Subfractionation of Inside-Out Thylakoid Vesicles*

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Thylakoid membranes can be fragmented by press treatment and separated by centrifugation and aqueous two-phase partition12 into photosystem I- and photosystem II-rich vesicles.3,4 From these studies it was concluded that the two photosystems are segregated into two separate domains in the native thylakoid membrane.4

The photosystem I vesicles originate from the non-appressed, stroma-exposed region of the thylakoid system, while the photosystem II vesicles originate from the grana partition.5 The isolated photosystem II-rich vesicles have an inside-out conformation6,7 and have been used as very powerful tools in studies of the transverse and lateral organization of the thylakoid membrane and of oxygen evolution.8

In this work we have further fragmented the inside-out, photosystem II-enriched vesicles by sonication. This yields a population of smaller vesicles of heterogeneous composition.9,10 Using aqueous two-phase partition and counter-current distribution we have separated the vesicles into different populations. Their composition with regard to chlorophyll a and b content and their photosystem I activity (P 700) was determined.

Materials and methods

Inside-out vesicles from spinach were prepared as described in Ref. 11. The vesicles were included in a 7.5 g phase system comprising 5.6 % (w/w) Dextran 500, 5.6 % (w/w) PEG 4000, 10 mM sodium phosphate buffer (pH 7.4), 3 mM NaCl, 1 mM MgCl₂ and 20 mM sucrose. The sample was sonicated in a Branson Sonifier, Model B 30 (equipped with a 1/2-inch tip), for 2×30 s with a resting interval of 1 min and with 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 distribution10 using a 60 cavity thin-layer apparatus. The phase system used was the same as that described above. Chlorophyll was determined according to Arnon.11 The concentration of P 700 was measured directly from the magnitude of the light-minus-dark absorbance change at 700 nm.13 A differential extinction coefficient of 64 mM⁻¹ cm⁻¹ was used.14 The reaction mixture contained 0.02 % (w/w) sodium dodecylsulfate, 200 µM methyl viologen, 2 mM sodium ascorbate and chloroplast membranes yielding about 20 µM chlorophyll.

Plotting the data. If a membrane consists of two domains, one rich in a component A and the other in a component B, then there is a linear relation between the concentration of A (amount of A per membrane area) and the concentration of B (amount of B per membrane area) in fragments obtained by disintegrating the membrane

\[ \frac{[A]}{[B]} = - \frac{[A_0]}{[B_0]} [B] + [A_0] \]

where \([A_0]\) is the concentration of A in a pure A fraction ([B] = 0) and \([B_0]\) is the concentration of B in a pure B fraction ([A] = 0).10
Results and discussion

A counter-current distribution of sonicated inside-out (B3) vesicles is illustrated in Fig. 1. In agreement with previous results,\textsuperscript{8,9} fractions with different chlorophyll a/b ratio and P 700 content were found. The fractions in the left part of the diagram had the lowest chlorophyll a/b ratio and the lowest P 700 content. Compared to previous results, we have now obtained fractions with lower chlorophyll a/b ratio (1.8) and greater depletion of P 700. We used the concentration of P 700 as representative of the photosystem I- and that of chlorophyll b as representative of the photosystem II-rich domain since the latter is dominated by the chlorophyll b-rich, light-harvesting protein II. Total chlorophyll concentration was chosen as representative of membrane area.

![Graph showing chlorophyll a/b ratio and P 700 content](image)

Fig. 1. Counter-current distribution of sonicated inside-out (B3) thylakoid vesicles. On top of the diagram are shown the chlorophyll a/b ratio (mol/mol) and chlorophyll/P 700 ratio (mol/mol) of pooled fractions. The chlorophyll a/b ratios of the Yeda press homogenate of thylakoids and of the inside-out (B3) vesicles were 2.8 and 2.15, respectively. Corresponding values of the chlorophyll/P 700 ratio were 440 and 800, respectively.

![Graph showing chlorophyll b/Chl ratio against Chl a/mmol/mol](image)

Fig. 2. Plot of P 700/chlorophyll ratio (mmol/mol) against chlorophyll b/total chlorophyll ratio (mol/mol) of different thylakoid vesicles. The points represent, from left to right: Yeda press homogenate of thylakoids, pooled fractions (Fig. 1) 49–56 and 42–28, inside-out vesicles (B3) and pooled fractions (Fig. 1) 30–36, 20–28, 11–17 and 2–10.

We plotted the concentration ratio P 700/total chlorophyll against chlorophyll b/total chlorophyll. As seen in Fig. 2, a straight line is obtained which can be extrapolated to a chlorophyll b/total chlorophyll ratio of 0.37 at zero P 700/total chlorophyll. This implies that if there is a region with zero P 700 concentration it should have a chlorophyll a/b (mol/mol) ratio of 1.7, which is in good agreement with previous extrapolation\textsuperscript{10} and with what one should expect for a membrane preparation containing photosystem II reaction centres plus their antenna. In some of the sonicated fractions such low a/b ratios were indeed found; they also had extremely low P 700 concentrations, near the detection limit of the method for analysing for P 700. Thus, it can be concluded that sonication leads to fragmentation of inside-out thylakoid vesicles into smaller vesicles, some
of which lack P 700. Since these vesicles have a
diameter of about 0.3 μm⁹ it can be concluded
that in vivo there are regions rich in PS II which
do indeed lack P 700. These results therefore sup-
port the suggestion³ that inside-out PS II vesicles
(B3) originate from regions in the native thy-
lakoid membrane containing only PS II, and they
also support a model of the thylakoid membrane
in which PS I is excluded from the partition re-
gion.¹⁵,¹⁶ The PS I contaminant in inside-out ves-
icles obtained by press treatment is therefore not
intermixed at the molecular level with PS II, as
suggested by Atta-Asafo-Adjei and Dilley,¹⁷ but
is located in separate contaminating domains
probably originating from stroma membranes.

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