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Fine structure of granal thylakoid membrane organization using cryo electron tomography

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ABSTRACT

The architecture of grana membranes from spinach chloroplasts was studied by cryo electron tomography. Tomographic reconstructions of ice-embedded isolated grana stacks enabled to resolve features of photosystem II (PSII) in the native membrane and to assign the absolute orientation of individual membranes of granal thylakoid discs. Averaging of 3D sub-volumes containing PSII complexes provided a 3D structure of the PSII complex at 40 Å resolution. Comparison with a recently proposed pseudo-atomic model of the PSII supercomplex revealed the presence of unknown protein densities right on top of 4 light harvesting complex II (LHCII) trimers at the lumenal side of the membrane. The positions of individual dimeric PSII cores within an entire membrane layer indicates that about 23% supercomplexes must be of smaller size than full $C_2S_2M_2$ supercomplexes, to avoid overlap.

1. Introduction

Chloroplasts play a central role in the plant energy metabolism known as photosynthesis. They enclose the thylakoid membrane, which forms a unique three-dimensional network with regular stacks of thylakoids, called grana, which are interconnected by single membranes, the stroma thylakoids. The distinct parts of the thylakoid membrane specifically accommodate individual components of photosynthetic apparatus. Photosystem II (PSII) and the light-harvesting complex II (LHCII) are confined mainly to grana stacks, whereas most of Photosystem I (PSI), the light-harvesting complex I and ATP synthase are localized in unstacked stromal thylakoids and grana margins. Distribution of cytochrome b_6f complex in the thylakoid membrane seems to be unrestricted [1].

PSII consists of a dimeric core complex and a peripheral antenna system composed of 6 different complexes, belonging to the Lhcb (Light-harvesting complex) multigenic family [2]. The major antenna complex, LHCII, is organized in heterotrimers composed of the products of the *Lhcb1-3* genes. The three other subunits, CP29 (Lhcb4), CP26 (Lhcb5) and CP24 (Lhcb6) are present as monomers. A variable number of the peripheral antenna proteins can associate with dimeric PSII core complexes to form the so-called PSII-LHCII supercomplexes [3]. Many supercomplexes observed in spinach and *Arabidopsis thaliana* contain a dimeric core (C_2), 2 LHCII trimers (trimer S) strongly bound to the complex on the side of CP43 and CP26, and 2 more trimers, moderately bound (trimer M), which are in contact with CP29 and CP24. This complex is known as the $C_2S_2M_2$ supercomplex [3]. Occasionally, spinach supercomplexes loosely bind a third trimer (trimer L) around CP24 [4]. A 3D reconstruction of a smaller supercomplex containing only one trimer per reaction center and lacking CP24 (C_2S_2) was obtained by cryo-EM at about 17 Å resolution [5,6]. More recently, a 2D map at 12 Å resolution was obtained and used to generate a pseudo-atomic 3D model. This allowed determination of the location and orientation of individual light-harvesting components and the approximate position of pigments [7].

The lateral distribution of protein complexes within grana membranes is an intriguing topic and was a subject of many structural studies using electron microscopy of either freeze-fractured or negatively stained grana membranes (see [3,8] for reviews), atomic force microscopy [9] or, very recently, by cryo electron tomography [10]. Although in most grana membranes PSII supercomplexes are not highly organized, semi-crystalline domains of PSII supercomplexes or core complexes appear in a minority of the membranes (see [8] for a list of native crystalline arrays, [10]). A study of paired inside-out grana membranes indicated that crystalline arrays of adjacent layers can have a specific interaction in which orientations of opposing PSII complexes have preferred angles. This was found for spinach [11] as well as for *Arabidopsis* membranes [12]. A tomographic study on isolated spinach thylakoid membranes revealed the presence of the PSII crystalline arrays exclusively in stacked grana membranes, indicating the importance of a specific interaction between the stroma membrane surfaces of two adjacent membrane layers for the formation of regular PSII arrays [10]. In addition, recent experiments indicate that the PsbS protein controls the frequency of this crystalline macro-organization in the grana membrane [13]. High levels of PsbS disrupt the macro-organization. However, many other aspects of the PSII distribution,

Abbreviations: PSII, photosystem II; LHCII, light harvesting complex II; C, photosystem II core complex; S, strongly bound trimeric LHCII; M, moderately bound trimeric LHCII

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such as possible changes upon state transition or photoinhibition are not known in detail. Recently, however, the mobility of grana membrane proteins was studied by fluorescence recovery after photobleaching [14]. In intact, wild-type chloroplasts a mobile population of grana membrane proteins increases significantly after photoinhibition, which is consistent with a role of protein diffusion in the PSII repair cycle. It was shown that protein phosphorylation switches the membrane system to a more fluid state, thus facilitating the PSII repair cycle [14].

There is a generally accepted idea that the entire thylakoid membrane is folded in such a way that it encloses a single aqueous space, the thylakoid lumen. The space between the thylakoid membrane and the chloroplast envelope is called the stroma. The details of the thylakoid membrane folding and the interconnection of grana stacks with stromal thylakoids have not been totally solved. Early electron microscopy studies of serial sections of chemically fixed thylakoid membranes led to the proposition of several 3D models of thylakoid membrane architecture (reviewed in [15]). Afterward, some of these models were questioned by an electron microscopy study of freeze-substituted chloroplast sections [16]. These models include (i) "the folded membrane model", which proposes a way of a dynamic reversible folding of the thylakoid membrane [17] and (ii) "the helical model", in which the stroma membranes are wound around the granal stacks [15]. It was further proposed that a bifurcation of stromal membranes and a subsequent membrane bending and fusion is involved in grana stack formation [16]. These novel viewpoints of the grana architecture initiated a further discussion with a common consensus that current data still require further refinements to clarify discrepancies between individual models [18–20]. Refining of chloroplast membrane topology models will depend very much on improving of electron microscopy hardware to reach better data acquisition and on perfecting tomography reconstruction techniques, necessary to image complete chloroplasts.

Over the last decade the application of cryo electron tomography on intact frozen-hydrated samples has become a popular technique to visualize cell structures because the method is free of fixation artifacts [21]. In combination with sub-volume averaging, it is nowadays a very feasible approach for studying macromolecules and membrane architecture inside cells, cellular organelles and structures of membrane protein complexes in their native membrane environment [10,22–25]. Because electron tomography is most efficient with thin objects and since there is a direct relation between the size of an object and the theoretical resolution [26], it works the best for small objects up to about 0.5–1 μm. Unfortunately, chloroplasts are substantially larger and also densely packed with membranes. Hence, intact chloroplasts are currently just too large to yield a resolution of about 50–60 Å, which is necessary to unambiguously elucidate structural features of the thylakoid membrane, including its precise folding. In this study, we applied cryo electron tomography to isolated grana membrane stacks. Working with smaller tomography volumes, in contrast to e.g. intact chloroplasts, allowed pushing up the resolution to see interpretable densities of PSII complexes in the granal membrane. This enabled averaging sub-volumes containing PSII and seeing characteristic features of PSII core complexes in the natural membrane at 40 resolution. In addition, single particle 3D averaging revealed a novel density associated with the PSII core complex in multiple copies, which has not been observed before in isolated forms of PSII complexes. Comparison of the 3D structure of the PSII core complex with the recently proposed pseudo-atomic model of the complete, $C_2S_2M_2$ PSII supercomplex [7] revealed a striking fit of the extra densities with the position of LHCII trimers. A possible origin of the novel density is discussed.

2. Materials and methods

2.1. Preparation of the granal thylakoid membranes

Thylakoid membranes were isolated from market, dark adapted spinach according to [27]. The isolated membranes were resuspended in 20 mM Bis-Tris (pH 6.5) with 5 mM $MgCl_2$ at a final concentration of 0.5 mg of Chl/mL and partially solubilized with digitonin at a final concentration of 0.5% (w/v) for 20 min at 4 °C with a slow stirring, followed by centrifugation in an Eppendorf table centrifuge for 15–20 min [28]. The pellet, which contained the non-solubilized granal thylakoid membranes, was used for cryo electron tomography.

2.2. Cryo electron tomography

Granal thylakoid membranes were mixed with 10-nm gold particles as fiducial markers and applied to glow discharged 200 mesh Quantifoil specimen support grids (Quantifoil Micro Tools GmbH) coated with a thin carbon film. Vitrification was performed in liquid ethane using a Vitrobot Mk3 (FEI company, Eindhoven) operating under a 100% humidified atmosphere at room temperature. Electron tomography was performed on a 300 kV G2 Polara electron microscope (FEI) equipped with a Gatan post-column energy filter. Images were recorded with a 2 k×2 k CCD camera (Gatan) at 8 μm underfocus and 51,750x final magnification, resulting in a pixel size of 0.58 nm at the level of the specimen. Single axis tilt series were recorded at 2° increments over a range of ±68° with a total dose of about 80 $e/Å^2$.

2.3. 3D reconstruction and image analysis

Tomograms were calculated using IMOD software [29] and further denoised with 20 iterations by non-linear anisotropic diffusion [30]. Averaging of manually selected sub-volumes with a box size of either 232×232 Å or 319×319 Å was performed using PEET program (a part of IMOD package). Central coordinates of the sub-volumes were selected using 3dmod program. Surface views of averaged sub-volumes were produced using 3dmod program (a part of the IMOD package). The resolution of the final averaged sub-volume was estimated by Fourier Shell Correlation [31] at 0.3 threshold with EMAN software [32].

3. Results

3.1. Cryo electron tomography of granal thylakoid membranes

Suspensions of isolated granal thylakoid membranes (Supplemental Fig. 1) were directly investigated by cryo electron tomography. Fig. 1 shows four slices of a typical electron tomogram of one granal membrane stack, where four membrane layers were clearly resolved (Fig. 1A to D). This and other tomographic reconstructions revealed distinct densities in all membrane layers, which could be unambiguously assigned to the dimeric PSII complex (see e.g. white arrows in Fig. 1A). Tomographic slices of the middle part of the reconstruction indicate that the membranes form two vesicles, as evident from a pair of strong rims resolved at the edge of the granal membrane (Figs. 1B and C). Close investigation of the tomographic data revealed other interesting features that will be discussed below.

Creation of surface views of individual granal stacks gave good insight into the overall 3D organization. Fig. 2 shows different angular surface views of two granal vesicles like in Fig. 1, with marked positions of individual PSII complexes (Figs. 2A to C, and D to F, respectively). Both vesicles have the shape of a sac and each one is formed by a pair of continuous membranes, which does not have any direct contact with the other. The outer membrane, depicted in green, accommodates PSII complexes in both the upper and bottom membrane layers (Fig. 2, green and blue spheres, respectively). The outer membrane is not (anymore) continuous at the vesicle margin, which is mainly obvious from the top-views (Figs. 2A and D). The inner membrane, depicted in brown, encloses the inner space of the vesicle and accommodates PSII complexes in two layers (Fig. 2, cyan and red spheres). At this moment, it is not clear (due to a resolution-limited membrane tracking) if the inner membrane fully encloses the