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Heterogeneity among Photosystem II_α. Isolation of thylakoid membrane vesicles with different functional antennae size of Photosystem II_α

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The functional antenna size of different chloroplast membrane vesicles was determined from light-saturation curves of the oxygen evolution using paraphenylbenzoquinone as acceptor. Thylakoids showed a biphasic light-saturation curve indicative of the presence of at least two types of PS II unit, PS II_α and PS II_β. Inside-out thylakoids were isolated by press treatment and phase partition. These vesicles, which contain only PS II_α, were then further fragmented and separated into different classes of vesicles by a sonication-phase partition procedure (Svensson, P. and Albertsson, P.-Å. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. II, pp. 281–284, Martinus Nijhoff, Dordrecht). Three fractions enriched in PS II_α were obtained; these differ in the antenna size of PS II_α by up to 20%. It is suggested that the size of the light-harvesting complex of PS II_α from different PS II_α units changes in increments of about 25–50 chlorophyll molecules depending on localization of the units in the grana or different parts of the leaf.

Introduction

Classification by antenna size gives two classes of photosystem II in chloroplasts from higher plants [1–8]. One, PS II_α, is located in the grana partitions, the other, PS II_β, is located in the stroma-exposed membranes [3,9]. PS II_α has an antenna size which is 2–3-times larger than that of PS II_β [10]. In addition, there is also heterogeneity among the PS II centers regarding their primary electron acceptor [11–16].

Thylakoid membrane vesicles enriched in PS II_α and almost lacking in PS II_β can be prepared by press treatment of stacked thylakoids, followed by partition in an aqueous polymer two-phase system [9]. These so-called inside-out vesicles originate from the grana and are assumed to represent the grana partitions. See Ref. 17 for a review on inside-out vesicles.

We have subfractionated the inside-out, PS II_α-containing vesicles by sonication and phase partition into different membrane fragments in the form of vesicles which differ in their functional antenna size.

Materials and Methods

Chemicals

Dextran 500 was obtained from Pharmacia Uppsala, Sweden. Poly(ethylene glycol) 4000 (Car-

Abbreviations: PS I and II, Photosystem I and II; LHC II, light-harvesting chlorophyll *a*/*b* protein, ppBQ, paraphenylbenzoquinone.

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bowax PEG 3350) was obtained from Union Carbide, New York, NY, U.S.A.

Chloroplast isolation

Spinach plants (*Spinacia oleracea* L medania) were grown at 18°C under cool-white fluorescent light (incident intensity, 400 $\mu Em^{-2} \cdot s^{-1}$) 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 2000 × *g* for 2 min. The chloroplast pellet was resuspended in preparation medium and centrifuged at 2000 × *g* for 5 min. The chloroplasts were suspended and osmotically broken in 5 mM $MgCl_2$, then centrifuged at 2000 × *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 and once in 10 mM sodium phosphate buffer (pH 7.4) 5 mM NaCl, 100 mM sucrose, 1 mM $MgCl_2$. Finally, they were resuspended in 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.

Preparation of inside-out vesicles [17]

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 × *g* for 10 min). The broken thylakoid membranes were then partitioned in a phase system of 5.55% (w/w) Dextran 500, 5.55% (w/w) poly(ethylene glycol) 4000, 10 mM sodium phosphate buffer (pH 7.4), 3 mM NaCl, 1 mM $MgCl_2$ 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 5 ml of broken thylakoids in 10 mM sodium phosphate buffer (pH 7.4) 5 mM NaCl, 5 mM $MgCl_2$, 100 mM sucrose was added to 20 g of a polymer mixture to yield a phase system of the composition described above. The polymer mixture was premade by mixing 6.94 g 20% (w/w) Dextran 500, 3.47 g 40% (w/w) poly(ethylene glycol) 4000, 0.5 g 0.1 M NaCl, 1.0 g 0.2 M sodium phosphate buffer (pH 7.4) and 8.09 g water. After addition of 5 ml of the fragmented thylakoids, the phase system was mixed thoroughly and allowed to separate under low-speed centrifugation (1000 × *g* for 3 min). The upper poly(ethylene glycol) phase was discarded. The lower dextran phase (B1) was collected and repartitioned twice with fresh poly(ethylene glycol) phase to yield the final lower phase fraction B3. The B3 fraction was diluted 3–4 fold with buffer and the vesicles collected by centrifugation.

Sonication of inside-out vesicles

Fragmentation of inside-out thylakoid vesicles and separation of the fragments were performed essentially as described by Svensson and Albertsson [19]. The inside-out vesicles (fraction B3) suspended in the phase system were sonicated as follows: 15 ml of fresh upper phase, about one-third of it frozen, was added to 15 ml bottom phase containing inside-out vesicles (B3). The phase system, contained in an aluminum beaker suspended in ice-water, was sonicated with a Branson apparatus equipped with a 1/2-inch tip, for 30 s. The ultra-sonic exposure had an intensity output of 7 with 20% duty pulses. The phase system was thoroughly mixed at 3°C and allowed to separate by low-speed centrifugation (1000 × *g* for 3 min). The upper phase, designated 30S, was collected, and 15 ml fresh upper phase, about one third of it frozen, was added to the remaining lower phase.

This sonication–phase-partition cycle was repeated two times and the upper phase fractions were designated 90S and 120S, respectively. The lower phase remaining after the sonications was called BS. The fractions including BS were diluted 2-fold with 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl and 100 mM sucrose, and precipitated by centrifugation at 100000 × *g* for 45 min.

Assays

Oxygen evolution activity was measured by a Clark-type electrode obtained from Hansatech Limited Co., U.K. The reaction mixture was composed of 100 mM sodium phosphate buffer (pH 6.5), 10 mM NaCl, 200 mM sucrose, 0.2 mM ppBQ and 20 mM chlorophyll content of thylakoid

fragments. The light intensities [3] for illumination were varied and detected by Li-170 quantum/radiometer/photometer.

The relative functional antenna size was determined from the slope of the plot of V/I versus V, where V is the oxygen evolution in moles O_2 (mg Chl)$^{-1} \cdot h^{-1}$ and I is the light intensity in $\mu Em^{-2} \cdot s^{-1}$ [20,21].

Results

When oxygen evolution (V) is plotted versus light intensity (I), a typical light-saturation curve is obtained Fig. 1. At low light intensities the oxygen evolution increases almost linearly with light intensity, while it approaches a plateau value (V_{max}) at high light intensities. This behaviour is similar to an enzyme-catalyzed reaction following Michaelis-Menten kinetics if we replace light intensity with the enzyme substrate. The light intensity at which V is one-half of V_{max} is a measure of the antenna size, assuming the same turn-over rate of PS II. The smaller the light intensity at half-maximal oxygen evolution, the larger the antenna size. The saturation curves can be analyzed by a V/I versus V plot. For a population of uniform PS II units having the same antenna size, such a plot should give a straight line with a slope of $-1/k_m$, where k_m is the light intensity at half-maximal oxygen evolution. The line intercepts the X-axis at V_{max} and the Y-axis at V/I at zero light

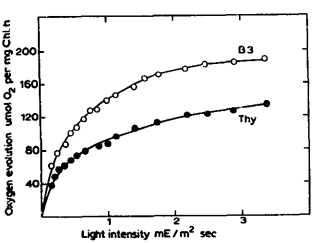

Fig. 1 Photosystem II activity (µmol O_2 per mg Chl h) as a function of light intensity for thylakoids (Thy) and inside-out membrane vesicles (B3) obtained after press treatment and phase partition.

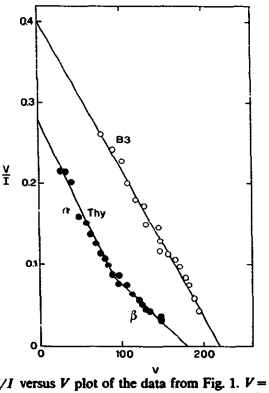

Fig. 2. V/I versus V plot of the data from Fig. 1. V = oxygen evolution, I = light intensity. The intercept on the abscissa is the maximal velocity and the slope is proportional to the inverse negative value of the antenna size. The thylakoids (Thy) curve is composed of two parts with different slopes, the steepest one corresponding to PS II_α and the other one to PS II_β.

intensity (proportional to the apparent quantum yield).

The V/I versus V plot for the thylakoids (Fig. 2) does not give a straight line, but an upward concave curve which demonstrates heterogeneity in the antenna size among the PS II units. As a first approximation, the curve can be divided into two parts, a steep one corresponding to PS II_α and a less steep one corresponding to PS II_β.

The V/I versus V plot for the inside-out vesicles (B3) fits well a straight line indicative of a more homogeneous population of PS II in this preparation as compared to the thylakoids. The slope is close to the steepest part of the thylakoid curve, hence the inside-out vesicles (B3) contain almost only PS II, in agreement with the work of Anderson and Melis [9].

Fig. 3 shows the result of the sonication–phase-partition procedure for fractionation of the inside-out vesicles. The inside out vesicles which are used as starting material for sonication have a chlorophyll a/b ratio of 2.4.

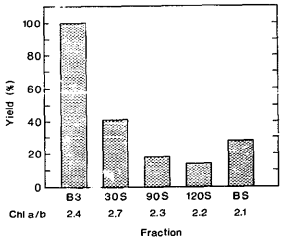

Fig. 3. Fractionation of inside-out (B3) thylakoid vesicles by sonication and phase partition. The starting material (B3) present in the lower phase of aqueous dextran-poly(ethylene glycol) two-phase system was first sonicated for 30 s. Part of the material partitioned into the upper phase and gave the fraction 30S. Next a new upper phase was added to the remaining bottom phase and the phase system sonicated for 60 s to give the 90S fraction. The same procedure was repeated with an additional 30S to give the 120S fraction. BS is the material remaining in the lower phase. Note the chlorophyll *a/b* ratios of each fraction.

The fraction found in the upper phase after 30 s sonication has a chlorophyll *a/b* ratio of 2.7. This step removes PS I-containing membrane material as described in a separate work [19], where the method is described in more detail. Further sonication gives fractions with chlorophyll

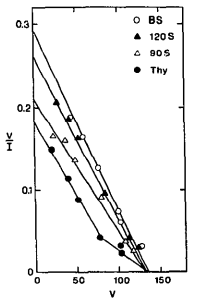

Fig. 4. *V/I* versus *V* plot for fractions from the experiment described in Fig. 3 and for thylakoids (Thy). The different slopes for the three fractions 90S, 120S and BS, show that the antenna size of their PS II_α differ.

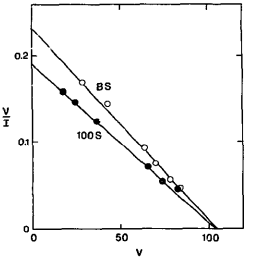

Fig. 5. *V/I* versus *V* plot for two PS II_α fractions obtained from another experiment similar, to that of Figs. 3 and 4.

a/b ratios lower than the starting material and hence more pure PS II membrane vesicles.

These fractions (120S, 90S and BS) all show PS II_α characteristics, i.e., their slopes in the *V/I* versus *V* plot are relatively steep, Fig. 4. However, they all differ from each other. The BS fraction has the steepest slope. The 120S fraction has a slope similar to that of the inside-out preparation (B3), while the 90S fraction has a slope which is somewhat less steep than that of the B3 preparation. The intercept on the vertical axis, i.e., the apparent quantum yield, is also different for the different fractions. Fig. 5 shows *V/I* versus *V* plots for two other fractions obtained by a similar

TABLE I

THYLAKOID MEMBRANE FRAC

The inverse negative value (k_m) of the slopes of the *V/I* versus *V* plots for different thylakoid membrane fractions, see Figs. 2, 4 and 5. The inverse value of k_m is proportional to the functional antenna size of PS II. The values for the thylakoids are from the two slopes of the curve and since they are composed of at least two components, these values cannot be taken as accurate measurements of the antennae sizes.

Fraction	chlorophyll *a/b*	k_m
B3	2.4	525
90S	2.32	619
120S	2.15	512
BS	2.09	462
100S	2.41	559
BS	2.03	464
Thylakoids	3.0	(550, 1280)

procedure as described in Fig. 3. Again, the plots give straight lines with different slopes.

Our fractionation procedure has therefore separated PS II_α units which differ in their antenna size. The k_m values, i.e., the light intensity at half-maximal oxygen evolution, are shown in Table I The different PS II preparations here named PS II_{α_1}, PS II_{α_2} and PS II_{α_3}, differ in their k_m values and hence their antenna size by about 10–20%. From the fractionation diagram of Fig. 3, it can be estimated that they each constitute 10–30% of the B3 material.

Discussion

When light is limiting, the antenna size determines the turn-over rate of the reaction centres in photosynthesis. It was therefore an important discovery when it was demonstrated that in plants there are two classes, PS II_α and PS II_β, of photosystem II which differ in antenna size [1–10]. Nature has developed different antenna sizes of photosystem II in order to permit the chloroplasts to adapt to changes in light conditions depending on both time and their location in the leaf.

A light-saturation curve can be used for the determination of the relative functional antenna size [3,20,21] provided a constant turn-over rate of PS II. The V/I versus V plot (Fig. 2) of the light saturation curve for the thylakoids shows that these consist of at least two components, one of which is saturated at lower light intensities than the other. Our interpretation is that these two parts represent PS II_α and PS II_β, and that the light saturation curve as measured by the oxygen evolution provides independent evidence of the presence of at least two types of PS II differing in antenna size.

When we isolate the grana membranes after press treatment and phase partition, we obtain inside-out vesicles (B3) which originate from the grana partitions [17]. The V/I versus V plot for these vesicles shows about the same slope as the steep part of the thylakoid plot, i.e., the vesicles contain PS II, in agreement with the work of Anderson and Melis [9].

Sonication of the inside-out vesicles (B3) produces new vesicles which are smaller in size than the inside-out vesicles [22]. While the average diameter of the thin sections of the inside-out vesicles is 0.3 μm, this diameter is reduced to 0.1–0.2 μm after sonication [22]. Our sonication-phase partition fractionation procedure has separated these vesicles into classes which differ in their antenna sizes. From this we conclude that in the intact thylakoid partition regions there are domains of about 0.2 μm in diameter which contain PS II units with an antenna size differing from the antenna size of other PS II domains. There is a specialization at the membrane domain level in the sense that in different domains the antenna size of PS II differs. In this context it is of interest that counter-current distribution of sonicated inside-out vesicles show peaks in the separation diagram indicating that the PS II regions of the thylakoid contain discrete regions with different membrane properties [18,22–24].

We consider it unlikely that the different antenna sizes are induced by the sonication phase partition treatment for the following reasons:

(1) The sonication–phase-partition procedure is a relatively mild method, since it preserves the oxygen evolving capacity of the vesicles [19] due to protection by the polymers and the low ambient temperature during sonication (see Materials and Methods).

(2) The average antenna size of the 90S, 120S and BS fractions, which have been produced by sonication, is about the same as that of the B3 fraction from which they originate and which has not been sonicated, indicating that no net change in antenna size has been induced by sonication.

(3) One would expect that if the sonication had destroyed the antenna systems, these would become smaller in their functional size and more so with longer sonication time. We find, however, that those vesicles which have been subjected to the longest sonication time (120S and BS) have the largest functional antenna size.

The antenna size of PS II_α reported so far in the literature is probably an average value of different classes of antenna sizes. Since the pigment molecules are bound to polypeptides, one would expect the antenna chlorophyll-protein complex to increase in discrete steps as the number of polypeptides in the complex is increased. We find in this study an increase, or decrease, of 10–20% in the functional antenna size of our

subfractions compared to the value of PS II_α of the inside-out vesicles. Since the antenna of PS II_α contains about 230 chlorophyll molecules [10], the increments in antenna size which we find in this study are equivalent to about 25–50 chlorophyll molecules. The light-harvesting chlorophyll *a/b* protein LHC II is composed of two different polypeptides with molecular weights of 25 and 27 kDa, respectively. Each of these binds about 15 chlorophyll molecules [25]. Our data therefore suggest that the increments of the antenna of PS II_α consist of a dimer or trimer of these polypeptides or other polypeptides of equivalent size.

The BS fraction has the largest antenna size and would therefore contain relatively more of the LHC II polypeptides than the other fractions. This is consistent with the lower chlorophyll *a/b* ratio of this fraction and also with the fact that the BS fraction is most resistant to sonication. After 90 and 120 s sonication, the 90S and 120 S fractions, respectively, are moved from the lower phase to the upper phase, probably because they have turned from the inside-out to the right-side out conformation [26]. Note that during sonication, the $MgCl_2$ concentration is 1 mM, which is barely able to keep the grana thylakoids stacked. If the stacking force originate from the LHC II polypeptides, then one would expect the BS with its larger content of LHC II to be most resistant to such a change. According to this view, the differing concentration of LHC II in the different domains of the thylakoid membrane is the very basis for the selective sonication–phase-partition fractionation.

Where are the different domains of PS II_{α_1}, PS II_{α_2} and PS II_{α_3} located in vivo? There are several alternatives to consider.

(1) They may all be a part of the same granum with the PS II_{α_1}, which has the largest antenna size, being located in the central core of each partition, the PS II_{α_3}, at the edge, and with the PS II_{α_2} in between [22].
(2) They may be located in different grana of (a) the same chloroplasts, (b) different chloroplasts of the same cell, or (c) different chloroplasts from different cells in the leaf.

Further studies are necessary to distinguish between these alternatives which are all plausible. The light intensity in a leaf changes during the day and it can be an advantage for the chloroplast to have a heterogeneity of the PS II_α units of the grana in order to carry out effective photosynthesis at both low and high light intensities. In addition, the light conditions and the average antenna size of the photosystems are different in different parts of a leaf [27]. Different PS II_α vesicles may originate from different parts of the leaf and the vesicles with the largest antenna size may originate from PS II_α domains located in chloroplasts from those parts of the leaf which are exposed to the lowest light intensity, for instance the cells close to the lower side of the leaf.

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