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Surface charges, the heterogeneous lateral distribution of the two photosystems, and thylakoid stacking *

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Spinach grown in light which preferentially excites Photosystem II (PSII-light) had increased amounts of PS I reaction centres on a chlorophyll (Chl) basis, a higher Chl *a*/Chl *b* ratio but less extensive thylakoid stacking, when compared with growth in light preferentially favouring excitation of PS I (PSI-light). Surprisingly, despite the marked difference in the extent of thylakoid stacking, the overall surface charge densities of thylakoid membranes were similar for plants grown in PSII- and PSI-light. The amounts of PS II reaction centres, cytochrome *f* and adenosine triphosphate synthase were also similar on a Chl basis for the two growth light qualities. Thylakoids of the Chl-*b*-less barley mutant had a smaller magnitude of surface charge density, although the extent of thylakoid stacking (Goodchild, D.J., Highkin, H.R. and Boardman, N.K. (1966) Exp. Cell Res. 43, 684–688) was much smaller, compared with wild-type barley. The results are interpreted in terms of the possible mechanisms governing the heterogeneous lateral distribution of the two photosystems. They suggest that PS I is laterally segregated to non-appressed thylakoid membrane domains because of the excess negative charges it carries on the outer membrane surface, whereas PS II is mainly sequestered into the appressed regions because of an association with its Chl *a*/*b*-proteins which appear to contribute more to van der Waals attraction than hitherto recognized.

Introduction

One of the remarkable features of the chloroplast thylakoid membrane network of higher plants is its extremely large area to chloroplast volume ratio, which makes possible, among other things, the close packing of pigment-protein complexes for effective light harvesting [1]. The large area to volume ratio is achieved by the formation of granal stacks, in which a number of thylakoid membranes are appressed. Interconnecting the grana are nonappressed stromal thylakoid membranes. The top and bottom membranes and margins of granal stacks also constitute non-appressed membrane domains.

In the absence of a granal structure, there would be a random, uniform lateral distribution of membrane proteins and lipids, owing to the fluid nature of thylakoid membranes. Concomitantly, the surface electric potential would be more or less uniform. When two negatively-charged membranes come together, as occurs during granal formation, there is an overlapping of two electric double layers, tending to increase the magnitude of the surface electric potential of the appressed membranes. By Le Chatelier's principal, components with net negative charge at their outer surface will diffuse out of the appressed membrane region, so as to restore a uniform surface electric potential [2]. In addition, the appression of membranes will occur at protein-rich domains formed from protein aggregation in the presence of good electrostatic screening [1,3]. In the artificial process of destacking, when divalent cations are removed from thylakoid suspensions containing low concentrations of monovalent cations, it is envisaged that the reverse lateral migration occurs; negatively charged membranes components, originally segregated to non-appressed regions, are induced to migrate into the appressed region, so as to 'unzip' two appressed membrane regions [4]. The electrostatic control of membrane

* Dedicated to the memory of Dr David J. Goodchild, our friend and colleague.

Abbreviations: CF_1, coupling factor 1; Chl, chlorophyll; Hepes, 4-(2-hydroxyethyl-1-piperazine)ethanesulfonic acid; P700, reaction centre chlorophyll of PS I; PS, photosystem.

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appression is further supported by the finding that increasing net negative charges by either phosphorylation [5,6] or trypsin treatment [7] of Photosystem II (PS II) light-harvesting Chl *a/b*-protein complexes promotes destacking of granal membranes.

In the above essentially electrostatic model of granal formation, surface charges are non-uniformly distributed in the thylakoid membrane system. So far, however, there is only circumstantial evidence supporting the notion that non-appressed thylakoid membrane domains have a greater abundance of negative charges exposed to the stroma compared with appressed membrane domains. Firstly, right-side-out vesicles derived from non-appressed stromal thylakoid membranes of grana-containing chloroplasts did not readily aggregate when they were electrostatically screened by non-binding cations [8]. This finding suggests that these vesicles may have abundant negative surface charges giving rise to a large electrostatic repulsion which is not sufficiently decreased by cation screening to allow aggregation to take place [8]. However, an alternative (non-electrostatic) explanation is that these vesicles with less light-harvesting Chl *a/b*-protein complexes may have substantially less van der Waals attraction, so that the net force is repulsive even in the presence of screening cations. A second piece of circumstantial evidence is that chloroplasts from plants grown under high irradiance have less extensive granal formation and a greater abundance of negative surface charges in their destacked thylakoid membranes (with a randomized distribution of proteins) than chloroplasts from plants grown under low irradiance [9,10]. In further analyzing the data of Davies et al. [9], Telfer [11] calculated that the magnitude of the net surface charge density (σ) of the appressed membrane region is approx. 10-times lower than that of the non-appressed membrane surface. The calculation, however, was based on the solution of two simultaneous equations and therefore on the assumption that σ was identical for appressed thylakoid membranes, in both high-light- and low-light-grown lettuce plants. Since this assumption is more likely than not to be incorrect, neither the first nor the second piece of evidence unambiguously favours non-appressed thylakoid membranes being more negatively charged than appressed membranes.

The present study attempts to obtain evidence in support of the electrostatic model of granal formation from a new angle. Given the heterogeneous lateral distribution of Photosystem I (PS I) in the non-appressed membranes, including the periphery of granal stacks [12–14], we postulate that such a non-uniform distribution of PS I has arisen because the complex may possess an abundance of net negative surface charges at its outer surface. If so, a growth treatment which changes the amounts of PS I may be expected to alter both the overall σ of the thylakoid membrane system, and the extent of grana formation. To bring about such an alteration in the PS I content, we have used a light-quality treatment whereby plants, grown under light which preferentially excites PS I or PS II, have less or more PS I complexes, respectively [15].

In addition to the lateral heterogeneous distribution of PS I, there is also a predominant localization of the majority of PS II complexes together with their light-harvesting Chl *a/b*-protein complexes (PS II_α) in appressed membranes [16]. To investigate the mechanism underlying the sequestering of PS II_α in appressed membranes, we have also compared the Chl-*b*-less barley mutant with wild-type barley in terms of the surface charge densities of their thylakoid membranes.

Taken together, our results are consistent with the notion that electrostatic properties strongly determine the segregation of PS I to non-appressed membranes, whereas van der Waals attraction may play a greater role in the sequestering of PS II_α into appressed membranes than hitherto assumed.

Materials and Methods

Growth of plants

Spinacia oleracea (Henderson's hybrid 102) was cultivated in a 1 : 1 mixture of vermiculite and perlite in a growth room under controlled conditions (16 h light/24° C; 8 h dark/14° C). The growth light was either fluorescent illumination filtered by yellow plexiglass PSII-light, about 69 μmol photons m^{-2} s^{-1}, 520–695 nm measured with a Li-Cor 195A quantum photometer), or incandescent illumination filtered by red plexiglass (PSI-light, 48 μmol photons m^{-2} s^{-1} from 580 to 695 nm plus 47 μmol photons m^{-2} s^{-1} from 695 to 740 nm). The relative spectral irradiance of each light regime, measured with a spectroradiometer (SR 3000 A, Macam Photometrics, Livingstone, U.K.) is shown in Fig. 1. Because of the lower quantum yield obtainable with the far-red component of PSI-light, these two light treatments gave approximately equivalent photosynthetically effective radiation. The plants were supplied with

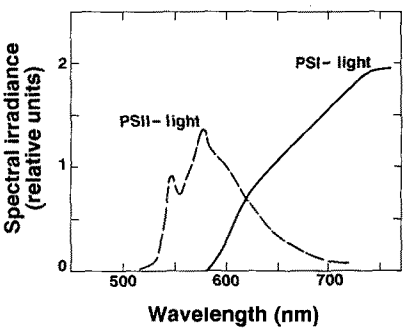

Fig. 1. Spectral distribution of irradiance in each growth light quality environment, designed to favour Photosystem I (PSI-light) or Photosystem II (PSII-light).

Hoagland's solution once weekly and with water daily. Leaves were sampled at 30 days after transplanting seedlings.

Barley seedlings were grown in a mixture of soil and perlite in a glasshouse. Leaves were sampled at 3 weeks after sowing.

Determination of thylakoid components

Chloroplasts were isolated as described previously [17] and frozen at 77 K until used for the determination of thylakoid components. The chlorophyll concentrations of thylakoid stocks were assayed in 80% acetone according to Ref. 18 using an Hitachi model U-3200 spectrophotometer.

The amounts of PS II reaction centres were determined by the oxygen evolved by leaf discs per single-turnover repetitive flash in the presence of background far-red light to ensure no limitation due to PS I turnover [19]. This method gave estimates of PS II content which were, on average, approx. 12% lower than the amounts of diuron-binding sites in isolated thylakoids [19].

The amounts of PS I reaction centres were determined by the light-induced absorbance changes of the PS I reaction centre chlorophyll *a* (P700) at 703 nm according to [17,20].

Cytochrome *f* was assayed from the hydroquinone-reduced *minus* ferricyanide-oxidized spectra in a Perkin-Elmer 557 double-beam spectrophotometer [21].

The Mg^{2+}-dependent ATPase activity of thylakoids was assayed in the presence of octyl glucoside as an activator [17,22]. The ATP-hydrolytic activity is taken as a relative measure of the content of CF_1, the coupling factor 1 of the chloroplast ATP synthase [23].

Electron microscopy

Tissue pieces (approx. 1 mm × 1 mm) were cut from leaves and fixed in 3% glutaraldehyde in 25 mM sodium phosphate buffer (pH 7.2) for 1.5 h at room temperature. To ensure uniform conditions during fixation, vials were kept in the dark. After several buffer washes the tissue pieces were post-fixed in 2% OsO_4 in the phosphate buffer for 1.5 h, washed, dehydrated through an alcohol series and embedded in Spurr's resin [24]. Thin sections were prepared from the embedded tissue, stained with uranyl acetate and lead citrate and examined in a JEOL 100S electron microscope at 60 kV.

Determination of the adsorption of divalent cations (diquat) to thylakoid membranes

Freshly-prepared chloroplasts were osmotically shocked in 20 mM KCl for about 10 s, and the suspension was mixed with an equal volume of double-strength medium to give a final composition of 100 mM sorbitol, 20 mM KCl and 0.5 mM NaOH (adjusted to pH 7.5 with the acid form of Hepes). The suspension was allowed to stand at 25 °C for 10 min to induce de-stacking [25], and then centrifuged at 3000 × *g* for 3 min. The pellet was resuspended in a small aliquot of the supernatant, and kept on ice.

Aliquots of the de-stacked thylakoid stock were diluted into an assay medium in an Eppendorf microfuge tube containing 100 mM sorbitol, 0.1 mM Na_3EDTA, 0.5 mM NaOH (pH 7.5/Hepes acid), thylakoids equivalent to 80 μM Chl (spinach) or 60 μM Chl (barley), and various concentration of [*ethylene*-^{14}C]diquat dibromide (Amersham, U.K.) that had previously been diluted with an unlabelled diquat sample to give a specific activity of 9 Bq $nmol^{-1}$. The concentration of the diluted radioactive diquat stock was determined using an extinction coefficient of 19.6 $mM^{-1}\,cm^{-1}$ at 308.3 nm which was derived from a pure sample of diquat dibromide. Below the 1 ml assay mix at the bottom of the microfuge tube was a silicone oil mixture (Wacker-Chemie, München) containing 6 parts of AR20 (0.985 g/ml) and 5 parts of AR200 (1.039 g/ml). After equilibrating in the dark for about 3 min, the microfuge tubes were spun for 3 min, depositing the thylakoid pellet which was separated from the supernatant by the silicone oil layer. The concentration of free diquat in the supernatant was determined by taking 0.7 ml and mixing with 5 ml Beckman Ready Value scintillation fluid. Standards were processed in the same way except that thylakoids were omitted. The amount of diquat adsorbed to thylakoids was obtained as the difference ($\Delta c''$) between the added concentration and the free concentration (c'') of diquat, where the double prime refers to the divalent nature of the cation.

The present method based on diquat is a more convenient variation of the previous method based on methyl viologen (paraquat) adsorption [26]. EDTA was included in the assay medium to complex any residual Mg^{2+}/Ca^{2+} that might be present.

Analysis of diquat adsorption to determine surface charge densities

In this analysis, diquat dibromide is treated as a salt consisting of a divalent cation and a divalent anion, $C^{2+}A^{2-}$, with the free concentration in the bulk solution denoted as c''. In addition, the medium contains monovalent cations, balanced by other anions which are taken as monovalent. The concentration of monovalent K^+ and Na^+ cations (c') in the assay mix was estimated to be approx. 1 mM (1 mol m^{-3}).

Applying Poisson's equation and Boltzmann's law in the Gouy-Chapman treatment of the electric double layer [1,27–30] in SI units, we obtain the following relationship for a negatively-charged surface bathing in a mixed solution containing monovalent : monovalent and divalent : divalent salts:

$$dy/dx = 2F(RT\epsilon_r\epsilon_o)^{-1/2}[c'(\cosh y - 1) + c''(\cosh 2y - 1)]^{1/2} \quad (1)$$

where $y = F\Psi/(RT)$, Ψ is the electric potential at a distance x from the charged surface, F the Faraday constant (96 487 C mol^{-1}), R the gas constant (8.3143 J K^{-1} mol^{-1}), T the absolute temperature (298 K), ϵ_r the dielectric constant (78.5), and ϵ_o the absolute permittivity of free space ($8.85415 \cdot 10^{-12}$ C V m).

The excess quantity of divalent cations per unit surface area (above the bulk solution value) present in the diffuse layer is

$$n'' = \int_0^d [c'' \exp(-2y) - c''] \, dx \quad (2)$$

where the upper limit of integration, d, is the distance of the plane of shear from the charged surface during centrifugation of the thylakoids. The value of d was taken as 50 nm, at which the electric potential has declined to approx. −0.5 mV or closer to zero. Eqns. 1 and 2 can be combined to give

$$n'' = [c''(RT\epsilon_r\epsilon_o)^{1/2}/(2F)] \int_{y_0}^{y_d} [\exp(-2y) - 1] dy \Big/ [c'(\cosh y - 1) + c''(\cosh 2y - 1]^{1/2} \quad (3)$$

In this equation, the lower limit of integration $y_0 = F\Psi_o/(RT)$, (where Ψ_o is the surface electric potential) is determined by the surface charge density, σ, and the salt concentrations in the bulk solution according to

$$\sigma = -(4RT\epsilon_r\epsilon_o)^{1/2}[c'(\cosh y_0 - 1) + 2c''(\cosh^2 y_0 - 1)]^{1/2} \quad (4)$$

For any trial value of σ, y_0 is given by Eqn. 4. The upper limit of integration $y_d = F\Psi_d/(RT)$, where Ψ_d is the electric potential at the plane of shear, can be determined by noting that the distance x from the charged surface is related to the value of y at x by

$$x = [(RT\epsilon_r\epsilon_o)^{1/2}/(2F)] \times \int_{y_0}^{y_x} [c'(\cosh y - 1) + 2c''(\cosh^2 y - 1)]^{-1/2} \, dy \quad (5)$$

The upper limit of integration, y_x, is selected as y_d when numerical integration in Eqn. 5 yields 50 nm on the right-hand side. Having determined the limits of integration, y_0 and y_d, for any given σ, c' and c'', we evaluate n'' by numerical integration in Eqn. 3, using Simpson's Rule and increments of 0.005 in y. The parameter n'' is directly proportional to $\Delta c''$, the difference between the added concentration and the free concentration of diquat. Thus, the experimental plot of $\Delta c''$ against c'' can be fitted by a theoretical plot of n'' against c''.

To perform curve fitting, the data points (Figs. 3 and 4) in a series of free concentrations c'' were first fitted by a rectangular hyperbola of the form

$$\Delta c'' = (\Delta c''_{max} c'')/(K + c'') \quad (6a)$$

or

$$\Delta c''/c'' = \Delta c''_{max}/K - \Delta c''/K \quad (6b)$$

The data points in a series of free concentrations of c'' were transformed into the form in Eqn. (6b) and fitted by linear regression, yielding the constants K and $\Delta c''_{max}$. These two constants allowed the 'smoothed' value of $\Delta c''$ to be calculated for any given value of c''. At two well-spaced values of c'' (0.01 and 0.04 mol m^{-3}), the corresponding 'smoothed' values of $\Delta c''$ were calculated and their ratio was obtained.

Next, some trial-values of σ were used to calculate n'' for $c'' = 0.01$ or $c'' = 0.04$ mol m^{-3} ($c' \approx 1$ mol m^{-3} and fixed), according to Eqn. 3. The value of σ, obtained by graphic interpolation, giving the same ratio of n'' as that of $\Delta c''$ at these two c'' values, was taken as the surface charge density of the de-stacked thylakoid membranes. Curve-fitting using a closer plane of shear ($d = 15$ nm) yielded much the same values of σ.

Once σ was determined, the corresponding constant of proportionality between n'' and $\Delta c''$ allowed the calculation of the effective area per unit chlorophyll, the surface charge per unit chlorophyll, or the adsorption curves in Figs. 3 and 4. The calculated adsorption curves generally tended to deviate from the (more-scattered) experimental points at $c'' \geq 70$ μM (Figs. 3 and 4). This deviation could be associated with a tendency of de-stacked thylakoid membranes to re-stack in the presence of increased divalent cation concentration when the monovalent cation concentration was fixed at a low value of 1 mol m^{-3}. In an attempt to avoid this possible complication, curve fitting was performed at $c'' = 0.01$ and c'' 0.04 mol m^{-3}, i.e. 10 μM and 40 μM, respectively. It has been known that with a background concentration of monovalent cations at 1 mol m^{-3}, the addition of 50 μM $MgCl_2$ induced negligible re-stacking of thylakoid membranes [31].

Results and Discussion

Membrane components and stacking of spinach thylakoids

To test the hypothesis that PS I is laterally segregated into non-appressed regions of thylakoids because of the excess negative charges it carried, we employed two contrasting light quality regimes [15,32] under which spinach leaves developed different amounts of PS I reaction centres to compensate for the imbalance of excitation energy partitioned between the two photosystems. As shown in Table I, growth in PSI-light led to a lesser amount of PS I per unit Chl, when compared with growth in PSII-light. Apart from the Chl composition (see below), the amount of each of the other major thylakoid components examined (PS II, ATPase and cytochrome f) remained largely unaltered on a Chl basis when spinach plants were grown under the two

TABLE I

The composition of thylakoid components isolated from spinach grown under light which preferentially excites PS I or PS II

Results are presented as the mean ± S.E. (n).

	PSI-light thylakoids	PSII-light thylakoids
Chl a/Chl b	2.99 ± 0.02 (5)	3.72 ± 0.01 (5)
PSI, mmol (mol Chl)$^{-1}$	1.34 ± 0.01 (3)	1.84 ± 0.04 (4)
PSII, mmol (mol Chl)$^{-1}$	2.88 ± 0.04 (5)	2.84 ± 0.06 (5)
PSII/PSI	2.15	1.54
ATPase, mmol s^{-1} (mol Chl)$^{-1}$	194	175
Cyt f, mmol (mol Chl)$^{-1}$	1.12 ± 0.04 (4)	1.04 ± 0.02 (4)

light regimes. Because of the differing amounts of PS I on a Chl basis, the PS II/PS I reaction centre ratio was markedly different between the two light treatments, being higher in PSI-light (Table I).

Since PS I is laterally segregated into non-appressed regions of thylakoid membranes [12–14], one may expect that a greater abundance of PS I may be associated with a lesser extent of thylakoid stacking. Indeed, the electron micrographs in Fig. 2 show that, when grown in PSII-light, spinach leaves exhibited smaller granal stacks and greater non-appressed membrane domains as compared with growth in PSI-light. As a measure of the extent of granal stacking, we measured the absolute

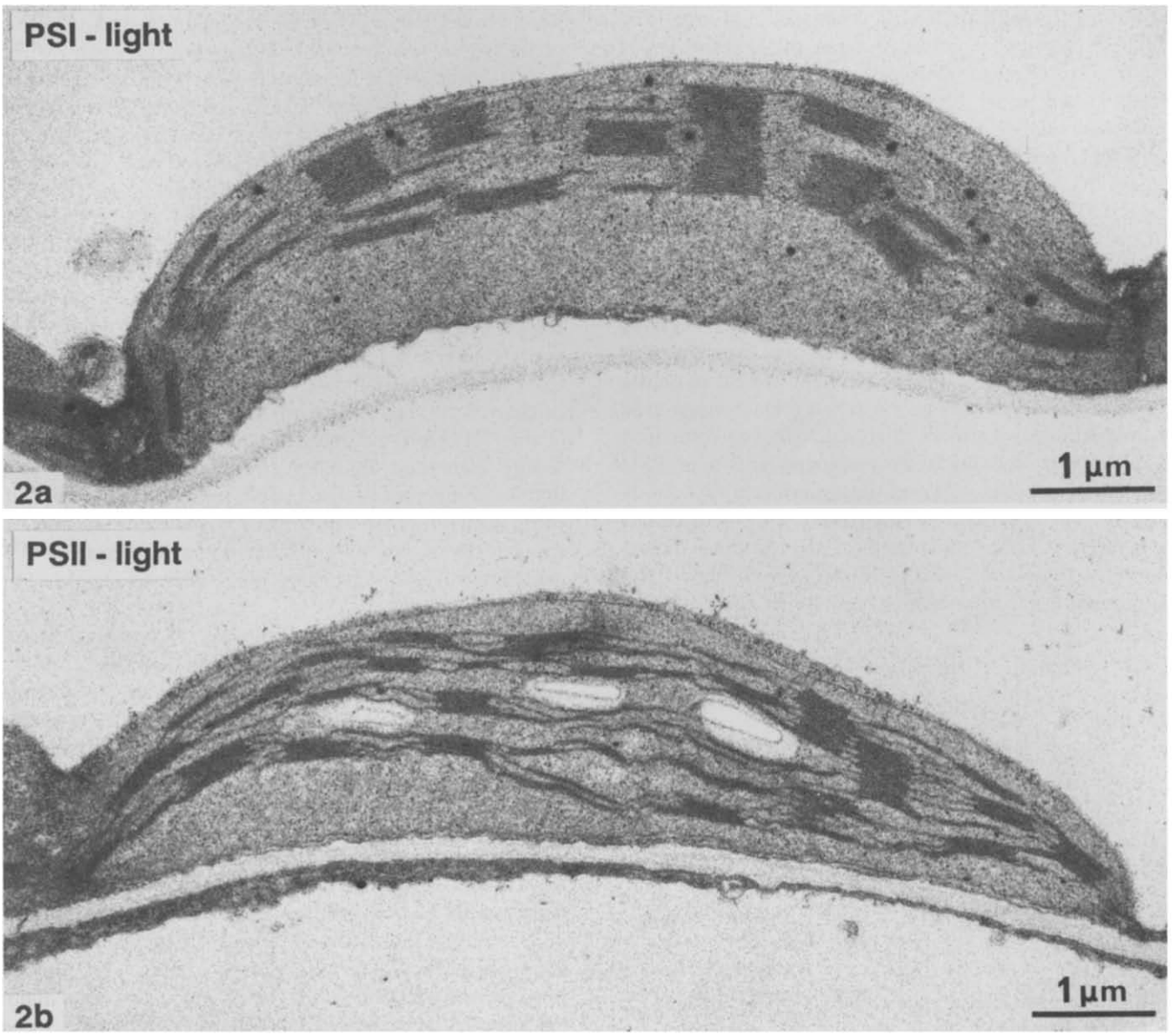

Fig. 2. Electron micrographs of chloroplasts in palisade cells of spinach grown in PSI-light and PSII-light.

TABLE II

The extent of grana formation in spinach grown under light which preferentially excites PS I or PS II

Results are presented as the mean ± S.E. ($n = 5$ chloroplasts from palisade cells). Any cross-sectional area of starch grains was excluded from the evaluation of stromal cross-sectional area.

	PSI-light thylakoids	PSII-light thylakoids
Total granal cross-sectional area per chloroplast $(\mu m)^2$	2.97 ± 0.20	1.89 ± 0.10
Ratio of granal to stromal cross-sectional area	0.25 ± 0.02	0.19 ± 0.02
Cross-sectional area per chloroplast $(\mu m)^2$	15.1 ± 1.0	12.3 ± 0.7

TABLE III

The surface charge density and effective surface area of de-stacked thylakoids from spinach grown in PSI- or PSII-light, and from wild-type or Chl-b-less barley

The values are derived from curve fitting of data in Figs. 3 and 4.

	Spinach		Barley	
	PSI-light	PSII-light	wild-type	*b*-less mutant
σ, C m^{-2}	−0.028	−0.032	−0.0175	−0.010
$10^6 \times$ effective surface area, m^2 (mol Chl)$^{-1}$	3.72	3.07	7.50	27.4
Net negative charges, equiv (mol Chl)$^{-1}$	1.08	1.02	1.36	2.84

cross-sectional area of granal stacks per chloroplast, or the ratio of granal to stromal cross-sectional area [33]. Table II shows that either of these two parameters is smaller (i.e., less granal stacking) in chloroplasts from plants grown under PSII-light as compared with PSI-light. The smaller extent of granal formation in PSII-light compared with PSI-light spinach leaves confirms the results reported for spinach cotyledons developed under PSII- or PSI-light [32].

Surface charge density of de-stacked spinach thylakoids

If the distribution of PS I exclusively in non-appressed domains is attributable to the excess negative charges on its outer surface, then one may expect that in de-stacked thylakoids, a higher content of PS I in plants grown in PSII-light may give rise to a greater magnitude of the net negative surface charge density (σ) compared to de-stacked thylakoids from spinach grown in PSI-light. The surface charge densities of spinach thylakoids from the two light quality treatments were determined by analysis of the adsorption of the divalent diquat cation to thylakoids (see Methods). Fig. 3 shows the experimental adsorption as a function of the free concentration of divalent cation, together with the calculated curves for appropriate values of σ and effective surface area per unit Chl. Perhaps surprisingly, there was little difference in the diquat-adsorption characteristics of the de-stacked thylakoids from the two light treatments. As shown in Table III, PSI-light spinach thylakoids, though having a much reduced amount of PS I reaction centres (Table I), were only about 10% less negatively charged compared with PSII-light thylakoids. Given the scatter in the data points in Fig. 3, this small difference is probably not significant.

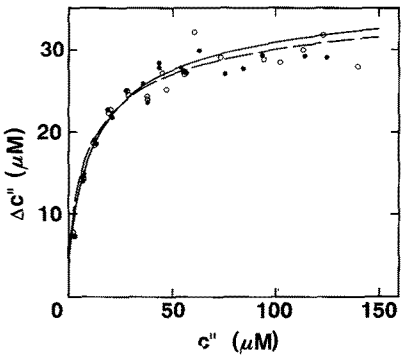

Fig. 3. Adsorption of the divalent cation ,diquat, to destacked thylakoids of spinach grown in PSI-light (●——●) or PSII-light (○- - -○). Thylakoid suspensions were equivalent to 80 μM CHl. The curves were calculated by assuming the values of surface charge density and effective surface area in Table III.

The values of σ obtained for spinach (Table III) are comparable to that assumed previously [30]. From σ and the effective surface area per unit Chl obtained by curve-fitting, the amount of net negative charges is calculated to be approximately 1 per Chl molecule (Table III). Interestingly, this amount of net negative charge estimated for the stromal side of de-stacked thylakoid membranes (accessible to diquat) is somewhat comparable to that estimated for the lumenal side of spinach thylakoids by an independent technique (~ 0.6 net negative Donnan charge per Chl molecule [34,35]).

Contribution of light-harvesting Chl a / b-proteins to negative surface charge of barley thylakoids

At first sight, the close similarity between the surface charge densities of the spinach thylakoids from plants grown in the two light-quality regimes seems puzzling. However, it should be born in mind that the other major difference between PSI- and PSII-light thylakoids was in the Chl *a*/Chl *b* ratio (Table I); this ratio was 2.99 for PSI-light thylakoids, but 3.72 for PSII-light thylakoids. Assuming, for example, that the PS II light-harvesting Chl *a*/*b*-proteins have a Chl *a*/Chl *b* ratio of 1.38 [18], it can be readily calculated that 60% of the total Chl is in the light-harvesting Chl *a*/*b*-proteins of PSI-light thylakoids, but only 50% in PSII-light thylakoids. An increased amount of PSII light-harvesting Chl *a*/*b*-protein complexes implies a greater abundance of the apoproteins, which, if contributing to the

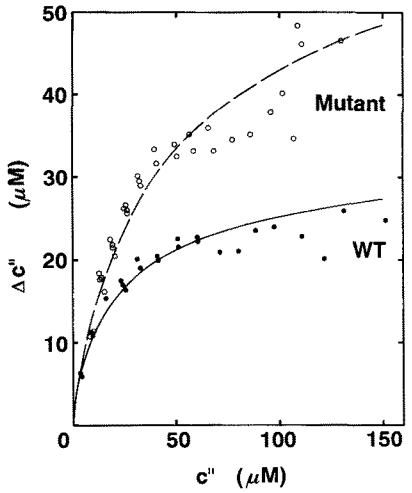

Fig. 4. Adsorption of the divalent cation, diquat, to destacked thylakoids of wild-type barley (●———●) and Chl *b*-less mutant barley (○- - -○). Thylakoid suspensions were equivalent to 60 μM Chl. The curves were calculated by assuming the values of surface charge density and effective surface area in Table III.

negative surface charges of thylakoids, would tend to compensate for any reduced contribution from the lower amounts of PS I on a chlorophyll basis in PSI-light thylakoids.

We therefore investigated the possible contribution of light-harvesting Chl *a/b*-proteins to the negative surface charges of thylakoid membranes. This was done by comparing the surface charge density of wild-type barley and that of the Chl-*b*-less barley mutant. Fig. 4 shows the diquat adsorption data, and the calculated curves based on appropriate values of σ and effective area per unit Chl. The fitted value of σ was -0.0175 C m^{-2} for wild-type thylakoids, a value more negative than for the Chl-*b*-less mutant (-0.010 C m^{-2}, Table III). Since the thylakoids of the Chl-*b*-less mutant are already enriched in the negatively-charged phosphatidylglycerol and diacylsulphoquinovosylglycerol compared with other acyl lipids [36], a lower overall magnitude of surface charge density strongly suggests that the absence or at least much-reduced amounts of the apoproteins of the PS II light-harvesting Chl *a/b*-proteins has rendered the mutant thylakoid membrane much *less negatively charged*. Indeed, a contribution of the apoprotein to the net negative charges on the outer stromal surface of thylakoid membranes is expected, whether the apoproteins have three [37] or four [38] membrane-spanning α-helices. Thus, we suggest that the lower amount of PS II light-harvesting Chl *a/b*-proteins in PSII-light thylakoids tends to render the membranes less negatively charged as compared with PSI-light thylakoids, but that the greater amount of PS I has a compensatory effect. The net result is that the surface charge density of de-stacked membranes is rather similar between thylakoids of spinach plants grown in the two light quality treatments.

Contribution of light-harvesting Chl a/b-proteins to van der Waals attraction

Despite the similar surface charge densities, the extent of granal stacking was obviously greater in PSI-light thylakoids (Fig. 2) with their greater abundance of light-harvesting Chl *a/b*-protein complexes. The greater extent of granal formation is attributable to a greater contribution to van der Waals attraction by the increased amounts of PS II light-harvesting Chl *a/b*-protein complexes. Whereas the previous models depicted stacking between membrane surfaces of low magnitudes of negative surface charge density [4,11,39], perhaps a more realistic model is one in which enhanced van der Waals attraction, particularly that conferred by the PS II light-harvesting Chl *a/b*-proteins, induces stacking *despite* a substantial but not excessive electrostatic repulsion.

Furthermore, the important role of the PS II light-harvesting Chl *a/b*-proteins in contributing to van der Waals attraction is consistent with the findings that granal formation is greatly reduced in the Chl-*b*-less barley mutant [36,40], despite a relatively low magnitude of surface charge density (Table III) and, by implication, a relatively low electrostatic repulsion between thylakoid membranes.

Previous calculations of the van der Waals force yielded an attraction which could counteract electrostatic repulsion only if the surface charge density was substantially lowered, either by neutralization of negative charges due to cation binding or by a redistribution of surface charges between appressed and non-appressed regions [41]. While both of these processes could occur to some extent in vivo to facilitate thylakoid stacking, it is also possible that the previously calculated van der Waals attraction [41] could have been underestimated. As shown in Ref. 41, this attractive force is highly dependent on the dielectric constant assumed for membrane proteins, and also to some extent, on the proteins as a percentage of the total membrane mass. A better knowledge of the dielectric constant of thylakoid proteins, particularly that of the PS II light-harvesting Chl *a/b*-proteins, is needed, in order to reevaluate the van der Waals attraction. Nevertheless, it is quite possible that the PS II light-harvesting Chl *a/b*-proteins may contribute more to van der Waals attraction than hitherto realized. This possibility is consistent with our present findings that (1) the greater abundance of light-harvesting Chl *a/b*-proteins in PSI-light thylakoids (compared with PSII-light thylakoids) was associated with more extensive granal formation, even though σ was similar; and (2) the much reduced granal formation in the Chl-*b*-less barley mutant (com-

pared with wild-type barley) occurred despite a lower magnitude of σ.

Given that PS II light-harvesting Chl *a/b*-proteins promote thylakoid stacking, it follows that the core complex of PS II is sequestered into the appressed region because of its association with its light-harvesting Chl *a/b*-proteins to form PS II_α. However, the lateral heterogeneous distribution of PS II is not extreme, since PS II_β reaction centres with a reduced light-harvesting antenna size are found in non-appressed membrane regions [16], suggesting decreased van der Waals attraction associated with a smaller antenna.

Lateral distribution of other thylakoid membrane complexes

Growing spinach plants in PSI-light or PSII-light did not lead to marked alterations in the amounts of cytochrome *b/f* complex (as measured by cytochrome *f*) or ATP synthase (as measured by ATPase activity) on a chlorophyll basis (Table I). Therefore, our present results do not answer the question of how different amounts of these two complexes might have influenced thylakoid stacking.

Quite early on, it was known that coupling-factor 1 (CF_1) of chloroplasts was exclusively located in non-appressed thylakoid membrane regions [42]. Subsequently, it was suggested that, owing to steric hindrance which would result from the inclusion of the bulky CF_1 in appressed regions, the lateral heterogeneous distribution of CF_1 was more energetically favourable [9]. However, the possibility that negative charges carried by CF_1 also contribute to its exclusion from the appressed region cannot be ruled out.

Unlike ATP synthase and PSI, the cytochrome *b/f* complex is present in both appressed and non-appressed thylakoid membrane regions [43–46]. Presumably, its non-heterogeneous distribution results from a low or zero amount of net charge on the stromal side of its exposed surface, a possibility that can be tested once the molecular organization of the complex in the thylakoid membrane is known.

Concluding remarks

Both electrostatic repulsive and van der Waals attractive forces participate in determining the resultant appressed and non-appressed domains of the thylakoid membrane network, and the associated lateral heterogeneous distribution of the two photosystems. Our results suggest that PS I is laterally segregated into the non-appressed membrane domains because of the excess negative charges it carries on the stroma-facing side of the membrane, whereas PS II_α is sequestered into the appressed membrane regions because of an association with its light-harvesting Chl *a/b*-proteins. We suggest that these PS II Chl *a/b*-proteins, despite their contribution to net negative surface charge density and therefore to electrostatic repulsion, play an important role in promoting thylakoid stacking by substantially increasing van der Waals attraction.

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