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Entropy-assisted stacking of thylakoid membranes

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Abstract

Chloroplasts in plants and some green algae contain a continuous thylakoid membrane system that is structurally differentiated into stacked granal membranes interconnected by unstacked thylakoids, the stromal lamellae. Experiments were conducted to test the hypothesis that the thermodynamic tendency to increase entropy in chloroplasts contributes to thylakoid stacking to form grana. We show that the addition of bovine serum albumin or dextran, two very different water-soluble macromolecules, to a suspension of envelope-free chloroplasts with initially unstacked thylakoids induced thylakoid stacking. This novel restacking of thylakoids occurred spontaneously, accompanied by lateral segregation of PSII from PSI, thereby mimicking the natural situation. We suggest that such granal formation, induced by the macromolecules, is partly explained as a means of generating more volume for the diffusion of macromolecules in a crowded stromal environment, i.e., greater entropy overall. This mechanism may be relevant in vivo where the stroma has a very high concentration of enzymes of carbon metabolism, and where high metabolic fluxes are required.

Keywords: Chloroplast ultrastructure; Depletion attraction; Entropy; Grana; Macromolecular crowding; Thylakoid stacking

1. Introduction

The photosynthetic thylakoid membranes of higher plants and more recently-evolved green algae are extensively folded into two domains. Grana, consisting of regular stacks of appressed thylakoids (diameter 400–600 nm), are ubiquitous and intriguing structures in chloroplasts [1–3]. They are inter-connected via non-stacked membranes, the stromal lamellae. This structural differentiation of thylakoids into appressed granal and non-appressed stromal lamellae is accompanied by a functional differentiation; indeed, there is a well-defined lateral heterogeneity with respect to the location of the membrane protein complexes [1]. PSI occurs as a supercomplex consisting of monomeric PSI and four LHCI proteins [4] and ATP synthase possibly occurs as dimers [5]. As both PSI and ATP synthase have bulky protruding groups which cannot be accommodated between appressed membranes, they are located exclusively in non-appressed membrane domains (stromal thylakoids and the margins and end membranes of granal stacks) and are in direct contact with the stroma. PSII is found mainly in appressed granal domains as PSII supercomplexes which are dimers of PSII core complex together with tightly bound LHCII trimers $[PSII–(LHCII)_3]_2$ and more loosely-associated LHCII trimers [6,7]. Cytochrome *bf* complex occurs as dimers in both appressed and non-appressed domains.

Grana formation in chloroplasts, with a spatial separation between PSI on the one hand and PSII dimers and LHCII trimers on the other, is of great physiological relevance to photosynthetic energy transduction in land plants and green algae. Photosynthetic function is optimized in response to ever-changing environmental conditions, particularly light. For example, a dynamic variation in the extent of grana formation prevents excessive spillover of excitation energy

Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; Chl, chlorophyll; Fm, maximal PSII fluorescence yield when the traps are closed; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethansulfoinc acid; PSII, Photosystem II; PSI, Photosystem I; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase

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from PSII to PSI [8], alters the separation of kinetically "slow" PSII from "fast" PSI [9], regulates both light energy use and safe dissipation of excess excitation [10], enhances non-cyclic ATP synthesis [11], fine tunes the balance between linear and non-cyclic electron transport [12], and protects non-functional PSII from D1 protein turnover in high irradiance [13].

The granal configuration represents a minimum free energy state of thylakoids. In functional chloroplasts, the appression of thylakoid membranes to form grana is a consequence of a highly dynamic interplay between repulsive and attractive forces, coupled with thermodynamic constraints related to steric factors and the maximization of entropy [2]. Electrostatic repulsion arises from the opposing net negative charges on the outer thylakoid membrane surface mainly due to proteins and to a much lesser extent phosphatidyl glycerol and sulfolipid [14–16] whereas van der Waals attraction is associated with permanent and transient electric dipoles in molecules [17]. The electrostatic repulsion can be altered in vitro by varying the concentration and valency of cations in a suspension of envelope-free chloroplasts [18–20]. The van der Waals attraction can be varied in vivo by growth conditions that favour the accumulation of major thylakoid proteins, LHCII trimers, thereby increasing the abundance of permanent and transient electric dipoles. When van der Waals attraction exceeds electrostatic repulsion, membrane appression occurs spontaneously to form grana, with a regular distance of about 4 nm [21] between adjacent thylakoids. The separation between adjacent thylakoids, termed the partition gap, is defined by strong short-range 'hydration repulsion' associated with the need to remove ordered water molecules at the membrane surface at shorter separations [22]. At this separation, specific interactions between groups on opposite membranes may occur [23–25]. However, Kirchhoff et al. [26] recently demonstrated that energy transfer in grana between PSII dimers and loosely-associated LHCII trimers is mainly confined within individual appressed thylakoids rather than between opposing appressed thylakoids.

Are electrostatic repulsion, van der Waals attraction and hydration repulsion the only forces that shape the ultrastructure of grana-containing chloroplasts? Granal stacking confers local order in the form of (i) orderly regular stacks of membranes, and (ii) lateral segregation of PSII from PSI and ATP synthase. The generation of such local order in thylakoid membranes could be spontaneous if there were a sufficiently large increase in entropy overall, i.e., in the chloroplast as a whole. Chow [2] hypothesized that the generation of more disorder overall in the chloroplast could occur because the appression of thylakoid membranes allows more volume in which stromal enzymes can diffuse randomly. This increased molecular diffusion represents increased entropy [27], which is responsible, for example, for an attraction between large colloidal particles when small particles are also present [28], and for forming ordered structures in a suspension of rod-like viruses and sphere-like polystyrene latex [29]. Entropy-driven adhesion or aggregation is a general phenomenon, sometimes termed depletion attraction or macromolecular crowding [29]; it is a thermodynamic force whereby small particles, when mixed with large ones, maximize their own entropy by pushing the large ones together. Macromolecular crowding, though rarely explicitly discussed in entropy terms, is highly relevant to the crowded milieu of both the cytosol and organelles [30,31].

The aim of this study was to test the hypothesis that granal stacking in vitro is promoted by conditions in which excessive crowding in a macromolecular environment is alleviated by the adhesion of membranes, i.e., macromolecular diffusion is enhanced in the stroma. One way to mimic the very high contents of proteins and nucleic acids in the stroma in vivo is to add high concentrations of neutral macromolecules to a suspension of envelope-free chloroplasts. Here, we demonstrate for the first time that the addition of the water-soluble macromolecules, BSA or dextran, spontaneously induced thylakoid restacking in a suspension of thylakoids that had been artificially unstacked in the dark in a moderately-low-salt medium. The novel restacking of the thylakoid membrane system into grana and stroma thylakoids, induced by the addition of macromolecules without the addition of extra monovalent or divalent actions, generates greater order in the thylakoid structure at the expense of increased entropy in the chloroplast stroma, which is attributed to the generation of more space for free diffusion of macromolecules in a crowded environment.

2. Materials and methods

2.1. Growth of plants

Arabidopsis thaliana, cv. Columbia plants were grown in a cabinet at a day/night temperature of 23/18 °C with an 8-h photoperiod at an irradiance of 150 μmol photons m^{-2} s^{-1}.

2.2. Chloroplast isolation

Envelope-free chloroplasts were isolated largely according to Andersson et al. [32]. Leaves were homogenized in 20 mM Tricine–KOH (pH 8.4), 450 mM sorbitol, 10 mM EDTA, 10 mM $NaHCO_3$ and 0.5% BSA. The homogenate was filtered through 4 layers Miracloth/6 layers muslin and centrifuged at $3000 \times g$ for 30 s at 4 °C. After suspension in 20 mM Tricine–KOH (pH 7.6), 300 mM sorbitol, 5 mM $MgCl_2$ and 2.5 mM EDTA, the chloroplasts were osmotically shocked in 5 mM $MgCl_2$ for 30 s; then an equal volume of medium was added to give a final concentration of 50 mM HEPES–KOH (pH 7.6), 330 mM sorbitol, 2.5 mM $MgCl_2$ and 10 mM KCl prior to centrifugation at $1500 \times g$ for 2 min at 4 °C. The envelope-free chloroplasts were resuspended in 20 mM Tricine–KOH (pH 7.6), 300 mM sorbitol, 5 mM $MgCl_2$ and 2.5 mM EDTA (2 mM Chl) and kept on ice in the dark until use. Chl concentrations were

determined in buffered 80% acetone [33] using a Hitachi U-3200 Spectrophotometer (Tokyo, Japan).

2.3. Variation of the extent of restacking of artificially unstacked thylakoid membranes

Chloroplasts (60 μM Chl) were suspended in a basic medium containing 50 mM HEPES–KOH (pH 7.6, 26 mM K^+), 300 mM sorbitol and 1 mM EDTA with varied [KCl] either in the absence or presence of fatty acid-free BSA (5% w/v, M_r 68,000) (ICN Biomedicals Inc.) or dextran (15% w/v, M_r 72,000) (Sigma). For simplicity, the [KCl] added to the basic medium is mentioned in describing the effects of salt concentration on stacking. However, it should be remembered that there was a 26 mM background K^+ already present in the basic medium. Samples were incubated in the dark for 10 min at room temperature.

2.4. Sonication

Chloroplast suspensions, freshly prepared from leaves, were sonicated for 3×10 s with 10-s resting intervals on ice using a probe sonicator (MSE 100, Thomas Optical and Scientific, Sydney, Australia) at 6 μm peak amplitude. Sonicated samples were diluted 30-fold into the basic medium supplemented with 30 mM KCl and centrifuged at $10{,}000 \times g$ for 1 h to yield a granal-containing pellet and small vesicles of non-appressed membranes in the supernatant.

2.5. Transmission electron microscopy

Chloroplast suspensions were fixed in 4% formaldehyde and 2.5% glutaraldehyde for 2 h at room temperature, washed 3 times for 10 min in the basic medium and fixed in 1% OsO_4 for 1 h at room temperature. After washing 3 times for 10 min in water, samples were dehydrated in a graded ethanol series and embedded in LR White resin. Ultrathin sections were cut on a Reichert Ultracut ultramicrotome fitted with a glass knife and collected on formval-coated grids. They were stained by incubation in 6% aqueous uranyl acetate for 20 min, followed by incubation in Reinold's lead citrate for 10 min. Ultrathin sections were examined with a Hitachi H-7100 FA transmission electron microscope at 75 kV.

2.6. Measurements of conductivity

The electrical conductivity of buffer solutions was measured in a basic medium containing 50 mM HEPES–KOH (pH 7.6, 26 mM K^+), 300 mM sorbitol and 1 mM EDTA with various added [KCl] either in the absence or presence of 5% BSA or 15% dextran. For simplicity, the added [KCl] will be mentioned in the text, but it should be noted that there is an additional fixed background $[K^+]=26$ mM from the KOH used to adjust the pH of the basic medium. The conductivity meter (LC-81, TPS, Brisbane, Australia) was calibrated against a series of pure KCl solutions of known conductivities.

2.7. Fluorescence measurements

Room temperature Chl *a* fluorescence was determined with a PAM 101 fluorometer (Heinz Walz, Effeltrich, Germany). A saturation pulse of white light (10,000 μmol photons m^{-2} s^{-1}) was applied with a Schott KL-1500 lamp for the measurement of Fm. Chl *a* fluorescence emission spectra were measured at 77 K with an SLM-8100 spectrofluorometer (Spectronics, Rochester, NY, USA) and corrected for wavelength-dependent instrument response. Samples were diluted into each medium (10 μM Chl), excited at 440 nm (slit-width 8 nm), and measured (emission slit-width 2 nm). Data were normalized to the PSI peak emission at 735 nm.

3. Results

3.1. Restacking of initially unstacked thylakoids on adding macromolecules

To test the hypothesis of entropy-assisted thylakoid stacking [2] and to test whether the phenomenon is independent of the type of water-soluble macromolecules, we chose two different macromolecular entities. One was BSA, a globular protein that carries net negative surface charges, whereas the other was dextran, an electrically neutral polysaccharide. First, we note that a suspension of envelope-free chloroplasts in a medium containing a low concentration of a monovalent cation but no multivalent cations results in unstacking of the thylakoid membranes [18], and in a random mixing of all thylakoid protein complexes. Fig. 1A confirms this phenomenon in *Arabidopsis* thylakoids suspended in the basic buffer medium supplemented with 30 mM KCl. For simplicity, the added [KCl] is mentioned in the text, but it should be noted that there is an additional fixed background $[K^+]=26$ mM from the KOH used to adjust the pH of the basic medium. This is a moderately-low-salt medium, in which the thylakoids, though unstacked, were poised to respond to small increases in attractive force or decreases in repulsive force. Thus, an increase in electrostatic screening of net negative charges on raising the concentration of a monovalent cation (e.g., by adding 100 mM KCl) induced thylakoid restacking, as demonstrated in Fig. 1B. Significantly, we have found another way to induce thylakoid stacking in initially unstacked *Arabidopsis* thylakoids in vitro: When a suitable concentration of a large water-soluble protein (BSA) or polysaccharide (dextran) was present in an otherwise unstacking medium, granal stacks were readily observed in a few minutes. For example, 5% BSA (Fig. 1C) or 15% dextran (Fig. 1D), when added to the basic buffer medium supplemented with 30 mM KCl, both induced

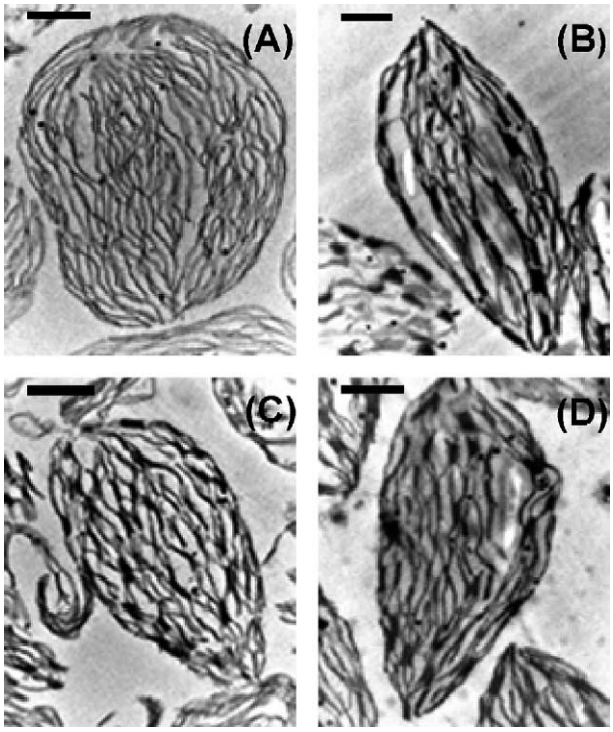

Fig. 1. Transmission electron micrographs of envelope-free *Arabidopsis* chloroplasts suspended in a basic medium to which was added (A) 30 mM KCl, (B) 100 mM KCl, (C) 30 mM KCl + 5% BSA or (D) 30 mM KCl + 15% dextran. The basic medium contained 50 mM HEPES–KOH (pH 7.6, 26 mM K^+ in the background), 300 mM sorbitol and 1 mM EDTA. Each bar represents 1 μm.

thylakoid stacking to an extent similar to that observed in the basic medium containing an added 100 mM KCl (Fig. 1B).

A quantitative measure of the extent of thylakoid restacking was achieved by summing the cross-sectional areas of the grana in each envelope-free chloroplast (Table 1). In the unstacking medium containing only 30 mM added KCl, the total cross-sectional area of the grana was 0.15 μm^2 per chloroplast. Significantly, the total cross-sectional area of the grana per chloroplast increased, by almost an order of magnitude when either 5% BSA or 15% dextran was included, to a value similar to that obtained in the presence of a high concentration of KCl (100 mM) alone which induced thylakoid restacking (Table 1). Moreover, there was no further increase in the extent of thylakoid stacking in 100 mM KCl + BSA or 100 mM KCl + dextran (Table 1).

Another measure of the extent of thylakoid restacking was obtained by sonicating the suspension to yield a mixture of granal stacks and small vesicles derived from the detached stromal lamellae that interconnected the original grana [34]. In effect, more granal stacks in the original suspension produce more granal fragments after sonication. These granal fragments were pelleted by centrifugation at 10,000 × *g* ("10 K pellet") and separated from the supernatant containing vesicles derived from stromal lamellae and small granal fragments. Table 1 shows that the chlorophyll content of the 10 K pellet was small (46%) when the original thylakoid suspension contained only 30 mM added KCl and there was no grana restacking. In contrast, the inclusion of either 5% BSA or 15% dextran increased the Chl content of the 10 K pellet to 59% or 75% of the total Chl, respectively. This finding, for a far larger population of envelope-free chloroplasts than was feasible by transmission electron microscopy, confirms the macromolecule-induced increase in thylakoid restacking seen directly in individual chloroplasts (Fig. 1).

3.2. The local order induced by the restacking of thylakoids is associated with lateral segregation of the two photosystems

Another noteworthy finding from the sonication of envelope-free chloroplasts is the relative abundance of Chl *a* and Chl *b* in the 10 K pellet containing the granal stacks. The inclusion of 5% BSA or 15% dextran in the 30 mM KCl-buffered suspension of envelope free-chloroplasts led to lower Chl *a*/Chl *b* ratios in the 10 K pellet (Table 1). Since the main LHCII trimers are enriched in Chl *b*, a lower Chl *a*/Chl *b* ratio in the 10 K pellet, together with the greatly increased chlorophyll content (Table 1), signifies a marked enrichment of PSII in the pellet, and hence in the grana. In

Table 1
The extent of grana formation and of lateral separation of the two photosystems in *Arabidopsis* envelope-free chloroplasts treated with KCl+BSA or KCl+dextran

	30 mM KCl	30 mM KCl+ 5% BSA	30 mM KCl+ 15% dextran	100 mM KCl	100 mM KCl+ 5% BSA	100 mM KCl+ 15% dextran
*Total granal cross-sectional area chloroplast^{-1} ($\mu m)^2$ (±S.E.; n=20 chloroplasts)	0.15±0.03	1.22±0.08	1.48±0.08	1.36±0.10	1.38±0.07	1.37±0.07
#Percent (%) Chl in 10 K pellet (±S.E.; n=3 preparations)	45.7±4.2	58.7±2.3	75.3±3.2	70.9±2.0	63.7±3.9	69.2±2.6
#Chl *a*/Chl *b* in 10 K pellet	3.59±0.01	3.30±0.06	3.40±0.03	3.37±0.06	3.26±0.02	3.37±0.03
F685/F735 (±S.E.; n=4 preparations)	0.22±0.02	0.28±0.01	0.34±0.03	0.42±0.06	0.36±0.05	0.35±0.03

The extent of grana formation was monitored by *transmission electron microscopy or by #sonication, followed by centrifugation at 10,000 × *g* to sediment a pellet enriched in granal fragments (10 K pellet). The Chl *a*/Chl *b* ratio for whole chloroplasts was 3.65±0.025. Chlorophyll fluorescence from PSII (F685) and PSI (F735) was measured at 77 K, the ratio F685/F735 being higher when spillover from PSII to PSI is restricted due to lateral segregation of PSII from PSI in the restacked thylakoid membranes.

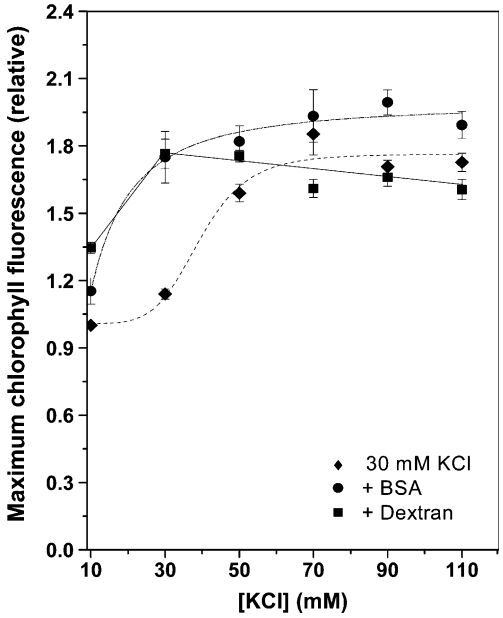

Fig. 2. Maximum room-temperature chlorophyll fluorescence yield in *Arabidopsis* thylakoids in relative units as a function of [KCl] added to the basic buffer medium (see Fig. 1 legend), either in the absence (control) or presence of the macromolecules, BSA (5% w/v) or dextran (15% w/v). Values are the means (±S.E) of 4 replicates.

contrast, the addition of 30 mM KCl alone to the basic medium yielded a Chl a/Chl *b* ratio that was similar to that of the original chloroplasts; in this case, the 10 K pellet represented mainly large fragments of unstacked thylakoid membranes with randomized PSII and PSI.

The lateral segregation of the two photosystems, with PSII complexes located mainly in appressed granal domains and PSI complexes exclusively in non-appressed membrane domains, was confirmed by measurements of chlorophyll fluorescence in the BSA- or dextran-induced granal stacks. At room temperature, the maximum yield of PSII chlorophyll *a* fluorescence (Fm), obtained when the primary quinone acceptor (Q_A) was fully reduced, increased with a rise in [KCl] from 10 to 110 mM (control, Fig. 2). This is consistent with the spatial separation of most PSII from PSI complexes at high [KCl], restricting the spillover of excitation energy from PSII to PSI and, therefore, enhancing fluorescence emission from PSII. With the addition of 5% BSA, importantly, the increase in Fm occurred at lower KCl concentrations. Similarly, on adding 15% dextran, Fm was maximal already at 30 mM KCl; higher concentrations of KCl led, if anything, to a slightly lower value, perhaps due to other effects of dextran on Chl fluorescence yield. At 30 mM added KCl, the effects of BSA and dextran were most striking (Fig. 2).

At 77 K, the Chl fluorescence emission from PSII has a main maximum at 685 nm (F685), and PSI fluorescence emission has a peak at 735 nm (F735). It is known that poor spillover of excitation energy from PS II to PS I corresponds to a high F685/F735 ratio [35]. If the spillover of excitation energy from PSII to PSI were indeed limited by the lateral segregation of the two photosystems on the addition of BSA or dextran, we expect the relative fluorescence emission from PSII at 77 K (F685/F735) to be increased. Therefore, we determined the F685/F735 ratio in spectra normalized to the PSI emission maximum at 735 nm. Fig. 3 shows that the fluorescence ratio F685/F735 was lowest in 30 mM KCl alone, indicating maximum spillover of excitation energy from PSII to PSI with unstacked thylakoids. On adding 5% BSA or 15% dextran, the F685/F735 ratio increased significantly, though not to the full level observed in 100 mM KCl alone. In the presence of 5% BSA, the Chl fluorescence emission had a shoulder between the wavelengths 660 and 680 nm, presumably due to the partitioning of some Chl molecules as free pigment into BSA molecules. Table 1 shows the mean F685/F735 ratios obtained from four experiments. The F685/F735 ratio increased only moderately when 5% BSA was added to 30 mM KCl, but the other four treatments increased the ratio considerably relative to that with 30 mM KCl alone. Taken together, the fluorescence data indicate strongly that the segregation of PSII and PSI was induced by the addition of BSA or dextran to initially unstacked thylakoids in 30 mM KCl added to the basic medium.

3.3. Search for possible effects of BSA and dextran on the electrolytic properties of the assay buffers

In principle, the addition of large quantities of a co-solute such as BSA or dextran could affect the ionic strength of the

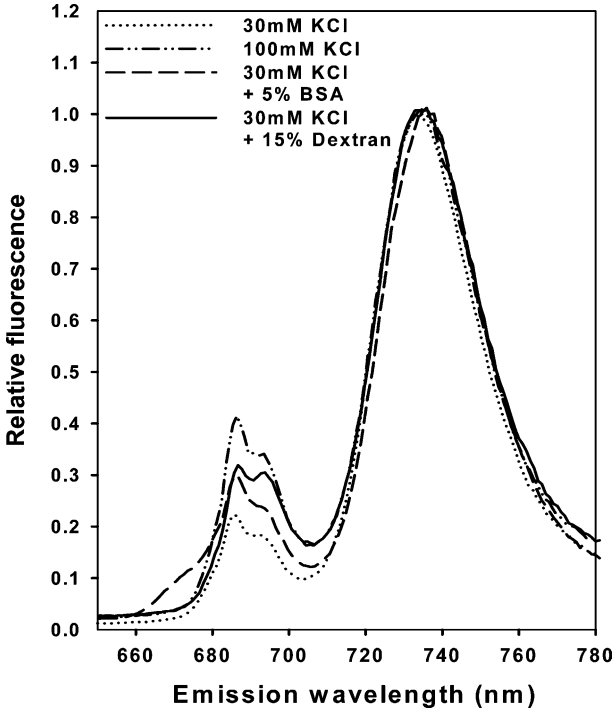

Fig. 3. Chlorophyll fluorescence emission spectra measured at 77 K of *Arabidopsis* envelope-free chloroplasts suspended in media with or without macromolecules added to a basic medium (see Fig. 1 legend). The spectra were corrected for wavelength-dependent instrument response and normalized at 735 nm.

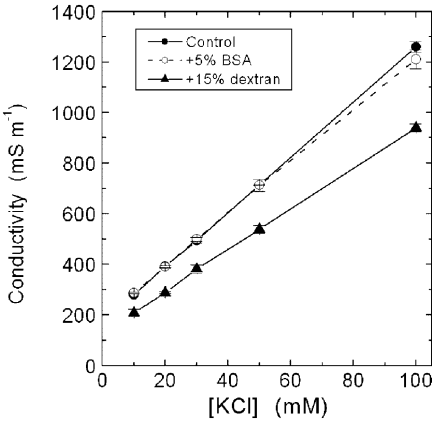

Fig. 4. The electrical conductivity of assay media containing varied concentrations of added KCl, in the presence or absence of 5% BSA or 15% dextran. The basic medium contained 50 mM HEPES–KOH (pH 7.6), 300 mM sorbitol and 1 mM EDTA.

assay buffers, thereby affecting the extent of thylakoid stacking. We therefore investigated the effects of BSA or dextran on the electrical conductivity of the assay media. Fig. 4 shows that 5% BSA had negligible effect on the conductivity at a given [KCl]. Dextran affected the conductivity slightly: At 30 mM KCl, 15% dextran decreased the conductivity to a value corresponding to about 20 mM KCl in the absence of dextran.

4. Discussion

4.1. Thylakoid restacking is driven by increased entropy in the stromal phase

When Good and Izawa invented low-ionic-strength buffers, they made the surprising observation that thylakoid membranes became completely unstacked in such buffers; the addition of high concentrations of K^+ or low concentrations of divalent cations resulted in a spontaneous increase in granal stacks similar to those occurring in vivo [18]. The classical effect of cations being responsible for enhanced electrostatic screening of thylakoid membrane surface charges was considered to be a dominant factor in thylakoid stacking [14–17].

We have made a novel finding that thylakoids restacked spontaneously when a water-soluble macromolecule, BSA or dextran, was added to initially unstacked thylakoids in envelope-free chloroplasts suspended in a buffer containing only 30 mM added KCl (Fig. 1, Table 1). At first sight, there could have been some osmotic effect associated with the added macromolecules. However, given the low concentration of BSA (0.7 mM) and dextran (2.1 mM) added to the basic buffer medium containing 380 mM solutes, any osmotic effect of the added macromolecules would have been negligible. Alternatively, the stacking that occurred at a low [KCl] in the presence of BSA or dextran could be simply due to the sequestration of water molecules bound to the macromolecules, thereby raising the thermodynamic activity of KCl. However, Hatters et al. [36] reported that the addition of 5 to 15% dextran to a solution of 100 mM sodium phosphate (pH 7.4) had no effect on the ionic conductivity or the pH, which are measures of the thermodynamic activity of ions in general and H^+ in particular, respectively. These authors suggested instead that macromolecular crowding accelerates the specific self-association of proteins to form amyloid fibrils. Similarly, we found that the addition of 5% BSA to our buffer containing 30 mM added KCl had negligible effect on the conductivity (Fig. 4). Indeed, dextran (15%) had a slight effect of lowering the conductivity; at 30 mM added KCl, the effect of dextran was equivalent to lowering [KCl] from 30 to 20 mM in the absence of dextran (Fig. 4). Hence, this effect of lowering the conductivity could not have caused the observed increase in thylakoid stacking. Instead, we propose that the granal formation induced by BSA or dextran, which enhances order in the thylakoid membranes, results from an increase in overall disorder (enhanced entropy) in the stroma. The basis of this proposal is that macromolecules, when added at a high concentration to mimic the very high protein and nucleic acid content in the stroma in vivo, initially suffered a restriction of their free diffusion; the envelope-free chloroplasts then underwent spontaneous thylakoid restacking to generate more space for diffusion of the macromolecules present.

The mechanism of entropy-assisted thylakoid stacking is illustrated in Fig. 5A and B. For simplicity, each macromolecule is considered as a hard sphere, the surface of which, at the closest approach to the thylakoid membrane, touches the ordered layer(s) of water molecules (indicated by dotted areas) at the thylakoid membrane surface. That is, the center of each sphere, unable to be any closer to the thylakoid

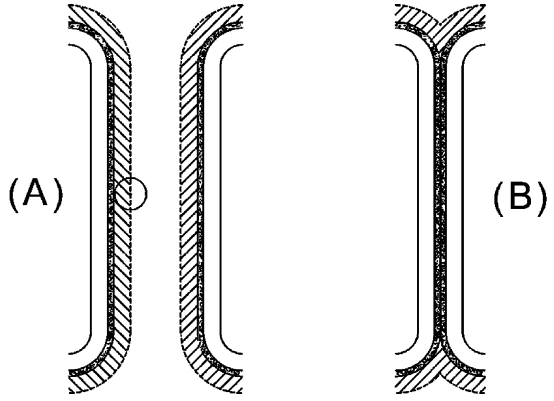

Fig. 5. A schema for the generation of more volume for the diffusion of macromolecules by the appression of thylakoid membranes. In Panel (A), each non-appressed thylakoid membrane (non-shaded) is surrounded by a hydration layer of ordered water molecules (dotted). The center of a macromolecular complex such as the octameric Rubisco (560 kDa, represented by the circle) cannot approach the membrane beyond the dashed line. The "excluded volume" is shown hatched. In Panel (B), two opposite membranes adhere to contact via the hydration layer; the excluded volume (hatched) is now less, yielding increased volume for free diffusion of Rubisco in the stroma.

membrane than indicated by the dashed line (Fig. 5A), is excluded from the volume indicated by the hatched area. When two adjacent thylakoids adhere, they are in contact through the layers of ordered water molecules. In this case, the hatched area is considerably smaller (Fig. 5B), thereby allowing more volume for free diffusion in the stroma. Accordingly, diffusion of each sphere is enhanced, resulting in an increase in overall entropy. In vivo, each sphere can represent a Rubisco enzyme complex that is present at extremely high concentrations in the stroma. Therefore, the in vitro observations made here with BSA and dextran can be extrapolated to Rubisco in vivo (see below).

4.2. Spatial separation of PSII and PSI (order within the membrane) is also assisted by an increase in entropy in the stroma

The addition of BSA or dextran to a suspension of envelope-free chloroplasts, in which the photosystems were initially randomized in the presence of 30 mM KCl added to the basic medium, resulted in the re-formation of grana with a concomitant lateral segregation of PSII from PSI, thereby mimicking the lateral heterogeneity in the distribution of PSII and PSI in vivo. This was demonstrated by an increase in the fluorescence emission (Fm) from PSII at room temperature (Fig. 2) and in the F685/F735 ratio at 77 K (Fig. 3) when the spillover of excitation energy from PSII to PSI was restricted by their spatial separation. The spatial separation of the two photosystems was further confirmed by sonication of envelope-free chloroplasts: The pellet collected at $10{,}000 \times g$, representing fragments of grana, was enriched in Chl *b*, which is mostly found in LHCII trimers. The sequestration of PSII supercomplexes and more loosely-associated LHCII trimers in grana, away from PSI and ATP synthase, represents enhanced thylakoid membrane order generated at the expense of an overall increase in entropy associated with increased free diffusion of the macromolecules added.

4.3. Implications of granal stacking on photosynthetic reaction rates in vivo

By analogy with granal stacking induced spontaneously by the addition of 5% BSA or 15% dextran to artificially unstacked thylakoids, we speculate that the diffusion of stromal enzymes and of the chloroplast gene expression machinery in vivo is enhanced by the stacking of thylakoids. Firstly, Rubisco, the most abundant protein complex in nature, is present at extremely high concentrations in the stroma (about 300–400 mg/ml). Consequently, any change in the ultrastructure of chloroplasts that enhances the diffusion of Rubisco and other stromal enzymes will be thermodynamically favoured. One can estimate the increase in volume for diffusion of Rubisco upon thylakoid stacking. For example, Arvidsson and Sundby [37] provide a cross-section of part of a spinach chloroplast (1.6 μm^2), from which we obtain the total length of appressed pairs of membranes as 22.6 μm. A layer of thickness 0.006 μm (the radius of Rubisco octameric complex) adjacent to each membrane surface is excluded for the diffusion of Rubisco prior to stacking. On stacking, the exclusion area decreases by a significant amount of $22.6 \times 0.006 \times 2 = 0.27$ μm^2 (i.e., 17% of 1.6 μm^2). The percentage decrease in area represents the same percentage decrease in exclusion volume since the areas are multiplied by the same depth. Similarly, we calculated the percentage (%) decrease in exclusion area upon thylakoid stacking for some other high-resolution electron micrographs in the literature, obtaining a mean value of about 17% (Table 2).

A decrease in exclusion volume by 17% in higher-plant chloroplasts would facilitate Rubisco diffusion, thereby increasing entropy. Given the high fractional volume occupancy by stromal enzymes, particularly Rubisco, all association reactions are diffusion limited [31]. Hence, alleviation of macromolecular crowding by thylakoid stacking should also enhance photosynthetic capacity. In addition, a decrease in exclusion volume may facilitate enzyme-catalyzed reactions of small molecules if the mechanism of catalysis involves significant conformational changes of the enzyme [31]. Taken together, these effects of thylakoid stacking tend to enhance metabolic rates in the stroma.

4.4. Steric factors contributing to the lateral heterogeneous distribution of thylakoid membrane complexes

Earlier considerations of what causes PSI and PSII to partition themselves between stromal and granal thylakoids, respectively, suggested that PSI carries more net negative

Table 2
Decrease in cross-sectional exclusion area for Rubisco diffusion upon thylakoid membranes stacking

Decrease in exclusion area for Rubisco as a percentage (%) of total cross-sectional area	Plant	Reference
11.2	Spinach	[42]
13.6	Maize	[43]
15.7	Maize	[44]
16.2	Barley	[45]
16.4	Arabidopsis	[46]
17.0	Spinach	[37]
19.7	Peperomia	[47]
24.1	Spinach	[48]
16.7 ± 1.4 (mean ± S.E.)		

A number of high-resolution electron micrographs of grana and stroma were taken from the literature. The cross-sectional area excluded for the diffusion of the octameric Rubisco complex (taken to be a sphere of radius 6 nm) was calculated as the product of total length of appressed membranes and radius of the Rubisco complex, and expressed as a percentage of the total cross-sectional area of the grana and stroma in each micrograph, neglecting any cross-sectional area of cytosol and starch grains. This percentage represents the gain in cross-sectional area for free diffusion of Rubisco upon thylakoid stacking, i.e., the gain in volume for free diffusion when both cross-sectional areas are multiplied by the same depth.

charge on its stroma-facing surface [14,38]. It was thought that when two membrane surfaces approach each other under the combined influence of van der Waals attraction and decreased electrostatic repulsion due to screening by cations, the two electric diffuse layers overlap, tending to make the surface electric potential more negative. By Le Chatelier's principle, components with more negative charges will diffuse into non-appressed membranes, thereby tending to restore a uniform distribution of surface electric potential [39]. In this view, one would expect PSI, located as it is in non-appressed membrane regions, would be more negatively charged than PSII. However, only circumstantial evidence existed for this idea [2].

Rather, it is more likely that steric factors play a more important role in the segregation of PSI and ATP synthase to non-appressed membrane regions. The early work of Staehelin [40] demonstrated that the bulky head groups of the ATP synthase could not be accommodated in the narrow partition gap. More recently, when the dimensions of PSI were revealed by X-ray crystallography [4,41], it was also clear that the bulky protrusions of PSI at the stroma-facing surface could also not be accommodated in the partition gap.

In summary, we have provided the first experimental support for Chow's hypothesis [2] that the spontaneous appression of adjacent thylakoid membranes to form granal stacks together with the lateral segregation of the photosystems is partly driven by a thermodynamic tendency to increase entropy overall within the chloroplast stroma. We have shown that the addition of the water-soluble macromolecules, BSA and dextran, at a high concentration to artificially unstacked thylakoids, caused spontaneous restacking of thylakoids. Restacking occurred without the need for the addition of extra monovalent or divalent cations or for an increase in ionic conductivity. Rather, the addition of these macromolecules created the need for greater freedom for diffusion of the macromolecules; this need was satisfied by thylakoid stacking which resulted in greater volume for diffusion of the macromolecules (greater entropy). Thus, we have extended the traditional view of thylakoid stacking as being simply the consequence of van der Waals attraction counteracted by electrostatic repulsion, hydration repulsion and steric factors.

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References

[1] B. Andersson, J.M. Anderson, Lateral heterogeneity in the distribution of chlorophyll–protein complexes of the thylakoid membranes of spinach chloroplasts, Biochim. Biphys. Acta 593 (1980) 427–440.
[2] W.S. Chow, Grana formation: entropy-assisted local order in chloroplasts? Aust. J. Plant Physiol. 26 (1999) 641–647.
[3] L.A. Staehelin, Chloroplast structure: from chlorophyll granules to supramolecular architecture of thylakoid membranes, Photosynth. Res. 76 (2003) 185–196.
[4] A. Ben-Shem, F. Frolow, N. Nelson, Crystal structure of plant Photosystem I, Nature 426 (2003) 630–635.
[5] S. Rexroth, J.M.W. Meyer zu Tittingdorf, H.J. Schwassmann, F. Krause, H. Seelert, N.A. Dencher, Dimeric H^+-ATP synthase in the chloroplast of *Chlamydomonas reinhardtii*, Biochim Biophys. Acta 1658 (2004) 202–211.
[6] B. Hankamer, J. Nield, D. Zhelva, E. Boekema, S. Jansson, J. Barber, Isolation and biochemical characterization of monomeric and dimeric Photosystem II complexes from spinach and their relevance to the organization of Photosystem II in vivo, Eur. J. Biochem. 243 (1997) 422–429.
[7] E.J. Boekema, H. van Roon, F. Calkoen, R. Bassi, J.P. Dekker, Multiple types of association of Photosystem II and its light-harvesting antenna in partially solubilized Photosystem II membranes, Biochemistry 38 (1999) 2233–2239.
[8] J.M. Anderson, The significance of grana stacking in chlorophyll b containing chloroplasts, Photobiochem. Photobiophys. 3 (1982) 225–241.
[9] H.-W. Trissl, C. Wilhelm, Why do thylakoid membranes from higher plants form grana stacks?, Trends Biochem. Sci. 118 (1993) 415–419.
[10] P. Horton, A.V. Ruban, R.G. Walters, Regulation of light harvesting in green plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996) 655–684.
[11] W.S. Chow, Electron transport, photophosphorylation and thylakoid stacking, in: C. Sybesma (Ed.), Advances in Photosynthesis Research, vol. III, Dr W. Junk Publishers, The Hague, 1984, pp. 83–86.
[12] J.M. Anderson, Distribution of the cytochromes of spinach chloroplasts between the appressed membranes of grana and stroma-exposed thylakoid regions, FEBS Lett. 138 (1982) 62–66.
[13] J.M. Anderson, E.-M. Aro, Grana stacking and protection of Photosystem II in thylakoid membranes of higher plant leaves under sustained high irradiance: an hypothesis, Photosynth. Res. 41 (1994) 315–326.
[14] J. Barber, W.S. Chow, A mechanism for controlling the stacking and unstacking of chloroplast thylakoid membranes, FEBS Lett. 105 (1979) 5–10.
[15] J.T. Duniec, S.W. Thorne, An analysis of the effect of mono- and di-valent cations on the forces between charged lipid membranes with special reference to the grana thylakoids of chloroplasts, J. Theor. Biol. 79 (1979) 473–484.
[16] J. Barber, Membrane surface charges and potentials in relation to photosynthesis, Biochim. Biphys. Acta 594 (1982) 253–308.
[17] M.J. Sculley, J.T. Duniec, S.W. Thorne, W.S. Chow, N.K. Boardman, Quantitative analysis of the balance of forces between thylakoid membranes of chloroplasts, and the role of divalent cations, Arch. Biochem. Biophys. 201 (1980) 339–346.
[18] S. Izawa, N.E. Good, Effect of salt and electron transport on the conformation of isolated chloroplasts II. Electron microscopy, Plant Physiol. 41 (1966) 544–552.
[19] J. Barber, G.F.W. Searle, Cation induced increase in chlorophyll fluorescence yield and the effect of electrical charge, FEBS Lett. 92 (1978) 5–8.
[20] B.T. Rubin, W.S. Chow, J. Barber, Experimental and theoretical considerations of mechanisms controlling cation effects on thylakoid membrane stacking and chlorophyll fluorescence, Biochim. Biphys. Acta 634 (1981) 174–190.
[21] I.M. Nir, D.C. Pease, Chloroplast organisation and the ultrastructural location of Photosystems I and II, J. Ultrastruct. Res. 42 (1973) 534–550.
[22] J.N. Israelachvili, R.M. Pashley, Molecular layering of water at

surfaces and origin of repulsive hydration forces, Nature 306 (1983) 249–250.
[23] J.E. Mullet, C.J. Arntzen, Simulation of grana stacking in a model membrane system. Mediation by a purified light-harvesting pigment–protein complex from chloroplasts, Biochim. Biphys. Acta 589 (1980) 100–117.
[24] J.F. Allen, How does protein phosphorylation regulate photosynthesis? Trends Biochem. Sci. 17 (1992) 12–17.
[25] D. Stys, Stacking and separation of Photosystem I and Photosystem II in plant thylakoid membranes: a physico–chemical view, Physiol. Plant. 95 (1995) 651–657.
[26] H. Kirchhoff, I. Tremmel, W. Haase, U. Kubitscheck, Supramolecular Photosystem II organization in grana thylakoid membranes: evidence for a structures arrangement, Biochemistry 43 (2004) 9204–9213.
[27] L. Onsager, The effects of shape on the interaction of colloid particles, Ann. N.Y. Acad. Sci. 51 (1949) 627–659.
[28] A.D. Dinsmore, A.G. Yodh, D.J. Pine, Entropic control of particle motion using passive surface microstructures, Nature 383 (1996) 239–242.
[29] M. Adams, Z. Dogic, S.L. Keller, S. Fraden, Entropically driven microphase transitions in mixtures of colloid rods and spheres, Nature 393 (1998) 349–351.
[30] R.J. Ellis, Macromolecular crowding: an important but neglected aspect of the intracellular environment, Curr. Opin. Struct. Biol. 11 (2001) 114–119.
[31] A.P. Minton, The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media, J. Biol. Chem. 276 (2001) 10577–10580.
[32] J. Andersson, R.G Walters, P. Horton, S. Jansson, Antisense inhibition of the photosynthetic antenna proteins CP29 and CP26: implications for the mechanism of protective energy dissipation, Plant Cell 13 (2001) 1193–1204.
[33] R.J. Porra, W.A. Thompson, P.E. Kriedemann, Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophyll *a* and *b* with four different solvents: verification of the concentration of chlorophyll by atomic absorption spectroscopy, Biochim. Biophys. Acta 975 (1989) 384–394.
[34] G. Jacobi, Subchloroplast preparations, in: A. Trebst, M. Avron (Eds.), Photosynthesis: I. Photosynthetic Electron Transport and Photophosphorylation, Springer-Verlag, Berlin, 1977, pp. 543–562.
[35] W.S. Chow, R.C. Ford, J. Barber, Possible effects of the detachment of stromal lamellae from granal stacks on salt-induced changes in spillover. A study by sonication of chloroplasts, Biochim. Biophys. Acta 635 (1981) 317–326.
[36] D.M. Hatters, A.P. Minton, G.J. Howlett, Macromolecular crowding accelerates amyloid formation by human apolipoprotein C-II, J. Biol. Chem. 277 (2002) 7824–7830.
[37] P.-O. Arvidsson, C. Sundby, A model for the topology of the chloroplast thylakoid membranes, Aust. J. Plant Physiol. 26 (1999) 687–694.
[38] W.S. Chow, S.W. Thorne, J.T. Duniec, M.J. Sculley, N.K. Boardman, The stacking of chloroplast thylakoids: evidence for segregation of charged groups into nonstacked regions, Arch. Biochem. Biophys. 216 (1982) 247–254.
[39] J.N. Israelachvili, The packing of lipids and proteins in membranes, in: D.M. Deamer (Ed.), Light Transducing Membranes: Structure, Function and Evolution, Academic Press, New York, 1978, pp. 91–107.
[40] A. Staehelin, Reversible particle movements associated with unstacking and restacking of chloroplast membranes in vitro, J. Cell Biol. 71 (1976) 136–158.
[41] N. Nelson, A. Be-Shem, The complex architecture of oxygenic photosynthesis, Nat. Rev. 5 (2004) 1–12.
[42] K.R. Miller, M.K. Lyon, Do we really know why chloroplast membranes stack? Trends Biochem. Sci. 10 (1985) 219–222.
[43] K.R. Miller, A chloroplast membrane lacking Photosystem I changes in unstacked membrane regions, Biochim. Biphys. Acta 589 (1980) 143–152.
[44] K.R. Miller, The photosynthetic membrane, Sci. Am. 241 (1979) 100–111.
[45] D.J. Simpson, Freeze-fracture studies on barley plastid membranes V. *viridis-n*^{34}, a Photosystem I mutant, Carlsberg Res. Commun. 47 (1982) 215–225.
[46] A. Haldrup, D.J. Simpson, H.V. Scheller, Down-regulation of the PSI-F subunit of Photosystem I (PSI) in *Arabidopsis thaliana*. The PSI-F subunit is essential for photoautotrophic growth and contributes to antenna function, J. Biol. Chem. 275 (2002) 31211–31218.
[47] J.T.O. Kirk, The nature of plastids, in: J.T.O. Kirk, R.A.E. Tilney-Bassett (Eds.), The Plastids, W.H. Freeman and Company, London, 1967, pp. 1–91.
[48] L. Stryer, Photosynthesis, in: L. Stryer (Ed.), Biochemistry, Third edition, W.H. Freeman and Company, New York, 1988, p. 517.