FREEZE-FRACTURE EVIDENCE FOR THE ISOLATION OF INSIDE-OUT SPINACH THYLAKOID VESICLES

by

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An aqueous dextran/polyethylene glycol two phase system is used to separate spinach chloroplast thylakoid vesicles obtained by Yeda press treatment of a grana-enriched fraction. It is shown by freeze-fracturing and freeze-etching that the majority of the vesicles in the dextran-rich bottom phase are turned inside-out with respect to the surfaces of the thylakoid in the chloroplast. Most of the vesicles in the top phase had the normal orientation of the two thylakoid surfaces. These results confirm the previous proposal that the bottom phase vesicles show light-induced proton extrusion instead of uptake because their membranes are turned inside-out.

The polypeptide pattern of the bottom phase thylakoid fraction is enriched in the light harvesting chlorophyll a/b protein complex and the reaction centre protein of photosystem II, which is consistent with the origin of these thylakoids from granal regions enriched in photosystem II. A mechanism is suggested to explain the formation of inside-out vesicles from grana.

1. INTRODUCTION

In previous studies, a grana-enriched fraction isolated from spinach chloroplast thylakoids was disrupted by suspension in low salt medium and passage through a Yeda press. The membrane vesicles produced could be divided into two populations on the basis of their different affinity for the two phases of an aqueous dextran/polyethylene glycol system (4, 12). The

membrane material that partitioned into the dextran-rich bottom phase was highly enriched in photosystem II properties, while the thylakoids in the polyethylene glycol-rich top phase were slightly enriched in photosystem I properties (12). The two thylakoid populations translocated protons in opposite directions when illuminated and supplied with the photosystem II acceptor phenyl-*p*-benzo-

quinone (4). The bottom phase membranes showed reversible light-induced proton pumping into the surrounding medium, while under identical conditions the top phase showed the usual proton uptake. These observations suggested that a majority of the closed thylakoid vesicles in the bottom phase were turned inside-out.

The present work investigates this hypothesis. Freeze-fracture and freeze-etch examination of the vesicles separated by phase partition revealed that most of the simple vesicles in the bottom phase were turned inside-out.

2. MATERIALS AND METHODS

2.1. Preparation of thylakoid vesicles

Spinacia oleracea L. (cv. Viking riksort, Weibulls, Landskrona) was grown at 18-22°C in nutrient solution with a light period of 12 h. Washed class II spinach chloroplasts i.e. naked lamellar systems (6) were isolated (3), suspended in 150 mM-NaCl, 50 mM-sodium phosphate buffer, pH 7.4 and disintegrated by passage through a Yeda press at a nitrogen gas pressure of 10 MPa. The grana-enriched pellet obtained after centrifugation for 30 min at 40,000 g was

resuspended in 10 mM-sodium phosphate buffer, pH 7.4, 5 mM-NaCl, 100 mM-sucrose (low salt buffer) and passed twice through the Yeda press. This treatment is designed to break up the grana into vesicles.

After removal of starch grains and residual unbroken lamellae by a low speed centrifugation (15 min at 1000 g) the thylakoid fragments suspended in low salt buffer were fractionated by phase partition (1) as illustrated in Figure 1. Five ml of the thylakoid fraction (800 µg chl/ml) was added to 20 g of a polymer solution of the following composition: 5.7% (w/w) dextran 500, 5.7% (w/w) polyethylene glycol 4000, 10 mmol/kg sodium phosphate buffer, pH 7.4, 5 mmol/kg NaCl, 20 mmol/kg sucrose, and chloroplast material corresponding to 4 mg of chlorophyll. The phase system was carefully mixed and allowed to separate. Centrifugation in a swinging bucket rotor for 3 min at 1500 g was performed to facilitate phase separation. The top phase (T1) and bottom phase (B1) were collected and repartitioned with fresh bottom phase and top phase respectively, yielding fractions T2 and B2. The B2 fraction was further purified by one more partition step. The thylakoids in the B3 fraction thus obtained were removed from the viscous bottom phase by a final partition into a top phase containing the positively charged trimethylaminopolyethylene glycol. All operations were carried out at 3-4°C. The chlorophyll distribution during the phase partition procedure was estimated by absorption at 680 nm. Dextran 500, batch no. 3447, was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden, and polyethylene glycol 4000 from Union Carbide, New York, NY, USA. Trimethylaminopolyethylene glycol 4000 was synthesized according to JOHANSSON et al. (8).

Figure 1. Scheme for fractionation of chloroplast thylakoid vesicles by phase partition. Numerical values represent chlorophyll content, expressed as a percentage of the total chlorophyll in the starting material.

2.2. Electron microscopy

Thylakoid preparations were fixed in 2% glutaraldehyde in low salt buffer, washed in 10

Abbreviations: B2 = bottom phase, second partition; B3 = bottom phase, third partition; DTT = dithiothreitol; EF = endoplasmic fracture; ES = endoplasmic surface; PF = protoplasmic fracture; PS = protoplasmic surface; SDS = sodium dodecyl sulphate; T2 = top phase, second partition.

Figure 2. Freeze-fracture electron micrograph of the membranes in the 40,000 g pellet. Note the presence of stacked (EFs) and unstacked (EFu) membrane regions. Arrow represents the direction of shadowing in this and all freeze-fracture micrographs. $\times 115,000$ (Bar = 0.5 μm).

Figure 3. Thin section of the membrane vesicles in the bottom phase (B3). Most vesicles appear to consist of two membranes, more or less closely appressed. $\times 26,500$ (Bar = 1.0 μm).

Figure 4. Freeze-fracture appearance of the vesicles shown in Figure 3. Note the predominance of concave EF and convex PF faces, indicating that these vesicles are inside-out (cf. Figure 10). A concavo-convex vesicle (asterisk) has fractured to reveal both EF and PF fracture faces, although this type of behaviour was not frequently seen. $\times 110,000$ (Bar = 0.5 μm).

mM-sodium phosphate buffer, pH 7.4 and pelleted for freeze-etching. For freeze-fracturing, membranes were either fixed in glutaraldehyde, or resuspended in 20% glycerol in 10 mM-sodium phosphate buffer, pH 7.4, 5 mM-NaCl without fixation. The pelleted membranes were frozen in Freon 22 cooled in liquid nitrogen and replicas made using a Balzers BAF 301 freeze-etching machine (Balzers Aktiengesellschaft, Liechtenstein). Specimens were fractured at -110°C and etched at -100°C for 2 min. Replicas were cleaned in concentrated sodium hypochlorite and chromic acid and examined in a Siemens Elmiskop 102 operated at 80 kV. Survey photographs, used for calculating the surface orientation of vesicles were taken of areas of replicas selected at random at a magnification of 20,000 $\times$. Fixation for thin-sectioning was carried out on the same material used for freeze-etching, according to the schedule described by ÅKERLUND et al. (12).

2.3. Gel electrophoresis

Unfixed thylakoids were solubilized in 2% SDS dissolved in 50 mM- Na_2CO_3 -50 mM-DTT. Gel electrophoresis was performed using the conditions described by CHUA and BENNOUN (5), with the exception that an 11-15% acrylamide gradient was used.

3. RESULTS

The grana-enriched fraction comprised about 94% of the chloroplast lamellae. Figure 1 shows the distribution of this material during

the phase partition procedure, as determined by absorption at 680 nm. Two membrane fractions were isolated: the bottom phase material (B3) showed reversible light-induced proton extrusion under conditions where chloroplast thylakoids normally show proton uptake (4). On the other hand, exposure to light induced proton uptake in the top phase thylakoids (T2).

The freeze-fracture appearance of the grana-enriched pellet revealed thylakoids in which stacking had been maintained by high salt concentration (Figure 2). The B3 fraction was shown by thin-sectioning to consist largely of vesicles (Figure 3). These vesicles frequently appeared in one plane as sections of collapsed spheres with a concavo-convex outline and varying degrees of appression between the two membranes (Figures 5 a, b). Vesicles sectioned at right angles to this plane were seen as two concentric membrane profiles (Figure 5c). What appeared to be simple vesicles consisting of a single spherical membrane were only occasionally seen (Figure 5d). When this material was freeze-fractured, typical EF and PF faces were present (Figures 4 and 6). Only those faces for which it could be decided whether they were concave or convex were

Table I

Characterization of the fracture faces of vesicles from phase partitioned thylakoids.

Fraction	Convex EF+ concave PF(%)	Concave EF+ convex PF(%)	Total no. counted
T2	89	11	151
B3	26	74	183

Figures 5a-d. Vesicle profiles seen in the bottom phase (B3) membranes. These have been interpreted as indicating that most vesicles have a three dimensional concavo-convex configuration. $\times 125,000$ (Bar = $0.2 \mu\text{m}$).

Figure 6. Electron micrograph of bottom phase membranes demonstrating a freeze-fractured concavo-convex vesicle (arrow). $\times 110,000$ (Bar = $0.5 \mu\text{m}$).

Figure 7. Freeze-fractured vesicles from the top phase (T2). The predominance of convex EF and concave PF fracture faces indicate that most of these vesicles are right-side out (cf. Fig. 10). $\times 110,000$ (Bar = $0.5 \mu\text{m}$).

scored. A majority of these faces from B3 membranes showed concave EF and convex PF faces (Table I). Freeze-etching of B3 membranes revealed convex surfaces covered with tetrameric particles (Figure 8a), corresponding to the ES (10).

In contrast, freeze-fracturing of the vesicles in the T2 fraction (Figure 7) produced a majority of convex EF and concave PF faces (Table I). Freeze-etching of these thylakoids (Figure 8b) showed convex surfaces characterized by the large particles typical of the PS (10).

The polypeptide patterns produced by gel

electrophoresis of the thylakoids in the grana-enriched fraction and the B3 and T2 fractions are shown in Figure 9. The differences between samples were quantitative, with an enrichment of the light harvesting chlorophyll a/b protein complex, and polypeptides numbered 2, 3, 4, 5 and 6, in the B3 membrane fraction. The T2 thylakoids are enriched in chlorophyll complex I and polypeptide 1, these being relatively depleted in the B3 membranes. It should be noted that the alpha and beta subunits of coupling factor were present in the B3 membranes, although in reduced amounts compared with the other two fractions.

Figure 8a. Freeze-etched vesicle from the bottom phase, showing a convex membrane surface covered with the tetrameric particles characteristic of the ES, and therefore inside-out. $\times 206,000$ (Bar = $0.2 \mu\text{m}$).

Figure 8b. Freeze-etched vesicle from the top phase, revealing a convex membrane surface covered with large particles, typical of the PS, and therefore right-side out. $\times 206,000$ (Bar = $0.2 \mu\text{m}$).

Figure 9. Polypeptide patterns in a gel electrophoretogram of the three membrane preparations. Chlorophyll-protein complex I (CPI), the α and β subunits of coupling factor (CF_1) and the light-harvesting chlorophyll a/b protein complex (CPII) are indicated. Quantitative differences between the three preparations are found with respect to the amounts of polypeptides labelled 1 to 6. Polypeptide 2 is probably the reaction centre protein for photosystem II.

4. DISCUSSION

The original suggestion that the B3 phase contained sealed inside-out vesicles was based on proton flux measurements (4). In order to eliminate alternative explanations, another method for determining the orientation of the membrane surfaces was required. Freeze-fracturing and freeze-etching are suitable techniques because they are not dependent upon assumptions about membrane permeability, and enable one to examine individual vesicles rather than make a bulk measurement on a population of vesicles.

If a normal right-side out spherical vesicle is freeze-fractured, it will produce a concave PF face and a convex EF face, as shown in Figure 10 a. An inside-out vesicle, in which the outer surface has become the inner surface will produce a concave EF face and a convex PF face (Figure 10 b). Concavo-convex vesicles were predominant in thin sections of B3 membranes. Right-side out vesicles of this type freeze-fracture to reveal either a single concave PF face or a single convex EF face (as for a spherical vesicle), or a convex PF face surrounded by a cross-fractured membrane, or a concave EF face surrounded by a cross-fractured membrane (Figure 10 c). An inside-out vesicle of the same topography behaves in the opposite manner (Figure 10 d). Freeze-fracture faces surrounded by a cross-fractured membrane were rarely encountered (e.g. Figure 4), probably because such fracture behaviour is energetically less favoured. As a consequence thereof concavo-convex vesicles yield a majority of fracture images indistinguishable from spherical vesicles of the same membrane sur-

Figure 10. Diagrammatic representation of the freeze-fracture behaviour of inside-out and right-side out spherical vesicles, and of inside-out and right-side out concavo-convex membrane vesicles, derived from chloroplast thylakoids.

face orientation (compare Figures 10 a with c and 10 b with d).

When electron micrographs are scored for vesicle membrane sidedness, 74% of such vesicles in the B3 fraction are inside-out, while only 11% are inside-out in the T2 phase, indicating the effectiveness of phase partition as a method for separating the two vesicle populations. The sidedness of vesicles in the two fractions is confirmed by freeze-etching.

Previous studies on the photochemistry of the T2 and B3 fractions (12) have established that B3 thylakoids are highly enriched in

photosystem II properties, and depleted in photosystem I activity, as evidenced by their low chlorophyll a/b ratio, low fluorescence emission ratio (F_{735}/F_{685}), high Mn content and high chlorophyll/P700 ratio. Analysis of the polypeptide patterns agrees with these findings. The enrichment of the light harvesting chlorophyll a/b protein complex is correlated with the low chlorophyll a/b ratio, and the reduced amount of chlorophyll-protein complex I is consistent with the high chlorophyll/P700 ratio. A photosystem II enriched fraction obtained by Triton X-100 treatment of spinach

Figure 11. Proposed mechanism for the formation of inside-out membrane vesicles from chloroplast granal regions subjected to shear forces.

by KLEIN and VERNON (7) was enriched in a polypeptide with an apparent molecular weight of 44,000 daltons, which is similar to that designated polypeptide 2 in Figure 9. The functions of other polypeptides (3, 4, 5 and 6) enriched in the B3 fraction are unknown.

ÅKERLUND et al. (12) have postulated that the B3 membranes originate from grana regions rich in photosystem II. The high density of the freeze-fracture particles on the EF face of these membrane vesicles is consistent with a granal origin, although some movement of particles between grana and attached stroma lamellae (10) may have occurred in the low salt buffer. Loose grana stacks are known to be stable in 30 mM-NaCl (9), and STAEHELIN (10) has shown that 50 mM-NaCl induces restacking of destacked thylakoids without subsequent particle redistribution.

A plausible origin of inside-out vesicles is presented in Figure 11. Grana discs rupture at their edges due either to osmotic shock or shearing, or a combination of these processes. Resealing of a disc membrane with the nearby appressed membrane from the adjacent disc would thereby form an inside-out membrane vesicle. Such paired thylakoid regions have been prepared by osmotic shock of isolated thylakoid systems by KAHN and VON WETTSTEIN (6) who proposed a similar mechanism. These structures were stable during subsequent manipulation and such a mechanism explains the presence of appressed curved membrane vesicles in the B3 fraction. Slow leakage of ions through the membrane may subsequently lead to separation of the membranes into more spherical concavo-convex vesicles. A ruptured appressed grana disc membrane which does not seal with its neighbour will subsequently separate from its neighbour and has then the option of sealing into an inside-out or right-side-out spherical vesicle (Figure 11).

This mechanism can explain the enrichment of grana membranes in the B3 phase, since stroma lamellae, not being stacked, could not form inside-out vesicles in this manner. When thylakoids are completely destacked and then subjected to shearing followed by phase partition, all membranes partition into the upper

phase (ÅKERLUND and ANDERSSON, unpublished results).

A population of inside-out photosynthetic membrane vesicles is valuable for localization of electron transport chain components (11) and surface polypeptides (2). The results presented demonstrate that a mixture of inside-out and right-side out thylakoid vesicles can be obtained from chloroplasts and separated by phase partition utilising the differences in the properties of the inner and outer surfaces of the thylakoid membrane.

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