Structure of photosystem II
van Bezouwen, Laura

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

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

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 5

Location of phycobilisomes in whole cyanobacteria analysed by cryo-electron tomography

Laura S. van Bezouwen, Gert T. Oostergetel and Egbert J. Boekema

Author contribution
L.S.v.B., G.T.O and E.J.B. designed the research and wrote the chapter. L.S.v.B. and G.T.O performed the data collection, L.S.v.B analysed the data.
Abstract

In cyanobacteria photosynthesis takes place in thylakoid membranes. The primary steps of this process are carried out by two photosystem proteins, photosystem I (PSI) and photosystem II (PSII) and their light harvesting complexes, the phycobilisomes (PBSs), which are attached to the membranes. The PBSs are large multisubunit protein-pigment complexes, which are connected in an unknown way to PSII and PSI. In this study several different cyanobacteria were studied with electron tomography to solve the structure of the PBSs \textit{in vivo} and their connection with PSII and/or PSI. We could visualise the concentric arrangement of pairs of thylakoid membranes. Based on the used technique we could determine the location of the PBSs, but no further structural details. One important limiting parameter is the large size of the cyanobacteria with respect to the penetration power of the electron beam. To solve the structure of the PBSs \textit{in vivo} and their connection with PSII and PSI we extensively discuss which technique can be used.

Introduction

Cyanobacteria are believed to be the oldest group of organisms in evolution that can perform oxygenic photosynthesis. The photosynthetic reactions are light driven and convert light into chemically fixed energy (Heathcote \textit{et al.} 2002). Oxygen is a side-product of water splitting. Photosynthesis takes place in specialized membranes, the thylakoid membranes. Because cyanobacteria have no compartmentalization, the thylakoid membrane is contiguous with the cytoplasmic membrane system, where respiration reactions are catalysed (Vermaas 2011). Plants on the other hand have a specialized organelle, the chloroplast, in which the thylakoid membranes are located. The thylakoid membranes in chloroplasts of higher plants were first described by Wilhelm Menke as unstacked and stacked flattened vesicles, surrounded by a membrane made of proteins and lipids (Menke 1962). Many studies involved the structure of the chloroplasts and how the membranes were interconnected, as well as the distribution of the photosynthetic proteins, photosystem I (PSI) and photosystem II (PSII). A major milestone was the discovery that PSII is mainly located in the stacked membranes and PSI only in the unstacked membranes (Andersson and Anderson 1980). To visualize the structure of the thylakoid membranes and their interconnection, electron microscopy has been used in many studies. Over the years several different models have been proposed to show how the thylakoid membranes are organized. In these studies, whole cells or whole chloroplasts were freeze-substituted before embedding in plastic. The embedded samples were sliced into thin sections and these were analysed with electron tomography (Arvidsson and Sundby 1999, Mustardy and Garab 2003,
Shimoni et al. 2005, Austin and Staehelin 2011). In the study of Daum et al. (2010) thin cryo-sections were prepared and analysed with electron cryo-tomography (Daum et al. 2010). The model proposed by Austin and Staehelin (2011) shows how the grana thylakoid membrane stacks are interconnected by unstacked stroma thylakoids (reviewed by Daum and Kühlbrandt 2011).

The thylakoid membranes of cyanobacteria, compared to the chloroplasts of higher plants, have a completely different ultrastructure. There is neither a division between stacked and unstacked membranes, nor a clear spatial separation between the photosystems. With evolution the complexity of the thylakoid membrane organization increased. Many cyanobacterial species have membranes, which run as concentric shells, but the membranes can interconnect as well (Nevo et al. 2007, Ting et al. 2007, Liberton et al. 2011, Nevo et al. 2012). Not only are the thylakoid membranes different between plants and cyanobacteria, but the light harvesting systems as well. Where plants have light harvesting complexes (LHC) as peripheral antenna, which are membrane-integral, most cyanobacteria have phycobilisomes (PBSs), which are associated with the thylakoid membrane surface. In none of those membrane studies the location of the phycobilisomes is discussed. Because of the large size of the phycobilisomes the pairwise thylakoid membranes are spaced by specific distances. The PBSs absorb light between 550 and 650 nm wavelength range and transfer the energy efficiently towards PSI and PSII to drive the primary photosynthetic reactions (Glazer 1983, Arteni et al. 2009). The connection between the PBSs and PSI and PSII is still largely unknown, although there is a schematic model that proposes how PBSs interact with PSII core complexes (Mullineaux 2008, Arteni et al. 2009). In a review the possible connection between the PBSs and PSI in cyanobacteria is described (Mullineaux 2008). A connection between a PBS and PSI is shown with negative stain in Anabaena (Watanabe et al. 2014). In a study of Liu et al. (2013) a megacomplex of PBS-PSII-PSI was isolated. Based on the energy transfer of the megacomplex it was concluded that the PBS excites both PSII and PSI. The energy transfer rates between PBS-PSII and PBS-PSI are different, the transfer of energy to PSI is delayed, and therefore the suggested model of the megacomplex involves a full coverage of PSII by a close association of the PBSs and a side-on association of the PBSs with PSI (Liu et al. 2013). However, none of these models can be supported by electron microscopy data due to easily disrupted interactions between the PBS, PSI and PSII (Liu et al. 2013).

In order to verify the findings of Liu et al. (2013), electron microscopy data are necessary on intact PBSs and preferably on the megacomplex PBS-PSII-PSI. Moreover, the currently proposed models of PBSs are based on negative stain electron microscopy. In the study of Arteni et al. (2008) intact PBSs are visualized
with single particle analysis but only a clear structure was visible on membrane-bound PBS in an orientation as in the membrane plane. Side views or free PBSs are difficult to visualize (Arteni et al. 2008). In none of the studies of Arteni et al. (2008 and 2009), there is a connection visualized between the PBSs and PSII or PSI. These interactions can easily be disrupted during isolation; therefore it is difficult to study the full intact megacomplex. A technique to overcome the isolation problem would be the use of electron tomography. With this technique the in-situ structure of the thylakoid membranes has been visualised of both plants and cyanobacteria. Smaller volumes of internal structures can be extracted computationally, if they are present in a sufficient number of identical copies in the tomograms and can be solved by so-called subtomogram averaging. But there are some limitations as well; one is due to the thickness of the sample. The maximum thickness of a sample depends on the amount of phase contrast in an image and the amount of inelastic scattering that occurs. The more inelastic scattering events and even multiple scattering events of electrons, the smaller the fraction of the unscattered beam can go through. Therefore a sample should be thin. At an operational acceleration voltage of 300 kV this leads to a maximum sample thickness of 500 nm (Gan and Jensen 2012).

Overall most cyanobacteria, which contain phycobilisomes, are substantially thicker than 500 nm, even bacteria that are described as pico-cyanobacteria. Nevertheless, based on literature the smallest copies of some fresh-water and salt-water species could be appropriate for an ultrastructure study by electron tomography. The described study is performed on whole bacteria to avoid making thin sections and thereby damage the fine cellular details.

In this chapter, we describe electron tomography experiments performed on three different cyanobacteria of a thickness that might be suitable for electron tomography reconstruction. The internal thylakoid membrane structure at the tip of these bacteria was visualized. The membranes occur pairwise at a distance of approx. 50 nm. Between these membranes, we could locate the phycobilisomes for the first time in intact cyanobacteria. A critical discussion is presented on how to interpret internal structure in relatively large bacteria by electron tomography, as well as how future instrumental developments and sub-tomogram averaging may be of help to ultimately solve the interaction of PSII and/or PSI with the phycobilisomes.

**Materials and Methods**

*Cyanobacteria*

*Synechococcus* sp. CCY9504 and CCY9505 cultures, originally isolated from the Baltic Sea, were obtained from the NIOZ collection and grown on a Baltic Sea medium,
modified from Ferris and Hirsch (Ferris and Hirsch 1991). They were maintained at 16°C and under a long day regime (16h). A *Cyanobium gracile* PCC6307 culture from the Collection of Cyanobacteria from Pasteur Institute (Rippka and Cohenbazire 1983), grown on a B11 medium (Stanier *et al.* 1971) was kept at 20°C under a normal day regime (12h).

*Electron cryo-tomography of the cyanobacteria*

The cyanobacteria were concentrated before adding 10 nm gold particles as fiducial markers. Aliquots of 2.8 µl were applied onto a glow-discharged 200 mesh holey carbon copper grid (Quantifoil R2/2). Grids were vitrified using an FEI Vitrobot. After blotting at 100% humidity for 10 seconds, they were plunge-frozen in liquid ethane. Data were collected with a 300 kV G2 Polara electron microscope (FEI) equipped with a Gatan post-column energy filter. Images were recorded with a Gatan 2k CCD camera at 8 or 15 µm defocus at a final magnification of 41,400 or 31,050 and with a pixel size of 0.725 nm or 0.966 nm respectively 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 100-170 e−/Å².

*3D reconstruction and image analysis*

IMOD (Kremer *et al.* 1996) was used for alignment of the tilt series and reconstruction of the tomograms, based on SIRT. No CTF correction was performed.

*Statistical analysis*

In 8 cyanobacteria the distance between the pairwise membranes was measured. A total of 66 data points were taken, and the distances were averaged. This was done in ImageJ.

*Results*

Two different specimen of *Synechococcus sp.* CCY9504 and CCY9505 were analysed in order to determine their thickness, and therefore the possibility to perform tomography on these sample. In the *Synechococcus sp.* CCY9504 two different types of bacteria were present, large bacteria, wider and longer than 500 nm (Figure 1A), and bacteria, which were less than 500 nm wide (Figure 1B). The first tomograms were taken on the smaller bacteria. While analysing the tomograms, it became clear that these bacteria did not have any apparent internal thylakoid membranes, and therefore did not have the phycobilisomes (Figure 1B). The other bacteria did have visible internal membranes (Figure 1A), and were the cyanobacteria, which contain the phycobilisomes. When measuring the ice thickness close to the bacteria, and the
width of the bacteria it became clear that these bacteria were over 500 nm thick. A few image series were collected but after reconstruction none of these tomograms showed internal structures due to thickness of the sample.

*Figure 1. Two different cyanobacteria used for tomography.* (A) *Cyanobacterium Synechococcus* sp. CCY9504 which is too thick for tomography but does contain the thylakoid membranes. The black dots are gold fiducials of 10 nm, which are used for alignments of the tilt series. (B) A thin cyanobacterium with a good thickness for tomography. This cyanobacterium has no discernable thylakoid membranes. Scale bar is 200 nm (adapted from figure 4 in [Boekema et al. 2013].)

To improve the quality of the tomograms and be able to find internal structures a different cyanobacterium was used. *Cyanobium gracile* PCC6307 is a fresh water bacterium and smaller in size than the salt-water cyanobacteria. When the width of the bacteria was measured they were still over 1 µm wide (Figure 2A, red asterisks). However, these bacteria are pointy instead of rounded at one of the tips, and therefore a lot thinner in that area. As long as the pointy end was less than 800 nm wide (Figure 2A, red dots), the thylakoid membranes were visible, and an ice thickness measurement close to the edge of the bacteria indicate a thickness between 300 and 500 nm, an image series could be collected.
Figure 2. A *Cyanobium gracile* PCC6307 cyanobacterium used for tomography. (A) This cyanobacteria species is pointy at one tip and therefore tomograms could be collected from this part of the bacteria. Thylakoid membranes are visible and the black structures are carboxysomes. The red asterisks indicate where the bacterium is 1 µm wide, the red dots indicate where the bacterium is 800 nm wide. Blue lines indicate where the distance between the pairwise membranes has been measured. The black dots are gold fiducials of 10 nm. Scale bar is 200 nm. (B) Reconstructed tomogram of the same bacterium. A z-slice of 97 nm thick is shown. The yellow arrows indicate the location of the structures between the thylakoid membranes.

In most parts of the tomograms single membranes are visible. They mostly appear pairwise. The distance between the pairs of membranes was measured in several images (Figure 2A, blue lines). On average, the distance between the membranes is 51 ± 10 nm (SD, n=66), see also Figure 3). After reconstruction of these image series the tomograms showed some internal structures between the thylakoid membranes (Figure 2B). These structures are roughly hemi-spherical but no further structural details could be detected. To improve the details of these hemi-spherical structures, the chosen bacteria was reconsidered, due to its thickness, but none of the bacteria was thinner than shown in Figure 2. It is likely that these hemi-spherical structures are phycobilisomes, due to the location, their rough shape, and the distance between the pairwise membranes.
Figure 3. Histogram of the distance distribution between the pairwise membranes in increments of steps of 5 nm. The average distance between the pairwise membranes is 51 nm with a SD of 10 nm.

Discussion

The primary function of phycobilisomes is to absorb photons and transfer the energy towards the photosynthetic reaction centre of PSII or PSI. To understand the energy flow and regulation within the photosynthetic apparatus of cyanobacteria, it is important that the complete structure of phycobilisomes and their connection with PSII and PSI in the natural thylakoid membrane is known. The aim of this research was to study the PBSs in situ and to solve the connection between PBSs and PSII and/or PSI by electron tomography and subtomogram averaging. As shown in Figure 2B internal structures of bacteria could be detected. Based on the distance between the pairwise membranes, which is on average 51 nm (n=66), the size of a full phycobilisomes (33 nm (Ohki et al. 1985, Chang et al. 2015), the PBSs can be located between the membranes in several different configurations as indicated schematically in Figure 4. As the distance between the pairwise membranes is a range between 33 nm and 75 nm (Figure 3), both options are likely to be present in the cyanobacteria (Figure 4). However, based on an average distance of 51 nm Figure 4A is the most likely option, where the PBSs are alternately between the membranes.

Although structures were present between the thylakoid membranes, none of these hemi-spherical structures showed clear structural details. Based on the location, the distances between the pairwise membranes, the size of the PBSs, and previous
Phycobilisomes in whole cyanobacteria studies (Giddings et al. 1983, Tsekos et al. 1996, Olive et al. 1997) it is believed that these structures are the phycobilisomes.

Figure 4. Schematic overview of how the phycobilisomes can be located between the pairwise membranes. (A) The PBSs are located between the membranes alternately. (B) The PBSs are opposite of each other. Distance and height of PBSs are scaled.

Both types of bacteria used in this study are thicker than 500 nm, and therefore above the tolerance for multiple scattering a microscope with an acceleration voltage of 300 kV. For this reason it has been impossible to solve the structure of the phycobilisomes and their connection with PSII and/or PSI with the currently used technique. However, there are different approaches that could be used. Several options are available; cryo-scanning transmission electron tomography (CSTET) on whole bacteria or cryo sectioning or cryo-focused-ion-beam (cryo-FIB) milling can be applied to create thin sections or lamellae from the bacteria. To solve the interactions and the structure of the PBSs subtomogram averaging needs to be applied as well. The last option would be cryo-EM followed by single particle analysis of isolated complexes.

With CSTET, a probe is used which scans the specimen. The unscattered or scattered transmitted electrons are detected with a bright-field or dark-field detector respectively. This technique can be very useful for thicker samples (~600 nm), as it provides a better signal-to-noise ratio (Wolf et al. 2014). As there are no other results published with this technique and most cyanobacteria are over 600 nm thick, this technique could be difficult to use, and it is not clear yet if it will improve the visibility of relevant details sufficiently.

As it is difficult to visualize internal structures in whole cyanobacteria another option would be to use thin sections of the bacteria. A technique that has been used since 1965 is cryo sectioning. Over the years it has been used, and since the eighties it has
been applied in combination with electron cryo microscopy (review Dubochet et al. 1988). Since the start of the use of the technique it became clear that while applying the technique artefacts could occur. Sections as thin as 100 nm are relatively easily obtained (Richter 1994) but thicker sections are problematic. And sections thicker than 100 nm are necessary with electron tomography, as thin sections likely do not contain enough 3D volume to cover at least two intact membranes plus PBSs in between. An artefact in thicker sections has to do with the deformation or compression of the sample, which can be up to 60% of the sample (Dubochet et al. 1988, Richter 1994). Another artefact is the formation of crevasses and although it is now described how to overcome it for thicker samples it remains a problem (Hsieh et al. 2006). Overall this technique could be used, but thicker sections then 100 nm are necessary and because of the many artefacts this technique is not the preferred approach for this research.

FIB milling has the ability to precisely control the preparation by removing material from the surface of the sample (Giannuzzi 2005). As stated by Rigort and Plitzko (2015) FIB milling can be applied on frozen-hydrated biological samples, but some alterations need to be done compared to the system setup for material science (Rigort and Plitzko 2015). Several studies showed that tomography can be done on FIB thinned frozen-hydrated biological samples (Dubrovsky et al. 2015, Engel et al. 2015, Harapin et al. 2015, Narayan and Subramaniam 2015). The most interesting research compared to ours is done by Engel et al. (2015). In this study a structure of thylakoid membranes of *Chlamydamonas* has been investigated. Small structures with size of 6 nm have been detected on these membranes. In the same study a low resolution model showing the hexagonal packing of RuBisCO has been obtained by subtomogram averaging. As most of the research is done with a CCD camera it is stated that with the new direct-detection cameras the resolution of the tomograms could be improved further (Engel et al. 2015).

Based on the results of the study of Engel et al. (2015) and the size of the phycobilisomes, FIB milling could be a technique of help to visualize the connection of PBSs with PSII and/or PSI. However, for this research a larger bacterium would be recommended, compared to the currently used bacteria. This has to do with the optimal thickness of the final lamellae, which is between 200 and 500 nm. If the bacteria are too thin, creating lamellae will be difficult. The optimal lamellar thickness to study the phycobilisomes needs to be established. And to improve the resolution of the structure of the PBSs with PSII and/or PSI, subtomogram averaging will have to be applied on the analysed tomograms.

Comparing the different approaches involving tomography and based on the known thickness of the cyanobacteria, cryo-FIB milling followed by tomography would be the best technique to solve the structure of the PBSs and their connection with PSII or PSI.
The remaining option would be single particle analysis of isolated PBSs. This option has been tried before in the studies of Arteni et al. 2008, 2009 but without success. But recently Chang et al. (2015) published a low resolution negative stain 3D structure of the PBSs of *Anabaena* sp. strain PCC 7120 and a model of the connection with a PSII dimer. In this model it is shown that the binding of the PBSs with PSII is a close interaction, which is necessary for fast energy transfer. The latter model supports the data of Liu et al. (2013) that the connection between PBSs and PSII is occurs at a specific site. To confirm the connection of PSII with the isolated PBSs immunoblotting and immunogold labelling in the EM on core subunits was applied (Chang et al. 2015). Although the structure is not at high resolution, the first questions with respect to the connection of PSII could be answered. But it remains unclear how the PBSs are connected with PSI and how the energy transfer towards PSI is regulated. To improve the structure of Chang et al. 2015, the same technique could be used, but instead of using negative stain at room temperature the advice would be to do this at cryo temperature without a heavy metal stain. In this case the protein would stay in a more native condition, as it won’t be embedded in uranyl acetate. And therefore there is no resolution limit by the stain.

The approaches described in this chapter could be of help regarding the unsolved interactions of PBSs connected with PSI, to solve the megacomplex of PSII-PSI-PBSs, and to improve the proposed model of the PBSs with PSII. It is shown that with tomography the phycobilisomes could be detected, and their location on the pairwise membranes was determined. To solve these structures at a sufficient resolution a different preparation technique is required as well. And in this case the advice would be to apply cryo-FIB milling in combination with tomography and subtomogram averaging.