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Entropy and biological systems: Experimentally-investigated entropy-driven stacking of plant photosynthetic membranes

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According to the Second Law of Thermodynamics, an overall increase of entropy contributes to the driving force for any physicochemical process, but entropy has seldom been investigated in biological systems. Here, for the first time, we apply Isothermal Titration Calorimetry (ITC) to investigate the Mg^{2+} -induced spontaneous stacking of photosynthetic membranes isolated from spinach leaves. After subtracting a large endothermic interaction of $MgCl_2$ with membranes, unrelated to stacking, we demonstrate that the enthalpy change (heat change at constant pressure) is zero or marginally positive or negative. This first direct experimental evidence strongly suggests that an entropy increase significantly drives membrane stacking in this ordered biological structure. Possible mechanisms for the entropy increase include: (i) the attraction between discrete oppositely-charged areas, releasing counterions; (ii) the release of loosely-bound water molecules from the inter-membrane gap; (iii) the increased orientational freedom of previously-aligned water dipoles; and (iv) the lateral rearrangement of membrane components.

Stacking of photosynthetic membranes (thylakoids) to form grana interconnected by non-stacked thylakoids, bathed in an aqueous stroma, is ubiquitous in plants, being required for fine-tuning (a) photosynthesis, (b) photoprotection and (c) acclimation to environments^{1–4}. Advantages of granal stacking include: (1) an extremely large membrane surface-to-volume ratio to accommodate a high density of chlorophyll (Chl) to promote light-harvesting⁵; (2) a spatial separation of the two photosystems⁶ which limits excessive spillover of excitation energy from Photosystem II (PS II, responsible for oxygen evolution) to PS I^{1,2,7}; (3) enhancement of non-cyclic photophosphorylation to increase the “energy currency”⁸; (4) regulation of non-photochemical dissipation for protection against excess light⁹; (5) delay of premature degradation of D1 protein in the PS II reaction centre at sustained high irradiance¹⁰; (6) regulation of linear versus cyclic electron transport¹¹; and (7) a potential increase in photosynthetic capacity for a given chloroplast composition⁴.

Here we monitored Mg^{2+} -induced stacking of spinach photosynthetic membranes to address the question of what thermodynamic attractive force drives granal stacking. Granal stacking should depend on the net sum of (1) hydration repulsion, (2) electrostatic repulsion, (3) van der Waals attraction, and (4) entropy-driven attraction. Hydration repulsion is very short-ranged, originating from the displacement of structured water molecules around charged groups on membranes surfaces¹². It partly determines the separation between stacked thylakoids, recently estimated as 3.2 nm¹³.

Electrostatic repulsion, caused by the net negative charge on the outer surface of thylakoid membranes, is a major factor opposing granal stacking^{5,14–18}. A net negatively-charged membrane in an electrolyte is surrounded by a diffuse layer of counterions and is therefore electrically neutral; however, bringing two such membranes together would squeeze the diffuse counterion layers which then resist with an osmotic pressure. This is the origin of the electrostatic repulsion between two membrane surfaces in an aqueous ionic medium, as distinct from air. Notably, the electrostatic repulsion can be decreased by adding ionic solutes to provide charge screening^{4,15–20}.

However, since even in the presence of cations the electrostatic force is still repulsive, an attractive force that exceeds the electrostatic repulsion is required for granal stacking. van der Waals attraction is long-range, often dominated by the London dispersion force among instantaneously-induced dipoles. There has been only one published attempt at calculating it for thylakoids^{18,21}. Significantly, the calculated van der Waals attraction is vastly

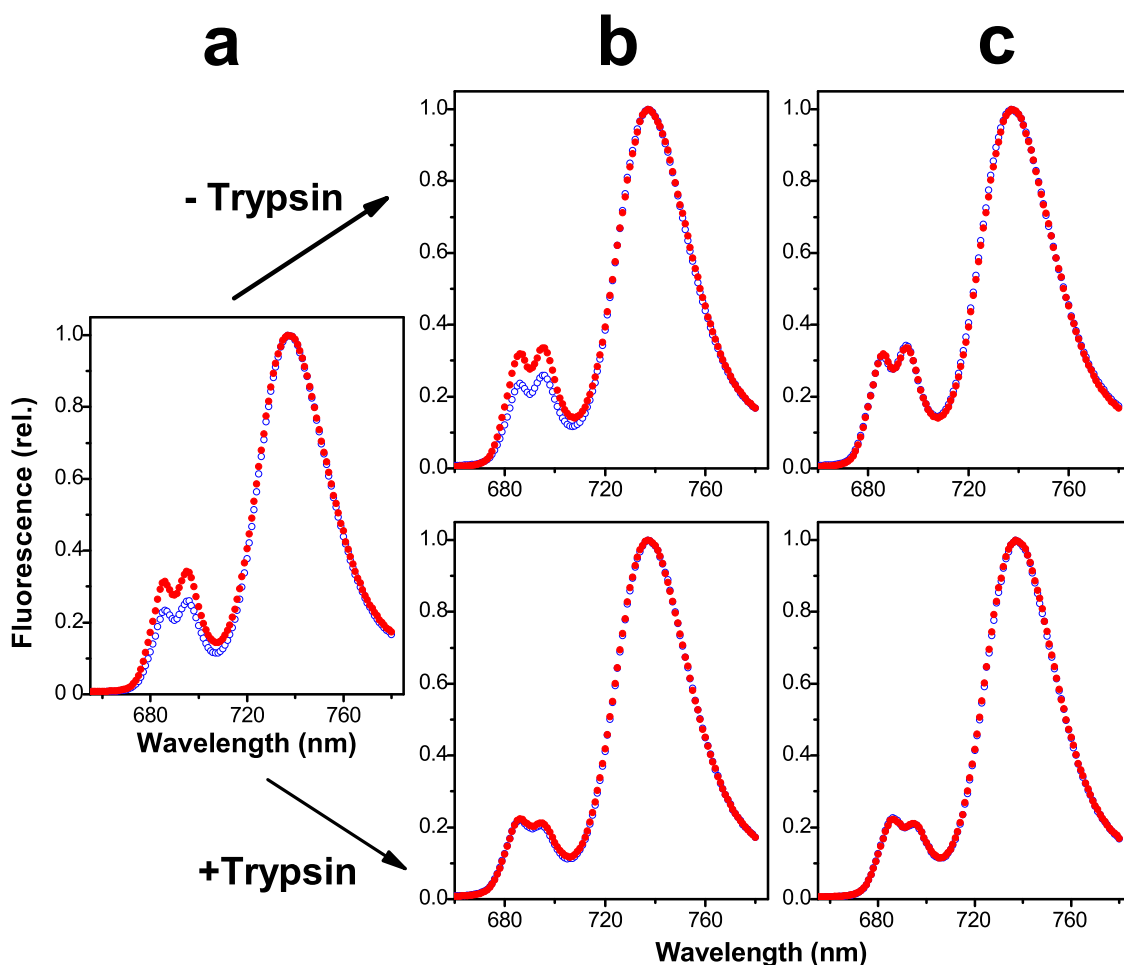


Figure 1 | Fluorescence emission spectra at 77 K of previously-unstacked membranes placed in the absence (blue open circles) or presence (red closed circles) of 5 mM MgCl_2 . Samples were taken (a) from the thylakoid stock suspension before addition to the ITC sample cell; (b) from the ITC sample cell immediately after addition to the cell but before the commencement of calorimetric titration; and (c) from the ITC sample cell at the end of titration. The excitation wavelength was 440 nm. Spectra were obtained as described in Methods.

weaker than the electrostatic repulsion; therefore, the van der Waals attraction might not effect membrane stacking. What then is the main attractive force?

Chow¹⁴ hypothesized that a high concentration of stromal macromolecules, such as the abundant ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) complex or ribosomes, results in an entropically-driven force (depletion attraction): granal stacking allows greater volume for free diffusion of stromal macromolecules in a crowded environment. This was observed in thylakoids *in vitro*, using negatively-charged bovine serum albumin or neutral dextran as the macromolecules¹⁵. Nevertheless, the question remains as to what attractive force overcomes the electrostatic repulsion when MgCl_2 , rather than macromolecules, is added to unstacked thylakoids.

To investigate the nature of the attractive force driving Mg^{2+} -induced granal stacking, we used the highly sensitive technique of ITC to directly measure the enthalpy change (ΔH_{exp}). We also obtained the theoretical maximum and minimum sum of the component enthalpy changes ($\Delta H_{\text{tot max}}$ and $\Delta H_{\text{tot min}}$) from published component forces between thylakoid membranes as functions of inter-membrane separation. The results show that the theoretical enthalpy change is slightly positive and that the experimental enthalpy change, while the mean value obtained from several experiments is slightly negative, could either be zero, slightly positive or slightly negative within experimental error. This is the first direct experimental evidence which strongly suggests that an entropy

increase might indeed drive the spontaneous, Mg^{2+} -induced stacking of membranes in this intriguingly ordered biological structure. Possible mechanisms for the entropy increase are discussed.

Results

Monitoring the stacking of thylakoids in isolated envelope-free chloroplasts. Chl fluorescence emission spectra of a dilute suspension of envelope-free chloroplasts at 77 K exhibit peaks: a double peak emitted by PS II at 686 and 696 nm, and a single peak emitted by PS I at 736 nm (Figure 1). The double peak is lowered relative to the single peak upon unstacking of the thylakoids prior to freezing at 77 K. This is because unstacking induces a random mixing of the two photosystems within the membrane system, and increases “spillover” of excitation energy from PS II to PS I, thereby lowering the Chl fluorescence emission by PS II. Therefore, the height of the double peak relative to that of the single peak is a convenient measure of the extent of granal stacking, a higher double peak indicating a greater extent of granal stacking. Figure 1 shows the lower double peak exhibited by unstacked membranes (blue open circles). Before and during the ITC experiments, small aliquots of the membrane suspensions were taken from three sampling times. Figure 1 shows that in the presence of MgCl_2 (red closed circles) at any sampling time, the membranes were stacked (high double peak) in the absence but not the presence of a low molar ratio of trypsin to Chl.



Table 1 | Experimental ΔH_{exp} and theoretical values of the component enthalpy changes of Mg^{2+} -induced stacking of thylakoid membranes. Values of the enthalpy changes are in kJ mol^{-1} Chl. Endothermic changes are positive, and exothermic changes negative. Column 1 (ΔH_{exp}) is the experimental enthalpy change of thylakoid stacking, calculated as described in Methods, averaged from a total of six days' experiments, displayed as mean \pm S.D. Columns A-D refer to ΔH after integration of the electrostatic repulsion calculated at constant surface charge density (σ) or constant surface potential (ψ). The values in the rows marked 2 nm, 3 nm and 4 nm were obtained by integrating the curves from membrane separations (Gap) of 2 nm, 3 nm and 4 nm, respectively, to the maximum distance in the calculations²¹; the values shown in square brackets were obtained by extrapolation to separations below those in the calculations²¹ (see Methods). The row marked 3 nm has been shaded because the most recent estimate yielded 3.2 nm for the inter-membrane gap¹³. ΔH_{tot} is the algebraic sum of the electrostatic, van der Waals and hydration enthalpy changes: $\Delta H_{\text{tot}}(\text{max}) = \Delta H_{\sigma}(1) + \Delta H_{\text{hyd}} + \Delta H_{\text{vdW}}(2)$. $\Delta H_{\text{tot}}(\text{min}) = \Delta H_{\psi}(2) + \Delta H_{\text{hyd}} + \Delta H_{\text{vdW}}(1)$. V_{pr} is the volume fraction of protein in the membrane, and ϵ is the assumed dielectric constant

ΔH_{exp}	Gap	(A)	(B)	(C)	(D)	(E)	(F)	(G)	ΔH_{tot} (max)	ΔH_{tot} (min)
		ΔH_{σ}	ΔH_{ψ}	ΔH_{σ}	ΔH_{ψ}	ΔH_{hyd}	ΔH_{vdW}	ΔH_{vdW}		
		(1)	(1)	(2)	(2)		(1)	(2)		
		1 mM K ⁺ 50 μM Mg ²⁺	1 mM K ⁺ 50 μM Mg ²⁺	10 mM K ⁺ 5 mM Mg ²⁺	10 mM K ⁺ 5 mM Mg ²⁺	10 mM K ⁺ 5 mM Mg ²⁺	$\epsilon = 3$ $V_{\text{pr}} = 0.6$	$\epsilon = 2.5$ $V_{\text{pr}} = 0.4$		
-0.20 \pm 0.51	2 nm	[1.37]	[0.68]	[1.21]	[0.66]	0.015	-0.037	-0.021	1.36	0.64
	3 nm	[0.68]	[0.45]	[0.49]	[0.35]	0	-0.013	-0.006	0.67	0.34
	4 nm	0.36	0.29	0.22	0.19	0	-0.007	-0.002	0.36	0.18

Experimental enthalpy change ΔH_{exp} associated with granal stacking. MgCl_2 was titrated into suspensions of unstacked thylakoids, some of which had had minute concentrations of trypsin (1:3,000 molar ratio of trypsin to chlorophyll) added to them (trypsin is known to render thylakoid membranes incapable of stacking^{22,23}), at a membrane concentration equivalent to 0.2 mM Chl (see Methods). Subsequent Chl fluorescence analysis of samples frozen at 77 K revealed that the membrane suspensions without trypsin had stacked during the course of the ITC experiment while those with added trypsin had not. The positive enthalpy signal of the raw ITC plot (see Methods) reveals that the overall interaction of MgCl_2 with the thylakoid membranes was endothermic. The enthalpy change of the interaction was about 3.4 kJ per mol Chl at a membrane concentration equivalent to 0.2 mM Chl, decreasing markedly to 1.25 kJ mol^{-1} Chl at 1 mM Chl, and 0.33 kJ mol^{-1} Chl at 3.3 mM Chl (data not tabulated). At 1 mM Chl, the interaction was 50% complete at about 1 mM MgCl_2 (data not shown). Notably, virtually identical plots were obtained regardless of whether the membranes stacked or did not stack (see Methods). This shows that the endothermic interaction is predominantly unrelated to stacking but is presumably due to the interaction of Mg^{2+} ions (or Cl^- ions) with membrane components.

We determined the enthalpy change of membrane stacking (at 0.2 mM Chl) by subtracting the ΔH values obtained from the ITC plots for those membranes which were shown by subsequent Chl fluorescence spectral analysis not to have stacked during the course of the experiment from those obtained from the ITC plots for those membranes which were shown to have stacked. This yielded an average enthalpy change of $\Delta H_{\text{exp}} = -0.20 \pm 0.51 \text{ kJ mol}^{-1}$ Chl (first column of Table 1).

Thus, the magnitude of ΔH_{exp} was very small: while the mean value in the first column of Table 1 was marginally below zero, the actual value could either be zero or marginally above or below zero within experimental error. Since the Gibbs free energy change (ΔG) for the spontaneous stacking of thylakoids upon adding Mg^{2+} at an absolute temperature T must be appreciably negative, this observation implies that an increase in entropy (ΔS) probably makes a significant contribution to the negative Gibbs free energy change $\Delta G = \Delta H - T \Delta S$.

Theoretical sum of component enthalpy changes ΔH_{tot} associated with granal stacking. The theoretical calculations of the electrostatic repulsion made by Rubin *et al.* (1981)²¹ and the hydration repulsion and the van der Waals attraction between thylakoid membranes calculated by Sculley *et al.* (1980)¹⁸ have been reproduced in a linear plot in Figure 2 as functions of the distance between

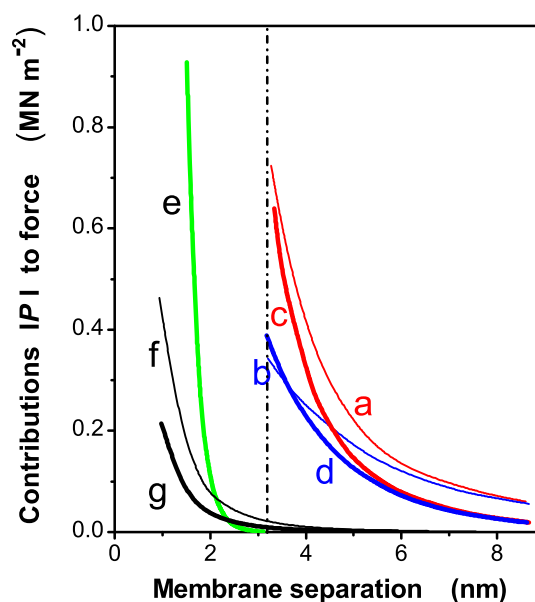


Figure 2 | Theoretical calculations of the contributions, $|P|$, to the force between two membranes as a function of separation. Re-plotted on a linear scale from Figure 8 of Rubin *et al.*²¹. The curves a, b, c and d represent the electrostatic repulsion in two ionic media (one stacking and the other unstacking), calculated at either constant surface charge density or constant surface potential. Curve e denotes the hydration repulsion. Curves f and g denote the van der Waals attraction, corresponding to dielectric constants of 3 and 2.5, respectively, with volume fractions of protein (V_{pr}) in the membrane of 0.6 and 0.4, respectively. The dashed vertical line denotes a very recent estimation of membrane separation¹³.



membranes; the linear plot gives a better visual impression than a semi-log plot^{18,21} of the magnitudes of these component forces. The various electrostatic forces (curves a, b, c and d) and the hydration force (curve e) are repulsive while the two van der Waals curves (f and g) are attractive. The theoretical enthalpy changes of stacking produced by these forces were calculated by integrating the curves in Figure 2 to three membrane-separations (gap). The theoretical ΔH_{tot} consists of endothermic ΔH components associated with electrostatic repulsion and hydration repulsion, and an exothermic component associated with van der Waals attraction. Table 1 depicts the theoretical values of the total enthalpy (ΔH_{tot}) for membrane stacking calculated for three inter-membrane gaps. For a gap of 3 nm (shaded row in Table 1), the total enthalpy change (ΔH_{tot}) for membrane stacking ranged from a lower limit, ΔH_{tot} (min), of 0.34 kJ mol^{-1} Chl to an upper limit, ΔH_{tot} (max), of 0.67 kJ mol^{-1} Chl. For a gap of 4 nm (Table 1), the lower limit was 0.18 kJ mol^{-1} Chl while the upper limit was 0.36 kJ mol^{-1} Chl. The experimentally determined value, $-0.20 \pm 0.51 \text{ kJ mol}^{-1}$ Chl, falls just outside the range for a 3 nm gap but within the range for a 4 nm gap, which is consistent with the recently estimated gap distance of 3.2 nm^{13} . Further, if the dielectric constant ϵ of the thylakoid membrane were greater than 3, ΔH_{vdw} (Table 1) would be more negative, making the ΔH_{tot} less positive and the experimental value more within the range of theoretical estimates. Hence, the *positive* theoretical (ΔH_{tot}) enthalpy change and the *near zero* experimental (ΔH_{exp}) enthalpy change strongly imply that a significant entropic driving force is required to achieve an appreciably negative ΔG and to effect spontaneous stacking on adding MgCl_2 .

Discussion

How could an entropy-driven attraction arise on adding Mg^{2+} to unstacked isolated thylakoids? Here, we suggest four ways whereby entropy can be increased.

Firstly, the light-harvesting complex II (LHC-II) is a major component of higher plant thylakoid membranes. The outer stroma-facing surface of an LHC-II trimer consists of discrete areas of net positive charge separated from areas of net negative charge²⁴. A positive area on one LHC-II trimer could interact *non-specifically* with a negative area on an LHC-II trimer in the opposite membrane; this “Velcro effect” could play a major role in granal stacking²⁴. A similar idea²² was advanced earlier, though its “contact mechanism” requires interaction between *specific* groups of opposite charge. Each positively-charged or negatively-charged area is surrounded by a diffuse aqueous layer containing counterions. When two membranes experience less electrostatic repulsion upon adding screening cations such as Mg^{2+} , and begin to approach each other such that areas of opposite charge face each other (Figure 3a), counterion pairs are released from the inter-membrane gap to diffuse freely as individual ions in the bulk medium, thereby gaining entropy²⁵.

Secondly, upon adding MgCl_2 , as two domains of opposite charge come closer, loosely-bound water molecules could be released from the inter-membrane gap to the bulk medium (Figure 3b), thus increasing entropy. Such release occurs because the end of a water dipole pointing away from one domain is attracted by an approaching domain of opposite charge.

Thirdly, in an unstacking medium (without Mg^{2+}), the local electric field strength (E) near a negatively-charged (or a positively-charged) area may be high enough to align water dipoles locally, thereby restricting their orientational freedom (Figure 3c). Assuming that the local surface charge density of a negatively-charged area is 10 times the overall *net* negative surface charge density (σ) of the stroma-facing membrane surface, the estimated magnitude of E near the charged area in an unstacking electrolyte can be calculated. Figure 4 depicts the variation of E with distance from a negatively-charged area of LHC-II for the two ionic compositions specified in the figure legend. In the unstacking Z^+/Z^- electrolyte, the

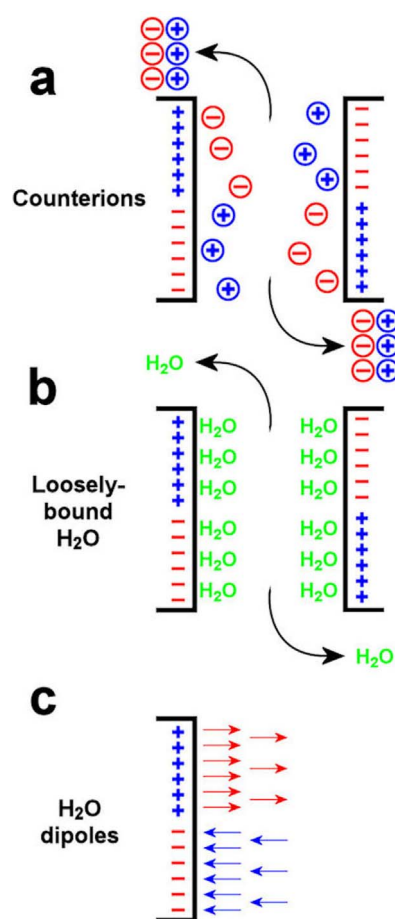


Figure 3 | Mechanisms by which entropy can be increased by the stacking of two thylakoid membranes. (a), areas of opposite charge approach each other in a Velcro effect, expelling pairs of counterions which then diffuse freely as individual ions. (b), loosely-bound water molecules are released from the inter-membrane gap when areas of opposite charge approach each other, thereby diffusing freely. (c), in an *unstacking* ionic medium, the electric field strength adjacent to the membrane surface is sufficient to align water dipoles, but is diminished on adding Mg^{2+} (details in Methods and Discussion); loss of alignment increases the orientational freedom of water dipoles.

local electric field strength E at the negatively-charged area surface is approximately 1.14 GV m^{-1} (upper curve). A water molecule with a dipole moment of $6.17 \times 10^{-30} \text{ C m}$ will have minimum potential energy $1.14 \text{ GV} \times 6.17 \times 10^{-30} \text{ C m} = 7.0 \times 10^{-21} \text{ J}$, with a magnitude almost twice the kinetic energy per water molecule of $kT = 4.1 \times 10^{-21} \text{ J}$, where k is Boltzmann’s constant. Therefore, the electric field strength near a negatively-charged area on the stroma-facing surface of LHC-II could be high enough to align water dipoles against their random orientational motion. Upon adding Mg^{2+} , however, E is reduced by a factor of about 4 (lower curve), and the alignment is almost certainly lost. Similarly, a positively-charged area of the stroma-facing surface of LHC-II could induce an electric field strong enough to align water molecules in an unstacking buffer, this time in the opposite orientation; E could again be much diminished in a stacking medium. In either case, the gain in orientational freedom of water dipoles upon adding MgCl_2 could then contribute to an increase in entropy that drives the stacking of thylakoids. The increased orientational freedom of water molecules on adding MgCl_2 should compensate for the rise in potential energy of water dipoles (reaching a less negative value) in a diminished electric field; in turn, the rise in potential energy of water dipoles should promote

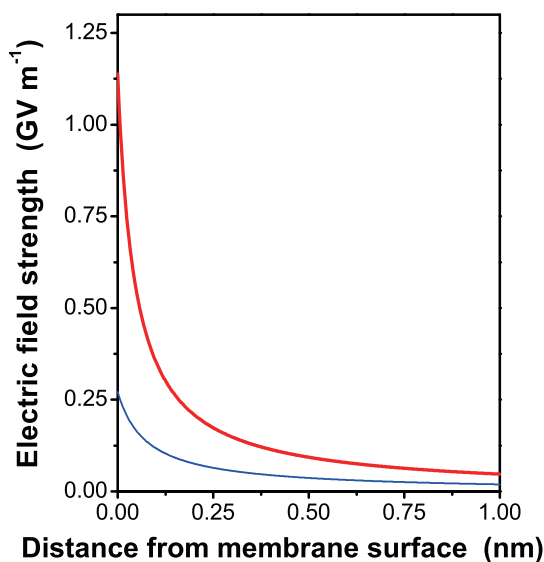


Figure 4 | The electric field strength as a function of distance from a charged surface with a local surface charge density of -0.25 C m^{-2} , calculated in Methods. This value is assumed to be ten times the *net* negative overall surface charge density on thylakoid membranes. The upper thick curve refers to an unstacking ionic medium (20 mM monovalent cation, 20 mM monovalent anion), and the lower thin curve to a stacking medium (20 mM monovalent cation, 30 mM monovalent anion and 5 mM divalent cation).

the above-mentioned release of loosely-constrained water molecules into the bulk medium, thereby increasing entropy.

Fourthly, there is the entropy-driven attraction (depletion attraction²⁶) by which large membrane-spanning complexes ('large particles'), such as PS II and LHC-II, are pushed together to form dimers and trimers, respectively, within a single membrane, so as to free up volume for diffusion of smaller intra-membrane 'small particles' (lipids and plastoquinone)¹⁴. The aggregation of PS II and LHC-II will position the protein complexes on opposite membranes for the Velcro effect. Additionally, there is more entropy to gain from the aggregation of PS II and LHC-II if the acyl chains of the lipids are highly unsaturated compared with saturated acyl chains: the bend in unsaturated acyl chains due to *cis*-double bonds contributes more to entropy if the unsaturated acyl chains are given more space in which to flex freely, when compared with saturated acyl chains. Consistent with this expectation, granal stacking is enhanced in thylakoids with highly-unsaturated lipids²⁷.

Finally, it is intriguing how, in previously-unstacked thylakoids, stacking may be *initiated* by adding Mg^{2+} . The membranes have to be brought to a sufficiently small separation for the Velcro effect to operate. We envisage that an unstacked membrane system "wobbles", such that occasionally a localized protein-rich domain on one membrane comes sufficiently close to one on the opposite membrane. The gain in entropy from a localized Velcro effect would lower the free Gibbs energy, enabling the adhesion to propagate to other regions of the membrane system.

Mg^{2+} and K^{+} being abundant in the chloroplast stroma²⁸, the *in vitro* effect of Mg^{2+} on inducing thylakoid stacking observed here, and of high concentrations of monovalent cations reported elsewhere^{21,29}, should also apply *in vivo*. Our study of what strongly appears to be an entropy-driven attraction in Mg^{2+} -induced granal stacking, together with entropy-driven depletion attraction in the presence of macromolecules¹⁵, highlights the importance of the unifying concept of an entropy-driven attraction that determines chloroplast ultrastructure generally, *in vivo* and *in vitro*, thereby regulating chloroplast function indirectly. This and other hitherto

rare investigations of the contributions of entropy, as formulated by the Second law of Thermodynamics, to the driving forces of biological processes will lead to a deeper understanding of biological systems.

Methods

Plant growth. *Spinacea oleracea* L. (cv. Yates hybrid 102) plants were grown in a glasshouse during spring at approximately 28/15°C (day/night) under about 10% natural light to minimize starch content.

Preparation of thylakoid membranes. Leaves were homogenized at 6°C in a buffer containing 20 mM Tricine-KOH (pH 8.4), 0.3 M sorbitol, 10 mM EDTA, 10 mM NaHCO_3 , 5 mM MgCl_2 , 10 mM KCl, 4.5 mM sodium ascorbate and 0.5% bovine serum albumin. The homogenate was filtered through 6 layers of muslin and centrifuged at $3,000 \times g$ for 2 min at 4°C. After re-suspension in 20 mM Tricine-KOH (pH 7.6), 0.3 M sorbitol, 5 mM MgCl_2 and 2.5 mM EDTA, the chloroplasts were osmotically shocked in 5 mM MgCl_2 for 1 min. An equal volume of medium was added to give 50 mM Hepes-KOH (pH 7.6), 0.3 M sorbitol, 2.5 mM MgCl_2 and 10 mM KCl. Following centrifugation at $3,000 \times g$ for 2 min at 4°C, the thylakoid membranes were re-suspended in a small volume of buffer containing 20 mM Tricine-KOH (pH 7.6), 2.5 mM EDTA and 0.3 M sorbitol.

Chlorophyll determination. Chlorophyll was determined in buffered 80% acetone according to Porra *et al.*³⁰.

Monitoring stacking of the thylakoid membranes by Chl fluorescence spectral analysis. Small aliquots of the membrane suspensions were taken: (a) from the thylakoid stock suspension before addition to the ITC sample cell; (b) from the ITC sample cell immediately after addition to the cell but before the commencement of calorimetric titration; and (c) from the ITC sample cell at the end of titration (Figure 1). Each aliquot was divided into two portions. To one portion was added MgCl_2 (final concentration 5 mM, Figure 1, red closed circles) which would induce stacking of the membranes if they were capable of stacking. The other portion had no MgCl_2 added (blue open circles). Each portion was diluted to 2 μM Chl in the ITC buffer containing 20 mM Hepes (pH 7.6), 0.3 M sorbitol and 10 mM KCl, with or without 5 mM MgCl_2 , and frozen for 77 K fluorescence spectral analysis to determine the extent of membrane stacking.

A spectrofluorimeter (SLM-8100, Spectronic, Rochester, NY) excited the membrane samples at 440 nm, to yield the PS II double emission peak (686 nm and 696 nm) and the PS I peak (736 nm) at 77 K, corrected for wavelength-dependent instrument response. The ratio PS II peak/PS I peak represents the partitioning of absorbed light energy between the two photosystems: the greater the extent of stacking, the higher the PS II double peak relative to the PS I peak.

Isothermal titration calorimetry (ITC). ITC experiments were conducted using a Microcal VP-ITC (MicroCal, Northampton, USA). The stock membrane suspension was diluted to 0.2 mM Chl, unless otherwise specified, in the ITC buffer. The membrane suspension was degassed, loaded into the ITC sample cell (volume 1.44 mL), and kept in darkness. Aliquots of a 50 μM trypsin solution were added to some samples (molar ratio of 1 trypsin:3,000 Chl). Incubation with trypsin was for ~30 minutes before the addition of MgCl_2 (25 mM) from a syringe (capacity 300 μL). Each day, at least three experiments were performed without added trypsin and at least two were performed with added trypsin. The experiment was conducted at 25°C with the syringe needle stirring at 260 rpm, and consisted of 3 injections, each of 95 μL , with a duration of 190 s, spaced 10 minutes apart, unless otherwise specified (Fig. 5a).

Calculation of the experimental enthalpy change of membrane stacking from the ITC data. The pre-injection and post-injection baselines were extrapolated across each injection (Figure 5a, b, d and e). Linear fits were made to the segments of the plot immediately before, and at the end of, each injection (Figure 5b and e) and the slopes measured. A cubic polynomial continuous with the pre-injection and the post-injection segments was calculated, the instantaneous slopes of the polynomial equalling the slopes of the pre-injection and the post-injection segments. The extrapolated baseline was then subtracted from the raw heat capacity plot to give the heat capacities ($\mu\text{J s}^{-1}$) (Figure 5c and f). The enthalpy change of each injection was obtained by integration. The interaction of Mg^{2+} ions with the thylakoid membrane was deemed complete by the end of the second injection; therefore, the raw enthalpy of the third injection was taken to represent the heat of dilution of the third injection of MgCl_2 solution. This value, corrected for the progressive dilution of the contents of the sample cell, was subtracted from the enthalpies of both the first and the second injections, to give the enthalpies of interaction of Mg^{2+} ions with the thylakoid membrane of the first and the second injections. The values for the first and second injections were summed and divided by the amount of chlorophyll in the sample ($1.44 \text{ mL} \times 0.2 \text{ mM} = 288 \text{ nmol}$), to give the molar enthalpy of the total interaction of Mg^{2+} ions with the thylakoid membrane. The values for all experiments with no added trypsin performed on a particular day were averaged, as were those obtained on that same day with added trypsin. The average molar enthalpy of experiments performed with trypsin (shown by subsequent fluorescence analysis not to have stacked) was then subtracted from that of experiments performed without trypsin

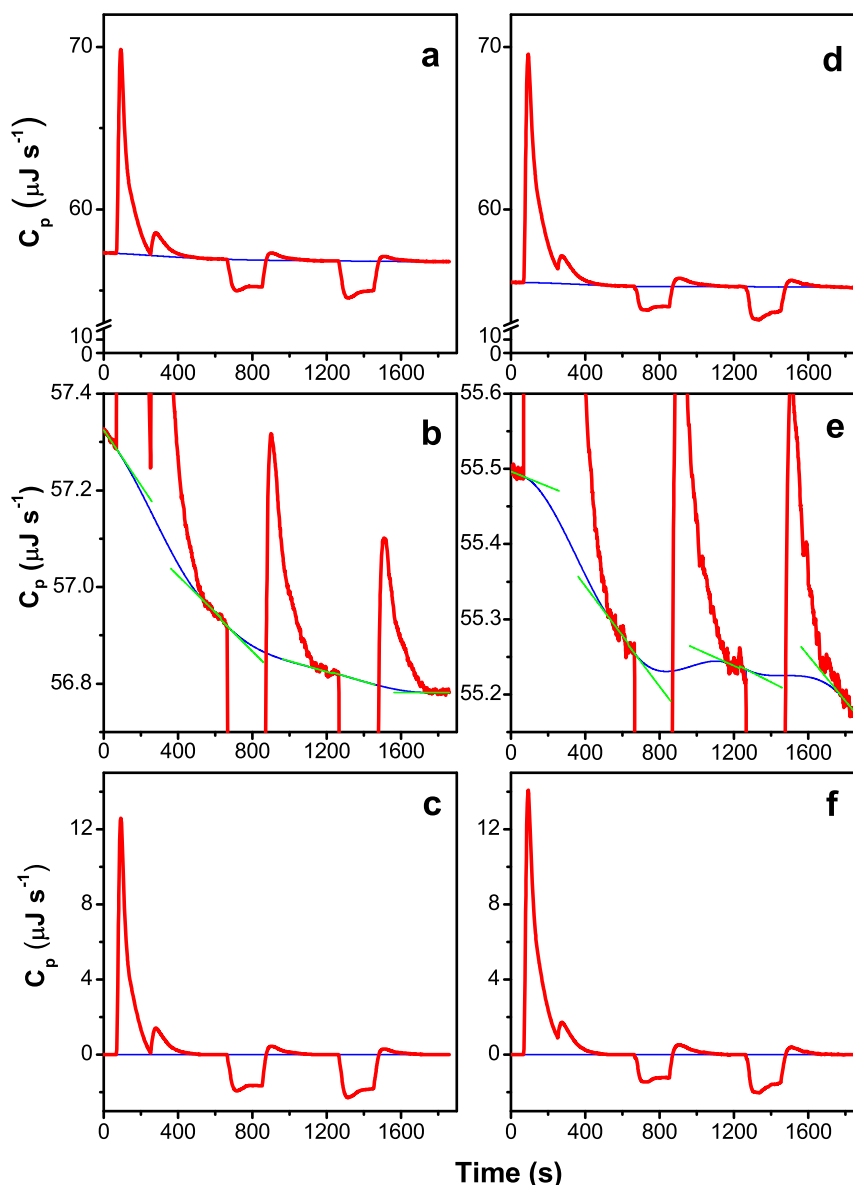


Figure 5 | Heat-capacity (C_p) plots and extrapolated baselines obtained from injection of $MgCl_2$ into thylakoid suspensions which did not contain trypsin or which did contain trypsin. [Chl] in the suspensions was 0.2 mM. [$MgCl_2$] in the syringe was 25 mM. Three injections, each of 95 μ Ls and duration 190 s, were made at 60 s, 660 s and 1,260 s. Heat capacity plots obtained from thylakoid suspensions which (a) did not contain trypsin or (d) contained trypsin at a 1 : 3,000 molar ratio of trypsin to chlorophyll. (b) and (e) are expanded segments of the same plots shown in (a) and (d). The thick red curves denote the raw ITC data and the thin blue curves denote the extrapolated baselines, calculated as described in Methods. The green straight lines in (b) and (e) denote the linear fits made to the segments of the plot immediately before each injection and at the end of each injection. (c) and (f) are the plots shown in (a) and (d) after the baselines have been subtracted. The thick red curves denote the data plots and the thin blue lines denote the x-axes.

(shown by subsequent fluorescence analysis to have stacked) to give the average enthalpy change of membrane stacking for that day. The enthalpy changes of membrane stacking for six days were averaged (Column 1, Table 1). The large relative error is due to the enthalpy change being actually the near-zero difference of two large enthalpies which must be subtracted one from the other, resulting in an absolute error which is large in comparison.

Calculation of the theoretical component enthalpy changes of membrane stacking. The theoretical calculations made by Sculley *et al.* (1980)¹⁸ and Rubin *et al.* (1981)²¹ of the various forces between thylakoid membranes as functions of inter-membrane distance are reproduced on a linear scale in Figure 2. The theoretical enthalpy changes of stacking produced by these forces, represented by the areas under the curves, were calculated by integration of these curves, yielding values in units of $J (nm)^{-2}$ of membrane area. Each Chl molecule corresponds to a thylakoid membrane area of $1.71 \pm 0.14 nm^2$ for spinach³¹. However, the units of membrane area in Figure 2 are really units of area for a pair of stacked membranes and therefore represent double units of membrane surface area. Thus, $1.71 nm^2$ of the double

membrane of Figure 3 really corresponds to 2 chlorophyll molecules. The enthalpy changes obtained by integrating the curves in Figure 2 were therefore multiplied by $0.5 \times 1.71 nm^2 molecule^{-1}$ Chl and then multiplied by Avogadro's number to give units of $J mol^{-1}$ Chl.

Approximate estimates of the electrostatic enthalpies at membrane separation (Gap) distances lower than for which the forces were calculated by Rubin *et al.* (1981)²¹ were obtained by extrapolation. The slopes of the semi-log plots shown in Figure 8 of Rubin *et al.*²¹ were measured at the lowest Gap distances for which they had been calculated: 3.3 nm for the electrostatic forces. The semi-log plots were then, using straight lines, extrapolated back to 2 nm. Enthalpies were obtained by integration.

Calculation of the electric field strength near negatively-charged areas of the stromal surface of LHC-II trimers. For a negatively-charged surface (surface charge density of magnitude $|\sigma|$) bathing in a mixed electrolyte of valence types (Z^+/Z^- ; Z^{2+}/Z^-), such as (KCl, bulk monovalent cation concentration $c_{1,265}$ $MgCl_2$, bulk divalent cation concentration $c_{2,265}$), the Gouy-Chapman theory (Equation A17 in Rubin and



Barber, 1980¹⁷) gives the magnitude of the electric field strength E as a function of distance x from the surface:

$$E = \sqrt{(2RT\gamma^2)/(\epsilon_0\epsilon_r)} \left(\frac{v}{v^2 - c_{2,\infty}} \right) \sec h^2(\phi_0 + \kappa x) \quad (1)$$

In this equation $\gamma = c_{1,\infty} + 3c_{2,\infty}$

$\kappa = \sqrt{(F^2\gamma)/(2RT\epsilon_0\epsilon_r)}$ where F is the Faraday constant, R the gas constant, T the absolute temperature, ϵ_0 the permittivity of a vacuum, and ϵ_r the dielectric constant.

$\phi_0 = \tanh^{-1}(v_0/\sqrt{\gamma})$ where $v_0 = \sqrt{c_{2,\infty} + (c_{1,\infty} + 2c_{2,\infty})e^{-(F\psi_0)/(RT)}}$, ψ_0 being the surface electric potential (Equation 8 of Rubin and Barber, 1980¹⁷). For a Z^+/Z^- electrolyte at a bulk concentration 20 mM (= 20 mol m⁻³, unstacking medium), ψ_0 is given by

$$|\sigma| = \sqrt{2RT\epsilon_0\epsilon_r(20e^{-F\psi_0/(RT)} + 20e^{F\psi_0/(RT)} - 2)} \quad (2)$$

For a Z^+/Z^- ; Z^{2+}/Z^{2-} electrolyte (10 mM KCl + 10 mM NaOH added to adjust the pH of the 20 mM Hepes buffer; 5 mM MgCl₂, stacking medium), ψ_0 is given by:

$$|\sigma| = \sqrt{2RT\epsilon_0\epsilon_r[(20e^{-F\psi_0/(RT)} - 1) + (30e^{F\psi_0/(RT)} - 1) + (5e^{-2F\psi_0/(RT)} - 1)]} \quad (3)$$

An estimate of the net σ for the whole thylakoid membrane is -0.025 C m⁻², resulting from a mix of negative and positive surface charges^{5,32}. Assuming that the local $|\sigma|$ of a negatively-charged area on the stroma-facing surface of LHC-II is ten times the net σ , the local ψ_0 is the value that results in the right-hand expression in Equation (2) or (3) being equal to $|\sigma|$.

$$\text{Finally, } v = \sqrt{\gamma} \tanh(\phi_0 + \kappa x) \quad (4)$$

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Author contributions

H.J., J.R.L. and W.S.C. designed research; H.J. and J.R.L. performed research; H.J., J.R.L. and W.S.C. analyzed data; and J.R.L. and W.S.C. wrote the paper.

Additional information

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