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The domain organization of the chloroplast thylakoid membrane. Localization of Photosystem I and of the cytochrome b_6-f complex

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The relative distribution of chlorophyll (Chl) *a* and Chl *b*, of Photosystem I (PS I), and of the cytochrome b_6-f (Cyt b_6-f) complex in different subchloroplast membrane vesicles is presented. Spinach chloroplasts (Chl *a*/Chl *b* = 2.7, Chl/PS I = 435:1, Chl/Cyt *f* = 970:1) were fractionated by Yeda press treatment and by sonication. Counter-current distribution of the resulting thylakoid vesicles in dextran-polyethylene glycol aqueous two-phase systems yielded a laterally extended range of chloroplast thylakoid membranes without loss of chloroplast material from the initial sample. In this analysis, PS I-enriched membrane vesicles moved in the leading edge of the counter-current distribution diagram, whereas PS II-enriched vesicles remained close to the trailing edge of the counter-current distribution diagram. Vesicles of mixed composition and vesicles depleted in both PS I and PS II were isolated from intermediate positions. The Chl/PS I ratio in the various fractions approximated a hyperbolic function of the Chl *a*/Chl *b* ratio with limiting values of Chl/PS I = 190:1 attained at a Chl *a*/Chl *b* = 6 and a Chl *a*/Chl *b* = 1.67 attained when the Chl/PS I ratio approaches infinity ([PS I] = 0). Such hyperbolic distribution is consistent with the exclusive localization of PS I in stroma-exposed thylakoids and of PS II in the membrane of the grana partition region. A depletion of the Cyt b_6-f complex from the membrane domain of PS I was detected (Chl *a*/Chl *b* = 5.1, Chl/PS I = 210:1, Chl/Cyt *f* = 1510:1). Moreover, a chloroplast thylakoid membrane was identified, distinct both from that of PS II and from that of PS I which was enriched in the Cyt b_6-f complex (Chl *a*/Chl *b* = 2.67, Chl/PS I = 460:1, Chl/Cyt *f* = 590:1). Our results suggested that such a Cyt b_6-f domain is localized in the region between the appressed and the non-appressed membranes, possibly in the membrane of the fret region.

Introduction

The thylakoid membrane of higher plant chloroplasts shows a distinct structural differentiation in areas of grana where disc-shaped thylakoids are appressed against each other at the 'partition region', and in areas of stroma-exposed thylakoids that interconnect the grana [1–3]. Thus, the membrane system of chloroplasts forms an elaborate network in which each stroma-exposed thylakoid interconnects several grana thylakoids in different grana stacks.

A general principle applicable to the structural-functional organization of biological systems is that a structural differentiation evolves strictly in order to facilitate a specialized function. The

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Abbreviations: Chl, chlorophyll; P-700, reaction center of PS I; Cyt, cytochrome; CCD, counter-current distribution; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PS I, II, Photosystem I, II.

evolution of grana in higher plant chloroplasts and in certain algae probably constituted a distinct functional advantage for the overall process of $NADP^+$ reduction and/or ATP formation. A clear understanding on the advantage derived by higher plant chloroplasts from the formation of grana is currently beyond our comprehension. In spite of this shortcoming in our understanding of chloroplast function, great progress in recent years has helped in elucidating certain functional aspects of this differentiation. Chief among them is the realization that different photosystems are localized in separate regions of chloroplast membranes [4–7]. Thus, it is now believed that PS II_α is segregated in the thylakoid membrane of the grana partition region [7–9], whereas PS II_β has been isolated with a light membrane fraction following Yeda press treatment of spinach thylakoids [7]. The ATP synthase complex and the enzyme ferrodoxin-$NADP^+$ reductase were found localized exclusively on thylakoid membranes exposed to the chloroplast stroma medium [10–12].

Our understanding with respect to the localization of PS I has shifted dramatically over the last five years. It was originally assumed that PS II and PS I existed in stoichiometrically equal amounts and they both operated in the grana partition regions where they shared excitation energy from the same Chl pigment bed. Photosystem I complexes were also found localized in the intergrana lamellae and it was commonly assumed that the function of this pool of PS I was to generate ATP via cyclic photophosphorylation. The introduction of the aqueous polymer two-phase separation method by Albertsson [13,14] allowed the isolation of inside-out vesicles derived mainly from the membranes of the grana partition regions [15–17]. Such vesicles had a substantially lower PS I activity [4] and were enriched in PS II Chl-protein complexes [5]. Subsequent quantitation of PS II and PS I complexes in the inside-out vesicles revealed a PS II/PS I ratio of about 10 [6,7]. On the basis of kinetic evidence, Anderson and Melis [7] suggested that all PS I is excluded from the membrane of the grana partition region and that the low PS I content found in the inside-out vesicles simply indicated that portions of stroma-exposed lamellae participated in the formation of inverted thylakoid vesicles. However, a concern voiced by colleagues against the use of inside-out vesicles for the determination of PS I localization in the thylakoid membrane is that such preparations may be atypical presumably because a substantial PS I depletion is obtained only in a relatively small fraction of the total membrane sample [18].

A somewhat greater uncertainty currently exists with respect to the localization of the Cyt b_6-f complex in the thylakoid membrane of higher plant chloroplasts. The distribution of the Cyt b_6-f complex in grana and stroma thylakoids has been investigated in the past upon fractionation of thylakoid membranes with detergent, mechanical treatment and aqueous polymer two-phase separation. Chloroplast fractionation with Triton X-100 yielded resolved membranes from the grana partition region [19] that contained little or no Cyt b_6-f complex [9] suggesting the exclusion of this complex from the membrane of the grana partition region. The measurements with inside-out and right-side-out vesicles obtained by mechanical disruption suggested an even distribution of the Cyt b_6-f complex between stacked and unstacked membranes [20–22] which is also suggested by immunocytochemical studies [23,24]. Based on a study with maize mesophyll (grana containing) and bundle sheath (grana lacking) chloroplasts, Ghirardi and Melis [25] concluded that both PS I and the Cyt b_6-f complex are excluded from the membrane of the grana partition region. They suggested that the Cyt b_6-f complex might be localized in a domain of the thylakoid membrnae occurring in the vicinity of the PS II-containing grana partition regions but not as an integral component of the partition region itself [25]. Finally, vesicles obtained upon sonication of inside-out thylakoids (the latter originate from the membrane of the grana partitions regions) were resolved by counter-current distribution into three populations differing in the Chl a/Chl b ratio. The Cyt b_6-f content was fairly evenly distributed among these fractions with some preference for the PS II-enriched fractions [26].

In the present work we addressed the question of the localization of PS I and of the Cyt b_6-f complex in the thylakoid membrane. We used mechanical disruption and sonication followed by counter-current distribution [26] to obtain vesicles

of different composition. Our result is consistent with the exclusive localization of PS I in stroma-exposed thylakoids and suggest the presence of a domain rich in cytochrome b_6-f.

Materials and Methods

Chemicals

Dextran 500 was obtained from Pharmacia, Uppsala, Sweden. Polyethylene glycol 4000 (Carbowax PEG 3350) was obtained from Union Carbide, New York, NY., U.S.A.

Chloroplast isolation

Spinach plants (*Spinacia oleracea* L.) were grown at 18°C under cool-white fluorescent light (incident intensity, 40 $W \cdot m^{-2}$) with a light/dark cycle of 12 h. Chloroplasts were isolated by grinding freshly harvested leaves in 'preparation medium' (50 mM sodium phosphate buffer, (pH 7.4)/5 mM $MgCl_2$/300 mM sucrose). The slurry was filtered through four layers of nylon mesh (25 μm) and centrifuged at $1000 \times g$ for 1 min. The chloroplast pellet was resuspended in preparation medium and centrifuged at $1000 \times g$ for 10 min. The chloroplasts were suspended and osmotically broken in 5 mM $MgCl_2$ followed by centrifugation at $2000 \times g$ for 10 min. The envelope-free thylakoids obtained were washed three times in a buffer containing 10 mM Tricine (pH 7.4)/5 mM $MgCl_2$/300 mM sucrose. Finally, they were resuspended in 'Yeda press medium' (10 mM sodium phosphate buffer (pH 7.4)/5 mM $MgCl_2$/5 mM NaCl/100 mM sucrose) to give a chlorophyll concentration of about 4 mg/ml.

Thylakoid membrane fractionation

The thylakoids were fragmented by passing the suspension twice through a Yeda press at a nitrogen gas pressure of 10 MPa. Starch and unfragmented membranes were removed by low speed centrifugation ($1000 \times g$ for 10 min). The broken thylakoid membranes were then partitioned in a phase system of 5.7% (w/w) Dextran 500, 5.7% (w/w) polyethylene glycol 4000, 10 mM sodium phosphate buffer (pH 7.4)/5 mM NaCl/20 mM sucrose at 3°C in order to separate inside-out vesicles (B3 fraction) from right side-out vesicles (T1 fraction), as follows.

An aliquot of 1 ml of broken thylakoids was added to 24 g of a polymer mixture to yield a phase system of the composition described above. The polymer mixture was premade by mixing 7.13 g 20% (w/w) Dextran 500/3.56 g 40% (w/w) polyethylene glycol 4000/1.2 g 0.1 M NaCl/1.2 g 0.2 M sodium phosphate buffer (pH 7.4)/0.14 g sucrose/24 g water. After addition of 1 ml of the fragmented thylakoids, the phase system was mixed thoroughly and allowed to separate under low speed centrifugation ($1000 \times g$ for 3 min). The upper polyethylene glycol phase (T1) was retained and used later in the sonication procedure. The lower Dextran phase (B1) was collected and repartitioned twice with fresh polyethylene glycol phase to yield the final lower phase fraction B3.

The thylakoid vesicles in both the B3 and T1 fractions were diluted 3-fold with 5 mM $MgCl_2$ in order to facilitate the precipitation of the vesicles by centrifugation at $35000 \times g$ for 20 min. This procedure resulted in the complete precipitation of the vesicles from the B3 sample, yielding the B3-pellet. However, a green supernatant remained after a similar centrifugation of the T1 sample. Thus, a second centrifugation at $100000 \times g$ for 45 min was necessitated to precipitate these 'light' membrane fragments from T1. This 'light' membrane fraction was termed T1-supernatant in order to distinguish it from the T1-pellet obtained after the first step centrifugation. Only the membrane vesicles obtained as B3-pellet and T1-pellet were subjected to sonication and counter-current distribution analysis: the corresponding B3 and T1 pellets were resuspended in 3.6 ml of buffer containing 10 mM sodium phosphate (pH 7.4), 3 mM NaCl and 1 mM $MgCl_2$. An aliquot of 3 ml from this suspension was added to a phase system to give 8.5 g of the same phase composition as the one used for phase partition described above.

Sonication and counter-current distribution

The crude Yeda press fraction, the B3-pellet and the T1-pellet, after being suspended in 8.5 g of the phase system, they were sonicated (separately) in a Branson Sonifier, Model B 30 equipped with a 1/2-inch tip, for variable multiples of 30 s with resting intervals of 1 min under continuous cooling. The ultrasonic exposure had an intensity output of 7 with 20% duty pulses.

The sonicate was then subjected to liquid interface counter-current distribution [13,26] using a 60 cavity thin layer apparatus. The phase system used was the same as that described above. All cavities were loaded with 0.71 ml lower dextran phase (90% of the lower cavity volume) supplemented with a 0.71 ml upper polyethylene glycol phase. Cavities nos. 0–4 also contained the sonicated thylakoid vesicles. The apparatus mixing time was set 40 s with a settling (resting) time of 15 min between transfers. The total number of transfers was 56.

Spectrophotometric analysis

Measurements of chlorophyll concentration and the determination of the Chl *a*/Chl *b* ratio of the various samples was implemented according to Arnon [27]. The relative chlorophyll content of the various counter-current distribution fractions was tested directly by measuring the absorbance of the suspension at 680 nm. Absorbance difference measurements for the quantitation of P-700 (reaction center of PS I) and of Cyt *f* (Cyt b_6-*f* complex) were obtained with an Aminco spectrophotometer operated in the split-beam mode. The concentration of P-700 was measured directly from the amplitude of the light-minus-dark absorbance change at 700 nm [28]. A differential extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ was used [29]. The reaction mixture contained 0.02% (w/w) SDS, 2 mM methyl viologen, 2 mM sodium ascorbate and chloroplast membranes to yield about 20 μM Chl($a + b$). The concentration of Cyt *f* was determined from the reduced-minus-oxidized absorbance change at 554.5 nm in Triton-solubilized thylakoids [30]. The reaction mixture contained 1% (w/v) Triton X-100, 200 μM potassium ferricyanide and chloroplast membranes to yield 100–150 μM Chl($a + b$). Following registration of the baseline, sufficient hydroquinone (from a 100 mM stock) was added to yield a concentration of 2.0 mM in the sample cuvette only. An equal volume from a 20 mM potassium ferricyanide stock was added in the reference cuvette.

Theoretical

Counter-current distribution in aqueous polymer two-phase systems is useful in the separation of biological membranes having different surface properties. Of particular interest is the separation of different 'membrane domains' derived upon mechanical fractionation of the continuous membrane of cells and organelles. The resolution of different 'membrane domains' by counter-current distribution depends largely upon the precision of the fractionation procedure in breaking off domain X from domain Y in the continuous sheet of the biological membrane under question. In general, the fragmentation and separation of a biological membrane into any two fully resolved domains is experimentally difficult to achieve. For example, consider a membrane configuration in which two different domains are discerned, each having a diameter L, contain either protein X or protein Y, as shown in Fig. 1. Assume that mechanical fractionation by Yeda or French press treatment, or by sonication, would break the membrane in uniform size fragments of diameter D_1 ($D_1 = L$). Then, a mixture of fragments will result with a composition ranging from pure X to pure Y (lower portion of Fig. 1). If domains X and Y of the biological membrane have sufficiently different surface properties, then application of counter-current distribution in aqueous polymer two-phase systems will generate an X–Y profile in which the X-dominated fragments will be differ-

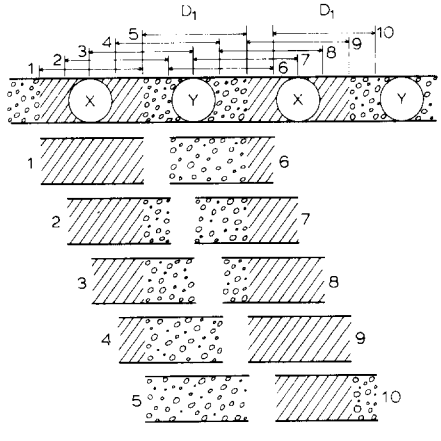

Fig. 1. Hypothetical biological membrane containing the distinct domains of protein X and Y, respectively. It is assumed that mechanical fractionation of the biological membrane will result in a continuous gradient of membrane fragments. Such uniform size but mixed composition fragments of diameter D_1 are shown in the lower part of the figure.

ently separated from the Y-dominated fragments. The concentration of protein Y in the various counter-current distribution samples (defined by the amount of this protein per membrane-fragment area) could then be described as a function of the concentration of protein X as shown in Eqn. 1 below:

$$\frac{X}{X_0} + \frac{Y}{Y_0} = 1 \tag{1}$$

where Y_0 and X_0 is the concentration (defined as the amount of protein per membrane area) of proteins Y and X in their fully resolved domains, respectively. Thus, in any given membrane fragment, the following limiting values would apply:

$$0 \leqslant X \leqslant X_0 \quad \text{and} \quad 0 \leqslant Y \leqslant Y_0 \tag{2}$$

In the inverse form, the limiting values are converted to:

$$\frac{1}{X_0} \leqslant \frac{1}{X} \leqslant \infty \quad \text{and} \quad \frac{1}{Y_0} \leqslant \frac{1}{Y} \leqslant \infty \tag{3}$$

An inverse plot is shown in Fig. 2. Please observe that when the diameter of individual fragments is equal to or smaller than the diameter of domains Y and X, the limiting values of Y and X in Eqn. 1 will be always Y_0 and X_0 which correspond to the fully resolved domains of proteins Y and X, respectively. Such fully resolved domains will be differentially separated in the leading and trailing edge of the counter-current distribution, respectively. All other intermediate composition fragments will distribute themselves in intermediate positions in the counter-current distribution.

In practice, it may prove difficult to achieve the idealized membrane fractionation assumed above. This is because the resulting membrane fragments may be larger than individual membrane domains. As an exmaple, considering the situation in which the area of the membrane fragments is 1.5 × larger than the surface area of the individual domains, i.e., having a diamter $D_2 = 1.225L$ (not shown). It is obvious that no fully resolved membrane domains will be derived by the counter-current distribution analysis in that case. Nevertheless, membrane fragments enriched in proteins Y and X will be differentially separated from each

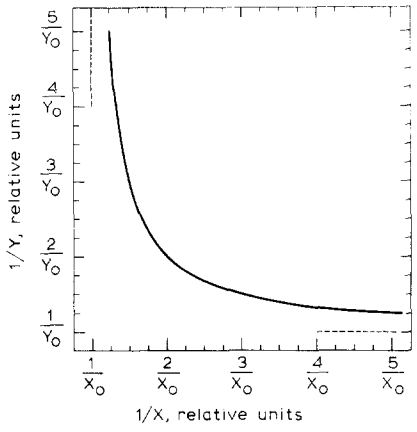

Fig. 2. Double reciprocal plot of the concentration per membrane area of protein X and protein Y in the various membrane fragments having a diameter D_1. The hyperbolic function has limiting values $1/X_0$ and $1/Y_0$, corresponding to membrane fragments containing exclusively protein X and protein Y, respectively.

other. In this case, Eqn. 1 remains valid for the phase separation of the resulting membrane fragments. The only difference from the case examined previously is that the protein concentration (Y or X) will not reach the maximum possible in the leading and trailing edges of the CCD (which is Y_0 and X_0, respectively). Instead, if the resulting membrane fragments are 1.5-times larger in the surface area, the most enriched membrane

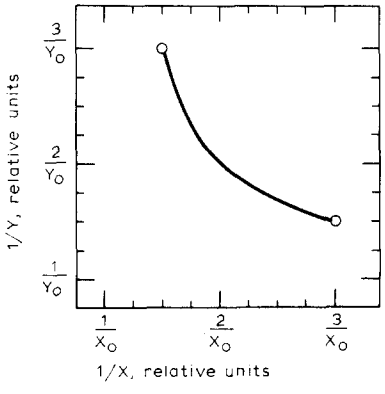

Fig. 3. Double reciprocal plot of the concentration per membrane area of protein X and protein Y in the various membrane fragments having a diameter D_2 ($D_2 = 1.225 \times D_1$). The restricted hyperbolic function has the same limiting values as in Fig. 2.

fragments isolated with the CCD method will contain, on per fragment area basis, the proteins Y and X at the reduced concentrations of $Y_0/1.5$ and $X_0/1.5$, respectively. Thus, the limiting values for the varialbes Y and X in Eqn. 1 would be $1.5/Y_0$ and $1.5/X_0$, respectively. The inverse plot of Eqn. 1 for the limiting values $1/X = 1.5/X_0$ and $1/Y = 1.5/Y_0$ is shown in Fig. 3.

The practical significance of the above derivations is that they point to the applicability of hyperbolic functions in counter-current distribution analysis. Hyperbolic functions may be used in predicting the properties of fully resolved membrane domains without actually having attained complete purification. This could be implemented experimentally with membrane domains that were partially separated by mechanical fractionation and/or by other means, and partially resolved via counter-current distribution analysis. The results of the counter-current distribution analysis could be fitted with a specific hyperbolic function. Once the hyperbolic function is experimentally derived, it could be used, via the process of data extrapolation, to define the limiting values, hence, the predict the biochemical properties of the fully resolved membrane domains. In this publication, we used hyperbolic function analysis to define, following mechanical fragmentation and counter-current distribution, the domain of Photosystem I in the thylakoid membrane of spinach chloroplasts.

Results

Unfractionated spinach chloroplast thylakoids showed a Chl *a*/Chl *b* ratio of about 2.7 and had average Chl/P-700 = 435 : 1 and Chl/Cyt *f* = 970 : 1 ratios (see Table I). Compared to field and greenhouse-grown spinach, our chloroplasts had lower Cyt *f* and higher P-700 contents in their thylakoid membranes. This is consistent, however, with a chloroplast response to the particular light conditions during plant growth, i.e., they are due to a combination of low-intensity light and far-red depleted illumination conditions [31–33].

Following the Yeda press treatment of spinach thylakoids we applied a first step aqueous polymer two-phase separation which resulted in the separation of about 50% of the total Chl in the bottom dextran phase (B1). The remaining 50% of the Chl was collected in the top polyethylene glycol phase (T1). The thylakoid vesicles of the B1 fraction were subjected to further aqueous polymer two-phase separation. The resulting B3 fraction contained about 40% of the total Chl. Thus, the samples used in the analysis described below contained about 90% of the initial amount of Chl (40% B3 and 50% T1).

In agreement with the results obtained from earlier preparations of inside-out (B3) and right-side-out (T1) vesicles [4–7] we observed substantial enrichment in Chl *b* and depletion of P-700 in the B3 samples (Table I). Simultaneously, depletion of Chl *b* and enrichment in P-700 content was observed in the T1 samples (Table I). Also in agreement with earlier reports [20–22], there was a nearly uniform distribution of Cyt *f* among B3 and T1 samples (see Table I).

P-700 distribution

In spite of substantial depletion of PS I from the B3 samples, considerable amount of P-700 remained. We reasoned that most, if not all, of the residual PS I occurred either in the small right-side-out vesicles that were 'tagged' along with the inside-out vesicles into the bottom phase or was localized in the inverted-thylakoid vesicles as a

TABLE I

CHLOROPHYLL *a*/*b*, PHOTOSYSTEM I AND CYTOCHROME *f* DISTRIBUTION IN SPINACH SUBCHLOROPLAST FRACTIONS

Subchloroplast fractions were obtained following Yeda press treatment, sonication, and aqueous polymer two-phase separation of spinach chloroplast thylakoids [26]. B3 is inside-out vesicles derived mainly from the grana partitions. T1 is right-side-out vesicles CCD1–CCD4 are fractions from the counter-current distribution shown in Fig. 5. T1-supernatant is obtained after collection of the T1 vesicles by centrifugation (see Materials and Methods).

	Chl *a*/ Chl *b*	Chl/ P-700	Chl/ Cyt *f*
Thylakoids	2.7	435	970
B3	2.25	700	890
T1	3.10	300	990
CCD1	2.37	650	720
CCD2	2.67	460	590
CCD3	3.25	300	970
CCD4	3.60	260	1440
T1-supernatant	5.10	210	1510

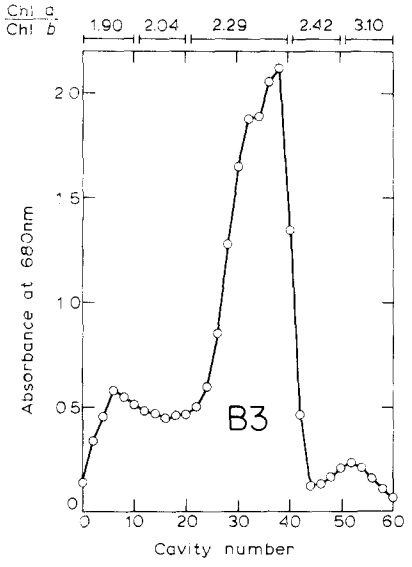

Fig. 4. Counter-current distribution diagram of sonicated inside-out vesicles (B3). Note the Chl *a*/Chl *b* ratio of samples pooled together from several adjacent CCD cavity positions (top of figure).

portion of stroma-exposed PS I-containing lamella that was apparently included in the formation of the inside-out vesicle [26]. The latter is a distinct possibility, since it is not clear whether inside-out membrane formation occurs exclusively from the appressed membranes of the grana partition regions. A further selective depletion of PS I from the inside-out vesicles can be obtained by sonication of the B3 samples followed by counter-current distribution [26].

Counter-current distribution of the sonicated B3 fractions (Fig. 4) yielded a profile of vesicles suggesting further separation of PS II-enriched inside-out vesicles from PS I-containing membranes in agreement with earlier results [26]. This was evident by the much lower Chl *a*/Chl *b* ratio (1.9) of the samples pooled together from the trailing edge of the CCD and by the higher Chl *a*/Chl *b* ratio (3.10) of the samples pooled together from the leading edge of the counter-current distribution. By varying the sonication time of the B3 samples and also by varying the polymer composition in the counter-current distribution analysis, we were able to alter the relative amplitude of the three counter-current distribution bands shown in Fig. 4.

A similar analysis following sonication and counter-current distribution was applied to the T1 samples. Prior to sonication, thylakoid membranes from T1 samples were precipitated by centrifugation at 35 000 × *g* for 20 min (see Materials and Methods). This pellet was designated as T1-pellet and, upon resuspension, was subjected to sonication and counter-current distribution analysis. Unlike the B3 samples, which were subjected to the same centrifugation, we noticed that such centrifugation was not sufficient to precipitate all chlorophyll from the T1 samples. A second centrifugation at 100 000 × *g* for 45 min precipitated a 'light' membrane fraction from T1. This membrane fraction was designated as T1-supernatant and was *not* subjected to further sonication or counter-current distribution analysis.

Counter-current distribution of the sonicated T1-pellet fractions (Fig. 5) yielded a profile of vesicles suggesting the presence of inside-out thylakoids enriched in PS II (Chl *a*/Chl *b* ratio = 2.37) and of right side-out vesicles enriched in PS I (Chl *a*/Chl *b* = 3.60).

The relative distribution of PS I (P-700) and of Chl *b* in various CCD fractions derived from B3

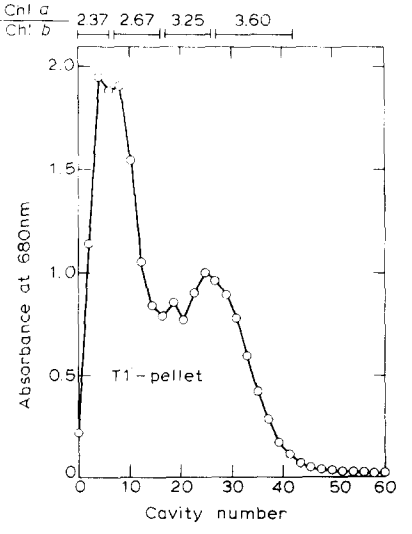

Fig. 5. Counter-current distribution diagram of sonicated right-side out vesicles (T1). A T1-supernatant containing very small vesicles enriched in PS I but depleted both in Cyt *f* and in PS II was not applied to the counter-current distribution analysis. Note the Chl *a*/Chl *b* ratio of samples pooled together from adjacent CCD cavity positions (top of figure).

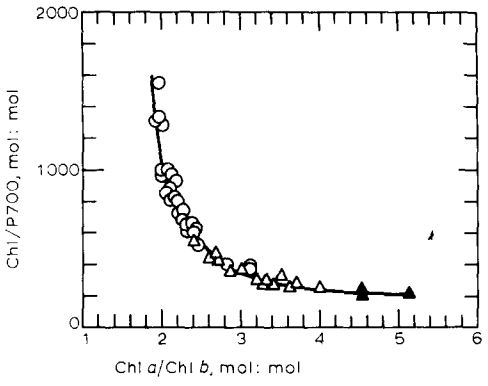

Fig. 6. The ratio of Chl/P-700 plotted as a function of the Chl *a*/Chl *b* ratio in the various counter-current distribution fractions derived from several B3 (○) and T1-pellet (△) samples. Also shown are the coordinate values obtained with T1-supernatant samples (▲). Note the good approximation of a hyperbolic-function relationship between the two variables plotted (solid line).

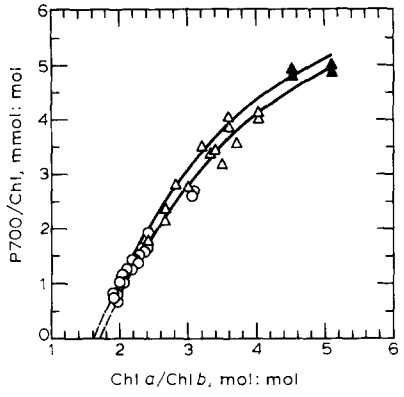

Fig. 7. The empirical relationship between the P-700/Chl ratio and the Chl *a*/Chl *b* ratio of the thylakoid vesicles derived from the counter-current distribution analysis of B3 (○) and T1-pellet (△) samples. Also shown are the coordinate values obtained with the T1-supernatant samples (▲). Extrapolation of the experimental values to a P-700/Chl = 0 defined a Chl *a*/Chl *b* = 1.67 ± 0.05 (dashed lines).

and T1 samples is summarized in Fig. 6 which provides an illustration of the hyperbolic relationship between the parameters Chl/P-700 and Chl *a*/Chl *b*.

It is worthwhile noting that the results of Fig. 6 represent the distribution of P-700 and Chl *b* in about 90% of the original chloroplast material obtained from the Yeda press treatment. We determined the limiting values of the hyperbolic function shown in Fig. 6. This was implemented from the empirical relationship of the P-700/Chl ratio plotted as a function of the Chl *a*/Chl *b* ratio, shown in Fig. 7. Extrapolation of the experimental values to a P-700/Chl = 0 (dashed lines in Fig. 7) defined an intercept value of Chl *a*/Chl *b* = 1.67 ± 0.05. Thus, PS II complexes in the fully resolved membrane of the grana partition region must have a Chl *a*/Chl *b* = 1.67 ± 0.05. This value has been measured independently in resolved membrane preparations from the grana partition region obtained after treatment of spinach thylakoids with the detergent Triton X-100 [9,19]. A similar process of data extrapolation defined the domain of PS I with a Chl/P-700 = 190 (± 20) : 1 at a Chl *a*/Chl *b* ratio of about 6. Thus, the properties of the domain of PS I are in good agreement with measurements of the functional PS I antenna size established from direct kinetic measurements [34,35].

Cytochrome f distribution

The distribution of the Cyt b_6-*f* complex in the various counter-current distribution fractions derived from B3 samples was essentially identical to that presented by Albertsson [26]. In summary, further depletion of P-700 from the B3 samples resulted either in a very small enrichment or negligible change in Cyt *f* content (not shown). Qualitatively different results were obtained following sonication and counter-current distribution analysis of the T1 samples. There, we observed that certain membrane fractions were substantially enriched in Cyt b_6-*f* content, while others were substantially depleted of this complex. Table I sumamrizes the Cyt b_6-*f* distribution in counter-current distribution fractions (CCD1–CCD4) derived from the sonicated samples. It is evident that substantial Cyt *f* enrichment occurs in the CCD1 and CCD2 samples (a 30% and 60% enrichment, respectively). Simultaneously, a 45% Ct *f* depletion is observed in the CCD4 sample and 53% depletion in Cyt *f* content was observed in the T1-supernatant. Please observe that the CCD2 sample (Chl *a*/Chl *b* = 2.67) was the most enriched in the Cyt b_6-*f* complex and that it represented a substantial fraction of the total material. Since this fraction was collected from counter-current distribution cavities intermediate to the PS

II-most-enriched (CCD1) and to the PS I-most-enriched (CCD4), it suggested the existence of a Cyt b_6-f domain in the thylakoid membrane intermediate to the domains of PS I and PS II. It is also of interest to observe that both the T1-supernatant and the PS I-enriched CCD4 fractions were low in Cyt b_6-f content. This observation bears on the mechanical fractionation of chloroplast thylakoids by Yeda press and by sonication. If it is assumed that thylakoid membranes break at the point of least resistance during mechanical fractionation, then it must be concluded that most of such points occur between the Cyt b_6-f and PS I complexes. This observation is also of importance in terms of the mechanism of inside-out vesicle formation (see Discussion section).

To strengthen the notion of a distinct Cyt b_6-f complex domain in the chloroplast thylakoid membrane, we repeated the chloroplast fractionation steps via Yeda press treatment, sonication and counter-current distribution analysis, thus by-passing the first-step aqueous polymer two-phase separation of thylakoid vesicles into B3 and T1 samples. We reasoned that such approach will both simplify the procedure and alleviate a partial Cyt b_6-f complex randomization that might occur in the T1-samples. The distribution of total Chl, the Chl a/Chl b and the Chl/Cyt f ratios in the various CCD fractions from the Yeda press treated and sonciated class II chloroplasts is summarized in Fig. 8. As expected, low Chl a/Chl b ratios (2.07) were measured in the samples from the trailing edge of the counter-current distribution (cavity nos. 1–20). High Chl a/Chl b ratios (3.54) were measured in the samples from the leading edge of the counter-current distribution (cavity nos. 38–52). Thus, the relative distribution of Chl b is consistent with a PS II enrichment in cavity nos. 1–10 and a PS I enrichment in cavity nos. 40–52. The distribution of Cyt f in the various counter-current distribution fractions followed a pattern similar to that shown in Table I for the T1 samples: a significant Cyt f enrichment was observed in samples from cavity nos. 20–30 and a simultaneous Cyt f depletion in samples from cavity nos. 40–52 (see Fig. 8, top). It must be noted that the absolute Chl/Cyt f values in the CCD samples varied from preparation to preparation; however, the pattern of differential Cyt f distribution shown in Fig. 8 was reproduced consistently. We found no evidence for a solubilization of the Cyt f as a result of press treatment, sonication or counter-current distribution.

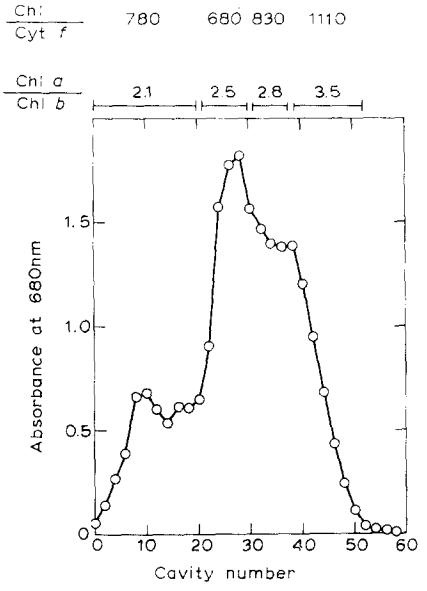

Fig. 8. Counter-current distribution of fragmented class II spinach chloroplasts. Envelope-free thylakoid membranes were forced through a Yeda press and subsequently sonicated prior to counter-current distribution analysis in a Dextran-polyethylene glycol aqueous polymer two-phase system. Note the Chl a/Chl b ratio and the corresponding Chl/Cyt f ratio of samples pooled together from adjacent CCD cavity positions (top of figure).

Discussion

The results from this work support the notion of the domain organization of the thylakoid membrane in higher plant chloroplast. This concept is applicable in the organization of the main three thylakoid membrane integral complexes that are involved in electron transport (PS II, Cyt b_6-f and PS I). We demonstrated that the Chl/P-700 ratio approximated a hyperbolic function of the Chl a/Chl b ratio with limiting values of Chl/P-700 = 190 : 1, attained at Chl a/Chl b = 6, and Chl a/Chl b = 1.67 attained when Chl/P-700 approaches infinity. These results are consistent with a chloroplast thylakoid membrnae model in which there are two different membrane domains con-

taining chlorophyll and P-700. One membrane domain contains only PS I (Chl/P-700 = 190 : 1 an Chl *a*/Chl *b* = 6, partitions with a high coefficient in the counter-current distribution, and is apparently derived from stroma-exposed thylakoids. A second membrane domain from which PS I is entirely excluded (P-700/Chl = 0 and Chl *a*/Chl *b* = 1.67) partitions with a low coefficient in the CCD, contains exclusively PS II_α, and is apparently derived from the grana partition region. The good fit of a hyperbolic function on the results of the CCD analysis, obtained over a wide range of the Chl *a*/Chl *b* ratio with a broad range of thylakoid membrane fractions, support the notion of extreme lateral heterogeneity in chloroplast membranes with grana partition regions being the exclusive domain of PS II_α, whereas stroma-exposed thylakoids is the domain of PS I. Thus, the application of hyperbolic functions to counter-current distribution analysis helps in the process of data extrapolation and in the definition of two limiting values (a) of the Chl *a*/Chl *b* ratio in the domain of the grana partition region (PS II_α) and (b) of the Chl/P-700 ratio in the domain of the intergrana lamellae (PS I).

The detection of a Cyt b_6-*f* domain was elusive until now because the Cyt *f* distribution in B3, T1 and the supernatant of a Yeda press homogenate (Y-100) all contained, on a Chl basis, approximately equal amounts of Cyt *f* [20–22]. We believe that such earlier results, albeit correct, are indicative of the pattern of thylakoid fragmentation rather than of the equal distribution of Cyt b_6-*f* complex among the appressed and non-appressed membranes. A Yeda press treatment will break the continuity of the thylakoid membrane by tearing off stroma-exposed regions from the grana along the contour lines of least resistance. It is known that each granum thylakoid extends directly into a stroma-exposed region (stroma lamella) through the membrane of the fret region [1–3]. Thus, it is reasonable to expect that contour lines of least resistance occur at or very near the region of the thylakoid membrane where an intergranum lamella narrows prior to its conversion into a granum thylakoid.

Our results with the counter-current distribution of T1 and class II chloroplasts clearly demonstrated depletion of Cyt b_6-*f* complex in the PS I enriched fractions and Cyt enrichment in the PS II enriched fractions (Table I and Fig. 8). A similar tendency, though not so pronounced, was observed upon sonication of B3 vesicles [26]. Of particular interest, however is the observation of the highest Cyt b_6-*f* enrichment in the fraction between the PS II most-enriched and the PS I most-enriched fractions. If it is assumed that the aqueous polymer two-phase separation of the various vesicles depends on their surface properties [14,15], then we must conclude that the surface properties of the Cyt most-enriched vesicles are intermediate to those of the PS II most-enriched and the PS I most-enriched vesicles. It is then tempting to conclude that the vesicles enriched in the Cyt b_6-*f* complex originate from discrete domains in the thylakoid membrane which are enriched in this complex and are localized between the grana partition regions and the stroma-exposed regions of the chloroplast membrane. Our results, therefore, support the notion of Ghirardi and Melis [25] that Cyt-b_6-*f* complexes are localized in the fret region of the thylakoid membrane. However, our results also demonstrates an enrichment of Cyt b_6-*f* complex in the vesicles containing PS II, pointing to a localization of the Cyt b_6-*f* complex in the grana partition region in agreement with an earlier work on counter-current distribution of sonicated inside-out vesicles [26]. Further experiments are needed to determine the exact distribution of cytochrome b_6-*f* between the fret region and the grana region.

The lateral localization of the Cyt b_6-*f* complex in the thylakoid membrnae is of importance in terms of our understanding of the linear electron-transport process from PS II to PS I. As discussed by Haehnel [36], an even distribution of the Cyt b_5-*f* complex among the partition region of grana and stroma thylakoids poses certain constraints on the coordination of electron transport from PS II to PS I. On the other hand, localization of this complex in the narrow membrane of the fret region will support the assignment of an important role to both plastoquinone and plastocyanine in the long-distance electron-transport process. Thus, plastohydroquinone will diffuse between PS II and the Cyt complex in the membrane of the grana partition region only. There, electrons will

be intercepted by the Cyt complex. Subsequently, the role of electron transport between the Cyt complex and PS I in stroma-exposed lamellae is assumed by plastocyanine [25].

The emerging model on the electron transport complex organization in higher plant chloroplasts suggests PS II_α localization strictly in the grana partition region and PS I localization strictly in the intergrana lamellae. There is an apparently intermediate domain in the thylakoid membrane which is enriched in the Cyt b_6-f complex (see Fig. 7 in Ref. 25). This domain appears to be in the vicinity of PS II_α but it may not be an integral part of the membrane in the grana partition region. Given the constraints imposed by the structural configuration of grana thylakoids, the locus assigned to the Cyt b_6-f complex is the membrane of the fret region. In essence then, our present results corroborate the concept of extreme lateral heterogeneity of electron-transport complexes in the thylakoid membrane of photosynthesis and advance the notion of the domain organization of the electron-transport complexes in the thylakoid membrane of chloroplasts.

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