Probing the biogenesis pathway and dynamics of thylakoid membranes

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How thylakoid membranes are generated to form a metabolically active membrane network and how thylakoid membranes orchestrate the insertion and localization of protein complexes for efficient electron flux remain elusive. Here, we develop a method to modulate thylakoid biogenesis in the rod-shaped cyanobacterium *Synechococcus elongatus* PCC 7942 by modulating light intensity during cell growth, and probe the spatial-temporal stepwise biogenesis process of thylakoid membranes in cells. Our results reveal that the plasma membrane and regularly arranged concentric thylakoid layers have no physical connections. The newly synthesized thylakoid membrane fragments emerge between the plasma membrane and pre-existing thylakoids. Photosystem I monomers appear in the thylakoid membranes earlier than other mature photosystem assemblies, followed by generation of Photosystem I trimers and Photosystem II complexes. Redistribution of photosynthetic complexes during thylakoid biogenesis ensures establishment of the spatial organization of the functional thylakoid network. This study provides insights into the dynamic biogenesis process and maturation of the functional photosynthetic machinery.
Oxygenic photosynthesis produces energy and oxygen for life on Earth and is arguably the most important biological process. In phototrophs, such as cyanobacteria, algae, and plants, photosynthetic light reactions are performed by a set of light-harvesting complexes, reaction center protein complexes, as well as other electron transport complexes and cofactors, which are accommodated in specialized intracellular membranes, termed thylakoid membranes. Unlike the thylakoids in plant chloroplasts, cyanobacterial thylakoid membranes do not form tightly appressed grana stacks; they sit between the plasma membrane and the central cytoplasm, leading to intricate cellular compartmentalization and providing the site for both photosynthetic and respiratory electron transport chains. The photosynthetic electron transport chain comprises four membrane-bound protein complexes: photosystem I (PSI), photosystem II (PSII), cytochrome (Cyt) b6f, and ATP synthase (ATPase), along with the electron carriers plastoquinone and plastoquinanin. The respiratory electron transport chain involves type-I NADH dehydrogenase-like complex (NDH-1), succinate dehydrogenase, and terminal oxidases. The interplay of these electron transport complexes and molecules ensures the physiological direction of electron flows and the required stoichiometry of ATP/NADPH synthesis in the cell.

An outstanding fundamental question is how the thylakoid membranes and photosynthetic machinery are constructed and maintain functionality. Despite considerable efforts in determining the atomic structures and spectroscopic properties of individual thylakoid protein complexes, we are only beginning to understand the assembly and membrane locations of photosynthetic complexes, as well as the spatial organization of thylakoids in cyanobacteria. For example, early genetic and biochemical analyses have indicated that the multi-subunit PSII and PSI complexes undergo sophisticated stepwise assembly processes that are finely regulated in time and space. Recent atomic force microscopy (AFM) studies have revealed the native organization of different photosynthetic complexes in thylakoids of the cyanobacterium Synechococcus elongatus PCC 7942 (Synechococcus), . The photosynthetic complexes are densely packed in the thylakoid lipid layer, which facilitates the association of functionally coordinated complexes and electron flow. Likewise, the photosynthetic assemblies exhibit high heterogeneity in structure and membrane arrangement, highlighting their structural and assembly variations in situ.

Little is known about where the biogenesis of thylakoid membranes originates in cyanobacterial cells and whether there are physical connections or fusions between the intracytoplasmic thylakoid membranes and the plasma membrane. Electron microscopy (EM) on the cyanobacterium Synechocystis sp. PCC 6803 (Synechocystis) has shown that the thylakoid membranes converge at some sites close to the plasma membrane. Recently, cryo-electron tomography (cryo-ET) of Synechocystis cells did not identify any direct fusion of the plasma membrane and thylakoid membranes, instead, cells possess so-called “thylapse” contacts where thylakoid convergence membranes form tight connections with the plasma membrane. At the convergence regions, distinct layers of thylakoids interconnect with each other and form a contiguous membrane network, which potentially facilitates diffusion of constituents between adjacent thylakoid lumens and the electron fluxes throughout the thylakoid network. While these studies provided static pictures about the assembly of photosynthetic complexes and the organization of thylakoid membranes, a systematic characterization of the molecular processes concerning the origin and development of thylakoid membranes remains to be established for a complete understanding of the pathways and dynamics of thylakoid biogenesis.

Cyanobacterial thylakoid membranes are truly plastic and adapt rapidly to changes in growth conditions. Previous studies have revealed that the thylakoid content could be reduced by dark acclimation or nitrogen starvation and could be recovered by exposing cells to light or nitrate. The physiological modulations of thylakoid formation, protein composition, and organization provide the foundation for exploring in detail, the biogenesis process and the functions of photosynthetic apparatus in thylakoid membranes. Here, we developed a method to modulate in vivo thylakoid biogenesis of Synechococcus, by regulating light intensity during cell growth. Unlike the irregular Synechocystis thylakoid organization, the thylakoid membranes in the rod-shaped Synechococcus cells form regular concentric cylinders aligned along the plasma membrane, suitable for quantitative analysis. Using thin-section EM, cryo-ET of cell lamella produced by cryo-focused ion beam (cryo-FIB), live-cell confocal microscopy, proteomics, and biochemical approaches, we probed the initial sites of thylakoid biogenesis and the stepwise membrane integration, assembly, and dynamic localization of photosynthetic complexes during thylakoid biogenesis. The results enabled us to delineate the biogenesis pathway of cyanobacterial thylakoid membranes, which may inform strategies for engineering functional photosynthetic systems to underpin bioenergy production.

**Results**

**Manipulation of thylakoid membrane biogenesis by varying light intensity.** To control thylakoid membrane biogenesis, Synechococcus cells were first subjected to high light (HL, 300 μmol photons m⁻² s⁻¹) for 14 days to greatly reduce the content of intracellular thylakoids compared with the cells under growth light (GL, 40 μmol photons m⁻² s⁻¹), as indicated by the reduction of pigment content per cell (Supplementary Fig. 1). Thin-section electron microscopy (EM) revealed that, unlike the GL-grown cells that possess generally 3–4 layers of mostly intact, concentric thylakoid membranes (Fig. 1a, b, Supplementary Fig. 2), the number of thylakoid layers in the HL-adapted cells was greatly reduced. There is typically one continuous thylakoid layer that was accompanied occasionally by a thylakoid membrane fragment close to the plasma membrane (Fig. 1a). The distance between the inner and peripheral thylakoid layers (109.2 ± 1.6 nm, n = 50) was ~39% larger than the space between thylakoid layers in the GL-grown cells (78.4 ± 3.4 nm, n = 50) (Fig. 1c). The HL-adapted cells were then transferred to low light (LL, 20 μmol photons m⁻² s⁻¹) to promote the development of thylakoid membranes. The changes in pigment content and thylakoid membrane ultrastructures were assayed at distinct time points during LL adaptation (Fig. 1, Supplementary Figs. 1, 2). In the cells after one-day LL adaptation (LL day 1), one additional thylakoid layer appeared on the same longitudinal side of the cell where the single membrane fragment was observed, giving rise to an asymmetrical distribution of thylakoid membranes (Fig. 1a, b). The distance between the thylakoid layers (110.6 ± 5.5 nm, n = 50) remained relatively consistent with that of HL-adapted cells (Fig. 1c). In LL day 2, the existing thylakoid fragments expanded, generating fascicles of lamellar sheets or spiral-like thylakoid membranes lining the plasma membrane in the cell, reducing the asymmetric distribution of thylakoids (Fig. 1a, b). After LL day 2, the content and organization of thylakoid membranes become closer to those of the GL-grown cells; on LL day 5 the cells exhibited a GL-like phenotype (Fig. 1, Supplementary Fig. 1). Thus, by regulating light intensity and duration, we could specifically control thylakoid expansion to characterize the membrane construction process.
In situ organization of thylakoid membranes in *Synechococcus*.

To dissect in-depth thylakoid membrane organization at the early stage of biogenesis, we applied in situ cryo-ET to image the *Synechococcus* cells grown under HL and LL day 1. The HL-induced decrease in the number of thylakoid layers was confirmed by cryo-ET (Fig. 2a, b). No apparent connection or extended continuity between the plasma and thylakoid membranes was detected in the *Synechococcus* cells (Fig. 2, Supplementary Figs. 3, 4, Supplementary Movies 1, 2), in agreement with earlier studies from other cyanobacterial species.\(^{14,15,21}\) Alternatively, the tips of thylakoid extension appeared in close vicinity of the plasma membrane (Fig. 2e, f, i, j, Supplementary Movie 3), resembling the "thylapse" domains as reported in *Synechocystis*\(^{15}\). Likewise, small membrane segments were visualized near the thylakoid membrane "breakages" close to the plasma membrane (Supplementary Fig. 5, Supplementary Movies 4, 5). These segments were connected with the thylakoid layer and approached the plasma membrane without direct fusion, probably representing the convergence zones of thylakoid membranes. Similar thylakoid domains have also been observed in *Synechocystis*\(^{15}\).

Some small, tubular-like structures were also discerned close to the plasma membrane, with a diameter of 20–30 nm and a length varying between 30 and 48 nm (Fig. 2g, white arrow; Fig. 2h, red; Supplementary Figs. 6, 7; Supplementary Movies 1, 6). Some of these structures sit between the thylakoid tips and the plasma membrane and no apparent connection between these structures and the thylakoid membrane was determined (Fig. 2h, Supplementary Fig. 7, and Supplementary Movies 1, 5). These unknown structures are reminiscent of the VIPP1 (vesicle-inducing protein in plastids 1, or inner membrane-associated protein of 30 kDa—IM30) multimers\(^ {22–27}\) that are deduced to be related to thylakoid membrane biogenesis in cyanobacteria\(^ {26}\) and chloroplasts\(^ {29}\). Whether these tubular-like structures are Vipp1 assemblies or involved in thylakoid biogenesis remains to be explored.
Closer views of the tomogram revealed that the extra thylakoid fragment close to the plasma membrane did not form connections with the adjacent continuous thylakoid layer (also refers to the pre-existing thylakoids) that is curved towards the central cytoplasm (Fig. 2i, j). This indicates that thylakoid biogenesis in *Synechococcus* occurs most likely at the peripheral sites of the cytoplasm close to the plasma membrane. Expansion of the new thylakoid layer likely pushes the existing thylakoid lamellae toward the central cytoplasm to develop sacs of thylakoid layers. The thylakoid membranes of *Synechococcus* are often discontinuous, as several perforations were observed in the thylakoid lamellae (Fig. 2g, h, Supplementary Fig. 8, and Supplementary Movie 7), consistent with the previous electron tomography of thylakoids in *Synechococcus* and *Chlamydomonas*.

This membrane organization may provide a means for the transport of ribosomes and synthesized proteins to...
specific cellular regions\textsuperscript{36}. By contrast, the thylakoid perforations were not defined in the recent cryo-ET study of \textit{Synechocystis}\textsuperscript{15}.

**Sequential membrane integration of photosynthetic complexes during thylakoid biogenesis.** To address how different photosynthetic complexes are integrated and assembled into thylakoid membranes during the redevelopement of thylakoids, we isolated the thylakoid membranes from the \textit{Synechococcus} cells grown at different time points during thylakoid biogenesis. Label-free mass spectrometry\textsuperscript{1,2,3} was used to determine the composition and relative abundance of photosynthetic proteins integrated into the thylakoid membranes. Using mass spectrometry, it is possible to examine a vast number of target proteins, but the quality of obtained spectra may vary between proteins, especially the membrane-integral protein complexes. Thylakoid samples were digested and then loaded in mass spectrometry with an equal protein amount. A total of 1200 proteins were detected (Supplementary Data 1). The trajectories of the changes in protein abundance, normalized by the GL-samples, were fitted well with fourth-degree polynomial functions (Fig. 3, Supplementary Table 1), likely due to the combination of protein biosynthesis and degradation processes.

Proteomic results confirmed the reduced abundance of photosynthetic complexes in the HL-adapted thylakoid membranes (Fig. 3b–d, Supplementary Data 1). The decrease in the PSI amount was represented by the reduced content of PSI reaction center proteins PsAa and PsBb, as well as PsCa (the docking site for ferredoxin or flavodoxin at the cytoplasmic side of PSI\textsuperscript{32,33}) and PsLa (required for the formation of PSI trimer\textsuperscript{34}) (Fig. 3b). When cells were transferred to LL, the relative content of PsAa, PsBb, PsCa, and PsLa increased at comparable rates. After two days of LL adaptation, the PsCa and PsLa contents appeared to increase faster than those of PsAa and PsBb, corroborating the stepwise assembly of PSI proteins in PSI complex biogenesis\textsuperscript{10}. Compared to other PSII subunits, the content of PsbF that forms part of cytochrome \textit{b}$_{559}$ was less reduced under HL, and there was a remarkable increase in the PsbF content at the initial stage of thylakoid biogenesis (Fig. 3c). A greater accumulation of PsbL also occurred at the initial thylakoid biogenesis process compared to PSII reaction center proteins PsbA (D1) and PsbD (D2), as well as the core antenna proteins PsbB (CP47) and PsbC (CP43). Thus, our results indicate that the stepwise biogenesis of PSI is promoted by the accumulation of PsbF and PsbL, which do not interact in the PSI holoenzyme, prior to D1 and D2\textsuperscript{9}. Consistently, it has been shown that cytochrome \textit{b}$_{559}$ functions as a nucleation factor for the early steps of PSI assembly\textsuperscript{35}, and the expression of the \textit{psbEFLJ} operon is a prerequisite for D1 and D2 accumulation\textsuperscript{36,37}. Among the Cyt \textit{b}$_{55}$ subunits, PetA (Cyt \textit{b}) had a greater accumulation at the early step of thylakoid biogenesis compared to others (Fig. 3d), suggesting that cyanobacterial Cyt \textit{b}$_{55}$ also possesses a stepwise assembly.

Unlike the decrease in the abundance of photosynthetic complexes, NDH-1 complexes, which have different forms and participate in respiration, cyclic electron transfer, and CO$_2$ uptake in cyanobacteria\textsuperscript{38}, were highly accumulated in thylakoid membranes induced by HL (Fig. 3e), consistent with previous studies\textsuperscript{8}. NdhE and NdhV are common subunits for all the forms of NDH-1\textsuperscript{39,39}, and their abundance under HL was noticeably higher compared to GL (Fig. 3e, Supplementary Fig. 9). Likewise, NdhF3 and CupA, which are the specific subunits of the low-CO$_2$-inducible, high-affinity NDH-1$_{3}$ complex for CO$_2$ uptake\textsuperscript{40}, were accumulated to levels 3.8 and 5.7 times than seen under GL, respectively (Fig. 3e). During thylakoid development, the relative amount of NdhE, NdhF3, CupA, and NdhV gradually declined (Fig. 3, Supplementary Fig. 9). By contrast, the expression of NdhF4 and NdhD4, which are the specific subunits of constitutively expressed, low-affinity CO$_2$ uptake system NDH-1$_{4}$ complex\textsuperscript{41}, was downregulated by \textsim{}60% compared to GL (Fig. 3e), in agreement with the previous transcriptional studies\textsuperscript{41}; their relative amounts increased constantly up to the levels in the GL cells during thylakoid recovery (Fig. 3e). Our results demonstrate the distinct regulatory changes in the NDH-1 abundance during thylakoid biogenesis, which are dependent on the forms and functions of NDH-1.

The changes in the abundance of photosynthetic proteins during thylakoid biogenesis were further confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) combined with immunoblot analysis (Supplementary Fig. 9). Determination of ATPase composition and stoichiometry by mass spectrometry was unreliable, due to high signal variation between biological samples. However, the specificity of the antibody against ATP-\(\beta\), encoded by \textit{atpD}, allowed us to monitor the changes in the ATPase content. The content of ATP-\(\beta\) subunits increased by 14% under HL compared to GL and was then gradually increased during LL adaptation (Supplementary Fig. 9), likely to meet with the increasing demand of energy for thylakoid membrane biosynthesis.

To examine the assembly of functional photosynthetic complexes during the thylakoid biogenesis, blue native-polyacrylamide gel electrophoresis (BN-PAGE) combined with immunoblot analysis was applied on the isolated thylakoid membranes. The contents of PSI trimers, PSII dimers, and PSI/PSII monomers were decreased drastically under HL compared to GL, and then were increased during thylakoid biogenesis (Fig. 4a). During this process, PSI monomers accumulated faster than PSI trimers, PSII dimers, and PSI monomers (Fig. 4b–d). Almost no PSI trimers were detected on LL day 1 (Fig. 4b, d), consistent with the lower abundance of Psal that mediates PSI trimerization\textsuperscript{34} on LL day 1 (Fig. 3b). Along with thylakoid biogenesis, the relative amount of PSI trimers increased rapidly (Fig. 4b, d). Consistently, the monomer/oligomer ratio of PSI was higher than that of PSII at the early stage of thylakoid biogenesis and then became comparable at the later phase (Fig. 4e). These results indicated that during thylakoid biogenesis the assembly of PSI monomers in thylakoid membranes occurred before the accumulation of PSI trimers and PSIII complexes. PSI monomers were also detected in HL-adapted thylakoid membranes (Fig. 4b), ensuring PSI-mediated cyclic electron flow throughout cell growth\textsuperscript{19}. In contrast, there were only a low amount of PSII monomers in the HL-adapted thylakoid membranes (Fig. 4c, d). Instead, the thylakoids accumulated a large amount of PSII core complexes lacking CP43, termed RC47 complexes\textsuperscript{36,42}. On LL day 1, the RC47 complexes remained dominant in the PSIII assemblies (Fig. 4c, d). During thylakoid biogenesis, PSII monomers and dimers accumulated at the expense of RC47, indicative of the stepwise assembly of PSI and the stoichiometric changes of distinct PSIII assemblies. Recent studies showed that the binding of the assembly factor Psb28 to RC47 protects PSII from photodamage during PSI assembly\textsuperscript{43}.

In agreement with the relative quantification of individual proteins (Fig. 3c) and levels of complexes (Fig. 4c), PSII activity was reduced almost 88% by HL treatment (Supplementary Fig. 10a) when most of the PSI complexes are in the form of RC47 (Fig. 4c), in relative to GL. Under LL treatment, PSII activity gradually recovered when PSII monomers and dimers started to accumulate and become dominant (Supplementary Fig. 10a, 4c). After 4-day LL treatment, PSII activity reached about 87% of that in the GL-adapted cells. These results indicate that the recovery of PSII activity lags behind Chl synthesis (Supplementary Fig. 1) and there might be a pool of Chls, which do not associate with functional photosystems in the recovering
cells but could associate with IsiA forming dynamically the PSI—IsiA supercomplexes\textsuperscript{11} or could be redistributed between photosystems\textsuperscript{44}. In contrast, PSI activities were reported to be less variable than the PSII activity during the light-triggered thylakoid biogenesis\textsuperscript{19}, consistent with our results indicating that the integration of PSI monomers into thylakoid membranes occurred before the assembly of PSII complexes during thylakoid biosynthesis (Fig. 3). Moreover, 77 K fluorescence emission spectra with Chl excitation (435 nm) showed that the fluorescence at 695 nm specific for PSII was not distinguishable in the HL-grown cells but clearly reappears as a distinct peak in LL day 1 (Supplementary Fig. 10b). This indicates the biosynthesis of PSII and thylakoid membranes, as confirmed by the proteomics (Fig. 3c) and BN-PAGE results (Fig. 4). There was a blue-shift of
the PSI fluorescence peak after the HL phase (Supplementary Fig. 10b), consistent with the presence of a large amount of PSI monomers as observed in BN-PAGE (Fig. 4b). 77 K fluorescence emission spectra, when excited at 600 nm, revealed that there was a notable increase in the fluorescence at 685 nm (phycobilisomes and PSII) relative to the fluorescence at 695 nm (PSII) under HL compared to GL (Supplementary Fig. 10c), indicating the functional decoupling of phycobilisomes and photosystems. A further increase in the phycobilisomes fluorescence at LL day 1 suggested the presence of newly synthesized free phycobilisomes in cells. These phycobilisomes started to be energetically coupled with the photosystems at LL day 2, when more functional PSII complexes were assembled in the thylakoid membranes.

Membrane location and redistribution of photosynthetic complexes during thylakoid biogenesis.

We further utilized super-resolution confocal fluorescence imaging to visualize the in situ distribution of photosynthetic complexes during thylakoid biogenesis, using fluorescently labeled Synechococcus mutants PSI-eGFP (tagged to PsaE), PSII-eGFP (tagged to PsbB (CP47)), Cyt b/f-eGFP (tagged to PetA), and ATPase-eGFP (tagged to AtpB). Cells at different thylakoid biogenesis stages were imaged by a Dragonfly spinning disk confocal microscope with the super-resolution radial fluctuations (SRRF)-stream technology. The eGFP-tagged Synechococcus cells exhibited similar Chl/OD750 ratios as the WT cells under the corresponding light conditions (Supplementary Fig. 11), confirming that fluorescent tagging had limited effects on cell physiology as reported previously. Under HL, PSI-eGFP exhibited both the thylakoid and cytosolic locations (Fig. 5a), probably due to the HL-induced detachment of the peripheral PSI subunit PsaE from thylakoids that was tagged with eGFP. This was confirmed by immunoblot analysis (Supplementary Fig. 12). Transferring the HL-adapted cells to LL rescued the thylakoid location of PSI-eGFP (Fig. 5a, Supplementary Fig. 12). At the initial stage of thylakoid biogenesis, the Chl fluorescence in all studied strains was brighter on one longitudinal side of the cell than the other side (Fig. 5), demonstrating the asymmetrical biosynthesis of thylakoid membranes as revealed in thin-section EM (Fig. 1a, b) and cryo-ET (Fig. 2). PSI, PSII, Cyt b/f, and ATPase also displayed an asymmetrical distribution on LL day 1 and GFP signals were concentrated on the same longitudinal side of the cells as Chl signals (Fig. 5, Supplementary Fig. 13), further confirming the asymmetrical biosynthesis of thylakoid membranes in Synechococcus cells. Compared to the other three photosynthetic complexes at this stage, PSI showed a higher degree of asymmetrical distribution (Fig. 5c, white arrows; Fig. 5d, Supplementary Fig. 13), suggesting that a great amount of PSI complexes were integrated into the newly synthesized thylakoid membranes. During the synthesis of thylakoid membranes, PSI displayed more clustered distribution in mature thylakoid membranes, frequently at the poles of thylakoids (Fig. 5a, orange arrows; Fig. 5b), giving rise to a more uneven distribution in thylakoid membranes (Supplementary Fig. 14a), whereas PSII, Cyt b/f, and ATPase complexes exhibited a relatively even distribution in the thylakoid membranes at the later stage of thylakoid biogenesis (Fig. 5, Supplementary Fig. 14b–d). These results provide evidence for the membrane redistribution of photosynthetic complexes during thylakoid biogenesis, ensuring the formation of specific membrane domains for function. Likewise, phycobilisomes localized largely with Chl fluorescence during thylakoid membrane reconstruction (Supplementary Fig. 15).
Fig. 5 Expression and intracellular localization of photosynthetic complexes in vivo in Synechococcus during thylakoid membrane biogenesis. a PSI:eGFP; c PSII:eGFP; e Cyt b$_{5}$;f eGFP; and g ATPase:eGFP Synechococcus cells imaged by Dragonfly spinning disk confocal microscope with the super-resolution radial fluctuations (SRRF)-stream technology. Figures are representative of three biologically independent experiments. First row: GFP emission, second row: Chl fluorescence, third row: merge. b, d, f, h GFP profile analysis of PSI-, PSII-, Cyt b$_{5}$- and ATPase-eGFP around the cell in these strains. Profiles in red are the averages from 30 individual imaged cells, shown in yellow on the background, from three biologically independent experiments. In the schematic model shown on the top, L1 in green: longitudinal cell side 1, P1 in orange: pole 1, L2 in yellow: longitudinal cell side 2, P2 in pink: pole 2. In each cell tracking of eGFP signal was started from the same site (indicated by the red vertical line) and to the same direction (indicated by red arrow). A higher difference between the minimum and maximum of the signal indicates the more clustered distribution of proteins. Cells were grown under growth light (GL), high light (HL), and HL-grown cells were then transferred to low light (LL) conditions for five consecutive days.
**Discussion**

In this study, we systematically characterized the dynamic process of thylakoid membrane biogenesis in *Synechococcus*. By applying classical chemical fixation and thin-section EM of cyanobacterial cells, we showed the regulation of thylakoid membrane abundance in cyanobacteria in response to the changing light intensity during cell growth. Consistent with the finding derived from conventional EM, our study by combining cryo-focused ion beam (cryo-FIB) milling and in situ cryo-ET further revealed that no physical fusion was discerned between the plasma membrane and regular thylakoid concentric cylinders in vivo, in line with recent observations on *Synechocystis*.

The newly synthesized thylakoid membrane layers appear asymmetrically on one longitudinal side of the cell between the plasma membrane and pre-existing thylakoids. The assembly and integration of photosynthetic complexes in thylakoids proceed via a stepwise fashion and vary in temporal order and spatial location. The integrated photosynthetic complexes are reorganized in membranes in the course of thylakoid biogenesis. These results allow us to propose a model to illustrate the biogenesis pathway of thylakoid membranes in *Synechococcus* (Fig. 6).

Where thylakoid biogenesis initiates and how the biogenesis process proceeds are poorly understood. Three distinct models for cyanobacterial thylakoid membrane biogenesis are currently being debated:

1. The synthesis of photosynthetic components occurs at the plasma membrane before migrating to thylakoids via their fusion sites, based on membrane fractionation combined with immunoblot and proteomic studies;
2. The synthesis of photosynthetic components (including protein, pigment, and lipid) occurs in situ in pre-existing thylakoid membranes; and
3. The photosynthetic components are synthesized at ribosome-rich sites and are assembled in specialized regions in the thylakoid membrane.

The thylakoid membrane structures in cyanobacteria vary among different species. It has been well documented that in the spherical unicellular cyanobacterium *Synechocystis*, thylakoid membranes converge at several sites close to the plasma membrane.

Figure 6: A schematic model representing the generation and distribution of both thylakoid membrane and photosynthetic protein complexes, including their oligomerization state, at different stages of thylakoid biogenesis in *Synechococcus*. Note that the number of individual complexes has no direct links to their real stoichiometry in situ. Existing membrane parts are in green and new ones in orange during the initial steps of the thylakoid biogenesis process. The schematic models of photosynthetic complexes were generated based on their PDB structures. PDB ID: PSI trimer and monomer, 1JB0; PSI dimer and monomer and RC47, 3WU2; Cyt b₈f, 2E74; ATPase, 6FKF.
how thylakoid biogenesis is initiated in rod-shaped Synechococcus cells that have regular thylakoid sacs that form concentric cylinders without convergence sites (Figs. 1, 2).

Our cryo-ET results showed no direct connections between the plasma membrane and regular thylakoid membranes in Synechococcus at the initial stage of thylakoid biogenesis (Fig. 2, Supplementary Figs. 3, 4), similar to the observations in Synechocystis13,14. Moreover, we observed the extension of thylakoid lamellae towards the plasma membrane, and the thylakoid tips are in close vicinity of the plasma membrane (Fig. 2, Supplementary Fig. 5, and Supplementary Movie 3), reminiscent of the thylapses characterized in Synechocystis15. To our knowledge, this is the first characterization of the “thylapse”-like structures in the cyanobacterial cells possessing regular thylakoid cylinders. Whether these “thylapse”-like structures are involved in thylakoid biogenesis remains to be experimentally verified. It is worth noting that the thylapse structures observed in Synechococcus are mainly located at the cell poles, and thus are not likely to be involved in cell division.

We also observed that additional thylakoid layers representing the newly synthesized thylakoids appear specifically on one longitudinal side of the cell, resulting in the asymmetrical distribution of thylakoid lamellae at the early stage of thylakoid biogenesis (Figs. 1, 2, 5). These thylakoid sheets sit between the plasma membrane and the pre-established continuous thylakoid layer (Figs. 1, 2), suggesting that thylakoid biosynthesis in Synechococcus likely commences from the regions close to the plasma membranes rather than from the inner cytoplasmic side of the cell. Consistent with this, some small membrane segments that have one side connected with the thylakoid lamellae and the other end approaching the plasma membrane have been seen (Supplementary Fig. 5, Supplementary Movies 4, 5). Likewise, some tube-like structures were observed next to the plasma membrane and sometimes close to the thylakoid tips (Fig. 2g–i, Supplementary Figs. 6, 7, and Supplementary Movies 1, 6). The extension of the peripheral thylakoid fragments will compress the cytoplasmic space (Figs. 1a, 2b, j), resulting in the development of cylinders without convergence sites (Figs. 1, 2).

Our confocal images showed explicitly that during the initial stages of thylakoid biogenesis, compared to other photosynthetic organisms, the RC47 complexes appeared predominantly as PSI monomers undertook a faster accumulation than PSI trimers and PSII holoenzymes. Recent AMF images have demonstrated that the PSI trimers, monomers, and some dimers coexist in the Synechococcus thylakoid membranes11. The ratio of PSI monomer/trimer varied during thylakoid biogenesis, along with the changes in the Psal content (Figs. 3b, 4b, d, e). These results outlined the stepwise de novo assembly process of PSI and the flexibility of PSI oligomerization that is regulated by Psal.34 The functional discrepancy of different PSI oligomers and how their stoichiometries are physiologically regulated in cyanobacterial cells remain elusive. AFM also revealed the association of PSI with PSII in native thylakoid membranes11, highlighting the possible roles of PSI in PSII biogenesis37 and photoprotection via efficient energy spillover38.

The temporal stepwise assembly of photosynthetic complexes from building modules was also demonstrated by the early accumulation of PsbA and PsbB compared to other PSI components (Fig. 3b), and there was a notable increase in the relative amounts of PsbF and PsbL compared to the PSII core proteins D1 and D2 during the initial stages of thylakoid biogenesis (Fig. 3c). As D1 protein is strongly involved in PSII photoinhibition, the lack of a considerable increase in the D1 amount at the early stage of thylakoid biogenesis could reflect the balanced synthesis and degradation of D1 in the PSII repair cycle39,60. Moreover, a large amount of RC47 complexes were accumulated under HL and LL day 1, prior to the formation of PSII monomers and subsequent dimers that are considered as the final photochemically active form of PSI39,59 (Fig. 4c–d).

NDEH-1 complexes in cyanobacterial thylakoid membranes are crucial for both photosynthetic cyclic electron transfer and respiration38. In vivo observations illustrated that in thylakoid membranes, NDEH-1 complexes could reorganize to be clustered in respiratory membrane zones under a lower light irradiance to facilitate respiratory electron flow, or to be distributed throughout the thylakoid membranes adjacent to PSI for cyclic electron transfer.8 In addition to the biochemical evidence,8 the tight association of NDH–1–PSI supercomplexes has been directly visualized in cyanobacterial thylakoid membranes31. Cyanobacteria possess different isoforms of NDH-1 that vary in protein composition and function.62 Our results indicated that the stoichiometric regulation of different NDH-1 isoforms in response to the changes in light conditions differs. The total NDH-1 complex, including the low-CO2-inducible, high-affinity NDH-1,3, were accumulated under HL in agreement with previous studies,8,11, and then the relative amount decreased during the LL-induced thylakoid biosynthesis (Fig. 3e). In contrast, the constitutively expressed, low-affinity NDH-14 exhibited sharply opposite responses to light regulation. The underlying mechanisms and physiological significance in CO2 assimilation remain to be determined.

In addition to the orders of assembly of photosynthetic complexes, our study also provides clues about the de novo membrane integration and assembly sites of photosynthetic complexes and the dynamics of their subcellular locations. The thylakoid membranes,63 thylapse membrane domains15 or PratA-defined membrane (PDM) regions,64–66 in Synechocystis, and the translation zones in the chloroplast of Chlamydomonas67,68 have been proposed to be the principal sites for the initial biogenesis and/or assembly of photosynthetic complexes, in particular PSII. Our confocal images showed explicitly that during the initial stages of thylakoid biogenesis, compared to other photosynthetic complexes, PSII complexes exhibited a greater accumulation on one longitudinal side of the Synechococcus cell, where the newly synthesized thylakoid fragments appear (Fig. 5, Supplementary Figs. 13, 14). Since eGFP was tagged to CP4712 and the PSII complexes appeared predominantly as RC47 at the initial stage of thylakoid biogenesis (Figs. 4c, 4d), our results indicate that the initial steps of RC47 assembly occur predominantly in these newly generated thylakoid fragments. In a very recent study, we have revealed that the mRNA molecules encoding the core subunits of PSI and PSII clustered at the thylakoid surfaces that are mainly adjacent to the central cytoplasm.66 We showed that the locations of mRNA molecules correspond to the sites of translation. After translation, these synthesized photosystem proteins...
could be directed to unknown mechanisms, to the newly formed thylakoid layers at the peripheral side of the cytoplasm. Indeed, the perforations in the thylakoid lamellae that we observed in cryo-ET (Fig. 2g, h, Supplementary Fig. 8, and Supplementary Movie 7) could facilitate rapid protein transport between spatially distinct cellular regions through the thylakoid membranes. During the expansion of thylakoid membranes, PSI assemblies spread throughout the thylakoid membranes (Fig. 5c, d, Supplementary Fig. 14) and heterogeneous clustering and arrays of PSI dimer rows have been observed in the thylakoid membranes of Synechococcus, suggesting the presence of PSI repair "zones." Unlike PSI, the initial membrane insertion and assembly and distribution of PSI complexes seems to occur more evenly in both newly synthesized and existing thylakoid membranes (Fig. 5a, b, Supplementary Fig. 13), suggesting that there might be different protein targeting mechanisms or assembly sites of PSI and PSII. During the thylakoid biogenesis process, PSI complexes could redistribute to exhibit more polar membrane distribution (Fig. 5a, b, Supplementary Fig. 14a, consistent with previous studies). PSI-enriched thylakoid membranes of Synechococcus have been observed in previous AFM studies. Therefore, our results provide evidence for the dynamic membrane location of photosynthetic and respiratory complexes during the thylakoid biogenesis.

In summary, our study provides insights into the spatial-temporal assembly and biogenesis of photosynthetic complexes and thylakoid membranes in the rod-shaped unicellular cyanobacterium Synechococcus. We demonstrate that thylakoid membrane formation is a highly dynamic process, involving the assembly and functional modulation of photosynthetic protein complexes. It is still unclear how pigment and lipid biosynthesis are functionally coordinated with protein biosynthesis and assembly during thylakoid biogenesis. We believe that our methodologies are suited to assess the biogenesis and development of morphologically distinct thylakoid membranes in diverse cyanobacterial species and chloroplasts as well as other biological membrane systems.

Methods

Strains and culture conditions. Synechococcus elongatus PCC 7942 (WT) and eGFP-expressed Synechococcus strains were grown in BG11 medium under the continuous white light of 40 µmol photons m⁻² s⁻¹ at 30 °C in culture flasks with constant shaking. Experimental cultures were grown in a photobioreactor MultiCultor MC 1000 (Photon Systems Instruments, Brno, Czech Republic). Parallel 80 ml cultures were started from pre-experimental cultures by inoculating cells to a final OD₇₅₀ of 0.2 in fresh BG11 medium. Cells were diluted to OD₇₅₀ ∼0.8 in their culture medium before plunge freezing. 3 µl of suspended cells in culture medium were applied in dilution of 1:10,000 and 1:1000, respectively. Cells were dehydrated with increasing alcohol concentrations (30–100%), embedded in resin, and cut in thin sections (70 nm). These were post-stained with 3% lead citrate. Images were recorded using a Tecnai G2 Spirit BioTWIN transmission electron microscope (Field Electron and Ion Company, FEI) equipped with a Gatan Rio camera. Distance between the thylakoid membrane layers was determined from TEM images with FIJI image processing package (ImageJ, NIH).

Cryo-electron tomography. Cell vitrification and cryo-focused ion beam (cryo-FIB) milling. Synechococcus cells were diluted to OD₅₇₀ = 0.8 in their culture medium before plunge freezing. 3 µl of suspended cells in culture medium were applied to the front side of glass-discharged R2/R2 carbon-coated copper grids (Quantifoil MicroTools), with additional 1 µl cells added to the back side. The grids were blotted for 4 s before vitrified in liquid ethane using a Leica GP2 plunge freezer. The vitrified grids were subsequently loaded to an Aquilos focused ion beam/scanning electron microscope (FIB/SEM) (Thermo Fisher Scientific) to prepare thin lamellae of cells. To reduce overall specimen charging, the grids were sputter-coated with platinum before milling. The ion beam current was gradually adjusted to lower values as the lamellae thinned progressing (first using 0.1 nA until 0.75 µm thick, and then 50 pA until 250 nm thick).

cryo-ET data collection: tomography tilt-series were acquired from lamella using a Titan Krios microscope (Thermo Fisher Scientific) operated at 300 kV. The tilt-series data were acquired with a stage pre-tilt of −7° to account for the angle of lamellae, meaning the lamella should be highly perpendicular to the beam. Tilt-series were collected from −42 to 42° in 2° increments by a group of three using dose symmetric scheme in SerialEM, with a target defocus of −10 µm. The tilt-series alignment was performed using IMOD. The black dot-like stains on the surface of lamella were selected as fiducial markers, which were manually traced to create the fiducial
model. Tomogram was reconstructed using 4x binned (24.92 Å/pixel) micrographs without CTF correction with 5 cycles of SIRT. The automated segmentation/annulation of 3D volumes was performed using a neuronal network implemented in EMAN2, with additional manual adjustments. These volumes were visualized in 3D using UCSF Chimera.

Mass spectroscopy. In-solution digestion of thylakoid membrane samples for MS: thylakoid membrane pellets were reconstituted, reduced, alkylated, and digested as described in [1], with the exception that trypsin to protein ratio was 1:50. The following day sample was removed by centrifugation of 0.5% (v/v) TFA and incubation at 37 °C for 45 min. Digests were centrifuged at 17,200 g for 30 min and the clarified supernatants aspirated. Samples were stage-tipped on C18 filters to remove chlorophyll prior to LC-MS/MS analysis.

LC-MS/MS: analysis data-dependent LC-MS/MS analyses were conducted on a QExactive quadrupole-Orbitrap mass spectrometer coupled to a Dionex Ultimate 3000 RSLC nano-liquid chromatograph (Dionex/Thermo Fisher Scientific). An equivalent of 100 ng peptides per sample was injected for mass spectrometry. The mass spectrometer was operated in DDA mode with survey scans between m/z 300–2000 acquired at a mass resolution of 70,000 (FWHM) at m/z 200. The ten most intense precursors ions with charge states of between 2+ and 5+ were selected for MS/MS with an isolation window of 2 m/z units.

Database search and Protein identification: raw data files were searched against the UniProt proteomes database of Synechococcus elongatus 7942 (UniProt ID: UP00002717) using the Mascot Discoverer software (Thermo Fisher Scientific version 2.4.1) connected to an in-house Mascot server (Matrix Science, version 2.4.1). A precursor ion tolerance of 10 ppm and a fragment ion tolerance of 0.01 Da were used with carboxymethyl cysteine set as a fixed modification and oxidation of methionine as a variable modification.

Label-free quantification in Progenesis QI for MS: raw mass spectral data files were processed using Progenesis QI (v1.1; Nonlinear Dynamics) to determine total protein abundances. All raw files were initially automatically aligned, according to the retention time, to produce an aggregate LC-MS map, from which peptide feature charge-states +1 and +2 were selected. A precursor ion tolerance of 10 ppm and a fragment ion tolerance of 0.01 Da were used, with carboxymethyl cysteine set as a fixed modification and oxidation of methionine as a variable modification. Trypsin was the specified enzyme, and one missed cleavage was allowed. A pooled extract was used for Mascot and searched against the UniProt proteomes database of Synechococcus elongatus 7942 (UniProt ID: UP00002717) using the Mascot search engine (version 2.4.1; Matrix Science, UK).

Confocal fluorescence microscopy and imaging analysis. To immobilize cells for imaging 10 µl of culture was pipetted on top of agar and dried drop was cut out. Live-cell super-resolution spinning disc confocal fluorescence microscopy and imaging analysis were performed on a Zeiss microscope equipped with a 100X Plan-Apochromat oil immersion lens and a 488 nm laser. Images were acquired at 2000 acquired at a mass resolution of 70,000 (FWHM) at m/z 200. The ten most intense precursors ions with charge states of between 2+ and 5+ were selected for MS/MS with an isolation window of 2 m/z units.

Whole-cell absorption spectra and inherent Chl determination. Whole-cell absorption spectra were measured at room temperature with 1 nm increments using a customized Aminco DW2000 UV/Vis spectrophotometer (Olis, USA), which places the sample cuvettes very close to a large detector window, hence minimizing distortions due to turbidity. Chlorophyll concentration was estimated from the cell absorption spectra using the formulae as described previously [8].

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