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Distribution of Thylakoid Proteins between Stromal and Granal Lamellae in Spirodela[1]

Dual Location of Photosystem II Components

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ABSTRACT

We have quantified the lateral distribution of 12 thylakoid proteins of *Spirodela oligorrhiza* by immunoblot analysis of detergent-derived granal and stromal lamellae. The immunological, ultrastructural, cytochemical, and biophysical measurements each indicated the expected overall separation of photosystem II (PSII) and photosystem I (PSI) components; however, certain proteins were not completely localized to one lamellar fraction. The apoproteins of the light harvesting chlorophyll *a/b* complex, subunit 1 of PSI and the components of the PSII reaction center (the 32 kilodalton, D2, and cytochrome b_{559} proteins) were dually located between granal and stromal lamellae. Proteins associated exclusively with one of the membrane types were: in granal lamellae, the 43 and 51 kilodalton PSII proteins, and in stromal lamellae, the α and β subunits of the proton ATPase.

The photosynthetic membranes of chloroplasts are comprised of discrete multienzyme complexes—PSII, Cyt b_6-*f*, PSI, ferredoxin-NADP reductase, proton ATPase—which together with plastoquinone and plastocyanin operate in series to harvest light energy, catalyze the primary charge separations, and produce ATP and NADPH (27). Biochemical and morphological data indicate that these complexes are distinctly segregated on thylakoids such that granal lamellae are enriched in PSII proteins, while PSI and the proton ATPase are primarily localized in stromal lamellae (27, 28). Immunocytochemical studies (31) have corroborated this view of the steady state lateral distribution of thylakoid protein complexes. However, other reports suggest the existence of dynamic changes in various protein components of PSII. For instance, phosphorylation of the LHCP[4] results in the separation of a 'mobile' LHCP from granal lamellae and its relocation to stromal lamellae (28). Also, the 32 kD PSII reaction center protein has been shown to be synthesized, processed, and integrated within the stromal lamellae and then translocated to the granal lamellae (18, 19).

In this paper we have determined the relative distribution of 12 thylakoid proteins in granal and stromal lamellae using immunological techniques and a double detergent fractionation method (15) that yields highly resolved granal and stromal lamellae from minimal amounts of plant tissue. Based on their distribution, thylakoid proteins can be classified into two types. One class constitutes those proteins that undergo lateral diffusion within the thylakoids and show dual location. The second class comprise proteins that do not appear to translocate and are associated exclusively with one of the lamellar types.

MATERIAL AND METHODS

Plant Material

Axenic cultures of *Spirodela oligorrhiza* were grown phototrophically (15–20 μmol m^{-2} s^{-1}, 400–700 nm light, 25°C) for 10 to 15 d in mineral medium containing 0.5% sucrose as previously described (25).

Membrane Isolation and Fractionation

Spirodela plants were washed several times with distilled water prior to grinding and isolation of thylakoids (15). Whole thylakoids (T) were fractionated into granal (G) and stromal (S) lamellae by extraction with digitonin and Triton X-100 and by differential centrifugation (15). Final pellets were resuspended at 1 to 2 mg Chl/mL in 0.4 M sorbitol, 10 mM $MgCl_2$, 10 mM NaCl, 0.1 M Tricine-NaOH (pH 7.8). In certain experiments, whole thylakoids were fractionated into highly active, O_2-evolving granal lamellae using only Triton X-100

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[4] Abbreviations: LHCP, light harvesting Chl *a/b* apoprotein; CPI, Chl-protein complex of PSI; CPII, Chl-protein complex of PSII; G, granal; LDS, lithium dodecyl sulfate; S, stromal; T, thylakoids; TMF-2, oxygen-evolving, Triton X-100-prepared PSII membrane fragments.

(24). These latter preparations are referred to as TMF-2. All procedures were carried out at 4°C in dim light.

The yields on a total Chl basis relative to whole thylakoids were: S = 0.06 to 0.10; G = 0.25 to 0.35; TMF-2 = 0.40 to 0.45.

Rate Measurements of Photosynthetic Oxygen Evolution

O_2 evolution was measured polarographically in saturating, filtered light (24). Reaction mixtures previously optimized for assay of whole thylakoids (7) and TMF-2 (24) were employed. Granal and stromal lamellae were assayed in the reaction mixture used for TMF-2. All rate measurements were carried out with freshly isolated membrane preparations.

Polyacrylamide Gel Electrophoresis and Protein Blotting Procedures

Chl-protein complexes were resolved by LDS-PAGE (11). Polypeptides associated with the various membrane fractions were analyzed on SDS/10 to 20% polyacrylamide gradient gels (17). All membrane fractions were solubilized by addition of sample application buffer (17) to a final SDS/Chl ratio (wt/wt) of 160 and incubation at 25°C for 1 h. Samples were vortexed 3 to 4 times during solubilization and immediately prior to loading on the gel. Following SDS-PAGE, proteins were transferred electrophoretically onto nitrocellulose paper (0.1 μ, Schleicher and Schuell[5]) using a standard apparatus (Hoefer). Transfer buffer consisted of 25 mM Tris, 0.192 M glycine, 0.02% SDS, and 20% methanol. A voltage of 40 to 50 was maintained throughout the transfer (12–16 h, 20°C). The nitrocellulose paper containing the transferred proteins was incubated for 2 h with phosphate-buffered saline containing 1% milk powder (Carnation) and 0.5% bovine serum albumin. Blots were then washed in phosphate-buffered saline containing 0.05% Tween-20 and incubated for 2 h with the indicated antibody. Each primary antibody tested (as described in figure legends) was diluted in phosphate-buffered saline containing 0.1% milk powder and 0.05% bovine serum albumin. Each blot was subsequently washed as above before incubation for 2 h with either goat-anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) using 4-chloro-1-naphthol as the chromogenic substrate or ^{125}I-Protein A (New England Nuclear). All manipulations of blots were carried out at room temperature with mild shaking. Prestained markers (Bethesda Research Laboratories) and ^{14}C-methylated mol wt standards (Amersham) were electrophoresed alongside membrane samples for subsequent realignment of the immunodecorated blots and original nonblotted Coomassie stained gels.

Electron Microscopy and Cytochemical Assay of PSI Activity

Tissue preparation for ultrastructural examination and cytochemical localization of PSI activity was modified from previously published procedures (32). Intact plants as well as isolated membrane preparations were fixed in a drop of 3% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4), and cut into 2 to 3 mm segments. All tissues were then transferred to darkness for subsequent processing. Following fixation for 1.5 h, the samples were washed and placed in an incubation medium consisting of 1 mg/mL diaminobenzidine in phosphate buffer. Photoactivity was initiated by illumination with fluorescent and incandescent lights. Control samples were kept in darkness. Following incubation for 1 h, all samples were returned to the dark, washed three times in Sorenson's buffer, and postfixed in 2% buffered osmium tetroxide for 2 h. The samples were then dehydrated in an ethanol series, and infiltrated with Spurr's low viscosity resin. All procedures were carried out at room temperature. Silver-gray sections of either intact plants or membrane preparations were cut with a diamond knife on a Sorvall MT 5000 Ultramicrotome and mounted on uncoated copper grids. The sections were stained with 2% aqueous uranyl acetate for 10 min and then with lead citrate for 5 min. Thin sections were viewed in a Hitachi H-500 transmission electron microscope operated at 75 kV.

Chl Determination

Chl *a*/*b* determinations were made following extraction of membrane fractions in 80% acetone (3).

RESULTS AND DISCUSSION

Electron Microscopic and Biochemical Analyses of Granal and Stromal Lamellae

The electron micrographs (Fig. 1) illustrate ultrastructural and cytochemical characteristics of the isolated granal and stromal lamellae as compared to chloroplasts of a leaf (frond) section. The chloroplasts in the frond section (Fig. 1A) display an extensive network of thylakoids organized into stacked-granal and unstacked-stromal lamellae surrounded by a soluble milieu. The cytochemical staining (32) seen in the unstacked-stromal lamellae and their extensions bordering the stacked-grana (see enlargement, Fig. 1B) indicates PSI activity in these regions. Such localization of PSI agrees with proposed models of the thylakoid structure derived from freeze fracture (27, 28) and immunocytochemical (31) studies.

The integrity of thylakoid structure is largely maintained in the isolated chloroplasts (Fig. 1C) following lysis and washing to remove soluble proteins. Detergent fractionation of the isolated chloroplasts and differential centrifugation yielded granal (Fig. 1D) and stromal (Fig. 1E) lamellar fractions composed of double membrane sheets *versus* single membrane vesicles, respectively. Contamination of one type of membrane with the other was no greater than 2% based on visual examination in the electron microscope. The physical characteristics (vesicles and double membrane sheets) of these fractions (Fig. 1, D and E) are similar to previously published observations of stromal and granal lamellae isolated by either detergent or mechanical shearing techniques (16, 26). The isolated stromal lamellar fraction stained heavily for PSI activity (*cf.* Fig. 1, E *versus* F). Very little PSI activity stain was associated with the grana fraction, which is consistent with observations *in situ* (Fig. 1B).

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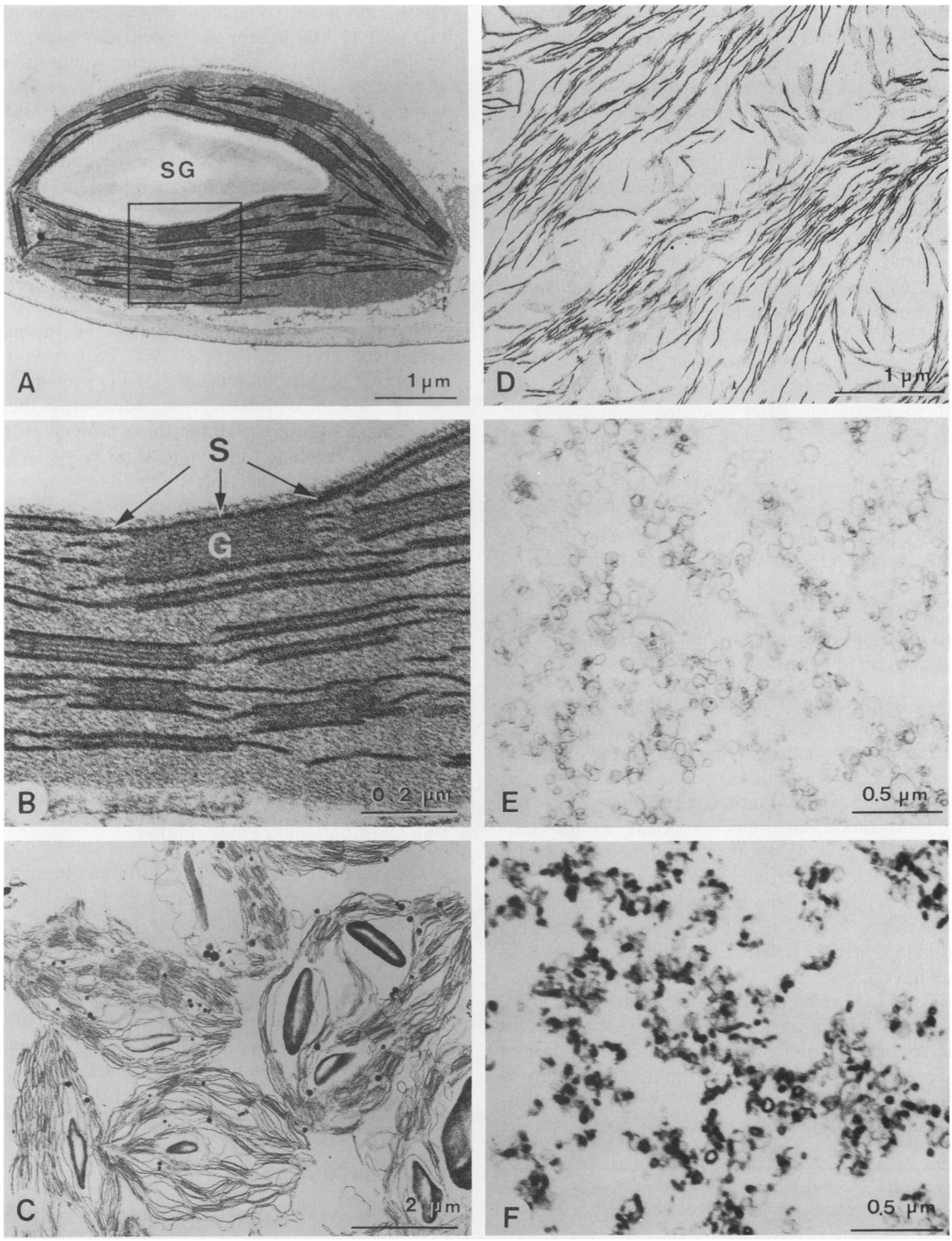

Figure 1. Ultrastructural and cytochemical characteristics of Spirodela chloroplast fractions. A, Chloroplast of frond section. Electron opaque regions of the thylakoids correspond to PSI activity as determined by cytochemical assay ("Materials and Methods"); B, boxed area of A; C, isolated chloroplasts after lysis and washing; D, isolated granal lamellae; E, isolated stromal lamellae; F, isolated stromal lamellae stained for PSI activity. Abbreviations: SG, starch grain; S, stromal lamellae; G, granal lamellae.

Other characteristics of the granal and stromal lamellar fractions are given in Table I. The stromal lamellae were highly enriched in Chl *a* and CPI complex. The Chl *a*/*b* and CPI/CPII ratios for the stromal and granal lamellae fractions (Table I) compare favorably with ratios obtained for similar fractions by other techniques (1, 2, 12). The recovery of PSII activity in the grana was 36% of that found in the whole thylakoids. Low recovery of PSII activity in granal lamellae derived by digitonin fractionation has been noted previously (5, 26). TMF-2 preparations (see "Materials and Methods"), on the other hand, were enriched in PSII activity. SDS-PAGE analyses of granal lamellae *versus* TMF-2 revealed equivalent polypeptide profiles except for a decreased abundance of the extrinsic 23 and 17 kD proteins of the water oxidizing complex (4) in the granal preparation. Thus, the lowered PSII activity in the granal lamellae is probably due to deleterious effects of digitonin on the water oxidizing complex *per se*. The data in Figure 1 and Table I show that a high degree of separation of PSI and PSII components exists in the stromal and granal lamellae fractions isolated by our procedure. We have previously shown that granal and stromal lamellae of Spirodela differ in lipid characteristics at the level of individual molecular species of mono- and digalactosyldiacylglycerol (23).

Distribution of Polypeptides in Stromal and Granal Lamellae Based on Immunoblot Analyses

A battery of 12 antibodies raised against thylakoid proteins was used to determine the steady state distribution of polypeptides between the stromal and granal lamellae of Spirodela chloroplasts. Many of these same antibodies previously served in studies identifying *in vitro*-synthesized spinach gene products and subunits of various multicomponent enzyme complexes in the thylakoid (14). Proteins associated with thylakoids, granal lamellae, and stromal lamellae were electrophoresed on SDS-polyacrylamide gels and transferred onto nitrocellulose sheets. Parallel sets of nitrocellulose strips were then immunodecorated each with one of the 12 antibodies as summarized in Figure 2.

The extrinsic 33 kD protein of the water oxidizing complex and the 32 kD and D2 proteins of the PSII reaction center showed similar electrophoretic mobilities. A false doublet or 'ghost,' which corresponded to the electrophoretic mobility of the extrinsic 33 kD protein, was repeatedly observed in immunoblots using D2 antibody as a probe. We found that the ghost band is absent in those granal preparations where the extrinsic 33 kD protein was specifically removed by $CaCl_2$ washing (29) and in stromal lamellae where the extrinsic 33 kD protein is in low abundance (Fig. 3). Conversely, the ghost band persists in NaCl-washed granal lamellae where only the 23 and 17 kD proteins are specifically released (24). Thus, while the D2 and extrinsic 33 kD polypeptides from Spirodela often have indistinguishable mobilities in the SDS-PAGE system used here, they can be readily identified by the strength of their association with the thylakoids.

Table I. *Comparison of Some Biochemical and Biophysical Characteristics of the Isolated Membrane Fractions*

Rates of photosynthetic oxygen evolution (V_{O_2}) and ratio of Chl *a* to Chl *b* were determined as described in "Materials and Methods." Three replicates of two experiments were analyzed. Similar patterns were consistently obtained. The ratio of CPI/CPII was estimated from densitometric scans of LDS-polyacrylamide gels (11). T, whole thylakoids; S, stromal lamellae; G, granal lamellae; TMF-2, oxygen-evolving, Triton X-100-extracted PSII membrane fragments (24).

Parameter	T	S	G	TMF-2
V_{O_2} (μmol·mg Chl^{-1} h^{-1})	252	0	91	309
Chl *a*/Chl *b*	2.80	6.20	2.00	1.90
CPI/CPII	0.46	6.32	0.05	0.04

PSII Reaction Center Polypeptides

A summary of the distribution of the various thylakoid proteins is given in Table II. The PSII reaction center is comprised of three proteins: the 32 kD protein, D2, and Cyt b_{559} (21). All three proteins are encoded within the chloroplast (6). The distribution of these three proteins in the stromal and granal lamellae was found to be 12 to 14% and 86 to 88%, respectively (Table II).

The 32 kD protein in synthesized as a 33.5 to 34.5 kD species and is posttranslationally processed to the mature 32 kD form exclusively on the stromal lamellae (18, 25). Following processing, the mature protein translocates to the grana (8, 19). The equivalent relative abundances of the three PSII reaction center proteins between stromal and granal lamellae suggest that D2 and Cyt b_{559} also translocate from stromal lamellae during their integration into grana. Preliminary results support such an intramembrane translocation of the D2 protein (8); thus, the PSII reaction center complex may be assembled on stromal lamellae and then translocated as a unit to grana.

PSII Chl-Containing Polypeptides

Using monoclonal antibodies (10), an abundance of 19 to 23% for two different denatured 25 to 26 kD LHCPs was found in the stromal lamellae, *versus* 77 to 81% in the granal lamellae (LH1 and LH2; Fig. 2; Table II). The major denatured LHCP (LH1), once imported into the chloroplast, is directly integrated within the granal lamellae (9). However, this protein is known to disengage from the granal lamellae upon phosphorylation (28). Detection of significant amounts of denatured LHCP in the stromal lamellae is consistent with the contention that the mobile form of LHCP might migrate from the granal to stromal lamellae.

Other PSII Polypeptides

In contrast to the dual lamellar location of LHCP and PSII reaction center proteins, the 43 and 51 kD PSII Chl proteins were found almost exclusively in the granal lamellae (Fig. 2; Table II). These proteins were earlier thought to be reaction center proteins; however, they are not integral components of the PSII reaction center core and their removal does not impair primary charge separation at PSII (18, 21, 30). The results in Figure 2 suggest to us that the 43/51-kD Chl proteins are immobile and may serve as anchors for the integration and functional assembly of the 32 kD/D2/Cyt b_{559} PSII reaction center proteins in the grana. In this context, it is known that PSII reaction centers located in the stromal la-

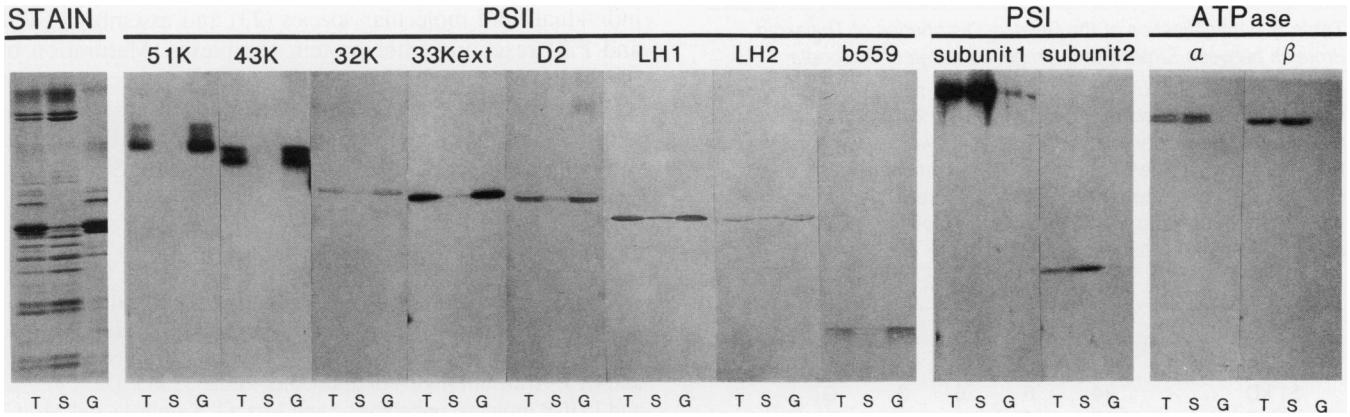

Figure 2. Immunological identification and localization of the major thylakoid proteins in Spirodela. Whole thylakoids (T), granal (G), and stromal (S) lamellae were resolved by SDS-PAGE and blotted onto nitrocellulose paper as described in "Materials and Methods." Blots were immunodecorated with antibodies specific for individual proteins. Gel lanes were loaded on the basis of equivalent total Chl in amounts (2 μg Chl) predetermined to give linearity with regard to reaction with the various antibodies. A control portion of each gel was stained with Coomassie R-250 instead of blotting. Abbreviations are described in the text.

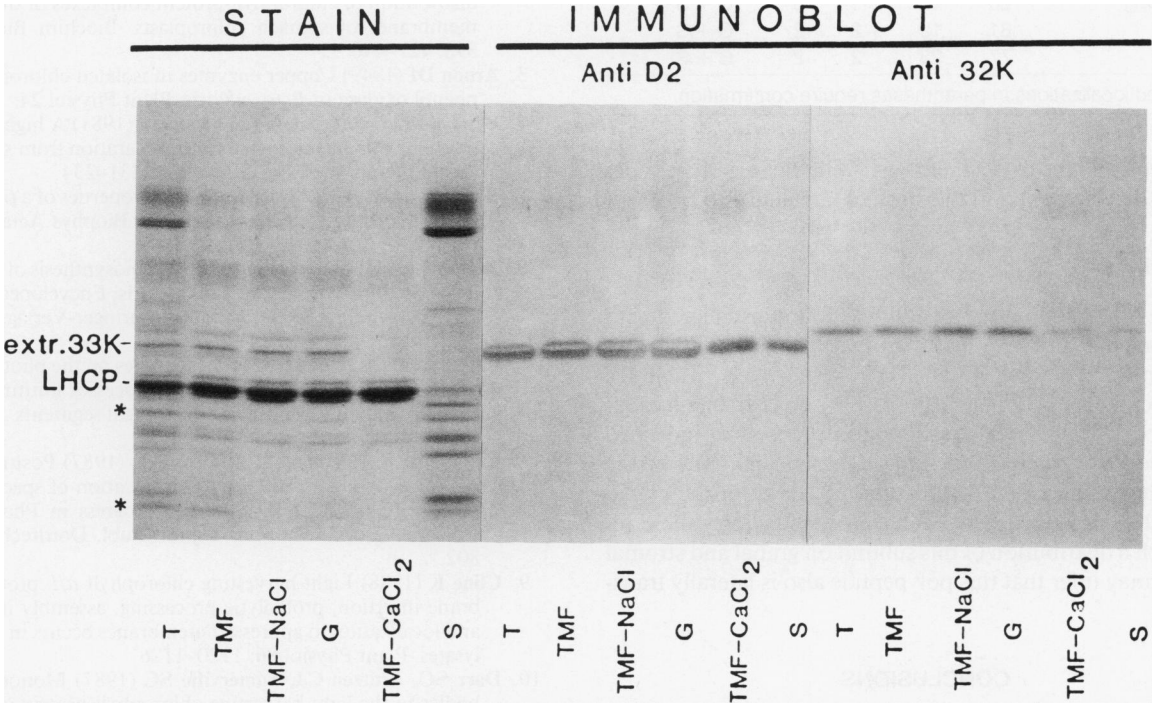

Figure 3. Relative electrophoretic mobilities of the 33 kD and D2 PSII reaction center proteins as compared to the 33 kD extrinsic polypeptide of the water-oxidation complex. Proteins of the indicated membrane fractions were resolved by SDS-PAGE and blotted onto nitrocellulose. Blots were reacted with either anti-D2 (Anti D2) or anti-32 kD (Anti 32K) antibodies and compared to a nonblotted, Coomassie stained section of the gel. TMF-NaCl and TMF-$CaCl_2$ refer to TMF-2 ("Materials and Methods") membranes which were either washed with NaCl (24) for specific removal of only the 23 and 17 kD extrinsic polypeptides, or with $CaCl_2$ (29) for removal of 33, 23, and 17 kD extrinsic polypeptides of the water-oxidizing complex. Other abbreviations are as described in Table I legend.

mellae are capable of undergoing primary charge separation and reducing exogenously added quinones (20). Although stromal lamellae were shown not to bind the herbicide azidoatrazine (33), they possibly do retain the herbicide binding site, as suggested by the reports of stoichiometry of total Chl/atrazine-binding sites (13).

The 33 kD extrinsic polypeptide of the oxygen evolving complex (Fig. 2A; Table II) is a granal lamellae protein which also exhibited a fractional presence on stromal lamellae; however, the degree of variation in our measurements in this case precluded clear conclusions concerning its dual location.

CF_α and CF_β (ATPase) Subunits

The α and β subunits of the proton ATPase were found exclusively associated with the stromal lamellae (Fig. 2; Table

Table II. *Quantification of the Relative Distribution of Thylakoid Proteins between Stromal and Granal Lamellae of Spirodela*

Several replicates of experiments such as shown in Figure 2 were analyzed by densitometric scanning (LKB, 2202 Ultroscan) of the immunodecorated, dry blots. In all cases the values were determined from responses in the linear range of the immunoblots as described in the legend to Figure 2. Values for peak areas were normalized to the steady state ratio in whole thylakoids of the 43 kD protein to subunit 2 of PSI. *n*, Number of experiments.

Protein	Relative Distribution				Inferred Localization
	G	S	SD	*n*	
	%				
51 kD	94	6	6	3	G
43 kD	97	3	2	3	G
CF α	1	99	1	3	S
CF β	5	95	3	3	S
PSI (subunit 2)	9	91	3	4	S (+G)[a]
PSI (subunit 1)	17	83	1	2	G + S
33 kD extrinsic	88	12	12	3	G (+S)[a]
32 kD (D1)	88	12	2	4	G + S
D2	86	14	4	3	G + S
Cyt b_{559}	88	12	5	3	G + S
LH1	81	19	2	2	G + S
LH2	77	23	2	2	G + S

[a] Inferred localizations in parentheses require confirmation.

II). In pulse-chase experiments as well, these polypeptides could be detected only on the stromal lamellae (19).

PSI Subunits

About 17% of subunit 1 of PSI was found associated with granal lamellae and 83% with stromal lamellae. The values for subunit 2 (22) were 9% and 91%, respectively (Table II). Immunocytochemical observations (31) of PSII membranes derived by Triton X-100 fractionation procedures indicated that some PSI protein is present at the end margins of the bilayer sheets. The level of PSI-subunit 1 seen in the granal preparations described here (Table II) substantiates these results. Such a distribution of this subunit on granal and stromal lamellae may infer that this polypeptide also is laterally translocated.

CONCLUSIONS

Our studies reveal two classes of thylakoid proteins: class 1 proteins which are associated exclusively with either granal or stromal lamellae, and class 2 proteins which are largely localized to one membrane domain but occur in lesser abundance in another distinct membrane region. Several class 2 proteins are known to undergo lateral diffusion between stromal and granal regions of the thylakoid.

The immunological and biochemical analyses shown here validate the method of Leto *et al.* (15) for obtaining purified stromal and granal lamellae from a single preparation of whole thylakoids using small amounts of plant tissue. Purified stromal and granal lamellae preparations can be used in conjunction with pulse-chase experiments for studying protein trafficking in the thylakoids (9, 18, 19), biogenesis of individual lipid molecular species (23) and assembly of PSI and PSII reaction center protein complexes. Maturation of the 32 kD PSII reaction center protein *in vivo* occurs on the stromal lamellae (19). In this case, the isolated stromal lamellae offer an enriched starting material for identifying and purifying the peptidase involved (FE Callahan, SK Sopory, unpublished data).

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