescence associated with a decrease in energy transfer from PSII to PSI.

6. Predictions of the model

6.1. *Control of membrane stacking and chlorophyll fluorescence yield by electrostatic screening*

Accepting the model in fig.1, then the cation-induced chlorophyll fluorescence and thylakoid membrane stacking changes should show the following properties:

(i) Be sensitive to the 'fluidity' of the lipid matrix. In support of this it has been shown that decreasing fluidity of the thylakoid membrane (monitored by fluorescence depolarization changes of the lipophylic probe, diphenyl hexatriene), by ageing or lowering the temperature of isolated membranes and by treatment with cholesterol, inhibits the two cation-induced processes [27,51,64].

(ii) Inhibited if significant levels of net electrical charge occurs in the lipid matrix so that areas of close membrane appression are not possible. Introduction of negative charge into the lipid matrix by treating thylakoids with linolenic acid inhibits both the cation-induced fluorescence and stacking effects [27,65] in accordance with this prediction.

(iii) Affected or inhibited by disturbing the distribution of electrical charge on the chlorophyll *a*/*b*–PSII LHC complex. Treatment with trypsin and pronase severely inhibits the cation-induced fluorescence and stacking changes by a mechanism which involves the removal of a portion of a polypeptide from the exposed surface of the LHC [38,39]. The treatment required to remove this component leads to a net increase in the surface charge density of the membrane [40]. It is predicted by the model that the exposure of net negative charge on the LHC will not allow the normal close appression of membranes to occur (i.e., within 40 Å) and no domain formation of the type indicated in fig.1b will be possible. As a consequence both grana formation and chlorophyll fluorescence changes will be inhibited in line with the experimental findings [40,54].

(iv) As predicted from the model in fig.2, domain formation leading to changes in energy transfer between PSII to PSI will be inhibited if the electrical charges on the PSI complexes are neutralized. On the other hand coulombic repulsion between adjacent membranes will be minimised so that stacking can occur. Such effects are observed on addition of polylysine and other binding cations [24,26,27,40,54].

(v) There should be a decrease in the chlorophyll *a*/*b* ratio of the grana induced by cation addition as compared with unstacked membranes or stromal lamellae. This distribution in chlorophyll *a* and *b* is well estabished [27,42].

(vi) The grana will contain PSII and PSI but the stroma lamellae will predominantly contain PSI. Again this distribution in photosynthetic activity is well established [42].

(vii) According to the model in fig.1, the partial seggregation of PSII and PSI leads to a decrease in energy transfer from PSII to PSI but possibly an increase in energy transfer between the PSII light-harvesting chlorophyll complexes. There is evidence in support of the closer interaction between PSII units when spillover from PSII to PSI is a minimum [5,6,17].

(viii) The unstacking of salt-induced grana requires the migration of PSI complexes into the appressed regions [14]. In support of this, it has been shown that the unstacking of grana does not readily occur when the stromal lamellae are removed by digitonin treatment [54]. Stacked grana, free of stromal lamellae, can be unstacked however, by introduction of the charged fatty acid, linolenic acid, into the lipid matrix of the membrane [54].

6.2. *Control of membrane stacking by electrostatic neutralization*

In contrast, the electrostatic neutralization model in fig.2 should show the following properties:

(i) Be independent of lipid fluidity and in the case of protonation, maximum stacking should occur at the isoelectric point of the membrane, that is, in the region of pH 4.3. These predictions have been experimentally verified [27].

(ii) The stacked membranes should contrast with normal salt-induced grana membranes in having a high chlorophyll *a*/*b* ratio similar to that of unstacked membranes. There is evidence in support of this [27].

(iii) Since stacking due to charge neutralization does not involve long range lateral diffusion of protein through the lipid matrix then it should be less sensitive to temperature than the normal cation-induced stacking. Such a result has been found when comparing pH and salt-induced stacking [27].

(iv) May be expected to give rise to a more extensive area of membrane/membrane contact than normal cation-induced stacking. This tends to be true but, for example, lowering to pH ~4.3 or by addition of La^{3+} does not lead to 100% stacking [27,66]. This indicates that the surface charge properties are not homogeneous.

(v) The stacking induced by charge neutralization should not be inhibited by treatment with trypsin assuming that the action of the enzyme is to expose net electrical charge on the LHC. Indeed membranes treated with trypsin sufficiently to inhibit cation-induced grana formation, will stack on lowering the suspending medium to pH ~4.3 [54].

6.3. *Migration of chlorophyll protein complexes should be observable in the electron microscope*

Freeze-fracture studies lend support to the general concepts presented in fig.1. The chlorophyll fluorescence yield and membrane stacking changes induced by changing cation levels in the suspending medium is associated with a migration and redistribution of particles in the thylakoid lipid matrix.

The first evidence for this stems back to the work of Wang and Packer [67] and Ojakian and Satir [68]. It was shown that the large particles on the EF freeze fracture face concentrate in the appressed regions of the grana. However, when the grana were unstacked by suspending thylakoids in a low salt medium, the large EF particles became randomly distributed and intermixed with the smaller PF particles. Further investigations of this phenomena have been undertaken by Staehelin and his colleagues [16,58,69]. They were able to show that particle migration was reversible and closely related with salt-induced grana formation. However, they could not closely correlate changes in the chlorophyll fluorescence yield with the long distance lateral diffusion observed in their electron micrographs [16]. This does not seem unreasonable, as explained in section 7.

7. Consequence of the model

The concepts presented in this paper lead to the conclusion that various functional activities which take place in and on the chloroplast thylakoid membrane can be controlled by changes in the interaction between different types of membrane proteins. That is, the thylakoid membrane should be considered as a fluid-mosaic structure as visualized in the general membrane model of Singer and Nicolson [52,56].

I have considered here only two types of chlorophyll–protein complexes, but clearly there are other membrane proteins which may also undergo lateral displacement when salt conditions are varied. A good example of this is the diffusion of the coupling factor component CF$_1$ to the stromal lamellae on induction of grana formation on adding salts [17,70].

The question arises as to the physiological significance of the lateral pigment–protein diffusion which underlies the basic concept of the model presented in fig.1. It has been realized for many years now that the cation-induced chlorophyll fluorescence changes are in someway related to an in vivo control mechanism for maintaining optimal delivery of quanta to PSII and PSI. The existence of this mechanism stems back to the work of Murata [71], Bonaventura and Myers [72] and Duysens and Talens [73]. In intact photosynthetic tissue, the low fluorescence/maximum spillover state is known as state 2 and can be induced by illumination preferentially absorbed by PSII. On the other hand, PSI light drives the system into state 1 which is characterized by minimum spillover and a high yield of chlorophyll fluorescence [5,6]. Thus the physiological role of the state 1–state 2 mechanism is to use changes in the degree of energy transfer between PSII and PSI to give an optimal rate of electron flow for changes in spectral quality of the incident radiation under light-limiting conditions [5].

Although the cation-induced chlorophyll fluorescence changes observed with isolated thylakoids can be correlated with the fluorescence changes associated with state 1–state 2 transitions, the dramatic stacking/unstacking observed with the isolated membranes does not occur in vivo. Thus, how can the model in fig.1 be used to understand the in vivo state 1–state 2 control of spillover? To answer this ques-

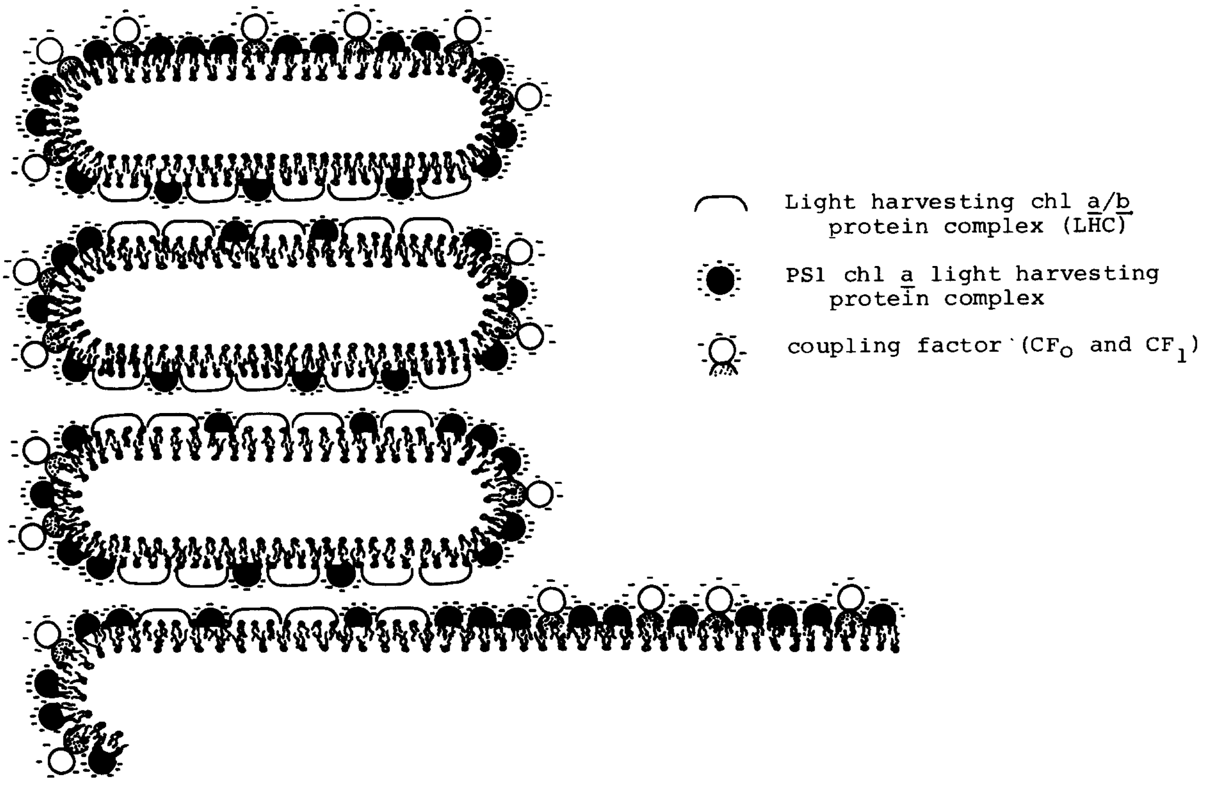

Fig.3. A model of the distribution of chlorophyll–protein complexes in normal chloroplast grana which also emphasises that the coupling factor, like the PSI complex, is mainly located outside the appressed membrane regions. Introduction of negative charge onto the surface of the white, chlorophyll *a/b* complex near the periphery of the partition gap would induce some unstacking and increase energy transfer to the PSI complexes. Such a mechanism could give rise to the state 1–state 2 changes.

tion it is necessary to refer to fig.3 and recognise the fact that changes in energy transfer between the PSII and PSI light-harvesting chlorophylls requires changes in the spacial separation between the complexes of only tens of Ångstroms while the drastic stacking changes involve lateral protein diffusion over much larger distances. Thus the state 1–state 2 changes need only involve spacial changes between the PSII and PSI complexes at the periphery of the grana stacks. Even so, there should be some change in the degree of stacking and in agreement with this, Bennoun and Jupin [74] found that there was a decrease in the thylakoid stacking in *Chlamydomonas* of ~20% on going from state 1 to state 2 (i.e., from minimum to maximum spillover).

With isolated thylakoids, the spillover changes are brought about by changing the ionic conditions of the medium. However, the model in fig.1 also relies on the difference in the surface charge density of the two pigment–protein complexes. It has been argued, that by exposing negative charge on the LHC surface increases mixing of PSII and PSI so that spillover is at a maximum and inhibits stacking (e.g., trypsin treatment). It seems very unlikely that it is changes in the cation levels at the thylakoid surface which controls the in vivo state 1–state 2 changes. This is because there is good evidence that there is sufficient free Mg^{2+} in the intact chloroplast to adequately screen the negative charges on the thylakoid membrane surface so that under dark conditions there is maximum grana formation and minimum spillover indicative of state 2 [49]. Based on the model in fig.1 and 3, the most likely mechanism for the control of the state 1–state 2 transitions is the introduction of electrical charge onto the exposed surface of some of the LHC–PSII complexes close to the periphery of the grana so that for energetic reasons these modified components would tend to disrupt the stacking at grana/stroma lamellae interface and become more intimately mixed with the stromal PSI particles. The observation that light can induce the phosphorylation of LHC [75] and that this is dark reversible and can effect the degree of spillover between PSII and PSI [76] is consistent with this idea. Thus the control mechanism of the state I–state II transition could be intimately involved with photophosphorylation and the balance of kinase and phosphatase activity [77].

Although there are many experiments which support the general ideas presented in this paper there are still more to be done. The identification of the two different forms of complexes in fig.1 with specific types of freeze-fracture particles is an over simplification since there is still controversy in the literature to the origin of various particles observed in the thylakoid membranes [17,38]. Also, in addition to the PSI particles it is necessary for formulating a complete model to include other integral proteins of about the same size such as the hydrophobic component of the coupling factor and the cytochrome f–b_6-containing complex [17]. Because the coupling factor is also mobile and displaced to the grana edges and stromal lamellae when stacking occurs [17,70, 79] it can be assumed that, like the PSI complex, this protein carries net negative charge on its exposed surfaces and thus could play a role in the energetics of grana formation. It should also be mentioned of course that some membrane stacking can occur in the absence of LHC [43,78,80] and thus the assumptions presented above are only valid for normal chlorophyll *a*/*b*-containing thylakoids. Nevertheless the model presented here emphasises the dynamic nature of the thylakoid membrane in terms of its ability to undergo large changes in its organisation. The existence of lateral diffusion of integral protein complexes is well established for many membrane systems [81–83] and elegantly demonstrated by new techniques such as fluorescence photobleaching recovery [83–86]. It must now be recognised that this type of reorganisation of functional proteins of the thylakoid membrane is likely to play an important role in the overall efficiency of photosynthesis. Because of this, more attention should be placed not only on the functional properties of the membrane protein complexes but also on the physical and chemical properties of the lipids in which they are located [87–89]. Accepting that the properties of the thylakoid lipids, such as fluidity, can affect photosynthetic activity has implications in the study of plant growth and productivity under various environmental conditions as already being explored [87–89].

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