Chapter 5
Dynamic Architecture of Plant Photosynthetic Membranes

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Abstract The energy converting photosynthetic machinery in plants is harbored in the thylakoid membrane system inside the chloroplast. The structural flexibility of thylakoid membranes is a central feature that allows sessile plants to adapt to very different and highly fluctuating environmental conditions. This chapter surveys structural attributes of the thylakoid membrane system and its dynamics. It connects structural alterations to the functionality of the energy converting apparatus and its regulation. Two structural levels are addressed that cover different length scales. The first deals with changes in the overall membrane architecture (micrometer length scale) with a special emphasis on stacked grana regions. The second part focuses on the organization of many protein complexes in thylakoid membranes (100 nm length scale). It turns out that thylakoid membranes evolved a remarkable degree of plasticity that fine-tune different aspects of photosynthetic energy transformation.

Keywords Thylakoid membrane · Grana thylakoid · Macromolecular crowding · Protein arrays · Supramolecular level

Abbreviations

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AFM	Atomic force microscopic
CLSM	Confocal laser scanning microscopy
cyt b6f	Cytochrome *b6f*
DLVO	Derjaguin-landau-verwey-overbeek
FRAP	Fluorescence recovery after photobleaching
LHCI	Light-harvesting complex I
LHCII	Light-harvesting complex II
OEC	Oxygen-evolving complex
PC	Plastocyanin
PMF	Protonmotive force
PSI	Photosystem I
PSII	Photosystem II
ROS	Reactive oxygen species
VDE	Violaxanthin deepoxidase

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5.1 Introduction

The conversion of solar radiation into biologically useful energy by photosynthesis is confined to highly specialized membrane systems. In plants, the thylakoid membrane inside the chloroplast harbors the nanomachines that constitute the photosynthetic apparatus for sunlight-driven generation of $NADPH + H^+$ and ATP accompanied by water splitting and molecular oxygen production. Although the overall reaction equation is simple, the underlying molecular processes are extremely complex and fascinate thousands of researchers in many disciplines of natural sciences ranging from quantum mechanics to ecophysiology. Learning how nature optimizes and regulates photosynthetic energy transformation is a high priority research field with significant social impact because it might provide a key to solving global energy and food problems in a challenging climate. Understanding the primary processes in photosynthesis requires not only detailed knowledge of the molecular structure of the individual nanomachines, but it is also essential to know how the overall thylakoid system is structured and how its numerous protein complexes work together to establish functional networks for light-harvesting and electron transport. Recent work has given new insights into the structural plasticity of thylakoid membranes on the nanometer and micrometer length scale. This data reveals a high degree of structural flexibility in photosynthetic membranes, which is necessary to tackle the multiple challenges dictated by environmental changes that occur day-to-day on very different time scales.

A striking structural feature that is characteristic for the thylakoid system is that part of the membrane forms tightly stacked cylindrical domains called grana thylakoids [31, 90, 111]. Grana discs are interconnected by unstacked stroma lamellae forming a continuous membrane system that separates two aqueous reaction spaces: the chloroplast stroma and the intrathylakoid lumen space (see also Fig. 5.1). Embedded in the thylakoid membrane are six main transmembrane protein complexes that establish the energy transforming apparatus: photosystem II (PSII) with light-harvesting complex II (LHCII), photosystem I (PSI) with light-harvesting complex I (LHCI), the cytochrome *b6f* (cyt *b6f*) complex, and a F-type ATPase complex (ATPase). In addition to these major protein complexes, low abundance transmembrane proteins are present like the *psbS* protein or *stn* kinases. It is well established that differentiation into stacked and unstacked membrane regions leads to a lateral heterogeneity in protein complex distribution [2, 14, 35, 111]. The main part of PSII and LHCII is localized in stacked grana. In contrast, PSI with LHCI and the ATPase are excluded from stacked grana, most likely by steric hindrance because the stroma-facing protein moieties of PSI and ATPase are too large to enter the narrow gap between adjacent grana membranes (see Sect. 5.2.1.3). It is assumed that the cyt *b6f* complex is the only complex that is homogeneously distributed [111] but there is also evidence for its depletion in stacked grana [41, 122] as well as for its concentration in this subcompartment [2]. However, the lateral protein distribution within the thylakoid network is not static but highly dynamic. The dynamic redistribution of protein complexes within and between stacked and unstacked thylakoid regions could play a key role to adjust photosynthetic performance to environmental
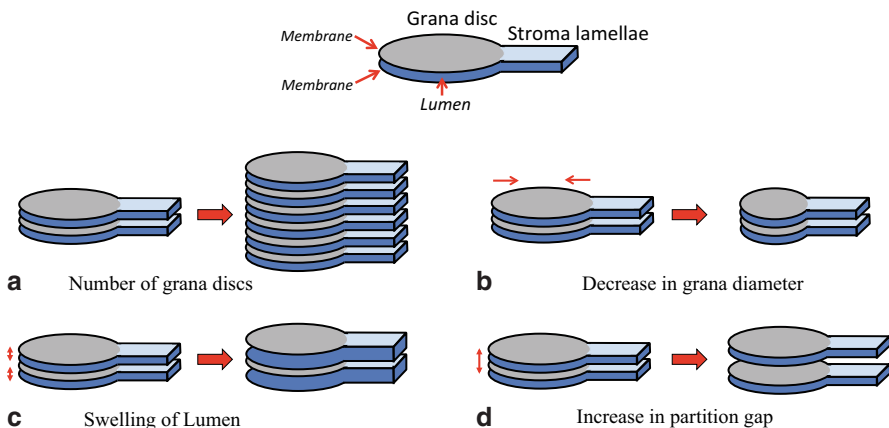
Fig. 5.1 Levels of protein organization in thylakoid membranes. Proteins can arrange themselves into different assembly levels. The example shows these levels for the LHCII-PSII complex. Different *psb*-subunits form the PSII (multi-subunit complex). This PSII complex associates to a dimer that binds two trimeric LHCII complexes (*red*) forming the LHCII-PSII supercomplex. Further trimeric LHCII attach to the supercomplex generating megacomplexes that can further form extended supramolecular networks. The different types of complexes are characterized by different binding strengths indicating a hierarchy in protein-protein interactions. For example, the two trimeric LHCII binds stronger to the dimeric PSII than the additional trimers in megacomplexes. As indicated by the arrows, the different assembly levels are in equilibrium and are inter-convertible. Intact thylakoid membranes contain all assembly levels [30] and the state of equilibrium is controlled by environmental factors. This inter-convertibility is critical for the PSII repair cycle (see text for further details)

changes. Thus, unraveling this interdependency is central to understanding the plasticity of the energy transforming machinery.

In the last few decades, considerable progress in solving the structure of photosynthetic protein complexes (for recent reviews see [8, 18, 21, 54, 121]) leads to an almost complete set of high-resolution models making the thylakoid membrane one of the best-characterized biomembranes. Furthermore, low-resolution data deduced from single-particle analysis of solubilized thylakoid membranes reveals that photosynthetic protein complexes form larger aggregates named supercomplexes (Fig. 5.2). It seems that protein complexes in thylakoid membranes are mainly organized into supercomplexes, i.e. dimeric cyt *b6f* complex, trimeric LHCII, PSI with four LHCI, and dimeric PSII with two trimeric LHCII [35]. In particular, the PSII supercomplex in grana thylakoids was extensively studied [25, 70, 91]. There is now an understanding that the dimeric PSII-LHCII supercomplex represents the structural building block in stacked grana (but see [115] for a different view). Furthermore, evidence exists [35] that in grana membranes isolated by mild-detergent treatments, supercomplexes can arrange into higher associations called megacomplexes (Fig. 5.2). It has to be clarified whether these higher associations represent a native state or that they are generated by the detergent treatment [88]. Under some conditions, the PSII-LHCII supercomplex can further associate to highly ordered semicrystalline supramolecular networks consisting of many proteins ([32, 35, 70],

| Single subunit | Multi-subunit Protein complex | Supercomplex | Megacomplex | Supramolecular level |
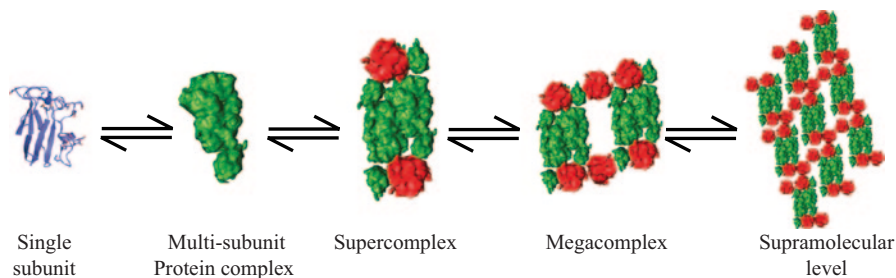
Fig. 5.2 Survey of geometrical changes in the overall grana architecture. **a** Light intensity is the major determinant that controls the number of grana thylakoid membranes constituting the grana cylinder. **b** The grana diameter seems to be a relative robust quantity. However, it was reported that it shrinks under certain unfavorable conditions. This leads to inter-conversion of stacked (*grey*) to unstacked thylakoid membrane regions (*light blue*). **c** Recent data suggests that the thylakoid lumen in grana (*dark blue*) swells in light that has impact on the molecular mobility of lumen-hosted proteins. **d** Although experimental evidence is weak, it was suggested that the distance between neighbored grana membranes on the stroma side (partition gap) can change under high-light stress

see Sect. 5.3.2). Since these semicrystalline arrays are found in intact thylakoid membranes that never had contact with detergents [32], it is safe to conclude that they represent an *in vivo* state of the photosynthetic machinery. Thus, a remarkable degree of order and hierarchy in protein organization is realized in particular in grana membranes (Fig. 5.2). Although this has been recognized for a long time, we are just now understanding the functional significance and dynamics of structural ordering in photosynthetic membranes. Interestingly, a similar complexity and hierarchy is realized for membrane proteins in the respiratory membranes in mitochondria that also form supercomplexes [37, 38, 40] and supramolecular rows [33, 38, 39, 112]. It seems that both bioenergetic membranes share common structural organization motifs indicating that learning the principles of protein organization in thylakoid membranes could be valuable for respiratory membranes too and *vice versa*. For example, electron transport in both membranes depends on diffusion of small electron carriers (plastoquinone, ubiquinone, plastocyanin, cytochrome c). Supercomplexes and supramolecular ordering as found photosynthetic as well as in respiratory membranes could have strong impact on the mobility of these carriers [53, 72, 59].

A current research challenge is to determine the supramolecular arrangement in native membranes and to understand how it dynamically responds to environmental changes. Furthermore, the overall thylakoid architecture is highly flexible and undergoes dynamic swelling and shrinkage processes that seem to be correlated with the adaptation of photosynthetic energy transformation. This review focuses on the flexibility of the whole thylakoid system (see Sect. 5.2) as well as on the supramolecular protein arrangement in thylakoid membranes (see Sect. 5.3) of higher plants. The reader is also referred to excellent review articles on related fields, i.e. on the supramolecular PSII organization [70] and on 3D models for the overall thylakoid membrane architecture derived from electron tomography [31, 90].

5.2 Overall Thylakoid Architecture

The structure of the thylakoid membrane system in chloroplasts was mainly studied by diverse electron microscopic techniques [86, 89, 111] and atomic force microscopy [55]. Most recently, electron tomography was established for visualizing the 3D thylakoid architecture [16, 32, 69, 87, 105]. An intensively discussed point is how the stroma lamellae insert into the grana stacks. Basically, two models have been proposed. One postulates that the stroma lamellae spiral up around the grana cylinder in the form of a right-handed helix and insert into the grana cylinder by slit like connections called "frets." The other model hypothesizes that bifurcation of stroma lamellae leads to grana stacks ("fork" model). This open aspect in the thylakoid structure is not addressed in this review and the interested reader is referred to [16, 31, 87, 90]. The following section focuses on the architecture of grana stacks that constitute about 80 % of the thylakoid membrane [3].

5.2.1 Structural Organization of Grana Thylakoids

5.2.1.1 Grana Diameter

The 3D ultrastructural models derived from electron microscopy reveal the complexity but also the high degree of organization realized in thylakoid membranes. For example, it is striking that the diameters of the numerous grana cylinders in a chloroplast (typically 40–60) are in a narrow size range, i.e. between 300 and 600 nm [35, 111]. This size range seems to be a robust quantity because it does not change significantly under different growth conditions [59]. The grana diameter is particularly relevant for processes that are based on lateral diffusion between stacked and unstacked thylakoid membrane regions because it determines the diffusion distance and consequently the diffusion time. Diffusion dependent processes that are affected include the PSII repair cycle, *state transition* or electron shuttling between PSII and PSI by small electron carriers'. For a detailed discussion, see [59]. It is not clear why no broad statistical distribution in the grana diameters is realized, e.g. grana with a few tens of nm and µm sized grana. This points to physicochemical driving forces determining the size of stacked thylakoids. A possible driving force that is involved in grana organization is the phosphorylation level of PSII and/or LHCII. PSII and LHCII can be reversible phosphorylated by *stn7 and stn8* kinases [24, 42, 96, 116]. In *stn7/stn8* Arabidopsis double mutants with a very low PSII/LHCII phosphorylation level, the grana diameter is about 50 % wider compared to wild-type plants [43]. Based on these findings, it was suggested that the phosphorylation level of grana hosted PSII determines the grana diameter [44]. This correlates with the hypothesis that stacking is a result of mutual electrostatic attraction between positive and negative surface charges localized on the stroma-facing N-terminal part of the LHCII complexes located on adjacent grana discs (for a model see [21]). In the context

of this model, the introduction of negative charges by protein phosphorylation is expected to disturb the electrostatic balance leading to repulsion and destacking. The idea of stacking mediated by electrostatic interactions goes back to earlier models based on the *Derjaguin-Landau-Verwey-Overbeek* (DLVO) theory that combine electrostatic repulsion with van-der-Waals attraction [19]. In addition, at short distances (<2.5 nm), strong hydrostructural repulsion hinders further approximation of adjacent grana membranes [103]. In difference to this "surface-charge model," other models were postulated for explaining grana stacking including the molecular recognition of complementary protein surface regions [4, 5, 7] or entropic effects [26, 27]. It needs to be elucidated how all these factors work together for establishing tightly stacked grana. However, the observation that differences in the protein phosphorylation level of grana hosted protein complexes induce changes in grana diameter points to an important role of this post-translational protein modification for the structural organization of thylakoid membranes. A critical role of protein phosphorylation on the stromal LHCII site for thylakoid stacking is in accordance with studies showing that cleavage of a 2 kDa sized N-terminal domain of LHCII prevents stacking [81]. Since the activity of the *stn*-kinases are tightly controlled by the redox state of the photosynthetic electron transport chain (i.e. the plastoquinone pool), reversible protein phosphorylation allows dynamic adjustment of structural boundary conditions of the thylakoid membrane system to the needs determined by an ever-changing environment (see Sect. 5.2.2).

5.2.1.2   Grana Distribution in Chloroplasts

Another example for the high degree of organization found in the overall thylakoid architecture is that the many grana stacks in a chloroplast are remarkably homogenously distributed. We analyzed the grana distribution by confocal laser scanning microscopy (CLSM) in combination with mathematical analysis tools and found that the separation distance between adjacent grana stacks fall in a narrow range that varies between 450 and 750 nm with a mean at about 550 nm (unpublished results). As for the grana diameter, it is striking that a broad range of separation values is not realized. The non-random equal-distant separation by about $\sim$550 nm of $\sim$500 nm wide grana discs could be advantageous for absorption of visible light. This point has not been examined so far.

The constancy of the grana diameter and separation indicates that structural organizing forces are at work leading to a high level of order found in the overall thylakoid membrane architecture. Because cytoskeleton-like elements are very likely missing in chloroplasts, the capability to arrange the thylakoid network is most likely determined by physicochemical features of the membrane itself. The potential for self-organization can be demonstrated by *in vitro* destacking and restacking experiments of isolated stacked thylakoid membranes induced by changing salt concentrations [55, 65, 108, 109]. Destacking by low salt treatment not only leads to unfolding of grana stacks but also to randomization of the protein complexes
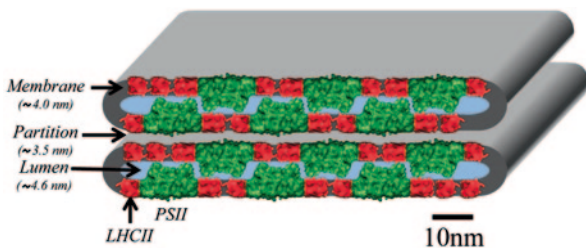
Fig. 5.3 In-scale model of a grana stack (*cross-section*). The model is based on recent cryo-EM data (see text for details). It represents the situation in dark- or dim light-adapted plants. The numbers change considerably in light-adapted plants. The model shows four grana membranes enclosing two lumen spaces and separated by one partition gap. The lateral grana dimension is not in-scale. Note that adjacent PSII complexes sharing the same lumen cannot adopt a face-to-face arrangement due to steric restrictions of the lumenal PSII protrusions

within the thylakoid membrane. Re-addition of salt to destacked thylakoids leads to almost full refolding to the complex grana network that is accompanied by the same inhomogeneous protein distribution between stacked and unstacked membrane regions as found for stacked control membranes. It follows that the complex folding and protein organization is self-organized.

5.2.1.3 Transversal Grana Structure

The transversal geometry of grana stacks recently came into focus of photosynthesis research since technical improvements (cryo-sample preparations and cryo-EM) allow determination of structural thylakoid attributes in near-native states. The pioneering work of Murakami and Packer [84] on thin-sections of room-temperature chemical fixed samples gives a first comprehensive quantitative picture of the transversal grana thylakoid structure. However, it was recognized that chemical fixation of thylakoids at ambient temperatures and using isolated thylakoids can be a source of artifacts [89]. Recently, cryo-EM tomography [32, 70] and cryo-EM [68] was applied to reexamine structural attributes of grana thylakoids. While the studies of Kouřil and co-workers were performed with isolated grana membranes, Daum et al. used ruptured chloroplasts and Kirchhoff et al. used intact leaf discs. The latter two studies gave a consistent picture of the transversal grana architecture summarized in Fig. 5.3. In difference to the values given in Fig. 5.3, Kouřil and co-workers found significant higher numbers for the luminal width (14–16 nm) and a variable width of the stroma partition gap. These differences may indicate that preparation of isolated grana lead to alterations in the native membrane organization and highlights the importance for analyzing membrane attributes with intact material.

Given that high-resolution information about photosynthetic protein complexes is available now, it is appealing to combine them with ultrastructurual data of stacked grana as shown in Fig. 5.3 for the LHCII-PSII supercomplex. These quantitative

models give an intuitive access to the structural organization of the photosynthetic machinery and allow estimations of steric restrictions. From Fig. 5.3 it follows that the aqueous stroma space between two grana discs (partition gap) available for diffusion is less than 3 nm (taking into account that PSII extends ~1 nm into the stroma). It follows that the ATPase complex and PSI must be excluded from stacked grana by steric reasons because their stroma protrusions are too large (~10 nm for the ATPase [1] and ~3.5 nm for PSI [9, 55]). In turn, both PSII and LHCII have very flat stromal surfaces (Fig. 5.3) that enable tight grana stacking. The situation for the cyt *b6f* complex is less clear since stromal protrusions may cause steric hindrance and exclusion from stacked regions. These structural conclusions based on new EM data taken from material in a near native state correspond with earlier models [7, 35]. The overall picture derived in Sect. 5.2.1 represents the situation for dark-adapted samples or samples in dim light. However, as detailed in the next section the structural attributes of the thylakoid architecture are not static but can change significantly, in particular by illumination.

5.2.2   Flexibility of the Grana Architecture

5.2.2.1   Classification of Structural Changes in the Grana Architecture

Before summarizing the current knowledge on the structural flexibility of the thylakoid membrane system, it is worthwhile to classify possible geometrical changes for grana stacks. Figure 5.1 gives a schematic overview about these changes that are all realized in native thylakoid membranes. As detailed below, the four different types of structural changes have different functional consequences. Therefore, general statements like "destacking of grana" without specifying what exactly is meant by this may be not sufficient to understand the different functional implications associated with the different types of destacking.

   The most obvious and best-documented change in the overall thylakoid architecture is alteration in the number of thylakoid membranes that constitute a grana stack (Fig. 5.1a). It has long been known that there is an inverse correlation between light intensity and the number of thylakoids per grana stack (for reviews see [11, 12, 111]). For example, shade plants form higher grana stacks (more membrane layers) than sun plants. An extreme example is the shade plant *Alocasia microrrhiza* growing in the deep shade in rainforests with more than 100 thylakoid membranes per grana stack [46]. Increasing the number of membrane layers could be a strategy to increase the probability for absorbing the limiting number of light quanta that reach the plant in the shadow [12]. A simple example illustrates this. A single (grana) membrane layer with a chlorophyll concentration of ~0.3 M [61] and a thickness of 4 nm (Fig. 5.3) would absorb only about 1 % of the incident light of 680 nm (extinction coefficient for chlorophylls at 680 nm is 53,000 mM^{-1} m^{-1}, [119]). (The wavelength of 680 nm was arbitrarily chosen). Although this is a rough estimate because the optical properties in intact membranes are much more complex, the number of 1 % is in agreement with more elaborate models [93]. Due to this low probability

to harvest sunlight by one membrane layer, the stacking of many membrane layers to grana could be an important optimization for collecting limiting light quanta in shaded habitats. Besides changing the number of membranes per grana stack, other geometrical alterations in response to environmental cues are realized that are summarized in the following sections.

5.2.2.2 Lateral Changes in Grana Diameter (Fig. 5.1b)

As discussed in Sect. 5.2.1.1, the diameter of the grana cylinder seems to be fairly constant in non-stressed plants. However, under certain conditions, the diameter of grana stacks can change. An example is *state transition*. The broad outline of *state transition* [4, 6, 56, 75] is that it dynamically adjusts the functional antenna size of PSII and PSI (number of chlorophylls coupled to a photosystem reaction-center) to balance the energy distribution between them and in that way to synchronize the photochemical rates of the two photosystems. Under conditions where PSII receives more light energy than PSI, the imbalance is sensed by a higher reduction level of the intersystem PQ pool. The reduced PQ pool activates the *stn7* kinase that phosphorylates LHCII subunits (LHCII-P). LHCII-P uncouples from PSII and migrates to unstacked thylakoid regions where it serves as light harvester for PSI. This process is reversible, i.e. if the PQ pool gets oxidized again, LHCII-P is dephosphorylated and redistributes back from PSI to PSII. State 1 is defined when LHCII preferentially binds to PSII, state 2 when it binds to PSI. State transition is an important mechanism to optimize energy conversion under low light, i.e. under conditions where each light quantum should be used for photochemistry. Under high light, the regulation by state transition is turned off because the *stn7* kinase is inactivated by thiol-modulation [97]. Comparative EM micrographs of pea thylakoids in state 1 and 2 reveal that the grana diameter shrinks in state 2, leading to a 23% decrease in the amount of stacked membranes and consequently to a corresponding increase in unstacked membranes [72, 110]. This lateral grana shrinkage under state 2 was recently confirmed [28].

What could be advantages to reduce the grana diameter? As discussed in detail in 5.3.1, grana thylakoids are heavily crowded by proteins that challenge lateral transport of membrane integral protein complexes. This interferes with the redistribution of LHCII between PSII in stacked grana to PSI in unstacked thylakoid parts. In this respect, decreasing the grana diameter has several advantages. First, LHCII localized in grana areas that are subjected to destacking reaches unstacked regions without long-range diffusion. Second, the diffusion distance of proteins that are still in stacked regions to reach unstacked membrane parts is shortened. Consequently, their diffusion time will be reduced. Third, the grana perimeter to area ratio increases after shrinkage of the grana diameter. Therefore, it is expected that the contact zones between stacked and unstacked membranes increases. In summary, lateral destacking by reducing the grana area could be relevant to improve lateral protein traffic between stacked and unstacked thylakoid membranes as required for *state transition*.

Another prime example where lateral protein traffic is essential is the PSII repair cycle. Plants have to deal with the problem that primary photochemical processes in the reaction center of PSII have an intrinsic probability for producing toxic reactive oxygen species (ROS) that mainly damage the D1 subunit of PSII [73, 79, 83, 92]. This cannot be completely avoided and becomes a severe problem under high-light or temperature stress. Plants addressed this challenge by the evolution of a sophisticated PSII repair cycle that is one of the fastest repair machineries in nature [73, 79, 83]. An open question concerning the PSII repair cycle is how damaged PSII in stacked grana becomes mobilized and can escape from crowded grana to find its repair machinery in distant (several 100 nm) stroma lamellae. As discussed above for *state transition*, reduction of the grana diameter could be an elegant way to solve this problem because it facilitates diffusion dependent steps in the PSII repair cycle. Recently, we analyzed structural grana attributes by mathematical analysis of CLSM images of dark-adapted and light-stressed *Arabidopsis* protoplasts (unpublished results). A key finding is that high-light stress causes a shrinkage of the grana diameter by about 21 % (from 380 nm to 300 nm). This contrasts with EM studies in which no change in grana diameter was observed by high light stress [43]. However, the error bars in the EM study are in the order of 35 %. Thus, it could be that the more subtle changes determined by CLSM were simply not detected in the EM study because the error bars are too large. It is striking that both *state transition* and the PSII repair cycle includes reversible phosphorylation of LHCII or PSII subunits (D1, D2, CP43, psbH) and lateral destacking of grana. This supports the concept that the protein phosphorylation level of grana hosted proteins determines the grana diameter (see Sect. 5.2.1.1).

5.2.2.3   Swelling of Thylakoid Lumen (Fig. 5.1c)

Until recently, the accepted view was that the thylakoid lumen shrinks in light compared to dark-adapted samples. The concept of a light-induced shrinkage mainly goes back to observations made by Murakami and Packer on samples prepared by classical room temperature fixation techniques [84]. In contrast to this mainstream concept, we could show in a collaborative study with Dr. Ziv Reich by applying two different cryo-EM techniques on intact leaf discs that the lumen expands in light-adapted Arabidopsis leaves from about 4.6 nm to about 9.2 nm, i.e. by about 100 % [68]. We interpreted the discrepancy of our study to the work of Murakami and Packer by differences in sample preparations and by working with intact leaf material instead of isolated thylakoids (see [88] for a detailed discussion). Therefore, recent data indicates that the lumen expands in light. A controlled swelling and shrinkage of the lumen introduces interesting types of regulation of electron transport reactions as well as photoprotective and protein degradation processes that are localized in the lumen.

What are the functional implications of a swelling or shrinkage of the thylakoid lumen? Before addressing this question it is essential to appreciate the high protein density in this narrow reaction space. In dark-adapted samples, a luminal width

of only ~4.6 nm excludes the possibility that the protruding luminal parts of PSII complexes localized in adjacent grana thylakoid membranes adopt a face-to-face arrangement and therefore must be staggered (Fig. 5.3). This is because the height of the luminal protrusion of 4–5 nm [91] is too large to allow a face-to-face organization. From a staggered arrangement, it follows that the area occupied by luminal PSII protrusions in the middle of the lumen is doubled compared to a face-to-face arrangement. Taking measured PSII densities and mid-resolution models of PSII, we generated molecular landscapes of the lumen of dark-adapted Arabidopsis plants [68]. These models reveal that the lumen in grana is an extremely crowded space. About 70% of the area is occupied by OECs. Estimation of the available diffusion space of the electron carrier plastocyanin (PC) shows that it is trapped in small diffusion microdomains. PC can escape from these microdomains only by rearrangements of the overall protein network in grana, which is a very slow process [62]. Consequently, long-range diffusion of PC in dark-adapted thylakoids is expected to be slow; this was supported by functional electron transport measurements [68]. In contrast, the significant swelling of the lumen in light-adapted thylakoids leads to a switch from highly localized diffusion (in microdomains) to long-range diffusion [68]. Thus, light-induced dynamic swelling and shrinkage of the thylakoid lumen can control photosynthetic electron transport by controlling the diffusion radius of plastocyanin.

The highly restricted diffusion in dark-adapted plants observed for PC is likely to hold also for other lumenal proteins. Steric restrictions for other proteins can even be more pronounced because they are often larger than PC. For example, the violaxanthin deepoxidase (VDE) is about 40 kDa [102]. It is assumed that the functional VDE-form is a dimer. Thus, the physical size (diameter) of functional dimeric VDE is ~twice that of PC ($(80/10.5 \text{ kDa})^{1/3}$) assuming a spherical shape of both molecules. It follows that there is hardly any space in the lumen of stacked grana to accommodate VDE or that the enzyme is trapped in a few places in stacked grana only. VDE catalyzes the conversion of the xanthophyll violaxanthin to zeaxanthin [50]. The latter is an important activator of photoprotective high-energy quenching (qE). Size exclusion of VDE from stacked grana or a highly localized VDE within grana implies that the xanthophylls in grana have to migrate through the lipid membrane phase to and from the enzyme. Since the membrane is also a highly crowded environment, it is expected that this diffusion is slow, as was shown as well for plastoquinone [60, 74]. Taken together, it could be that activation of qE by zeaxanthin formation is kinetically limited by slow diffusion of xanthophylls through crowded grana to a tethered VDE in dark-adapted samples. This restriction could be reversed in the light by luminal swelling that could lead to acceleration of qE due to higher VDE mobility. Similar conclusions as for VDE can be drawn for luminal *Deg* proteases (molecular weight ~35 kDa [102], ~1.5 times larger than PC) that are involved in degradation of photodamaged PSII (see also FtsH proteases in Sect. 5.2.2.4). Further experiments have to prove these possibilities of dynamic restricted accesses to stacked grana and/or trapping in microdomains of lumen hosted VDE and proteases.

Another process that could be influenced by changes in the luminal width is the assembly/disassembly of the oxygen-evolving complex (OEC) of PSII. It has been

postulated that changes in the luminal width can control the proper assembly of the four subunits that constitute the OEC for catalyzing water splitting [13]. Although the authors assumed shrinkage of the lumen in the light (based on the work of Murakami and Packer, 84], the concept of a dynamic switch in OEC activity controlled by swelling/shrinkage processes is an interesting possibility to regulate PSII activity and in consequence linear electron transport from water to PSI. A related mechanism is the disassembly of photodamaged PSII. It is known that the damaged LHCII-PSII holocomplex in grana dismantles, including the removal of the luminal OEC subunits. This removal may be required to mobilize PSII in stacked grana to make it accessible for its repair machinery in stroma lamellae (see Sect. 5.2.2.2 above). It is likely that this disassembly is facilitated by the expansion of the lumen. So far, this expansion was seen only for moderate light intensities (500 µmol quanta m^{-2} s^{-1}). It has yet to be determined whether this also takes place in high-light stressed plants.

At this point, we can only speculate about the factors that determine changes of the luminal width. A plausible scenario is that light-induced protonmotive force (*pmf)* drives an influx of chloride anions that in turn leads to osmotic swelling of the lumen. Thylakoid membranes contain at least two chloride channels [107]. At least one of these channels is voltage-gated [101]. Thus, the electrical potential gradient ($\Delta\varphi$) generated in the light could activate voltage-gated chloride channels leading to a *pmf*-driven influx of chloride anions into the lumen and to osmotic swelling. The important consequence of this chloride-channel regulated swelling is that the luminal width is not directly light-dependent but rather controlled by $\Delta\varphi$. Any factors that either manipulate the magnitude of the total *pmf* or that alter the partition between the chemical and electrical *pmf* parts would also control the luminal width. For example, metabolic control of the ATPase activity by thiol-modulation of the γ-subunit [69] would not only regulate the magnitude of the trans-thylakoid *pmf* but also structural attributes of the thylakoid system, i.e. the width of the lumen. Thus, there could be an indirect link between regulation of ATPase activity and control of PC-mediated electron transport and VDE-dependent photoprotection.

5.2.2.4   Vertical Destacking of Grana (Fig. 5.1d)

Vertical destacking (i.e. increase in the partition gap) was recently discussed in the context of the PSII repair cycle. It was postulated that widening of the stromal gap allows FtsH proteases to enter the grana region enabling degradation of damaged PSII [43, 123]. The height of the stromal protrusion of the hexameric FtsH proteases is ~6.5 nm [113]. This large protrusion may exclude the FtsH proteases from stacked regions in dark-adapted samples by steric hindrances (Fig. 5.3). Therefore, a significant widening of the stromal gap in light-stressed plants could allow FtsH proteases to access stacked grana regions for swift degradation of damaged PSII [123, 124]. It was also suggested that transversal destacking could be a factor to mobilize damaged PSII in stacked grana to reach its repair machinery in distant stroma lamellae [43]. The effect of vertical destacking on PSII mobility in grana

could be two-fold. First, an increase in the stromal partition gap lowers attractive van-der-Waals forces between PSII in adjacent membrane discs that holds PSII in grana [20]. Second, transversal separation also diminishes steric hindrances since the stromal part of PSII sticks out ~1 nm from the membrane surface. The "effective" protrusion could be even larger due to structured water layers. Thus, steric hindrances on the stroma side could restrict PSII mobility in dark-adapted samples where the partition gap is only 3–4 nm (Fig. 5.3). These considerations make plausible that transversal destacking could facilitate the degradation of damaged PSII localized in stacked grana. According to the "surface charge model" of grana stacking (see Sect. 5.2.1.1) the driving force for separation of grana membranes is an increase in electrostatic repulsion by introduction of negative charges by protein phosphorylation of PSII (and/or LHCII) subunits. Although these ideas describing how widening of the stromal gap can facilitate accessibilities of FtsH proteases and the mobility of PSII are sound, the experimental evidence for a high light induced widening of the stromal gap in stacked grana is weak. There is evidence that grana partially destack under photoinhibitory conditions [43, 58]. But as mentioned above, grana destacking can mean different things and direct evidence for an increase in partition gap under light stress is missing. For example, no cryo-EM data exists on thylakoids from high-light stressed plants.

Another aspect of dynamic changes in grana partition gap concerns the excitonic energy transfer between protein-bound pigments. It was hypothesized that grana stacking allows for transversal energy transfer between LHCII-pigments localized in opposite grana membranes [7]. The significance of this vertical energy transfer from one grana disc across the stromal partition gap to the adjacent membrane disc for light harvesting by PSII is unclear. Experimental evidence supports [118] or contradicts [63] a significant contribution for vertical exciton transfer in grana. Theoretically, it is expected that lateral energy transfer between pigments in the same grana membrane is faster (and consequently more efficient) than the transversal transfer, because the pigment-pigment distances are shorter [63]. Within the membrane, the mean chlorophyll-chlorophyll distance is expected to be 2 nm or shorter as was estimated for adjacent trimeric LHCII in thylakoid membranes [21]. By contrast, the closest transversal chlorophyll-chlorophyll distance in dark-adapted plants is ~4 nm [estimated from [21, 32]. Assuming that inter-protein energy transfer can be described by Förster theory and assuming that pigment-pigment orientation factors and refractive indexes are the same for transversal and lateral exciton transfer pathways, the difference between 2 and 4 nm would reduce the energy transfer rate more than 60 fold. However, we do not have reliable information for the pigment orientation factors and for the refractive index (in particular for energy transfer across the aqueous partition gap) that can change this number considerably. Our recent cryo-EM studies show a tendency for a reduction in the partition gap in plants illuminated under non-photoinhibitory conditions [68]. Thus, there is a possibility for a light induced switch in transversal energy transfer, i.e. that is activated in illuminated samples by narrowing of the adjacent grana discs. This interesting possibility has to be proved by e.g. analyzing the partition gap under different light intensities.

Finally, the vertical stromal distance in grana thylakoids could control the sublocalization of the cyt *b6f* complex. The stromal protrusion of this complex is 1.5–2.0 nm (Dr. William Cramer, personal information). This is an interesting value because it is between the protrusion of PSII ($\sim$1 nm) and PSI ($\sim$3.5 nm). It could be that small changes in the partition gap have significant impact on the sublocalization of the cyt *b6f* complex in thylakoid membranes, i.e. a widening of the gap would give access to stacked grana whereas a narrowing would exclude the cyt *b6f* from this region. This in turn could determine whether the cyt *b6f* complex is involved in linear electron transport (localization in stacked grana) or in cyclic flow around PSI (localization in unstacked thylakoid regions).

5.3   The Supramolecular Level

Structural flexibility is not only apparent for the whole thylakoid membrane system but also realized on the supramolecular level, i.e. for the arrangement of many proteins in membranes. It is interesting that photosynthetic membranes in more ancient autotrophic organisms are less dynamic. For example, LHCs and reaction centers in purple bacteria form relative rigid highly ordered supramolecular assemblies [17]. It is likely that supramolecular flexibility in photosynthetic membranes was an important evolutionary selection criterion to adapt land plants to highly-dynamic terrestrial habitats [82]. For example, sunlight can be an elusive energy source for land plants because its intensity can change by orders of magnitude on time scales ranging from seconds to months. Photosynthetic energy conversion must compensate for these fluctuations. In low light, the absorption of light quanta and its conversion into chemical energy must be optimized to energetically fuel the cellular metabolism of the plant. In contrast, in high light, it could be necessary to switch the system to an energy dissipating state that minimizes severe damage by toxic side reactions of the primary photochemical reactions. The situation in nature is even more complex because plants are integrated in a highly dynamic network of different biotic and abiotic factors that determine different requirements on photosynthetic energy conversion. Thus, flexibility of photosynthetic functionality is central for plants. Recent finding suggest that a main part of the capability for adjustment of photosynthetic performance is realized by dynamic rearrangements of the protein network in grana thylakoids. Before describing the current knowledge on the supramolecular flexibility, it is essential to understand that photosynthetic membranes are densely packed by protein complexes.

5.3.1   Macromolecular Crowding

An important and long known tool to study supramolecular protein arrangements in photosynthetic membranes is EM with freeze-fractured or freeze-etched membranes [111]. In freeze-fracture EM, the lipid bilayer is split into the two monolayers.

Transmembrane protein complexes visibly stick out of the lipid monolayer as small knobs. In early freeze-fracture and freeze-etched EM studies, it was recognized that thylakoid membranes must be densely packed with photosynthetic protein complexes, especially in grana membranes (reviewed in [109]). Recently, the protein packing density was quantified for grana thylakoids [61, 62]. The study showed that ~70% of the membrane area in grana belongs to proteins and only ~30% to the lipid bilayer. Visualization of 70% protein packing density show that there is hardly any lipid space left. Indeed, both theoretical [61] as well as EPR studies with spin-labeled lipid probes [76, 77] indicate that a high fraction of thylakoid lipids (>50%) have direct protein contact (so-called boundary lipids). Thus, photosynthetic energy conversion takes places in an extremely crowded environment. The consequences of macromolecular crowing on the functionality of photosynthetic membranes are less considered so far, although it is known from theoretical considerations (such as percolation theory) that physicochemical membrane properties can be significantly affected by high protein packing densities [98–100]. Some aspects of high protein densities on photosynthetic energy conversion are discussed below. Again, it is interesting to look on related biomembrane systems. The protein/lipid ratio (as a measure of protein crowding) is 0.34 (w/w) in respiratory membranes [15, 29] that is almost identical to thylakoid membranes [61, 77, 85]. Furthermore, EM studies on photosynthetic membranes in cyanobacteria, red algae, purple bacteria, or green algae indicate a similar high protein packing density as in thylakoid membranes of higher plants. It appears that macromolecular crowding is a common structural feature of bioenergetic membranes.

Why are thylakoid membranes so densely packed with proteins? An obvious advantage is that it allows a tight packing of light-harvesting pigments bound to the proteins. On the molecular scale, even full sunlight is a dilute energy source [23]. Therefore, a high concentration of light-absorbing chromophores is a prerequisite for efficient photosynthetic energy conversion. This is realized by macromolecular crowding. However, the situation is more complex since the high chlorophyll concentration in thylakoid membranes (0.3 M) can lead to detrimental excimer formation that leads to an almost complete conversion of collected sunlight energy into heat if the pigments are randomly organized [22]. Energy quenching by high pigment concentrations is avoided by exact chlorophyll positioning in rigid protein scaffolds in LHCs and PSs. The evolutionary optimization of pigment-pigment distances, angles, and dielectric constants within the hydrophobic regions of light-harvesting proteins prevents unwanted energy losses and ensures ultrafast and thus very efficient energy transfer.

Recently, we could identify a further advantage of macromolecular crowding in grana thylakoid membranes [49]. The organization of the PS-II light-harvesting system in grana is modular. That means that multiple layers of LHCII complexes with different binding strengths surround the PSII core [35, 70]. In detail, the PSII core contains two core antenna complexes (CP43 and CP47) that are complemented by minor LHC (CP26, CP29) and one trimeric major LHCII forming the dimeric LHCII-PSII supercomplex. This supercomplex is named C2S2 (C=PSII *core*, S=*strongly* bound trimeric LHCII). Further LHCII-trimers with the minor LHCII

CP24 can be attached to the C2S2 supercomplex forming a C2S2M2 supercomplex (M = *m*edium bound trimeric LHCII) that can further bind up to eight additional weakly attached major LHCII complexes. The consequence of this modular antenna organization is that it critically depends on efficient intermolecular energy transfer between LHCIIs. In our study [49], we diluted the natural high protein packing density by fusing isolated grana membranes with protein-free lipid-liposomes with the natural lipid composition. The data shows that dilution leads to uncoupling of peripheral LHCII trimers not tightly bound to the C2S2 supercomplex and consequently to a reduction in the functional antenna size of PSII. This result indicates that high protein packing densities in stacked grana are required to bring weakly interacting LHCII and PSII in close contact to enable efficient intermolecular exciton energy transfer. Thus, protein crowding is advantageous for light-harvesting by PSII surrounded by its multi-layer light-harvesting system. In contrast to advantages of protein crowding, there can be serious problems associated with high protein packing densities addressed in the next chapters.

5.3.2   Disordered Versus Ordered

Early EM studies in the 1960s on thylakoid membranes revealed that proteins in stacked grana can arrange themselves in highly ordered semicrystalline arrays [94, 95]. In the following decades, numerous publications described this remarkable self-ordering by using electron microscopy (reviewed in [35, 109]). Very recently, semicrystalline PSII arrays were visualized also by atomic force microscopy ([114] and Fig. 5.4). A deeper structural analysis of the protein arrays in grana reveals that they are composed of C2S2 supercomplex [32, 35]. Furthermore, it was recognized that there is variability in the crystal structure. The crystals can have different lattice constants and can also be composed of C2S2M or C2S2M2 supercomplexes [35].

Semicrystaline protein arrays are often seen under non-optimal growth conditions. This includes low temperatures, low light, high sugar concentrations, or special buffers [45, 64, 78, 80, 104, 106, 120]. Under these conditions, the arrays can cover a whole grana disc. The observation that different abiotic factors induce a similar switch from disordered to ordered is significant because it indicates that changes in the protein ordering level in grana could be a universal response to non-optimal growth condition that highlights its physiological relevance. Recently, EM tomography on ruptured chloroplast shows that PSII arrays are also formed under non-stressed conditions but with low abundance [32]. It correlates with the observation that in grana with disordered protein organization, a tendency for a parallel PSII alignment is already apparent [59, 66]. These findings are indicative for a dynamic equilibrium between disordered and semicrystalline states controlled by the environment. This equilibrium could represent a metastable state as suggested recently [70], i.e. small changes can trigger large-scale reorganizations from disordered to ordered and *vice versa*.

The factors that determine the equilibrium between disordered and ordered protein organizations in grana are unknown. One possibility is that properties
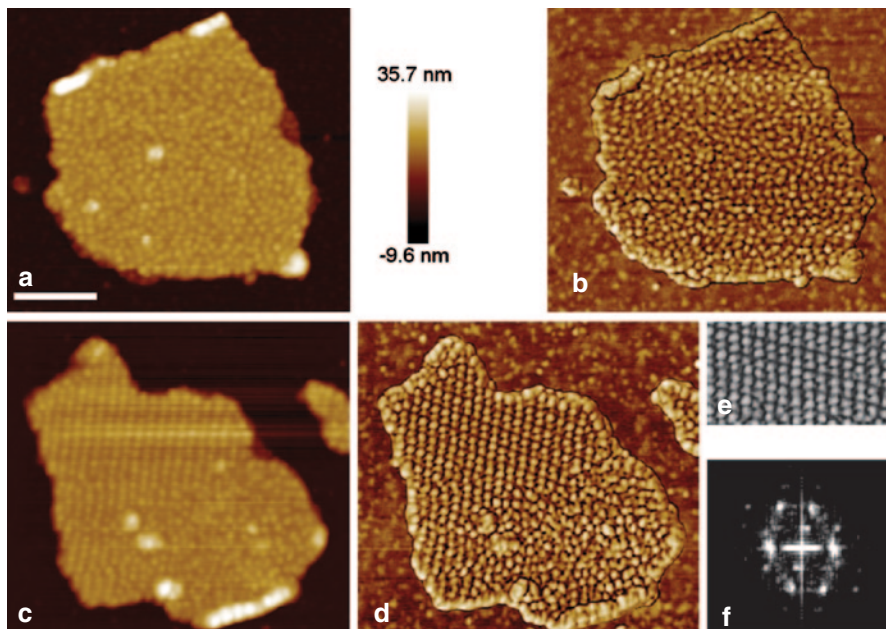
Fig. 5.4 Atomic force microscopic (AFM) micrograph of isolated grana membranes (unpublished results). The micrographs show the topography (**a**, **c**) and the phase image (**b**, **d**) of grana isolated from Arabidopsis. The numerous (*whitish*) particles represent the lumenal protrusion of PSII (mainly the water-splitting complex). Particles are better identifiable in the phase images. PSII rarely organizes into semicrystalline arrays (**c**, **d**, **e**). E is a zoom into the arrayed region. The lattice constants for the semicrystalline arrays can be derived from the Fourier transform (**f**). Scale bar 200 nm

of the lipid bilayer (hydrophobicity, hydrophobic matching, curvature forces) could govern the supramolecular protein organization. A lipid-controlled switch between disordered and ordered shines new light on the xanthophyll cycle (see Sect. 5.2.2.3) because the catalytic formation of zeaxanthin from violoxanthin changes physicochemical properties of the lipid bilayer phase (reviewed in [50]). This would enable control of the xanthophyll-cycle on the supramolecular protein arrangement in grana. Another possibility is that proteins could alter membrane properties. For example, it was suggested that the amount of the *psbS* protein controls the abundance of semicrystalline protein arrays in grana, i.e. the more *psbS* present in thylakoid membranes the more the proteins in grana adopt a disordered configuration [48, 57]. Although this 'inhibitory' effect of *psbS* on semicrystalline array formation is apparent, no mechanistic suggestions how *psbS* controls the supramolecular protein arrangement are around. Interestingly, both zeaxanthin and the *psbS* protein play key roles in photoprotective qE. It was also shown that qE is accompanied by large-scale protein reorganizations in grana [51]. Thus, changes in xanthophylls or *psbS* could trigger supramolecular switches required for photoprotection.

5.3.3 Functional Significance of Protein Ordering

Although macromolecular crowding seems to be required for efficient light-harvesting by the modular organized PSII complex (see Sect. 5.3.1), it can have severe impacts on all processes that require lateral diffusion in the thylakoid membrane system. The mobility of components of the photosynthetic machinery in thylakoid membranes is of vital importance. Not only the small electron carrier PQ has to diffuse through crowded grana to functionally connect PSII and the cyt *b6f* complex (for PC see Sect. 5.2.2.3), but *state transition* and the PSII repair cycle also require lateral traffic of proteins. Finally, thylakoids membranes remodel their protein composition in response to environmental changes. The site of new protein insertion is the stroma lamellae (ribosomes are size-excluded from stacked grana see Fig. 5.3). Therefore, changing the protein composition in grana requires migration over several 100 nm. Although it is intuitively evident that it is easier to laterally transport a molecule in a pure lipid bilayer compared to a bilayer fully packed with diffusion obstacles (proteins), the details of the correlation between obstacle density, size or shape, and diffusion can be very complex. Percolation theory describes this interdependency [98–100] and a main outcome of this theory is that at obstacle area occupations of ~70 %, small enclosed microdomains are formed that restrict long-range diffusion processes. Monte Carlo computer simulations on PQ diffusion [117] and PSII diffusion [62] reveal severe retardation of long-range diffusion for both molecules in accordance with percolation theory. This highlights the potential problem of macromolecular crowding.

An interesting discrepancy is that measurements of protein diffusion in grana by fluorescence recovery after photobleaching (FRAP) is heterogeneous [47, 67] whereas the Monte Carlo simulation show a homogenous very slow diffusion. In FRAP measurements, 15–25 % of granal proteins are moving relatively fast (e.g. they can cross the whole grana diameter in a few seconds). A main difference between simulation and experiment is that the simulation assumes a pure random protein distribution. Thus, it is likely that ordering of the proteins in grana can explain why a certain fraction is diffusing faster than others. It is important to recognize that even in disordered grana, proteins are not purely randomly arranged as demonstrated by mathematical analysis of freeze-fractured EM micrographs [62]. Very recently, we could show that lipid diffusion is faster in a mutant which constitutively forms semicrystalline arrays in grana (unpublished results). These observations point to a role of protein ordering for diffusion processes in grana thylakoids. Protein ordering could be a strategy to ensure high protein packing required for light harvesting by PSII and at the same time allows efficient protein/metabolite (PQ, xanthophylls) traffic required for ET function and regulation processes.

5.3.4 Stroma Lamellae

In contrast to grana thylakoids, stroma lamellae are less crowded by proteins as indicated by a higher lipid to protein ratio (0.64 for stroma lamellae, 0.16 for grana,

[85]). Furthermore, as discussed above, the protein composition is quite different, i.e. stroma lamellae are enriched in PSI-LHCI supercomplexes and ATPase and in addition contain cyt *b6f* complexes and a low amount PSII [2, 111]. In contrast to the pronounced protein heterogeneity between grana thylakoids and stroma lamellae, the four main acyl lipids (monogalactosyl-diacylglycerol, digalactosyl-diacylglycerol, sulfoquinovosyl-diacylglycerol, phosphatidyl-diacylglycerol) are evenly distributed between the two subcomparments [36]. So far, no ordered protein arrays were reported to exist in stroma lamellae indicating that physiochemical and/ or structural properties of proteins in stroma lamellae (i.e. the PSI-LHCI supercomplex) do not facilitate organization in higher supramolecular assemblies. It seems that PSI-LHCI functions as an isolated entity in contrast to PSII-LHCII in grana thylakoids that form extended networks that exchange exciton energy (named connectivity, [52, 71]). Furthermore, compared to PSII in grana the antenna system of PSI is less modular, i.e. most of the 167 chlorophylls of the PSI-LHCI supercomplex are bound to the central *psaa/psab* subunits [10]. Thus, it could be that the missing (evolutionary) pressure to pack PSI and LHCI tightly (as realized for PSII/ LHCII) to ensure efficient light harvesting (see Sect. 5.3.1) leads to lower protein packing densities that is advantageous for diffusion processes.

A striking difference exists for the organization of the ATPase in unstacked thylakoid regions compared to its mitochondrial counterpart. In respiratory membranes, the ATPase is organized as a dimer that in turn can form extended rows of dimers [33, 39, 112]. It has been discussed that this supramolecular ATPase arrangement is important for shaping the overall structure of the inner mitochondrial membrane (cristae formation) and for conversion of the protonmotive force to ATP [33]. No such structural or functional role is reported for the ATPase in stroma lamellae. The comparison between respiratory and photosynthetic membranes shows that very different structural principles are realized that govern the overall membrane architecture. In plants, grana are formed by electrostatic/van-der-Waals interactions between flat surfaces of LHCII (PSII). In respiratory membranes, ATPase dimer formation could exert a bending force to the lipid bilayer that causes cristae formation.

5.4   Outlook

After the first publication of the crystal structure of a photosynthetic protein complex in 1985 [34], the following two decades were dominated by unraveling the atomic structures of all photosynthetic complexes. We are now in the unique position to have an almost complete set of high-resolution structures. There are still essential tasks to do, i.e. no high-resolution structure of the PSII-LHCII supercomplex exists. Until appropriate PSII supercomplex crystals are available that allows generation of near atomic-resolution models, medium-resolution EM in combination with single-particle analysis has proved to be an excellent alternative. However, one challenge for the future is to study how many proteins interact in the intact membrane to form functional networks and to analyze the dynamics of these networks in response to environmental cues. The first glimpse of information we have

on the supramolecular level reveals that thylakoid membranes, although they are densely packed with proteins, are very dynamic and can switch their protein organization from disordered to crystalline. What are the molecular mechanisms that determine these rearrangements? As suggested by Kouřil and co-workers [70], it could be small perturbations of structural or physicochemical parameters that trigger large-scale rearrangements. Since these reorganizations determine photoprotection, electron flow (by PQ and PC), adaption mechanisms (*state transition*), and repair processes (PSII repair cycle), it is a prime task to unravel the determinants for these protein rearrangements.

A further important research field for the future is the development of dynamic supramolecular models for photosynthetic membranes that ideally allow the tracking of individual molecules. Available methods related to this challenge have specific advantages and drawbacks. EM produces high-resolution molecular images but they are static, i.e., they give only a snapshot in time. It is expected that EM tomography will develop in the near future to allow drawing protein landscapes within whole thylakoid membranes. However, EM tomography will not give access to the dynamics of the protein assemblies. Conventional and high-speed AFM can visualize molecular mobility with a certain time resolution. However, AFM probes only surfaces and does not allow to track proteins in stacked grana of intact thylakoids. Methods that measure protein dynamics (diffusion) like FRAP do not have the resolution to visualize single molecules. A further drawback with FRAP is that it is established only for measuring bulk chlorophylls that does not allow to distinguish different protein types, i.e. between LHCII and PSII. Therefore one future task will be the design of specific labels for individual protein complexes that can be applied to high-resolution light microscopy, e.g. single particle tracking. However, although establishing this advanced light microscopic methods for photosynthetic membranes would be a technological breakthrough, they do not allow tracking of a whole protein assemble. A possible way to solve these limitations is to combine ultrastructural data (EM, AFM) and diffusion data in a dynamic computer simulation program. If the output parameters of these simulations are chosen carefully, i.e. that they can be tested by measurements, the combination between simulation and experiment could be extremely powerful.

The enormous progress made in the last few years with EM tomography are very encouraging and promise to study the thylakoid membrane architecture in intact chloroplasts. This tool would be very valuable to address the multiple questions raised in this review on the overall changes in grana architecture triggered by environmental challenges. The combination of ultrastructural data with functional measurement will allow unraveling dynamic structural attributes of the thylakoid system and how it affects photosynthetic performance. This will be the basis for designing strategies to improve plants for producing food and energy.

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