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A MECHANISM FOR THE FORMATION OF INSIDE-OUT MEMBRANE VESICLES

PREPARATION OF INSIDE-OUT VESICLES FROM MEMBRANE-PAIRED RANDOMIZED CHLOROPLAST LAMELLAE

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Summary

Inside-out thylakoid membrane vesicles can be isolated by aqueous polymer two-phase partition of Yeda press-fragmented spinach chloroplasts (Andersson, B. and Åkerlund, H.-E. (1978) Biochim. Biophys. Acta 503, 462—472). The mechanism for their formation has been investigated by studying the yield of inside-out vesicles after various treatments of the chloroplasts prior to fragmentation. No inside-out vesicles were isolated during phase partitioning if the chloroplasts had been destacked in a low-salt medium prior to the fragmentation. Only in those cases where the chloroplast lamellae had been stacked by cations or membrane-paired by acidic treatment did we get any yield of inside-out vesicles. Thus, the intrinsic properties of chloroplast thylakoids seem to be such that they seal into right-side out vesicles after disruption unless they are in an appressed state. This favours the following mechanism for the formation of inside-out thylakoids. After press treatment, a ruptured membrane still remains appressed with an adjacent membrane. Resealing of such an appressed membrane pair would result in an inside-out vesicle.

If the compartmentation of chloroplast lamellae into appressed grana and unappressed stroma lamellae is preserved by cations before fragmentation, the inside-out vesicles are highly enriched in photosystem II. This indicates a granal origin which is consistent with the proposed model outlined. Inside-out vesicles possessing photosystem I and II properties in approximately equal proportions

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Abbreviation: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

could be obtained by acid-induced membrane-pairing of chloroplasts which had been destacked and randomized prior to fragmentation. Since this new preparation of inside-out thylakoid vesicles also exposes components derived from the stroma lamellae it complements the previous preparation.

It is suggested that fragmentation of paired membranes followed by phase partitioning should be a general method of obtaining inside-out vesicles from membranes of various biological sources.

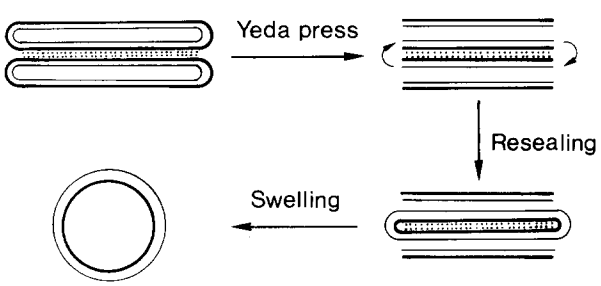
Introduction

Inside-out vesicles have recently been isolated from spinach chloroplast lamellae [1—4] allowing a direct study of the inner thylakoid surface. The isolation has been accomplished by partition of Yeda press-disrupted chloroplasts in an aqueous dextran/poly(ethyleneglycol) two -phase system. Evidence for the reversed sidedness of the vesicles includes studies on the direction of their proton [1,2] and electrical gradients [3] and freeze-fracture appearance [4].

The inside-out vesicles originated from the grana partition regions as judged by their photosystem II enrichment [4,5] and freeze-fracture appearance [4]. This led us to the conclusion that stacked granal membranes were essential for the production of the inside-out vesicles. The following mechanism for their formation was therefore suggested (scheme 1) [4]. Due to the press treatment the grana stacks rupture at their margins but still remain appressed. Resealing of such a membrane with a membrane from a neighbouring grana disc would form an inside-out vesicle. To test this hypothesis we have studied the yield and properties of inside-out thylakoid vesicles isolated under various conditions effecting the stacking behaviour of the chloroplast thylakoid membrane. Only in the cases when the membranes were stacked by cations or membrane-paired by a low pH value, did we obtain any amount of inside-out vesicles supporting the proposed mechanism.

Materials and Methods

Poly(ethyleneglycol) 4000 was obtained from Union Carbide, New York, and Dextran 500, batch No. 3447 from Pharmacia Fine Chemicals AB, Uppsala, Sweden.



Scheme 1. Proposed mechanism for the formation of inside-out vesicles by press treatment starting from appressed membranes. The dots represent attractive forces between two adjacent membranes, such as those occurring in chloroplast grana stacking.

Spinach (*Spinacea oleracea*) was grown in nutrient solution [8] at approx. 18°C with a light period of 12 h. The light intensity was 400 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Preparation and fragmentation of chloroplast lamellae

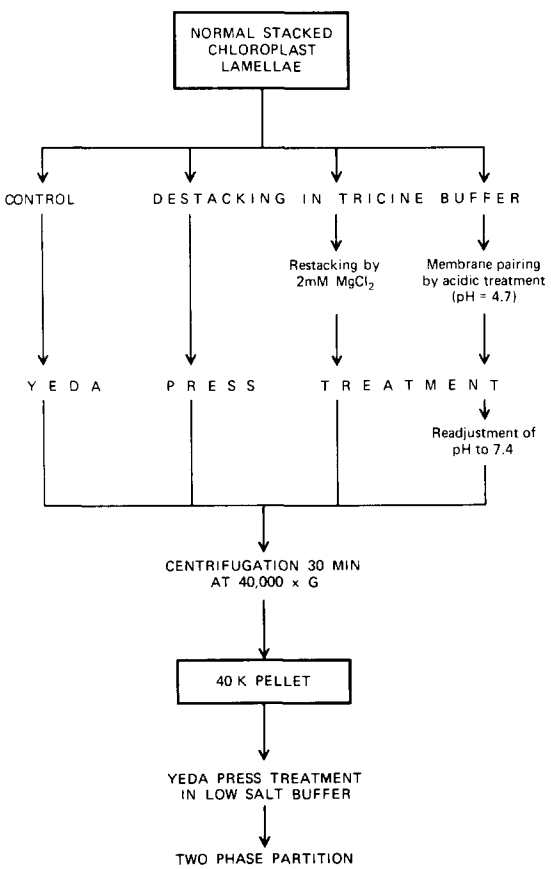
Washed chloroplast lamellae (class II) were prepared as previously described [9]. Prior to Yeda press [10] fragmentation the chloroplasts were treated in the following ways (scheme 2) to obtain lamellae of various stacking appearance.

(1) Normal stacked chloroplast lamellae (control) were obtained by suspension in either 50 mM sodium phosphate (pH 7.4)/150 mM NaCl/100 mM sucrose or 10 mM Tricine (pH 7.4)/2 mM $MgCl_2$/100 mM sucrose.

(2) Destacked chloroplast lamellae were experimentally obtained by incubation in 10 mM Tricine (pH 7.4)/100 mM sucrose for 1.5 h at 4°C.

(3) Restacked chloroplast lamellae were obtained by addition of 2 mM $MgCl_2$ to the destacked chloroplast lamellae (preparation 2) and allowed to stand for 1.5 h at 4°C.

(4) Artificially membrane-paired chloroplast lamellae were obtained by



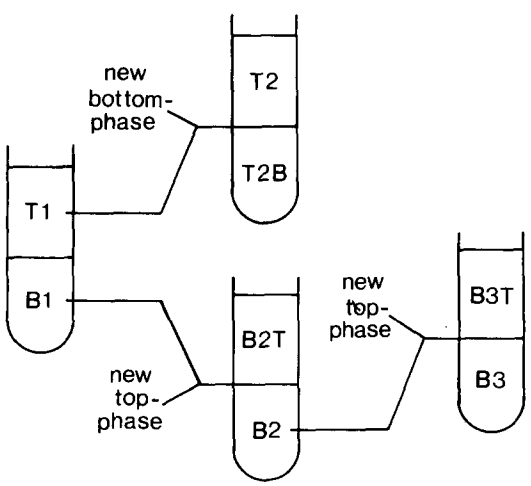
Scheme 2. Scheme for the preparation and Yeda press fragmentation of normal stacked chloroplasts, destacked chloroplasts, restacked chloroplasts and membrane-paired, randomized chloroplasts.

lowering the pH of a destacked chloroplast preparation to 4.7, using 0.1 M HCl.

The fragmentation procedures are illustrated in scheme 2. Disruption was obtained by two consecutive passages through a Yeda press at a nitrogen pressure of 10 MPa (i.e., 10^7 N/m^2). After centrifugation of fragments at 40 000 × *g* for 30 min the pellets were resuspended in a buffer containing 10 mM sodium phosphate (pH 7.4)/5 mM NaCl/100 mM sucrose and passed twice more through the press. This material, designated 40 K, was centrifuged at 1000 × *g* for 10 min to remove starch grains before being subjected to phase partitioning. In the case of low pH-treated lamellae, readjustment to physiological pH (7.4) was performed using 0.1 M NaOH immediately after the first set of Yeda press treatments.

Phase partition [6,7]

Each of the four vesicle populations (40 K) were fractionated by phase partitioning in the following way as outlined in scheme 3. 5 ml of chloroplast suspension (4 mg Chl) were added to the 20 g of polymer mixture to yield a two-phase system of the following composition per kg of mixture. 5.7% (w/w) dextran 500, 5.7% (w/w) poly(ethyleneglycol) 4000, 10 mmol sodium phosphate, pH 7.4, 5 mmol NaCl and 20 mmol sucrose. The phase partition procedure and designation of fractions are outlined in scheme 3. The phase system was thoroughly mixed by several inversions at 3°C and allowed to settle. To facilitate the phase settling, centrifugation at 1500 × *g* for 3 min was usually performed. The material in the top (T1) and bottom phase (B1) was collected and partitioned a second time after the addition of new bottom and top phase, respectively. The B2 fraction was partitioned a third time to yield the B3 fraction.



Scheme 3. Scheme for the fractionation of chloroplast thylakoid vesicles by phase partitioning, including designation of fractions. The main fractions, T2 and B3, are characterized for sidedness and photochemical properties.

Chlorophyll determination and photochemical activities

For crude chlorophyll estimations the absorbance at 680 nm was used. Chlorophylls *a* and *b* were determined according to the method of Arnon [13]. Oxygen was measured with a Clarke-type oxygen electrode in the presence of the photosystem II acceptor, phenyl-*p*-benzoquinone. *P*-700 was determined by the photochemical method using the instrumentation described by Öqvist [14]. For the proton translocation studies the membranes were freed from buffers and polymers by two consecutive centrifugations at 100 000 × *g* for 1—3 h. The external pH changes accompanying light-induced phenyl-*p*-benzoquinone reduction were monitored by a combined glass electrode in a thermostatically controlled reaction vessel (20°C). Light was provided from two 375 W tungsten lamps placed on opposite sides of the reaction vessel. Both lamps were fitted with $CuSO_4$-filters.

Electron microscopy

Chloroplast membranes suspended in buffer were fixed with 2% glutaraldehyde. After 2 h they were pelleted, washed with buffer and post-fixed with 2% OsO_4 for another 2 h. The specimens were dehydrated step-wise in ethanol at 4°C and finally propylene oxide before embedding at room temperature. Sections were cut and finally stained with uranyl acetate and lead citrate (aqueous solution).

Results

Influence of grana stacking on the yield of inside-out vesicles

To see whether membrane stacking is a necessity for the formation of inside-out thylakoids, destacking and restacking of chloroplast lamellae were performed prior to Yeda press disruption. The typical compartmentation of spinach chloroplasts into stacked grana thylakoids and unstacked stroma thylakoids was maintained by suspension of chloroplast lamellae in buffers containing either 150 mM NaCl or 2 mM $MgCl_2$ (Fig. 1a). When chloroplasts are suspended in a low-salt medium the granal stacked membranes separate and form non-appressed membrane sheets [11]. Such destacked chloroplast lamellae (Fig. 1b) were obtained by incubation of the normal stacked chloroplasts in Tricine buffer (pH 7.4) for 1.5 h. Restacking of the destacked chloroplast lamellae (Fig. 1c) could be achieved by addition of $MgCl_2$ to a concentration of 2 mM followed by 1.5 h incubation. The chlorophyll distribution after phase partitioning of 40 K fragments obtained from the three chloroplast populations is shown in Table I. After phase partition of press-treated material from the normally stacked chloroplast, 67% of the material is found in the final poly(ethyleneglycol)-rich upper phase (T2), while approx. 12% partitioned to the final dextran-rich lower phase (B3). This B3 fraction had earlier been shown [1—4] to contain inside-out, photosystem II-enriched thylakoid vesicles. This is confirmed by their reversed proton pumping presented in Fig. 2 and their low chlorophyll *a*/*b* ratio (Table I). On the other hand, the top phase (T2) contains right-side-out material slightly enriched in photosystem I. This result is obtained in both cases when stacking is maintained by buffers containing either 150 mM NaCl or 2 mM $MgCl_2$. When destacked chloroplasts are

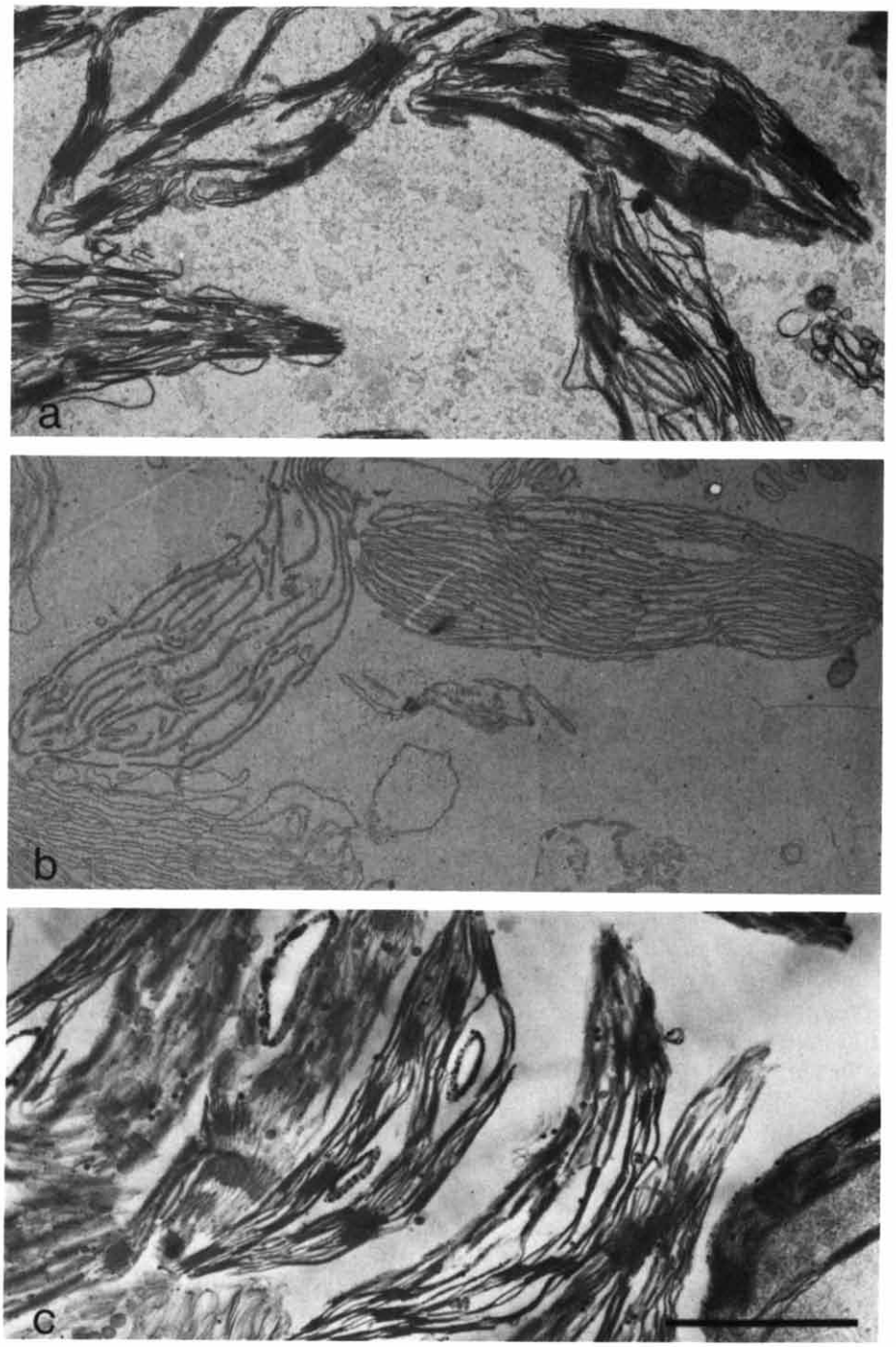


Fig. 1. Electron micrographs of: (a) normal stacked chloroplasts; (b) chloroplasts destacked in 10 mM Tricine (pH 7.4)/100 mM sucrose; (c) chloroplasts restacked by addition of 2 mM $MgCl_2$ to the Tricine buffer. The bar represents 2 μm.

TABLE I

CHLOROPHYLL DISTRIBUTION AND CHLOROPHYLL *a/b* RATIOS OF MAIN FRACTIONS OBTAINED BY YEDA PRESS TREATMENT AND PHASE PARTITIONING OF NORMAL STACKED, DESTACKED AND RESTACKED CHLOROPLAST LAMELLAE

Subchloroplast fractions were obtained and designated as illustrated in schemes 2 and 3. The chlorophyll content of each fraction is expressed as percentage of the 40 K fractions. The chlorophyll *a/b* ratios are shown in parentheses.

Fraction	Percentage chlorophyll		
	Normal stacked	Destacked	Restacked
T2	67 (3.8)	98 (3.1)	83 (3.5)
B3	12 (2.3)	1.6 (—)	10 (2.5)

disrupted by the Yeda press, almost no material partitions to the bottom phase (Table I) indicating that no formation of inside-out vesicles has taken place. When restacked chloroplasts are used as starting material, photosystem II-enriched material showing reversed proton translocation is again found in the bottom phase (Table I, Fig. 2). The yield of inside-out vesicles is somewhat lower than that when starting from normal stacked chloroplasts and is probably due to incomplete restacking. Experiments (not shown) on fragments from unstacked bundle sheath chloroplast/lamellae isolated from the C_4 plant, *Digitaria sanguinalis*, gave no inside-out vesicles which is consistent with the observations above. Thus, inside-out vesicles are isolated only under conditions when grana partitions are present in the chloroplasts. During the second press treatments,

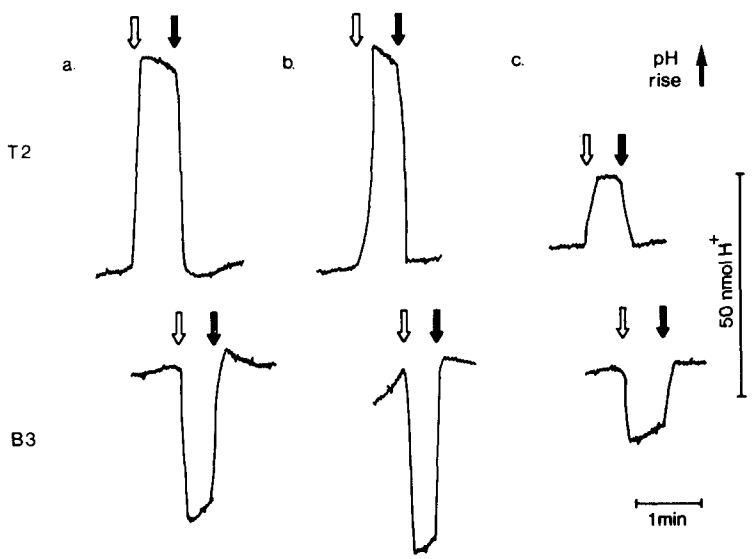


Fig. 2. Proton translocation associated with light-induced phenyl-*p*-benzoquinone reduction of vesicles obtained by press treatment and phase partitioning of; (a) normal stacked chloroplasts; (b) restacked chloroplasts; (c) membrane-paired, randomized chloroplasts. The assay medium was composed of: 40 mM KCl, 0.37 mM phenyl-*p*-benzoquinone and chloroplast material corresponding to 150 μg of chlorophyll. Initial pH in the medium was 6.5.

variations in salt composition can be made without affecting the yield of inside-out vesicles. This step therefore does not seem to be actively involved in the formation of the inverted vesicles, but rather makes them separate from other membrane fragments. It also further disintegrates remaining large fragments interfering with the subsequent partition steps.

Isolation of inside-out vesicles after induction of membrane pairing of destacked chloroplasts by low pH

When chloroplast lamellae are suspended in low-salt media at neutral pH, not only will the grana stacks disappear but also the components of grana partitions will diffuse by means of lateral movements into other regions of the membrane, giving rise to a random distribution of membrane particles [12]. As shown above, such destacked and randomized chloroplast lamellae did not give rise to any inside-out vesicles after press treatment. By lowering the pH close to the isoelectric point of such lamellae [15,16], membrane-pairing occurs due to diminished electrostatic repulsion. Such artificially low pH-stacked chloroplast thylakoids show membrane pairing all along the lamellae (Fig. 3). A similar membrane-pairing pattern has been seen after methylation of thylakoid carboxyl groups [17]. If such destacked and randomized chloroplast thylakoids, membrane-paired by low pH, were used as starting material for the press treatment, vesicles partitioning into the bottom phase would be obtained (Table II). This new bottom-phase material showed a reversed light-induced proton gradient (Fig. 2) similar to that of the vesicles prepared from normal stacked chloroplasts which have been proved to be inside-out [1—4]. The gradient of the low pH-treated vesicles is smaller than that for the vesicles

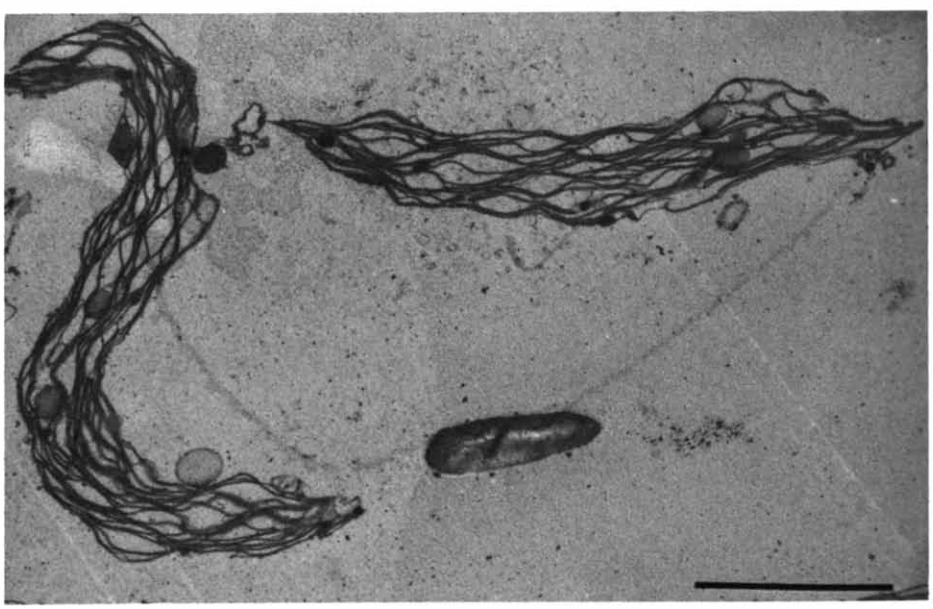


Fig. 3. Membrane-paired, randomized chloroplast lamellae obtained by experimentally destacking and randomization in Tricine buffer for 1.5 h followed by lowering the pH from 7.4 to 4.7. The bar represents 2 μm.

TABLE II

CHLOROPHYLL DISTRIBUTION AND PHOTOSYSTEM II ACTIVITIES OF FRACTIONS OBTAINED BY YEDA PRESS TREATMENT AND PHASE PARTITIONING OF NORMAL STACKED AND MEMBRANE-PAIRED, RANDOMIZED CHLOROPLAST LAMELLAE

Subchloroplast fractions were obtained and designated as illustrated in schemes 2 and 3. Assay of photosytem II: oxygen evolution was measured with a Clarke-type oxygen electrode in an assay medium containing 0.2 μmol phenyl-*p*-benzoquinone, 30 μmol sodium phosphate buffer (pH 6.5), 5 μmol NaCl and chloroplast material corresponding to 10 μg chlorophyll. Total volume 1.0 ml. The specific photosytem II activities are expressed as μmol O_2 produced/mg chlorophyll per h. Values within parentheses are the percentage of the total activity in each fraction calculated from the total activity of the 40 K material. P_pBQ, phenyl-*p*-benzoquinone; PS, photosystem.

Fraction	Normal stacked chloroplasts		Membrane-paired randomized chloroplasts	
	Chlorophyll (%)	PS II activity ($H_2O \rightarrow P_pBQ$)	Chlorophyll (%)	PS II activity ($H_2O \rightarrow P_pBQ$)
40 K	100	100 (100)	100	81 (100)
T1	76.5	83 (62)	88.7	78 (85)
B1	23.5	124 (29)	11.2	79 (11)
T2	68.0	94 (63)	82.2	78 (78)
T2B	8.5	120 (10)	6.5	64 (5)
B2T	5.8	116 (6)	5.0	81 (5)
B2	17	145 (25)	6.2	78 (6)
B3T	3.3	123 (4)	1.6	70 (1)
B3	14.5	150 (21)	4.6	80 (5)

pressed at physiological pH. This is probably explained by the loss of electron-transport activity during the acidic treatment (Table II). The presence of membranes in the B3 fraction with a reversed proton gradient shows that artificial membrane-pairing is sufficient also for the formation of inverted thylakoid vesicles. Comparisons of the chlorophyll *a*/*b* and chlorophyll/*P*-700 ratios and photosystem II electron-transport activity of the new inside-out vesicles with the previous ones, obtained from normally stacked lamellae, are shown in Tables II and III. As evident from Table III, fractions T2 (right-side-out material) and B3 (inside-out) from the randomized artificially stacked chloroplast lamellae show appproximately the same chlorophyll *a*/*b* ratios (3.3 and 3.1, respectively) whilst the B3 fraction from normally stacked chloroplasts is enriched in photosystem II compared to fraction T2 (2.3 and 3.2). Similar

TABLE III

CHLOROPHYLL/*P*-700 AND CHLOROPHYLL *a*/*b* RATIOS OF MAIN FRACTIONS OBTAINED BY YEDA PRESS TREATMENT AND PHASE PARTITIONING OF NORMAL STACKED CHLOROPLASTS AND MEMBRANE-PAIRED, RANDOMIZED CHLOROPLAST LAMELLAE

The fractions were obtained and designated as illustrated in schemes 2 and 3. *P*-700 was measured photochemically. The assay medium contained in a final volume of 1 ml: 20 μmol sodium phosphate buffer, pH 7.4, 2.5 μmol NaCl, 10 μmol sucrose, 0.05 μmol benzyl viologen, 0.01 μmol 2,6-dichlorophenol indophenol and 20 μmol ascorbate. Values within parentheses are the chlorophyll *a*/*b* ratios.

Fraction	Normal stacked chloroplasts	Membrane-paired, randomized chloroplasts
T2	518 (3.2)	513 (3.3)
B3	1136 (2.3)	699 (3.1)

relationships are found for the chlorophyll/*P*-700 ratios (Table III). Also the specific photosystem II activity (Table II) is equally distributed between top- and bottom-phase fragments isolated from the artificially stacked chloroplasts (80 and 78 μmol O_2/mg chlorophyll per h, respectively). In contrast, fraction B3 from normally stacked chloroplasts shows high activity (150) compared to the T2 material (94).

Some photosystem II activity is lost due to the low pH treatment. It should be emphasized that the pH must not drop below 4.7 to avoid severe irreversible inactivation of the photosystem II activity. The activities are, however, well preserved during phase partition, as seen from the total activities shown in Table II.

Thus, the inside-out vesicles prepared from thylakoid membranes paired by low pH contain components from both grana and stroma lamellae; as expected due to the randomization of components preceding the fragmentation.

Discussion

After mechanical rupture, the inner surface of biological membranes will be exposed to the surrounding medium. During the concomitant resealing process, right-side-out and inside-out vesicles can be formed in various proportions depending on the membrane used. For example, sonication of inner mitochondrial membranes produces a high proportion of inverted vesicles [18], while breakage of erythrocytes gives right-side-out membranes unless certain manipulations are made [19]. For the chloroplast thylakoid membrane the previous non-availability of the inside-out vesicles suggested that the intrinsic properties in this membrane were such that no inside-out vesicles could be created after fragmentation. However, by combinding Yeda press treatment and phase partitioning it has been shown that approx. 20% in terms of total chlorophyll of the thylakoids can indeed be isolated as inside-out vesicles. The mechanism for the formation of right-side-out and inside-out thylakoid vesicles after press disruption does not seem to be the result of a random resealing process. Instead, the present study shows that ruptured thylakoids tend to reseal into right-side-out vesicles unless the membranes are appressed by the stacking forces. In the latter case, inside-out vesicles can be formed probably by the mechanism proposed and illustrated in scheme 1. Due to the stacking forces some grana membranes are kept closely appressed even after press disruption. Resealing of such appressed membranes will form inside-out vesicles. Such a mechanism gives satisfactory explanations for several observations from the present and earlier phase partition studies on chloroplast lamellae. (i) The inside-out vesicles originate from the grana partitions as is evident from their high photosystem II enrichment [4,5] and the large number of particles on their EF freeze-fracture faces [4]. (ii) Only right-side-out vesicles have been obtained after phase partitioning of vesicles derived from the unstacked stroma lamellae [9]. (iii) Destacking of the chloroplast lamellae prior to Yeda press treatment gives only right-side-out vesicles (Table I). (iv) Restacking of destacked chloroplasts again allows inside-out vesicles to be formed (Table I). Thus, membrane stacking is a necessary prerequisite for the formation of inside-out thylakoid vesicles. It is therefore likely that in early

fractionation studies on the chloroplast lamellae using press treatments or sonication [20] some inside-out vesicles were formed. But since separation methods depending only on size and density were used, the inverted vesicles could not be isolated from fragments of normal sidedness. Instead, phase partitioning which separates particles according to membrane surface differ- [6,7] enables the discrimination of membrane vesicles differing only in sidedness. The phase partition technique has also been successfully applied to the isolation of inside-out erythrocytes [19,21].

Also, artificial membrane-pairing induced by reduction of the electrostatic repulsive forces between thylakoid membranes prior to Yeda press treatment yielded inside-out vesicles which could be isolated by phase partitioning. Thus, even artificially induced membrane-membrane interactions are sufficient to create appressed membrane pairs which can reseal into inside-out vesicles. The yield of inside-out vesicles from artificially membrane-paired chloroplasts was lower than that obtained from normal stacked lamellae. This lower yield may be due to both the randomization and the low pH prior to membrane-pairing which leads to a different type of membrane-membrane contact compared to normal stacking. Thus, for example, coupling factors and other bulky proteins might be differently distributed and cause steric hindrance. The inside-out thylakoids obtained in this way possess photosystems I and II in approximately the same proportions as the chloroplast lamellae, in contrast to the previous type being highly enriched in photosystem II. This is expected since the incubation of the chloroplast lamellae in low-salt medium for 1.5 h, which preceded the low pH treatment, not only leads to destacking but also to randomization movements of the components by movement in the lateral plane of the thylakoid [12]. Thus, in such a way, stacked randomized lamellae are obtained prior to the press treatment. This new type of inverted thylakoids in which components derived from the stroma lamellae are also exposed should be an important complement to the previous preparation which mainly originates from the grana partitions.

The transverse asymmetry of biological membranes has been best characterized in those cases where sealed inside-out vesicles were readily available, such as for erythrocytes and the inner mitochondrial membrane. For the chloroplast thylakoid membrane, improved knowledge of its transverse asymmetry has now been obtained from recent studies on the inside-out vesicles. An internal localization of the water-splitting site has been demonstrated from trypsinization experiments [22]. Lactoperoxidase-catalyzed radioactive iodination has demonstrated an asymmetric distribution of several polypeptides (Winget, G.D., personal communication). Kinetic and immunological studies on the inside-out vesicles have revealed an internal localization of plastocyanin (Haehnel, W., Berzborn, R.J. and Andersson, B., unpublished results).

For several membrane systems the lack of inside-out vesicles still hampers the study of their transverse organization. It is therefore of great importance to find general and rational methods for the formation and isolation of inverted membrane vesicles. The present study suggests such a general procedure, provided that membranes other than thylakoids would reseal into inside-out vesicles during disintegration if membrane pairing were induced. Their subsequent isolation should depend on a separation method such as phase par-

titioning which utilizes membrane surface properties for separation rather than size and density. Examples where the suggested approach should be worth trying are membranes from the endoplasmic reticulum, Golgi apparatus and rod outer segment discs.

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