Three-Dimensional Organization of Higher-Plant Chloroplast Thylakoid Membranes Revealed by Electron Tomography

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The light-harvesting and energy-transducing functions of the chloroplast are performed within an intricate lamellar system of membranes, called thylakoid membranes, which are differentiated into granum and stroma lamellar domains. Using dual-axis electron microscope tomography, we determined the three-dimensional organization of the chloroplast thylakoid membranes within cryo-immobilized, freeze-substituted lettuce (Lactuca sativa) leaves. We found that the grana are built of repeating units that consist of paired layers formed by bifurcations of stroma lamellar sheets, which fuse within the granum body. These units are rotated relative to each other around the axis of the granum cylinder. One of the layers that makes up the pair bends upwards at its edge and fuses with the layer above it, whereas the other layer bends in the opposite direction and merges with the layer below. As a result, each unit in the granum is directly connected to its neighbors as well as to the surrounding stroma lamellae. This highly connected morphology has important consequences for the formation and function of the thylakoid membranes as well as for their stacking/unstacking response to variations in light conditions.

INTRODUCTION

The chloroplast-based photosynthetic apparatus is located within a membranous system of flattened vesicles called thylakoids (Menke, 1962, 1990), which accommodate all the molecular complexes that perform the light-driven reactions of photosynthesis and provide a medium for energy transduction. In higher plants and green algae, thylakoids are differentiated into two distinct morphological domains: cylindrical stacked structures, called grana, and unstacked membrane regions, called stroma lamellae, which interconnect the grana. These two domains are organized in a complex three-dimensional (3D) lamellar network that is believed to enclose a single compartment called the thylakoid lumen (reviewed in Mustardy, 1996).

The differentiation of thylakoids into granum and stroma lamellar domains is thought to be a morphological reflection of the uneven distribution of the major photosynthetic complexes within the constituent membranes. Photosystem I (PSI) and the CF$_1$/CF$_2$ ATP synthase concentrate in nonappressed thylakoid within the constituent membranes. Photosystem II (PSII) and its light-harvesting antenna complex LHCII are clustered in the appressed regions of the granum stacks (Andersson and Anderson, 1980; for recent reviews, see Albertsson, 2001; Staehelin, 2003). The resulting asymmetry in structure and protein composition has been proposed to serve several functions, including minimization of spillover of excitation energy from PSII to PSI and regulation of light energy distribution between the two photosystems, maximization of light trapping by PSII and protection of PSII complexes containing inactivated D1 molecules against degradation at sustained high irradiance (reviewed in Anderson, 1999). Thus, in addition to providing a scaffold and a medium for light harvesting and energy transduction, the unique organization of the thylakoid membranes is important for the ability of the photosynthetic machinery to adjust to changes in illumination conditions and for protection against light-induced damage.

Clearly, a full understanding of the function of the chloroplast-based photosynthetic machinery requires not only that the structure of individual complexes be known, a task which has largely been achieved (reviewed in Nelson and Ben-Shem, 2004; Merchant and Sawaya, 2005), but also a precise knowledge of the spatial organization of the thylakoid membranes that host these complexes. In this work, we used dual-axis electron microscope tomography to examine the architecture of the thylakoid membranes of higher-plant chloroplasts in three dimensions in rapidly frozen, freeze-substituted leaves. The structures derived deviate from existing models of chloroplast thylakoid membranes and provide new insights into their formation and function as well as into the extent of structural alteration that they can undergo during adaptation to variations in light quality and intensity.
RESULTS

To determine the architecture of the thylakoid membranes of the chloroplast, we used dual-axis electron microscope tomography, which provides a powerful means to obtain high-resolution details in three dimensions for cellular structures and organelles (Mastronarde, 1997; Grunewald et al., 2003). To preserve the ultrastructure of the chloroplasts, leaf samples were cryo-immobilized by high-pressure freezing, followed by freeze substitution and resin embedding. Tomography was performed on four serial sections with a total thickness of almost 1 μm. Studies were conducted on dark-adapted leaves. In this state, the two photosystems are highly segregated, and heterogeneities due to light-induced membrane unstacking and redistribution of LHCII (and possibly PSII) between grana and stroma thylakoids are minimized.

Figure 1A shows a low-magnification overview of the chloroplast. The grana appear as cylindrical stacks measuring ~300 nm in diameter and 200 to 600 nm in height. These stacks are connected by multiple stripes of paired membranes—the stroma lamellae, which run roughly parallel to each other. The stroma lamellae also make rare connections with the inner envelope membrane of the chloroplast. One such serendipitously viewed connection is shown in the inset. Ribosomes, which appear as heavily stained particles, are localized at the periphery of the thylakoids or in empty spaces between neighboring stroma lamellae or near grana-end membranes (the top- and bottom-most layers of the grana).

Figure 1B depicts a granum-stroma lamellae assembly viewed in a plane perpendicular to the granum layers. The grana membranes are readily distinguishable from the stroma membranes, as they appear highly granulated. This granulation results...
from the presence of densely packed, moderately stained particles, which protrude slightly into the intergranal space. These particles are most likely PSII-LHCII supercomplexes, which are concentrated in appressed granum domains and which do not extend significantly into the partition region between the granum layers. Indeed, no such particles are observed at the nonappressed end membranes of the granum. At the resolution of the tomograms, the stained particles seem to be distributed homogeneously across the granum layers, with no apparent changes in pattern between the layers. The average thickness of the granum layers is 20 nm ± 2 nm, which is also the thickness of the flanking stroma lamellae. Within the granum, the surfaces of adjacent layers or discs are separated by 3 to 4 nm, in agreement with previous reports (Nir and Pease, 1973; Arntzen and Briantais, 1975) and in line with predictions made by electrical double-layer theories (Rubin and Barber, 1980; Dubois et al., 1991). Note that the granum layers, while presenting a distinct granular texture, are topologically continuous with the stroma membranes that penetrate the granum at multiple levels. Some of the stroma lamellae appear to branch off into the granum, forming forks at the interface, whereas others appear to continue directly into the granum body without splitting. In fact, all the stroma lamellae that intersect the granum bifurcate at the granum-stroma boundary. As will be further elaborated below, the reason these bifurcations cannot all be observed simultaneously is that they do not occur in a single plane.

Figure 1C presents a tomographic slice made in a direction roughly parallel to the plane of a layer located at the middle of the granum stack depicted in Figure 1B. Viewed from above, the granum layer appears as a flat circular structure with a grainy surface. The stroma lamella (yellow), which is continuous with the granum layer, appears as a sheet that flanks the granum body. By contrast, stroma lamellae that intersect the granum above (light blue) or below (blue and orange) the granum layer appear as ribbons. While this may give the impression that the stroma membranes loop around the granum, the reason for this appearance is that these layers ripple in and out of the section plane.

Figure 2A shows the 3D structure of a granum-stroma lamellae assembly that was generated from the segmented tomograms. As evident in the tomographic slices, the stroma lamellae intersect the granum perpendicular to the cylinder axis in roughly parallel planes. At the boundary, where the two membrane structures merge, the stroma membranes bifurcate into stacked layers that form the granum body. Entry and exit of the stroma lamellae into and out of the granum occur in approximately the same plane. Notably, the layers that constitute the granum stack are internally interconnected. Looking at the left-hand side of the structure, one can see that the second and third layers from the bottom of the granum are fused to each other at their edges (arrow). These two layers are also interconnected at the right-hand side through what appears as a wide stalk (arrowhead). These internal connections are better visualized in Figures 2B

**Figure 2.** 3D Organization of a Granum-Stroma Assembly.

The structure was generated from the tomographic data shown in Figure 1. 

(A) The granum layers are contiguous with the stroma membranes that bifurcate at the granum-stroma interface. Internal connections between adjacent layers are indicated by an arrow and an arrowhead. G, grana; SL, stroma thylakoids.

(B) and (C) To better visualize the connectivity of the assembly, the structure shown in (A) was enlarged, clipped, rotated by ~20° (B) and 40° (C), and the upper layer of the stack has been removed. Connections between adjacent layers that are not marked by an arrow or an arrowhead are bifurcations of the stroma lamellae. In all panels, the structure was expanded along the z axis to provide a clear view of the interior of the granum.
and 2C, in which the structure shown in Figure 2A was rotated and enlarged, and the upper layer of the stack has been removed. For another, more vivid, illustration of the assembly, see Supplemental Movie 2 online, which shows the tomographic slices that were used to derive the structure.

The exact way in which the grana layers relate to and connect to each other can be realized by inspection of Figure 3, which presents a schematic model of the grana. The structure is made of repeating units that consist of paired layers formed by bifurcations of stroma lamellae sheets that fuse within the grana body. In each unit, part of the top layer bends upwards at its edge and fuses with the layer above it, whereas the other layer bends downwards at the opposite side and merges with the layer below. This basic unit repeats itself along the grana. Proceeding from one unit to the one above it involves a counterclockwise rotation of ~25° around the axis of the grana cylinder. This rotation and the bending of the granum layers underlie the inability to visualize all the bifurcations of the stroma thylakoids with single cross-sectional views. The combination of bifurcations, bending, and fusion between adjacent granum layers would be exceedingly difficult to discern by conventional two-dimensional electron microscopy methods. Topologically, this combination leads to a highly connected structure that encloses a single continuous lumen, which is joined to the lumen of the stroma lamellae at multiple levels via the bifurcations. As discussed below, this extensive multilevel interconnectedness has important implications for the formation and function of the thylakoid network.

Finally, it is useful to have a larger view of the system, where the relationship between grana and stroma thylakoids can be observed over a longer range. Figure 4 shows a small ensemble consisting of two grana interconnected by several stroma thylakoids. To better illustrate the spatial relationship between the two membrane domains, the two grana are presented as surface-rendered objects such that internal features are not visible. The stroma lamellae run almost perpendicular to the axes of the grana, intersecting them in multiple parallel planes. As opposed to commonly used descriptions, the stroma membranes do not form tubular structures or frets, nor do they wind around the grana. Instead, they form wide, seemingly flexible, parallel sheets that surround the grana, while the grana act as clearly defined regions of sheet consolidation and increased connectivity.

**DISCUSSION**

Since Menke’s observation of thylakoids in electron micrographs of chloroplast cross sections (Menke, 1960), several models have been proposed to describe the structure and interconnections of granum-stroma assemblies in the thylakoid membranes of higher-plant chloroplasts (Weier and Thomson, 1962; Heslop-Harrison, 1963; Weier et al., 1963; Wehmeyer, 1964; Paolillo, 1970; Brangeon and Mustardy, 1979; Arvidsson and Sundby, 1999). At present, two models dominate the field. The first model was proposed by Wehmeyer (1964) and modified by Paolillo (1970) and subsequently by Brangeon and Mustardy (1979) (see also Mustardy, 1996; Mustardy and Garab, 2003). This model suggests an essentially bipartite structure consisting of a cylindrical grana body, made of discs piled one on top of the other, around which the stroma lamellae are wound as right-handed helices. The grana discs connect to each other through the stroma lamella helices that make multiple contacts with successive layers through slits located at the rim of the grana cylinder.

The second model, referred to as the folded-membrane model, was originally used to illustrate the segregation of the two photosystems into grana and stroma lamellar domains (Andersson and Anderson, 1980; Anderson and Andersson, 1988). More recently, it was extended by Arvidsson and Sundby (1999) to explain how the thylakoid network may be constructed by the folding of a single continuous membrane and to account for the complete or almost complete unstacking of grana layers seen in isolated thylakoids subjected to low ionic strength conditions. In this model, grana are formed by symmetrical invaginations of stroma thylakoid pairs into piles of three discs (see Figure 2 in Arvidsson and Sundby, 1999). This arrangement gives rise to a regular folded structure, which is stabilized solely by surface interactions between appressed grana membranes and, hence, can be readily dismantled.

The structure derived in this work deviates qualitatively from both models. The grana layers are formed by bifurcation and subsequent fusion of the membranes rather than by invagination or folding. Adjacent layers in the grana are not connected to each other through the stroma lamellae. Instead, they are interconnected directly through their edges, which bend toward and fuse with neighboring layers. In the absence of high-resolution 3D images, these bends and fusions, which occur at the grana-stroma interface and which spiral along the grana cylinder, might give the impression that they belong to stroma

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**Figure 3. A Topological Model of the Granum.**

The grana is made of repeating units, each consisting of two layers (red and yellow), which are formed by bifurcations of the stroma lamellae (gray). In each unit, part of the top layer (red) bends upward and fuses with the layer above it, whereas the other layer (yellow) bends downward at the opposite side and fuses with the layer below. As indicated by the blue dashed lines, going from one unit to the one above it involves a counterclockwise rotation of ~25° around the axis of the grana cylinder. Note that the spacing between the three units that constitute the stack has been grossly exaggerated for clarity and that the bent regions that interconnect them appear to take a significant part of the area of the grana layers. In reality, all the layers in the stack are closely appressed and run parallel to each other along almost their entire area; bending occurs only at the edge of the layers.
across or along the granum and the diffusion of protons to facilitate equilibration of proton and electron gradients arising throughout the granum volume. This, in turn, should act to

The continuity between the granum layers should facilitate the organization of the membranes that make up the assembly. The unique structure derived in this work readily accounts for the long-range chirality. Specifically, the successive rotation of paired granum layers with distance along the granum cylinder axis gives rise to a cholesteric-like organization with right-handed asymmetry.

Lamellar sheets that are wound around the granum. Finally, the extensively connected nature of the structure does not allow for massive unstacking of the granum layers and should be quite immune to disintegration.

Chloroplast thylakoid membranes have been shown to exhibit anomalous circular dichroic bands, which were assigned to LHCII-containing macromdomains (Garab et al., 1988; Garab, 1996) and which suggest the presence of long-range chiral order or handedness in the granum (Keller and Bustamante, 1986). The structure derived in this work readily accounts for the long-range chirality. Specifically, the successive rotation of paired granum layers with distance along the granum cylinder axis gives rise to a cholesteric-like organization with right-handed asymmetry.

The extensively connected morphology of the granum-stroma assembly as derived here is likely to have many implications for our understanding of the formation and function of the thylakoid network of higher-plant chloroplasts. Here, we discuss three of them. We start with the effect of the structure on the trafficking of molecules within, into, and out of the assembly. The unique organization of the membranes that make up the assembly requires that all the layers inside the granum be directly connected to each other as well as to the stroma lamellar sheets from which they emerge. This is in contrast with other models where neighboring discs in the granum are either not connected at all or are linked to each other at the periphery by the stroma lamellae. The continuity between the granum layers should facilitate the traffic of solutes, including protons and mobile electron carriers, throughout the granum volume. This, in turn, should act to facilitate equilibration of proton and electron gradients arising across or along the granum and the diffusion of protons to the

granum-end membranes, which host a significant portion of the proton-driven ATP synthase molecules. The connections between the granum cylinder and the stroma lamellae that intersect it at multiple levels provide additional pathways for these processes and ensure rapid exchange of water- and lipid-soluble molecules and macromolecules between the two membrane domains. Mediated by the stroma lamellae, the high connectivity of the structure extends to neighboring assemblies, ensuring continuity of the whole network.

Another consequence of the bifurcated and internally interconnected nature of the structure concerns the way the thylakoid network is formed and stabilized. Inspection of the structure of the granum-stroma assembly depicted in Figures 2 and 3 indicates that, in spite of the continuity of the system, the thylakoid network cannot be formed by the folding of one continuous membrane as proposed (Arvidsson and Sundby, 1999). Such a scheme of formation is clearly topologically impossible. Rather, the observed architecture of the assemblies requires that their makeup involve multiple mechanisms, including expansion, splitting, and fusion. This picture is generally in line with a model proposed by Brangeon and Mustardy for grana development in which grana formation is initiated at specific perforation sites within the parental lamellar sheets and proceeds through a complex process involving membrane overgrowth, branching, and bridging via fusion (Brangeon and Mustardy, 1979; Mustardy, 1996). The interwoven nature of the grana endows them with substantial mechanical strength, which is required since the wide stroma lamellar sheets are likely to be quite fragile and are unlikely to contribute significantly to the stability of the network.

Finally, the interlinked topology of the granum-stroma assembly has implications for the unstacking and restacking of the grana membranes during state transitions (short-term regulatory processes that occur in response to variations in illumination conditions and are accompanied by changes in membrane stacking). While complete unstacking of granum layers into lamellar sheets has been reported for isolated thylakoids suspended in low-salt solutions (Izawa and Good, 1966; Murakami and Packer, 1971), changes in membrane stacking that occur in vivo during state transitions typically do not exceed 10 to 20% (Drepper et al., 1993; Delosme et al., 1996; Rozak et al., 2002) and are mostly confined to grana margins (Drepper et al., 1993). Excessive unstacking is likewise not observed when isolated de-enveloped chloroplasts are induced to undergo state I → state II transitions by PSII-specific light (S. Chuartzman, unpublished results). It was also shown that excessive unstacking of thylakoids is associated with lesions and ruptures of the membranes (Brangeon, 1974; Briantais, 1984). Our model of the grana readily accounts for these observations. Possessing sufficient degrees of freedom and an extensive interface with the stroma lamellae, the structure readily allows for a significant level of unstacking at the edges of the grana. However, the symmetrical bifurcations and fusions of adjacent granum layers set strong topological constraints, which prevent unstacking from proceeding in an uncontrollable manner that could potentially lead to irreversible long-range structural distortions. Rather, they facilitate restacking by limiting conformational freedom. In fact, complete unstacking of the grana layers can occur only by physically

Figure 4. Thylakoid Network.

Architecture of an ensemble consisting of two grana interconnected by multiple stroma lamellae. The figures shown in (B) and (C) are rotated views of the structure shown in (A), with two (B) or four (C) of the lamellae removed. The stroma lamellae form wide slightly curved sheets that run parallel to each other and intersect the grana at an angle that is roughly perpendicular to the axis of the granum cylinder. The grana (surface-rendered gray objects) act as defined regions of sheet consolidation and increased connectivity. The arrows in (C) define the plane of the layers inside the granum.
breaking multiple connections in the grana, possibly leading to the aforementioned lesions and ruptures in the membranes.

This work reveals the structural organization of dark-adapted thylakoid membranes. Future research should concentrate on the structure of thylakoids exposed to PSII-specific light, where the exact nature of membrane unstacking and other rearrangements associated with state transitions can be determined. One may also use electron microscopy techniques to determine the organization of photosynthetic complexes and supercomplexes in the thylakoid membranes under different conditions of illumination (Boekema et al., 2000; Ford et al., 2002; Kirchhoff et al., 2004). The information obtained from such analyses can be combined with data derived from other high-resolution imaging methods, such as atomic force microscopy (Kaftan et al., 2002), as well as from x-ray crystallography to provide a detailed picture of the macroscopic and microscopic makeup of the photosynthetic apparatus, which can then be correlated to its activity and light adaptability.

METHODS

Discs with a diameter of 2 mm were punched out of overnight dark-adapted lettuce (Lactuca sativa) leaves, using an ophthalmic puncher (Greishaber and Co.) and were mildly degassed in 50 mM Tricine buffer, pH 7.4, containing 15% dextran (40 kD; Sigma-Aldrich). Samples were then slowly warmed up to 0°C. Following acetone rinses, the samples were polymerized at 60°C overnight.

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