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Correlation of Structure and Function of Chloroplast Membranes at the Supramolecular Level

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Freeze-fracture electron microscopy has revealed that different size classes of intramembrane particles of chloroplast membranes are nonrandomly distributed between appressed grana and nonappressed stroma membrane regions. It is now generally assumed that thylakoid membranes contain five major functional complexes, each of which can give rise to an intramembrane particle of a defined size. These are the photosystem II complex, the photosystem I complex, the cytochrome f/b_6 complex, the chlorophyll a/b light-harvesting complex, and the CF_0-CF_1 ATP synthetase complex. By mapping the distribution of the different categories of intramembrane particles, information on the lateral organization of functional membrane units of thylakoid membranes can be determined. In this review, we present a brief summary of the evidence supporting the correlation of specific categories of intramembrane particles with known biochemical entities. In addition, we discuss studies showing that ions and phosphorylation of the membrane adhesion factor, the chlorophyll a/b light-harvesting complex, can affect the lateral organization of chloroplast membrane components and thereby regulate membrane function.

Key words: membrane proteins, lateral organization, chloroplast, chlorophyll, phosphorylation

Thylakoids of higher plant chloroplasts contain five integral membrane protein complexes [1]. These are (1) the photosystem II (PSII) complex, which includes the reaction center chlorophyll (chl) protein P680, the tightly bound chl a antenna molecules of PSII, the components required for oxygen evolution, and the immediate electron donors and electron acceptors of PSII; (2) the photosystem I (PSI) complex, which includes the P700 reaction center chl proteins, the chl a antenna molecules, and the immediate electron-donor and electron-acceptor molecules of PSI; (3) the cytochrome f/b_6 complex, which also includes the Rieske nonsulfur iron protein and bound plastocyanin; (4) the chl a/b light-harvesting complex (chl a/b LHC) that delivers excitation energy primarily to PSII; and (5) the CF_0-CF_1 ATP synthetase complex. Detergent fractionation [1–4] and morphological [5] studies have provided evidence for a nonrandom distribution of all of these complexes between grana and

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stroma thylakoids, with the possible exception of cytochrome f/b_6. Much of our research has been devoted to obtaining improved supramolecular maps of the lateral distribution of protein complexes in thylakoid membranes and learning more about the functional significance of the spatial differentiation of protein complexes in chloroplast membranes.

FREEZE-FRACTURE ARCHITECTURE OF THYLAKOID MEMBRANES

A typical example of freeze-fractured pea thylakoid membranes is shown in Figure 1. Four different fracture faces can be distinguished based on the size and density of their intramembrane particles [6]: two associated with *s*tacked, grana regions (referred to as EF*s* and PF*s*), and two with *u*nstacked, stroma membrane regions (EF*u* and PF*u*). EF stands for fracture *f*ace of the *e*xoplasmic membrane leaflet, ie, the half of the bilayer membrane adjacent to the thylakoid lumen; PF, for fracture *f*ace of the *p*rotoplasmic membrane leaflet, ie, the half of the bilayer membrane adjacent to the chloroplast stroma (equivalent of *p*rotoplasm). The differences in particle composition of the four faces provide direct morphological support for the lateral differentiation of thylakoid membranes between grana and stroma regions.

IDENTIFICATION OF PROTEIN COMPLEXES IN THYLAKOID MEMBRANES

Photosystem II (PSII) Complexes

Although positive identification of PSII complexes on freeze-fractured thylakoid membranes has yet to be achieved, the amount of indirect evidence equating the large (10–18 nm) EFs and EFu particles with PSII complexes is nearly overwhelming. Thus the distribution of EF particles between grana and stroma thylakoid regions (85% to 15%) [6] closely matches the values obtained from biochemical fractionation experiments [7–9]. In addition, several freeze-fracture studies of PSII mutant thylakoids have revealed either a significant reduction in the number of EFs and EFu particles or a lack of such particles [10,11]. In the case of the Chlamydomonas mutant F34SU3, Wollman and coauthors [11] observed that a 50% decrease in PSII centers was paralleled by a 50% decrease in EF particles, thereby suggesting that each particle contained one PSII reaction center.

One initially bothersome aspect of thylakoid EF particles was their great variability in size. This variability seemed at first to be contradictory to the idea of relating a single, specific functional role to these particles. The initial breakthrough in the interpretation of these particles came when it was shown that all EF particles were transmembrane complexes that protruded from the lumenal surface of thylakoid membranes [6]. Since all of the protruding particles exhibited the same substructure consisting of four subunits, it was proposed that the EF particles may consist of a constant core unit and variable amounts of other associated proteins [5]. This model was also consistent with earlier reports of a gradual increase in the size of EF particles during the light-induced greening of barley [12] and Euglena chloroplasts [13].

The biochemical basis for the light-induced change in particle size was elucidated by Armond and co-workers [14], who examined the greening of pea seedlings grown under intermittent illumination. Plastids of such seedlings have complete photochemical activities but lack chlorophyll b (chl b) and the chl b-containing pigment proteins [15]. The plastid membranes are also essentially devoid of grana

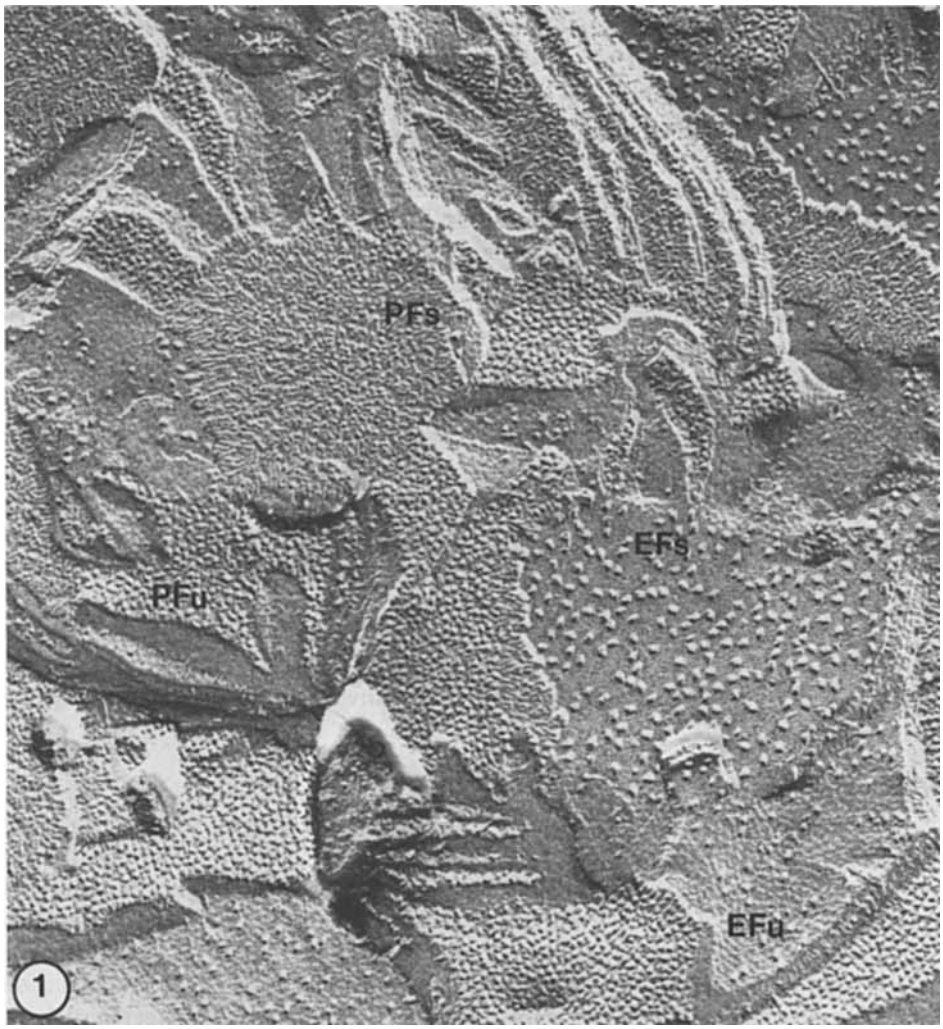

Fig. 1. Freeze-fractured pea thylakoids illustrating the four types of fracture faces typical for such specimens. The faces EFs and PFs belong to stacked, grana membrane regions; the faces EFu and PFu, to unstacked, stroma membrane regions. ×85,000.

stacks. During subsequent greening in continuous illumination, large quantities of the chlorophyll-containing light-harvesting pigment proteins (chl a/b LHC) were incorporated into the thylakoid membranes. Concomitantly a major increase in the diameter of EF particles was observed, and typical grana membranes began to form. In addition, the size of the EF particles was found to increase in steps of ~2.5 nm, from 8 nm in intermittent light plastids to ~10.5 nm, ~13 nm, and ~16 nm. These results were interpreted to suggest that the EF particles of higher plant thylakoids consist of an 8-nm PSII core complex that can be tightly associated with either one, two, or four units of chl a/b LHC (now referred to as "bound" chl a/b LHC

[60,61]). Further support for this interpretation of EF particles has come from studies of a chl b-less barley mutant [16], and chl b-deficient Chlamydomonas [17] and soybean [18] mutants, all of which possess EF particles of reduced size. Consistent with the low amounts of chl b and thus chl a/b LHC in normal, light-grown Euglena cells [19], the thylakoids of this alga contain EF particles averaging only 12 nm in diameter [20].

Based on the fact that EFu particles were more uniform in size and smaller ($\sim$10.5 nm average diameter) than EFs particles, which varied in diameter from 10 to 18 nm, it was postulated that the stroma PSII units had a smaller antenna size and may differ from grana PSII units in other respects [5]. This prediction has been confirmed by several investigators. Armond and Arntzen [21] found that PSII activity of isolated stroma lamellae requires much higher light intensities for saturation than that of grana membrane preparations. Analysis of the biphasic kinetics of PSII by Melis and Homann [22] showed a fast and nonexponential α component ($PSII_\alpha$) and a slower β component ($PSII_\beta$). The two types of PSII reaction center complexes differ both in the effective light-harvesting antenna size and in the apparent midpoint redox potential of their primary electron acceptor Q [23–25]. Since Mg^{++} ions affect the organization of $PSII_\alpha$ but not $PSII_\beta$ in the membrane, it was proposed that $PSII_\alpha$ could be localized in grana partitions and $PSII_\beta$ in the stroma exposed thylakoids [26]. Direct support for this hypothesis has come from the analysis of fractionated spinach thylakoids, which revealed appressed grana membranes to be highly enriched in $PSII_\alpha$ and nonappressed stroma thylakoids in $PSII_\beta$ [9]. These findings are consistent with the idea that EFs particles are the structural equivalents of $PSII_\alpha$ units, and EFu particles of $PSII_\beta$ units.

Photosystem I (PSI) Complexes

Since only PSII complexes appear to be able to give rise to EF particles, it has been generally assumed that PSI complexes have their structural counterparts in PF particles [1, 5]. Two types of studies have produced correlative evidence linking 10–12-nm PF particles with PSI complexes. Freeze-fracture micrographs of thylakoid membranes of the PSI maize mutant 1481 [27] and of the PSI barley mutants viridis-n^{34} [28] and viridis-zb^{63} [28a], both of which are deficient in the P700 reaction center chl a protein, show a loss of a category of large (10–12 nm in diameter) particles on the PFu face. When purified PSI complexes are reconstituted into artificial membranes, the average size of the PSI particles in freeze-fracture replicas is 10–11 nm [29]. The nearly exclusive location of 10–12 nm PF particles in unstacked stroma thylakoids is consistent with results of biochemical fractionation studies, which show approximately 85% of PSI to be localized in stroma membranes [8].

It should be noted, however, that in intermittent light plastids, which exhibit normal PSI activity but lack chl b-containing light-harvesting complexes, only 6–7-nm particles are seen in lieu of 10–12-nm PF particles [14]. Transfer of the intermittent light seedlings to continuous illumination, which triggers the formation of light-harvesting pigment proteins, also leads to the appearance of larger PFu particles. This finding suggests that the large PFu particles could consist of an $\sim$8 nm PSI core surrounded by light-harvesting pigment proteins. The combined PSI core-light harvesting complex (PSI-LHC) then, would give rise to 10–12-nm PFu particles. Recent

studies on the composition of the PSI light-harvesting pigment proteins has revealed that they too contain some chl b [29]. Whether normal thylakoids contain a sizable population of ~8-nm PSI core particles, eg, in grana membranes, besides the larger PSI-LHC units has not been determined.

Cytochrome f/b_6 Complex

The exact location of cytochrome f/b_6 complexes in thylakoid membranes has yet to be resolved. Although two biochemical fractionation studies [30, 31] have produced results consistent with the notion that cytochrome f/b_6 complexes are equally distributed between grana and stroma thylakoids, a third one [31a] has found virtually no such complexes in grana membranes. Based on a kinetic analysis of components of the electron transport chains of mesophyll and bundle sheath chloroplasts of maize, Ghirardi and Melis [31b] have proposed that cytochrome f/b_6 complexes could be located at the interface between grana and stroma thylakoids. Since one of these studies has produced unambiguous results, it has not been possible to obtain a clear correlation between cytochrome f/b_6 complexes and a specific category of PF particles in either stacked or unstacked membrane regions. Mörschel and Staehelin [32] have recently obtained freeze-fracture micrographs of purified cyctochrome f/b_6 complexes reconstituted into phospholipid and digalactolipid liposomes. In both types of lipids, the complexes produce intramembrane particles with a diameter of approximately 8.5 nm. Using anticytochrome f antibodies it was possible to aggregate the complexes into geometrical arrays with a repeat distance of 8.5 nm. Based on a particle diameter of 8.5 nm and a measured height of 11 nm, a molecular weight of about 280,000 was calculated for the isolated complex, consistent with its possessing a dimeric configuration. The 8.5-nm size of the reconstituted cytochrome f/b_6 complex is well within the range of the freeze-fracture particles found on PFs and PFu faces of thylakoid membranes.

Chlorophyll a/b Light-Harvesting Complex

As mentioned above, the size of EFs and EFu particles of thylakoid membranes varies with the amount of chl a/b LHC that is bound to the 8-nm PSII core particles [14]. More recent studies have revealed an additional pool of chl a/b LHC units that are *not* tightly bound to PSII particles [11, 33, 34]. These isolated units give rise to ~8-nm PF particles and have recently been termed "mobile" chl a/b LHC particles [61] to distinguish them from the "bound" chl a/b LHC units that are tightly bound to PSII complexes.

Several lines of evidence have revealed that the mobile chl a/b LHC is responsible for thylakoid adhesion and therefore is preferentially associated with grana regions. Plants grown under intermittent illumination exhibit photochemical activity of PSI and PSII, but lack both chl a/b LHC and grana stacks. Upon transfer to continuous illumination, chl a/b LHC peptides (23–29 kilodaltons) are synthesized and inserted into thylakoid membranes, and grana formation is observed [14]. Mutants that lack, or contain a modified, chl a/b LHC, exhibit modified membrane adhesion [15, 17]. Selective trypsin cleavage of chl a/b LHC peptides in chloroplast membranes disrupts normal membrane adhesion [35–38]. Finally, purified chl a/b LHC reconstituted into liposomes is capable of mediating membrane adhesion at the same salt

TABLE I. Spatial Distribution of Chloroplast Membrane Components

Component	Stacked (grana) membranes (%)	Unstacked (stroma) membranes (%)	References
PSII	~85	~15	[4, 6, 8]
PSI	<15	>85	[4, 8]
Cyt f/b_6	0-50(?)	50-100(?)	[30, 31, 31a]
Chl a/b LHC	More (70-90) (Phosphorylation dependent)	Less (10-30) (Phosphorylation dependent)	[4, 58]
ATP synthetase	0	100	[46]

concentrations (>2 m $MgCl_2$ or >100 mM NaCl) as required for in vitro stacking of thylakoid membranes [39].

The CF_0-CF_1 ATP Synthetase

The first complex to be positively identified and localized in thylakoid membranes of higher plants was the coupling factor complex (CF_1). This peripheral membrane complex was initially shown to be accessible to antibodies in isolated thylakoid preparations [40, 41]. Subsequent electron microscopical studies correlated the CF_1 complex with knoblike structures that protruded from the stromal surface of the thylakoid membranes [42–45]. The diameter of these knobs was reported to be ~9 nm in negatively stained samples, ~10 nm in thin-sectioned material, and 14–15 nm in freeze-etch preparations. Finally, Miller and Staehelin [46] demonstrated that CF_1 was limited to nonappressed, stroma thylakoid membrane regions (including the grana margins). The same study also revealed the presence of some bound complexes of ribulose 1, 5 bisphosphate carboxylase on the exposed surfaces of stroma thylakoids.

Information on the hydrophobic CF_0 portion of the CF_0-CF_1 ATP synthetase complex is more limited both because the intact complex is more difficult to isolate [47], and because the CF_0 particle has not yet been identified on freeze-fractured thylakoid membranes. Following reconstitution of purified CF_0-CF_1 complexes into phospholipid [48] and digalactolipid [32] liposomes, the CF_0 basepieces give rise to ~9.5 nm freeze-fracture particles. Particles of this size range make up a considerable proportion of the PFu particles of chloroplast membranes, but more work is needed to prove that the CF_0 complexes give rise to ~9.5 nm particles in vivo.

DYNAMIC ASPECTS OF THYLAKOID MEMBRANE ORGANIZATION

Effects of Salt on Thylakoid Structure

Table I summarizes our current information on the distribution of the five major functional protein complexes of thylakoid membranes between grana and stroma membrane regions. This lateral differentiation of chloroplast membranes both arises from and is dependent on the presence of adhering membrane regions. Experiments involving particle electrophoresis, surface potential measurements, and reaction rates of charged electron donors and acceptors (reviewed in [49]) have revealed that at neutral pH thylakoids carry a net negative charge. Adhesion between thylakoid membranes requires that the electrostatic repulsive forces on the surface of these membranes be screened by cations [49,50]: monovalent cations are the least effective;

and trivalent cations, the most [51]. Transfer of isolated thylakoids into a low salt medium (eg, 50 mM Tricine or 10 mM NaCl) leads not only to a loss of stacked membrane regions but also to complete intermixing of *all* intramembrane protein complexes [6]. This intermixing can be completely reversed by the addition of >3 m $MgCl_2$ or >150 mM NaCl to the suspension, which leads to the formation of new grana stacks and concomitantly to the resegregation of the complexes between stacked and unstacked membrane regions [6,46]. It should be stressed here that this low-salt-induced unstacking is *not equivalent* to unstacking produced by phosphorylation of the chl a/b LHC ([34, 58]; see also next section).

Phosphorylation-Dependent Changes in Thylakoid Structure

Optimization of noncyclic electron flow in the photosynthetic membranes of higher plants and algae is achieved by regulating the distribution of absorbed excitation energy between PSII and PSI reaction centers [52–54]. Illumination of plants with >700 nm light, preferentially absorbed by PSI, stimulates structural reorganizations in thylakoids that increase the proportion of absorbed excitation energy delivered to PSII (state I). Conversely, exposure to PSII-light (650 nm), results in an increased proportion of absorbed excitation energy being distributed to PSI (state II). This regulation of excitation energy distribution between PSII and PSI has been correlated with the state of phosphorylation of peptides of chl a/b LHC [55,56]. Under state II conditions chl a/b LHC becomes phosphorylated; under state I conditions dephosphorylation of the chl a/b LHC occurs. Studies of the effect of phosphorylation of the chl a/b LHC on excitation energy distribution have revealed that both the absorptive cross section of PSI and the energy transfer from PSII to PSI increased in phosphorylated membranes [57,58].

Until recently the structural basis of state I-state II transitions has been an enigma. Bennoun and Jupin [59] reported that during the conversion of Chlamydomonas thylakoids from a state I to a state II condition, the extent of appressed membrane regions decreased by approximately 20%. Based on this finding and the analogy with low-salt-induced changes in thylakoid membrane structure discussed above, Barber [49, 50] proposed the following mechanism for state I-state II changes: Phosphorylation of the chl a/b LHC, the membrane adhesion factor, would increase the electrostatic repulsion between the membranes containing these proteins and thereby reduce membrane stacking. In turn, the reduction in stacked grana membrane regions would lead to a net transfer of chl a/b LHC and PSII complexes to stroma thylakoids, and thereby to increased intermixing of PSII, PSI and chl a/b LHC particles.

Our freeze-fracture studies of state I-state II transitions [58] have confirmed that phosphorylation of the chl a/b LHC leads to a reversible decrease in thylakoid stacking (a 23% decrease for our in vitro phosphorylated pea thylakoids). However, quantitative analysis of particle sizes and particle densities of unphosphorylated, phosphorylated, and phosphorylated/dephosphorylated thylakoids revealed that the changes in the amount of stacked membranes were accompained only by a reversible net transfer of 8 nm PF particles between stacked and unstacked membrane regions. These 8 nm PF particles were subsequently correlated with mobile chl a/b LHC units. Thus, in the nonphosphorylated state (state I) most mobile chl a/b LHC particles accumulate in stacked, grana membrane regions that contain 85% of the PSII complexes. Upon phosphorylation of chl a/b LHC peptides (state II), there is a net transfer

of mobile chl a/b LHC particles from the PSII-rich grana membranes to PSI-rich stroma membranes. Dephosphorylation allows the chl a/b LHC particles to return to the stacked membrane regions. Based on these and other observations we have proposed that regulation of excitation energy distribution between PSII and PSI is brought about by the reversible lateral migration of a population of mobile light-harvesting antennae [60,61].

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