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Fractionation of the thylakoid membrane from *Dunaliella salina* – heterogeneity is found in Photosystem I over a broad range of growth irradiance

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Abstract

Thylakoids from the green alga, *Dunaliella salina*, were fragmented by sonication and the appressed grasna membranes separated from stroma lamellae by partitioning in aqueous two-phase systems. The concentration and antenna size of Photosystem I in the two membrane domains were determined for cultures grown at three different light intensities. Although the antenna size of both PS is decreased with increasing growth irradiance, the antenna size of Photosystem I in the grana was approximately 25–30% greater than the antenna size of Photosystem I in the stroma lamellae. Counter-current distribution analysis of sonicated thylakoids revealed that the amount of stroma lamellae increased whereas the amount of the stacked membranes decreased at higher growth irradiance. The overall decrease in the antenna size of PS I, in *D. salina*, at higher light intensities can therefore be explained by the combined effect of a decrease in the antenna size of Photosystem I, both in the grana and the stroma lamellae, and a relative increase in the amount of stroma lamellae which has smaller Photosystem I antennae than the Photosystem I centers found in the grana. Light-induced protein phosphorylation increased the relative amount of the stroma lamella fraction. This is interpreted as a result of partial unstacking of the grana. It is suggested that this may be a mechanism for increasing the cyclic electron transport around Photosystem I.

Keywords: Aqueous two-phase partitioning; (*Dunaliella salina*); Membrane domain; Thylakoid membrane organization; Protein phosphorylation; Photosystem

1. Introduction

Fragmentation and separation analysis provides a powerful tool in the elucidation of the structure–function relationship of biological membranes. This approach has been particularly successful in the case of the photosynthetic membrane, the thylakoid, of higher plants [1,2].

The thylakoid consists of at least three different domains, the stroma lamellae, the grana margins and

Abbreviations: CCD, counter-current distribution; Chl, chlorophyll; cyt, cytochrome; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMSO, dimethylsulfoxide; LHC II, light harvesting complex II; PS, photosystem; P-700, reaction center of Photosystem I; PpBQ, phenyl-*p*-benzoquinone; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MV, methyl viologen.

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the grana core (partitions). These can now be isolated by a combination of sonication and aqueous two-phase partitioning. The three membrane domains have different biochemical compositions and have specialized functions [3,4]. They exhibit heterogeneity with respect to antenna size in Photosystem I (PS I) and Photosystem II (PS II). Thus, PS IIα localized in the grana core has an antenna which is about twice as large as that of PS IIβ in the stroma lamellae [3,5]. The PS I in the grana, PS Iα, is located in the periphery (margins) of the grana and its antenna size is 30–40% greater than that of PS Iβ in the stroma lamellae [6].

In the thylakoid of unicellular green algae, one can also distinguish between stacked grana regions and single paired stroma lamellae albeit the difference in appearance between the grana and stroma lamellae is not as spectacular as in the case of higher plants. In a green alga, *Dunaliella salina*, it has been demonstrated that the antenna of both PS II and PS I change with growth irradiance [7]. In higher plants, however, the antenna size of PS I is not greatly affected by light intensity, while the number of electron transport carriers is markedly increased on chlorophyll basis at higher light intensities [8]. It was therefore of interest to study the membrane domain organization of the thylakoid from a green alga by the same approach as we have used for spinach thylakoids [2,3].

The aim of this work was to investigate the presence of any heterogeneity in the antenna size of PS I in the unicellular green alga, *D. salina*. Kim et al. [7] have reported changes in both the concentration per Chl and the antenna size of PS I, as a result of changes in growth irradiance, in *D. salina*. Here we investigate if there is a difference in the antenna size of PS I in grana and stroma lamellae and, furthermore, how the antennae in these two compartments are affected by growth irradiance.

Our results show that the antenna of PS I in *D. salina* is heterogeneous. At all light intensities investigated, the antenna size of PS I is 25–30% greater in the grana fraction. The antenna size of both subpopulations of PS I in *D. salina*, PS Iα and PS Iβ, decreases with increasing growth irradiance. Furthermore, the relative amount of stroma lamellae increases with increasing growth irradiance. The combined effect of the relative increase in the stroma lamellae and the decrease in the antenna size of both sub-populations of PS I, PS Iα and PS Iβ, explains the overall decrease in antenna size of PS I.

2. Materials and methods

2.1. Chemicals

Dextran T500 was purchased from Pharmacia (Uppsala, Sweden). Poly(ethylene glycol) 4000 (Carbowax PEG 3350) was obtained from Union Carbide (New York, NY, USA).

2.2. Growth conditions

D. salina cells were grown in hypersaline medium with $NaHCO_3$ as a carbon source [9], in flat, half-liter flasks (3 cm light path). The medium was composed of 1.5 M NaCl, 5 mM $MgSO_4$, 0.3 mM $CaCl_2$, 0.1 mM KH_2PO_4, 20 mM EDTA, 2 mM $FeCl_3$, 5 mM NH_4Cl, and 40 mM Tris-HCl (pH 7.5), 25 mM $NaHCO_3$, and micronutrients. The cultures were grown at 25°C at three light intensities; 100, 500 and 1000 μmol photons/m^2/s under continuous mixing to the late log phase as determined by absorbance at 678 nm.

2.3. Preparation of chloroplast thylakoids and subthylakoid membrane fractions

2.3.1. Thylakoid membrane isolation

Cell harvesting and thylakoid isolation took place at 4°C. Cells were collected by centrifugation, 1500 × *g* for 3 min, and the pellets washed in 20 mM Tris-HCl (pH 8.0) and 15% (v/v) glycerol followed by centrifugation, 1500 × *g* for 3 min. The pellet was resuspended in 5 ml of the same buffer followed by addition of 45 ml glycerol-free buffer to break the cell membrane. The mixture was incubated on ice for 10 min. under gentle agitation and chloroplasts collected by centrifugation, 1100 × *g*, for 2 min. The chloroplast envelopes were osmotically broken in 5 mM $MgCl_2$ and thylakoids collected by centrifugation at 5000 × *g* for 5 min.

Thylakoids were washed twice in 10 mM Tricine buffer (pH 7.4), 5 mM $MgCl_2$, 300 mM sucrose, once in 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl, 1 mM $MgCl_2$, 100 mM sucrose and

resuspended in the same medium to a chlorophyll concentration of approximately 2 mg/ml.

2.3.2. Sonication

Fragmentation of the membranes by sonication and separation of grana and stroma lamellae compartments by aqueous two-phase separation were carried out as described in [3] with some modifications. Thylakoid suspension, 2.00 g (approx. 2 mg Chl/ml), was added to 9.66 g of a polymer mixture to give the final concentrations: 6.0% (w/w) Dextran T500, 6.0% (w/w) PEG 4000, 10 mM sodium phosphate buffer (pH 7.4), 3 mM NaCl, 1 mM $MgCl_2$ and 20 mM sucrose. This mixture, now constituted the sample system, and was incubated on ice for 45 min and sonicated using a Vibra-cell ultrasonic processor Model VC 500 (Sonics and Materials, Danbury, CT, USA) equipped with a 1.3-cm horn. The sample system was sonicated six times for 30 s, with resting intervals of 1 min, in a cylindrical aluminium tube immersed in ice/water. The ultrasonic exposure had an intensity output setting of 7, with 20% duty pulses.

After sonication, 6.43 g of pure lower phase and 5 g of pure top phase from an aqueous two-phase system comprised of 6.0% (w/w) Dextran T500, 6.0% (w/w) PEG 4000, 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl and 20 mM sucrose were added to the sonicated sample system. The temperature was adjusted to 4°C and the system mixed prior to separation by a batch procedure or by counter-current distribution.

2.3.3. Batch procedure

In the batch procedure, phases were separated by centrifugation, $2000 \times g$ for 3 min. The upper and lower phases, enriched in thylakoid fragments from stroma lamellae and grana, respectively, were separated and each washed twice by 10 ml of fresh lower or upper phase, respectively. The final upper phase fraction, after being washed twice with fresh lower phase, constituted the stroma lamellae membrane fraction (T3) and the final lower phase fraction, after two washes by fresh upper phase, constituted the grana lamellae membrane fraction (B3). Membrane fractions were then washed free of the polymers with dilution in a medium consisting of: 100 mM sucrose, 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl (dilution medium) and centrifugation at 100 000 $\times g$ for 90 min. The fractions were then resuspended in the same medium +5% DMSO and stored in liquid nitrogen.

2.3.4. Counter-current distribution

Quantitative separation of the grana and stroma laemllae compartments was achieved by using a centrifugal counter-current distribution apparatus [10]. Three cavities, out of sixty, were each loaded with 1.8 ml of the sonicated sample system. The remaining 57 cavities were each loaded with 1.8 ml of the phase system described above (0.9 ml of the top phase and 0.9 ml of the lower phase). Twenty-five operative cycles, comprising 30 s mixing, 90 s centrifugation and one transfer each, were carried out. After completion of the counter-current distribution, the contents of each of the cavities were collected in a tube. Each fraction was diluted three times in dilution medium (see batch procedure above) and the absorbance at 680 nm recorded for each sample. Tubes, containing vesicle populations representing the structural domains of the thylakoid membrane, were pooled, and membranes pelleted at 100 000 $\times g$ for 90 min and resuspended in dilution medium.

2.4. Analysis

Chlorophyll concentrations were determined according to Arnon [11]. Polypeptides were separated by SDS-PAGE using the buffer system of Laemmli [12] in the presence of 4 M urea with an acrylamide gradient from 12 to 18%.

The concentration of P-700 (reaction center of PS I) was obtained with an Aminco DW-2 spectrophotometer operated in a split-beam mode with a slit width of 2 nm. The amplitude of the light-minus-dark absorbance change at 700 nm was measured using an extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ [13]. The reaction buffer contained 0.02% (w/w) SDS, 2 mM methyl viologen, 2 mM sodium ascorbate, 15 mM Tricine (pH 7.8), 5 mM $MgCl_2$, 10 mM NaCl, 400 mM sucrose and thylakoid vesicles to obtain a final chlorophyll concentration of 20–30 μM.

The rate of photo-oxidation of P-700 was measured according to Melis [14] using an Aminco DW-2 spectrophotometer working in the dual wavelength

mode. The measuring wavelength was set at 700 nm and the reference beam was set at 730 nm. The optical path length of the cuvette for the measuring beam was 10 mm and for the actinic beam 4 mm. An RG 665 Schott filter was placed in front of the photomultiplier, a 566.9 nm bandpass filter, a cut-off 600 nm filter, and a neutral filter were placed in front of the actinic light source so that the light intensity was 29 $\mu E \cdot m^{-2} \cdot s^{-1}$. Signal averaging was performed with a Nicolet instrument corporation model 527 instrument. Samples were first dark-adapted for 30 min on ice and then plastocyanin inhibited by incubation for 2 h on ice in a buffer containing 150 mM potassium cyanide, 90 mM Tricine-KOH (pH 7.8), 1 mM $MgCl_2$, 100 mM sucrose and 300 μM Chl [15]. After incubation the sample was diluted to obtain the a final sample concentration of 30 μM Chl.

Electron transport measurements, from H_2O to PpBQ, were made polaro-graphically using a Clark-type O_2 electrode [3].

2.5. Fractionation of phosphorylated thylakoids

Thylakoids from *D. salina* were phosphorylated, fragmented and the sub-thylakoid vesicle populations, grana (B3) and the stroma lamellae (T3) vesicles isolated according to [16] except that 5.8% (w/w) of both polymers, PEG and Dextran, were used in the counter-current distribution experiments.

2.6. Light-induced protein phosphorylation in isolated sub-thylakoid vesicles

Protein phosphorylation in sub-thylakoid vesicles, grana (B3) and stroma lamellae (T3), was induced by light and ATP as described in [16].

2.7. Electron microscopy

Samples for electron microscopy were fixed for 2 h at 4°C with 1% osmium tetroxide. The samples were dehydrated with acetone and embedded in epoxy resin (Agar 100, Agar Scientific, UK). Thin sections were stained with 5% uranyl acetate at room temperature for 45 min.

3. Results

3.1. Thylakoid fragmentation

3.1.1. Stacking in relation to growth irradiance

Counter-current distribution with aqueous polymer two-phase systems can be used to separate membrane vesicles according to their surface properties [2,10]. The procedure is analogous to liquid-liquid chromatography. The peaks of the counter-current distribution diagram represent membrane vesicles with different affinities for the two phases, the polyethylene glycol-rich top phase and the dextran-rich lower phase. Peaks to the right in the diagram represent vesicles which prefer the upper phase while those to the left prefer the lower phase.

The diagrams for counter-current distribution of thylakoids and sonicated thylakoids from *D. salina*, grown at different light intensities, are shown in Table 1. Unfragmented thylakoids (Fig. 1a) display a peak to the right-hand side of the diagram, i.e., thylakoids prefer the upper phase. A small peak in the middle of the diagram may represent damaged thylakoids.

After sonication the counter-current distribution diagram shows two well separated peaks, Fig. 1b–d. The two separated vesicle populations, the peak to the left and the peak to the right, have different chlorophyll a/b ratios. Those vesicles which prefer the upper phase (right-hand peak) have higher chlorophyll a/b ratios and are enriched in PS I (P-700), while the vesicles with lower chlorophyll a/b ratios (left-hand peak) are depleted in P-700 compared with the thylakoids. From this, we conclude, in analogy with the results from separation experiments with higher plants [3,17], that the vesicles represented by the right-hand peaks of Fig. 1b–d are derived from single-paired lamellae (stroma lamellae) while the vesicle populations represented by the left hand peaks are derived from the stacked, grana-like, regions of the thylakoid. This conclusion is also supported by the polypeptide composition of the different peaks, as revealed by gel electrophoresis (Fig. 2). The vesicles derived from the grana (peaks to the left in Fig. 1b–d) are depleted in PS I polypeptides but enriched in PS II and LHC II polypeptides, whereas the opposite is found for the vesicles derived from stroma lamellae, which are depleted in PS II polypeptides

Table 1
Properties of sub-thylakoid vesicles derived from cultures grown at different light intensities

	100 μmol photons · $m^{-2} \cdot s^{-1}$			500 μmol photons · $m^{-2} \cdot s^{-1}$			1000 μmol photons · $m^{-2} \cdot s^{-1}$		
	Thylakoids	Grana (B3)	Stroma lamellae (T3)	Thylakoids	Grana (B3)	Stroma lamellae (T3)	Thylakoids	Grana (B3)	Stroma lamellae (T3)
Chl *a* / *b*(mol/mol)	4.4	3.7	6.1	4.9	4.1	6.7	5.5	4.2	7.3
P-700/Chl(mmol/mol)	2.0	1.4	3.2	2.2	1.6	3.3	2.5	1.8	3.6
$K_{\text{P-700}}(s_{-1})$	n.d.	4.0 (0.3)	3.1 (0.1)	n.d.	3.3 (0.2)	2.7 (0.1)	n.d.	3.1 (0.2)	2.5 (0.1)
PS II V_{max}	59	38	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Thylakoids and sub-thylakoid vesicles representing the grana and stroma lamellae were isolated from *D. salina* cultures grown at three different light intensities as described in Section 2.3. The experimental values of the kinetic rate constant of photooxidation, $K_{\text{P-700}}$, is the average from three separate preparations. The standard deviation is given within parentheses. V_{max} for PS II is expressed as O_2 /mol chlorophyll per h.

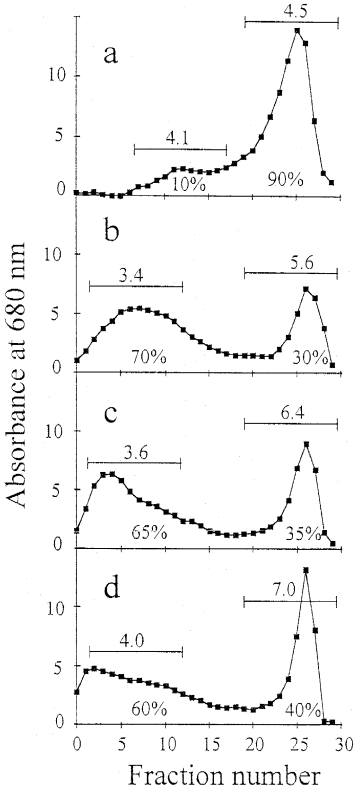


Fig. 1. Counter-current distribution diagrams for thylakoid membranes from *D. salina* grown at different light intensities. (a) Unfragmented thylakoids (growth irradiance 100 $\mu E \cdot m^{-2} \cdot s^{-1}$) (b–d), sonicated thylakoids, growth irradiance 100, 500 and 1000 μmol photons $m^{-2} \cdot s^{-1}$, respectively. Peaks to the left (b–d) represent membrane vesicles derived from the grana and the peaks to the right (b–d) represent vesicles representing the stroma lamellae. The relative amount of chlorophyll associated with the stroma lamellae fraction (percentage figures under the peaks, estimated by the total absorbance at 680 nm) increases with increasing growth irradiance of the cultures. The chlorophyll *a / b* ratios of samples pooled from cavities are given above each peak.

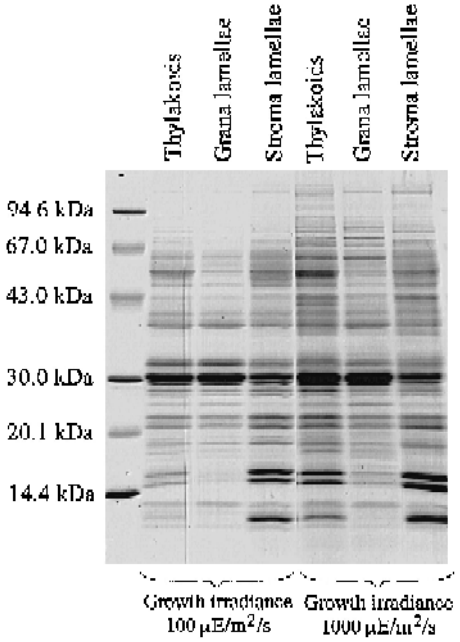


Fig. 2. SDS-PAGE of thylakoid and sub-thylakoid membranes from *D. salina* grown at two different light intensities, 100 and 1000 μmol photons $\cdot m^{-2} \cdot s^{-1}$. Each lane was loaded with 17 μg Chl.

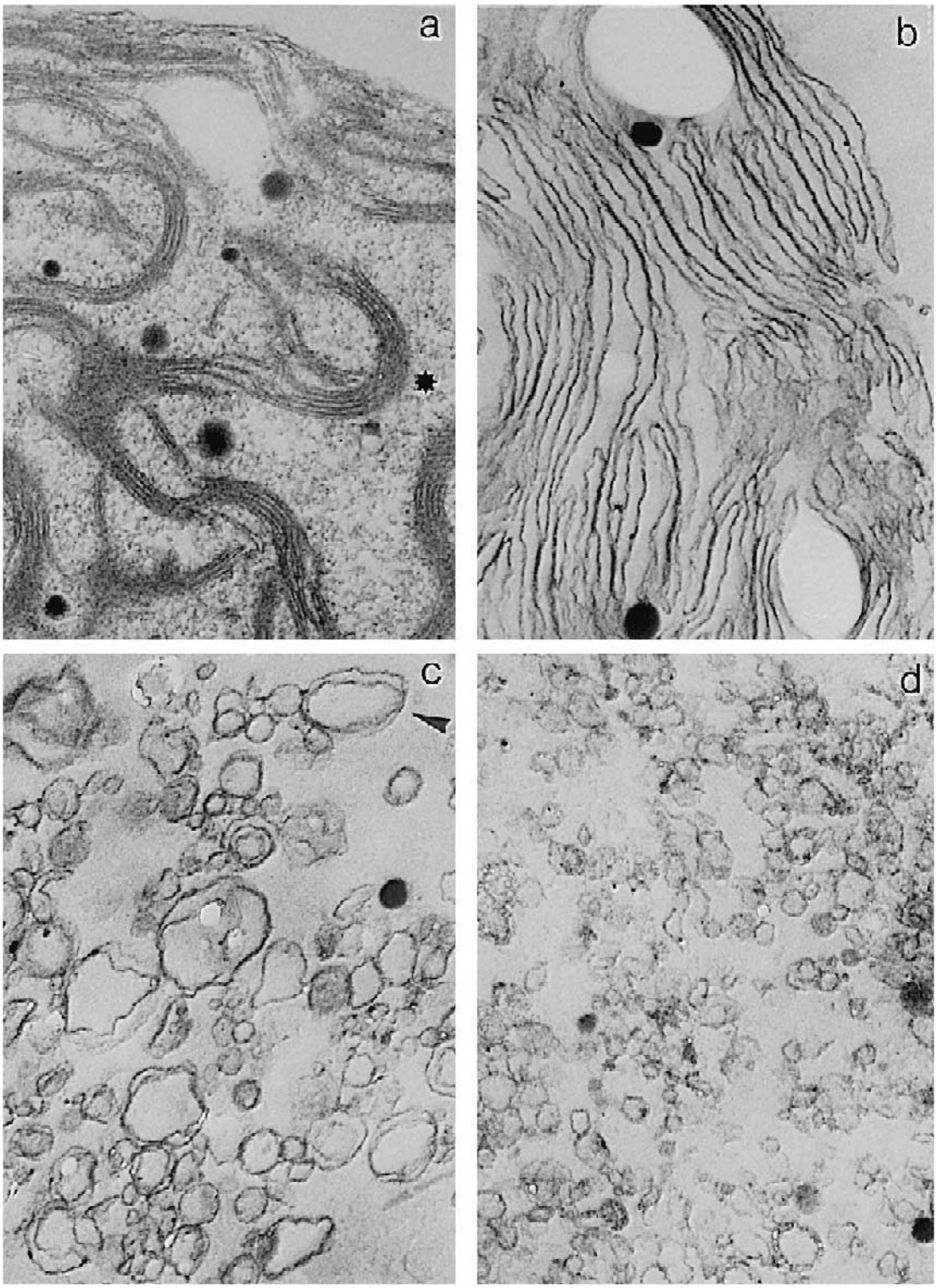
and enriched in PS I polypeptides. The same amount of chlorophyll was loaded in each well. Thylakoids and sub-thylakoid vesicles from cells grown at higher light intensities contain more protein on chlorophyll basis which explains the greater staining observed for thylakoids grown at high light intensity than those grown at lower light intensity.

Oxygen evolution is impaired in *D. salina* by the sonication process since sonicated thylakoids retain only 60% of their water splitting activity compared with intact thylakoids. Oxygen evolution in the stroma fraction is very poor, while in the grana fraction we observe oxygen evolution although the activity is lower than expected in comparison with whole thylakoids (Table 1).

The relative sizes of the two peaks, representing the grana and stroma lamellae, varies with growth irradiance Fig. 1b–d. The peak to the right in Fig. 1b (100 μmol photons $\cdot m^{-2} \cdot s^{-1}$ growth irradiance), representing the stroma lamellae, contains approxi-

Fig. 3. Electron micrograph sections of *D. salina*: intact cell (a), isolated chloroplast (b) and sub-thylakoid vesicles derived from grana (c) and stroma lamellae (d), all from culture grown at 100 μmol photons $\cdot m^{-2} \cdot s^{-1}$. Note the domains of appressed thylakoids in (a). The grana vesicles appear as circular or oval structures with double membranes, with varying degrees of appression, which may represent a cup-shaped form in analogy with grana vesicles from higher plants. (c) The circumference of the grana vesicles (arrow) is of the same order of magnitude as the length of the appressed regions (*). The stroma lamellae vesicles (d) are smaller than the grana vesicles, appear as more uniform spheres and consist of a single membrane.

mately 30% of the chlorophyll loaded, as indicated by the absorbance at 680 nm. The relative chlorophyll content of this peak increases with increasing growth irradiance. In Fig. 1c (500 μmol photons · $m^{-2} \cdot s^{-1}$ growth irradiance) 35% of the chlorophyll is in the stroma lamellae peak and this has increased



to 40% in Fig. 1d (1000 μmol photons $\cdot m^{-2} \cdot s^{-1}$ growth irradiance). The Chl *a*/*b* ratio of thylakoids and the sub-thylakoid populations increases with increasing growth irradiance.

3.1.2. Electron microscopy of sub-thylakoid vesicles

Electron micrographs of thylakoids *D. salina* (Fig. 3a) grown at 100 μmol photons $m^{-2} \cdot s^{-1}$ are dominated by appressed regions (*) involving 4–5 membrane pairs. We find that 70–80% of the membranes are stacked, the remaining being single paired. The electron micrograph of isolated chloroplasts (Fig. 3b) indicates less stacking but appressed membranes are shown (arrows). The sub-thylakoid vesicles, representing grana and stroma lamellae, differ in size and shape (Fig. 3c and d, respectively). The vesicles derived from the appressed regions are relatively large; the vesicle circumference being comparable to the length of the stacked regions in the intact thylakoid. The vesicles derived from the single-paired (stroma lamellae) membranes are smaller, circular and more uniform.

3.2. Heterogeneity in PS I

The functional antenna size of PS I can be determined by studying the kinetics of P-700 photo-oxidation [14]. The rate constant for P-700 photo-oxidation is proportional to the antenna size of PS I and is independent of the rate of electron transfer to P-700 when plastocyanin is inhibited by KCN [15]. Fig. 4 shows typical kinetic traces of the time course of the bleaching of P-700 as a result of photo-oxidation. Semilogarithmic plots for both sub-populations, PS Iα and PS Iβ, fit straight lines with different slopes. The slope for PS Iα is 25–30% steeper than the slope obtained for PS Iβ, i.e., the functional antenna size is 25–30% greater for PS I in grana compared to PS I in the stroma lamellae.

3.2.1. Effect of growth irradiance on the antenna size of PS I

It has been reported that the overall antenna size of PS I declines with increasing growth irradiance in *D. salina* [7]. It was therefore of interest to see if both PS I sub-types, PS Iα and PS Iβ, were affected by growth irradiance or if only the stoichiometry of the α and β forms of PS I were changed by changing the

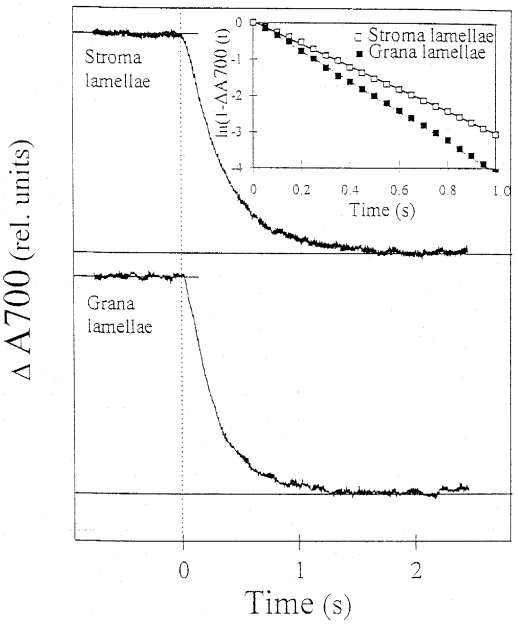


Fig. 4. Kinetic traces of the time course of P700 photo-oxidation for grana and stroma lamellae vesicles from *D. salina* thylakoids (growth irradiance of the culture 100 μmol photons $\cdot m^{-2} \cdot s^{-1}$). Irradiance with actinic green light, 29 μmol photons $\cdot m^{-2} \cdot s^{-1}$ s, started on at time 0 s. The slope of the line, ln [ΔA700(*t*)] vs. *t* (inserted figure), gives the negative value of the rate constant for P-700 photo-oxidation which is proportional to the antenna size of the P-700 units analyzed, providing that the actinic light is constant and limiting. A steeper negative slope therefore reflects a larger antenna.

degree of stacking. This was determined by measuring the rate of photo-oxidation of P-700 for the two sub-fractions from cells grown at different light intensities. Data for cells grown at 100, 500 and 1000 μmol photons $\cdot m^{-2} \cdot s^{-1}$ together with relative concentrations of P-700/Chl are given in Table 1. The sub-thylakoid fractions derived from grana (B3) and stroma lamellae (T3) were isolated using the batch procedure of partitioning (see Section 2.3). Both PS I sub-types, α and β, were affected by growth irradiance. The kinetic rate constant $K_{P\text{-}700}$ for the sub-thylakoid vesicle populations is plotted against the growth irradiance in Fig. 5. Higher growth irradiance revealed smaller antenna sizes for both the stroma lamellae fraction (T3) and the grana lamellae fraction (B3). The antenna size in the grana fraction (B3) was approximately 26% greater at all light intensities compared with the stroma lamellae fractions (T3).

The concentration of PS I changes in relation to growth irradiance. The highest P-700/Chl value for

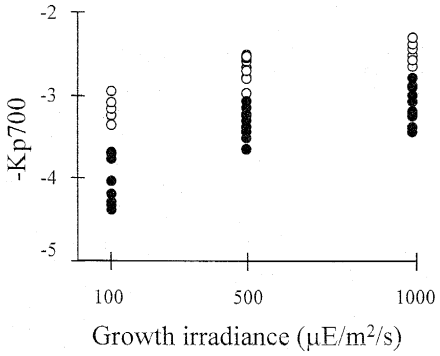


Fig. 5. Rate constant for P700 photo-oxidation in grana and stroma lamellae vesicles from *D. salina* cultures grown at 100, 500 and 1000 μmol photons·m^{-2}·s^{-1}. Subthylakoid vesicles derived from the grana (filled circles) exhibit 25–30% larger antenna size than vesicles derived from the stroma lamellae (open circles) regardless of growth irradiance.

both grana and stroma lamellae vesicles was obtained with thylakoids grown under 1000 μmol photons · $m^{-2} \cdot s^{-1}$ and the lowest P-700/Chl values were found for sub-thylakoid vesicles grown at 100 μmol photons · $m^{-2} \cdot s^{-1}$ (Table 1).

3.3. Effect of light-induced protein phosphorylation in D. salina thylakoids on domain organization

In Fig. 6, counter-current distribution diagrams of sonicated thylakoids, from control and phosphorylated thylakoids, are compared (phosphorylation was carried out with 5 mM $MgCl_2$ in the medium). As before, the peaks to the left represent the grana vesicles and the peaks to the right the vesicles derived from stroma lamellae. Light-induced protein phosphorylation prior to sonication accounts for an increase in the stroma lamellae fraction of approximately 20% on chlorophyll basis. The Chl *a*/*b* ratios for the right hand half of the grana peak and the stroma fraction decrease from 3.8 to 3.6 and from 4.6 to 4.5, respectively, whereas the Chl *a*/*b* ratio of the left hand half of the grana peak is not affected.

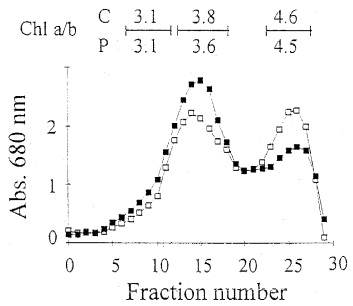


Fig. 6. Counter-current distribution diagram for sonicated control thylakoids (filled squares) and phosphorylated (open squares) thylakoids. The appressed grana membranes are represented by the peak to the left and stroma lamellae membranes by the peak to the right. The yield of the stroma lamellae population increases by 20% upon phosphorylation and the Chl *a*/*b* ratio decreases.

3.4. Protein phosphorylation in isolated grana and stroma lamellae vesicles

Sub-thylakoid vesicles derived from grana (B3) and stroma lamellae (T3), were prepared from *D. salina* cultures (growth irradiance 100 μmol photons · $m^{-2} \cdot s^{-1}$) by the batch procedure, as described in Section 2.3. Thylakoids and the sub-thylakoid vesicles were then incubated in the presence of light and [γ-^{32}P]ATP and 5 mM $MgCl_2$. SDS-PAGE was

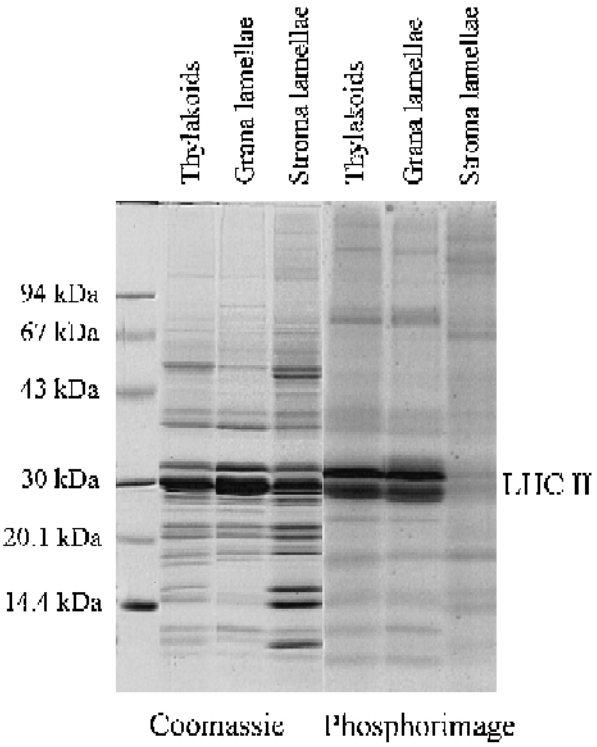


Fig. 7. Light induced protein phosphorylation with sub-thylakoid vesicles. The isolatred sub-thylakoid vesicles were allowed to phosphorylate after fragmentation and separation of the thylakoid membrane. SDS-PAGE and subsequent phosphor-imaging of grana (B3) and stroma lamellae (T3) vesicles.

carried out on the phosphorylated samples, the protein was visualized by coomassie staining, and protein phosphorylation by phosphor imaging (Fig. 7).

PS I and PS II polypeptides were found in both the grana and the stroma lamellae fraction; PS II polypeptides enriched in the former and PS I polypeptides in the latter. However, most of the LHC II and PS II protein kinase activity was found in the grana fraction. Specific labeling of LHC II and PS II polypeptides in the grana fraction was comparable to that which was observed in thylakoids (Fig. 6). This shows that although the sub-thylakoid vesicles, representing grana and stroma lamellae, partly have the same polypeptide composition, they are functionally different since no phosphorylation of the PS II polypeptides is observed in the stroma lamellae vesicles.

No phosphorylation was observed in the stroma lamellae fraction when either NADPH or DPC was added in order to help reduce the plastoquinone pool since water splitting is poor in the stroma fraction (results not shown).

4. Discussion

4.1. Fractionation of D. salina thylakoids

Our fragmentation and separation method works equally well with *Dunaliella salina* thylakoids as with higher plant thylakoids [3,16]. However, the separation is not as clear cut with *D. salina* which may be due to the less distinct stacking of grana in *D. salina*. The peaks to the left in Fig. 1b–d represent vesicles derived from the grana and those to the right the stroma lamellae vesicles (vesicles derived from the end membranes of the grana may also be present in the stroma lamellae fraction). That the peaks to the left represent grana vesicles is indicated by the low chlorophyll *a*/*b* ratio, relatively low P-700 content, PS II activity (Table 1), gel electrophoresis pattern (Fig. 2) and the electron micrographs (Fig. 3).

The separation is based on differences in surface properties [17]. For spinach thylakoids it has been shown that the vesicles of the two peaks in the counter current-distribution diagram differ with respect to their sideness, the grana vesicles being inside-out and the stroma lamellae vesicles right-side-out [3,18]. By analogy, one would expect that the grana vesicles of *D. salina* also would be of the inside-out and the stroma lamellae fraction of the right side-out conformation.

The chloroplasts were osmotically broken in 5 mM $MgCl_2$ and the thylakoids were isolated with the same magnesium chloride concentration to ensure grana stacking. The concentration of magnesium chloride was then reduced to 1 mM during sonication and the subsequent separation of the sub-thylakoid vesicles in order to avoid their aggregation. It has been shown that particularly the inside out grana vesicles aggregate at 5 mM magnesium chloride [19].

4.2. PS II activity in the grana and stroma lamellae fractions

The sonication process utilized for separation of the grana and the stroma lamellae compartments impairs the PS II oxygen evolving capacity of the photosynthetic membrane in *D. salina* (Table 1). Very little oxygen evolving activity is observed in isolated stroma lamellae vesicles and the activity observed in the grana vesicles is less than in intact thylakoids. This is in contrast with what we observed for spinach thylakoids, where the identical sonication procedure did not affect the oxygen evolving capacity of PS II [3]. We have no explanation for this difference in stability in PS II activity between *D. salina* and spinach.

4.3. Antenna size of Photosystem I in relation to growth irradiance

The antenna size of Photosystem I of both grana and stroma lamellae decreases as the growth light intensity increases (Table 1). At all light intensities the antenna of grana PS I is about 25–30% larger than that of stroma lamellae PS I (Fig. 5). The stroma lamellae population increases when the growth light intensity increased (Fig. 1b–d) from 30% for cells grown at 100 μmol photons $\cdot m^{-2} \cdot s^{-1}$ to 40% for cells grown at 1000 μmol photons $\cdot m^{-2} \cdot s^{-1}$. This means that the overall antenna size of PS I is a function of both the antenna sizes of PS Iα and PS Iβ, and the relative proportions of stacked and unstacked membranes in the thylakoid.

The P-700 content in the stroma lamellae changes from 3.2 to 3.6 (mmol P-700/mol Chl) betveen the low-light (100 μmol photons $\cdot m^{-2} \cdot s^{-1}$) to the

high-light (1000 μmol photons · m^{-2} · s^{-1}) thylakoids (Table 1). For the grana vesicles, the corresponding increase is from 1.4–1.8 (mmol P-700/mol Chl). This agrees with the concomitant reduction in the antenna size. Since the corresponding relative proportion of stroma lamellae increases from 30% to 40% the relative proportion of P-700 in the stroma lamellae will also increase at the higher light conditions. From Table 1 and Fig. 1, one can calculate that 48% of the P-700 is in the stroma lamellae fraction of cells grown at low light (100 μmol photons · m^{-2} · s^{-1}). This value increases to 58% for cells grown at high light (1000 μmol photons · m^{-2} · s^{-1})

Heterogeneity among the photosystems located in the different compartments or domains of the thylakoid membrane and limited lateral transport of reducing equivalents between the grana and stroma lamellae compartments are important facets in our model for domain organization of the thylakoid membrane [1,20]. We have proposed a three-domain model for the thylakoid membrane where grana core, containing PS IIα, has a large contact surface with the grana margin domain, containing PS Iα, allowing for efficient electron transport between the two photosystems. In addition, there is an overall excess of Chl associated with PS I compared with that of PS II, this excess beeing located together with the PS I of the stroma lamellae (1). According to this model, the grana perform oxygenic non-cyclic electron transport, whereas cyclic electron transport takes place in the stroma lamellae. If we apply this model to *D. salina* it means that the larger portion of stroma lamellae at higher irradiances leades to a higher capacity for cyclic electron transport.

The *D. salina* cultures are grown under continuous stirring to provide uniform illumination of all cells. In leaves, however, there are chlorophyll-containing cells on both the sun and the shade side of the leaf and leaves may cover each other. The thylakoids of higher plants will therefore be exposed to different amount of light depending on whether they are located on the sun or shade side of the leaf, covered or exposed. One would therefore expect more heterogeneity in the antenna size of the photosystems in thylakoids from higher plants. However, much the same heterogeneity is found in the antenna of PS I in the unicellular green algae, *D. salina*, as we find in higher plants, i.e., 30–40% larger antenna for PS I in the grana fraction compared with PS I in the stroma lamellae fraction [3,6].

4.4. Effect of light-induced protein phosphorylation on domain organization

D. salina is able to adapt to both long-term and short-term changes in irradiance. The state 1–state 2 transitions, mediated by redox-dependent phosphorylation of the light-harvesting complex of of PS II, increases the relative amount of the stroma lamellae fraction to the grana fraction, Fig. 6. The same was observed for spinach thylakoids and we have interpreted these results as being the consequence of partial unstacking of grana, i.e., loosening of the stacking forces in the annular peripheries of the circular grana membranes. This may promote the exposure of the granal PS Iα centers to the chloroplast stroma, thereby enhancing their participation in cyclic electron transport activity [16]. Partial destacking of the grana after protein phosphorylation may therefore serve to regulate the proportions of linear and cyclic electron flow and thereby balance the NADH/ATP ratio.

4.5. Phosphorylation of isolated sub-thylakoid vesicles representing grana and stroma lamellae

In isolated sub-thylakoid vesicles from spinach, the LHC II and PS II kinase activity was found predominantly in the grana fraction. However, the incorporation of ^{32}P into LHC II polypeptides was only 20% of that observed for intact thylakoids [16]. In *D. salina* the same qualitative results were obtained; only the grana compartment is capable of performing light-induced phosphorylation of LHC II and PS II polypeptides. The kinase activity, however, differs from spinach since the specific labeling of LHC II and PS II polypeptides is similar in thylakoids and isolated grana vesicles from *D. salina* (Fig. 7).

Unlike higher plant chloroplasts, the dark state in green algae is state 2. State 2 in the dark is achieved by reducing the plastoquinone pool by products of the chlororespiratory pathway through a membrane bound NADH-plastoquinone oxidoreductase [21]. Furthermore, *D. salina* is markedly more effective than higher plants in its ability to adjust to a wide

range of light intensities. Different regulation mechanisms may therefore explain the different kinase activity observed in isolated grana vesicles from spinach and *D. salina* thylakoids.

The high kinase activity (estimated from phosphor images of SDS gels) in the grana vesicles makes them suitable as starting material for the isolation of the LHC II protein kinase and for protein phosphorylation experiments in Photosystem-I-depleted membrane vesicles.

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References

[1] P.-Å. Albertsson, Photosynth. Res. 46 (1995) 141–149.
[2] P.-Å. Albertsson, E. Andreasson, H. Stefánsson, L. Wollenberger, Methods Enzymol. 228 (1994) 469–482.
[3] E. Andreasson, P. Svensson, C. Weibull, P.-Å. Albertsson, Biochim. Biophys. Acta 936 (1988) 339–350.
[4] L. Wollenberger, C. Weibull, P.-Å. Albertsson, Biochim. Biophys. Acta 1230 (1995) 10–22.
[5] J. Naus, A. Melis, Plant Cell Physiol. 32 (1991) 569–576.
[6] P. Svensson, E. Andreasson, P.-Å. Albertsson, Biochim. Biophys. Acta 1060 (1991) 45–50.
[7] J.H. Kim, J.A. Nemson, A. Melis, Plant Physiol. 103 (1993) 181–189.
[8] W.S. Chow, L. Quian, D.J. Goodchild, J.M. Andersson, Aust. J. Plant Physiol. 15 (1988) 107–122.
[9] U. Pick, L. Karni, M. Avron, Plant Physiol. 81 (1986) 92–96.
[10] H.-E. Åkerlund, J. Biochim. Biophys. Methods 9 (1984) 133–141.
[11] D.I. Arnon, Plant Physiol. 24 (1949) 1–15.
[12] U.K. Laemmli, Nature 227 (1970) 680–685.
[13] A. Melis, J.S. Brown, Proc. Natl. Acad. Sci. USA 77 (1980) 4712–4716.
[14] A. Melis, Arch. Biochem. Biophys. 217 (1982) 536–545.
[15] R. Outrakul, S. Izawa, Biochim. Biophys. Acta 305 (1973) 105–118.
[16] H. Stefánsson, L. Wollenberger, S.-G. Yu, P.-Å. Albertsson, Biochim. Biophys. Acta 1231 (1995) 323–334.
[17] Albertsson, P.-Å. (1986) Partition of Cell Particles and Macromolecules, 3rd edn., Wiley, New York.
[18] H.-E. Åkerlund, B. Andersson, Biochim. Biophys. Acta 725 (1983) 34–40.
[19] P.-Å. Albertsson, FEBS Lett. 149 (1982) 186–192.
[20] P.-Å. Albertsson, E. Andreasson, P. Svensson, FEBS Lett. 273 (1990) 36–40.
[21] P. Bennoun, Proc. Natl. Acad. Sci. USA 79 (1983) 4352–4356.