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QUANTITATIVE SEPARATION OF SPINACH THYLAKOIDS INTO PHOTOSYSTEM II-ENRICHED INSIDE-OUT VESICLES AND PHOTOSYSTEM I-ENRICHED RIGHT-SIDE-OUT VESICLES

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Yeda press disruption of thylakoids in the presence of magnesium followed by aqueous polymer two-phase partitioning fractionated the total thylakoid membrane material into two distinctly different fractions. One fraction comprised approx. 60% of the material on a chlorophyll basis and contained inside-out vesicles while the other fraction (40%) contained right-side-out vesicles. The sidedness of the vesicles was determined from the direction of their light-induced proton translocation. The inside-out vesicles showed a pronounced Photosystem (PS) II enrichment as judged by their high PS II and low PS I activities. Moreover, they showed a high ratio between the PS II reaction centre chlorophyll-protein complex and the PS I reaction centre chlorophyll-protein complex (CP I). The chlorophyll *a*/*b* ratio was as low as 2.3 compared to 3.2 for the starting material. In contrast, the right-side-out vesicles showed a pronounced PS I enrichment. Their chlorophyll *a*/*b* ratio was 4.3–4.9. The tight stacking induced by Mg^{2+} allows a quantitative formation of inside-out vesicles from the appressed thylakoid regions while mainly non-appressed thylakoids turn right-side-out. The possibility of fractionating all of the thylakoid material into two sub-populations with markedly different composition with respect to PS I and PS II argues against a close physical association between the two photosystems and in favour of their spatial separation in the plane of the membrane. This fractionation procedure, which can be completed within 1 h and gives high yields of both PS II inside-out thylakoids and PS I right-side-out thylakoids, should be very useful for facilitating and improving studies on both the transverse and lateral organization of the thylakoid membrane.

Introduction

There is increasing evidence that appressed and non-appressed regions of stacked thylakoids are structurally [1,2] and functionally [2–11] different. Apart from this lateral heterogeneity there is a pronounced transbilayer asymmetry [12,13]. Both the transverse and lateral organization of the thylakoid membrane can be studied using inside-out vesicles obtained by aqueous polymer two-phase partitioning following press disruption [14,15]. By comparing the effects of membrane-impermeable probes on inside-out and right-side-out thylakoids the transverse asymmetry of several components has been clarified [15,16]. The merit of the inside-out vesicle for studies on the thylakoid lateral organization became evident when it was shown that they originated mainly from the appressed thylakoid region [17,18]. Therefore, comparison of the composition of the inside-out vesicles with isolated stroma lamellar vesicles originating from non-appressed thylakoids [2,19] gives infor-

Abbreviations: Chl, chlorophyll; PS, photosystem; CP, chlorophyll-protein complex; DCIP, 2,6-dichlorophenolindophenol.

mation about differences between the appressed and non-appressed regions [4,6,9–11]. Such studies have shown that PS I and the coupling factor are mainly absent from the appressed thylakoids, and restricted to the non-appressed thylakoids, while most of PS II and the light-harvesting complex are located in the appressed regions [16].

A major limitation with all previous preparations of inside-out vesicles [9,14,18] and stroma lamellar vesicles [2,4,20] has been the quite low yield obtained. This is not only an experimental inconvenience but may also cast doubts on whether these subfractions are representative of the native thylakoid membrane.

This paper describes a high-yield fractionation procedure for the thylakoid membrane which is based upon Yeda press disruption of thylakoids tightly stacked by magnesium followed by phase partitioning. By this procedure the thylakoid membrane could be fractionated into two distinct sub-populations of quite different properties. One population comprising approx. 60% of total chlorophyll consisted of inside-out vesicles highly enriched in PS II while the remaining population (40%) consisted of membranes of normal sidedness with a pronounced PS I enrichment. These results give further support for the model, suggesting a heterogeneous distribution of PS I and PS II in the lateral plane of the thylakoid membrane [6,21–23]. Moreover, the formation of inside-out vesicles in such a high proportion enabled the rapid isolation of such vesicles in a large amount.

Materials and Methods

Spinach was grown hydroponically under artificial light (20 000 lx). Leaves were harvested when 6 weeks old. Dextran T-500, batch No. 2836, was otbained from Pharmacia Fine Chemicals AB, Uppsala, Sweden, and poly(ethylene glycol) 4000 (Carbowax PEG 3350) from Union Carbide, New York, NY, U.S.A.

Washed thylakoid membranes [4] were suspended in 5 mM $MgCl_2$, 5 mM NaCl, 10 mM sodium phosphate buffer (pH 7.4), 100 mM sucrose to give a chlorophyll concentration of about 4 mg/ml. The thylakoids were disintegrated by passage twice through a Yeda press [4] at a nitrogen gas pressure of 10 MPa. The material was then passed twice more through the Yeda press after addition of EDTA (0.1 M, pH 7.4) to give a final concentration of 5 mM. Starch and unfragmented material were removed by a low-speed centrifugation ($1000 \times g$ for 10 min). The supernatant, designated thylakoid homogenate, was fractionated by phase partitioning, either for analytical purposes by counter-current distribution as described earlier [4], or preparatively by a batch procedure in a few steps. In both types of experiment, a two-phase system of the following composition was used: 5.7% (w/w) dextran 500, 5.7% (w/w) poly(ethylene glycol) 4000, 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl and 20 mM sucrose. For the preparative method 1 ml of thylakoid homogenate (4 mg Chl/ml) was added to 24 g of a polymer mixture to yield a phase system of the described composition. The mixture was pre-made by mixing 7.13 g of 20% (w/w) dextran, 3.56 g of 40% (w/w) poly(ethylene glycol), 1.2 g of 0.1 M NaCl, 1.2 g of 0.2 M sodium phosphate buffer, pH 7.4, 0.14 g sucrose and finally H_2O to 24 g. After addition of the thylakoid sample the phase system was carefully mixed and allowed to settle. (To facilitate phase settling centrifugation was performed at $1500 \times g$ in a swing-out centrifuge.) The upper phase (T1) and lower phase (B1) were collected and repartitioned with pure lower phase (B0) and upper phase (T0), respectively, yielding fractions T2 and B2. These were further purified by another partitioning step using B0 and T0, yielding the final T3 and B3 fractions. T0 and B0 were obtained from a 250 g bulk-phase system prepared by mixing 71.3 g of 20% (w/w) dextran, 35.6 g of 40% (w/w) poly(ethylene glycol), 12.5 g of 0.1 M NaCl, 12.5 g of 0.2 M sodium phosphate buffer, 5.7 g of 30% sucrose and H_2O up to 250 g. The mixture was shaken and allowed to settle overnight. The upper phase (T0) and the lower phase (B0) were collected and stored separately until use. It is crucial that the phase partitioning is performed at 3–4°C. The polymer concentrations needed for optimal separation may vary depending on differences in average molecular weights for various commercial polymer batches and should be adjusted as described in Ref. 24. When required the thylakoid vesicles were removed from the polymers by 2–3-fold dilution in a suitable buffer and

pelleted at 100 000 × *g* for 30 min.

Light-induced external pH changes associated with PS II reduction of phenyl-*p*-benzoquinone were measured with a combined glass electrode in a vessel maintained at 20°C. PS I electron transport from ascorbate/DCIP to methyl viologen and the PS II electron transport from water to phenyl-*p*-benzoquinone were followed using a Clark-type oxygen electrode. The ratio of the PS I chlorophyll-protein complex (CP I) and the PS II chlorophyll-protein complex (CP a) was determined by a mild SDS-polyacrylamide gel electrophoresis [25]. Cytochrome *f* was determined from reduced – oxidized (hydroquinone – ferricyanide) difference spectra as described in Ref. 26. The medium was composed of 15 mM sodium phosphate buffer, pH 6.5, 30 mM sucrose, chloroplast material corresponding to 170 μg Chl/ml and Triton X-100 to a final concentration of 0.8%.

For electron microscopy the thylakoid membranes were fixed with glutaraldehyde and post-fixed with OsO_4. Embedding was done by gradually introducing Agar 100 in acetone solution [27]. Thin sections were post-stained with uranyl acetate and lead citrate.

Results

In order to increase the yield of inside-out thylakoid vesicles advantage was taken of the finding that formation of inside-out vesicles requires appressed thylakoids prior to disruption [18]. A tighter stacking would therefore be expected to increase the proportion of thylakoid membranes forming inside-out vesicles after disruption. Therefore, the 150 mM NaCl used in the original Yeda press disruption procedure [4] was replaced by 5 mM $MgCl_2$. Thus, in the new procedure the thylakoids were fragmented by two consecutive Yeda press treatments in a buffer containing 5 mM $MgCl_2$, followed by addition of 5 mM EDTA and two more press treatments. The EDTA was introduced to complex the Mg^{2+} prior to the two final press treatments, which have to take place under low ionic conditions [4]. Thus, the 30 min centrifugation at 40 000 × *g* and subsequent suspension in the low-salt buffer used in the original procedure could be omitted. When the thylakoid fragments obtained by this modified procedure were fractionated by phase partitioning two membrane populations were obtained: one population with a high affinity for the upper phase (T3) and another with high affinity for the lower phase (B3). The former comprised approx. 30% of the thylakoid fragments and the latter as much as 45%. The remaining 25% recovered in the wash phases during the two repartitioning steps was discarded. The fragmentation and phase partitioning steps were completed within 1 h. Typically, 100 g of spinach leaves yielded material corresponding to 5–7 mg Chl for both the T3 and B3 fractions.

The two membrane populations were tested for sidedness by measuring their direction of light-induced proton translocation (Fig. 1). The T1 vesicles (partly purified T3) showed normal proton uptake. In contrast, the B3 vesicles showed pronounced proton ejection. This clearly demonstrated that the bottom-phase material consisted of vesicles turned inside-out while the top-phase material consisted of right-sided material. Notably, the unfractionated vesicles showed already proton ejection, demonstrating that a high proportion of the vesicles

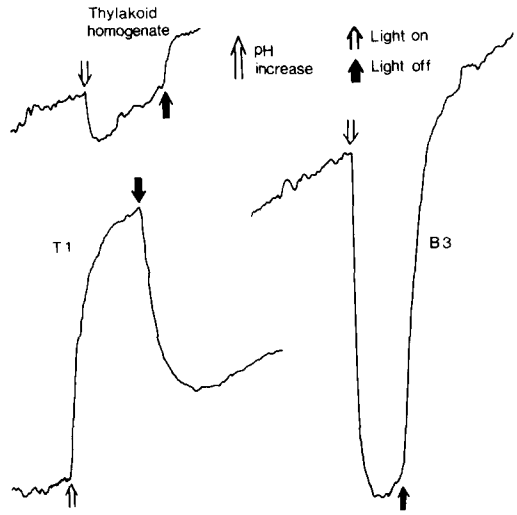

Fig. 1. Light-induced proton translocation associated with phenyl-*p*-benzoquinone reduction measured with a combined glass electrode. The assay medium was composed of 40 mM KCl, 0.37 mM phenyl-*p*-benzoquinone, and thylakoid material corresponding to 100 μg Chl/ml. Initial pH in the medium was set to 6.5 and the extent of proton uptake or extrusion was determined by addition of 50 mM NaOH. The steady-state level of proton transport (nmol H^+/mg Chl) was for: Thylakoid homogenate, −18; T1, 64; B3, −150.

TABLE I

PROPERTIES OF FRACTIONS ISOLATED BY PHASE PARTITIONING FOLLOWING YEDA PRESS DISRUPTION OF THYLAKOIDS IN THE PRESENCE OF MAGNESIUM

Chlorophyll was determined in 80% acetone. PS II was measured in a medium containing 0.2 mM phenyl-*p*-benzoquinone (PBQ), 30 mM sodium phosphate buffer, pH 6.5, and 5 mM NaCl. PS I activity was measured polarographically in 20 mM sodium phosphate buffer, pH 7.4, 2.5 mM NaCl, 10 mM sucrose 0.05 mM benzyl viologen (BV), 0.01 mM DCIP and 20 mM ascorbate. Both activities are expressed as μmol O_2/mg Chl per h. The ratio between the P-700-Chl *a*-protein complex of PS I (CP I + CP Ia) and the Chl *a*-protein of PS II (CP a) was determined by mild SDS-polyacrylamide gel electrophoresis according to the method of Anderson et al. [25]. Cytochrome *f* was determined spectrophotometrically from the benzoquinol-minus-ferricyanide difference spectra in the presence of 0.8% Triton X-100. The spectra were recorded on an Aminco DW-2 spectrophotometer.

Fraction	Chlorophyll		PS II activity ($H_2O \rightarrow PBQ$)	PS I activity ($DCIPH_2 \rightarrow BV$)	CP I/CP a	Cytochrome *f* (mol/10^3 mol Chl)
	%	*a/b*				
Thylakoids	100	3.2	117	71	0.61	1.1
T3	30	4.3	40	129	3.2	1.0
B3	45	2.4	167	39	0.21	1.1

formed by the press treatments were turned inside-out. This is consistent with the very high yield of B3 material. The original preparation of inside-out vesicles also showed a marked PS II enrichment while the right-sided vesicles only showed a slight PS I enrichment [4,6]. As shown in Table I, the present preparation not only gives PS II-enriched inside-out thylakoids but also right-side-out thylakoids with a marked PS I enrichment. The Chl *a/b* ratio of the inside-out vesicles was as low as 2.4 while that of the right-side-out vesicles was quite high (4.3). The inside-out vesicles showed a high PS II activity and low PS I activity, leading to a PS I/PS II activity ratio of 0.23. This ratio was 3.2 for the T3 vesicles and 0.61 for the starting thylakoids. Although the deactivations during the fractionation seemed small it may be argued that the observed activity differences could be due to selective inhibitory effects. There could also be differences in accessibility of certain effectors, since the two membrane populations have opposite sidedness. Another way to estimate the PS I/PS II ratio is to analyze by SDS-polyacrylamide gel electrophoresis [6,25] two Chl *a*-protein complexes, CP I and CP a, which have been ascribed to PS I and PS II, respectively. These analyses confirmed the PS II enrichment of the inside-out vesicles and PS II enrichment of the right-sided vesicles. In marked contrast to the pronounced differences in composition between the two fractions described above is the almost equal content of cytochrome *f*, which has also been seen in previous subfractionation studies [2,28–30].

The ultrastructural appearance of the T3 and B3 material is shown in Fig. 2. The T3 fraction consists mainly of simple vesicles of varying sizes (Fig. 2a). The B3 material shows the typical structure seen for previous preparations of inside-out thylakoids [17,27]. They appear as cup-shaped structures which in thin sections often give rise to double vesicular images, with the volume between the membranes representing the internal space of the vesicle (Fig. 2b). Addition of magnesium creates vesicular or rod-shaped structures where the internal volume no longer can be resolved (Fig. 2c). This can be explained by induction of internal appressions due to the attractive stacking forces which are acting on the inside of an everted thylakoid.

The results from this batch procedure suggest that press treatment in the presence of 5 mM $MgCl_2$ gives two distinct thylakoid populations, one highly PS II enriched and turned inside-out and another PS I enriched and of normal sidedness. The true size of these populations could not be determined from the three-step partitioning experiments, since the material in the wash phases was not repartitioned. This could, however, be done by counter-current distribution, where all material can be accounted for. Fig. 3 shows the distribution of the material after 115 transfers, in the phase system described, using the automatic

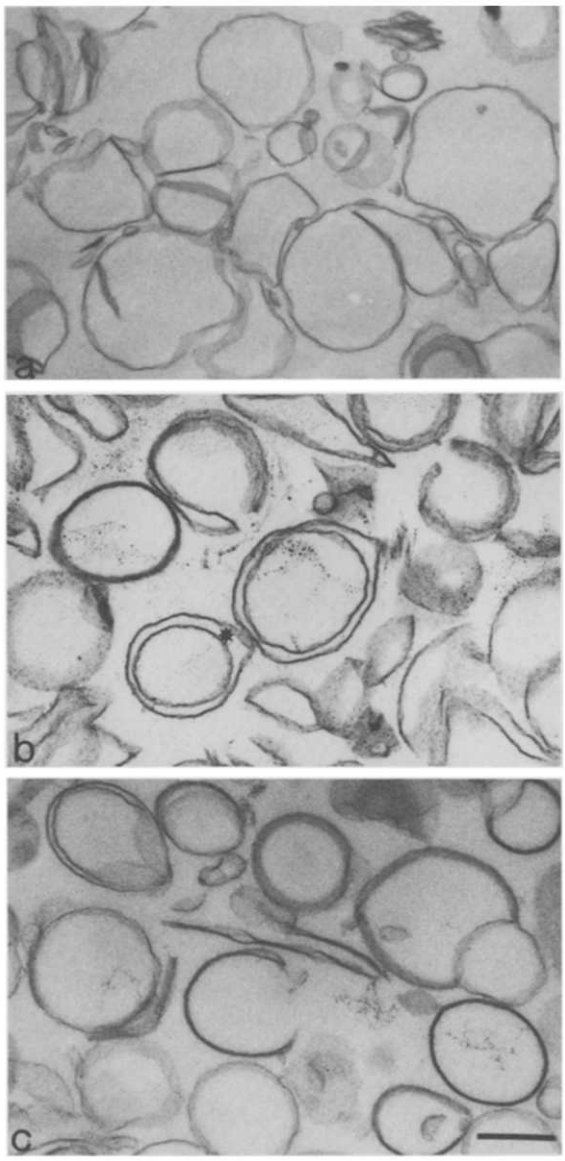

Fig. 2. Electron micrographs of: (a) T3 fraction, (b) B3 fraction, (c) B3 fraction + 5 mM $MgCl_2$. The asterisk marks the internal space of an inside-out vesicle. (Bar = 0.2 μm.)

thin-layer counter-current apparatus [24,31]. The material under the left peak, representing inside-out vesicles partitioning into the lower phase, comprised approx. 63% of the total chlorophyll with a Chl *a/b* ratio as low as 2.3. The rest of the material (37%) recovered under the right peak represents right-side-out thylakoids and showed a quite high Chl *a/b* ratio of 4.9.

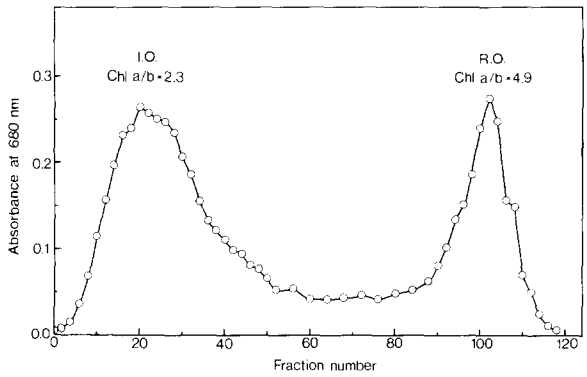

Fig. 3. Counter-current distribution digram of thylakoids disrupted by Yeda press treatment in the presence of magnesium. The amount of material in each fraction was estimated by the absorbance at 680 nm which was plotted against fraction number. On a chlorophyll basis the left peak contains approx. 63% of the total thylakoid material while the right peak contain approx. 37%. R.O., right-side-out; I.O., inside-out.

Discussion

The counter-current distribution experiment (Fig. 3) shows that thylakoids disrupted in the presence of 5 mM $MgCl_2$ give rise to two distinct sub-populations of different surface properties. The lower-phase material (left peak) was highly enriched in PS II and turned inside-out while the upper-phase material (right peak – was PS I enriched and of normal sidedness. It has previously been demonstrated that inside-out vesicles are only formed from appressed thylakoids by a mechanism where an appressed grana thylakoid ruptured at its margin remains appressed to and reseals with an adjacent thylakoid [17,18]. In contrast, non-appressed thylakoids always turn right-side-out [18]. These previous observations and the present high-yield isolation of the two opposite thylakoid populations suggest that the fragmentation in the presence of Mg^{2+} gives rise to quantitative formation of inside-out vesicles from appressed thylakoids and right-side-out vesicles from non-appressed thylakoids which can be discriminated by the phase system. Probably, the tight stacking induced by the Mg^{2+} during disintegration leads to a nearly total formation of inside-out vesicles from the appressed regions. Thus, no or very few right-sided vesicles can be formed from the appressed thylakoids. This means that the right-sided

upper-phase material is largely derived from the non-appressed regions, explaining its high PS I enrichment. Interestingly, the proportion of PS II inside-out vesicles (63%) and PS I right-side-out vesicles (37%) corresponds quite well to the amount of appressed and non-appressed thylakoid surfaces in low-light-grown spinach determined by electron microscopy [8]. However, in making such a comparison one has to consider the limitation in using chlorophyll as a measure of thylakoid membrane area.

The rapid and high-yield isolation of both PS II inside-out vesicles and PS I stroma lamellae is a considerable improvement compared to previous preparations which have given quite low yields of both types of subthylakoids [2,9,14,20]. The inside-out vesicles (B3) from the present procedure show nearly the same PS II enrichment as that of the original procedure but the yield is 2–5-times greater. The upper phase material (T3) gives a 2–7-times higher yield than stroma lamellar vesicles isolated by traditional centrifugation methods [2,4,20]. However, the present T3 fraction does not show the same extreme PS I enrichment as these low-yield stroma lamellar preparations. Whether this is due to contamination of the T3 fraction by membranes from the appressed region or the stroma lamellae as a whole have this composition is not clear. The counter-current distribution results (Fig. 3) would, however, argue for the first alternative.

The new inside-out vesicles show approx. 1.5-times higher PS II activity which is probably due to the omission of high concentration of NaCl previously used during disintegration. High concentration of NaCl releases from the inner thylakoid surface polypeptides essential for oxygen evolution [32]. Inside-out vesicles from $MgCl_2$-stacked thylakoids have previously been isolated by French press disruption and phase partitioning [9]. These vesicles showed a very high PS II enrichment but were obtained in a very low yield.

The high proportion of inside-out thylakoids formed by the present Yeda press treatment was demonstrated by the reversed proton translocation seen already in the unfractionated press homogenate (Fig. 1). Thus, in experiments where one needs access to the inner thylakoid surface without any demands for purity, the press homogenate may be used without any subsequent phase partitioning steps.

The low yield of previous preparations of stroma lamellae (maximum 15%) and inside-out vesicles (maximum 20%) may have cast some doubts on whether they are representative of the native thylakoid membrane and consequently on the model where PS I is mainly located in the non-appressed thylakoids and PS II mainly in the appressed thylakoids [16]. However, the present fractionation of the total thylakoid material into two distinct sub-populations with a markedly different proportion of the two photosystems clearly argues against a close physical association between the two photosystems and in favour of their spatial segregation in the plane of the thylakoid membrane. How extreme this spatial segregation is remains to be established.

The present procedure should greatly increase the availability of PS II inside-out thylakoids and PS I right-side-out thylakoids, thereby facilitating their use in future studies on the lateral and transverse organization of the thylakoid membrane, and also as starting material for purification of PS I and PS II components.

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