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Antenna protein composition of PS I and PS II in thylakoid sub-domains

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Abstract

Spinach thylakoids were separated into grana core, grana margin, and two different stromal lamella fractions in the absence of detergents. The levels of all light-harvesting chlorophyll *a*/*b*-binding (LHC) proteins were determined in all fractions, and were normalised to the amount of Photosystem I (PS I) and Photosystem II (PS II) centres. PS Iβ in the stroma lamellae was found to have a full complement of Lhca polypeptides and, probably, one attached LHC II trimer. PS Iα binds additional LHC II trimers, but PS I centres located in the inner parts of the grana stack lack Lhca1 and are depleted in Lhca4. PS IIβ, found in grana margins and stroma lamellae, seems to associate one monomer each of Lhcb4, Lhcb5 and Lhcb6 (CP29, CP26 and CP24, respectively) and one LHC II trimer consisting of two Lhcb1 and one Lhcb3 subunit. PS IIα has additional LHC II trimers (consisting of Lhcb1 and Lhcb2) attached. We also find evidence for the existence of both PS I and PS II centres in the extreme stroma (probably centres being synthesised or repaired), that lack all LHC proteins. © 1997 Published by Elsevier Science B.V.

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1. Introduction

In the photosynthetic light reaction, energy from sunlight is converted to reducing power and a proton gradient over the thylakoid membrane, which is subsequently used to produce carbohydrates in the photosynthetic dark reactions. According to the Z-scheme [1], two photosystems (PS I and PS II) are required to move electrons from water to NADPH. The two photosystems of higher plants are not, however, homogenous and over the years more and more evidence has accumulated indicating that different populations of PS I and PS II centres exist, which are not evenly distributed in the thylakoid membrane. Most notably, PS II is enriched in the appressed regions (the grana stacks) and PS I in the non-appressed, stroma-exposed regions of the thylakoid [2]; the stroma lamellae, the grana margins and the end membranes.

Numerous studies have investigated the heterogeneity among PS II centres (reviewed in [3,4]), and different PS II centres clearly vary in antenna size; the PS II in the grana, PS IIα, has a much larger

Abbreviations: Chl, chlorophyll; ECL, enhanced chemoluminescence; PS I, Photosystem I; PS II, Photosystem II; LHC, light-harvesting chlorophyll *a*/*b*-binding protein.

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antenna than PS IIβ, which is preferentially located in the stroma lamellae [5]. Different PS II centres also vary in activity, ability to reduce Q_B, susceptibility to photoinhibition, and other photo- and biochemical characteristics. In moderately high light, the D1 protein of the PS II reaction centre is also continuously turned over. This process has been modelled in the so-called PS II repair cycle [5] and, furthermore, PS II exists sometimes as a dimer [6–8]. Heterogeneity of PS I has also been demonstrated and it seems clear that its antenna size is variable [9,10]. It has also been suggested that only PS I in the appressed regions participates in the linear electron transport from water to NADPH, whereas PS I centres in the stroma lamellae are simply devoted to cyclic electron transport and, thereby, production of a proton gradient [11].

Some of the heterogeneic qualities mentioned above are linked to variations in the photosynthetic light antennae. In higher plants, these antennae are composed of Chl *a/b*-carotene binding proteins, close to the reaction centre, and the proximal antenna, consisting of the light-harvesting chlorophyll *a/b*-binding (LHC) proteins. These LHC bind the vast majority of the photosynthetic pigments; Chl *a*, Chl *b* and the xanthophylls lutein, neoxanthin, violaxanthin, antheraxanthin, and zeaxanthin [12]. They do not simply capture light energy and deliver it into the reaction centre; the antenna function is highly regulated and if excitation energy is present in excess, surplus energy is dissipated as heat to avoid formation of harmful radicals (reviewed in [13]). There are ten distinct types of LHC proteins encoded by the different *Lhc* genes [12] which have been highly conserved during at least 345 million years of evolution [14]. This means that all ten must have specific functions within the light-harvesting apparatus, since random genetic drift would have eliminated some of the corresponding genes without positive selective pressure for their preservation. However, not very much is known about the specific functions of the different LHC proteins. The antenna proteins of PS I are encoded by the *Lhca1-4* genes. *Lhcb1* and *Lhcb2* encode the most abundant proteins of the LHC II trimers (which can associate with either PS I and PS II), and *Lhcb3-6* encodes PS II-specific antenna proteins [15]. Although not confirmed by firm experimental evidence, it seems as if three of the PS II-specific proteins (Lhcb4, 5 and 6, also named CP29, CP26 and CP24) are monomeric proteins [12] whereas the fourth (Lhcb3) is found in trimers together with Lhcb1 (Jackowski and Jansson, unpublished results). The Lhca proteins seem to be organised as dimers [16] and Lhcb1 and Lhcb2, as mentioned above, as trimers.

To investigate the functions of the different LHC proteins, a complete set of monospecific antibodies has been raised against the different LHC proteins. These antibodies have proved useful in screening barley mutants lacking antenna polypeptides [17,18]. In this study, we have used the antibodies to study the antenna protein composition of PS I and PS II in the different thylakoid compartments. In contrast to previous studies addressing the same heterogenic qualities (e.g., [19,20]) we have used specific antibodies against all LHC proteins, and not prepared PS I or PS II particles with the use of detergents. Although pure PS I and PS II particles can be very useful for some studies, the necessary addition of detergents can lead to uncontrolled subunit dissociation. We have, instead, used the non-detergent methods developed to separate different thylakoid compartments in the form of membrane vesicles [21], and analysed the protein content in each of these fractions. The following sub-populations were isolated from the thylakoid membranes: grana core regions (BS), grana margins, and two fractions from the stroma lamellae-'T3' [22] and 'Y100'. Y100 is the most extremely 'stromal' fraction, having significantly less PS II and more PS I than the T3 fraction (see below). The T3 fraction probably also contains vesicles originating from the

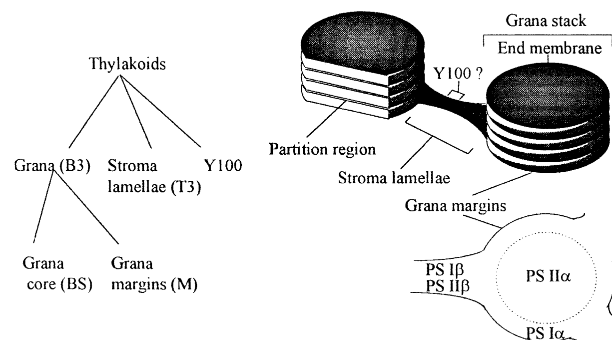

Fig. 1. Left: schematic representation of the fractionation procedure. Right: structure of the thylakoid membrane showing the different domains, and the origin of the thylakoid fractions.

end membranes [23]. The preparation scheme and origin of the various fractions are outlined in Fig. 1. It should be pointed out that both the grana core and grana margin fractions are derived from the same initial vesicle population (B3), which are inside-out vesicles formed by the appressed regions of the thylakoid after sonication.

In this report, we show that PS I and PS II in the different thylakoid compartments have differences in their antenna polypeptides, and we speculate about the properties of the various types of reaction centres.

2. Material and methods

2.1. Preparation of chloroplast thylakoids and subthylakoid membrane fractions

Spinach (*Spinacia oleracia* L.) was grown at 20°C with a light period of 12 h and incident light intensity of 300 $\mu E \cdot m^{-2} \cdot s^{-1}$. The leaves were dark-adapted for 24 h before harvesting. Chloroplasts were isolated in 50 mM sodium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 300 mM sucrose and were osmotically broken in 5 mM $MgCl_2$ as described in [10]. The thylakoids were washed twice in 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, 1 mM $MgCl_2$, 100 mM sucrose. They were then resuspended in the latter medium to a chlorophyll concentration of about 4 mg/ml.

2 g of the thylakoid suspension was added to 9.66 g of a polymer mixture to give the following final concentrations: 5.7% (w/w) Dextran, 5.7% (w/w) PEG 4000, 10 mM sodium phosphate buffer (pH 7.4), 20 mM sucrose, 3 mM NaCl and 1 mM $MgCl_2$. Dextran T500 was obtained from Pharmacia (Uppsala, Sweden). Poly(ethylene glycol) 4000 (Carbowax PEG 3350) was supplied by Union carbide (New York, NY). The thylakoids were subjected to sonication using a Vibra-cell ultrasonic processor Model VC 500 (Sonics and Materials, Danbury, CT, USA) equipped with a 1/2-inch horn. The sample system was sonicated in six 30-s bursts, alternating with 60-s resting intervals, in a cylindrical aluminium tube immersed in ice and water. The ultrasonic intensity output setting was 7, with 20% duty pulses. This sonication procedure essentially breaks apart the two main compartments, grana and stroma lamellae [10].

After sonication, 6.43 g of pure lower phase and 5 g of pure top phase from an aqueous two-phase system composed of 5.7% (w/w) Dextran T500, 5.7% (w/w) PEG 4000, 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl and 20 mM sucrose were added to the sonicated sample system. The sample system was mixed at 4°C and centrifuged at 2000 *g* for 3 min to separate the phases. The upper and lower phases, enriched in thylakoid fragments from stroma and grana lamellae, respectively, were separated and washed twice by 10 ml of fresh lower and upper phase, respectively. The final upper phase fraction, after being washed twice by fresh lower phase, constituted the stromal lamella fraction (T3) and the final lower phase fraction, after two washes by fresh upper phase, constituted the grana lamella fraction (B3).

The grana cores and grana margins were separated by sonicating the grana fraction further (12×30 s), and the resulting fragments were partitioned, as above, to yield new upper and lower phase fractions representing the grana margins and grana cores, respectively [22]. The upper and lower phases containing the sub-thylakoid vesicles were diluted three times with 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl, 100 mM sucrose, and they were centrifuged at 100,000 *g* for 90 min. The pellets were resuspended in the dilution medium to a chlorophyll concentration of approximately 1 mg/ml.

For preparation of Y100 particles, isolated thylakoids (2 mg chlorophyll/ml) in a buffer solution composed of 10 mM sodium phosphate (pH 7.4), 5 mM NaCl, 5 mM $MgCl_2$ and 100 mM sucrose were disintegrated in a Yeda press at a nitrogen pressure of 10 MPa [24]. The Yeda press homogenate was diluted $5\times$ with $MgCl_2$-free buffer and was centrifuged at $40\,000 \times g$ for 30 min. The membrane vesicles in the supernatant were sedimented by centrifugation at $100\,000 \times g$ for 45 min to yield the Y100 fraction.

2.2. PS I isolation

PS Iα was isolated from grana and PS Iβ from stroma lamellae (B3 and T3, respectively) basically as described by Williams et al. for thylakoids [25].

0.5 mg chlorophyll/ml of the B3 vesicle population was incubated in 25 mM Tris-HCl (pH 8.3) in Triton X-100 concentrations ranging from 0.48 to 0.56% (w/v). The mixture was incubated at room temperature for 45 min, constantly stirred. PS I particles were collected by centrifugation at $30\,000 \times g$ for 30 min at 4°C. The pellets were resuspended in 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl and 100 mM sucrose.

2.3. SDS-PAGE and densiometric scanning

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was carried out according to Laemmli [26], with linear gradients of 12–22.5% acrylamide in the presence of 4 M urea. Gels were stained with Coomassie Brilliant Blue.

2.4. Quantitative immunoblotting

Thylakoid membrane fractions corresponding to 1–3 μg of Chl were solubilised in 36 mM DTT, 3.6% SDS, 0.108 M Tris pH 6.8 at 37°C for 10 min. The samples were loaded on 1 mm Mini-protean gels (Bio-Rad) prepared according to Fling and Gregerson [27], except that homogenous 15% gels were used. After electrophoresis (at 200 V for 1 h), proteins were transferred to 0.2 μm Nitrocellulose membranes (Bio-Rad) in a semi-dry blotting apparatus (Pharmacia) using the discontinuous buffer system according to the vendors' recommendations. Membranes were air dried and blocked for 1–2 h in TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl) buffer supplied with 0.05% Tween-20 and 5% low-fat dried milk powder at 37°C. After washing in Wash buffer (TBS, 0.05% Tween-20), primary antibodies were added in Antibody buffer (TBS, 0.25% Triton X-100, 2% dried milk), and the membranes were incubated for 1–3 h at 37°C. After thorough washing, secondary antibody in Antibody buffer was added and the membranes were incubated for 1 h at room temperature. The membranes were washed four times in Wash buffer, once in deionized water, and the protein composition was analyed using an ECL (enhanced cheminoluminescence) detection kit (Amersham) according to the manufacturer's instructions. A Phosphor imager (GS-250 Molecular Imager™, Bio-Rad) with Chemiluminescent Imaging Screen was used to quantify the luminescence signals. Antibodies raised against PsaC [28], Lhca1, Lhca3 and Lhca4 [17], Lhca2 [29], Lhcb4 [30], Lhcb5 [31], Lhcb6 [17,31], cytochrome *b*-559 and PsbS [32] were used for detection. To assay Lhcb1, Lhcb2 and Lhcb3, new antibodies were raised with the same antigens used in [29] and [33].

3. Results

3.1. Polypeptide pattern of the thylakoid subdomains

The polypeptide pattern and the Chl *a/b*-ratio of the four different preparations are shown in Fig. 2. As noted earlier [22], the 33 kDa PsbO protein of the oxygen-evolving complex (OEC), LHC II and Chl b are most abundant in the grana core fraction, and are successively less abundant in the grana margins, the T3 and the Y100 fractions, whereas the opposite pattern is found for PS I and ATP synthase.

3.2. Quantitative immunoblotting

Quantification of membrane proteins is not a trivial task. Most quantitative immunological methods can not be used, since detergents must be present in

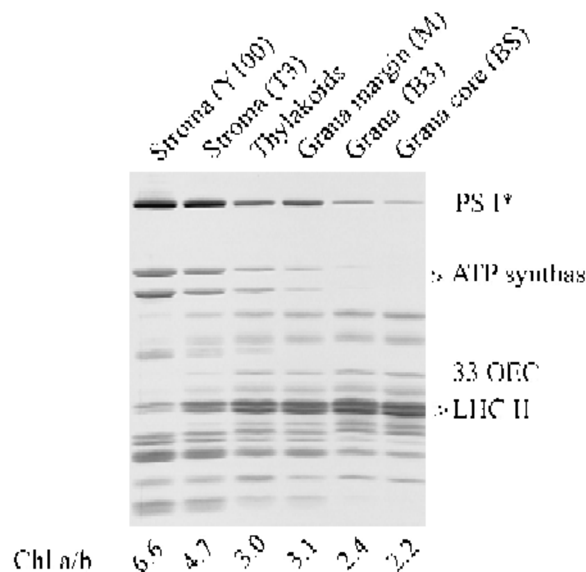

Fig. 2. SDS-PAGE in the presence of urea showing the polypeptide pattern in thylakoids and subthylakoid vesicles. Chl *a/b*-rations are given under each fraction.

the extracts to keep the highly hydrophobic proteins in a soluble form, and these detergents are likely to induce substantial systematic errors in the assay. To our knowledge, the most straightforward method is to perform quantitative immunoblotting with ECL detection, and analysis using a phosphor imager [34]. Since we have a complete set of monospecific antibodies raised against the LHC proteins, we exploited this experimental strategy. There are, however, many potential problems associated with this analysis. The final quantification by a phosphor imager is likely to be accurate over a wide range [34], but the strength of the signal from the luminescent products is not likely to be very reproducible. Inaccuracies in loading, incomplete transfer to the blotting membrane, non-linear binding to the membrane, and un-even binding of the primary or secondary antibodies, or the substrate for luminescence can all result in experimental errors. We tried to circumvent these problems by varying the amount of protein extract loaded, varying the blotting procedure (using semi-dry apparatus, or blotting in a tank), incubating with different amounts of primary and secondary antibody, varying the time from addition of the ECL substrate to phosphor imager detection, and varying the method of phosphor imager data analysis (using volume or profile analysis, different methods to draw the baseline, and Gaussian deconvolution of peak areas) but we were never able to get fully reproducible results. One problem was that the phosphor imager used was quite insensitive to the visible light produced by the ECL detection system, so the signal had to be quite strong to get a satisfactory signal-to-noise ratio in the phosphor imager, which meant that we could not load small amounts of protein onto the gel. In our hands, the best results were obtained when protein extracts containing 1–3 μg of Chl were loaded in each lane, separated by electrophoresis and then subjected to semi-dry blotting. Relatively little primary antibody was added (at a dilution of 1:1000 to 1:30 000, depending on antibody strength), and the secondary antibody was diluted 1:10 000. The membranes were incubated in the luminescent substrate solution and subjected to autoradiography, where we found that blots causing a strong, but not overexposed, signal after 30 s exposure on Cronex 4 film gave the best results. Such blots were re-incubated in ECL solution, and were loaded immediately into the phosphor imager dock, where the imaging screen was exposed for 2 h. We found that reliable background subtraction was easier to perform using profile analysis of the images, rather than volume analysis. Still, even on blots that looked normal on autoradiography film, we found differences of up to 20% in measurements of duplicate protein samples loaded next to each other on the same gel. We also compared our phosphor imager data with densiometric scans of the autoradiographs of the same gels, but densiometry was even less reproducible.

To obtain confident data, we repeated each blot numerous times. To make sure that we did not saturate any step in the analysis, we loaded two different dilutions of each sample on every gel, and only used data from blots where the signal from the diluted samples decreased proportionally to the dilution factor. Finally, we did not use gels where the autoradiography pattern looked aberrant. The data presented below are the mean values from at least three gels satisfying these criteria.

We should also point out that this analysis only gives us the relative stoichiometry (on a Chl basis) of the proteins in the different samples. The actual stoichiometry of the LHC proteins on a reaction centre basis has not yet been determined for any LHC protein [12], so we are only measuring the relative amount of each protein in the different thylakoid compartments.

3.3. PS I in grana core have a smaller complement of Lhca polypeptides

Immunoblots obtained using antibodies against PsaC, Lhca1, Lhca2, Lhca3 and Lhca4 are shown in Fig. 3. This figure also illustrates the specificity of the antibody collection. All antibodies strongly recognise a single protein band without background reactions, with the exception of Lhca1 which faintly detects a Lhcb polypeptide. The general pattern is, as expected, that these proteins are depleted in the grana core and enriched in the Y100 fraction, but there were significant differences between the polypeptides. The protein levels were quantified using ECL and a phosphor imager, and the results are shown in Table 1. Unexpectedly, we were not able to identify Lhca1 in the grana core fraction. To obtain the relative stoichiometry of the Lhca proteins per PS

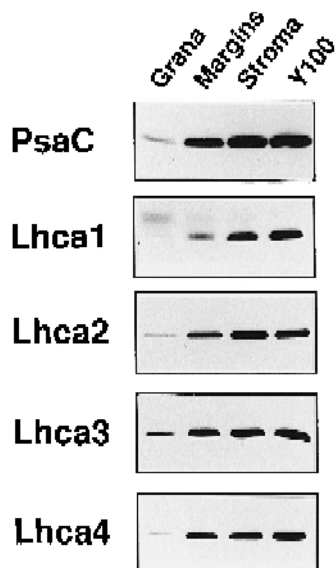

Fig. 3. Immunoblot analysis of the PS I polypeptide content in the subthylakoid vesicle populations.

I centre, we divided the value obtained from each protein with the value for PsaC in the same fraction. The resulting numbers were then normalised to obtain the highest relative stoichiometry in any fraction to 1 (Table 2). Evidently, Lhca2 and Lhca3 were present in approximately equal amounts per PsaC in all fractions, with the exception of Y100, where they were slightly depleted. As mentioned above, Lhca1 was absent in the grana core fraction, but was present in approximately equal amounts, on a PsaC basis, in the other fractions. Lhca4 was also depleted in the grana core fraction, where it was only about half as abundant as in the stroma lamellae.

Table 1
Quantitative immunoblotting of PS I and PS II polypeptides in the different thylakoid subdomains

	Grana core	Margins	Stroma	Y100
PsaC	0.15	0.53	0.78	1.00
Lhca1	0.00	0.55	0.80	1.00
Lhca2	0.18	0.67	0.89	1.00
Lhca3	0.17	0.65	0.99	1.00
Lhca4	0.09	0.54	0.91	1.00
b559	1.00	0.73	0.47	0.23
Lhcb1	1.00	0.66	0.31	0.17
Lhcb2	1.00	0.66	0.27	0.09
Lhcb3	1.00	0.64	0.40	0.13
Lhcb4	1.00	0.67	0.37	0.16
Lhcb5	1.00	0.75	0.47	0.13
Lhcb6	1.00	0.52	0.32	0.16
PsbS	1.00	0.59	0.35	0.11

The relative content is given on a Chl basis

Table 2
Relative amounts of the PS I and PS II polypeptides in the different thylakoid subdomains

	Grana core	Margins	Stroma	Y100
Lhca1	0.0	1.0	1.0	1.0
Lhca2	1.0	1.0	0.9	0.8
Lhca3	0.9	1.0	1.0	0.8
Lhca4	0.5	0.9	1.0	0.9
Lhcb1	1.0	0.9	0.7	0.8
Lhcb2	1.0	0.9	0.6	0.4
Lhcb3	1.0	0.9	0.9	0.6
Lhcb4	1.0	0.9	0.8	0.7
Lhcb5	1.0	1.0	1.0	0.6
Lhcb6	1.0	0.7	0.7	0.7
PsbS	1.0	0.8	0.7	0.5

Values are given on a reaction centre basis (see text)

3.4. Analysis of PS II polypeptides

Next, we performed the same analysis on the PS II antenna polypeptides (Fig. 4, Tables 1 and 2). We also included PsbS, a 22 kDa PS II protein of unknown function that is related to the LHC proteins, and also binds Chl *a*, Chl *b* and carotenoids [35]. The lower band in the Lhcb4 panel is Lhca2; the antibody used has a dual recognition. As noted before [36], the PsbS antibody also weakly recognises Lhcb4 (CP29) or Lhcb5 (CP26). The result obtained for the PS II proteins are more difficult to interpret. All Lhcb proteins decreased (per unit cytochrome *b*-559) from the grana core, through the margins to the stroma lamellae (Table 2). The two PS II proteins that exhibited the largest lateral heterogeneity are Lhcb2 and PsbS which, on a unit Chl basis, were roughly 10 times more abundant in the grana core than in Y100 fractions. On a reaction centre basis, Lhcb6 (CP24) seems to be under-represented in grana margins and stroma lamellae. This is somewhat uncertain, since we had more problems quantifying Lhcb6 than the other proteins, and in the following discussion we assume that the pattern of Lhcb6 abundance is similar to that of Lhcb4 and Lhcb5. The two antibodies used

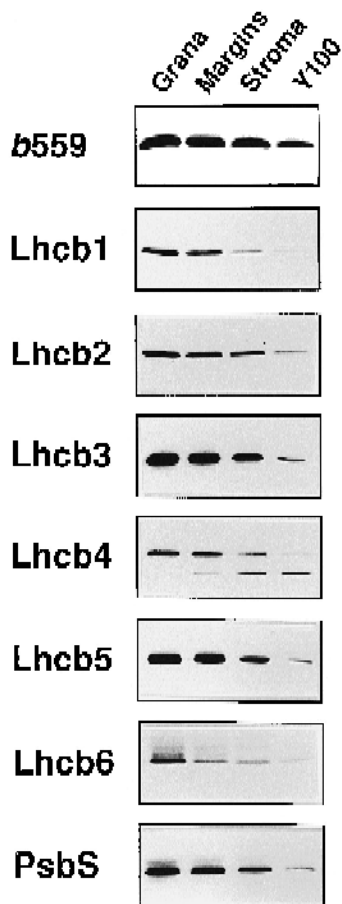

Fig. 4. Immunoblot analysis of the PS II polypeptide content in the subthylakoid vesicle populations.

gave inconsistent results and there was also a larger variation between experiments using these antibodies.

3.5. Lhcb1 and Lhcb2 is associated with PS Iα

When PS I preparations are performed in relatively low Triton X-100 concentrations, it is known that LHC II is not dissociated from the complex but is retained in the preparation [25,37]. However, the identity of the Lhcb polypeptides associated with PS I in vivo have not previously been carefully investigated. To address this, we analysed the Lhcb polypeptides which co-purified with PS I, as follows. PS Iα was prepared from B3 particles in three different Triton X-100 concentrations (0.50, 0.52 and 0.56%), and the resulting populations were analysed using the procedure described above. There was a small amount of PS II contamination in the PS I preparations, so we analysed the Lhcb and cytochrome *b*-559 content of the PS I preparations by quantitative immunoblotting, and compared the ratio between these polypeptides in the preparations to the ratios in the starting material (B3). Similarity in these ratios would indicate that the Lhcb protein content was due to PS II contamination in the preparation, whereas an elevated ratio would indicate that the surplus Lhcb1 was associated with PS Iα. The different Lhcb proteins showed three distinct patterns when analysed in this way. Lhcb1 and Lhcb2 were highly enriched in the PS Iα preparation, per unit cytochrome *b*-559; the 0.50% Triton X-100-preparation contained roughly six times more Lhcb/cytochrome *b*-559 than the starting material. However, in the preparation carried out in 0.56% Triton X-100, the Lhcb1 or Lhcb2/cytochrome *b*-559 ratios were close to the ones in the thylakoid, and the 0.52% preparation gave intermediate values. There was also a difference between the relative ratios of Lhcb1 and Lhcb2 in the different preparations. The Lhcb1/Lhcb2 ratio was lowest in the 0.50% Triton X-100 preparation, in which it was only a third of the ratio found in the 0.56% Triton preparation. This shows that LHC II trimers containing Lhcb2 were preferentially solubilised when the Triton X-100 concentration increased.

In contrast, very little Lhcb4 were found in any of the PS Iα preparations (Fig. 5). The lower band in

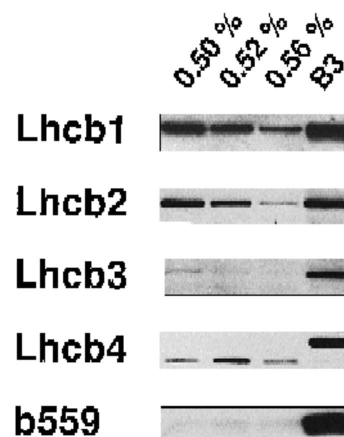

Fig. 5. Immunoblot analysis of the Lhcb protein content in different PS Iα preparations. PS Iα prepared in the presence of 0.50%, 0.52% or 0.56% Triton X-100, corresponding to 2 μg of Chl, or a B3 vesicle preparation, corresponding to 4 μg of Chl, was loaded in each lane.

the blot represents Lhca2 (see above). Lhcb5 and Lhcb6 showed a similar appearance (data not shown). The ratios of Lhcb4, Lhcb5 and Lhcb6/cytochrome *b*-559, measured with quantitative innmunoblotting, in the PS Iα preparations were similar or lower than in B3 showing that none of these proteins associated with PS I. A third pattern of occurrence was demonstrated by Lhcb3 (Fig. 5). Although Lhcb3 was not enriched per unit cytochrome *b*-559 in any of the preparations, the amount in the 0.50% Triton X-100 preparation was much higher than in the 0.52 and 0.56% preparations. This could be explained in two ways: (1) PS Iα could bind a minor fraction of trimers containing Lhcb3 that is easily solubilised; or (2) since Lhcb3 is depleted on a per cytochrome *b*-559-basis in the 0.52 and 0.56% preparations, it is possible that these trimers dissociated from the contaminating PS II complexes in the higher Triton X-100 concentrations. Taken together, this shows that Lhcb1 and Lhcb2, the components of the mobile LHC II trimers, were associated with PS Iα in significant amounts. Trimers containing Lhcb3 may also have been associated with PS Iα to a small extent, but the monomeric Lhcb4, Lhhcb5 and Lhcb6 proteins were not.

The actual number of LHC II trimers bound to each PS Iα could not be determined by this method, but the Chl *a/b*-ratios of the preparations can be used to get a rough estimate. Unfortunately, the stoichiometry of the pigments to the photosynthetic proteins are still unknown in detail. Even in LHC II (Lhcb1/Lhcb2), where the structure has been solved at atomic resolution [38], the number has not been unambiguously determined. The crystallised protein contains seven Chl *a*, five Chl *b* and two lutein molecules but in vivo, neoxanthin, violaxanthin and a few additional Chl molecules are also likely to be bound to the protein [39], as reviewed in [12]. The PS I-LHC I complex binds roughly 200 Chl molecules, and has a Chl *a/b* ratio of ≈ 7, though some recent estimates of the ratio are 6.1 [40], 7.2 [41] and 7.7 [42]. Using these figures, the PS I/LHC I complex with one LHC trimer (assumed to contain 24 Chl *a* and 18 Chl *b*) attached would have a Chl *a/b*-ratio of 4.6, with two trimers attached a ratio of 3.6, with three trimers 3.1 and with four trimers 2.8. If these numbers are compared to the measured Chl *a/b* ratios (2.6, 4.0 and 4.8 in the prepartions carried out in 0.50, 0.52 and 0.56% Triton X-100, respectively), PS Iα prepared in the presence of 0.50% Triton X-100 most likely contains four trimers, whereas the 0.56% sample contains only one.

3.6. PS Iβ also binds Lhcb1 and Lhcb2

We also wanted to determine whether the LHC II trimers in stroma lamellae are associated with PS I, PS II or both. To do this, we prepared PS Iβ from T3 vesicles in the presence of 0.48% Triton X-100. This concentration is lower than that normally used for preparation of the PS I-LHC II complex and we believe that PS I prepared in this way should retain most, if not all, of the LHC II initially bound to it. By comparing the amount of LHC II found in this preparation with the amount found in the starting material (T3), we derived an estimate of the fraction of Lhcb1 and Lhcb2 bound to PS I in stroma lamellae. Since both PS Iβ and PS IIβ are present in T3, we have argued that if the LHC II in T3 is primarily associated with PS I, it would become enriched in the PS I preparation compared to T3, but if PS IIβ has relatively more LHC II, it would be depleted. The result of the analysis is shown in Fig. 6. On a per unit Chl basis, both Lhcb1 and Lhcb2 were approximately equally abundant in T3 and PS Iβ prepared in 0.48% Triton X-100. We believe that this indicates that both Lhcb1 and Lhcb2 are approximately evenly distributed between PS I and PS II in stroma lamellae. When the PS I was prepared in the presence of 0.54% Triton X-100, the amount of Lhcb1 and Lhcb2 was, as found for granal PS I, smaller. As in PS Iα, Lhcb2 became more depleted than Lhcb1, indicating that trimers containing Lhcb2 were more prone to dissoci-

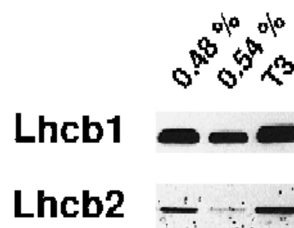

Fig. 6. Immunoblot analysis of the Lhcb1 and Lhcb2 in different PS Iβ preparations. PS Iβ prepared in the presence of 0.48% or 0.54% Triton X-100 or a T3 vesicle prepartion, corresponding to 1.5 μg of Chl was loaded in each lane.

ation from PS Iβ. The Chl *a/b* ratios (5.2 and 6.4 in 0.48 and 0.54% Triton X-100) indicated that the PS Iβ prepared in 0.48% Triton contained one LHC II trimer. In 0.54% Triton X-100, most of the PS I centres have lost this trimer.

4. Discussion

In this study, we have attempted to quantify the antenna proteins in PS I and PS II in the different domains of the thylakoid membrane. Although quantitative immunoblotting, at least in our hands, is not a highly accurate method, we have observed some unambiguous but unexpected differences between the different types of reaction centres. The results are, however, dependent on correct quantification of PS I and PS II centres. We are confident that the level of PsaC is an adequate measurement of the numbers of PS I reaction centres. PsaC binds the FeS-centres F_A and F_B, which are components of the PS I electron transport chain, and is thus indispensable for PS I function [43]. It is also an extrinsic protein bound to the PsaA and PsaB proteins at the stromal side, so PsaC proteins unattached to PS I reaction centres should not be obtained in our preparations. The levels of PsaC in the different fractions also closely reflect the numbers of PS I reaction centres measured by photooxidation of P700 ([44]; Stefánsson, unpublished results), as does the amount of the PsaE protein in the preparations (data not shown).

We are not equally convinced that our cytochrome *b*-559-measurements truly reflect the number of PS II reaction centres. Firstly, a PS II reaction centre is an ambiguous term. There is a large, well-established degree of heterogeneity among PS II centres [4], not only in terms of antenna proteins, the subject of our analysis, but also in terms of ability to reduce Q_B, fluorescence induction kinetics, and other photo- and bio- chemical characteristics, which may or may not be interdependent. Moreover, it has not, to our knowledge, been proven that the ratio of cytochrome *b*-559/RC is constant during all conditions and in all thylakoid compartments. The other components of the PS II reaction centre complex, the D1 and D2 proteins are, however, even less reliable as standards for PS II, since they, at least D1, are not found in PS II centres under repair. Thus, if we want to relate our data to ‘physical’ (rather than ‘active’) reaction centres, cytochrome *b*-559 is probably the best basis.

When our data from quantitative immunoblotting is compared to other characteristics, such as activity measurements, some discrepancies are apparent. According to our data, stroma lamellae (T3) contain almost half as much cytochrome *b*-559 as the grana core (BS). PS II activity measurements by electron transfer from H_2O to PpBQ indicate that the difference is only 3–4-fold [37]. However, if the activity is measured from DPC to PpBQ, the discepancy between the ratios is smaller (Stefánsson, unpublished results) Also, densiometric scanning of stained gels of our preparations indicate that the difference in the amount of the 33 kDa PsbO protein is approximately threefold. We might have overestimated the amount of PS II centres in stroma lamellae and Y100 but we consider this overestimation to be less that 20% for the stromal lamella (T3) fraction. If not, there would be a surplus of most Lhcb proteins/PS II in stroma lamellae compared to grana core, and it is unlikely that more CP29, CP26 and CP24 associate with each PS II centre in the stroma lamellae.

4.1. PS I heterogeneity

PS I centres are heterogeneous with respect to antenna size; PS Iα in grana core has approximately 40% larger antenna than PS Iβ in stroma lamellae [37]. Most of the granal PS Iα is found at the margin but there is also some PS Iα in the grana core fraction. Considering this, we were surprised to find that Lhca1 was absent, and Lhca4 severely depleted, in the grana core fraction. This has to be compensated by the presence of large amounts of Lhcb-proteins associating with PS I in the grana core. We have also shown that, among the Lhcb proteins, Lhcb1 and Lhcb2 are found in PS I preparations made in the presence of Triton X-100, but the monomeric Lhcb4, Lhcb5 and Lhcb6 proteins (CP29, CP26 and CP24, respectively) are not. Our results are not conclusive concerning trimers containing Lhcb3, which may associate with PS Iα. Nevertheless, our data shows that the LHC II proteins found in these PS I preparations were not merely contaminating PS II proteins, but were true constituents of the PS I holocomplex.

Lhca1 and Lhca4 are components of the LHCI-730 complex, characterised by an abnormal 77 K fluores-

cence emission at 730 nm [40,45]. There are two possible, speculative reasons for their low abundance in the grana core. Firstly, it was previously thought that LHC II associated with PS I was docked to one of the LHC I proteins. This might not be true; a PS I preparation from maize bundle sheath cells lacking LHC I but containing LHC II has recently been described [46]. Although this could be restricted to bundle sheath cells, it nevertheless indicates that LHC II could dock directly to the reaction centre subunits PsaA and PsaB. This has also been confirmed by cross-linking studies (Jansson and Sarvari, unpublished). If so, it can be speculated that LHCI-730 has to be removed before the full complement of LHC II trimers that are supposed to associate to PS I in grana core can bind, because of sterical hindrance. The second explanation is related to the possible function of LHCI-730. Since the 77 K fluorescence emission from LHCI-730 is red-shifted compared to the PS I reaction centre absorption, LHCI-730 could perhaps act as an energy sink, protecting the PS I reaction centre from over-excitation. If this is true, one could speculate that PS I in the grana core may not need this sink, since it probably never experiences donor side limitation due to the large number of surrounding PS II centres and a highly reduced PC pool. There are, however, recent data indicating that LHCI-730 is not a sink, but is involved in transfer of excitation energy to the PS I reaction centre by up-Hill Förster transfer [47] making this explanation less likely.

Biochemical studies have previously indicated that Lhca1 and Lhca4 are intimately associated in the LHCI-730 complex. The data presented here do not corroborate this. Only Lhca4 is present in the grana core, there is no Lhca1. Thus Lhca4 can not be invariably associated with Lhca1. Studies of barley mutants have given similar results [18]. The Lhca proteins seem to appear normally as dimers [16], so it would be interesting to find out whether the measured stoichiometry of Lhca4 in grana core (0.5 of the proportion in stromal lamellae) means that Lhca1 in grana core exists alone, that every second PS I centre binds a Lhca1 dimer, or if Lhca1 is associated with some other Lhc protein.

We found, on a reaction centre basis, slightly lower abundance of Lhca proteins in Y100 than in T3. We cannot exclude the possibility that this represents an experimental error, but it also seems quite possible that newly synthesised PS I centres, in which LHCI has not yet been assembled, could be present in this fraction, which only represents 1–2% of the thylakoid membrane.

Taken together, our data indicate that PS Iβ in the stroma thylakoids has a full complement of Lhca polypeptides, presumably two of each type. In addition, trimers of Lhcb1/Lhcb2 should associate with PS Iβ in the stroma lamellae, a conclusion also corroborated by spectroscopic data [37]. Taking into consideration the Chl a/b-ratio of the PS Iβ-preparation, it seems most likely that the number of trimers attached to each PS Iβ centre is one. PS Iα might bind up to four LHC II trimers, and we have also evidence for two distinct forms of PS Iα, one present in grana margins associating with the four Lhca proteins and LHC II trimers, and another form, located more in the interior of the grana stacks, that is depleted in Lhca4 and lacks Lhca1. Trimers containing Lhcb2 seem to have a higher tendency to dissociate from PS I in higher detergent concentrations, and there also seems to be a fraction of PS I centres in the stroma lamellae in which Lhca proteins have not yet been assembled.

4.2. PS II heterogeneity

Substantial heterogeneity of PS II centres has been reported, but it has been difficult to develop a coherent model of PS II heterogeneity because of the complexity of the thylakoid membrane. PS II centres are continuously being turned over, repaired, and transported between different thylakoid compartments [5]. Of importance for this study is the heterogeneity reported for the antenna size; PS IIα having larger (210–250 Chl) and PS IIβ smaller (approx. 130 Chl) antennae. The PS IIβ population has been suggested to be a precursor form of PS IIα [5]. The antenna size of PS II in the grana margins and stroma lamellae is known to be of the β-sub-type whereas the antenna size of PS II in the grana core fraction is of the α-sub-type [22].

In this study, we have tried to describe this heterogeneity at the molecular level. The results on Lhcb protein heterogeneity are, however, more difficult to interpret than the Lhca data. Since most data indicate that the minor Lhcb proteins are present in a unit

stoichiometry on a reaction centre basis [12], we will assume that this is the case in the following discussion, although firm evidence is lacking. When the figures are given relative to cytochrome *b*-559, Lhcb5 (CP26) was equally abundant in all compartments except in Y100, where the level was lower. Since mutant studies have previously indicated that Lhcb5 is the last antenna protein to disappear when Chl is limiting [17], we believe that our data show that most PS II centres in all compartments associate with one Lhcb5, but that a significant fraction of the PS II centres in the Y100 fraction are newly synthesised centres in which antenna polypeptides have not yet been assembled, or perhaps they are centres under repair. The pattern for Lhcb3 (type 3 LHC II), Lhcb4 (CP29) and Lhcb6 (CP24) resembles that of Lhcb5, although the relative amounts in grana margins and stroma lamellae are slightly lower. Due to the inherent inaccuracy of the method, it is possible that the patterns are really the same, but the same tendency is seen in most of our blots, so it is possible that there could be a minute fraction of PS II centres in margins and stroma lamellae binding only Lhcb5, but not Lhcb3, Lhcb4 and Lhcb6.

We also assayed the lateral distribution of PsbS, a Chl *a*/*b*-binding protein that is related to the LHC proteins. It has recently been suggested that this protein exhibits an extreme lateral heterogeneity; being 54 times more abundant in grana membranes than in stroma lamellae [35]. We found that PsbS has a distribution similar to that of the other Lhcb proteins, in fact Lhcb2 was slightly (perhaps insignificantly) more depleted in the stromal fractions. We offer no explanation for this discrepancy, the antibodies used are the same and the thylakoid sub-fractionation methods are comparable to each other. We believe that our data indicate that PsbS is an essential component of PS II, consistent with its presence even in plants severely deficient in chlorophyll [17] and in etiolated plants [35].

PS IIβ contains around 130 Chl molecules. Since the number of Chl molecules bound to the ‘core complex’ (D1/D2/CP43/CP47) seems to be 37 [48], approx. 90 chlorophylls are bound to the LHC proteins of PS IIβ. Assuming that 14 Chl molecules associate with each monomeric LHC protein, 90 Chl molecules would correspond to 6–7 LHC monomers. This indicates that PS IIβ consists of one Lhcb4, one Lhcb5 and one Lhcb6 monomer with one LHC II trimer. Our data corroborates the hypothesis, suggested by Peter and Thornber [49], that Lhcb3, together with two Lhcb1 subunits, form the LHC II trimer most tightly bound to PS II (and presumably present in PS IIβ), PS IIα in the grana should also associate with up to 3–4 additional Lhcb1/Lhcb2 trimers, according to antenna size determinations [50]. In plants grown at very low light intensities this figure could be even higher [51]. It should also be mentioned that PS IIα in itself is heterogeneous in antenna size [52], the number of additional LHC II trimers attached could be anything between 1–4. Moreover, according to recent models, the two PS II centres in a PS II dimer share an antenna [7]. This means that if one of these PS II centres binds 3 trimers and the other 4, the average antenna size of the dimer will be 3.5, introducing an additional level of heterogeneity.

Taken together, our data seem to be consistent

Table 3
Proposed antenna protein stoichiometry of different types of PS I and PS II centres

	PS Iα	PS Iβ	PS I under synthesis	PS IIα	PS IIβ	PS II under synthesis
Lhca1	0	2	0	0	0	0
Lhca2	2	2	0	0	0	0
Lhca3	2	2	0	0	0	0
Lhca4	1	2	0	0	0	0
(Lhcb1/2) trimers	< 4	1	0	< 4	0	0
$(Lhcb1)_2(Lhcb3)$ trimer	0	0	0	1	1	0
Lhcb4	0	0	0	1	1	0
Lhcb5	0	0	0	1	1	0
Lhcb6	0	0	0	1	1	0
PsbS	0	0	0	1	1	0

with a model with more than three different populations of PS II in terms of antenna polypeptides, as follows. Firstly, in grana, there is PS IIα-which includes the 'inner' antenna consisting of Lhcb4, Lhcb5, an Lhcb6, and up to five LHC II trimers. Secondly, there is PS IIβ, which binds one trimer consisting of one Lhcb3 and two Lhcb1 subunits, is probably monomeric, and is preferentially located in grana margins and stroma lamellae. There seems also to be a minor pool of PS II centres, enriched in the Y100 preparation, that do not associate with any Lhcb proteins at all, and most probably constitute centres under synthesis or repair.

To conclude, we have investigated the antenna protein composition of PS I and PS II in the thylakoid subdomains. We have found evidence (summarized in Table 3) that both PS I and PS II are present in at least three different forms in terms of antenna polypeptides. It is, however, clear that much remains to be learned about the structure and function of different types of PS I and PS II centres and their distribution in the different regions of the thylakoid membrane

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