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Published in:
Biochimica et Biophysica Acta

DOI:
[10.1016/j.bbabi.2011.05.024](https://doi.org/10.1016/j.bbabi.2011.05.024)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

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Citation for published version (APA):
Kouril, R., Dekker, J. P., & Boekema, E. J. (2012). Supramolecular organization of photosystem II in green plants. *Biochimica et Biophysica Acta*, 1817(1), 2-12. <https://doi.org/10.1016/j.bbabi.2011.05.024>

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Review

Supramolecular organization of photosystem II in green plants[☆]Roman Kouřil^a, Jan P. Dekker^{b,*}, Egbert J. Boekema^a^a Electron microscopy group, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands^b Faculty of Sciences, Division of Physics and Astronomy, VU University Amsterdam, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands

ARTICLE INFO

Article history:

Received 17 March 2011

Received in revised form 23 May 2011

Accepted 26 May 2011

Available online 23 June 2011

Keywords:

Photosystem II

Supercomplex

Thylakoid membrane

Electron microscopy

Tomography

ABSTRACT

Green plant photosystem II (PSII) is involved in the light reactions of photosynthesis, which take place in the thylakoid membrane of the chloroplast. PSII is organized into large supercomplexes with variable amounts of membrane-bound peripheral antenna complexes. These supercomplexes are dimeric and contain usually 2–4 copies of trimeric LHCII complexes and have a further tendency to associate into megacomplexes or into crystalline domains, of which several types have been characterized. This review focuses on the overall composition and structure of the PSII supercomplex of green plants and its organization and interactions within the photosynthetic membrane. Further, we present the current knowledge how the thylakoid membrane is three-dimensionally organized within the chloroplast. We also discuss how the supramolecular organization in the thylakoid membrane and the PSII flexibility may play roles in various short-term regulatory mechanisms of green plant photosynthesis. This article is part of a Special Issue entitled: Photosystem II.

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1. Introduction

Photosystem II (PSII) is one of the key proteins of the light reactions of photosynthesis. It is universally distributed throughout prokaryotes and eukaryotes capable of oxygenic photosynthesis, including higher plants, macroalgae, diatoms, dinoflagellates, and the oxyphotobacteria (cyanobacteria and prochlorophytes), but not in archaea [1]. In this review we will focus on the green plant system. The function of PSII and its role in the light reactions of photosynthesis cannot be understood without a detailed knowledge of its structure, including its peripheral antenna. Over the last two decades a substantial effort has been put on solving medium (4–10 Å) to high-resolution (<3 Å) structures of intact PSII complexes [2–7], single subunits [8–12] and light-harvesting complex II (LHCII) [13,14], which forms part of the peripheral antenna. Most of the high resolution information, however, has been obtained with cyanobacterial PSII complexes, which are generally more stable than the complexes from plants. There is also an increasing emphasis on the interaction of these complexes into higher order associates, like supercomplexes. This is not unique for the chloroplast, since also in the (plant) mitochondrion the existence of several supercomplexes has been described, such as a dimeric form of the ATP synthase [15] and a supercomplex consisting of monomeric complex I and dimeric complex III [16].

A good understanding of the reactions performed by PSII does, however, not only require a deep knowledge of its structure including the peripheral light-harvesting complexes, it requires also knowledge

of how PSII works together with the other main protein complexes such as photosystem I (PSI) and the cytochrome *b₆f* complex in the photosynthetic membrane to perform the primary light reactions. This is particularly relevant for the light-harvesting function and its regulation. During evolution green plants have adapted different strategies to develop and survive in very diverse environmental conditions characterized by high light or low light, strongly fluctuating light conditions, conditions in which the incoming light is spectrally altered due to shading by water or other photosynthetic organisms or certain types of stress conditions.

To understand how PSII works in the photosynthetic or thylakoid membrane we have to study this membrane in detail within the intact chloroplast. The thylakoid membrane forms a physically continuous three-dimensional network that encloses a single aqueous space, the thylakoid lumen. A characteristic feature of this membrane is its extensive folding. As a consequence, the thylakoid membranes of vascular plants and some green algae are structurally inhomogeneous. They consist of two main domains: the grana, which are stacks of thylakoids, and the stroma lamellae, which are unstacked thylakoids and connect the grana stacks. Three-dimensional models of the spatial relationship between grana and stroma thylakoids show that PSII and LHCII reside mainly in the grana membranes, while PSI and ATPase reside predominantly in the stroma and the cytochrome *b₆f* complex is distributed about evenly between the two types of membranes. It is important to realize that protein–protein interactions determine for a major part the shape and folding pattern of the thylakoid membrane [17]. In other words, we need to know the precise interactions of PSII with its neighbors within the intact chloroplast to fully comprehend how the light reactions of photosynthesis occur under changing light and environmental conditions.

[☆] This article is part of a Special Issue entitled: Photosystem II.

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This review focuses on the overall composition and structure of the PSII supercomplex of green plants and its organization and interactions within the photosynthetic membrane. Further, we present the current knowledge how the thylakoid membrane is three-dimensionally organized within the chloroplast and finally how this organization can play roles in the various photosynthetic regulation mechanisms.

2. Molecular organization of photosystem II

2.1. Peripheral antenna

In higher plants, the peripheral antenna consists of a number of pigment–protein complexes belonging to the Lhc super-gene family [18]. Two types of peripheral antenna proteins associated to PSII can be distinguished. The most abundant complex is the so-called “major” LHCII antenna complex. This complex occurs in a trimeric oligomerization state and consists of various combinations of three very similar proteins, encoded by the Lhcb1, Lhcb2 and Lhcb3 genes, which usually occur in a ratio of about 8:3:1 [19]. In addition, there are three “minor” antenna complexes, which are called Lhcb4 (CP29), Lhcb5 (CP26) and Lhcb6 (CP24) and usually occur in monomeric states. All these complexes bind various pigment molecules and the high-resolution structures of the major trimeric LHCII complex and CP29 have been solved [12–14]. It is likely that the minor complexes CP26 and CP24 adopt rather similar overall three-dimensional organizations.

2.2. PSII–LHCII supercomplexes

A variable number of peripheral antenna proteins can associate with dimeric PSII core complexes to form PSII–LHCII supercomplexes [17]. These supercomplexes have been denoted according to their composition. A dimeric core, C₂, can associate with up to four copies of peripheral LHCII trimers. Connection of the first two LHCII S-trimers extends a C₂ complex to a C₂S₂ supercomplex, and two further M-trimers are bound in a C₂S₂M₂ supercomplex [20]. Spinach supercomplexes may bind a third type of L-trimer, but the resulting C₂S₂M₂L_{1–2} supercomplexes are rare. The C₂S₂ supercomplex contains almost all PSII core proteins [21], but from the peripheral antenna the Lhcb3 and CP24 proteins appear to be absent [22]. A three-dimensional structure of this supercomplex from spinach was constructed using a low-resolution 3D electron density map obtained

by single particle cryo electron microscopy [23]. A recent analysis concerns the larger, C₂S₂M₂ supercomplex of *Arabidopsis thaliana* and shows details at 12 Å resolution [24], permitting a more accurate fitting of the peripheral antenna proteins, based on the known LHCII structure. The combination of high-resolution structures of components with the low-resolution provided by electron microscopy is useful because the precision of fitting an X-ray structure into an EM density map is much better than the resolution of the EM data [25]. The model is presented in Fig. 1 and shows how within the PSII–LHCII supercomplex the innermost LHCII S-trimer is attached to a dimeric PSII via CP29, binding to one PSII core monomer and CP26 to the other. Indeed, monomeric cores with a full set of peripheral antenna particles have never been observed in disrupted grana, although recently a particle consisting of a monomeric core plus CP26 and one LHCII trimer was found in *Arabidopsis* [24]. The need for dimeric core configuration also explains why the peripheral antenna proteins easily detach from PSII during the transition from dimer to monomer. No intact supercomplexes could be isolated from *Arabidopsis* plants expressing antisense constructs to CP29 [26], although recently small numbers of PSII–LHCII supercomplexes with empty Lhcb4 binding sites could be found [S. de Bianchi, N. Betterle, R. Kouřil, S. Cazzaniga, E. J. Boekema, R. Bassi and L. Dall’Osto, Plant Cell, in press]. This suggests that CP29 occupies a unique position in the PSII macrostructure and that in contrast to CP26 its presence is essential for the formation of PSII–LHCII supercomplexes. Both the CP29 and CP26 antisense mutants showed a rather normal photosynthetic performance, although the mutants showed slightly different fluorescence characteristics and an increased number of PSII centers [27]. This suggests that the organization of PSII and LHCII into supercomplexes is not absolutely required for photosynthetic performance, at least under normal physiological conditions and light levels.

A more peripheral LHCII trimer (M-trimer) is attached via CP24 and CP29. CP24 is necessary for binding the M-trimer because *Arabidopsis* plants depleted of CP24 do not form C₂S₂M₂ supercomplexes [28]. In the green alga *Chlamydomonas reinhardtii*, which lacks CP24, only C₂S₂ supercomplexes could be detected [17]. Some small core subunits, such as PsbW, are also necessary for intact supercomplexes [29], but their localization is not yet clear.

Analysis of supercomplexes isolated from *Arabidopsis* plants expressing an antisense construct to Lhcb2 revealed that the LHCII binding sites are not unique for the various types of trimers [30]. In these plants, not only was the synthesis of Lhcb2 almost completely

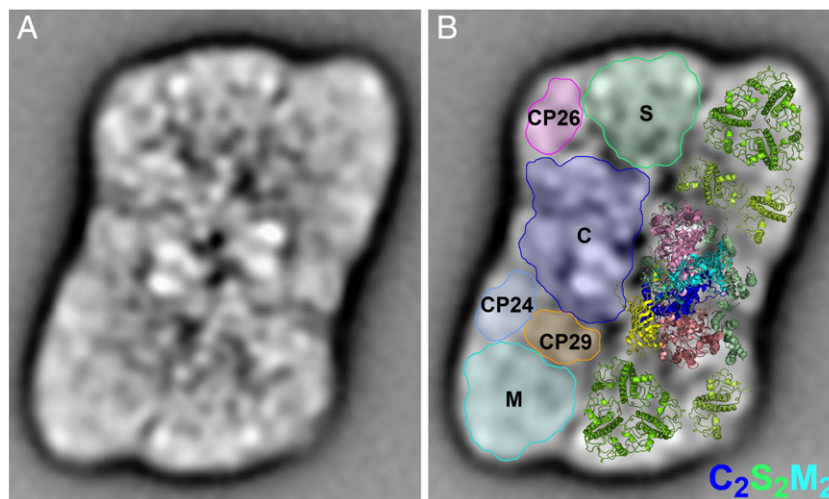


Fig. 1. Projection map and a structural model of the PSII C₂S₂M₂ supercomplex. (A) Top view projection map of the C₂S₂M₂ supercomplex from *Arabidopsis thaliana* obtained from single particle electron microscopy. (B) Assignment of the subunits of the supercomplex by fitting the high-resolution structures of PSII core [7] (subunits D1, D2, CP43, CP47 and extrinsic subunit PsbO are highlighted in blue, cyan, salmon, pink and yellow, respectively) and Lhcb [13] (trimeric LHCII and monomeric Lhcb in dark and light green, respectively). PSII core monomer, “C”-, “S”- and “M”-type of LHCII trimers and the minor antennas, CP24, CP26 and CP29, are schematically depicted in blue, light green, cyan, light blue, magenta and orange contours, respectively. Adapted from Ref. [24].

abolished, but also that of the strongly related Lhcb1 protein [31]. It appeared that in these plants, the expression of the antisense Lhcb2 construct resulted in strongly increased levels of CP26 and (to a minor extent) Lhcb3, and that supercomplexes were formed with trimers consisting of Lhcb5 and Lhcb3 at the S- and M-binding positions [30]. This replacement is unique, because expression of antisense constructs to the minor peripheral antenna proteins and to the peripheral antenna proteins of PSI did not lead to increased synthesis of other proteins, and stresses the importance of the particular organization of PSII and LHCII in supercomplexes.

2.3. Energy transfer

The recent structures of PSII–LHCII supercomplexes allow a more detailed description of the energy transfer routes from the peripheral antenna to the core antenna and the reaction center. Fig. 2 shows the chlorophylls and carotenoids of one half of the $C_2S_2M_2$ supercomplex, based on data reported in Ref. [24], with chlorophylls *a* in green and chlorophylls *b* in blue to point out the major routes of energy transfer between the pigment–protein complexes, based on a relatively close proximity of chlorophylls *a* in adjacent complexes (arrows Fig. 2). Chlorophylls *b* are probably only to minor extents involved in energy transfer between complexes, because their energies are at least $2 k_B T$ higher than those of chlorophylls *a*. The figure shows that within each PSII core monomer, there are two major routes of energy transfer, i.e., from CP47 to the RC and from CP43 to the RC (yellow arrows), and that within the supercomplex there are also two major routes of energy transfer to the PSII core (blue arrows). The first route goes from S-LHCII and/or CP26 to CP43 and the PSII core monomer on the left in Fig. 2, the other route goes from M-LHCII and CP24 via CP29 to CP47 and the other PSII core monomer on the right in Fig. 2. Along the sides of the PSII core, there is a rim of pigment-free low-molecular mass proteins (indicated by grey bars in Fig. 2), which prevents a close

proximity of chlorophylls between neighboring complexes and therefore makes energy transfer through this part of the structure less likely. This means that energy transfer to the supercomplexes within the membrane will predominantly flow through the peripheral antenna (green arrows) and not among dimeric PSII core complexes. This structural arrangement could be advantageous for the effectiveness of regulatory processes of light harvesting (such as high-energy quenching, see below).

3. Organization of supercomplexes in stacked grana membranes

There is quite some variation in the numbers and distribution of the PSII supercomplexes over the grana membranes. Most grana membranes show a rather random organization (Fig. 3A), but in some conditions, which are still not exactly understood, large parts of the membranes occur in semi-crystalline domains and row-like associations (Fig. 3B, C). The formation of semi-crystalline domains in the membrane requires a physical separation of PSII supercomplexes from the other membrane proteins, e.g. free LHCII proteins or PsbS (see also the Section 5.2), which can hamper a specific ordering of PSII supercomplexes into 2D arrays. However, the forces which lead to the protein separation, remain to be elucidated. It is likely that the ordered configuration is energetically favorable and that the random configuration is entropically favorable, and so it is possible that the network of forces at work in the membranes is balanced in such a way that relatively large structural changes (from disorder to order and vice versa) become possible with minimal energetic changes, thus allowing functional modifications.

Crystalline 2D arrays of dimeric PSII have been observed decades ago after freeze-etching and freeze-breaking of green plant photosynthetic membranes (see Refs. [17,22,32] for recent reviews). There is quite some variation in these crystalline arrays, which depends on factors like temperature and biochemical composition of the grana membranes. At least three types of supercomplexes can form the crystals (C_2S_2 , C_2S_2M and $C_2S_2M_2$), see Ref. [17] and moreover, these different types of particles can arrange in several different types of packing, which means that the interaction with neighbors is different. A recent novel type of packing of $C_2S_2M_2$ found in *Arabidopsis* is presented in Fig. 3D. The relevance of such crystals was not clear until recently when Kirchhoff et al. could relate the crystals to light conditions [33]. They found a remodeling of the photosynthetic machinery induced by growing spinach plants under low light intensities. This caused an up-regulation of light-harvesting complexes and down-regulation of PSII and cytochrome *b₆f* complexes in intact thylakoids and isolated grana membranes [33]. The antenna size of PSII increased by 40–60% as estimated by fluorescence induction and LHCII/PSII stoichiometry. These low light-induced changes in the protein composition were accompanied by the formation of ordered PSII complexes in grana thylakoids as detected by electron microscopy. They suggested that the supramolecular reorganization in ordered arrays in low light grana thylakoids is a strategy to overcome potential diffusion problems in this crowded membrane [33].

A further variation in the organization of supercomplexes is caused by the occurrence of pairs of supercomplexes. Image analysis of these pairs has indicated that the supercomplexes can laterally associate to each other in rather specific ways to form megacomplexes. In spinach three types of megacomplexes (dimers of supercomplexes) have been observed thus far, while in *Arabidopsis* a fourth type was found [17]. Some of these megacomplexes appear to be similar to two building blocks within the crystalline arrays [17].

Finally, the higher association of supercomplexes is not restricted to one grana membrane. It appeared that ordered PSII arrays are formed in two adjacent membranes, which are in a specific contact through their stromal sides. There are preferential angles between rows in opposing membranes, both in spinach and in *Arabidopsis*

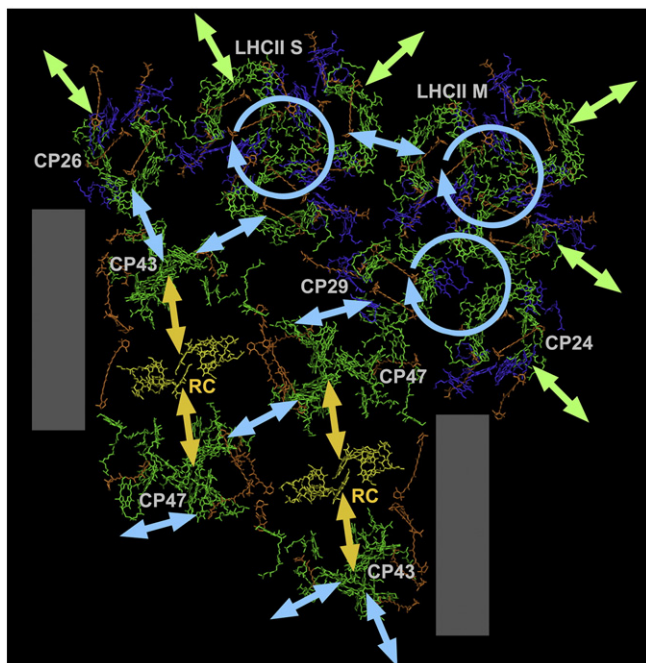


Fig. 2. Energy transfer routes within the PSII $C_2S_2M_2$ supercomplex. The chlorophylls and carotenoids of one half of the $C_2S_2M_2$ supercomplex are depicted, with chlorophylls *a* in green, chlorophylls *b* in blue and carotenoids in orange. The six central chlorophylls and pheophytins of the PSII RC are depicted in yellow. The arrows indicate major routes of energy transfer between (i) the RC and the core antenna proteins CP43 and CP47, (yellow arrows), (ii) within the supercomplex (blue arrows), and (iii) to or from possible neighboring (super)complexes within the membrane (green arrows). Grey bars indicate a rim of pigment-free low-molecular mass protein subunits. The model is based on data reported in Ref. [24].

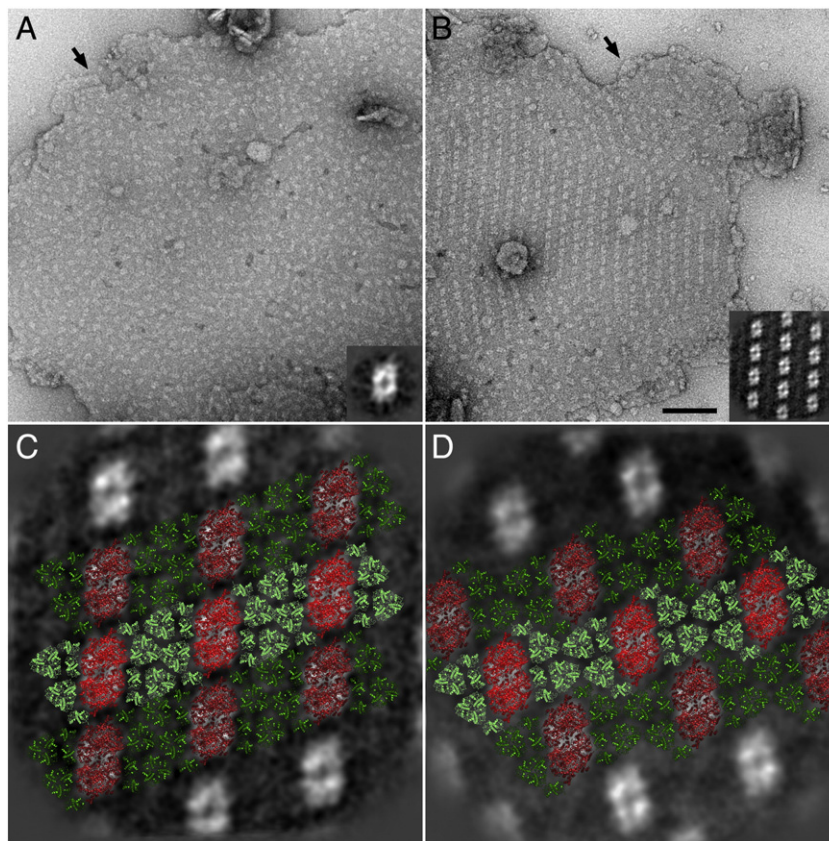


Fig. 3. The higher organization of PSII complexes in the thylakoid membrane. Examples of electron micrographs of negatively stained pairs of thylakoid membranes isolated from *Arabidopsis thaliana* with either a random (A) or ordered (B) organization of PSII complexes. Detergent treatment removed the margins; the black arrows point at remaining single membranes at the periphery. The inset in (A) shows the result of single particle analysis of 224 PSII particles selected from the electron micrograph. The projection map shows a typical density of the PSII core complex. The inset in (B) shows the result of single particle analysis of 96 sub-areas of ordered arrays of PSII complexes selected from the electron micrograph. (C) Assignment of the 2D array of PSII from (B) by the fitting of the structural model of the PSII $C_2S_2M_2$ supercomplex according to Ref. [24]. (D) A novel type of packing of the PSII $C_2S_2M_2$ supercomplexes. Scale bar for the electron micrographs in (A, B) is 100 nm.

[17,34]. At these angles the overlap of LHCII trimers is optimized, at least for the central part of the domains. For spinach the preferential angles were about 3° and 46° [35] or about 90° [34], and for *Arabidopsis* they were about 32° and 58° [36]. The fact that optimal angles are different for these two plant species is related to different basic units (C_2S_2M and $C_2S_2M_2$, respectively). These crystals indicate that the organization of the complexes in one membrane affects the organization of complexes in the opposing membrane and in particular, the preferential angles induce a strong overlap of LHCII trimers of adjacent layers (Fig. 4).

Much of the earlier work on the organization of PSII–LHCII supercomplexes in grana membranes was performed on detergent-derived membrane fragments, the so-called BBY preparations [37]. It was recently shown that the protein packing ratio in these membranes is about 80%, which is slightly higher than the ratio of 70% found in intact grana thylakoids [38], and that dilution of BBY membranes with lipids down to the ratio of 70% resulted in an increased antenna size and enhanced connectivity between reaction centers. Further dilution resulted in a functional disconnection between PSII and LHCII, suggesting that a packing ratio of about 70% brings about optimal photosynthetic performance, by keeping on one hand the distance between the various antenna and PSII core complexes as short as possible to allow fast excitation energy transfer kinetics, and on the other to allow sufficient structural flexibility to allow long-range transport of plastoquinone and to respond optimally to short-term physiological adaptations (e.g., high-energy quenching, state transitions, D1 repair).

In line of this, it was recently shown that excitation energy transfer in PSII is somewhat slower in intact thylakoids than in isolated PSII

membranes [39], suggesting on average a larger distance between PSII and a part of the peripheral antenna in physiological conditions. It was furthermore shown that PSII fluorescence lifetimes were similar in wild-type thylakoids and in thylakoids obtained from a mutant lacking CP26, whereas longer PSII fluorescence lifetimes were observed in mutants lacking CP29 or CP24 [39]. It was discussed above that mutants lacking CP26 retain the supramolecular organization of PSII and LHCII, whereas in mutants lacking CP29 the supercomplexes are much less stable [26], so this result suggests that an organization of PSII and LHCII in supercomplexes facilitates the transfer of excitation energy to the PSII core complexes. The absence of CP24 prevents the formation of $C_2S_2M_2$ supercomplexes but retains the possibility to form C_2S_2 supercomplexes [28], suggesting that in particular the additional M-LHCII–CP24–CP29 energy transfer route (Fig. 2) is important for allowing fast energy transfer between peripheral and core antenna.

4. Overall chloroplast membrane topology

The thylakoid membrane of higher plants forms a very characteristic network consisting of grana stacks interconnected by stroma thylakoids. The 3D architecture of the granum–stroma assembly and the entire thylakoid membrane network is intriguing, especially because of the ability of the membranes to undergo reversible changes in folding and organization under varying environmental conditions [40,41]. In the next sections we will focus on the most recent efforts to determine the overall 3D organization of the thylakoid membranes, of which the capacity to form grana stacks is a well-known aspect. In a previous review we discussed several aspects of the stacking, such as the factors

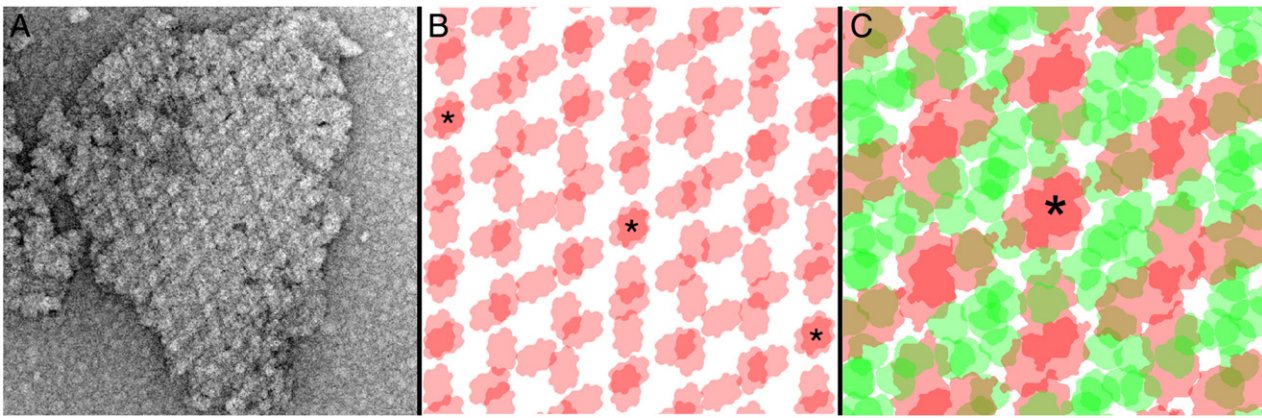


Fig. 4. Example of preferential stacking in paired inside-out grana membranes from *Arabidopsis thaliana*. (A) Electron micrograph of a negatively stained grana fragment of a 58°-type in which the layers make an angle of about 58°. (B) Simulation of the overlap pattern in the 58°-type crystal, based on 150 averaged crystals [36]. Asterisks indicate positions where PSII core complexes (in red) of adjacent layers match optimally. (C) The specific positions of the core complexes of two layers cause a strong local overlap of part of the LHCII trimers (light green).

that sustain the grana and stroma division the protein distribution within the stacks and the location of the cytochrome *b₆/f* complex [17]. Some are worth to repeat briefly, to better appreciate novel findings about changes in response to stress and other factors.

One of the main consequences of stacking is the physical separation of PSI and PSII. The stacking not only prevents spill-over of excitation energy, but it also provides the chloroplast the means to fine-regulate the light requirements for photosynthesis and provides PSII a very large functional antenna, in which excitation energy can flow within a thylakoid membrane and between two stacked membranes until an 'open' PSII reaction center is found. In addition, it provides an easy means to adapt to low light conditions, in which both the amount of LHCII and the extent of stacking have been shown to increase [17]. It is also possible that a physical separation of PSII and PSI is required to fine-tune the balance between linear and cyclic electron transport, as discussed before in more detail [17].

4.1. Architecture of chloroplast thylakoid membrane

Despite considerable efforts to get a detailed view of the 3D organization of the thylakoid membranes using different experimental approaches (employing conventional electron microscopy, state-of-the-art cryo electron tomography or atomic force microscopy), there is not yet a consistent view of the overall membrane architecture. Discrepancies in the interpretation of structural data can be caused by technical limitations of different experimental approaches and thus in the resolution of 3D reconstructions, or by the flexibility of the plant material. A major obstacle is the size of the entire thylakoid membrane system within an intact chloroplast. This size is just exceeding the limits of the penetration power of a medium-voltage transmission electron microscope and thus makes their direct visualization in an intact form unachievable. Analysis of either thin sections or partially solubilized thylakoid membranes can be applied to overcome the sample thickness limitation [42], however, at the cost of loss of intactness of the 3D membrane architecture.

In the last 40 years, several models of the thylakoid membrane organization have been proposed (see Ref. [43]). Two of them, the helical model and the fork or folded membrane model (Fig. 5), can be discussed as appropriate models of the thylakoid membrane architecture.

4.1.1. Helical model

In the helical model, which was derived from EM studies of thin sections and serial sections of chemically fixed thylakoid membranes [44], the tilted stroma thylakoids are wound around the granal stacks in the form of multiple right-handed helices, where each granum

thylakoid is connected to an average of seven stroma thylakoids (Fig. 5A). The helical arrangement of the stroma thylakoids around grana was further supported by other electron microscopy techniques including scanning electron microscopy [45] and freeze-fracture EM [46]. More recently further evidence came from a number of electron tomography studies: (i) on serial sections of chemically fixed membranes [47], (ii) on high pressure frozen and freeze-substituted sections of chloroplasts [48], and (iii) on high pressure frozen vitreous sections of isolated chloroplasts and thylakoid membranes [34]. In addition, these new tomography data revealed new and unexpected features. For instance, the periodicity of the connections between the stroma and grana thylakoids appeared to be more variable than previously proposed. Furthermore, there are fewer connections between one particular granum thylakoid and its surrounding stroma thylakoids [48]. Also the number of connected grana thylakoids in a stack by one spiral stromal thylakoid seems to be lower than previously thought [34,48]. Finally, dimensions of the connections between the stroma and grana thylakoids were found wider and also more variable in size [34,48]. Thus, their possible role in a regulation of the flow of ions, membrane molecules and proteins between stroma and grana thylakoids was suggested [48]. On the other hand, the observed variable dimensions of membrane connections can be accidental and thus these variations do not necessarily point to a regulatory role.

4.1.2. Fork model

In the fork or folded membrane model, which was originally proposed to illustrate the lateral heterogeneity in the distribution of PSII and PSI in the thylakoid membrane [49], the stroma membranes bifurcate and make a fork, which connects two adjacent grana thylakoids within a stack (Fig. 5B). The model became popular in the field especially due to its ability to illustrate in a simple and understandable way a principle of a dynamic reversible folding/unfolding of the thylakoid membrane [50]. Although the proposition of the fork model was not based on direct structural data, the fork motif was observed in many micrographs, including those of thin serial sections, which were, however, interpreted in favor of the helical model, due to a low depth resolution of serial sectioning [47]. Strong evidences for the existence of the fork at the granum–stroma assembly were provided by cryo tomography on cryo immobilized and freeze-substituted sections of intact leaves [42] and high pressure frozen vitreous sections [34]. The former work further indicates that the adjacent grana membranes fuse both at the margin of and within grana stacks, which is important for granum formation [42]. However, the formation of such membrane bridges would put significant constraints on the reversible membrane folding/unfolding. Structural

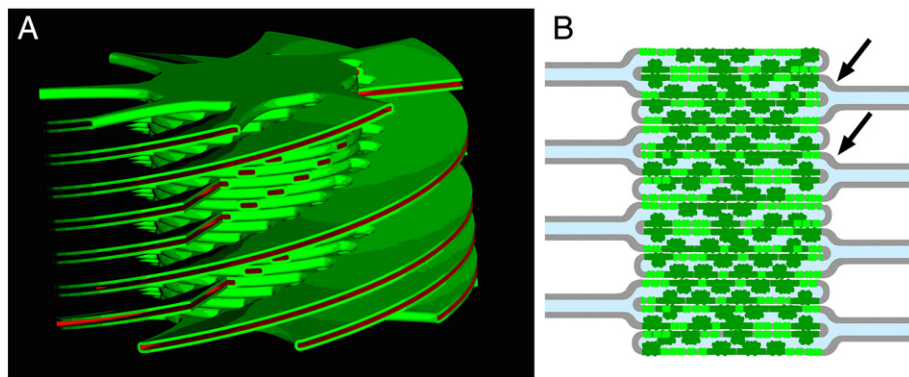


Fig. 5. Two current models for the 3D organization of thylakoid membranes in plant chloroplasts. (A) The helical model (from Ref. [43]). (B) The fork model (based on earlier drawings first presented in Ref. [49]) with a schematic presentation of PSII and LHCII trimers in dark and light green, respectively. The black arrows indicate a fork formation.

studies of thylakoid membrane rearrangements during state transitions indicate that a significant alteration of the membrane organization is accompanied by a reversible membrane fission and fusion [51]. Interestingly, recent tomography data suggest the existence of tubular interconnections between adjacent parallel stroma thylakoids [48].

4.1.3. Conclusions of overall 3D structure

In the light of these novel findings, it is evident that our current structural models are still rather limited and there is a clear need for further improvements of our structural knowledge of the thylakoid membrane architecture to better understand dynamic changes in the membrane folding. Earlier models of the thylakoid membrane organization, which have been obtained using conventional microscopy techniques, are now challenged by new tomography data [47,52,53]. Undoubtedly, a study of intact frozen-hydrated samples (free of fixation artifacts) using cryo electron tomography on a high-voltage electron microscope is an approach that could shed light on the grana structure, the granum–stroma assembly and the grana dynamics, because it visualizes the protein complexes within the membranes and therefore can provide insight in the molecular interactions that underlie the membrane architecture. However, any further refinement of current models will depend very much on the performance of electron microscopy hardware to reach better data acquisition and on perfecting tomography reconstruction techniques, necessary to image complete chloroplasts.

4.2. Dynamic rearrangements within the grana stacks

Besides the enigmatic 3D organization of the entire thylakoid membrane, the individual grana stacks possess other intriguing features. Firstly, although the diameter of single grana discs usually varies within a rather narrow range between 300 and 600 nm [43,54], the extent of grana membrane stacking is highly alterable under different and often varying environmental conditions [55]. Shade-type plants are characteristic for much broader grana with more stacked thylakoids per granum compared to sun-type plants [56]. Even a short exposure of plants to a lower light intensity results in a loss of small grana and formation of larger ones. The opposite is observed under a higher light intensity, although the total changes of the grana membrane stacking (a grana disc area) did not exceed 20% [57]. Surprisingly, a high light treatment induced a similar effect on grana membrane stacking, which was observed under a low light intensity. The observed effect of light quantity on the grana membrane stacking was attributed to a structural alteration that occurs in the thylakoid membrane during state transitions, as they were found to be controlled by the light intensity, not exclusively by a spectral light quality [58]. However, further studies showed that state transitions induced by light of a specific spectral composition can

bring about a structural rearrangement of the thylakoid membrane even at a larger scale [51]. It is also intriguing that the extent of *in vitro* thylakoid membrane unstacking can be further enhanced by e.g. low salt conditions or elevated temperature, when a complete reversible unstacking of the thylakoid membrane can be observed [50,54,59,60]. It clearly indicates that the thylakoid membrane represents a dynamic system, which can undergo under different conditions of highly variable structural rearrangements. It remains to be answered whether only one or more factors are involved in a regulation of the induced membrane rearrangements.

Secondly, the vertical distances between the stacked grana membranes are highly variable [41]. On the stromal side, the grana membranes are very flat, as their protein constituents (PSII, LHCII, cyt b_6/f complex) do not possess any protruding stromal domains. These structural features allow a very close stromal contact between two adjacent layers, which facilitates the stacking of these membranes (reviewed in Ref. [17]). The width of the stromal gap, estimated from thin sections of plastic-embedded chloroplasts, was found to vary between 2 nm and 4 nm. A value of 2.6 nm was determined from an atomic force microscopy study [61], which seems to represent a situation of displaced organization of PSII complexes in two adjacent membranes. Analysis of cryo tomography data indicates an average width of the stromal gap 3.2 nm [34], which most likely represents a spatial limit for a juxtaposition of PSII complexes. Calculation of electrostatic surfaces from the X-ray structure of LHCII [14] and their fitting into the tomography data proved that the stromal gap of 3.2 nm is sufficient to accommodate N terminal peptides of two adjacent LHCII trimers upon their interdigitation [34]. In contrast, at the luminal side, the distance between the membranes is (i) much larger due to the presence of large membrane-protruding subunits of the PSII core and cytochrome b_6/f complex and (ii) much more variable. The lumen width derived from plastic-embedded thin-sectioned chloroplasts varies roughly from 6 to 16 nm (reviewed in Ref. [17]). Recently, cryo tomography data reported a lumen width within the above range [42,62], although a tighter contact of about 5 nm was also reported in cryo tomography [34] and an atomic force microscopy study [61]. The observed variation is related to the light-induced acidification of the lumen, which was accompanied by a shrinking of the lumen volume [34,41,63,64]. However, the observed effect of light on the lumen width seems to represent a more complex response, as the opposite light effect was observed during state transitions. Under state 2 light conditions, a swelling of the lumen volume up to about 24 nm was observed [51], which strikingly differs from e.g. ~5 nm lumen width observed in plants adapted to growing light conditions [34]. It was hypothesized that the observed variation of the lumen volume is mediated by the light-induced structural rearrangement of the oxygen-evolving-complex (OEC) of PSII [65], which represents the largest membrane-protruding domain at the luminal side and thus the main spatial barrier determining the lumen

space. Under optimal light conditions, when PSII is photochemically active, a fully assembled OEC complex can closely interact in the lumen with the OEC of the opposite PSII complex. Under unfavourable light conditions, which lead to an inhibition of the photochemical activity of PSII complex, the OEC complex and its interaction with the opposite OEC are destabilized, which results in an expansion of the lumen. Namely PsbQ, an extrinsic subunit of OEC of PSII, was proposed to be a key subunit involved in the above-mentioned interactions as well as in the structure and dynamics of grana membrane stacking [65]. However, considering the height of OEC complex (about 4.5 nm), the hypothesis can only explain a shrinkage of the lumen up to 9 nm for a juxtaposition of PSII complexes. Reported values of the lumen width under 9 nm have to indicate a displacement of OECs of opposing PSII complexes. Although a mechanism and reason of the luminal volume variation is not fully understood yet, shrinkage of the lumen space could severely hinder the mobility of the complexes in both membranes [61] and also limit the available volume for luminal proteins. On the contrary, a lumen swelling under e.g. state 2 conditions [51] could facilitate a lateral diffusion of phosphorylated LHCII in the grana membrane. The finding of novel densities attached to the luminal side of LHCII trimers in supercomplexes (see below in Section 4.3.2 and Fig. 6) further constrains possible large-scale shrinkage of lumen widths.

4.3. Higher organization of PSII *in situ*

4.3.1. Possibilities and limitations of freeze-fracture EM and AFM

The possibility of studying the 3D organization of photosynthetic proteins *in situ*, within the thylakoid membrane has been a long standing dream, because it would give an opportunity to obtain information about their distribution and their higher organization. As will be explained in the next section, this possibility was only realized recently. For many years, freeze-fracture EM was the only way to obtain basic information about a lateral distribution of photosynthetic proteins within different compartments of thylakoid membrane from a metal replica of split lipid bilayer (see Ref. [32] for a review). Although the resolution of the technique is rather limited, it allows monitoring the protein density and distances of neighboring complexes in the membrane. Using this approach, an interesting

observation was recently made by a study of PSII rearrangements under either low light or high light intensities, which were found to induce a decrease in the density of PSII in the grana thylakoid membrane [66,67]. Moreover, the former study revealed the formation of rows of ordered PSII particles in low light grana thylakoids. Freeze-fracture EM was also used to show that the formation of a photoprotective state induced by a short-term exposure of plants to high light is accompanied by rapid and reversible structural rearrangements of photosynthetic proteins in the membrane. These rearrangements involve the dissociation of LHCII from PSII followed by LHCII aggregation and a tighter clustering of PSII particles [68]. Atomic force microscopy is another technique to study proteins in native membranes, by means of their surface properties. Because of a higher lateral resolution, in the range of about 4 nm, it can determine not only the position of individual PSII complexes within unfixed grana membranes, but also their orientation, which was found to be often in a parallel alignment [61, K. Sznee, J.P. Dekker, R.T. Dame, H. van Roon, G.J.L. Wuite and R.N. Frese, manuscript in preparation]. In particular, height differences can be determined quite accurately.

4.3.2. Emerging technique of cryo electron tomography

Although the AFM technique can provide structural data in three dimensions, it is basically limited to register the surface topology of objects with height differences. Hence the amount of structural information about, e.g., a three-dimensional membrane stack is rather limited. In contrast, cryo electron tomography can provide full 3D information and is currently the most promising technique with a great potential to fully extend our structural knowledge of intact biological objects in three dimensions close to *in vivo* conditions [69]. Cryo electron tomography is most efficient with thin objects because there is a direct relation between the size of an object and the theoretical resolution [70]. Objects up to about 0.5–1 μm can be studied at reasonable resolution, but for larger objects the thickness causes problems, engraved by multiple scattering of electrons. To overcome the specimen thickness limitation, large objects can be cut into a series of thin vitrious sections, which are examined separately. An alternative approach is to slightly disrupt organelles. The loss of *in vivo* conditions is compensated by a possibility to reach a higher resolution in a tomographic reconstruction. The latter approach was

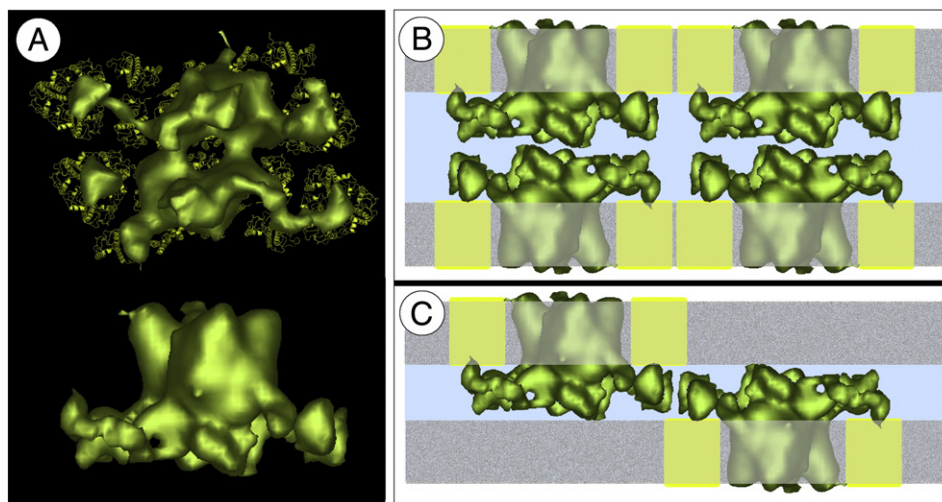


Fig. 6. 3D model of the PSII core complex and its possible organization in a pair of grana membranes. (A) 3D average of subvolumes of PSII complexes selected from tomographic reconstructions, viewed from the luminal side and from a side along the membrane plane (from the top to the bottom, respectively) [62]. Isosurface model shows the PSII core complex together with extrinsic subunits of oxygen-evolving complex resolved on the luminal side of the complex. Further, four additional spherical densities were revealed at the luminal side of the PSII complex. A comparison of the isosurface model of PSII complex with a pseudo-atomic model of the complete PSII supercomplex [24] viewed from the luminal side indicates that the spherical densities coincide with the position of LHCII trimers. (B, C) Possibilities in the variation of the luminal width at different positions of opposing PSII complexes. (B) A juxtaposition position of PSII complexes of two opposing grana membranes results in a large luminal space. Extrinsic subunits of oxygen-evolving complex and novel luminal densities revealed by cryo tomography represent a physical barrier of a closer contact between the opposing grana membranes. (C) Shrinkage of luminal space is only possible upon a full lateral displacement of opposing PSII complexes.

recently successfully used for the first study of PSII arrangement *in situ*, inside the isolated thylakoid membrane [34,62]. Typical densities of the extrinsic parts of PSII core complexes were clearly distinguished in tomograms of different preparations of stacked thylakoid membranes, including mild digitonin solubilization or osmotic shock. PSII core complexes were found to be either randomly distributed within the thylakoid membrane [34,62] or they were organized in 2D arrays of C_2S_2 supercomplexes. Interestingly, 2D arrays of PSII complexes were merely found in pairs, in two adjacent membrane layers, which were in a contact through their stromal sides [34]. Analysis of PSII interactions in those adjacent layers indicates multiple specific interactions between PSII subunits, including PSII reaction centers, LHCII trimers and minor antennae, which all might contribute to the grana stacking [17,34]. Analysis of positions of randomly distributed PSII cores and their distances from neighboring complexes within an entire membrane layer indicated that about one quarter of supercomplexes in spinach must be of smaller size than full $C_2S_2M_2$ supercomplexes, to avoid overlap [62]. This is consistent with earlier EM studies of grana membranes, where in case of crystalline arrays the C_2S_2M units were found to be predominant [35]. Tomography data further showed that regardless of a minor fraction of unassigned densities, there were several membrane areas, which were very flat and free of PSII supercomplexes or other protruding proteins [62]. Similar areas were found by AFM (Sznee et al., manuscript in preparation). It was suggested that they can be occupied by free LHCII proteins [17]. Calculations by Kirchhoff [71] on the numbers of lipids and proteins exclude the presence of large lipid-only areas. It should be pointed out that light growing conditions have a strong influence on the amount of free and bound LHCII [55], which can modify a relative ratio between different types of PSII supercomplexes (e.g. $C_2S_2M_2$, C_2S_2M , C_2S_2).

The fact that PSII core densities can be traced in tomograms offers the possibility to perform 3D averaging of hundreds of subvolumes containing such large proteins. In this way electron tomography can reveal details of protein structures *in situ* [34,62]. This opportunity will make sure that in the future electron tomography will fill the gap between high-resolution structures on the one side and low-resolution information of cellular structures on the other side. In the study of Kouřil et al., the PSII complex was resolved at 40 Å resolution, which revealed novel protein densities associated with the PSII supercomplexes, which were not observed before in isolated forms of PSII complexes (Fig. 6). Similar densities were also observed in recent AFM data (Sznee et al., manuscript in preparation). The nature of these protein densities, however, has to be established.

5. Structural rearrangements of protein complexes upon short-term adaptation

In most habitats, plants are exposed to a wide variety of irradiance intensities. There are two major short-term acclimations to changing light quantities and quality in which PSII supercomplexes are involved. A redistribution of absorbed light energy between PSI and PSII by means of a reversible redistribution of mobile LHCII trimers is called 'state transitions' (see Refs. [72–76] for reviews). Besides a redistribution, the formation of LHCII aggregates seems also to mediate the energy balance [76]. The other main fast acclimation is non-photochemical quenching (NPQ) when the light intensity exceeds the plant's capacity for photosynthesis [77]. Without proper protection, the accumulation of excited states will result in the accumulation of harmful oxygen species, which can damage the membranes, pigments and proteins of the photosynthetic organism [78,79]. The ability of plants in high light conditions to harmlessly dissipate excess excitation energy into heat is called high-energy quenching (qE) and is one of the main features of NPQ. Recent reports suggest that qE quenching involves dissociation of a complex consisting of CP24, CP29 and M-LHCII [80]. Thus it has been

speculated that the formation of energy-dissipative LHCII aggregates is relevant for both qE and state transitions, at least in the green alga *C. reinhardtii* [76]. If such energy-dissipative aggregation is facilitated by acidification of the lumen and/or reinforced by PsbS, the quenching could be classified as qE; if it is induced by redox-controlled LHCII phosphorylation, the quenching could be classified as a state transition [76,81,82].

5.1. State transitions

State transitions refer to a mechanism by which excitation energy is redistributed among PSI and PSII when they become exposed to light conditions that preferably excite either PSI or PSII. There is general consensus now about the basic features of this process. When PSII is preferentially excited by 'light 2', the plastoquinone pool becomes more reduced, which leads to a conformational change of the cytochrome b_6/f complex, which in turn activates a kinase bound to the b_6/f complex. A number of kinases have been characterized [75] and they all have in common a single transmembrane α -helix and a large extrinsic part at the stromal side of the membrane, suggesting that these kinases cannot occur in the grana stacks because of steric hinderance and thus are located in the stroma membranes. The kinase is then released from b_6/f , after which it can migrate to the margins between stroma and grana membranes, where it meets LHCII and promotes its phosphorylation. Phosphorylated LHCII has a decreased affinity for the grana stacks because of the charges on the phosphate groups and thus a lateral movement of phosphorylated LHCII from grana to stroma occurs where it can meet PSI, to form a PSI–LHCII supercomplex [83]. Such a move can explain the shift from 'state 1' (induced by 'light 1') to 'state 2' (induced by 'light 2') in plants.

There are several aspects of this mechanism that interfere with the supramolecular organization of PSII and PSI supercomplexes in the thylakoid membranes. Recent studies have focussed on the green alga *C. reinhardtii*, rather than on green plants, because the overall state transition effect is much larger in this particular species [84]. Gel filtration and electron microscopy of affinity-purified *C. reinhardtii* particles showed that a PSII–LHCII megacomplex was predominant in state 1, whereas the PSII core complex was predominant in state 2, indicating LHCII trimers dissociate from PSII upon a state 1-to-2 transition. Moreover, in state 2, strongly phosphorylated LHCII was found in the supercomplex, but not in the megacomplex. Phosphorylated minor LHCII (CP26 and CP29) were only found in the unbound form. The PSII subunits including the CP43 and D2 proteins were most phosphorylated in the core complex. Based on these observations, a 3-step model was proposed for the PSII remodeling during a state 1-to-2 transition [76]. Initially, unphosphorylated LHCII stabilize the megacomplex (State 1), then in step 1: the phosphorylation of LHCII in a LHCII trimer triggers the division of the megacomplex, resulting in individual C_2S_2 supercomplexes; step 2: the phosphorylation of CP26 and CP29, as well as the PSII core subunits D2 and CP43, induce the undocking of all the LHCII from PSII; and in step 3: the dissociated LHCII reassociate with the PSI–LHCI supercomplex yielding state 2.

Although this is a plausible mechanism for the state transitions in *C. reinhardtii*, we would like to make a few critical notes. First, there is to our knowledge and unlike in plants no solid evidence for the binding of trimeric LHCII to PSI in state 2. After the publication of our electron microscopic analysis of PSI complexes of *C. reinhardtii* [85] it appeared that these complexes were obtained from membranes poised in a mixture of state 1 and state 2 (J.A. Ihalainen, unpublished observations). In this analysis, we found two types of PSI supercomplexes, but the size difference was consistent with a monomeric LHC-type of protein, and certainly not by a trimeric complex. Second, it is clear that the PSII protein CP29 has considerable affinity for the PSI complex of *C. reinhardtii*, which in turn influences the association of the PSII–LHCII supercomplex. All association–dissociation processes are equilibria, and when CP29 (a complex that is crucial for the stability of

the PSII–LHCII supercomplex—see above) is taken away by binding to PSI, it will result in a shift of the equilibrium of PSII–LHCII formation towards the dissociated form. Third, free LHCII complexes don't need to be firmly bound to PSI to induce excitation energy transfer to PSI. In artificially unstacked thylakoid membranes from spinach, a considerable part of the energy transfer from LHCII to PSI was 'slow' (in the order of 150 ps), in contrast to 'fast' energy transfer from LHCII to PSI in a PSII–LHCII complex (in the order of 30 ps) [60], but both contribute to the increased absorption cross-section of PSI and both contribute therefore to the state transition.

Although green plants and *C. reinhardtii* have similar PSII complexes and peripheral antenna components, we speculate that the molecular mechanisms for the state transitions are quite different. In plants most of the LHCII is not accessible for the kinase, probably because a tight stacking of grana membranes restricts the LHCIIs that can be phosphorylated to those near the margins. In addition, the direct binding of an LHCII trimer to PSI induces a relatively large change in antenna cross section of PSI, and so the state transition mechanism is basically meant to provide PSI a larger absorption cross-section in state 2. In *C. reinhardtii*, a much larger part of PSII is accessible for the kinase, perhaps because of a less tight stacking of the thylakoid membrane. In view of the disappearance of the organization in supercomplexes, the main effect of the state transition is to provide PSII a smaller absorption cross-section in state 2, rather than to increase the absorption cross-section of PSI, because the permanent peripheral antenna of *C. reinhardtii* PSI is already much larger than in plant PSI.

Molecular details of the state transition mechanism have to be further studied, for instance, it is not yet clear how a protein like Lhcb3 (part of the M-LHCII trimer) modulates the state transitions [86].

5.2. High-energy quenching

The process of qE is triggered by acidification of the thylakoid lumen, which activates violaxanthin de-epoxidase, the enzyme that converts violaxanthin into zeaxanthin [87]. There is currently no consensus on the precise physical mechanism of quenching, nor on the site or sites in the PSII–LHCII macrostructure where the quenching occurs. Some authors favor a major role of the minor complexes (in particular CP24) [88,89], others prefer also an important role for the major trimeric complex LHCII [90].

How the structural changes underlying the quenching take place is not known in detail. It became clear, however, that the association and dissociation of a five-subunit membrane complex, composed of CP29 and CP24 and the trimeric LHCII-M, named B4C, is an important step [80]. Dissociation of this supercomplex seems essential for the onset of non-photochemical fluorescence quenching in high light, possibly because it makes quenching sites available for the switch to an energy-quenching conformation. These association and dissociation changes are reversible and do not require protein synthesis and degradation, thus allowing for rapid changes in light adaptation.

The process also requires the presence of the PsbS protein [91] as well as its protonation by the acidification of the thylakoid lumen [92]. There is no clear evidence yet for a direct structural role for PsbS in the quenching, also because it is not present in PSII–LHCII supercomplexes and also not in PSII–LHCII supercomplexes organized in 2D arrays [93]. A clear indirect effect of PsbS is in the macro-organization of PSII within the grana membranes. The amount of PsbS determines the proportion of supercomplexes that are organised into semi-crystalline arrays, and in addition determines the precise shape of the psi-type CD signal [94], that thus can be used as a spectroscopic fingerprint of ordered arrays in the thylakoid membranes (see also the discussion in Ref. [95]). In *Arabidopsis* mutants with overexpressed PsbS such arrays are absent [93,94] and the capacity for qE is increased by around two-fold [96], whereas in a mutant lacking PsbS there are significantly more crystals. Thus the vital role of PsbS in qE may be

explained in such a way that the changes in conformation and/or interactions between LHCII subunits that are required for the formation of the quenching state require a loose association of PSII supercomplexes. This may also be considered as the fully functional photosynthetic state that is fully competent in electron transport and able to rapidly and reversibly switch between the light harvesting and photoprotective state (see Fig. 3 from Ref. [93]). When the tendency for strong interaction is present, giving rise to an increased proportion of crystalline arrays, these changes in conformation are inhibited and qE prevented. Specifically, the dissociation of the aggregate of CP29, CP24 and the M-LHCII trimer, mentioned above and considered to be an obligatory event in qE formation [80], would be prevented. Further work is, however, necessary to test the hypothesis that qE involves a PsbS-catalyzed re-organisation of PSII supercomplexes in the grana membrane, mediating the transition between the unquenched and quenched states.

6. Outlook

With recent progress by single particle electron microscopy, atomic force microscopy and tomographic reconstructions of intact and fragmented chloroplasts we have provided insight in the supramolecular organization of PSII and how the thylakoid membranes are organized in the chloroplast. The final question is then what should come next? Tomography of complete chloroplasts is still at the level of membranes. One would like to see in the near future a completely reconstructed chloroplast at the level of individual proteins and protein–protein interactions. This is not impossible, but heavily relying on further improvements in hardware to increase resolution in electron tomography [97].

There is also a strong demand for higher-resolution data of the plant supercomplex, to understand in more detail excitation energy transfer and phenomena like state transitions and NPQ in the peripheral antenna. There are several questions to be solved in the core part of PSII but it should be emphasized that the high-resolution structures of the core complex of cyanobacteria cannot compensate for the lack of a high-resolution model of the plant core complex, because their proteins differ in their primary sequence and they have even a different subunit composition. For instance, the higher plants contain three small proteins (PsbR, PsbTn, and PsbW) that are not present in cyanobacteria [98], and both their localization and functional properties remain unclear. X-ray diffraction is the most obvious way to solve protein structures. However, for large, fragile structures like the PSII supercomplex this may be a very tedious enterprise. Single particle analysis has become an alternative in the case of large, water-soluble protein complexes [99] and large virus structures can be solved at high resolution. The $C_2S_2M_2$ supercomplex was, however, found to be flexible which limited the finally obtained resolution to only 12 Å [24]. Before single particle analysis became the most-popular way of performing EM analysis, electron crystallography was already an established technique and maybe this technique can provide higher resolution in the particular case of PSII. Noteworthy, this technique resulted in an LHCII structure [100], long before the X-ray structures became available.

In conclusion, structure determination has to go on to provide insight to one of the basic aspects of an enzyme, to fully understand and appreciate the light reactions of photosynthesis.

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