Biochimica et Biophysica Acta 1787 (2009) 25–36

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Fragmentation and separation analysis of the photosynthetic membrane from spinach

Ravi Danielsson, Per-Åke Albertsson *

Department of Biochemistry, Chemical Centre, Lund University, P.O. Box 124, S-221 00 Lund, Sweden

ARTICLE INFO

Article history:
Received 20 June 2008
Received in revised form 28 September 2008
Accepted 3 October 2008
Available online 17 October 2008

Keywords:
Photo system I
Photo system II
Thylakoid
Plastoglobule
Phase partition
Counter-current distribution
Antenna size

ABSTRACT

Membrane vesicles, originating from grana, grana core (appressed grana regions), grana margins and stroma lamellae/end membranes, were analysed by counter current distribution (CCD) using aqueous dextran-polyethylene glycol two-phase systems. Each vesicle population gave rise to distinct peaks in the CCD diagram representing different vesicle subpopulations. The grana vesicles and grana core vesicles each separated into 3 different subpopulations having different chlorophyll *a/b* ratios and PSI/PSII ratios. Two of the grana core subpopulations had a chlorophyll *a/b* ratio of 2.0 and PSI/PSII ratio of 0.10 and are among the most PSII enriched thylakoid vesicle preparation obtained so far by a non detergent method. The margin vesicles separated into 3 different populations, with about the same chlorophyll *a/b* ratios, but different fluorescence emission spectra. The stroma lamellae/end membrane vesicles separated into 4 subpopulations. Plastoglobules, connected to membrane vesicles, were highly enriched in 2 of these subpopulations and it is proposed that these 2 subpopulations originate from stroma lamellae while the 2 others originate from end membranes. Fragmentation and separation analysis shows that the margins of grana constitute a distinct domain of the thylakoid and also allows the estimation of the chlorophyll antenna sizes of PSI and PSII in different thylakoid domains.

1. Introduction

The photosynthetic membrane, the thylakoid, of higher plants consists of a system of paired membranes enclosing between them the lumen and separating it from the surrounding stroma of the chloroplast. In addition to the asymmetry across the membrane there is also a lateral asymmetry such that the membrane is differentiated into distinct domains (see Fig. 1 for nomenclature). Based on electron microscopy one can distinguish between two main compartments, the grana and the stroma lamellae. The grana in turn consist of appressed membranes (grana core) forming a grana stack together with the peripheral annuli of each grana disc (named margins). In addition there are two end membranes at the ends of each grana stack. The stroma lamellae (inter-grana lamellae), which account for about 20% of the thylakoid area [1] are single paired membranes forming helical networks around and between the grana stacks [2–6] Connected to the thylakoids are plastoglobuli which are surrounded by a single half-bilayer membrane connected to, and continuous with, mainly the edges of the stroma lamellae [7,8]. The lumen enclosed between the paired membranes contains a large number (>100) of different water soluble proteins [9].

Biochemical and electron microscopic studies have revealed a substantial specialisation of the different domains. Fragmentation and separation by aqueous two-phase partitioning resulted in isolation of membrane vesicles of about the same size as the grana discs and composed of mainly PSII as determined by photosynthetic activity [10]. This suggested a lateral heterogeneity and a model was presented according to which PSI and PSII are segregated such that PSII is mainly located in the appressed region of the grana while PSI is found in the stroma exposed membrane regions i.e. stroma lamellae, grana margins and the end membranes [11]. This model was later confirmed by analysis of the protein composition of the same type of vesicles [12]. The lateral separation of PSI and PSII was also confirmed by immuno-cytochemical studies in combination with electron microscopy [13].

Furthermore, both the two photosystems are heterogenous. PSI in the grana margins (PSIα) have larger antennae than the PSIβ in the stroma lamellae and the grana end membranes [14–16]. This difference is mainly due LHCII as an additional part of the PSI antennae in the grana margins [15,16]. PSII in the grana core (PSIIα) has a larger antenna than PSIIβ in the stroma lamellae [17,18]. In total, more chlorophyll is associated with PSI than with PSII, see [1] and references therein. Several studies have shown that the cytochrome *b6/f* complex is found all over the entire membrane system ([19], and references therein) with a slight, significant, preference for the

Abbreviations: CCD, counter current distribution; Chl, chlorophyll *(a+b)*; LHCII, light harvesting complex; PEG, polyethylene glycol; PpBQ, phenyl-p-benzoquinone; PSI, photo system I; PSII, photo system II

* Corresponding author. Tel.: +4646 2228190; fax: +4646 2224534.
E-mail address: per-ake.albertsson@biochemistry.lu.se (P.-Å. Albertsson).

0005-2728/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2008.10.002

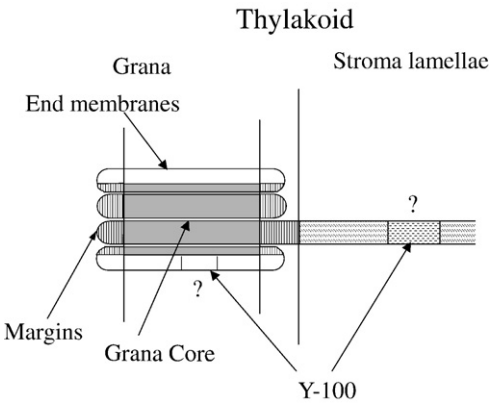


Fig. 1. Nomenclature of the different domains of the thylakoid membrane of higher plants. One can distinguish between two types of membranes, the stacked grana and the stroma lamellae (inter-lamellae) which connect the grana stacks. The grana stacks consist of piled circular disks. The disks contain two sub-domains, an appressed inner part, the grana core, and a peripheral annulus, the margin part, surrounding the core. The end membranes are single membranes and make the top and bottom of each grana stack. They constitute a third type of sub-domain in grana. The stroma lamellae, grana margins and end membranes are exposed to the chloroplast stroma. Vesicles originating from the different domains can be isolated more or less enriched after sonication and separation (see Materials and methods). The following nomenclature of the isolater fractions is used in this paper: Grana vesicles (originating from appressed + margins), grana core vesicles (originating from the appressed membrane), margin vesicles (origination from grana margins), "stroma lamellae/end membrane vesicles" originating from both stroma lamellae and end membranes. A special fraction, Y-100, is obtained from the supernatant after press treatment (Yeda press or French press) of the thylakoid and centrifugation at 100 000 g. The origin of this vesicle is not well known. It can be either stroma lamellae, end membrane or both. (In this paper we propose that Y-100 originates from the end membranes, see Discussion under "The Y-100 fraction".)

appressed grana membrane [20,21]. The dominating membrane lipids mono- and di-galactodiglycerol lipids are evenly distributed over the thylakoid membrane [22]. The carotenoids, lutein and β-carotene, and the xantophylls, violaxanthin and zeaxanthin, are also found over the entire membrane system but lutein, and particularly neoxanthin, are enriched in the PSII rich regions and β-carotene is enriched in the PSI rich regions while violaxanthin and zeaxanthin are fairly evenly distributed [23,24].

The biochemical composition of the different thylakoid membrane domains depends on the light intensities during growth [1,25–28] such that the concentration of PSII reaction centres decrease and their antenna size increase in the appressed domains at lower light intensities. By contrast the composition of the stroma exposed membrane domains is fairly constant. As a result the PSI/PSII reaction centre ratio varies considerably with light intensity during growth. Light conditions also affect the distribution of light absorption between the two photo systems as described by state transitions [29].

The different membrane domains can be isolated, more or less enriched, by mechanical fragmentation followed by separation by a combination of aqueous polymer two-phase partitioning and centrifugation [30], see Fig. 2. The result is sub-thylakoid membrane vesicle preparations originating from the grana (including grana margins), grana core, grana margins and stroma lamellae + end membrane vesicles. By press treatment and centrifugation one more preparation was obtained called Y-100 [31]. For nomenclature see Fig. 1. (In this paper "domain" refers to regions of the native, intact membrane while "subthylakoid vesicles" refers to membrane fragments produced by mechanical fragmentation such as sonication or press treatments).

Here we analyse the heterogeneity of the different populations of sub-thylakoid vesicles by counter current distribution using aqueous polymer two-phase system. The results show a considerable heterogeneity among the isolated membrane vesicles indicating a complex lateral heterogeneity of the thylakoid membrane. Moreover the results support the notion that the grana margins, i.e. the grana peripheral annuli, constitute a distinct domain which has a different composition from the surrounding membrane domains. In addition, we found that plastoglobules were highly enriched in two of the fractions originating from the stroma lamellae/end membranes. Furthermore, we propose that the stroma lamellae vesicles are separated from end membrane vesicles by counter-current distribution.

2. Materials and methods

2.1. Preparation of thylakoid membranes and its different fractions and sub-fractions

Spinach (*Spinachia oleracia L.*) was grown hydroponically under cool white fluorescent light at 20 °C with light–dark periods of 12 h and with the light intensity of 300 μE m^{-2} s^{-1}. The light source was dysprosium lamps of the type Osram Power star HQI-E 400W/DV, relatively close to day light and used in our previous publications [10–12,14–16,20,21,23,24,30–32].

Thylakoids from spinach grown in our dysprosium light have been compared to thylakoids from the same type of spinach grown under day light in the green house and there was no significant difference in the Chl *a/b* ratio of the thylakoids or the quality or quantity of isolated grana and stroma lamellae vesicles obtained by non detergent fragmentation and separation, see Table 2 of reference [32].

Thylakoids were prepared and fragmented by sonication to yield membrane vesicles originating from grana and stroma lamellae vesicles which were separated by aqueous two-phase partitioning as described in [14,30,31] except that the sonication was carried out 6 times instead of 8. The grana fraction was further sonicated to isolate "grana core" and "grana margins" vesicles [30]. Thylakoids were also fragmented by press treatment (Yeda Press), followed by centrifugation at 40 000 g for 30 min. The resulting supernatant contains small vesicles named Y-100, and these were pelleted at 100 000 g for 90 min [31].

2.2. Counter current distribution (CCD)

A dextran-polyethylene glycol (PEG) phase system was used for counter current distribution [30]. The polymer concentration used is given in the respective figure text. The composition of other components was in all experiments 20 mM sucrose, 10 mM Na-phosphate pH 7.4 and 10 mM NaCl (buffer A). The Åkerlund centrifugal counter current distribution apparatus [33–34] with 60 cavities was used.

Each of the first 5 cavities was loaded with the sub-thylakoid vesicle sample mixed in the two phase system to a volume of 1.8 ml (equal volumes of the two phases) and the rest of the cavities were loaded with 0.9 ml of top and 0.9 ml of bottom phase of the two phase system. The mixing time was 30 s and the centrifugation time was 90 s. After 53 transfers, the contents of each cavity was diluted with 1 ml of buffer A and mixed to convert the two-phase system to a one phase system. The relative Chl contents in the sample of each cavity were measured by absorption at 680. All separation procedures were performed at a temperature of 4 °C.

2.3. Analysis of the separated and pooled CCD fractions and sub-fractions

The Chl *a/b* ratio for each pooled peak was measured according to Arnon [35]. For correction to the method of Porra et al. see [36].

Steady state oxygen evolution rate was measured in 15 mM MES pH 6.5, 15 mM NaCl and 300 mM sucrose with a Clark electrode at 20 °C using saturating white light. 2 mM ferricyanide and 0.5 mM PpBQ were used as electron acceptors (Table 1).

The fluorescence emission spectrum for each pooled peak was measured at 77K with a Chl concentration of 10 μg/ml in 60% glycerol

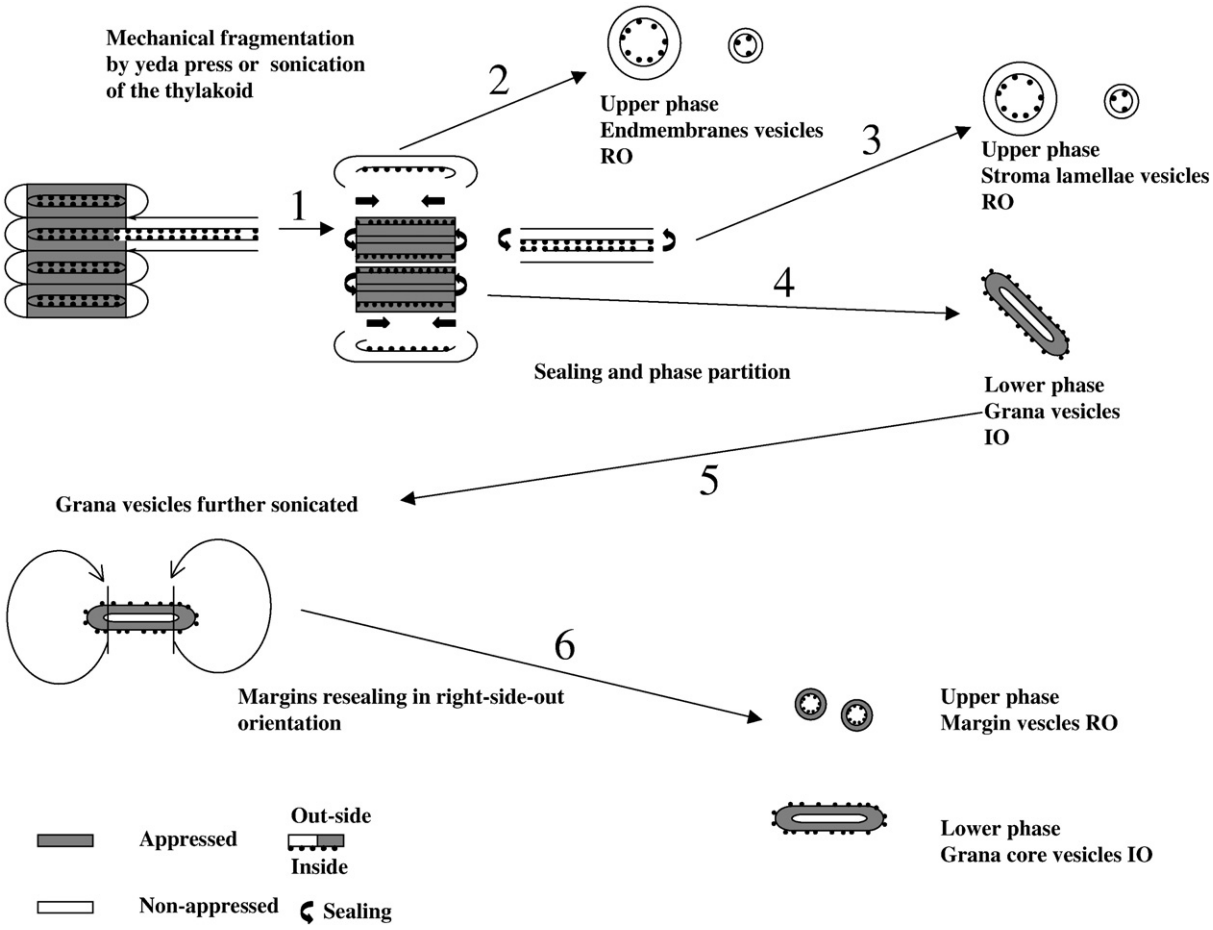


Fig. 2. Schematic illustration of fragmentation, re-sealing and phase partitioning of the thylakoid vesicles. Fragmentation is accomplished by two sonication steps. The first step results in 1–4 and the second in 5–6. 1. Sonication of stacked thylakoids leads to detachment of stroma lamellae and end membrane from grana. 2. End membranes are further fragmented resulting in right-side out vesicles which partition into the upper phase. 3. The same as 2. for stroma lamellae. 4. Part of the grana margins is split open and pairs of stacked grana discs form inside-out vesicles [10,11] partitioning into the lower phase. 5. Further sonication of grana vesicles detaches the grana margins which fuse into right-side out vesicles, and 6, partition into the upper phase while the remaining grana core stays inside-out and partition into the lower phase [30].

with an excitation wavelength of 485 nm. The measurements were made by a SPEX, fluoromax 2, spectrofluorometer and with software Data Max for Windows. A standard curve for measurements of the PSI/PSII ratios was constructed by plotting PSI/PSII ratios determined by EPR [31] against F_{735}/F_{695} (Fig. 4) on the same type of membrane vesicle preparations obtained from spinach grown with the same medium and under the same light conditions as in this study.

Table 1
Characterization of different sub-fractions of the thylakoid membrane

Fraction (Fig. 1)	O_2 evolution [μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}]	Chl *a*/*b* (mol/mol)	F_{735}/F_{695}	PSI/PSII from standard curve
Grana vesicles	249±26			
(Peak 1, Fig. 3B)		2.2	0.20	0.1
(Peak 2, Fig. 3B)		2.3	0.12	0.1
(Peak 3, Fig. 3B)		2.6	0.40	0.4
Grana core vesicles	271±16			
(Peak 1, Fig. 3C)		2.0	0.20	0.1
(Peak 2, Fig. 3C)		2.0	0.22	0.1
(Peak 3, Fig. 3C)		2.1	0.37	0.4
Margin vesicles	100±5			
(Peak 1, Fig. 3D)		3.2	1.26	1.9
(Peak 2, Fig. 3D)		3.0	0.95	1.4
(Peak 3, Fig. 3D)		3.1	1.01	1.5
Stroma lamella/end membrane vesicles	78±4			
(Peak 1, Fig. 3E)		4.4	2.03	3.2
(Peak 2, Fig. 3E)		4.5	2.25	3.6
(Peak 3, Fig. 3E)		4.3	1.87	2.9
(Peak 4, Fig. 3E)		4.3	1.68	2.6
Y-100	25±7			
(Peak 1, Fig. 3F)		4.8	1.91	3.0
(Peak 2, Fig. 3F)		5.5	3.91	6.4

The fifth column shows the PSI/PSII ratios obtained by a standard curve (Fig. 4) using the fluorescence ratios of column 4.

2.4. Electron microscopy

Pooled fractions from the CCD experiments were centrifuged at 100 000 g for 90 min. The pellets were fixed with 2.5% glutaraldehyd in 0.15 M kakodylate buffer, embedded in Epon, and stained with 3% uranyl acetate and lead citrate.

3. Results

3.1. Countercurrent distribution (CCD)

Sonication of stacked thylakoids yields two populations represented by two distinct peaks (Fig. 3A). The left peak with a Chl *a*/*b* ratio of 2.2 is enriched in PSII and represents the grana fraction (grana core+grana margins), while the right peak with a Chl *a*/*b* ratio of 4.3 is enriched in PSI and represents stroma lamellae together with grana end membranes. Both the Chl *a*/*b* ratios and the quantitative distribution of Chl (64% and 36% respectively) between the two peaks agree with results published earlier [1,14,30]. Peaks to the left in a CCD diagram prefer the lower, dextran rich, phase or the interphase while those to the right prefer the upper, PEG, rich phase [14,30].

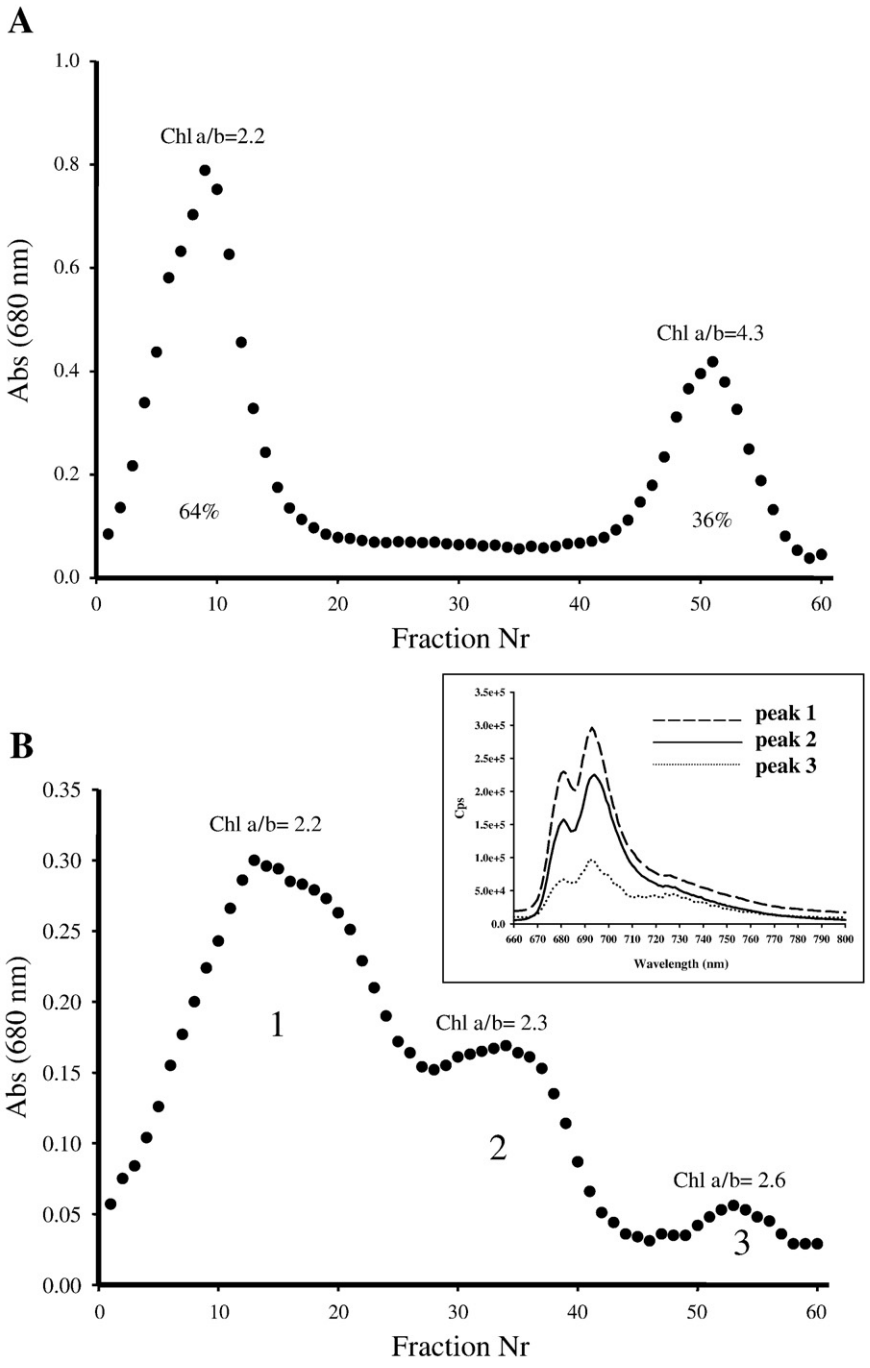


Fig. 3. CCD of sonicated thylakoid sub-thylakoid membrane vesicles. The fluorescence emission spectra for the pooled samples represented by the peaks numbered from the left to the right are shown in the insets. The fluorescence measurements were made at 77 K with the excitation wavelength of 485 nm. The same Chl concentration was used for each fraction in a CCD experiment. Ordinate: Cps0: counts per second. F_{680}, F_{685} and F_{695} emanate from LHCII, Cp43 and Cp47 respectively while F_{735} emanates from PSI. [56]. **(A)** CCD of sonicated stacked thylakoids showing the separation of grana vesicles (left peak) and "stroma lamellae/end membrane vesicles" vesicles (right peak) after mild sonication of stacked thylakoids and CCD using the two-phase system of 5.7% (w/w) of each of PEG and dextran. The fractions contributing to each peak were collected and pooled, and the Chl *a*/*b* ratios were measured. The Chl *a*/*b* ratio is 2.2 for the peak representing the grana vesicle fraction and 4.3 for the peak representing the "stroma lamellae/end membrane" fraction. The figures of the percentage in each peak represent the distribution of total chlorophyll between the two fractions. **(B)** Diagram showing the grana fraction, represented by the left peak in (A), further separated by CCD using a two-phase system with 5.5% (w/w) of each of PEG and dextran. The lower concentration of the two polymers shifts the partitioning more towards the upper phase concomitant with a shift of the position of the vesicles to the right in the CCD diagram. This leads to an increase in resolution and a separation of the grana fraction (left peak of A) into three different peaks, with different Chl *a*/*b* ratios. High fluorescence around 680–695 shows that PSII is present to a great extent compared to PSI with fluorescence at 735. **(C)** Grana core vesicles. The same two-phase system as in B. The curves show a similar heterogeneity for grana core as for grana (B). Fluorescence from PSII is dominating with high fluorescence around 680–695 and with low PSI fluorescence at 735. Note the small peak in the emission spectrum at 725 for fraction 3. **(D)** Grana margin sub-fractions. Phase system: 6.0% (w/w) PEG and 6.0% (w/w). The higher polymer concentration shifts the distribution more towards the bottom phase which results in improved resolution (compare with B). The curve for grana margins shows a heterogeneity with three different peaks having slightly different Chl *a*/*b* ratios. The fluorescence intensity is more equally distributed between PSI and PSII than in all other sub-fractions. **(E)** Stroma lamellae/end membrane fraction (represented by the right peak in A) further separated by a two-phase system, with 6.0% (w/w) of PEG and dextran respectively. Four peaks with different Chl *a*/*b* ratio show a heterogeneity in the stroma lamellae/end membrane fraction. The fluorescence from PSI at about 735 dominates in all four peaks (but with different intensity). Also the PSII fluorescence differs in all the four peaks. **(F)** The Y-100 preparation. Phase system as for the stroma lamellae/end membrane fraction (E). This curve shows one dominating peak with high Chl *a*/*b* ratio, and a shoulder on its right slope with still higher Chl *a*/*b* ratio. The fluorescence from PSI at about 735 dominates and the fluorescence from PSII at 680–695 differs in the two peaks.

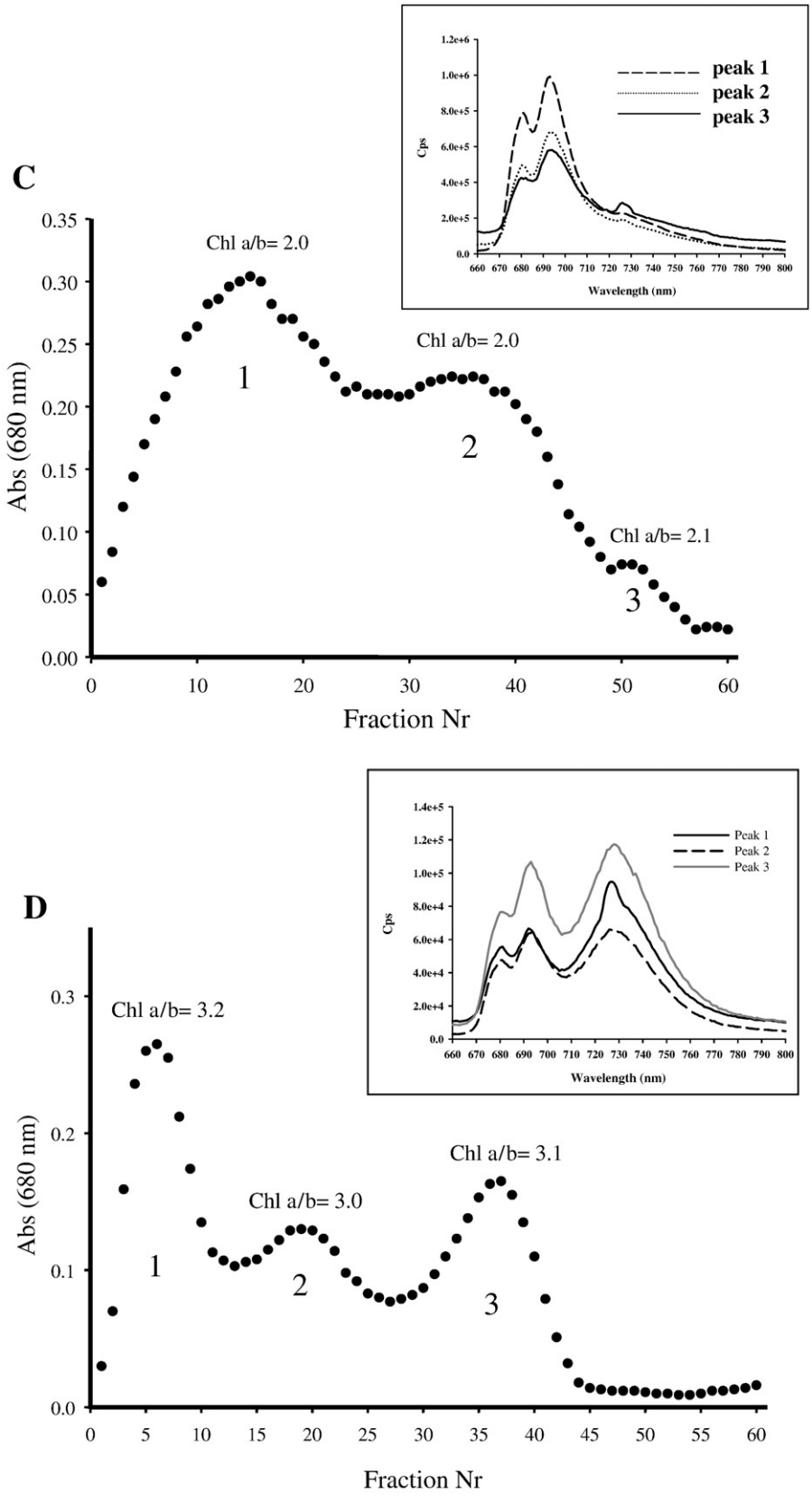
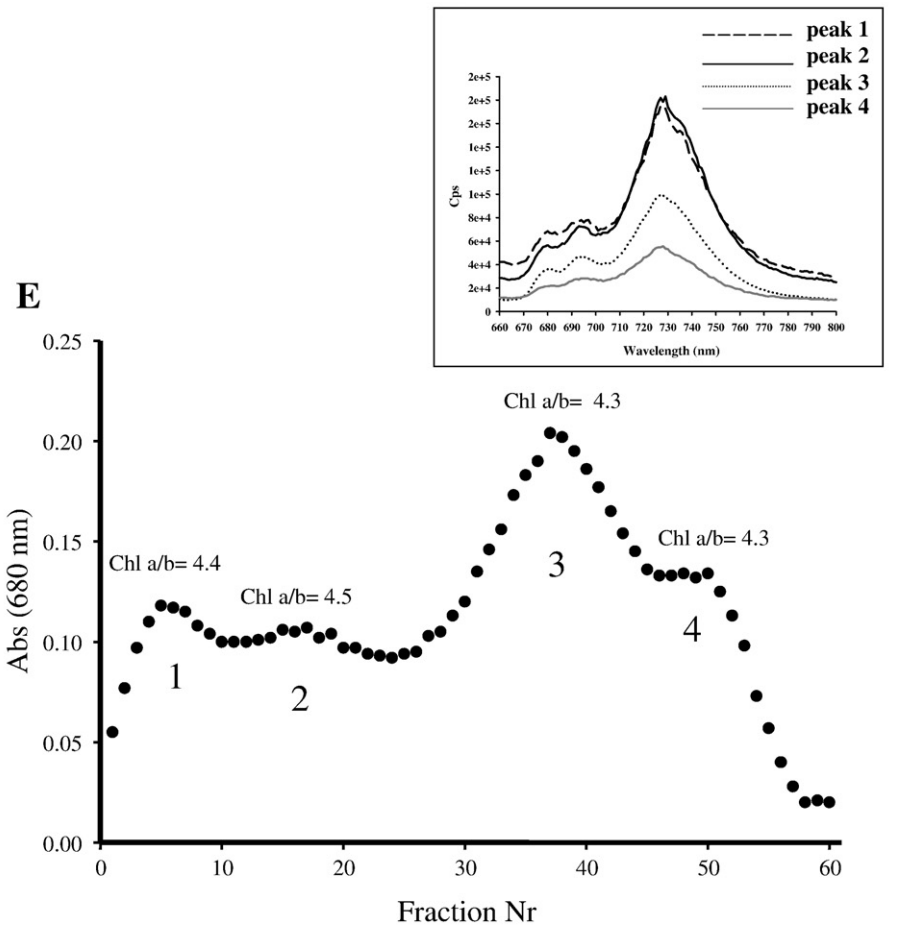


Fig. 3 (*continued*).

3.1.1. The grana fraction

Grana vesicles, obtained by batch preparation (see Materials and methods) and representing the vesicles from the left peak in Fig. 3A, were further analysed by counter-current distribution, without further sonication but with a new phase system having a polymer composition of 5.5% (w/w) PEG and 5.5% (w/w) dextran. This shifts the distribution of the vesicles more towards the upper phase giving optimal conditions for separation [37]. A considerable heterogeneity is



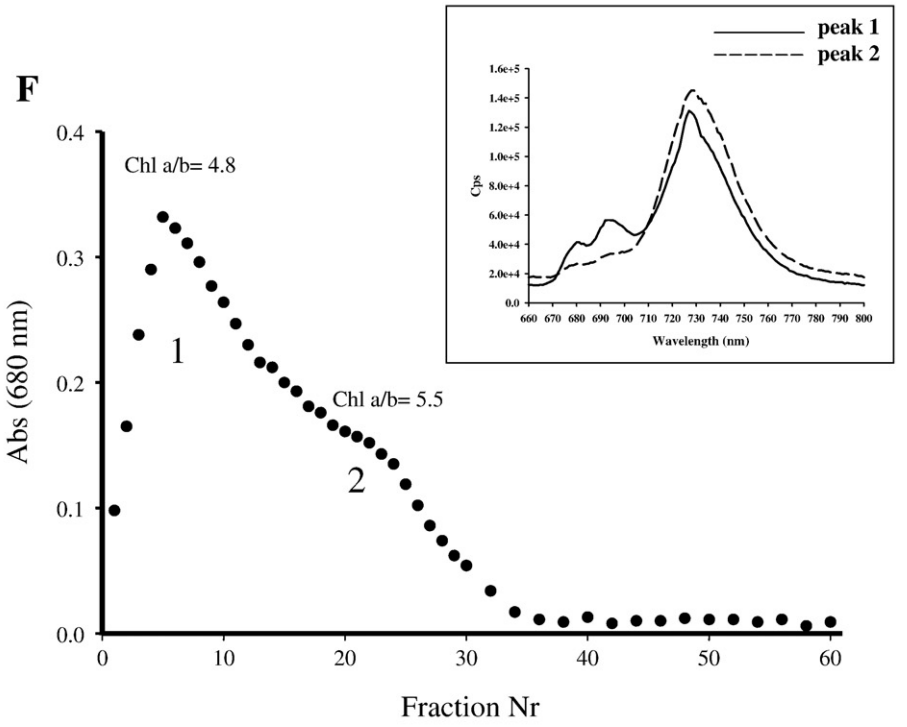


Fig. 3 (*continued*).

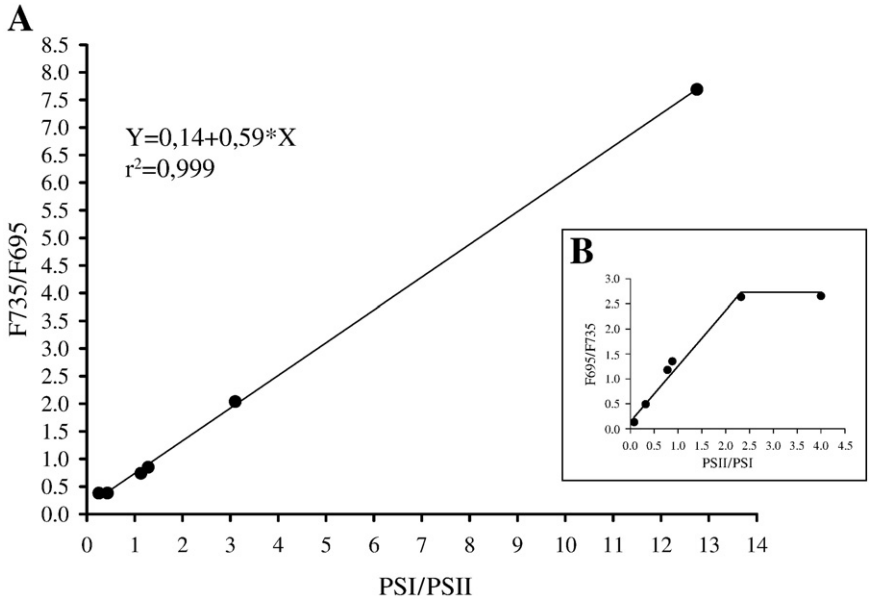


Fig. 4. (A) Standard curve for showing the ratio F_{735} (representing PSI)/F_{695} (representing PSII) plotted against PSI/PSII ratio determined by EPR measurements on the same sample of different thylakoid sub-fractions [31]. (B) The curve for the inverted ratios of A, indicating that the standard curve in A is misleading at very low PSI/PSII ratio.

thereby demonstrated with three peaks having different Chl *a/b* ratios, 2.2, 2.3 and 2.6 for peaks 1, 2 and 3 respectively (Fig. 3B), i.e. the Chl *a/b* ratios increasing with fraction number.

Also the fluorescence emission spectra for the three peaks are different (see inset Fig. 3B). The fluorescence emission spectra are dominated by PSII with high intensity at F_{680}, F_{685} and F_{695} emanating from LHCII, Cp43 and Cp47 respectively [56] while the fluorescence at F_{735} from PSI is low. By means of the standard curve (Fig. 4) the PSI/PSII ratio is estimated to be 0.1 for peaks 1 and 2, and 0.4 for peak 3 in Fig. 3B (Table 1).

3.1.2. The grana core fraction

The grana core fraction, prepared by a batch procedure (see Materials and methods), using the same polymer compositions for the grana fraction, also demonstrated heterogeneity with three peaks (Fig. 3C) having the Chl *a/b* 2.0, 2.0 and 2.1 for peaks 1, 2 and 3 respectively i.e. they are more close to each other compared to the grana sub-populations in Fig. 3B.

The fluorescence spectra show similarity to the spectra from the peaks of the grana fraction, with fluorescence from PSII dominating but with still higher fluorescence emission at F_{680}, F_{685} and F_{695}, and a low PSI fluorescence at F_{735}. For both the grana sub-fractions and grana core sub-fractions the fluorescence emission curves show a small increase at F_{725}, most clearly seen for the grana core sub-fractions and particularly so for peak 3 (see inset Fig. 3C). The PSI/PSII ratios for the three peaks of the grana core sub-fraction are 0.1, 0.1 and 0.4 respectively according to the standard curve (Fig. 4).

3.1.3. Grana margin-fraction

CCD of he grana margin fraction using a polymer composition of each 6.0 (w/w) PEG and 6.0 (w/w) dextran is shown in Fig. 3D. The higher polymer concentration shifts the distribution of the vesicles more towards the bottom phase giving optimal conditions for separation [30,37]. Also for the margin vesicles a considerable heterogeneity is demonstrated by the CCD diagram while the Chl *a/b* ratios for the three peaks do not differ so much, 3.2, 3.0 and 3.1 for peaks 1, 2 and 3 respectively. The fluorescence spectra show that peaks 2 and 3 have nearly the same fluorescence intensity from PSI (F_{735}) and PSII (F_{680}, F_{685}, F_{695}), while peak 1 shows more intensity from PSI than PSII. It can be noted that the peak from PSI has its maximum not at 735 but at about 725. The ratios PSI/PSII for peaks 1–3 of the margin sub-fractions are 1.9, 1.4 and 1.5 respectively.

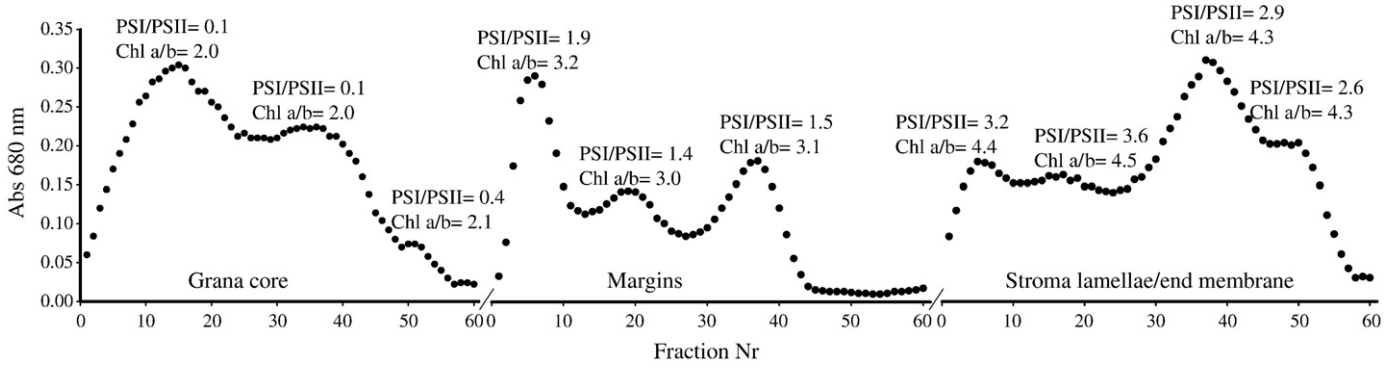


Fig. 5. Overall lateral heterogeneity of the thylakoid membrane. The diagram shows the combination of the curve for grana core fraction (Fig. 3C) with the curve for grana margins (Fig. 6D) and the curve for stroma lamellae/end membrane fraction (Fig. 3E). This combined curve account for the total lateral heterogeneity of the thylakoid, with ten different peaks representing vesicles with different surface properties and with different PSI/PSII and Chl *a/b* ratios.

3.1.4. Stroma lamellae/end membrane

Stroma lamellae/end membrane vesicles, prepared by a batch procedure (see Materials and methods), and representing vesicles of the right peak in Fig. 3A, were analysed without further sonication but using a polymer composition of 6.0% (w/w) of each of dextran and PEG i.e. the same as for grana margins. Fig. 3E shows 4 peaks. The two small peaks to the left, peaks 1 and 2, have both a higher Chl *a*/*b* ratio (4.4 and 4.5 respectively) than peaks 3 and 4 to the right (4.3). The fluorescence spectra of the four peaks (Fig. 3E) are dominated by emission from PSI at F_{735}, and only a small part of the fluorescence comes from PSII (at F_{680}, F_{685} and F_{695}).The PSI/PSII ratios of the four peaks in Fig. 3E, according to the standard curve, are 3.2, 3.6, 2.9 and 2.6, respectively.

3.1.5. Y-100

The Y-100, i.e. a sub-fraction with small vesicles obtained from press treatment and differential centrifugation, was analysed also using 6.0% (w/w) of each of dextran and PEG (Fig. 3F). The resulting diagram shows one major peak to the left (Chl *a*/*b*=4.8) with a shoulder (Chl *a*/*b*=5.5). The fluorescence emission spectra for these two peaks (Fig. 3F) show that the Y-100 fraction is even more enriched in PSI than "stroma lamellae/end membrane" fractions (Fig. 3E). According to the standard curve, the PSI/PSII ratio is 3.0 for peak 1, and 6.4 for peak 2 (the shoulder) which has the highest PSI/PSII ratio of all fractions (Table 1).

3.2. Electron microscopy

If subpanels C, D and E of Fig. 3 are put together as in Fig. 5, we get an overview of the total sub-thylakoid vesicles distribution as a curve containing 10 peaks. The three peaks to the left, with low Chl *a*/*b* and PSI/PSII ratios, represent the grana core sub fractions, the three middle peaks with medium Chl *a*/*b* and PSI/PSII ratios represent margin sub fractions, and the four peaks to the right, with high Chl *a*/*b* and PSI/PSII ratios, represent the stroma lamellae/end membrane sub fractions. Vesicles from all 10

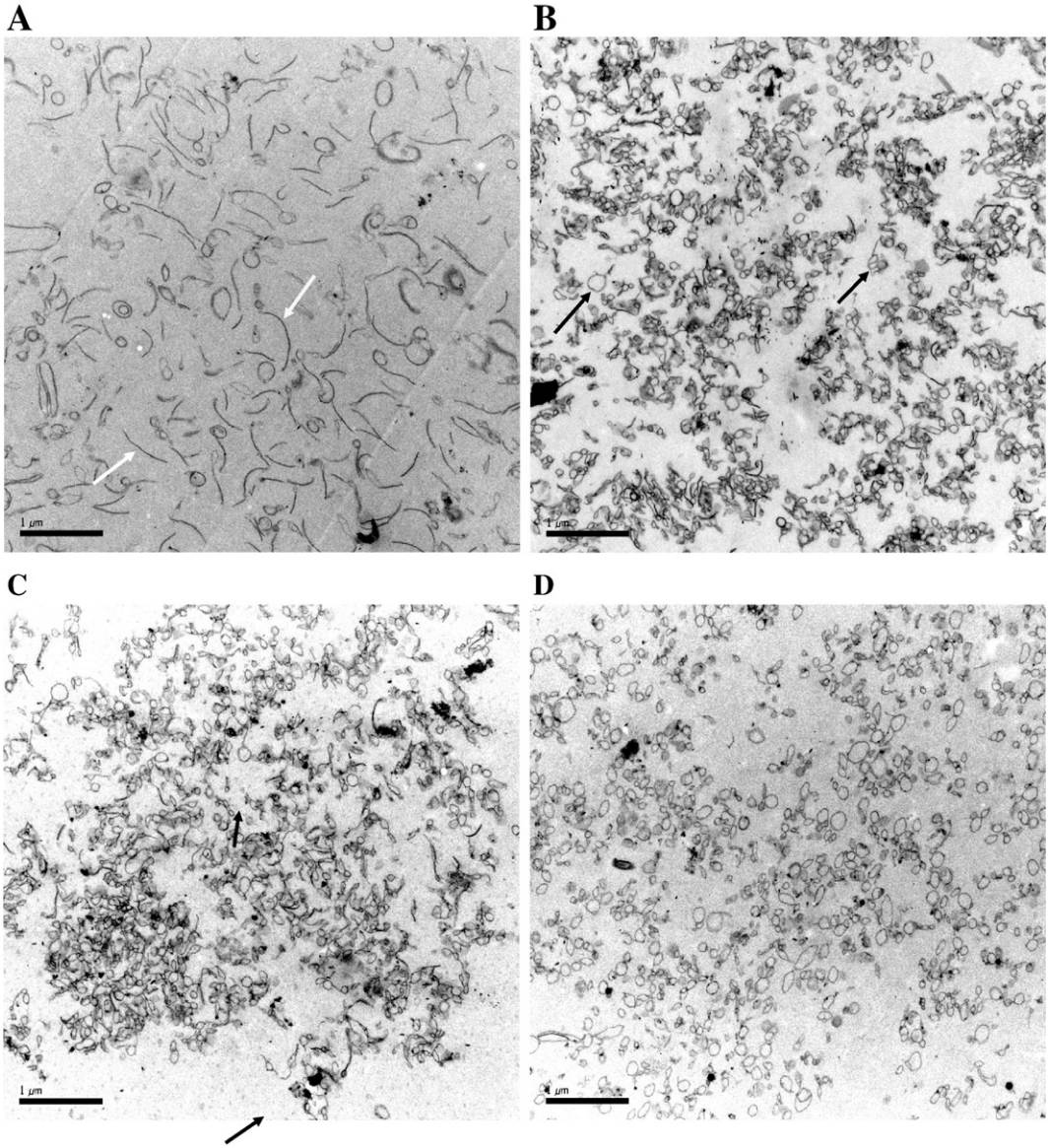


Fig. 6. Electron microscopy of sub-thylakoid vesicles from different fractions in Fig. 3C–E. (A) Grana core vesicles (peak 1 of Fig. 3C). The picture is dominated by thick lines representing two appressed membranes (arrows). (B) Grana margin vesicles (peak 1 o Fig. 3D); Small vesicles, often attached to small appressed pieces (arrows) (C). Grana margin vesicles (peak 3 of Fig. 3D). (D) Proposed end membrane vesicles (peak 1 of Fig. 3E, peak 2 of Fig. 3E look very similar); fairly uniform vesicles relative to the other vesicle fractions. (E) Stroma lamellae vesicles (peak 4 of Fig. 3E). The black bodies, represent, plastoglobules connected to, and supposedly continuous with, stroma lamellae membrane vesicles (arrows).

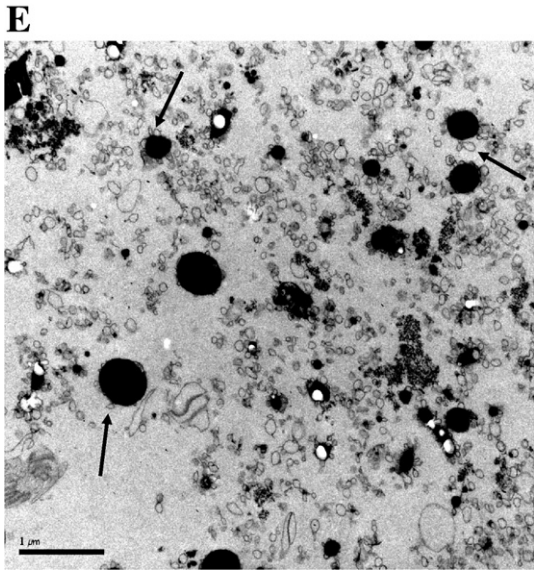


Fig. 6 (*continued*).

peaks were analysed by electron microscopy of which 5 are shown in Fig. 6.

3.2.1. Grana core vesicles, Fig. 6A

Vesicles from peak 1 in Fig. 3C are shown in Fig. 6A. These vesicles originate from the appressed region of grana (grana core) and have the highest PSII/PSI ratio. They are inside out vesicles, two membranes are appressed together and appear as one thick line (arrows). That inside-out grana vesicles consist of two appressed membranes has been demonstrated by freeze-fracture electron microscopy [38]. This fraction is among the most enriched PSII vesicle populations obtained so far by a non-detergent method. No plastoglobules were found in this fraction. The other fractions (peaks 2 and 3) look very similar as Fig. 6A and they also lack plastoglobules.

3.2.2. Grana margin vesicles, Fig. 6B and C

The margin vesicles represented by peaks 1, 2 and 3 in Fig. 3D look very similar. Vesicles from peaks 1 and 3 are shown in Fig. 6B and C respectively. They are relatively small and are often composed of one short appressed double membrane continuous with a circular vesicle (arrows) i.e. they seem to originate from the appressed marginal annulus of grana together with curved part of grana margins. Their Chl *a/b* ratio of about 3 is partly the result of LHCII connected to PSI (PSIα) in the margins. Very few plastoglobules were found in the pictures from these fractions.

3.2.3. Stroma/end membrane vesicles Fig. 6D and E

The vesicles from peaks 1 and 2 of Fig. 3E were fairly homogenous in size; no plastoglobules were found in peak 1 (Fig. 6D) and very few in peak 2. By contrast, several plastoglobules, connected to membrane blebs, were found in vesicles from peaks 3 and 4 and more so from those of peak 4 as shown in Fig. 6E which is loaded with plastoglobules both single and in clusters. The relative frequencies of plastoglobules were about <1, 1, 35 and 60 for peaks 1, 2, 3 and 4 respectively.

3.3. Plots of compositions of sub-thylakoid vesicles

Mechanical disruption of biological membranes yields fragments, of different sizes and compositions, and originating from different domains of the membrane. If the concentration of two components, say x/Chl and y/Chl, of the different fragments, can be determined, one can use this information for analysis of the domain structure of the membrane [21,39]. Thus, if a membrane consists of two domains a plot of the concentration of one component, say x/chl, against another component, say y/chl, gives a straight line provided the two components are differently distributed between the two domains. If the membrane consists of three domains a triangle is obtained; if four domains a quadrangle etc. See reference [21,39] for the theoretical basis. Thus, plots of the composition of different sub-thylakoid vesicles allow us to find the minimum of number of domains a membrane must consist of.

If we use the data from Table 1 and plot PSII/PSI+PSII against chl *b*/*a*+*b*, this does nor fit a straight line but rather two lines (Fig. 7A) supporting the notion of *at least* 3 distinct domains in the thylakoid membrane.

Using data based on EPR determination of PSI and PSII, on the same type of preparation of thylakoids used in this study and from plants grown under similar conditions [31], and plotting PSII/Chl against PSI/Chl we also get two lines 1 and 2 (Fig. 7B) again indicating that thylakoids consist of *at least* three domains, interpreted as grana core, grana margins and stroma lamellae, in agreement with a similar plot of PSI against cytochrome *b6f* [21]. Extrapolation of the two lines to zero concentration of PSI/Chl or PSII/Chl can be used to estimate the antenna size of the two photo systems (see Discussion below).

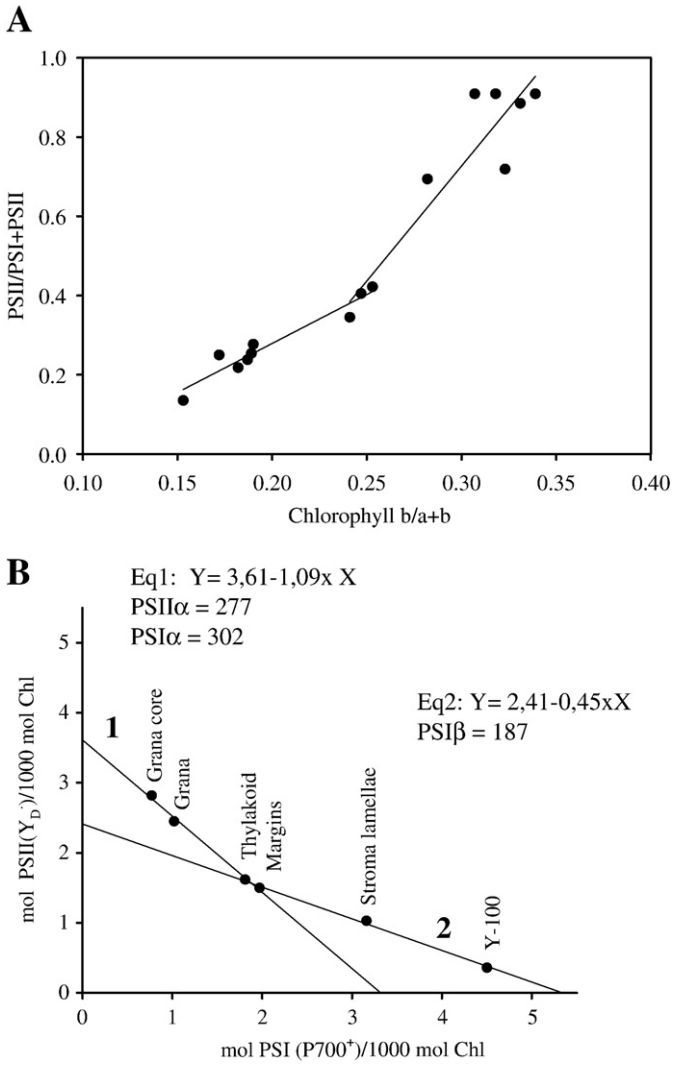


Fig. 7. Plots of compositions of different sub-thylakoid vesicles. (A) Data from Table 1. (B) Data from reference [31].

4. Discussion

4.1. General comments

Fragmentation and separation analysis is a classical method to study the structure of an object. In the case of the thylakoid membrane, this approach, combined with electron microscopy, has been very successful in giving us an important information on not only the structure but also the function of this complex membrane. It has provided us with a realistic picture of the architecture of the thylakoid membrane system i.e. one can distinguish between the stacked grana with its margin and its two end membranes in contrast to the single paired stroma lamellae connecting the grana [2–6].

Biochemical analysis of the different vesicles obtained by fragmentation allows a characterization of the different domains in the membrane which are necessary for construction of quantitative models which relate structure and function of the thylakoid membrane. For example: 1) Fragmentation and separation analysis shows that the grana margin consist of a distinct domain [21] which agrees with observation by electron microscopy [6], 2) It has been shown that more chlorophyll is associated with PSI than with PSII which support a model with cyclic electron transport in stroma lamellae concomitant with linear electron transport in grana [1,32,40].

The results of this work show that the thylakoid membrane is much more complex in that the different sub thylakoid vesicles isolated so far, mainly by batch procedures, are quite heterogeneous and separated into distinct, different vesicle populations. This can form the basis for further studies on the biochemical composition of the different vesicles and particularly modern proteomics techniques would be of interest in this context. Below follows discussion of the different sections of this work.

4.2. Light conditions

The spinach used was grown under dysprosium lamps which provide a light relatively close to day light. This was confirmed by comparing fragmentation and separation of thylakoids grown under dysprosium lamps with those grown under day light [32]. The same growth and light conditions as used here have been used in this laboratory for the last 30 years which allows a high reproducibility of the experiments and a meaningful comparison with earlier work. However, one should appreciate that, when comparing with studies from other laboratories, the biochemical composition of thylakoids depends on the light conditions used. Thus, the relative concentration of PSI, PSII, cytochrome *b6f* and ATP synthase varies with light conditions [26,27]. Likewise the proportion of end membranes decrease with lower light due to larger grana stacks while the proportion of stroma lamellae is fairly constant [1]. However, application of the fragmentation and separation methods used here on thylakoids from plants grown under different light conditions would probably give essentially similar results with regard to the overall complexity of the domain organisation of the thylakoid structure even though the composition of the different sub-thylakoid vesicles would depend on the light conditions. The application of proteomics on the different vesicle populations would certainly be of great interest.

4.3. The fragmentation procedure

When we started our first study on CCD of sonicated thylakoids included in the two-phase system, we found that the optimal concentration of magnesium chloride was 1 mM and also that the thylakoids were stacked [14]. It seemed somewhat surprising that this low magnesium concentration could ensure stacking conditions but recent studies have shown that the polymers themselves, at the concentrations used here, keep the thylakoids stacked as demonstrated by electron microscopy and fluorescence [41]. Thus, the combination of magnesium chloride and the two polymers dextran and PEG in the fragmentation medium ensures stacking conditions during sonication. It is known that the polymers protect biological material against denaturation such as surface denaturation by the air/liquid interface [37], that the photosynthetic activities by PSI and PSII are well preserved after sonication and separation [14,30,31] and that the envelope membranes of intact chloroplasts are preserved by the dextran-PEG phase system [42]. Thus, the medium used for sonication ensures both stacking conditions and protection of photosynthetic activities against the sonication and probably also keeps the half-bilayer membrane surrounding plastoglobules intact, attached to, and continuous with, the sub-thylakoid membrane vesicles (Fig. 6E).

4.4. Factors involved in aqueous two-phase partition

Partition of membrane vesicles in aqueous polymer two-phase systems depends on the properties of the phase system and the surface properties of the membrane vesicles. The factors involved include type, concentration and molecular weight of the polymers, ionic composition and the surface properties of the vesicles such as surface charge, isoelectric point, hydrophilic and hydrophobic interactions between exposed groups of the vesicles and the phase polymers [37].

The separation of grana vesicles from the stroma/end membrane vesicles, Fig. 3A, is due to the different sidedness of the two types of vesicles. The grana vesicles are inside-out and prefer the lower, dextran rich, phase while the stroma/end membrane vesicles are right-side out and prefer the upper, PEG rich, phase [43,44].

The heterogeneity observed within the grana vesicles, grana core vesicles, margin vesicles, and the stroma lamellae/end membrane vesicles (Fig. 3B–E) respectively is not due to different sidedness but probably to differences in the relative amount of different thylakoid proteins exposed to the surface.

It is remarkable that the separation diagrams (Fig. 3B–F) show distinct peaks indicating distinct classes of membrane vesicles and not just a random distribution of the surface properties of the vesicles. In the case of grana and grana core vesicles these may originate from different domains of the appressed grana membranes. Thus, the domains in the centre of the grana may be different from more peripheral parts of the appressed region which in turn differ from the regions close to the margins. That distinct peaks are obtained could be due certain domains, or border between domains, which are more easily and selectively broken by sonication and separated from neighbouring parts of the thylakoids membrane.

In the case of the stroma exposed membranes, one might expect different surface properties depending on differences in type and concentration of exposed proteins, such as PSI and ATP synthase, in the different regions reflecting their different functions. Assuming that end membranes and grana margins are involved in linear electron transport while stroma lamellae only in cyclic [32,40] one would expect differences in their surface properties. In fact, by using scanning force microscopy, in combination with immuno-gold labelling, it has been shown that the concentration of each of PSI and ATP synthase is different on the stromal side of end membranes, grana margins and stroma lamellae [6].

4.5. Grana core vesicles

The grana core vesicles represented by peaks 1 and 2 in Fig. 3C are highly enriched in PSII. A similar preparation obtained by repeated sonication and batch partitioning of grana vesicles has been characterized with respect to antenna size, absorption and fluorescence spectra [45]. Compared to PSII particles prepared by detergents such as “BBY” particles [46], the grana core vesicles have about the

same Chl *a/b* ratio, 2.0 compared to 1.9 for detergent particles [47], but a higher Chl/PSII ratio, 355 compared to 250 for detergent particles [48]. This is due to the small amount of PSI in the grana core vesicles and that chlorophyll containing proteins are extracted from thylakoids when using detergent treatment. Detergents also extract part of the membrane lipids and also some of the membrane proteins, such as the cytochrome *b6/f* complex [49]. Thus, compared to "BBY" particles the grana core vesicles are more intact with respect to membrane structure and composition but less pure with respect to PSII. It would be a challenge to try to purify the grana core vesicles further and remove the residual PSI without disturbing the integrity of the membrane structure. One reason for that the PSI is not easily detached from the grana vesicles may be that LHCII, which is considered to be one of the main factors behind the stacking forces in grana, is connected to the PSI of grana margins [15,16].

4.6. Plastoglobules and the separation of end membranes from stroma lamellae

Plastoglobules are surrounded by a membrane monolayer which is continuous with the outer, stroma exposed, half of the thylakoid bilayer [7,8]. This attachment occurs at curved areas of the thylakoid. Looking at several electron microscopy pictures in the literature, covering several decades, it appears that the plastoglobules are mainly attached to edges of the stroma lamellae and not so often to the flat regions of the end membrane. Often two or more plastoglobules are attached to each other in clusters via their monolayer membranes which is continuous with the outer layer of the thylakoid membrane [7,8]. In our CCD fractions, the plastoglobules are found almost exclusively in the two stroma/end membrane fractions represented by peaks 3 and 4 in Fig. 3E and, of these two, mainly in fraction 4. Since electron tomography and electron microscopy show that most plastoglobules are attached to the curved areas of stroma lamellae, often near the connections to the grana and very few to the end membranes [7], we propose that, of the four CCD fractions in Fig. 3E, fractions 1 and 2 originate from the end membranes and fractions 3 and 4 from the stroma lamellae.

The question then arises whether the proportion between the two populations of vesicles, i.e. those represented by peaks 1+2 versus 3+4, is what one would expect from the proportion of end membranes versus stroma lamellae in the chloroplast. The areas of end membranes and stroma lamellae have been determined by electron microscopy on the same type of spinach, grown under the same light conditions, as used in this study; see data on spinach ("Weibull, C personal communication") in Table 1 of reference [1]. End membranes accounted for 17.3% and stroma lamellae for 20.7% of the thylakoid area, e. a ratio of about 0.8. The mean value of the ratio end membrane/stroma lamellae for 6 different publications on spinach, as shown in Table 1 of reference [1], is about 0.6. The ratio of areas under the two populations in Fig. 3E i.e. the sum of the areas under curves 1+2 divided by the sum of the areas under curves 3+4 is about 0.6. Thus, the proportion between the two populations is compatible with the proposal that they represent vesicles from the end membranes and stroma lamellae respectively. In this context, it is of interest that the PSI/PSII ratios of fractions 1 and 2 of Fig. 3E are both higher than those of fractions 3 and 4 indicating an additional difference between the 1+2 and the 3+4 fractions. It would be of great interest to use proteomics for analysis of the material in the different peaks of Fig. 3E.

It has earlier been suggested that vesicles which originate from the end membranes differ from those originating from the stroma lamellae [50]. Experiments with tobacco thylakoids showed that the stroma/end membrane fraction, corresponding to the right hand peak of Fig. 3A, could be separated into two main peaks which were suggested to represent end membranes and stroma lamellae respectively, the end membranes having the highest Chl *a/b* ratio [50].

4.7. The Y-100 fraction

The result with the Y-100 fraction is particularly interesting. This is a fraction obtained by differential centrifugation after press treatment and consists of relatively small vesicles. It is usually considered as representative for stroma lamellae membranes [18]. However, a comparison of the diagrams of Fig. 3E and F shows that Y-100 is more close to the two left peaks of Fig. 3E. As a consequence of our earlier proposal, that the peaks 1 and 2 of Fig. 3E represent end membranes, we propose that the Y100 vesicles originate mainly from end membranes.

4.8. The grana margins as a distinct domain

The fragmentation and separation analysis (Fig. 7) shows that thylakoids consist of, *at least*, three domains. We propose that these are represented by the appressed grana core, the grana margins and the stroma lamellae/end membranes. Furthermore, the shape of the diagrams of Fig. 7 indicate that grana core is not in a continuous lateral contact with stroma lamellae/end membrane i.e. the grana margins are located between them, see reference [21].

With all thylakoid sub-fractions there is a clear correlation between Chl *a/b* ratio and PSI/PSII ratio; the higher the Chl *a/b* ratio the higher the PSI/PSII ratio (Table 1). However, this is not so if we compare the whole thylakoid and the margin fractions. The margin fractions have a much higher PSI/PSII ratio, 1.4–1.9, (Fig. 5, Table 1) compared to 1.13 for the whole thylakoid [31] yet they both have about the same Chl *a/b* ratios (2.9–3.2). This is due to that PSI in the margin vesicles (PSIα) is connected to LHCII [15,16] about 2 LHCII trimers per PSI reaction centre [31]. Since LHCII has a low Chl *a/b* ratio, 1.33 according to the crystal structure [51], its presence in PSIα will confer upon the margin vesicles a lower Chl *a/b* ratio than expected from their high PSI/PSII ratio. These data together with the fragmentation analysis (Fig. 7) therefore strengthen the notion that the margins constitute a distinct domain in the thylakoid [21,40] and not just a continuous transient between the appressed grana membrane and the stroma lamellae. This is also supported by scanning force microscopy [6].

4.9. Estimation of antenna sizes

Fig. 7B can also be used to calculate the antenna sizes of the two photo systems assuming that PSII is alone in the grana core and PSI alone in the stroma exposed domains i.e. stroma lamellae, end membranes and the grana margins. Extrapolation of line 1 to zero PSI gives a value of 3.61 PSII/1000 Chl=277 Chl/PSII i.e. the antenna of PSIIα in the appressed grana region which agrees with a previous work using EPR measurement of PSII [31]. Extrapolation of line 2 to zero PSII gives a value of 5.36/1000 Chl=187 Chl/P700 i.e. the antenna of PSIβ in the stroma lamella/end membrane. Extrapolation of line 1 to zero PSII gives a value of 3.31/1000 Chl=302 Chl/P700 which can be interpreted as representing the antenna of PSIα in the grana margins. This agrees with the antenna of PSIα determined experimentally from the kinetics of P700 photo oxidation of grana vesicle preparations [15]. Extrapolation of line 2 to zero PSI gives a value of 2.41/1000 Chl=415 Chl/PSII which can be interpreted as the antenna size of a PSII connected to a heptamer as described in [52,53] if we assume that each LHCII monomer contains 15 Chl and PSII without LHCII trimers contain 100 Chl [54]. This would then be a PSII domain located in the inner part of the margin annulus and containing either PSII with giant antenna or free floating LHCII heptamers. One might speculate that these heptamers are involved in the grana destacking process as a result of phosphorylation of LHCII leading to down-regulation of PSII and increase in cyclic electron transport due to an increase in destacked, single paired, thylakoid membranes, as described in [55] or, as suggested in [53], the heptamers are involved in energy

redistribution between the two photosystems as a result of phosphorylation of LHCII.

Acknowledgements

We thank the Royal Physiographic Society in Lund and the Carl Trygger Foundation for the financial support and Rita Wallén, Department of Zoology, Lund University, for taking the electron microscopy pictures.

References

[1] P.Å. Albertsson, E. Andreasson, The constant proportion of grana and stroma lamellae in plant chloroplasts, Physiol. Plant. 121 (2004) 334–342.
[2] W. Wehrmeyer, (Zur klärung structurellen variabilität der chloroplastengrana des spinats in profil und aufsicht), Planta 62 (1964) 272–293.
[3] D.J. Paulilo, The three-dimensional arrangement of intergrana lamella in chloroplasts, J. Cell Sci. 6 (1970) 243–255.
[4] L. Mustardy, Development of thylakoid membrane stacking, in: D.R. Ort, C.F. Yocum (Eds.), Oxygenic Photosynthesis: the Light Reactions, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1996, pp. 59–68.
[5] L. Mustardy, G. Garab, Granum revisited. A three-dimensional model, where things fall into place, Trends Plant Sci. 8 (2003) 117–122.
[6] D. Kaftan, V. Brumfeld, R. Nevo, A. Scherz, Z. Rech, From chloroplasts to photosystems: in situ scanning force microscopy on intact thylakoid membranes, EMBO J. 21 (6) (2002) 6146–6153.
[7] J.R. Austin, E. Frost, P.A. Vidi, F. Kessler, L.A. Staehelin, Plastoglobules are lipoprotein subcompartments of the chloroplast that are permanently coupled to thylakoids membranes and contain biosynthetic enzymes, Plant Cell 18 (2006) 1693–1703.
[8] C. Bréhélin, F. Kessler, K.J. van Wijk, Plastoglobules: versatile lipoprotein particles in plastids, Trends Plant Sci. 12 (2007) 260–266.
[9] T. Kiesselbach, W.P. Schröder, The proteome of the chloroplast lumen of higher plants, Photosynth. Res. 78 (2003) 249–264.
[10] H.E. Åkerlund, B. Andersson, P.Å. Albertsson, Isolation of photosystem II enriched membrane vesicles from spinach chloroplasts by phase partition, Biochim. Biophys. Acta 449 (1976) 525–535.
[11] B. Andersson, Separation of spinach chloroplast lamellae fragments by phase partition including the isolation of inside-out thylakoids, Thesis (1978) Lund University, Sweden
[12] B. Andersson, J.M. Anderson, Lateral heterogeneity in the distribution of chlorophyll–protein complexes of the thylakoid membranes of spinach chloroplasts, Biochim. Biophys. Acta 593 (1980) 427–440.
[13] O. Vallon, F.A. Wollman, J. Olive, Distribution of intrinsic subunits of the PS II protein complex between appressed and non appressed regions of the thylakoid membrane. An immunocytochemical study, FEBS Lett. 183 (1985) 245–250.
[14] E. Andreasson, P. Svensson, C. Weibull, P.Å. Albertsson, Separation of stroma and grana membranes — evidence for heterogeneity in antenna size of both photosystem I and photosystem II, Biochim. Biophys. Acta 936 (1988) 339–350.
[15] P. Svensson, E. Andreasson, P.-Å. Albertsson, Heterogeneity among photosystem I, Biochim. Biophys. Acta 1060 (1991) 45–50.
[16] E. Andreasson, P.-Å. Albertsson, Heterogeneity of photosystem I — the larger antenna of photosystem Iα is due to a special pool of LHCII, Biochim. Biophys. Acta 1141 (1993) 175–182.
[17] J.M. Anderson, A. Melis, Localization of different photosystems in separate regions of chloroplast membranes, Proc. Natl. Acad. Sci. U. S. A. 80 (1983) 745–749.
[18] T. Henrysson, C. Sundby, Characterisation of photosystem II in stroma thylakoid membranes, Photosynth. Res. 25 (1990) 107–117.
[19] J.M. Anderson, Cytochrome *b6f* complex: dynamic molecular organization, function and acclimation, Photosynth. Res. 34 (1992) 341–357.
[20] P.-Å. Albertsson, Subfractionation of inside-out thylakoid vesicles and the localization of cytochrome *f*, Physiol. Veg. 23 (1985) 731–739.
[21] P.Å. Albertsson, E. Andreasson, P. Svensson, S.G. Yu, Localization of cytochrome-*f* in the thylakoid membrane — evidence for multiple domains, Biochim. Biophys. Acta 1098 (1991) 90–94.
[22] S. Duchene, PA. Siegenthaler, Do glycerolipids display lateral heterogeneity in the thylakoid membrane? Lipids 35 (2000) 739–744.
[23] R.K. Juhler, E. Andreasson, S.-G. Yu, P.Å. Albertsson, Composition of photosynthetic pigments in thylakoid membrane vesicles from spinach, Photosynth. Res. 35 (1993) 171–178.
[24] P.O. Arvidsson, M. Carlsson, H. Stefansson, P.-Å. Albertsson, H.E. Åkerlund, Violaxanthin accessibility and temperature dependency for de-epoxidation in spinach thylakoid membranes, Photosynth. Res. 52 (1997) 39–48.
[25] H.K. Lichtenthaler, C. Buschmann, M. Döll, H.J. Fietz, T. Bach, U. Kozel, D. Meier, U. Rahmsdorf, Photosynthetic activity, chloroplast ultrastructure, and leaf characteristics of high-light and low-light plants and of sun and shade leaves, Photosynth. Res. 2 (1981) 115–141.
[26] J.M. Anderson, Photoregulation of the composition, function and structure of thylakoid membranes, Annu. Rev. Plant Physiol. 37 (1986) 93–136.
[27] W.S. Chow, A.B. Hope, The stoichiometries of supramolecular complexes in thylakoid membranes from spinach chloroplasts, Aust. J. Plant Physiol. 14 (1987) 21–28.
[28] H. Kirchhoff, W. Haase, S. Wegner, R. Danielsson, R. Ackermann, P.Å. Albertsson, Low-light induced array formation of photosystem II in higher plant chloroplasts, Biochemistry 46 (2007) 11169–11176.
[29] J.F. Allen, J. Forsberg, Molecular recognition in thylakoid structure and function, Trends Plant Sci. 6 (2001) 317–326.
[30] P.-Å. Albertsson, E. Andreasson, H. Stefansson, L. Wollenberger, Fractionation of the thylakoid membrane, Methods Enzymol. 228 (1994) 469–482.
[31] R. Danielsson, P.-Å. Albertsson, F. Mamedov, S. Styring, Quantification of photosystem I and II in different parts of the thylakoid membrane from spinach, Biochim. Biophys. Acta 1608 (2004) 53–61.
[32] P.Å. Albertsson, The structure and function of the chloroplast photosynthetic membrane—a model for the domain organization, Photosynth. Res. 46 (1995) 141–149.
[33] H.-E. Åkerlund, An apparatus for counter-current distribution in a centrifugal acceleration field, J. Biochem. Biophys. Methods 9 (1984) 133–141.
[34] H.E. Åkerlund, P.-Å. Albertsson, Thin-layer counter current distribution and centrifugal counter current distribution apparatus, Methods Enzymol. 228 (1994) 87–99.
[35] D.I. Arnon, Copper enzymes in isolated chloroplasts — polyphenoloxidase in *Beta vulgaris*, Plant Physiol. 24 (1949) 1–15.
[36] R.J. Porra, W.A. Thompson, P.E. Kriedemann, Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy, Biochim. Biophys. Acta 975 (1989) 384–394.
[37] P.Å. Albertsson, Partition of Cell Particles and Macromolecules, Wiley, New York, 1986.
[38] B. Andersson, D.J. Simpson, G. Höyer-Hansen, Freeze fracture evidence for the isolation of inside-out spinach thylakoid vesicles, Carlsberg Res. Commun. 43 (1978) 77–89.
[39] P.Å. Albertsson, Analysis of the domain structure of membranes by fragmentation and separation in aqueous polymer two-phase systems, Q. Rev. Biophys. 21 (1988) 61–98.
[40] P.Å. Albertsson, A quantitative model of the domain structure of the photosynthetic membrane, Trends Plant Sci. 6 (2001) 349–354.
[41] E.H. Kim, W.S. Chow, P. Horton, J.M. Anderson, Entropy-assisted stacking of thylakoids membranes, Biochim. Biophys. Acta 1708 (2005) 187–195.
[42] P.Å. Albertsson, H. Baltscheffsky, Counter-current distribution of spinach chloroplasts in an aqueous two-phase system, Biochem. Biophys. Res. Commun. 12 (1963) 14–20.
[43] B. Andersson, H.E. Åkerlund, P.Å. Albertsson, Light induced reversible proton extrusion by spinach-chloroplast photosystem II vesicles isolated by phase partition, FEBS Lett. 77 (1977) 141.
[44] B. Andersson, H.E. Åkerlund, Inside-out membrane-vesicles isolated from spinach thylakoids, Biochim. Biophys. Acta 503 (1978) 462–472.
[45] S.G. Yu, G. Björn, P.Å. Albertsson, Characterization of a non-detergent PSII-cytochrome *b/f* preparation (BS), Photosynth. Res. 37 (1993) 227–236.
[46] D. Berthold, G.T. Babcock, C.F. Yocum, A highly resolved, oxygen-evolving photosystem II preparation from spinach thylakoid membranes, FEBS Lett. 134 (1981) 231–234.
[47] Y. Yamamoto, T. Ueda, H. Shinkai, M. Nishimura, Preparation of O_2-evolving photosystem II sub chloroplasts from spinach, Biochim. Biophys. Acta 679 (1982) 347–350.
[48] L.-G. Franzen, Ö. Hansson, L.-E. Andreasson, The roles of the extrinsic subunits in Photosystem II as revealed by EPR, Biochim. Biophys. Acta 808 (1985) 171–179.
[49] E. Romanowska, P.Å. Albertsson, Isolation and characterization of the cytochrome *bf* complex from whole thylakoids, grana, and stroma lamellae vesicles from spinach chloroplasts, Plant Cell Physiol. 35 (1994) 557–568.
[50] R. Gadjieva, F. Mamedov, P.Å. Albertsson, Fractionation of the thylakoid membranes from tobacco. A tentative isolation of “end membrane” and purified “stroma lamellae” membranes, Biochim. Biophys. Acta 1411 (1999) 92–100.
[51] Z. Liu, H. Yan, K. Wang, T. Kuang, J. Zhang, L. Gui, X. An, W. Chang, Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution, Nature 428 (2004) 287–292.
[52] J.P. Dekker, H. van Roon, E.J. Boekema, Heptameric association of light — harvesting complex II trimers in partially solubilized photosystem II membranes, FEBS Lett. 449 (1999) 211–214.
[53] J.P. Decker, E.J. Boekema, Supramolecular organization of thylakoid membrane proteins in green plants, Biochim. Biophys. Acta 1706 (2005) 12–39.
[54] S. Jansson, The light-harvesting chlorophyll *a/b*-binding proteins, Biochim. Biophys. Acta 1184 (1994) 1–19.
[55] H. Stefánsson, L. Wollenberger, S.-G. Yu, P.Å. Albertsson, Phosphorylation of thylakoids and isolated subthylakoid vesicles derived from different structural domains of the thylakoid membrane from spinach chloroplast, Biochim. Biophys. Acta 1231 (1995) 323–334.
[56] G.H. Krause, E. Weis, Chlorophyll fluorescence and photosynthesis — the basics, Annu. Rev. Plant Physiol. Plant Mol. Biol. 42 (1991) 313–349.