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. 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-harvesting; (2) a spatial separation of the two photosystems (PS II, responsible for oxygen evolution) to PS I which limits excessive spillover of excitation energy from Photosystem II (PS II, responsible for oxygen evolution) to PS I; (3) enhancement of non-cyclic photophosphorylation to increase the "energy currency"; (4) regulation of non-photochemical dissipation for protection against excess light; (5) delay of premature degradation of D1 protein in the PS II reaction centre at sustained high irradiance; (6) regulation of linear versus cyclic electron transport; and (7) a potential increase in photosynthetic capacity for a given chloroplast composition.

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 surfaces. It partly determines the separation between stacked thylakoids, recently estimated as 3.2 nm.

Electrostatic repulsion, caused by the net negative charge on the outer surface of thylakoid membranes, is a major factor opposing granal stacking. 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. 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. Significantly, the calculated van der Waals attraction is vastly
weaker than the electrostatic repulsion; therefore, the van der Waals attraction might not effect membrane stacking. What then is the main attractive force?

Chow 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 macromolecules. Nevertheless, the question remains as to what attractive force overcomes the electrostatic repulsion when MgCl₂, rather than macromolecules, is added to unstacked thylakoids.

To investigate the nature of the attractive force driving Mg²⁺-induced granal stacking, we used the highly sensitive technique of ITC to directly measure the enthalpy change ($\Delta H_{\text{exp}}$). 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, Mg²⁺-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 I. 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 MgCl₂ (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.
Experimental enthalpy change $\Delta H_{\text{exp}}$ 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$^{12,23}$), 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 $\Delta H$ 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 $\Delta H_{\text{exp}} = -0.20 \pm 0.51$ kJ mol$^{-1}$ Chl (first column of Table 1).

Thus, the magnitude of $\Delta H_{\text{exp}}$ 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 ($\Delta G$) 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 ($AS$) 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 $\Delta H_{\text{tot}}$ 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 membranes.

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### Table 1 | Experimental $\Delta H_{\text{exp}}$ 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 ($\Delta H_{\text{exp}}$) is the experimental enthalpy change of thylakoid stacking, calculated as described in Methods, averaged from a total of six days’ experiments, displayed as mean ± S.D. Columns A-D refer to $\Delta H$ after integration of the electrostatic repulsion calculated at constant surface charge density ($\sigma$) or constant surface potential ($\psi$). 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 calculations$^{23}$; the values shown in square brackets were obtained by extrapolation to separations below those in the calculations (see Methods). The row marked 3 nm has been shaded because the most recent estimate yielded 3.2 nm for the inter-membrane gap$^{13}$. $\Delta H_{\text{tot}}$ is the algebraic sum of the electrostatic, van der Waals and hydration enthalpy changes: $\Delta H_{\text{tot}}$ (max) = $\Delta H_{e}$ (1) + $\Delta H_{\text{hyd}}$ + $\Delta H_{\text{vdW}}$ (2). $\Delta H_{\text{tot}}$ (min) = $\Delta H_{e}$ (2) + $\Delta H_{\text{hyd}}$ + $\Delta H_{\text{vdW}}$ (1). $V_{\text{pr}}$ is the volume fraction of protein in the membrane, and $\varepsilon$ is the assumed dielectric constant.

| Gap (nm) | $\Delta H_{\text{exp}}$ | $\Delta H_{e}$ | $\Delta H_{\text{hyd}}$ | $\Delta H_{\text{vdW}}$ | $\Delta H_{\text{tot}}$ |
|---------|-----------------|----------------|-----------------|-----------------|-----------------|
| 2 nm    | -0.20 ± 0.51    | 1.37           | 0.68            | 1.21            | 0.66            |
| 3 nm    | 0.68            | 0.45           | 0.49            | 0.35            | 0               |
| 4 nm    | 0.36            | 0.29           | 0.22            | 0.19            | 0               |

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**Figure 2 | Theoretical calculations of the contributions, $|P_i|$ 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 ($V_{\text{pr}}$) in the membrane of 0.6 and 0.4, respectively. The dashed vertical line denotes a very recent estimation of membrane separation$^{21}$. 
membranes; the linear plot gives a better visual impression than a semi-log plot 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 \( \Delta H_{\text{tot}} \) consists of endothermic \( \Delta H \) 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 (\( \Delta H_{\text{tot}} \)) for membrane stacking calculated for three inter-membrane gaps. For a gap of 3 nm (shaded row in Table 1), the total enthalpy change (\( \Delta H_{\text{tot}} \)) for membrane stacking ranged from a lower limit, \( \Delta H_{\text{tot}} \) (min), of 0.34 kJ mol\(^{-1}\) Chl to an upper limit, \( \Delta H_{\text{tot}} \) (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, \(-0.20 \pm 0.51\) 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. Further, if the dielectric constant \( \varepsilon \) of the thylakoid membrane were greater than 3, \( \Delta H_{\text{tot}} \) (Table 1) would be more negative, making the \( \Delta H_{\text{tot}} \) less positive and the experimental value more within the range of theoretical estimates. Hence, the positive theoretical (\( \Delta H_{\text{tot}} \)) enthalpy change and the near zero experimental (\( \Delta H_{\text{exp}} \)) enthalpy change strongly imply that a significant entropic driving force is required to achieve an appreciably negative \( \Delta G \) and to effect spontaneous stacking on adding MgCl₂.

**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 charge. 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 stacking. A similar idea 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 entropy.

Secondly, upon adding MgCl₂, as two domains of opposite charge come closer, loosely-bound water molecules could be released from the inter-membrane gap to diffuse freely as individual ions in the bulk medium, thereby gaining entropy.

Thirdly, in an unstacking medium (without Mg\(^{2+}\)), the 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\(^{-30}\) C m will have minimum potential energy 1.14 GV \times 6.17 \times 10\(^{-30}\) C m = 7.0 \times 10\(^{-21}\) J, with a magnitude almost twice the kinetic energy per water molecule of \( kT = 4.1 \times 10\(^{-21}\) 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 MgCl₂ could then contribute to an increase in entropy that drives the stacking of thylakoids. The increased orientational freedom of water molecules on adding MgCl₂ should promote 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...
rare investigations of the contributions of entropy, as formulated by the Second law of Thermodynamics, to the driving forces of biochemical 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 NaHCO3, 5 mM MgCl2, 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 × g for 2 min at 4 °C. After re-suspension of the thylakoid membranes, they 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.27.

**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 MgCl2 (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 MgCl2 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 MgCl2 and frozen for 77 K fluorescence spectral analysis to determine the extent of membrane stacking.

**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 degased, 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: 5,000 Chl). Incubation with trypsin was for ~30 minutes before the addition of MgCl2 (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 intercepted 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 (μJ s−1) (Figure 5c and f). The enthalpy change of each injection was obtained by integration. The interaction of Mg2+ 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 MgCl2 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 Mg2+ 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 mL × 0.2 mM = 288 nmol), to give the molar enthalpy of the total interaction of Mg2+ ions with the thylakoid membrane. The values for all experiments with no trypsin performed on a particular day were averaged, as were the values 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 stacking.
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Figure 5 | Heat-capacity ($C_p$) plots and extrapolated baselines obtained from injection of MgCl₂ into thylakoid suspensions which did not contain trypsin or which did contain trypsin. [Chl] in the suspensions was 0.2 mM, [MgCl₂] in the syringe was 25 mM. Three injections, each of 95 µLs 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.
Barber, 1980) gives the magnitude of the electric field strength E as a function of distance x from the surface:

\[ E = \sqrt{2RT} \Gamma / (\psi_0 \epsilon_0) \left( \frac{v}{\sqrt{T - c_{1/2}}} \right) \sec h \left( \frac{\psi_0 + kx}{2} \right) \]  

(1)

In this equation \( \gamma = c_{1/2} + 2c_\infty \)
\( \kappa = \sqrt{(RT)/2c_{1/2}} \)
where \( F \) is the Faraday constant, \( R \) the gas constant, \( T \) the absolute temperature, \( \epsilon_0 \) the permittivity of a vacuum, and \( \epsilon_0 \) the dielectric constant.

\[ \phi_0 = \tanh^{-1} \left( \frac{1}{\sqrt{T - c_{1/2}}} \right) \]

where \( v_0 = \sqrt{c_{1/2} + c_\infty - 2c_\infty} \left( e^{(F\phi_0)/RT} - 1 \right) \), \( \psi_0 \) being the surface electric potential (Equation 8 of Rubin and Barber, 1980). For a \( Z^- / Z^- \)
electrolyte at a bulk concentration 20 mM (Barber, 1980)
the magnitude of the electric field strength \( E \) is given by

\[ |E| = \sqrt{2RT} \Gamma / \left( 20 - \frac{F\phi_0}{RT} + 20e^{F\phi_0/(RT)} - 2 \right) \]  

(2)

For a \( Z^- / Z^- \) \( Z^- / Z^- \) electrolyte (10 mM KCl + 10 mM NaOH added to adjust
the pH to the 20 mM Hepes buffer; 5 mM MgCl$_2$, stacking medium), \( \psi_0 \) is given by:

\[ |\psi_0| = \sqrt{2RT} \Gamma / \left( 20 - \frac{F\phi_0}{RT} - 1 \right) + 30e^{F\phi_0/(RT) - 1} + 5e^{-2F\phi_0/(RT) - 1} \]  

(3)

An estimate of the net \( \sigma \) for the whole thylakoid membrane is \( -0.025 \) C m$^{-2}$,
resulting from a mix of negative and positive surface charges (1981). Assuming that the local \( \sigma \) of a negatively-charged area on the stroma-facing surface of LIIC-II is ten times the net \( \sigma \), the local \( \psi_0 \) is the value that results in the right-hand expression in Equation (2) or (3) being equal to \( |\sigma| \).

Finally, \( \psi_0 = \sqrt{v} \tanh(\phi_0 + kx) \)  

(4)

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