Localisation and characterisation of thylakoid membrane-shaping factors in *Synechocystis* sp. PCC 6803

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Table of content

Summary

Oxygenic photosynthesis converts light energy to chemical energy and releases molecular oxygen as a byproduct. Furthermore, the energy fixed during photosynthesis is the source for the main production of biomass on earth. Its evolutionary development goes back more than 2700 million years and has shaped a large part of life on our earth.

Photosynthesis is a highly complex process involving a large number of factors. The most important photosynthetic complexes are photosystem I, photosystem II, cytochrome b_6f and the ATP synthase. A co-evoled endomembrane system called thylakoids serves as carrier and reaction space for photosynthesis. While the photosynthetic complexes were relatively conserved in the course of evolution and differentiation of species, the shape and arrangement of the thylakoids were subject to constant change in adaptation to the respective environmental circumstances. This ranges from simple circular or star-shaped arrangements in cyanobacteria to the clear discrimination of stroma and grana lamellae in chloroplasts of today's plants.

In the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) the individual thylakoids are arranged in layers parallel to the plasma membrane and interrupted by convergence zones. These are regions where the thylakoid membranes partially fuse and curve towards the plasma membrane forming contact sites called thylapses. Due to the very high number of ribosomes in these areas, they have been shown to be biogenic regions.

CurT, the cyanobacterial homolog of the CURVATURE THYLAKOID1 protein family, is essential for the curvature of thylakoids in cyanobacteria as well as in chloroplasts of green algae and higher plants. A lack of CurT triggers dramatic alterations in the thylakoid architecture of *Synechocystis* 6803 including the loss of convergence zones. Moreover, photosynthetic activity and growth are reduced.

By whole-genome sequencing of a strain partially suppressing the photosynthetic *curT*$^-$ phenotype, the protein AncM (anchor of convergence membranes) was identified. AncM is an integral membrane protein located at thylakoid membrane convergence zones that form thylapses. An *ancM*$^-$ mutant is shown to exhibit an altered thylakoid ultrastructure with converged membranes detached from the plasma membrane and reduced photosynthetic performance. Furthermore, reduction in one protein, CurT or AncM, effects the other, which suggests an antagonistic function.

Another important factor for thylakoid architecture and maintenance is VIPP1. It has been shown that it belongs to a highly conserved superfamily of stabilizing and stress-counteracting proteins. Despite its essential role and apparent coevolution with thylakoids, the exact mechanism and action of VIPP1 has not been fully deciphered. However, by means of high-resolution microscopic techniques, the structure of homooligomeric supercomplexes could be shown in detail. Amphiphilic structures were identified and thereby a potential mechanism of how VIPP1 builds large hydrophobic columns to bind and curve membranes. In summary, the present work represents an extension of the previous knowledge about factors shaping the thylakoid membrane, their effects on photosynthesis and an expanded understanding of this important process.

Zusammenfassung

Die oxygene Photosynthese wandelt Lichtenergie in chemische Energie um und setzt als Nebenprodukt molekularen Sauerstoff frei. Darüber hinaus ist die bei der Photosynthese fixierte Energie die wichtigste Primärproduktion von Biomasse auf der Erde. Seine evolutionäre Entwicklung reicht mehr als 2700 Millionen Jahre zurück und hat einen Großteil des Lebens auf unserer Erde geprägt.

Die Photosynthese ist ein hochkomplexer Prozess, an dem viele Faktoren beteiligt sind. Die wichtigsten photosynthetischen Komplexe sind Photosystem I, Photosystem II, Cytochrom b_6f und die ATP-Synthase. Als Träger und Reaktionsraum für die Photosynthese dient ein ko-evolviertes Endomembransystem namens Thylakoide. Während die photosynthetischen Komplexe im Laufe der Evolution und Differenzierung der Arten relativ konstant blieben, veränderten sich Form und Anordnung der Thylakoide in Anpassung an die jeweiligen Umweltbedingungen ständig. Dies reicht von einfachen kreisförmigen oder sternförmigen Anordnungen bei Cyanobakterien bis hin zur eindeutigen Unterscheidung von Stroma- und Grana-Lamellen in Chloroplasten heutiger Pflanzen.

Im Cyanobakterium *Synechocystis* sp. PCC 6803 (im Folgenden *Synechocystis* 6803) sind die einzelnen Thylakoide in Schichten parallel zur Plasmamembran angeordnet und durch Konvergenzzonen unterbrochen. Dies sind Bereiche, in denen die Thylakoidmembranen teilweise verschmelzen und sich in Richtung der Plasmamembran krümmen und

Kontaktstellen bilden, die als Thylaps bezeichnet werden. Aufgrund der sehr hohen Anzahl von Ribosomen in diesen Bereichen haben sie sich als biogene Regionen erwiesen.

CurT, das cyanobakterielle Homolog der CURVATURE THYLAKOID1-Proteinfamilie, ist essentiell für die Krümmung der Thylakoide in Cyanobakterien sowie in Chloroplasten von Grünalgen und höheren Pflanzen. Ein Mangel an CurT löst dramatische Veränderungen in der Thylakoidarchitektur von *Synechocystis* 6803 aus, einschließlich des Verlustes von Konvergenzzonen. Außerdem werden die Photosyntheseaktivität und das Wachstum reduziert.

Durch Sequenzierung des gesamten Genoms eines *curT*$^{-}$ Suppressor Stammes wurde das Protein AncM (anchor of convergence membranes) identifiziert. AncM ist ein integrales Membranprotein, das sich an den Konvergenzzonen der Thylakoidmembran befindet und die Thylaps bildet. Es wird gezeigt, dass eine *ancM*$^{-}$ Mutante eine veränderte Thylakoid-Ultrastruktur mit zwar konvergierten Membranen aufweist, diese sind jedoch von der Plasmamembran abgelöst. Des Weiteren ist die Photosyntheseleistung verringert. Auch scheint die Reduktion eines Proteins, CurT oder AncM, das jeweils andere zu beeinflussen, was auf eine antagonistische Funktion hindeutet.

Ein weiterer wichtiger Faktor für die Thylakoidarchitektur und Wartung ist VIPP1. Es wurde gezeigt, dass es zu einer hochkonservierten Superfamilie von stabilisierenden und Stress entgegenwirkenden Proteinen gehört. Trotz seiner wesentlichen Rolle und der offensichtlichen Koevolution mit Thylakoiden sind der genaue Mechanismus und die Wirkung von VIPP1 noch nicht vollständig entschlüsselt. Mittels hochauflösender mikroskopischer Techniken konnte jedoch die Struktur homooligomerer Superkomplexe im Detail gezeigt werden. Amphiphile Strukturen wurden identifiziert und damit ein möglicher Mechanismus dafür, wie VIPP1 große hydrophobe Säulen bildet, um Membranen zu binden und zu krümmen.

Zusammenfassend stellt die vorliegende Arbeit eine Erweiterung des bisherigen Wissens über Faktoren, die die Thylakoidmembran formen, ihre Auswirkungen auf die Photosynthese und ein erweitertes Verständnis dieses wichtigen Prozesses dar.

I. Introduction

I.1 Biomembranes

There are many different requirements that together form life as we know and each one of them is necessary. One of the most subtle, but also important, of these is simply the surrounding shell, the membrane. An organism in its easiest form is a closed reaction space, i.e. an area separated from its environment and therefore capable of own chemical reactions separated from its environment as well as to build up a chemical and physical gradient between inner lumen and outer media. Only then metabolism and energy production is possible to counteract the surrounding entropy and make life possible (Davies et al., 2013).
In addition to the plasma membrane, there is especially in eukaryotes a very pronounced endomembrane system, which consists e.g. of the endoplasmic reticulum and Golgi apparatus, but also lysosomes, vacuoles and vesicles, endosomes, peroxisomes and many more (Hettema & Motley, 2009). However, even in bacteria there is a more and more researched membranous compartmentalisation. One example is the anammoxosome of anaerobic ammonium oxidation (anammox) bacteria which are essential for the world wide nitrogen turnover (Kuenen, 2008). A special and here in detail observed inner membrane system in cyanobacteria as well as chloroplasts are thylakoid membranes, as the photosynthesis carrying compartment of phototrophs.
In a closer look, biological membranes consist of a lipid bilayer with a hydrophilic surface and a hydrophobic core, thus amphiphilic molecules (Figure 1A). Each kind of biological membrane has its own specific lipid composition, which is tailored to its respective circumstances. The major part of membrane lipids are phospholipids. In thylakoids, these have been supplemented and partially substituted by galactolipids, mainly monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) (Dorne et al., 1990). It is precisely the commonality of this lipid composition in thylakoids which proofs the endosymbiotic origin of chloroplasts from early cyanobacteria (Vothknecht & Westhoff, 2001).

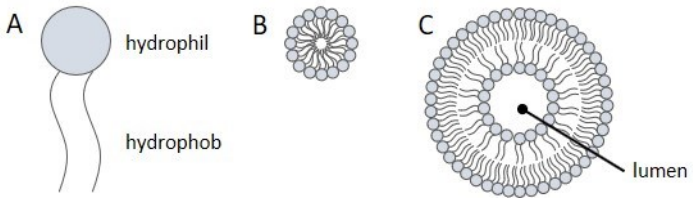

Figure 1: Membrane lipids and lipid arrangement.
A) Single membrane lipid with a hydrophilic head and a hydrophobic tail; B) micelle; C) liposome. Based on Bitounis et al. (2012).

One of the most important properties of biological membranes is that lipids form various aggregations by themselves from a certain concentration (Moroi, 1992). Their simplest but just single layered arrangement is in the form of micelles, in which the individual lipids are arranged in a spherical shape with the hydrophilic head facing outwards and the hydrophobic tail facing inwards (Figure 1B). Another kind of lipid arrangement are liposomes, which were first described in 1964 (Deamer, 2010). They are also spherical arranged, but in contrast to micelles they already consist of a lipid bilayer, which separates an outer space from an inner lumen (Figure 1C). This inner lumen can now contain another elemental composition than the surrounding area whereby a reaction space is created which can also enable separated chemical reactions. The space created in this way can be a whole cell, an organelle or simply a spatial delimitation which makes them good objects to carry out *in vitro* experiments with membrane-bound or associated proteins (Zoghi et al., 2018). With the help of membrane proteins like receptors, transporters, enzymes and other complexes, biological membranes represent an interaction point between their inner lumen and the environment (Guidotti, 1972). In the case of thylakoids, these proteins are the photosynthetic complexes together with the light harvesting complexes that generate a potential difference between thylakoid lumen and cytoplasm to drive energy production, the photosynthesis.

I.2 Structure and function of the photosynthetic apparatus

Each step in the evolution of life was, is and will be important. For photosynthesis, these were the evolution of cofactors such as quinones and chlorophylls, of protein complexes such as the reaction centres and ATP synthase, and of course groups of organisms such as bacteria and later eukaryotes. Thereby, step by step, life on earth has learned to turn the solar energy of its own star into chemical energy.

There are two kinds of photosynthesis, anoxygenic photosynthesis and oxygenic photosynthesis. Anoxygenic photosynthesis is used by green sulfur bacteria, purple bacteria, red and green filamentous phototrophs, acidobacteria and heliobacteria (George et al., 2020; Imhoff, 2008). Instead of chlorophyll they use bacteriochlorophyll a through g which absorbs in the near infrared (George et al., 2020). The redox potential of bacteriocholophyll is too low to split water and release molecular oxygen, which is also the eponymous difference between anoxygenic and oxygenic photosynthesis using chlorophyll (Blankenship & Hartman, 1998; Tamura et al., 2020).

Evolution of oxygenic photosynthesis and the resulting transition from anoxic to oxic conditions led to the singular Great Oxidation Event in the early Paleoproterozoic 2400 to 2000-million years ago (Hoffman, 2013; Holland, 2002). In this time the O_2 content in shallow waters and the atmosphere increased in a geological short time by several orders of magnitude (Eguchi et al., 2019; Lyons et al., 2014).

As a result, most of obligate anaerobic life has extinct. However, since oxidation with O_2 releases significantly more energy than it was the case with metabolic products through anaerobic photosynthesis, this also meant completely new paths for evolution. Only now it was possible for multicellular life to develop, mitochondria formed and finally the Cambrian explosion occurred (Albani et al., 2010).

Relevant for this work is purely oxygenic photosynthesis, which is directly related to the formation and evolution of cyanobacteria over 2700 million years ago (Brocks et al., 1999; Buick, 2008). In this process the supply of light energy is used to create glucose ($C_6H_{12}O_6$) and as a byproduct oxygen (O_2) from carbon dioxide (CO_2) and water (H_2O).

The overall equation is as follows:

$$6\ CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6\ O_2 \quad \Delta H^0 = +2870\ kJ/mol$$

The four major protein complexes of photosynthetic electron transport chain embedded in the thylakoid membrane are the photosystem I (PSI) and II (PSII), the cytochrome b_6f complex (Cyt b_6f), and the adenosine triphosphate (ATP) synthase (ATPase) (Lea-Smith et al., 2016; Liu, 2016; Nevo et al., 2012; Nickelsen & Zerges, 2013; Rast et al., 2015).

Oxigenic photosyntesis can be translated into a light-dependent and a light-independent reaction (Lerdau & Gray, 2003). In the light-dependent reaction (Figure 2), H_2O is split through photolysis with the help of a manganese cluster bound to PSII into electrons, protons and oxigen (Nugent et al., 2001; Yu, 1999). In order to be able to absorb as much of the

incident light as possible, chloroplasts have developed two light-harvesting complexes (LHC1 and LHC2), while cyanobacteria collect the energy via phycobilisomes (Adir et al., 2020; Croce & Van Amerongen, 2014; Nelson & Junge, 2015; Singh et al., 2015). These antenna collect and transfer the light energy to the reaction centre of PSII and expand the absorption spectra via Förster resonance energy transfer (FRET) (Jones & Bradshaw, 2019; Mirkovic et al., 2017; Şener et al., 2011).

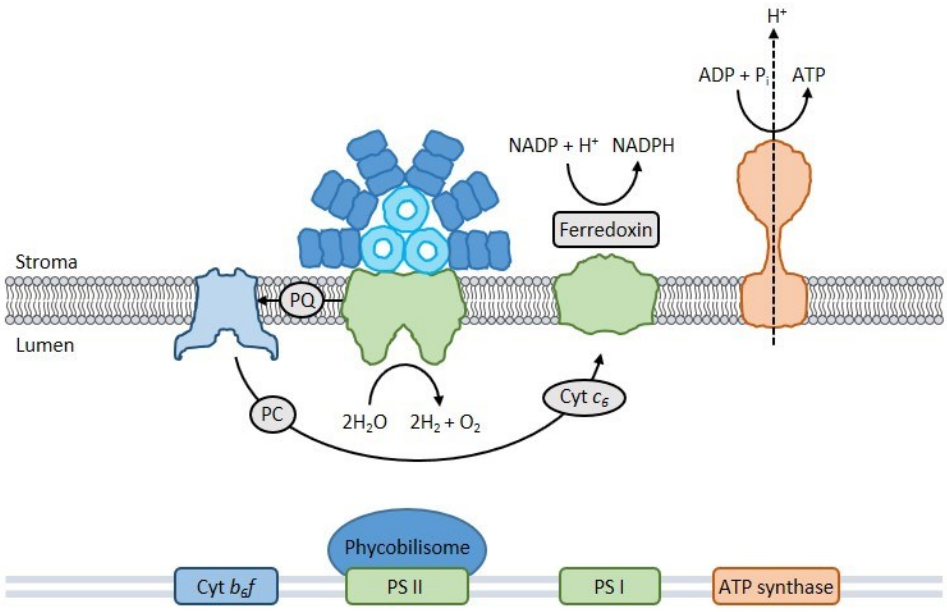

Figure 2: Photosynthetic complexes in cyanobacteria.

Schematic model of cyanobacterial thylakoid membrane (based on *Synechocystis* 6803 thylakoids) with the four major protein complexes of the photosynthetic electron transport from PSII over cyt b_6f to PSI, on the right side the proton driven ATPase.

Above the illustration, below the associated labelling of the complexes.

Abbreviations: ADP – adenosine diphosphate, ATP – adenosine triphosphate, cyt b_6f – cytochrome b_6f, cyt c_6 – cytochrome c_6, NADP – nicotinamide adenine dinucleotide phosphate, NADPH – reduced NADP, PC – plastocyanin, P_i – inorganic phosphate, PQ – plastoquinone, PSI – photosystem I, PSII – photosystem II.

Based on Liu (2016).

At the reaction centre of PSII the primary charge-separating event occurs at the "special pair", a chlorophyll dimer which ejects an electron in response to optical excitation (Barber, 2017; Narzi et al., 2016; Ozeki et al., 2004). A chain of consecutive redox reaction is then set in motion, which transfers the released electrons from PSII via a series of electron carriers such as plastoquinone (PQ) to a transmembrane proton pump, cyt b_6f (Berry et al., 2000; Havaux, 2020). Finally PSI reduce nicotinamide adenine dinucleotide phosphate (NADPH) in a noncyclic electron transport or it transfers the electrons back to cyt b_6f in a cyclic electron

transport (Allen, 2003; Arnon et al., 1957; Frenkel, 1954; Hill & Bendall, 1960; Walker, 2002). The released electrons from the water splitting finally refill the special pair (Hill & Bendall, 1960).

During electron transport, the protons transported into the thylakoid lumen and the resulting gradient drives an ATPase that regenerates ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i) (Arnon et al., 1957; Frenkel, 1954; Hill & Bendall, 1960).

In the light-independent reaction, also known as the Calvin-Benson-Bassham cycle, the ATP obtained is used to build up carbohydrates (glyceraldehyde 3-phosphate) from CO_2 by the key enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCo) (Biel & Fomina, 2015; Stanley & Dalton, 1982; Wedel & Soll, 1998). The NADPH from PSI is used here to reduce 1,3-bisphosphoglycerate to glyceraldehyde-3-phosphate, an anabolic and reductive intermediate in the cycle (Wedel & Soll, 1998).

PSII is a heterodimer containing of two homolog proteins, D1 and D2 (Loll et al., 2005; Zouni et al., 2001). D1 is the starting point of PSII biogenesis, the major target of photodamage and also completely replaced in the event of repair after photodamage (Kato & Sakamoto, 2009; Mattoo et al., 1981; Ohad et al., 1990). Therefore, when looking at photosynthesis and influencing factors, D1 is always a decisive factor. Processing of D1 starts with a precursor of D1 (pD1) with a C-terminal extension, which is incorporated into the thylakoid membrane cotranslational (Andersson & Aro, 2001; Zhang et al., 1999, 2000). The maturation of pD1 to D1 occurs during the early assembly of PSII by removing the C-terminal extension by a specific endoprotease (Anbudurai et al., 1994; Nickelsen & Rengstl, 2013).

During PSII repair, primarily D1 is degraded and resynthesised. Thereby it comes to a partial disassembly of PSII, followed by a new cotranslational insertion and maturing of D1 (Aro et al., 1993; Mulo et al., 2012; Nixon et al., 2010).

I.3 *Synechocystis* sp. PCC 6803 as a model organism

In 1990 life became divided into three domains, bacteria, archaea and eukarya (Woese et al., 1990). Bacteria and archaea were historically grouped together to form prokaryota, i.e. without a nucleus, but differ both in the composition of their cell membrane and in their metabolism (Woese et al., 1990). Since 1998, prokaryota have again been summarised as one unit under the term empire and later under the term superkingdom and contrasted with

the eukaryota (Cavalier-Smith, 1998; Ruggiero et al., 2015). Bacteria remain, however, as an independent group, whether they are classified as a kingdom or a domain (Sanchez-Baracaldo et al., 2005).

According to the current state of knowledge, oxygenic photosynthesis has evolved only once and this happened in the phylum cyanobacteria which belongs to the bacteria (Blankenship & Hartman, 1998; Garcia-Pichel, 1998). These early cyanobacteria or "protocyanobacteria" later transformed to the chloroplasts in green algae and plants through the event of endosymbiosis (Falcón et al., 2010; Whatley, 1993). As a result, the monophyletic group of cyanobacteria represent an very important branch in the development of life as we know on earth (Sanchez-Baracaldo et al., 2005; Soo et al., 2014).

Cyanobacteria are today still the only known bacteria capable of oxygenic photosynthesis, although there are very close sibling clades which are non-photosynthetic (Di Rienzi et al., 2013; Garcia-Pichel et al., 2020; Ley et al., 2005; Soo et al., 2014). A resent phylogenomic analysis defines the cyanobacterial lineage as "Organisms in the domain bacteria able to carry out oxygenic photosynthesis with water as an electron donor and to reduce carbon dioxide as a source of carbon, or those secondarily evolved from such organisms." (Garcia-Pichel et al., 2020; Sanchez-Baracaldo et al., 2005). Because of the blue-green colour of some species, which can be traced back to the blue photosynthetic pigment phycobilin, they were previously erroneously called blue-green algae. However, this is now misleading, as the term algae is now firmly assigned to the domain of the eukarya. Unfortunately, due to these historical circumstances, problems of nomenclature still arise (Oren, 2004, 2015; Palinska & Surosz, 2014).

Cyanobacteria play a major role as primary producers that are able to produce organic compounds from inorganic substances with the help of photosynthesis (Chisholm et al., 1988; Knoll, 2008; Li, 1994; Waterbury et al., 1979). Furthermore, they have conquered almost every habitat on earth. This applies to fresh water, salt water and on land, in deserts as well as in the eternal ice or in symbiosis with other species, e.g. in form of lichens on the walls of our cities (Chisholm et al., 1988; Perera et al., 2018; Rikkinen, 2002, 2013; Sanchez-Baracaldo et al., 2005; Stibal et al., 2006; Ward et al., 2012; Waterbury et al., 1979).

Even if many bacteria are unicellular, multicellularity occurs, especially with cyanobacteria (Herrero et al., 2016; Schirrmeister et al., 2011). This can result in the formation of filamentous structures with and without cell differentiation from vegetative cells to nitrogen-fixing heterocysts (e.g. *Anabaena* and *Nostoc*), but there are also colonial forms or flat

arrangements possible (Herrero et al., 2016; Komárek & Johansen, 2015; Schirrmeister et al., 2011; Wu & Song, 2008).

As gram-negative bacteria, cyanobacteria have a single peptidoglycan layer between the plasma membrane and the outer membrane. In the case of *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803), an unicellular fresh water cyanobacterium of about 2 µm in diameter, there is furthermore an S-layer, i.e. a superposed crystalline layer made of symmetrically arranged envelope proteins. In *Synechocystis* 6803 this protein seems to have an important function in growth and survival under different physiological conditions (Šmarda et al., 2002; Trautner & Vermaas, 2013).

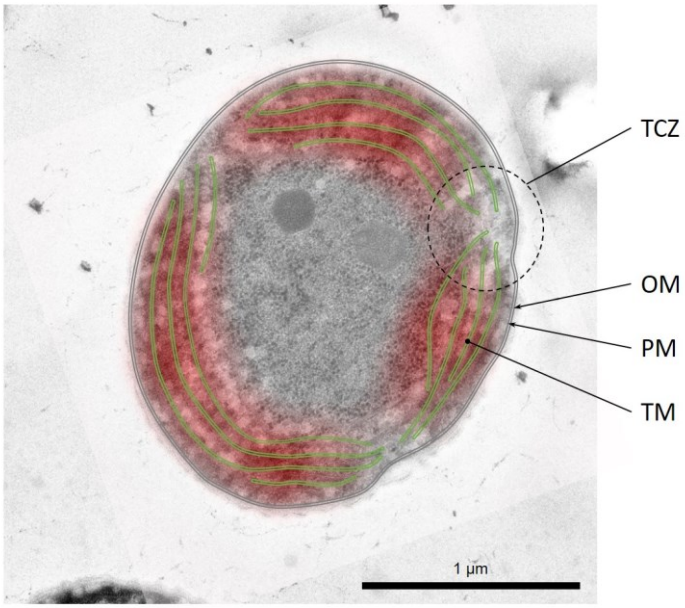

Figure 3: CLEM micrograph *Synechocystis* 6803.

Electron micrograph of *Synechocystis* 6803 superimposed with the autofluorescence of chlorophyll (red). The different membranes are additional marked, the thylakoid membrane in green and the plasma membrane and outer membrane in grey.

Abbreviations: OM – outer membrane, PM – plasma membrane, TCZ – thylakoid membrane convergence zone, TM – thylakoid membrane. Bar = 1 µm.

The thylakoid membranes of *Synechocystis* 6803 are located in individual interconnected fascicles of 3 to 5 lamellar layers at the cell periphery (Figure 3). These converge at their ends in the direction of the plasma membrane and partially merge together to form thylakoid membrane convergence zones (TCZ) (Heinz et al., 2016; Rast et al., 2019). A thylapse, a synapse-like closely appressed contact of 2-4 nm between thylakoid and plasma membrane (Figure 4), forms at the points where the thylakoid membrane and plasma membrane come

particularly close (Rast et al., 2019). These regions are proposed as regions for biogenesis of PSII due to its high accumulation of ribosomes (Rast et al., 2019; Stengel et al., 2012).

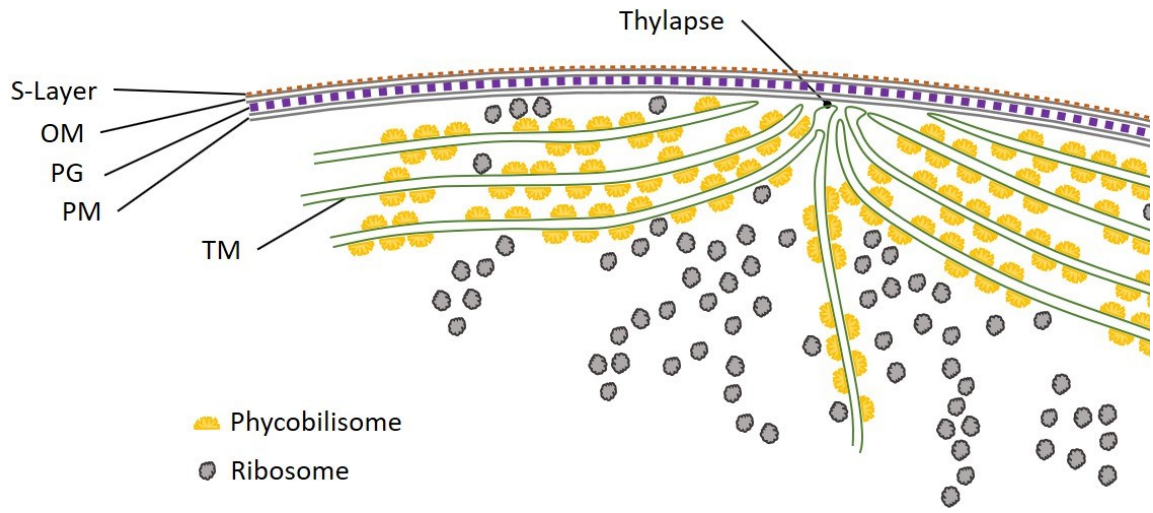

Figure 4: Thylakoid membrane convergence zone of *Synechocystis* 6803.

Graphic representation of the TCZ in *Synechocystis* 6803 based on current research results. The thylapse appears as a broadened formation of the thylakoids, which, like a synapse, approaches the PM down to 2-4 nm.

Abbreviations: TCZ – thylakoid membrane convergence zone, S-Layer – surface layer, OM – outer membrane, PG – peptidoglycan, PM – plasma membrane, TM – thylakoid membrane.

Based on Rast et al. (2019).

What makes *Synechocystis* 6803 to a good model organism that has been used for a long time in plant science is on the one hand its early and completely sequenced genome. In the year 1996, it was not only the fourth genome to be sequenced completely, but also the first of a phototrophic organism (Ikeuchi & Tabata, 2001; Kaneko et al., 1996). *Synechocystis* 6803 has around 12 copies of its genome, plus three small and four large plasmids (Kaneko et al., 2003; Labarre et al., 1989). Furthermore, it is natural genetically transformable, which makes it perfect for protein characterisation studies (Grigorieva & Shestakov, 1982; Williams, 1988). On the other hand *Synechocystis* 6803 can be grown photoheterotrophically with the addition of glucose as a source of carbon and therefore without functional PSII (Jansson et al., 1987). This is a key for photosynthetic research. With minimal changes in growth media, photoautotrophic and photoheterotrophic conditions can be compared, specific factors examined and specifically assigned to photosynthesis (Heinz et al., 2016; Yang et al., 2014).

I.4 Architecture of Thylakoids

Thylakoid membranes are the endomembrane system that carries the protein complexes relevant for photosynthesis. As an example of the function specificity the total amount of proteins can reach 70% to 80% of the total membrane content in granal membranes of chloroplasts (Kirchhoff et al., 2008).

Thylakoid membranes developed first in cyanobacteria. The unicellular photosynthetic cyanobacterium *Gloeobacter violaceus*, a very basal sister group to all other cyanobacteria, lacks internal thylakoids and the photosynthetic complexes are part of the inner surface of the plasma membrane where they are organised in distinct patches (Guglielmi et al., 1981; Herrero et al., 2008; Rexroth et al., 2011; Rippka et al., 1974). This indicates the formation of thylakoids through the plasma membrane (Kelly & Dörmann, 2004; Vothknecht & Westhoff, 2001). In plant chloroplasts for example, a proplastid matures in the absence of light into an etidioplast, which forms a semi-crystalline prolamellar body from its inner membrane (Pribil et al., 2014; Rosinski & Rosen, 1972). Than thylakoids develop as extensions of the prolamellar body complimented by lipids from the inner membrane (Adam et al., 2011; Pribil et al., 2014).

In unicellular but also filamentous basal branches of the phylogenetic tree of the cyanobacteria, thylakoids are mainly seen close to the inner surface of the plasma membrane, i.e. as peripheral thylakoids (Komárek & Kastovsky, 2003; Mareš et al., 2019). In *Synechocystis* 6803, these peripheral thylakoids are repeatedly interrupted and converge as interconnected fascicles of lamellar layers to the plasma membrane and form convergence zones (Nierzwicki-Bauer et al., 1983; van de Meene et al., 2006). Moreover, these areas show a high amount of ribosomes indicating a region of high translational activity. Thus, they are defined as regions of PSII biogenesis (Rast et al., 2019; Stengel et al., 2012). Furthermore, the close contact of thylakoid and plasma membrane forms a synapse-like attachment at a very small area called the thylapse for a hypothetically uptake path from the periplasmic space into the thylakoid lumen (Figure 4; Rast et al., 2019). The similar convergence zones but without proof for thylapses or detailed structure can be observed in *Agmenellum quadruplicatum* (Nierzwicki-Bauer et al., 1983).

In addition, there are a number of other thylakoid arrangements in cyanobacteria (Figure 5), from a radial arrangement in e.g. *Coleofasciculus chthonoplastes* PCC 7420, to a fascicular arrangement in e.g. *Arthrospira* sp. PCC 8005, irregular arrangement in e.g. *Stigonema ocellatum* SAG 48.90 and various others (Mareš et al., 2019).

The connection of cyanobacteria to chloroplasts represents the endosymbiosis, which in its beginnings as early as 1905 as a hypothesis by Mereschkowsky saw plastids as a reduced form of cyanobacteria (Falcón et al., 2010; Kutschera & Niklas, 2005; Martin & Kowallik, 1999). The endosymbiosis was in two different events responsible for i.) the formation of mitochondria as well as ii.) for the formation of chloroplasts (Gray et al., 1999; Kutschera & Niklas, 2005; Roger et al., 2017). In the latter case, a cyanobacterium was taken up by a eukaryotic cell, but not digested. The endosymbiont then gave parts of its genetic information to the host, which means that both symbiotic partners are more dependent on each other and the symbiont can concentrate more on a specific task (Glöckner et al., 2000; Sugiura, 1995). In the case of chloroplasts this is the production of glucose through photosynthesis (Glöckner et al., 2000).

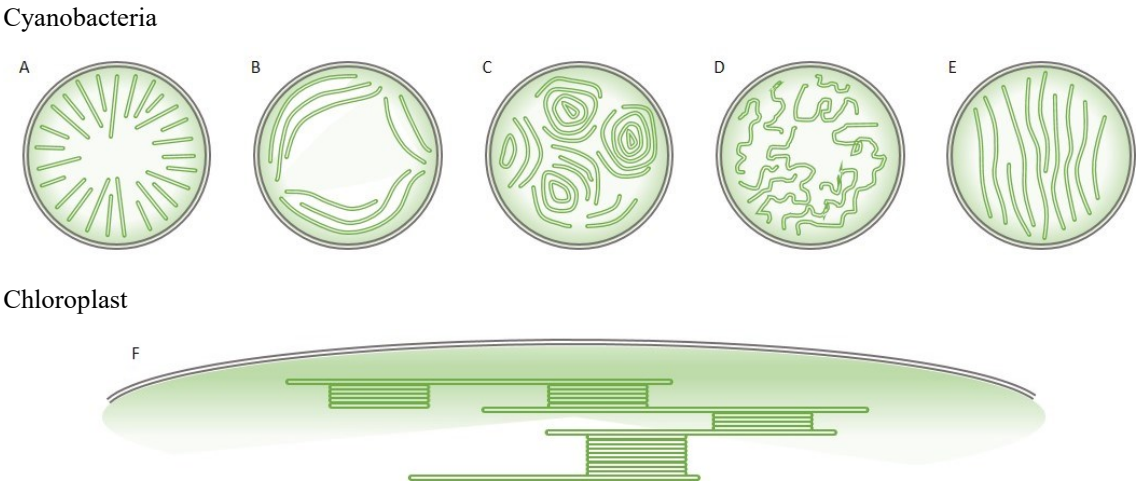

Figure 5: Schematic representation of different thylakoid arrangements.
Depicted are the different membranes in cyanobacteria. Outer and plasma membrane coloured in grey, thylakoid membranes in green. A) Radial arrangement; B) fascicular arrangement; C) fascicular type with dominant spherical formations; D) irregular arrangement; E) parallel arrangement; F) differentiation in stroma and stacked grana thylakoids.
Based on Mareš et al. (2019).

Together with this event, in green algae and thus in higher plants membrane-integrated light-harvesting complexes have been formed in the course of evolution, which, in contrast to phycobilisomes, which are not integrated into the membrane but sit on it, have a significantly lower steric hindrance (Lepetit et al., 2012; Rast et al., 2015). This in consequence led to

further restructuring and development of thylakoid membranes, which finally ended in the differentiation of stroma and grana of recent plants (Mullineaux, 2005). The core protein components of photosynthesis, which manage the splitting of water as well as the generation of energy and finally the fixation of CO_2, were highly conserved during this process (Allen et al., 2011; Rast et al., 2015).

For an optimal surface-to-volume ratio thylakoids in cyanobacteria and chloroplasts of green algae and plants exhibit extreme curvature at margins and convergent regions. This is also necessary for the close contact of individual thylakoid layers as well as for the much stronger contact of entire grana stacks. Different proteins such as MinE and epsin are suggested to play an important role for bending membranes (Shih et al., 2011; Yoon et al., 2010). However, responsible for thylakoid curvature is the CURVATURE THYLAKOID1 (CURT1) protein family, which was first discovered in *Arabidopsis thaliana* and is highly conserved in both cyanobacteria and chloroplasts (Armbruster et al., 2013).

In *Synechocystis* 6803, CurT is the sole homolog of the CURT1 protein family and it is required for the formation of the already mentioned biogenic regions (Rast et al., 2019). A knockout *curT*$^-$ leads, besides less growth rate, to a loss of TCZs with no interruption and convergence of the thylakoid membrane (Heinz et al., 2016). The marker for these biogenic regions is a tetratricopeptide repeat (TPR) protein, processing-associated TPR protein A (PratA), which is involved in the very early biogenesis of PSII by processing the C-terminus of pD1 to the mature D1 protein (Klinkert et al., 2004; Schottkowski, Gkalympoudis, et al., 2009). Furthermore PratA contains a high affinity to bind as well as efficient delivery of Mn^{2+}, the important and eponymous component for the H_2O splitting manganese cluster (Stengel et al., 2012). The sections on the thylakoid membranes where PratA is located are the PratA-defined membranes (PDMs) (Nickelsen et al., 2011; Rengstl et al., 2011). In *A. thaliana*, the task of PratA is taken over by the low PSII accumulation1 (lpa1) protein which also contains TPR motifs (Nickelsen & Rengstl, 2013; Peng et al., 2006). Interestingly, there are still biochemically isolable PDMs in *curT*$^-$, although PratA accumulates in smaller amounts (Heinz et al., 2016).

That means the biogenesis of PSII takes place in defined areas. In cyanobacteria, which possess an outer membrane, an inner plasma membrane and thylakoid membranes, *ne novo* assembly of PSII seems to be located at distinct membrane subdomains exclusively on the thylakoids (Nickelsen & Rengstl, 2013). In *Synechocystis* 6803 these points are defined as biogenic regions where thylakoids are curved to a very close contact at the plasma membrane and a high accumulation of ribosomes occur (Rast et al., 2019; Stengel et al., 2012).

In chloroplasts of the green algae *Chlamydomonas reinhardtii* the PSII *de novo* assembly occurs in translation zones (T-zones), discrete regions lateral to the pyrenoid, whereas D1 repair seems to occur throughout the entire thylakoid membrane system (Nickelsen & Zerges, 2013; Uniacke & Zerges, 2007).
In higher plants and some green algae where thylakoids are separated in stroma and grana lamellae, the insertion of D1 comes from polysomes bound to the stroma-lamella region of the thylakoid membranes, whereas the mature PSII dimer is located in the Grana lamellae (Rochaix, 2011; Rokka; Wollman et al., 1999).
Overall, it can be assumed that the architecture of thylakoids and formation of biogenic regions for the initial assembly of PSII goes hand in hand and is evolutionarily preserved, despite its different characteristics.

I.5 VIPP1

As mentioned, an important part in photosynthesis is to build up, alter and maintain thylakoids. This is where the vesicle-inducing protein in plastids 1 (VIPP1), also known as inner membrane-associated protein of 30 kDa (IM30), plays an important role. The first time it was identified in *Pisum sativum* as a 30 kDa protein associated with both of the envelope membranes of the chloroplast as well as the thylakoid membranes (Li et al., 1994). Immunogold localisation offered an unique localisation pattern where the single gold particles are localised as clusters in chloroplasts near either the thylakoid membrane or the envelope (Li et al., 1994). By mutational analysis of VIPP1 in *A. thaliana*, where it is also located in the inner envelope and the thylakoids, a harmful change in thylakoid formation was found when VIPP1 expression was reduced due to a T-DNA insertion in its promoter region (Kroll et al., 2001). The plants showed a strong albinotic phenotype, were incapable of autotrophic growth and did in the end not survive (Kroll et al., 2001; Zhang et al., 2016). In *C. reinhardtii* VIPP1 is suggested to play a role in biogenesis and assembly of thylakoid membranes by supplying structural lipids (Nordhues et al., 2012). In cyanobacteria VIPP1 is an essential protein and complete deletion under autotrophic growth is lethal for the cell (Gao & Xu, 2009; Westphal et al., 2001). A *vipp1* disruption mutant of *Synechocystis* 6803 also showed a loss in thylakoid structure and formation as well as decreased photosynthetic activity (Gao & Xu, 2009; Gutu et al., 2018; Westphal et al., 2001).

The findings in cyanobacteria as well as in chloroplasts of green algae and higher plants indicate a similar function concerning thylakoid formation of VIPP1 among different species and furthermore an evolutionary conservation (Heidrich et al., 2017; Kroll et al., 2001; Vothknecht et al., 2011). This means VIPP1 developed in prokaryotes, more precisely in cyanobacteria, and made its way into chloroplasts in coevolution with photosynthesis and thus thylakoid membranes.

A significantly similar protein was found in non-photosynthetic prokaryotes. This protein, PspA (phage shock protein A), is part of the Psp system that reacts both to the invasion of filamentous phages and to external stress factors (Brissette et al., 1990; Brissette et al., 1991; Li et al., 1994). Comparing with VIPP1 which works in thylakoid stress response and repair (Aseeva et al., 2007; Gutu et al., 2018; Westphal et al., 2001; Zhang & Sakamoto, 2015), PspA works in membrane stress response and repair of bacteria (Brissette et al., 1990; Joly et al., 2010; Kobayashi et al., 2007; McDonald et al., 2015; Yamaguchi et al., 2010). Furthermore VIPP1 contains in contrast to PspA an additional unique C-terminal domain of α-helical structure (Otters et al., 2013; Westphal et al., 2001). In *Gloeobacter violaceus* sp. *glr0898* shows higher sequence similarity to *vipp1* than to *pspA* but a region associated to the C-terminal domain of VIPP1 is missing, probably indicating an intermediate state between PspA and VIPP1 (Nakamura et al., 2003; Zhang et al., 2016). This development is of vital importance when considering VIPP1 as part of a family of proteins with PspA and a coevolution with thylakoid membranes (Liu et al., 2021; Thurotte et al., 2017; Zhang et al., 2016).

Another structural similar protein is ESCRT-III (endosomal sorting complex required for transport), which is responsible for forming composite polymers and multivescular bodies, plasma membrane repair and viral budding, was originally only known in eukaryots, but was finally discovered in archaea as well (Bertin et al., 2020; Lindås et al., 2008; McCullough et al., 2018; Nguyen et al., 2020; Samson et al., 2008). VIPP1, PspA and ESCRT-III all appear in their secondary structure to consist of five core helices plus a preceding N-terminal helix, which is involved in membrane binding, and a C-terminal extension (Buchkovich et al., 2013; Liu et al., 2021; McDonald et al., 2017; Otters et al., 2013). The N-terminal helix seems to has an amphipathic character and is responsible for a high affinity to lipids *in vitro* and *in vivo*, although there is no predicted transmembrane domain (Kroll et al., 2001; Li et al., 1994; Liu et al., 2021; McDonald et al., 2017; Otters et al., 2013). Furthermore, all these proteins self-assemble into large homo-oligomers (Hankamer et al., 2004; Huber et al., 2020; Liu et al., 2021; Saksena et al., 2009; Teis et al., 2008; Thurotte et al., 2017).

For VIPP1 there are indeed different structures known, depending on the species. *In vitro* observations in *Synechocystis* 6803 and *A. thaliana* showed primarily ring-shaped structures, in *C. reinhardtii* they form long rod-shaped structures (Aseeva et al., 2004; Fuhrmann et al., 2009; Liu et al., 2007; Saur et al., 2017; Theis et al., 2019; Zhang & Sakamoto, 2015). Microscopy reveals both diffuse and rare concentrated signals for VIPP1 (Bryan et al., 2014; Gutu et al., 2018; Nordhues et al., 2012; Zhang et al., 2012). Indeed VIPP1 is highly dynamic, continuously exchanging between a punctate fraction at the cell periphery that is concentrated at TCZs, and a diffuse fraction that is uniformly distributed in the cytoplasm (Gutu et al., 2018; Heinz et al., 2016).

The strong conservation and membership in a larger protein family as well as the lethality of a VIPP1 deletion shows how essential VIPP1 was and still is in the course of evolution. It seems as if there is a higher level of activity under stress, but the question of the exact function still seems unanswered.

II. Aims of this work

The aim of this work is to understand the structure and biogenesis of the thylakoid membranes and thus the photosynthetic apparatus more precisely. Special attention is on the TCZs, which are both strongly influenced by the thylakoid architecture and proposed as biogenic regions.

On the one hand, there is the newly discovered factor AncM, which plays an important role for thylakoid architecture in *Synechocystis* 6803. Furthermore, it appears to be present in at least most of the known cyanobacterial orders but not in chloroplasts of green algae or higher plants. However, there are also factors such as VIPP1, which have been described for some time, that are essential for the organism but are not understood completely.

To target these, microscopy plays a decisive role beside biochemical approaches, in the present work. The microscopy methods applied made it possible to gain completely new insights. With the help of fluorescence microscopy, clear membrane differentiations and protein distributions could be shown *in vivo*. Electron microscopy techniques were used to visualise a protein oligomer in the range of a few angstroms.

The focus is on the model organism *Synechocystis* 6803, which is compared with other cyanobacteria, chloroplasts of green algae such as *C. reinhardtii* or plants such as *A. thaliana*.

III. Results

The following chapter consists of two studies that are all published in international peer-reviewed journals. At the beginning of each study a small introduction, the main conclusions as well as the contributions of the author are summarised.

III.1 Thylakoid attachment to the plasma membrane in *Synechocystis* sp. PCC 6803 requires the AncM protein

Ostermeier, M., Heinz, S., Hamm, J., Zabret, J., Rast, A., Klingl, A., Nowaczyk, M.M., and Nickelsen, J. (2021). The Plant Cell 2021: 00: 1–24

The study focuses on the description, characterisation and localisation of AncM (anchor of convergence membranes). The protein first attracted attention when investigating a spontaneously formed suppressor mutation in *curT*$^-$ (*sucurT*$^-$). The suppressor showed both better growth and higher O_2 evolution than *curT*$^-$. Thereby *sucurT*$^-$ seems to rearrange the confused ultrastructure of *curT*$^-$ by silencing *ancM* through a STOP codon, which has brought this protein into the focus of the present work.

An *ancM*$^-$ mutant has a photosynthetic phenotype characterised by reductions in oxygen evolution, PSII accumulation and photosystem assembly and exhibits an altered thylakoid ultrastructure with additional sheets and convergent thylakoids detached from the plasma membrane. By genetic, biochemical and microscopic techniques, we show that AncM is an integral membrane protein attaching thylapses at TCZs. Moreover, AncM seems to have an antagonistic function to CurT in shaping thylakoid membrane ultrastructure.

In contrast to CurT, phylogenetic analysis showed that AncM occurs exclusively in cyanobacteria and neither in chloroplasts of green algae nor in higher plants.

I contributed to the paper the whole microscopic experiments. This includes several immunostaining approaches of WT and mutants as well as fluorescence microscopy of the CFP-tagged CurT line up to quadruple fluorescence analyses. To visualise the interdependency of AncM and CurT I reconstructed 3D models of stained cells. Furthermore, I took TEM micrographs with high-pressure-frozen samples of WT, *curT*$^-$, *sucurT*$^-$, *ancM*$^-$, *ancM*^{comp} and *ancM*^{164STOP}. In addition, I analysed the recordings and evaluated them statistically.

For a more detailed understanding of the cellular architecture and its relationship to photosynthetic activity, I took CLEM images of the different lines and compared their chlorophyllfluorescence distribution in contrast to their thylakoid organisation. Furthermore, I was responsible for the quantification of western blot signal intensities on BN/SDS-PAGE and partially for the phylogenetic analysis and creation of the phylogenetic tree of AncM. The manuscript was written by J. Nickelsen, S. Heinz and me, afterwards it was revised by all co-authors.

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III.2 Structural basis for VIPP1 oligomerization and maintenance of thylakoid membrane integrity

Gupta, T.K., Klumpe, S., Gries, K., Heinz, S., Wietrzynski, W., Ohnishi, N., Niemeyer, J., Spaniol, B., Schaffer, M., Rast, A., **Ostermeier, M.**, Strauss, M., Plitzko, J.M., Baumeister, W., Rudack, T., Sakamoto, W., Nickelsen J., Schuller J.M., Schroda, M., and Engel B.D. (2021). Cell 184: 1–17

The focus of this study was to determine the structure of the cyanobacterial VIPP1 ring with the help of cryo-electron microscopy. It was shown how the individual monomers are connected to each other to build up basket-like structures of different symmetry. Furthermore, it was shown how three individual monomers form a completely atypical nucleotide binding site. On the inside of the ring it was demonstrated how the combined N-terminal helices of various monomers form a hydrophobic column. This seems to enable VIPP1 to bind to a membrane and take it up like a suction cup. Mutations of single amino acids on this hydrophobic helix lead to a restructuring of the oligomer from the ring structure to the rod structure, which also occurs in *C. reinhardtii* (Theis et al., 2019). *In vivo*, these mutations in *Synechocystis* 6803 trigger swelling of the thylakoids in high light, which suggests an important role for VIPP1 in resistance to light stress.
With the help of in situ cryo-ET, VIPP1 structures could be detected within *Synechocystis* 6803. Moreover, VIPP1 enveloped tubules within the chloroplast of *C. reinhardtii* were shown by cryo-CLEM.
I contributed to the paper the culture preparation of the *Synechocystis* 6803 VIPP1 mutants under different light intensities as well as assistance for cryo-CLEM studies. T.K. Gupta, J.M. Schuller, M. Schroda, and B.D. Engel wrote the manuscript with input and revision from all authors.

Note: The structural homology between VIPP1 and PspA described in this work has already been considered extensively in two competing studies (Junglas et al., 2021; Liu et al., 2021). In order to avoid problems with the nomenclature, it should be mentioned that helix H1 corresponds to helix α0, H2 corresponds to helix α1, H3 corresponds to helix α2, etc.

https://doi.org/10.1016/j.cell.2021.05.011

IV. Discussion

IV.1 Structure and arrangement of thylakoids in *Synechocystis* 6803 depend on AncM, a protein that counteracts CurT

CurT is the decisive factor for the formation of TCZs in *Synechocystis* 6803. A *curT*$^-$ mutant is considerably disturbed in both photosynthetic performance and growth and has a clear ultrastructural phenotype (Heinz et al., 2016). In order to compensate this deficit, there was a mutation in *ancM* and suppression of the *curT*$^-$ phenotype resulting in *sucurT*$^-$, which ultimately led to more detailed investigations of the TCZs and the genetical background. It turned out that a base exchange at position 164 in the ORF *ancM* occurred, which led to a stop codon and partly loss of the protein (Figure 1 in section III.1, Ostermeier et al., 2021). Ultrastructural studies of *ancM*$^-$ have shown that a full lack of this protein leads to a very atypical membrane architecture in which individual thylakoids still converge, but these sites drift in the cell lumen, detached from the plasma membrane (Figure 5 in section III.1, Ostermeier et al., 2021). This means that the TCZ is no longer anchored to the plasma membrane.

A look at CurT, AncM and its mutants shows that a lack of one always results in major changes in thylakoid architecture. In any case, this leads to a reduced PSII activity and decreasing growth rate. Furthermore, the lack of one protein leads to both mislocalisation and altered expression of the other (Figures 3E and 9 in section III.1, Ostermeier et al., 2021). These changes are also reflected in the chlorophyll autofluorescence, which with the help of CLEM images can be used to discriminate between photosynthetically active and inactive areas within the cell (Figure 6 in section III.1, Ostermeier et al., 2021). In the WT, TCZs in particular have little to no chlorophyll fluorescence but concerning the current model, they harbour AncM. In *curT*$^-$ and *ancM*$^-$ there are no TCZs, but there are still distinct areas with nearly complete reduced chlorophyll fluorescence and especially in curT- it is shown that AncM migrates more inside the cell together with the chlorophyll free areas depicted in the CLEM micrographs. In addition, the reduction of chlorophyll can be associated with the biochemical localisation of PratA, a crucial manganese-binding protein and eponymous for the PDMs, that appears in the same biochemical fractions as AncM, which can be regarded as a marker for the biogenesis of PSII (Nickelsen et al., 2011; Rengstl et al., 2011).

In contrast to CurT, which is located in all membranes but concentrated in PDMs and TCZs, AncM is biochemically located exclusively in the PDMs and attaches TCZs to the plasma membrane. Therefore, it serves as a marker for thylapses and enables the biogenic regions to be defined more precisely (Figure 6).

Connection of CLEM images with immunofluorescence-marked samples result in the following picture:

In the WT the biogenic regions are located at the edge of the cells in regions with less chlorophyll, marked by AncM and CurT.

In *curT*$^-$, the TCZs disappear and the thylakoids wind their way through the cell lumen. Here areas with reduced chlorophyll fluorescence can still be observed and, in addition, a migration of AncM into the cell lumen, which points to a localisation of biogenic regions away from the periphery inside the cell. AncM still seems to attach the thylakoid membranes between each other.

In *ancM*$^-$, there was an increased amount of thylakoid layers with no defined TCZ as mentioned above, but a WT-like distribution of chlorophyll at the edge of the cell, partly not covering the margins of the thylakoids pointing inwards the cell. CurT as a marker for strong membrane curvature has also migrated more into the centre of the cells due to the arrangement of the thylakoids. Concerning the chlorophyll as a marker and the changes depicted in the electronmicrographs, it can be assumed that the biogenic regions are detached from the plasma membrane. Thus, due to the thylakoid membrane architecture and the resulting changes in photosynthetic areas, there is an explanation for the reduced oxygen evolution and lower growth in *curT*$^-$ and *ancM*$^-$.

In *sucurT*$^-$, this problem is less pronounced by both the loss of CurT and the factual inactivation of AncM. Here it seems as if unfavourable anchoring of the thylakoids in *curT*$^-$ has been loosened and enables them to be pressed towards the periphery due to the natural turgor pressure. This means, according to CLEM images, that the PDMs are peripheral again but there are still no TCZs. The same picture as in the *sucurT*$^-$ strain could be confirmed for the *curT*$^-$ *ancM*$^-$ double mutant (Supplemental Figures S3B and S8 in section VI.1, Ostermeier et al., 2021).

This means that both together, CurT and AncM, coordinate the formation of TCZs and the positioning of PDMs, one alone leads to an inefficient thylakoid arrangement caused by the other and only a lack of both normalises this situation partially (Figure 6). The direct interaction of CurT and AncM, apart from their localisation based on fluorescence

microscopy, is still unclear, but their behaviour indicates a mutual regulation and thus an antagonism in *Synechocystis* 6803.

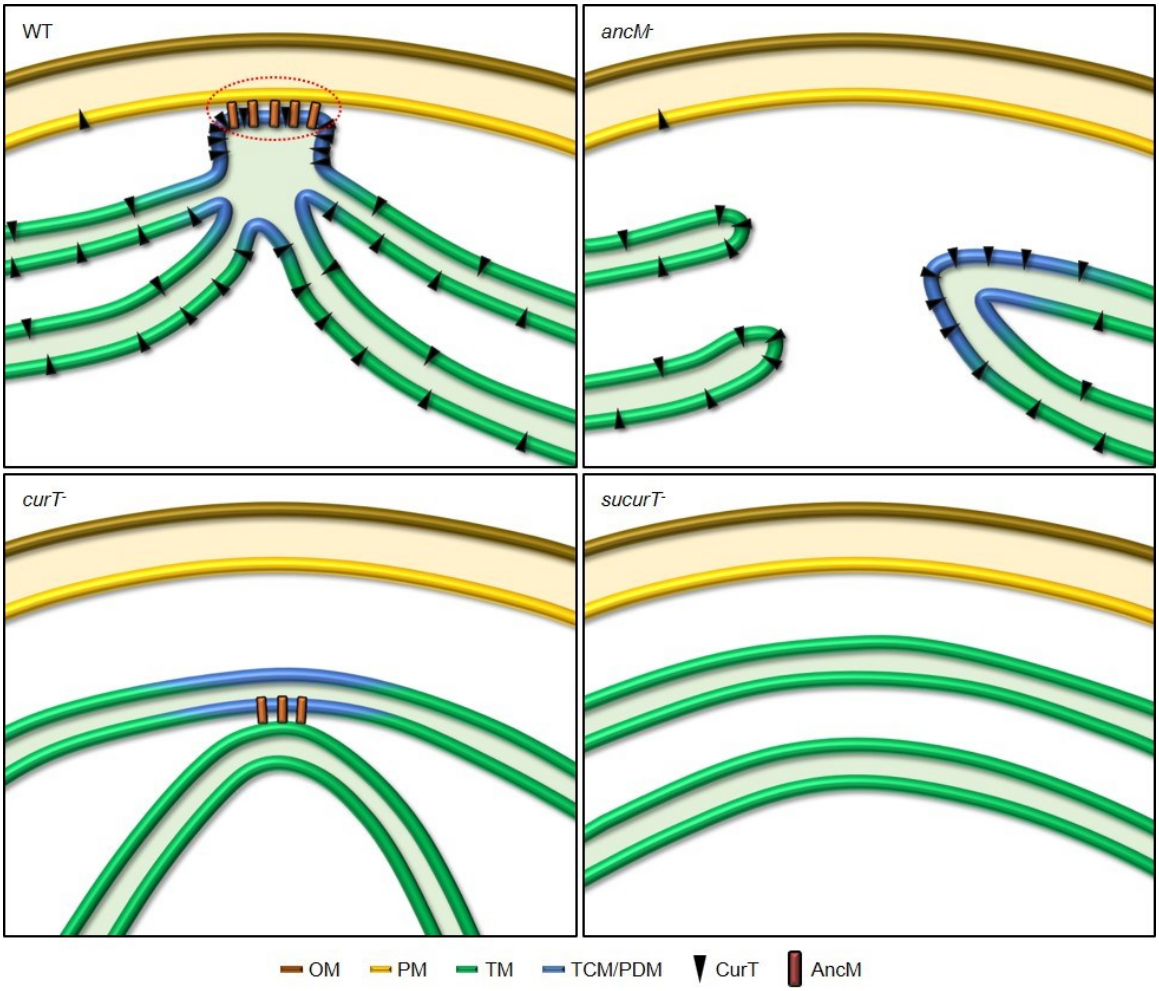

Figure 6: Working model of TCZs in *Synechocystis* 6803.

The model shows the distribution of AncM and CurT at TCZs based on localisation studies described in Ostermeier et al. (2021). Ultrastructure of the wild-type TCZ, based on the cryo-ET data in Rast et al. (2019), showing the thylapse (dashed red ellipse). CurT is distributed all over the thylakoid system, with local accumulations at TCZs where the thylakoids bend toward the PM. The AncM protein is an integral thylakoid protein bridging the thylapse gap to attach the thylakoids to the PM. In *ancM*-, thylakoid sheets still fuse but detached from the PM. The thylakoids in the *curT*- mutant are arranged in circular structures without any membrane fusions; however, they still possess biogenic regions. The TMs in *sucurT*- are peripherally organized into circular sheets lacking membrane fusion and TCZs.

Abbreviations: OM – outer membrane; PM – plasma membrane; TM – thylakoid membrane; TCM/PDM – thylakoid convergence membrane which corresponds to the biochemical isolated PratA-defined membrane.

Furthermore, AncM contains a DUF1868 domain which belongs to a superfamily of proteins which is involved in tRNA metabolism (Mazumder et al., 2002). One member of this superfamily, the mammalian 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), was found

to anchor microtubules to the plasma membrane (Bifulco et al., 2002; Laezza et al., 1997). If an attachment to the membrane takes place via this domain, this would underline the function of AncM too.

With a view to the phylogeny of AncM, it figures out that almost all species that have AncM also have CurT and, in fact, both proteins are co-expressed. Only in a few orders of Cyanobacteria, such as *Gloeobacterales* with no internal thylakoid membrane system, which also branch off very early, are both proteins completely absent (Supplemental Figures S1 and S2 in section VI.1, Ostermeier et al., 2021). This means that AncM might has undergone a joint evolution in its expression with CurT and may be associated with the evolution of thylakoids.

IV.2 Evolutionary conservation of AncM and its loss in Chloroplasts

Free oxygen, even if only a by-product of photosynthesis, is one of the most formative factors for life on our planet. Its story began when a single organism started to drive photosynthesis 2700 million years ago (Buick, 2008). This first cyanobacterium differentiated itself into a diverse phylum with a still unknown extent, from single-celled prokaryotes to filamentous species even with cell differentiation (Lyons & Kolter, 2015). Through primary endosymbiosis of an ancient cyanobacterium most closely related to *Chroococcales*, a branch in the phylogenetic tree developed step by step to the monophyletic group of chloroplasts and thus established all today's representatives from green algae to all recent plant species (Bhattacharya & Medlin, 1995; Douglas, 1998; Falcón et al., 2010; Martin et al., 2002; Yoon et al., 2004).

Very early in the evolution of cyanobacteria, specific proteins developed that made it possible for the thylakoids to maintain and stabilise the complex multiprotein apparatus of photosynthesis (Blankenship, 2010; Blankenship & Hartman, 1998; Xiong & Bauer, 2002). One of these is VIPP1, which will be discussed further below. The observed proteins required for the membrane architecture, CurT and AncM, developed at a basal point in the phylogenetic tree. This can be explained by the fact that almost every order of cyanobacteria known today contains a homologue of these proteins (Heinz et al., 2016; Mareš et al., 2019; Ostermeier et al., 2021).

In contrast to CurT, AncM does not or no longer occur in chloroplasts of both green algae and higher plants. Since it does not occur in *G. violaceus* but is co-expressed with CurT, it

seems, as already mentioned, to co-evolve with thylakoid membranes exclusively in cyanobacteria.

Furthermore, AncM is common in filamentous cyanobacteria such as *Snowella* sp., *Nostoc* sp. and many more (Supplemental Figures S1 and S2 in section VI.1, Ostermeier et al., 2021). A hypothetical consideration for this might be the necessity of a permanent membrane anchor to stabilise longer filaments and to ensure the exchange of nutrients from cell to cell (Flores, Nieves-Morión, et al., 2019). Nevertheless, this has to be investigated in more detail in further studies.

When comparing thylakoids of cyanobacteria and chloroplasts with regard to their architecture, there is a clear differentiation from largely uniform structures in cyanobacterial species to the division into stroma and grana lamellae in chloroplasts (Anderson, 1999; Austin & Staehelin, 2011; Mullineaux, 2005). This division in chloroplasts can be seen as a further differentiation of functional subdivision already existing in cyanobacteria. *Synechocystis* 6803 shows a smooth transition between the thylakoids and the PDMs where the membrane curves to TCZs but using CLEM micrographs, TCZs are highlighted by less chlorophyllfluorescence and therefore less photosynthetic activity (Figure 6 in section III.1, Ostermeier et al., 2021; Supplemental Figure S9 in section VI.1, Ostermeier et al., 2021). Furthermore, PDMs are also biochemically to be distinguished from the rest of the membranes, but there seems to be more of a smooth transition than clearly structurally differentiated membranes (Rengstl et al., 2011; Schottkowski, Ratke, et al., 2009). Moreover, in *Synechocystis* 6803, there is an increased occurrence of ribosomes at TCZs, which suggests increased translational activity (Rast et al., 2019). In comparison to higher plants, ribosomes bind exclusively to non-stacked stromal thylakoids (Hurewitz & Jagendorf, 1987; Minami & Watanabe, 1984; Yamamoto et al., 1981). That means in the area of translational intensity, TCZs can obviously be compared with stroma thylakoids. Integrating a protein such as AncM, which in *Synechocystis* 6803 creates a connection between TCZs and the plasma membrane that is quite advantageous for the bacterium, steric hindrances would definitely arise when bound to stroma thylakoids. This could suggest that loss of AncM was a necessary step in chloroplast evolution.

A noticeable change at the *ancM*$^-$ mutant is its increased number of thylakoid layers compared to the WT (Figure 5 in section III.1, Ostermeier et al., 2021). It can be concluded that due to the co-expression of AncM and CurT together with the presumed antagonism of both, the latter favours an increased formation of thylakoids. This means that CurT is no longer deadened by AncM and can enhance the number of layers in *ancM*$^-$. A comparison

can be made here with grana thylakoids in *A. thaliana*, in which the elimination of CURT1 leads to less stacking whereas the overexpression of CURT1 results in grana stacks with more margins and thylakoid membrane layers (Armbruster et al., 2013; Pribil et al., 2014). This does not exclude other possible factors that also may be involved in thylakoid stacking, but also shows that a thylakoid structure as seen in chloroplasts might not be possible with AncM.

Another difference of chloroplasts and cyanobacteria is the heterogeneity and variability of thylakoids in different kinds of plastids and their function (Pyke, 1999; Waters & Pyke, 2005; Yadav et al., 2019). Since the *ancM*$^-$ mutant also contains thylakoid membrane areas with a greatly reduced chlorophyll content apart from distinct regions, this could lead in the same direction (Figure 6B in section III.1, Ostermeier et al., 2021; Supplemental Figures S9D, E and F in section VI.1, Ostermeier et al., 2021). One possibility to investigate this more closely would be to observe AncM in filamentous and cell-differentiated cyanobacteria, whether there are differences in the protein content and function between vegetative cells and heterocysts.

Even if the comparison of stroma and grana lamellae with cyanobacterial structures is only based on assumptions, two points can very well be noted:

i.) In the evolution of cyanobacteria to chloroplasts, the anchoring of the thylakoids at certain points close to the the plasma membrane would have led to great steric hindrances, especially in the area of the grana stacks.

ii.) CurT seems to play an important role in the stacking of thylakoids and would be severely limited in its action by an antagonist.

This allows the hypothesis to be made that the last common ancestor of cyanobacteria and chloroplasts either had no AncM or, if present, it had to disappear in the earliest development of chloroplasts.

IV.3 Structures and lipophilic nature of VIPP1

As already described in the introduction, membranes serve both as a demarcation between cells and their environment and as a carrier for functional complexes. This means i) it is of crucial importance whether a protein has a lipophilic or lipophobic structure and ii) research *in vitro* on e.g. liposomes can help to understand protein-lipid interaction (Heinz et al., 2016; Hennig et al., 2015). This means, even if VIPP1 was exogenously expressed purely by E.

coli for the elucidation of the structure described in this work, conclusions can be drawn about reactions *in vivo*.

VIPP1 forms large oligomers *in vitro*, which either have a ring structure or a rod structure or, to be more precise, an equilibrium of these two structures that is shifted to one side or the other. In detail, this seems to depend on the respective organism, which means that in *syn*VIPP1 (VIPP1 homolog from *Synechocystis* 6803) more ring structures and in *cr*VIPP1 (VIPP1 homolog from *C. reinhardtii*) rather rod structures develop (Fuhrmann et al., 2009; Liu et al., 2007; Saur et al., 2017; Theis et al., 2019). The shift in the equilibrium between rings and rods seems to be depend on variations of the hydrophobic end, i.e. the N-terminal helix. However, the focus here is on the ring structure described by *syn*VIPP1.

VIPP1 consists of 7 helices (H1 to H7), whereas the described helices H1 (N-Terminal) to H6 (C-Terminal) are so closely related to PspA, a bacterial and not thylakoid membrane specific protein, that this can be functionally replaced by VIPP1 (DeLisa et al., 2004; Zhang et al., 2012). Moreover, the oligomerisation of these large heteromer structures of VIPP1 works via closely related interactions similar to PspA and ESCRT-III (Junglas et al., 2021). Furthermore, PspA is responsible for external stress responses, which could be a commonality for the function of VIPP1.

The binding affinity of VIPP1 to the membrane is mediated as mentioned by the helix H1 (McDonald et al., 2015; McDonald et al., 2017; Otters et al., 2013). The mechanism behind that is the amphipathic character of H1 with its hydrophobic side directed into the ring lumen (Figures 3 and 7 in section III.2, Gupta et al., 2021). This creates large hydrophobic surfaces that are responsible for drawing lipids into the *cr*VIPP1 rods and how *syn*VIPP1 rings can mediate liposome binding (Gupta et al., 2021; Hennig et al., 2015; Theis et al., 2019).

The binding affinity becomes prominent when taking a closer look at the structure and composition of membranes. Their ratio can not only change as a result of external stress, they also have a direct influence on physiological functions and can be a signal for specific effectors (Girotti, 1998; Lee, 2004; Pribil et al., 2014; Spector & Yorek, 1985). This could mean that depending on the thylakoid membrane structure and its lipids composition in different organisms under different conditions, VIPP1 must react differently in terms of structure maintenance and curvature of the thylakoid membranes.

In addition, the elucidation of the structure of VIPP1 oligomers made it possible to identify a non-canonical nucleotide binding site which is likely conserved with PspA (Ohnishi et al., 2018). Three VIPP1 monomers form a nucleotide binding pocket at one end of the ring (Figure 2 in section III.2, Gupta et al., 2021; Supplemental Figure S3 in section VI.2, Gupta

et al., 2021). Through that a nucleation on the membrane by sensing stored curvature elastic stress can lead to a "hydrolysis and release" mechanism to build up putative VIPP1 structures (Gupta et al., 2021; McDonald et al., 2015; McDonald et al., 2017).
Furthermore, the study shows what possibilities high-resolution microscopy can offer in biology and what findings complemented with previous knowledge can arise from it.

IV.4 VIPP1 counteracts environmental stress

Stress caused by various environmental factors is currently more present than ever. The climatic change caused by humans produces physiologically increasingly difficult hurdles, which have to overcome by life. This continues until organisms are forced into an evolutionary bottleneck that has to be mastered.
Light is a crucial stress factor for phototrophic organisms. PSII is subject to an constant repair cycle, which does not pose a problem for the organism, if it takes place under normal conditions (Aro et al., 1993). As soon as this cycle is required more and more, serious problems arise, including the breakdown of photosynthesis, which is lethal for the organism. In *C. reinhardtii* and *A. thaliana* light stress has been reported to lead to swollen thylakoids in VIPP1 knockdown lines (Nordhues et al., 2012; Zhang et al., 2012). This is caused by lumen acidification, which leads to an osmotic increase and therefore enhanced water absorption.
In addition, especially in high light, more photons are absorbed than the electron transport chain can utilize, which leads to the formation of reactive oxygen species. This in turn has a destabilising effect on proteins and lipids (Barber, 1995; Kirchhoff et al., 2011; Pospíšil, 2016; Telfer, 2014).
In vivo point mutations at two conserved H1 residues (F4E and V11E) which are shown to mediate lipid binding in PspA reveal direct impact on thylakoid convergence zones in *Synechocystis* 6803 (Gupta et al., 2021; Jovanovic et al., 2014; McDonald et al., 2017). Moreover, the thylakoids lacked connectivity and were detached from the plasma membrane (Figure 4 in section III.2, Gupta et al., 2021). These regions are now filled with round membranes, containing a variety of cytosolic material, including ribosomes, glycogen granules and phycobilisomes (Figure 4 in section III.2, Gupta et al., 2021; Supplemental Figure S7 in section VI.2, Gupta et al., 2021). Furthermore, in high light the F4E mutation

shows a decreased growth whereas V11E failed to grow at all (Figure 4A in section III.2, Gupta et al., 2021).

The findings in Chloroplasts as well as in Cyanobacteria implicate not only a constant reaction to light stress of thylakoid membranes but also the VIPP1's involvement in high light response via lipid binding mediated by its amphipathic N-terminal helix. Furthermore, it seems that in contrast to its related protein PspA, VIPP1 has this flexible C-terminal tail (H7) to deliver enhanced tolerance against membrane stress and to interact with external factors in the cellular environment (Liu et al., 2007; Zhang et al., 2016).

Recently it has been observed that the binding of VIPP1 to the membrane is enhanced by acidic conditions *in vitro*, suggesting that VIPP1 may be able to detect proton leaks caused by acidification in stressed thylakoid regions (Siebenaller et al., 2020).

In *C. reinhardtii*, VIPP1 rods were observed to encapsulate liposomes *in vitro* (Theis et al., 2019). In Synechocystis 6803, *in vitro* VIPP1 rings were observed how they bind and fuse to liposomes (Hennig et al., 2015). This could explain the putative *syn*VIPP1 coated membrane encapsulations that can be observed *in situ* by cryo-ET and which are react with a reduction in their abundance to light induced stress. These are oval structures that are always aligned in their longitudinal axis at right angles between the individual thylakoid membranes. In this way, VIPP1 could enable lipid transfer from one thylakoid membrane to another and thereby preserve its structure.

The reduction of *syn*VIPP1 coated membrane encapsulations under light stress could be interpreted as a shift from free membrane capsules as observed to direct membrane deposits due to an increased number of structural damage (Junglas & Schneider, 2018; Thurotte et al., 2017). However, this would have to be examined more precisely by, for example, a temporal resolution of the stress function.

One hint for this is the mentioned lipid binding affinity of VIPP1. Another one is that VIPP1 include a domain named 'Apolipophorin-III like' and this in turn is almost identical to the PspA-like domain of VIPP1 (Zhang & Sakamoto, 2015). Apolipophorin-III is a prototypical exchangeable apolipoprotein found in many insect species which functions in transport of diacylglycerol from a lipid storage depot to muscles (Ryan et al., 1990; Wang et al., 1995). That means it works as a lipid transporter, which might also apply to VIPP1 as a functional analogue.

IV.5 Conclusion

Photosynthesis is the driving force for life on earth, through the release of oxygen or the production of energy and biomass that will be used by living beings. This process has established in cyanobacteria and chloroplasts for more than 2700-million-years, has developed steadily and has always adapted to new environmental conditions.

When considering photosynthesis, it is essential to consider thylakoids as carriers of the photosynthetic complexes. Therefore, it is important to understand the factors that significantly influence the thylakoid architecture and / or ensure its maintenance under adverse circumstances.

AncM could be shown to be a crucial, structuring factor in *Synechocystis* 6803. It not only influences the architecture of thylakoid membranes by anchoring TCZs to the plasma membrane at the thylapse, but also acts as an antagonist to CurT, a factor required for the curvature of the thylakoids. In particular, this shows how important a specific relationship and balance between different factors is. AncM appears to be present in almost all branches of the cyanobacterial tree of life. The fact that AncM does not occur in chloroplasts, but CurT does, is a crucial point which raises the question of whether the elimination was a decisive evolutionary step for the formation of grana and stroma lamellae in chloroplasts by the loss of a membrane anchor.

For VIPP1, decisive new insights into its structure and its function *in vitro* and *in vivo* were obtained. It shows how small changes in a single helix can lead to completely changed oligomerisation and binding affinity. The study paves the mechanistic foundation to understand how VIPP1 mediates thylakoid biogenesis and stress resistance by its amphiphilic characteristic.

The microscopic techniques used have shown how important this tool can be in biology, from multicolour fluorescence techniques over localisation studies by CLEM to the detection of non-canonical nucleotide binding sites on nearly atomic level by Cryo-EM.

In summary, the understanding of the factors that form the thylakoid membranes in cyanobacteria has been expanded and this is essential to understand the process of photosynthesis more precisely.

IV.6 Future perspectives

The present work should not only expand the existing state of knowledge but also provide new possibilities and approaches for research. In the following, a few thoughts should be mentioned that could further explain the understanding and the mechanism of how AncM and VIPP1 work.

In order to better understand AncM, CurT, the interaction with PDMs and the biogenic regions that are important for PSII, it is necessary to compare this with existing findings.

One of the initial steps in assembly and repair of PSII is the maturation of D1. The precursor of D1 (pD1) is cotranslationally integrated into the thylakoid membrane (Zhang & Aro, 2002). The C-terminal end is then cut off and finally the maturated D1 is formed (Inagaki et al., 2001; Komenda et al., 2007; Nixon et al., 1992). In *Synechocystis* 6803, the transcription and subsequent translation of D1 is based on three mutually homologous genes, *psbA1*, *psbA2* and *psbA3* (Jansson et al., 1987). Under normal growth conditions as well as under most stress conditions *psbA2* and *psbA3* are sole responsible for D1, whereas *psbA2* accounts for 90% of total transcript (Mohamed et al., 1993).

Now it would be of interest to genetically mark *psbA2*, the most abundant homolog in *Synechocystis* 6803 with a fluorophore at the C-terminal end. The result should be that this label, together with the C-terminus, is split off during the development from pD1 to D1 and then accumulates at the regions of translation in detectable amounts. In this way, the initial phase of PS II assembly and / or repair can be identified. These images could then be correlated with an immunofluorescent counterstaining of AncM. The result should ultimately discriminate the biogenesis of PSII from the repair sites.

Another point is the localisation as well as the role of AncM in other cyanobacteria. Filamentous or even cell-differentiated representatives would be of interest here, in which on the one hand there is a strong anchoring at septal junctions between the individual cells and on the other hand a strongly differentiated thylakoid architecture (Flores & Herrero, 2010; Flores, Nieves-Morión, et al., 2019; Flores, Picossi, et al., 2019; Kieninger & Maldener, 2021). Specifically, the honeycomb structure of heterocysts in *Anabaena* sp. PCC 7120 is to be mentioned (Flores & Herrero, 2010; Merino-Puerto et al., 2011). Phylogenetically, these belong to the *Nostocales*, a sister group to the *Synechococcales* of which *Synechocystis* 6803 is a representative. By comparing the two species, crucial insights into the evolution of the thylakoids as well as the evolution of such an important process as bacterial nitrogen fixation could be obtained.

To further characterise the function of AncM, it could be transferred into AncM free species. It would be interesting to observe whether such new membrane connections are stabilised or even the growth of the cell is increased or decreased. In addition, it would be expected that CurT would react to this by regulating its expression. Here the role of VIPP1 could also be decisive in generating exact these membrane connections initially.

VIPP1 would also be worth observing in *Synechocystis* 6803 during the repair of PS II and the associated replacement of D1. Following the knowledge made in this work, VIPP1 should attach itself to strongly photo-damaged membranes or even membranes that expand during division. This could result in curvatures that increase the ratio of surface to volume at the corresponding area, which enables an increased exchange of nutrients. The resulting curvatures would break the thylakoids into pieces and with the help of CurT, they can merge toward the plasma membrane to form new TCZs, which are finally fixed by AncM and anchored to the plasma membrane. One could imagine that this could be the *de novo* formation of biogenic regions in *Synechocystis* 6803.

V. References

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VI. Appendix

Additional data for the work listed under Chapter III (Results) are stored below. These are not essential for the understanding of the same, but mentioned in the work and complement the research.

VI.1 Supplemental data - Thylakoid attachment to the plasma membrane in *Synechocystis* sp. PCC 6803 requires the AncM protein

The following information supplements the study described in section III.1. The figures include a phylogenetic analysis of AncM and depict homologs of AncM in the cyanobacterial taxa. Furthermore, they show the validation of the αAncM antibody in different strains and the construction of mutants. In addition, there is an enhanced TEM analysis of the ultrastructure of *ancM*$^{-}$, *ancM*^{comp}, *ancM*^{W164STOP} and *ancM*$^{-}$ *curT*$^{-}$ and an overview of additional correlative light-electron micrographs. Furthermore, there is an exemplified colocalisation analysis of AncM and CurT and additional immunofluorescence micrographs. The data contain a quantification of AncM puncta in immunofluorescence micrographs, the used primers and the sequence of the gBlock DNA fragment.
All supplemental data were arranged by me with assistance by S. Heinz. J. Nickelsen revised and approved the data file.

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VI.2 Supplemental data - Structural basis for VIPP1 oligomerization and maintenance of thylakoid membrane integrity

Following are key resource tables, experimental model and subject details as well as detailed methods used in the paper in section III.2. Furthermore, there are eight supplemental figures, which enhance the understanding of the article. This includes a *syn*VIPP1 protein purification, an interaction network for one monomer in the VIPP1 ring, analysis of the VIPP1 nucleotide density, mass spectrometry and nucleotide hydrolysis activity data, example negative-stain EM images from each *syn*VIPP1 protein, thylakoid protein levels and *in situ* cryo-ET, examples of defective convergence zones in the F4E mutant and putative *syn*VIPP1 structures in *Synechocystis* 6803 cells expressing VIPP1-GFP.
The supplemental data were arranged by T. K. Gupta.

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List of abbreviations

ADP	adenosine diphosphate
AncM	anchor of convergence membranes
ATP	adenosine triphosphate
CFP	cyan fluorescent protein
CLEM	correlative light-electron microscopy
CURT1	CURVATURE THYLAKOID1
CO_2	carbon dioxide
cyt b_6f	cytochrome b_6f
DGDG	digalactosyldiacylglycerol
DUF	domain of unknown function
EM	electron microscopy
ESCRT-III	endosomal sorting complex required for transport III
ET	electron tomography
FRET	Förster resonance energy transfer
IM30	inner membrane-associated protein of 30 kDa
LHC1	light-harvesting complex 1
LHC2	light-harvesting complex 2
MGDG	monogalactosyldiacylglycerol
$NADP^+$	nicotinamide adenine dinucleotide phosphate
ORF	open reading frame
P_i	inorganic phosphate
pD1	precursor of D1
PDM	PratA defined membrane
PQ	plastoquinone
PratA	processing associated TPR protein
PspA	phage shock protein A
PSI	photosystem I
PSII	photosystem II
RuBisCo	ribulose-1,5-bisphosphate carboxylase-oxygenase
TCZ	thylakoid membrane convergence zone
TEM	transmission electron microscopy
TPR	tetratricopeptide repeat
VIPP1	vesicle-inducing protein in plastids 1

Curriculum vitae

List of publications

Ostermeier, M., Heinz, S., Hamm, J., Zabret, J., Rast, A., Klingl, A., Nowaczyk, M.M., and Nickelsen, J. (2021). Thylakoid attachment to the plasma membrane in *Synechocystis* sp. PCC 6803 requires the AncM protein. The Plant Cell 2021: 00: 1–24

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„Alterius non sit, qui suus esse potest“
Philippus Theophrastus Aureolus Bombast von Hohenheim

“I don’t do regrets. Regrets are pointless. It’s too late for regrets. You’ve already done it, haven’t you? You’ve lived your life. No point wishing you could change it.”
Ian Fraser Kilmister

Eidesstattliche Erklärung und Erklärung

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, 13.Dezember 2021 _____

Matthias Ostermeier

Erklärung

Hiermit erkläre ich, dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist. Des Weiteren habe ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen.

München, 13.Dezember 2021 _____

Matthias Ostermeier