Molecular analysis of factors involved in biogenesis of the cyanobacterial thylakoid membrane

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SUMMARY

The reactions and factors participating during oxygenic photosynthesis in higher plants, algae and photoautotrophic bacteria are well known and understood. The high complexity of the photosynthesis reaction center subunits in the thylakoid membrane longs for a highly structured and organized assembly process. Several assisting factors have been described, which play important roles during biogenesis, which concerning its spatial organization is less understood. TPR proteins contribute to numerous steps during complex assembly and thylakoid biogenesis, by acting on RNA, protein or pigment level.

The present work aims to elucidate the function of two TPR proteins from the cyanobacterium *Synechocystis* sp. PCC6803 (*Synechocystis*).

The first one is the already described PratA factor, a periplasmic protein which was shown to interact with the C-terminal extension of the PSII core protein D1. Furthermore, the finding that the precursor of D1 accumulates in the absence of PratA, suggested a supporting role of PratA for the CtpA protein during the essential proteolytic processing of the C-terminal extension. To further characterize PratA and its interaction with D1 several molecular and biochemical approaches were carried out. The data, achieved by Yeast Two-Hybrid and GST-pulldown analyses, show that PratA interacts with the soluble C-terminus of the precursor as well as the mature D1 protein, indicating a different function of PratA from that stated earlier. Furthermore, the results suggest the existence of a novel membrane subfraction, to which 20 % of PratA is associated, due to its interaction with D1. This special membrane subfraction, which is neither plasma- nor thylakoid membrane, was analyzed in more detail. It seems that a connecting region between both membranes exists, where we propose several initial steps of thylakoid biogenesis in *Synechocystis* occur.

The second protein investigated in this work is the former undescribed TPR protein encoded by ORF *slr1644*, named Pitt. A systematic Yeast Two-Hybrid approach suggested an interaction between Pitt and the light-dependent protochlorophyllide oxidoreductase (POR) (Sato et al. 2007). The present work carries out the initial characterization of Pitt, concerning its possible role in photosynthesis. Analysis of the phenotype in a *pitt*-mutant reveals clear defects in the photosynthetic performance compared to wildtype cells. In addition, the resulting data validate the interaction of Pitt and POR and show that in the absence of Pitt the POR

content as well as the chlorophyll synthesis is reduced in the respective mutant. Finally, in a *pitt*-mutant pD1 accumulates in the special membrane subfraction, as also shown for a *pratA*-mutant, suggesting also for Pitt a role during assembly of photosynthetic complexes.

The present study gives insights in the mechanisms underlying the spatial organization of thylakoid membrane biogenesis in *Synechocystis* and reveals the roles of two TPR proteins during complex assembly with respect to polypeptide subunits and pigments, respectively.

ZUSAMMENFASSUNG

Die Abläufe der Photosynthese in höheren Pflanzen, Algen und photoautotrophen Bakterien sind weitestgehend aufgeklärt und die wichtigsten beteiligten Faktoren sind ausführlich beschrieben. Der Assemblierungsprozess der Untereinheiten der photosynthetischen Reaktionskomplexe in der Thylakoidmembran ist höchst komplex und verlangt einen strukturierten und organisierten Ablauf. An der Biogenese sind zahlreiche unterstützende Faktoren beteiligt, wobei jedoch die räumliche Organisation dieser Prozesse nicht aufgeklärt ist. TPR Proteine sind an vielen Stufen der Komplex-Assemblierung und der Biogenese der Thylakoidmembran beteiligt, sowohl auf RNA- und Protein- als auch auf der Ebene der Pigmentsynthese.

In der vorliegenden Arbeit sollten die Funktionen zweier TPR Proteine aus dem Cyanobakterium *Synechocystis* sp. PCC6803 (*Synechocystis*) untersucht werden.

Das erste Protein ist das bereits beschriebene PratA Protein, ein periplasmatisches Protein, das mit der C-terminalen Extension des PSII Kern-Proteins D1 interagiert. Die Tatsache, dass das Vorläuferprotein von D1 in der Abwesenheit von PratA akkumuliert, deutet auf eine unterstützende Funktion von PratA, während der essentiellen proteolytischen Prozessierung der Extension durch CtpA hin. Um PratA genauer zu charakterisieren und die Interaktion mit D1 zu bestätigen, wurden verschiedene molekulare und biochemische Versuchsansätze durchgeführt. Hefe-2-Hybrid- und „GST-Pull-Down“-Analysen zeigen, dass PratA mit dem löslichen C-Terminus sowohl des Vorläufer- als auch des maturen D1 Proteins interagiert, was auf eine abweichende als bisher vermutete Funktion von PratA schließen lässt. Weiterhin deuten die Ergebnisse darauf hin, dass etwa 20 % von PratA durch die Interaktion mit D1 an eine bisher unbekannte Membranfraktion assoziiert ist. Untersuchungen dieser Fraktion, die weder Plasma- noch Thylakoidmembran ist, werfen die Idee einer Verbindungsregion zwischen beiden Membrantypen auf, in der anfängliche Prozesse der Thylakoidmembran Biogenese in *Synechocystis* abzulaufen scheinen.

Das zweite in dieser Arbeit untersuchte TPR Protein ist das bisher unbeschriebene, durch den Leserahmen *slr1644* kodierte Protein, genannt Pitt. Ein umfassender Hefe-2-Hybrid Ansatz weist auf eine Interaktion zwischen Pitt und der licht-abhängigen Protochlorophyllid-Oxidoreduktase (POR) hin (Sato et al. 2007). Die

vorliegende Arbeit zielt darauf ab, eine erste Charakterisierung von Pitt bezüglich einer möglichen photosynthetischen Funktion durchzuführen. Phenotypische Untersuchungen der *pitt*-Mutante zeigen deutliche Beeinträchtigungen der photosynthetischen Aktivität im Vergleich zum Wildtyp. Zusätzlich bestätigen die Daten die Interaktion zwischen Pitt und POR und dass in Abwesenheit von Pitt sowohl der POR-Gehalt als auch die Chlorophyll Neusynthese reduziert sind. Schließlich, akkumuliert in der *pitt*-Mutante, wie auch in der *pratA*-Mutante, pD1 in der der „neuen“ Membranfraktion, was ebenfalls für Pitt eine Funktion während der Assemblierung der photosynthetischen Komplexe nahe legt.

In der vorliegenden Arbeit wird die Funktion zweier TPR Proteine bei der Assemblierung der Protein- und Pigmentuntereinheiten in die Photosynthese-Komplexe untersucht. Die Ergebnisse liefern Erkenntnisse über die räumliche Organisation, die der Biogenese der Thylakoidmembran in *Synechocystis* zugrunde liegt.

INTRODUCTION

Photosynthesis

The prerequisite for evolution of life on earth as we know it today was oxygenic photosynthesis. This essential process utilizes light energy to produce chemical energy on which all higher life on earth depends. Cyanobacteria invented this process 2.8 billion years ago and thus changed life on Earth to a maximum extent (Xoing & Bauer 2002). In the course of evolution, an ancestor of nowadays cyanobacteria was taken up by a primitive eukaryotic host cells and differentiated to the photosynthetic plastid, called chloroplast, of recent algae and higher plants. Even today cyanobacteria provide up to 30 % of the oxygen production on Earth per year and therefore are very important organisms for our ecosystem.

Photoautotrophic organisms absorb light energy, build up a proton gradient across the membrane and finally produce ATP. In further steps the ATP is used to produce sugar from carbon dioxide which is reduced to carbohydrate in the Calvin cycle. Especially sugars are used as energy and storage source for photoautotrophic organisms. The electrons needed for building up the proton gradient derive from the splitting of water and as a by-product oxygen is produced and released to the atmosphere. The formula for the photosynthesis occurring in plants, algae and cyanobacteria is as followed:

$$6CO_2 + 6H_20 \text{ --> } 6O_2 + C_6H_{12}O_6 + 6H_2O$$

The resulting carbohydrates provide the energy for respiration of all heterotrophic organisms and furthermore, it is an important source as fossil fuel for energy delivery for mankind.

The light reactions of photosynthesis which comprise capture of light energy, water-splitting, electron transport chain, proton gradient and production of ATP occur at a specialized membrane system, called thylakoid membrane. This membrane compartment in chloroplasts and cyanobacteria owes its name the greek word *thylakos,* meaning something like „sack“, due to its ultrastructure (Menke 1962). The thylakoid membranes enclose the thylakoid lumen. This membrane compartment

exhibits some ultrastructural differences between cyanobacteria and chloroplasts. While in cyanobacteria thylakoids are homogeneous arranged in 3 to 8 membrane bilayers, the membranes in chloroplast are organized in grana stacks and stromal lamella. Despite those structural differences thylakoid membranes of both house four big multi-subunit complexes, the photosystem I and II (PSI and PSII), cytochrome b_6f complex and the ATP-synthase (shown in Figure 1).

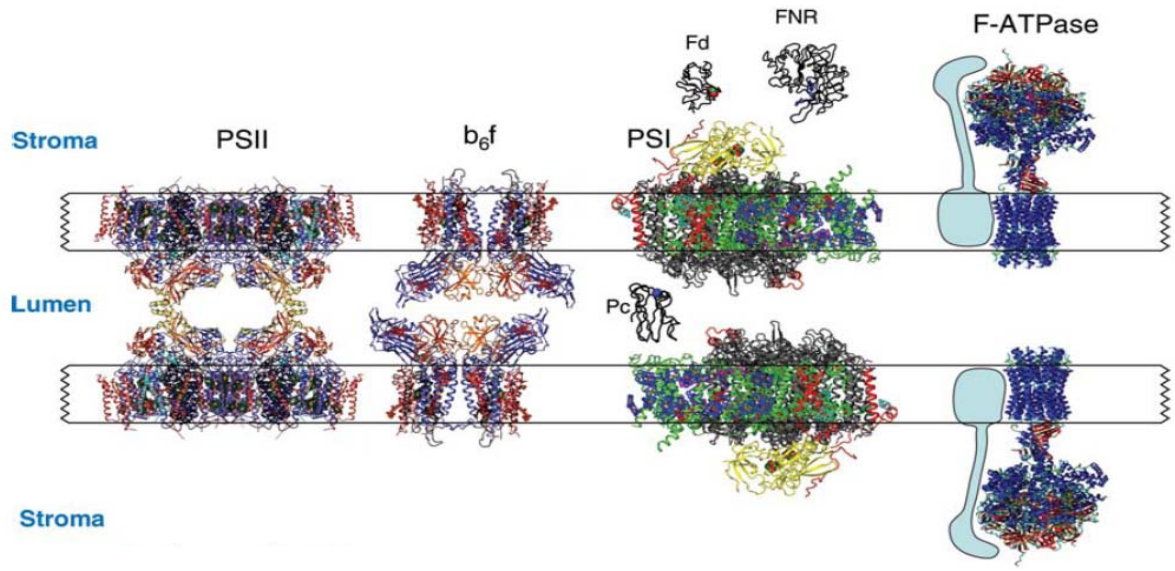

Figure 1: Model of the thylakoid membrane with embedded multi-subunit complexes PSII, cytochrome b_6f, PSI and ATPase. Additionally, the soluble electron carrier plasocyanin (Pc) and Ferredoxin (Fd) and the ferredoxin-NADP+ oxidoreductase (FNR) are shown (Nelson & Yocum 2006, modified).

Light energy is absorbed by a chlorophyll a-dimer, called P680, embedded in PSII. Electrons are released into the electron transport chain and transferred to plastoquinone A (Q_A). Then plastoquinone B (Q_B) is reduced and builds up the plastoquinone pool in the membrane. After that electrons are transferred to the luminal carrier plastocyanin via the cytochrome b_6f-complex (Kurisu et al. 2003; Stroebel et al. 2003). The next complex PSI is composed of 12 subunits. The major subunits are the proteins PsaA and PsaB, which share similar structure with the PSII core proteins D1/D2, indicating a common ancestor of both. Light-energy is captured by a chlorophyll pair, called P700, in PSI. This multi-subunit complex takes up the reduced plastocyanin and passes the electrons over to the stromal located ferredoxin. Electrons are then transferred to the ferredoxin-NADP+ oxidoreductase (FNR) (Saenger et al. 2002; Golbeck 2003). This enzyme drives the reduction of

NADP to NADPH. The fourth membrane-bound complex, the ATP-synthase, uses the proton-motive force, due to charge separation and electron transport to synthesize ATP. Finally, ATP is used in the photosynthetic dark reaction, where carbon dioxide is fixed to generate sugars.

Photosystem II

The multi-protein complex PSII, which is active as a dimer in the thylakoid membrane, provides the reactions occurring at the thylakoid membrane with electrons by splitting water and is therefore unique and absolutely necessary.

The PSII consists of at least 19 protein subunits and numerous co-factors, for example 35 to 40 chlorophylls and 8 to 12 carotenoids (Ferreira et al. 2004; Nickelsen et al 2007). The first crystals of PSII, generated from *T. elongatus*, delivered the first X-ray model of PSII (Zouni et al. 1998; Zouni et al. 2000). Today there are several structures of PSII available in a 3.7 to 3.0 A resolution (Fromme et al. 2002; Loll et al. 2005). All structural data provide insights into the structure of the core of PSII with the proteins D1 and D2 and additionally their antenna membrane proteins CP47 and CP43. Furthermore the characteristics of the water-splitting apparatus with the proteins PsbO, PsbP and PsbQ could be investigated.

The antenna associated to PSII captures the light energy and directs it to the reaction center (RC) where the electron transport chain is located. Antenna proteins in thylakoids of chloroplast are membrane-bound protein complexes, called light-harvesting complexes (LHC); on the other hand the light energy in cyanobacteria is absorbed by so called Phycobilisomes. These phycobilisomes are membrane attached proteins, which constitute to about 40 % of the dry mass of a cyanobacterial cell (Hankamer et al. 2001; Anderson & Toole 1998).

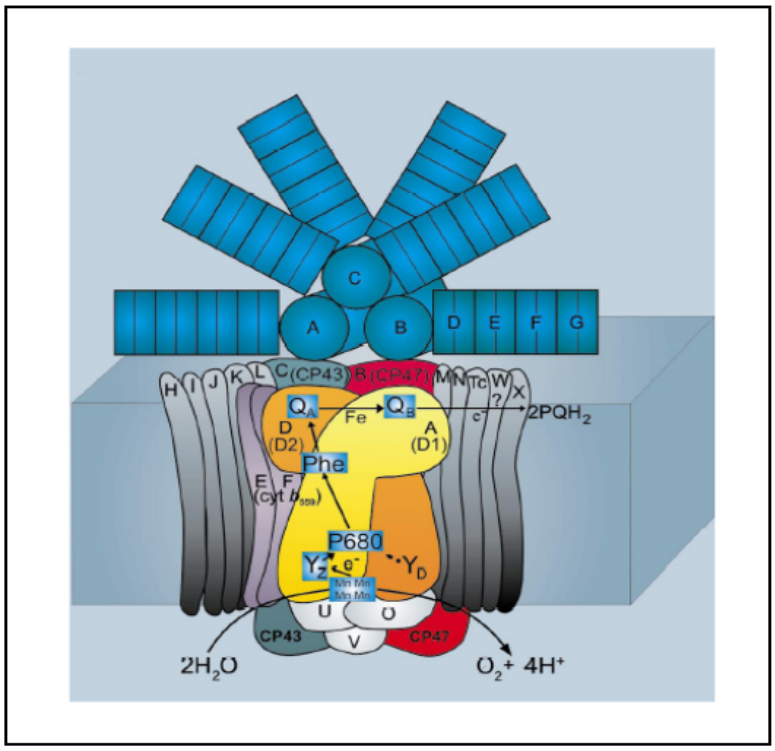

Figure 2: Structure and subunit composition of cyanobacterial PSII. The electron flow occurring at PSII is depicted with black arrows (Hankamer et al. 2001).

The core proteins D1 and D2 provide the energy conversion process with all needed redox active cofactors (Barber 2008). In detail, the exited P680 (4 chlorophylls in the center of the D1/D2 proteins) primary electron donor, provides the electrons to pheophytin (pheo) and plastoquinone (Q_A) and finally to Q_B. The resulting electron gap in P680 is filled by an electron derived from the redox active Tyrosin Z of the D1 protein (D1-Tyr161, Y_Z). The Tyrosin Z for its part is supplied by electrons from the water-splitting manganese-cluster, which consists of four Mn atoms and one Ca atom. The oxygen-evolving-complex (OEC) is located at the luminal side of PSII and drives the conversion of water into electrons and oxygen as a by-product (Barber & Murray 2008). But the mechanism of water oxidation and the exact structure of the OEC are still under debate.

Photosystem II reaction-center protein D1

The above described reactions, especially the high redox potential of P680 and the creation of reactive oxygen species, cause severe photodamage on the components of PSII. The core protein D1 is affected in highest degree, because it harbours the majority of the functional sites for the electron chain (Barber & Andersson 1992).

The *psbA* gene product D1 is synthesized as a precursor protein (pD1) with a C-terminal extension of 8-16 amino acids for most photoautotrophic organisms, except a few species (for a review see Satoh & Yamamoto 2007). This extension on the C-terminus seems to play a protective role against oxidative damage during assembly of the core protein into the PSII complex (Kuvikova et al. 2005). Albeit it could be shown that it is not a necessary feature of the D1 protein for the viability of the cells; mutants without this extension are also able to assemble fully functional PSII. In long-term mixed-culture approaches, otherwise, in which the fitness of extension-less mutants was compared to control strains, it turned out that especially under high-light conditions the presence of the extension enhances the fitness of the wild-type strain (Ivleva et al. 2000).

During maturation of PSII the D1-extension is posttranslational cleaved off by a C-terminal peptidase, called CtpA as shown in Figure 3 (Shestakov et al. 1994).

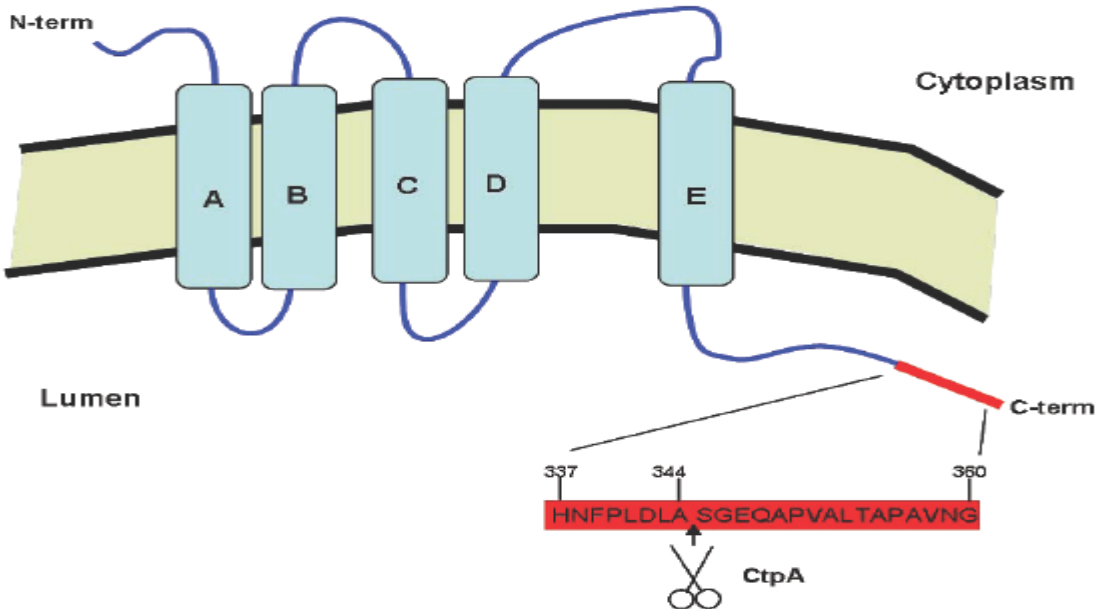

Figure 3: Membrane topology of the D1 protein. A-E mark the five transmembrane domains of the protein and furthermore the location of the N- and C-terminus are shown. The C-terminus is magnified and the cleavage-site for the CtpA protease is indicated.

The CtpA protein is soluble or loosely membrane-associated in the lumen of thylakoids (Bowyer et al. 1992; Fujita et al. 1995). Interestingly, in *Synechocystis* sp. PCC6803 (hereafter referred to as: *Synechocystis*) it is exclusively located at the plasma membrane and it was proposed that the periplasmic PratA factor supports CtpA in the D1-processing reaction (Zak et al. 2001; Klinkert et al. 2004). This led to new ideas concerning the location of complex assembly as discussed later.

The processing is an essential event in the cells. In the absence of the CtpA protease, pD1 is incorporated in the PSII complex, which on the one hand mediates electron transfer reactions, but is not able to catalyze water oxidation (Anbudurai et al. 1994). Today, the need of a processed D1 protein for attachment/assembly of the manganese-cluster to photosystem II is approved in general (Nixon et al. 1992; Cohen et al. 2007).

The core protein D1 is prone to extensive oxidative damage and has to be replaced by a newly synthesized copy. The light-induced turnover of this protein is intensively studied since decades in different organisms (Mattoo et al. 1981; Wettern & Ohad 1984; Ohad et al. 1985; Hoffman-Falk et al. 1983). During this repair cycle the PSII complex is disassembled, and specific proteases have access to the photodamaged D1 protein. After monomerization, it has been shown that Mn atoms, extrinsic PSII subunits of the OEC and CP43 are released during the repair process (Andersson & Styring 1991; Nixon et al. 2005). The damaged D1 protein then undergoes a conformational change and the stromal located DegP2 protease cuts D1 into a 23 kDa and a 10 kDa fragment. The 23 kDa fragment is then further degraded by the FtsH-protease, while DegP1, DegP5 and DegP8 cleave D1 fragments exposed to the luminal side. An alternative model is discussed, which proposes that FtsH alone is able to degrade damaged D1 protein (Adam & Clarke 2002; Huesgen et al. 2009). After removal of the D1 fragments, a newly synthesized copy of the D1 protein is inserted and finally the complex reassembles all subunits and cofactors and gains back its active dimeric form (Barber & Andersson 1992; Nixon et al. 2005; Huesgen et al. 2009).

Chlorophyll synthesis

The function of oxygenic photosynthesis not only depends on the supply of polypeptides for the complex subunits, the synthesis and incorporation of pigments is indispensable, too. Chlorophylls are the major pigments in PSII. They belong to the family of cyclic tetrapyrroles. In photosynthetic organisms the synthesis of chlorophyll is performed via a common tetrapyrrole biosynthetic pathway (Vavilin & Vermaas 2002). The precursor for all tetrapyrroles is 5-aminolevulinic acid (ALA), which is synthesized from glutamate in the C5-pathway (Beale 1999). In the course of several enzymatic reactions protoporphyrin IX (Proto-IX) is synthesized out of eight molecules ALA. The incorporation of Mg^{2+} finally leads to the chlorophyll synthesis branch (Chew & Bryant 2007). The binding of a monoethylester to Mg-proto-IX, which is performed by the Mg-protoporphyrin-IX methyltransferase under the presence of NADPH and oxygen forms protochlorophyllide (PChlide) (Porra 1997). The final steps of chlorophyll synthesis, namely the reduction of protochlorophyllide to chlorophyllide by the membrane-associated NADPH-PChlide oxidoreductase (POR) is the most studied reaction in this pathway. While in angiosperms the reaction is exclusively catalyzed in a light-dependent manner (Masuda et al. 1994), in cyanobacteria, algae and non-vascular plants an additional structural unrelated enzyme, the light-independent POR (DPOR) drives the reduction of PChlide in the dark (Armstrong 1998). The POR belongs to the family of short-chain alcohol dehydrogenases and catalyzes the trans addition of hydrogen from NADPH across the C17-C18 double bond in the D ring of PChlide (Baker 1994). Finally, the esterification of the resulting chlorophyllide with a phytyl by the chlorophyll synthase completes the chlorophyll a synthesis (Ruediger et al. 1977). At least for plants, it is shown that the biosynthesis of chlorophyll from glutamate takes place exclusively in the chloroplast. Most enzymes are either bound to the envelope or are soluble in the stroma. An exception is the last step; the esterification is located to the thylakoid membrane (Joyard et al. 1998).

(A) (B)

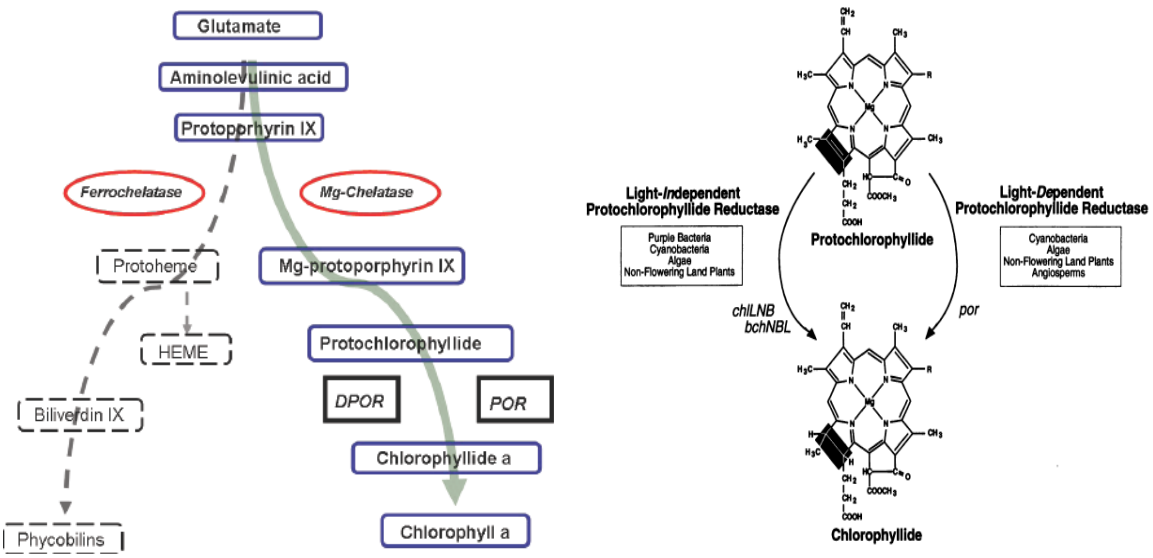

Figure 4: Chlorophyll synthesis pathway. (A) Complete pathway from glutamate to chlorophyll a. Incorporation of Mg leads to the chlorophyll branch, while incorporation of Fe to the heme branch. (B) Detailed view on the reaction performed by the POR and DPOR (encoded by chlL, N, B), respectively (Suzuki & Bauer 1995).

Biogenesis of thylakoid membranes

The assembly of the multi-subunit complexes must follow a concerted fashion of polypeptide and pigment subunits together with several trans-acting factors. These factors are not part of the fully assembled complexes, but are transiently bound to specific subunits during their assembly steps. Factors for both active complexes (PSI and PSII) are described and studied to a huge extent.

Assembly factors of PSI

For the cyanobacterial PSI the trans-acting factors Ycf4, Ycf3 and Ycf37 have been described recently. In the chloroplast of green algae and higher plants the factor Ycf4 was shown to be essential for stable accumulation of PSI (Boudreau et al. 1997; Ruf et al. 1997). The cyanobacterial homologue of the plastid-encoded factor was

identified in *Synechocystis*. It is encoded by the open reading frame *sll0226*, and also seems to be involved in PSI assembly in the cyanobacterium (Wilde et al. 1995). Astonishingly, in the absence of this protein fully active PSI complexes are formed in *Synechocystis*. This discrepancy between cyanobacterial and chloroplast Ycf4 protein might be due to a functional shift during evolution. Just recently, it was shown that in *Chlamydomonas reinhardtii* Ycf4 together with COP2 and several PSI subunits build a large complex of 1500 kDa that seems to represent a scaffold for PSI assembly (Ozawa et al. 2009).

The assembly factor Ycf3 belongs to the family of the so-called tetratricopeptide repeat proteins (for detail, see chapter “TPR proteins and photosynthesis”). It is an essential thylakoid membrane associated protein in *C. reinhardtii* and *N. tabacum* (Boudreau et al. 1997; Ruf et al. 1997). Interestingly, the PSI subunits PsaA and PsaD directly interact with the Ycf3 protein and temperature shift experiments revealed a function in assembly and not in stability of photosystem I (Naver et al. 2001). The cyanobacterial homologue is essential for PSI functionality, but in contrast to the chloroplast-encoded Ycf3 protein in *Synechocystis* it is associated to the plasma membrane, indicating a role during initial steps of complex assembly (Zak et al. 2001).

A second TPR protein, containing three TPR units, involved in biogenesis of cyanobacterial PSI is encoded by the open reading frame *slr0171*. In contrast to Ycf3, the Ycf37 protein does not exhibit essential functions in *Synechocystis* (Wilde et al. 2001). In a deletion mutant of Ycf37 a decrease of the trimeric PSI form is visible, but no significant effect on the photosynthetic activity could be estimated (Duehring et al. 2006). Recently, it was shown that in *Synechocystis* three monomeric forms of PSI exist, the complete one, the PsaL/PsaK-less and the monomer lacking the PsaK protein. Duehring and co-workers provide evidence that the subunit PsaK is incorporated as one of the last subunits into PSI (Duehring et al. 2007). The same is true for PsaK in *C. reinhardtii* (Hippler et al. 2002). Interestingly, both novel PSI monomers are missing in an *ycf37*-mutant, raising the idea that Ycf37 protein is involved in the latest steps of PSI assembly. The fact that, in contrast to the proteins Ycf3 and Ycf4, Ycf37 is exclusively associated to the thylakoid membrane supports its role as a trans-acting factor in the late steps of PSI formation.

Assembly factors of PSII

The photosystem II holds a greater complexity than PSI and thus, more factors important during assembly are described and studied.

One well studied factor is the luminal HCF136. This factor is essential for PSII activity in *A. thaliana* (Meurer et al. 1998). It was shown that HCF136 is part of an early PSII complex, consisting at least of D2 and cyt-b559 (Pluecken et al. 2002). The cyanobacterial homologue (*slr2034*) in *Synechocystis* is associated to this precomplex *in vivo*, too and there is some evidence that it is involved in the incorporation of newly synthesized pD1 protein into this complex. Komenda and co-workers showed in Yeast Two-Hybrid experiments that the HCF136 factor interacts with the precursor form of the D1 protein before its assembly to the precomplex and that in the absence of the factor HCF136 the pD1 is quite unstable. Furthermore, they found that HCF136 is also important for stability of PSII after assembly and replacement of damaged D1 during the repair cycle, indicating a dual role for the HCF136 protein in the early assembly and the repair of PSII (Komenda et al. 2008).

A further TPR-protein which acts as a biogenesis factor of thylakoid membranes is the PratA factor, encoded by *slr2048* in *Synechocystis* (Klinkert et al. 2004). In a *pratA*-mutant the content of PSII is reduced, but cells are still viable. The mutant cells accumulate a high amount of the unprocessed form of the pD1 protein, suggesting a role for PratA protein in the maturation of the D1 protein and a supporting function of the CtpA protein is discussed. Intriguingly, the periplasmic PratA protein was shown to directly interact with pD1 *in vitro*, supporting the idea that early steps of biogenesis occur at the plasma membrane.

Furthermore, the Oxa1 homologue of *Synechocystis* Slr1471p interacts with the pD1 as well (Ossenbuehl et al. 2006). It belongs to the family of Oxa1/Alb3/YidC proteins which appear to be involved in the integration and assembly of membrane complexes. In *Synechocystis* this protein is essential for thylakoid biogenesis (Spence et al. 2004) and further studies revealed a function for correct membrane integration, folding and assembly of pD1 to the early PSII complex (Ossenbuehl et al. 2006).

The complete and correct assembly of PSII depends on another protein, called Psb27. It is a bacterial lipoprotein and was detected in PSII complexes of *Synechocystis* (Roose & Pakrasi 2004). This factor is associated to the luminal side of PSII and is important for assembly of the Mn4-cluster to PSII during *de novo* and

repair cycle synthesis (Nowaczyk et al. 2006; Roose & Pakrasi 2007). Recent studies presented a model in which the Psb27 protein prevents the binding of the luminal, extrinsic PSII subunits PsbO, PsbU, PsbV and PsbQ to allow efficient access of manganese, calcium and chloride atoms to the Mn4-cluster. After complete assembly of the cluster Psb27 protein disassembles and the missing subunits attach to PSII (Roose & Pakrasi 2007; Cormann et al. 2009).

A factor, also detected in substantial amounts during PSII preparations is the Psb28 protein (Kashino et al. 2002). Contrarily, in recent structural studies of cyanobacterial PSII this protein was not identified (Ferreira et al. 2004; Loll et al. 2005). Nevertheless, it could be shown that Psb28 protein is part of the PSII complex lacking CP43 (RC47) in *Synechocystis* (Dobáková et al. 2009). In addition, Psb28 is not essential for photosynthetic activity, but in pulse-labelling experiments a retarded synthesis of the CP47 protein and interestingly, the PsaA and PsaB proteins was obvious. These findings indicate a role of the Psb28 protein for both PSII and PSI as well. Additionally, in the *psb28*-mutant an accumulation of intermediates during chlorophyll synthesis, especially protoporphyrin IX methylester, was shown. Taken together, a participation of the Psb28 protein for synthesis of chlorophylls and/or chlorophyll-binding proteins CP47 and PsaA/PsaB is possible (Dobáková et al. 2009).

A further protein detected in the analysis of PSII preparations was Psb29 (Kashino et al. 2002). This conserved 22 kDa protein is involved in PSII biogenesis in *Synechocystis* and *A. thaliana*. Its inactivation led to increased sensitivity to high-light and a lower PSII efficiency in both organisms (Keren et al. 2005). Thus, indicating a conserved function during evolution of photosynthetic organisms. Furthermore, caused by either incomplete assembly during biogenesis or incorrect disassembly during the repair cycle of PSII, the proximal antenna proteins accumulated (Keren et al. 2005). However, the exact function of the Psb29 protein still has to be elucidated.

The GUN4 protein of *Synechocystis* has been shown to be important for chlorophyll synthesis and assembly of photosynthetic complexes (Sobotka et al. 2008). This protein belongs to the family of porphyrin-binding proteins, activating the Mg-chelatase, which drives one of the first steps in the chlorophyll synthesis branch. In higher plants several GUN proteins (genome uncoupled) have been identified and seem to be involved in retrograde-signaling from plastid to nucleus (Susek et al. 1993). GUN4 related proteins are unique for photoautotrophs. Studies in

Synechocystis show that the GUN4 protein is essential for photoautotrophic growth (Wilde et al. 2004). Mutants are characterized by a decreased chlorophyll synthesis and incomplete assembly of both photosystems. The major deficiency was visible for PSII, due to a decreased accumulation of CP47 protein, which is affected by changes in the availability of chlorophyll in highest degrees (Sobotka et al. 2008). This study suggests, as already stated earlier (e.g. He & Vermaas 1998), a dependence of proper PSII assembly on the presence of pigments.

A more general factor involved in PSII assembly is MapA. The MapA protein is a methionine aminopeptidase (MetAP) important for the essential cleavage of the N-terminal methionine residue from newly synthesized proteins. This N-terminal excision (NME) pathway occurs in all kingdoms of life (Giglione et al. 2003). Most bacteria exhibit one *map* gene encoding a MetAP, in *Synechocystis*, there are even three, encoded by *mapA*, *mapB* and *mapC*. Activity assays revealed that the MapC protein is the major MetAP, while the MapB and MapA proteins displayed no or only a moderate activity, respectively (Atanassova et al. 2003). The MapA protein offers a specialized function during stress conditions. This protein is proposed to be important for the synthesis of functional photosynthetic complexes, if cells are exposed to nitrogen-starvation or high-light stress (Drath et al. 2009). It has already been shown that upon inactivation of the MetAP in the green algae *C. reinhardtii* the assembly of PSII was impaired (Giglione et al. 2003). Site-directed mutagenesis of the MapA protein in *Synechocystis* revealed an alteration in the Q_B binding pocket, even under non-stress conditions. This observation suggests a conformational change in the D1 protein or another PSII subunit, which indirectly affects the Q_B pocket (Drath et al. 2009). First biochemical approaches indicate an incomplete assembly and accumulation of free CP47 protein (Schottkowski, Forchhammer, Nickelsen, unpublished results).

Spatial organization of thylakoid biogenesis in cyanobacteria

Today a lot of structural data of the photosynthesis complexes is available (Nelson & Ben-Shem 2004). Even subunits and actions occurring along the thylakoid membrane are elucidated quite well. Otherwise, less is known about the spatial organization of thylakoid biogenesis. Of course, fully functional complexes are

integrated in the thylakoid membrane of cyanobacteria, but several recent studies provide evidence that the early steps of biogenesis may occur at the cyanobacterial plasma membrane.

Efficient separation techniques (sucrose density centrifugation in combination with aqueous two-phase partitioning) helped to yield pure plasma and thylakoid fractions and it was shown that the protein CtpA is present in the plasma membrane fraction, exclusively (Zak et al. 2001). As the CtpA protein is essential for the maturation of pD1 and thus for proper assembly of the oxygen-evolving-complex to PSII, this early step of biogenesis cannot occur in the thylakoids (Zak et al. 2001). In addition the complex subunits D2, cytochrome b559, PsaA and PsaB are found not only in the thylakoids but also in the plasma membrane fraction. The above mentioned assembly factors Ycf3 and Ycf4 are also located in the plasma membrane exclusively. Finally, contamination of cytoplasmic membrane preparations with thylakoids could be excluded because the PSII antenna proteins CP43 and CP47 are detected in the thylakoid fraction merely (Zak et al. 2001).

A further piece of evidence provides the study of the PratA factor (Klinkert et al. 2004). In proteomic analyses it was shown that PratA is present in the periplasmic space of *Synechocystis* and also the signal peptide of the protein suggest a transport of the PratA protein across the plasma membrane to the periplasm via the Sec-pathway (Fulda et al. 2000). The presence of the PratA protein in the periplasm was verified and a direct interaction with the pD1 protein was shown (Klinkert et al. 2004). Due to its location, the interaction has to occur at the plasma membrane during early steps of PSII assembly.

Pakrasi and co-workers prepared pure plasma and thylakoid membrane fraction and found partially assembled chlorophyll-containing PSII in the plasma membrane which can undergo light-induced charge separation and contains a functional electron acceptor side, but not a functional donor side (Zak et al 2001; Keren et al. 2005). For the complete assembly of the Mn4-cluster the presence of the thylakoid membrane protein CP43 and the contribution of necessary ligands to the OEC are required (Keren et al. 2005).

Summerization of the just depicted facts leads to the following model for the assembly pathway of cyanobacterial photosystem II (see Figure 5).

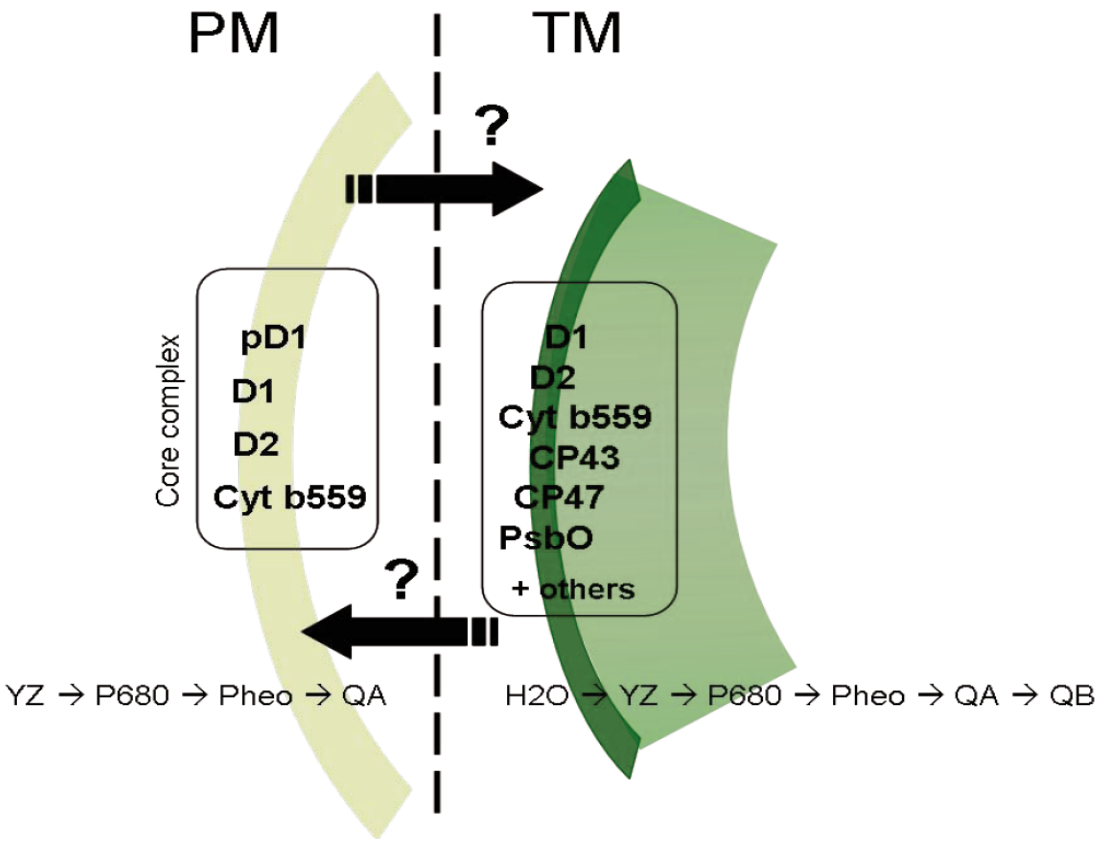

Figure 5: Schematic model describing the distribution of PSII subunits in plasma and thylakoid membrane. Details are given in the text.

The assembly starts with a precomplex in the plasma membrane, consisting of at least pD1/D1/D2 and cytochrome b559, which is in line with observation made in higher plants (Komenda et al. 2004; Zak et al. 2001; van Wijk et al. 1997). This PSII precomplex already contains the P680 chlorophyll and correct folded Q_A and Q_B binding pockets and thus, exhibits photochemical competence (Keren et al. 2005). During biogenesis the C-terminal extension of the pD1 protein exposed to the periplasm is cleaved by CtpA, which is a prerequisite for the assembly of the Mn4-cluster (Roose & Pakrasi 2004). Finally, the complex has to be transported to the thylakoids, where the last steps of biogenesis, the assembly of the missing subunits, e.g. CP43/CP47 and the OEC can occur.

Managing of the protein-complex transport from the plasma to the thylakoid membrane still needs to be elucidated. Today two major possibilities are discussed. On the one hand a vesicle transport might be imaginable. Such vesicles occur in chloroplasts of *A. thaliana* and are proposed to present transport vesicles (for a review see Vothknecht & Soll 2005). However, this observation seems to be restricted to land plants. In contrast, the vesicle-inducing protein in plastids 1 (VIPP1) is found in both, higher plants and cyanobacteria. In the absence of VIPP1 the vesicle transport disappeared and thylakoids in both organisms are reduced and unorganized drastically. This might indicate a connection between this transport system and thylakoid biogenesis (Aseeva et al. 2007; Fuhrmann et al. 2009).

On the other hand proteins may be transported from one to the other membrane by lateral fusions of both membrane systems. If these connections exist in cyanobacteria, they are hard to detect. Contradictory results propose either thylakoids as a discontinuous, separate membrane compartment with no connection to the plasma membrane in *Synechocystis* (Spence et al. 2003; Liberton et al. 2006) or both membrane systems to have occasional physical connections (van de Meene et al. 2006).

TPR proteins and photosynthesis

Several important cofactors for the biogenesis of thylakoid membranes belong to the family of so-called tetratricopeptide-repeat-protein (TPR). These proteins consist of 3-16 tandem-repeats of 34 conserved amino acids (D'Andrea & Regan 2003). There is a high homology in one motif concerning size and hydrophobicity and eight amino acids are highly conserved. One TPR pattern forms two anti-parallel α-helices and the tandem array of motifs generates a helical structure with an amphipatic character (Sikorski et al. 1990; Blatch & Laessle 1999). The first TPR protein was identified in yeast; here it plays a role in the cell division cycle (Sikorski et al. 1990, Hirano et al. 1990). Today it is known, that TPR proteins mediate protein-protein interactions and are often associated to multi-protein complexes. They function as a chaperone, in the cell-cycle, in transcription, splicing and protein transport in the different organelles (Goebl & Yanagida 1991). TPR proteins are ubiquitous and are present in bacteria,

fungi, plants, insects, animals and humans (Blatch & Laessle 1999). The organization of the TPR domains is given in Figure 6.

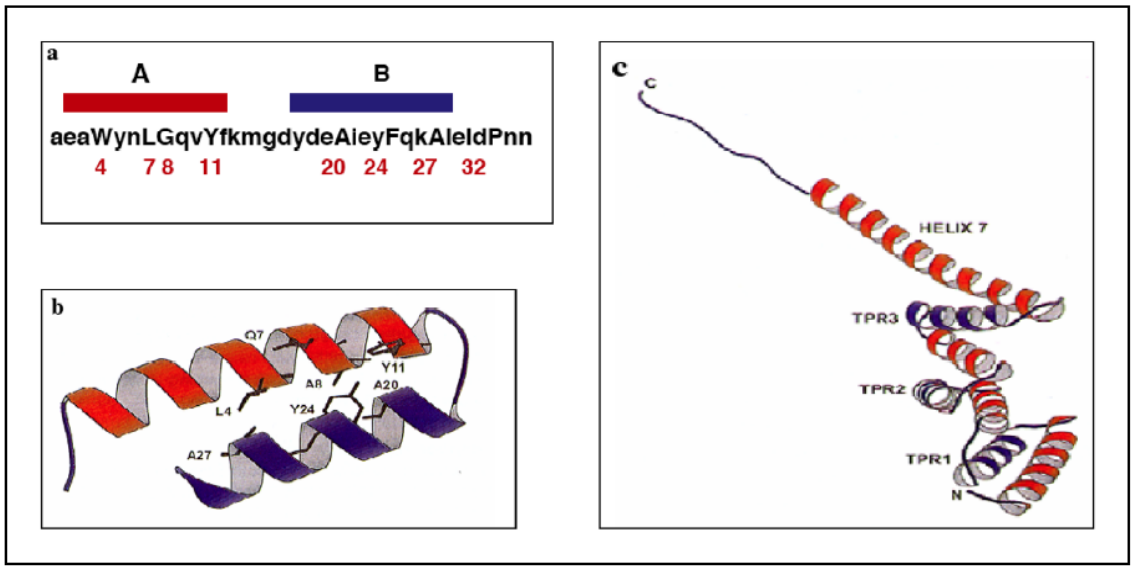

Figure 6: (A) Primary structure of one TPR motif. Blue and red lines indicate the two α-helices. Red numbers represent highly conserved amino acids. (B) Secondary structure of one and (C) tertiary structure with three TPR motifs (Blatch & Laessle 1999).

Besides the abovementioned TPR proteins, several more are described as factors involved in thylakoid membrane biogenesis (Tab. 1). Here they do not only mediate protein-protein interaction but also affect the stability of RNA. It was shown that in absence of specific factors, the transcripts of PSII subunits are rapidly degraded. This is true for the TPR proteins Nac2, Mbb1 and Hcf107 (Boudreau et al. 2000; Vaistij et al. 2000; Felder et al. 2001).

Another TPR protein, namely LPA1, is proposed to be involved in PSII assembly of *de novo* synthesized subunits in *A. thaliana* (Peng et al. 2006), but the homologue in *C. reinhardtii* REP27 seems to be important during the repair cycle and not the *de novo* synthesis of PSII (Park et al. 2007). This contrast suggests that the function of TPR proteins during evolution may change.

The factor FLU in *A. thaliana*, which contains two TPR motifs, regulates by interacting with enzymes of the tetrapyrrol synthesis pathway, the production of pigments (Meskauskiene et al. 2001). Taken together, the functions and targets of

TPR proteins in photoautotrophic organisms vary, but most of them have in common that they act during thylakoid membrane biogenesis.

Table 1: Overview of described TPR proteins from photoautotrophic organisms involved in thylakoid biogenesis.

FACTOR	TARGET	ORGANISM	REFERENCE
Mbb1	*psbB* RNA stability	*C. reinhardtii*	Vaistij et al. 2000
Hcf107	*psbH* RNA stability	*A. thaliana*	Felder et al. 2001
Nac2	*psbD* RNA stability	*C. reinhardtii*	Boudreau et al. 2000
Ycf3	PSI assembly	*C. reinhardtii* *N. tabacum*	Boudreau et al. 1997 Ruf et al. 1997
Ycf37	PSI assembly	*Synechocystis* *A. thaliana*	Wilde et al. 2001 Stockel et al. 2006
PratA	PSII assembly	*Synechocystis*	Klinkert et al. 2004
LPA1	PSII assembly	*A. thaliana*	Peng et al. 2006
REP27	PSII repair	*C. reinhardtii*	Park et al. 2007
FLU	chlorophyll biosynthesis	*A. thaliana*	Meskauskiene et al. 2001

Synechocystis sp. PCC6803 as a model organism

The prokaryote *Synechocystis* sp. PCC6803 belongs to the phylum of cyanobacteria, which ancestors have been the former free-living endosymbiont and nowadays chloroplast of higher plants and algae. *Synechocystis* is a gram-negative bacterium and its genome, which exists in 7-12 copies per cell, was the first fully sequenced (Kaneko et al. 1996) and comprises 3.5 Mbp, sequence data and additional info are available on the Kazusa homepage (http://genome.kazusa.or.jp/cyanobase). The proteome is encoded by 3.317 open reading frames and till today there are 143 proteins with known functions in photosynthesis and respiration (Kaneko & Tabata 1997). In addition to its fully sequenced genome, *Synechocystis* has a lot of advantages as a model organism. It is amenable of spontaneous transformation with exogenous DNA and this DNA recombines homologous, thus target-oriented mutations can be produced. Effects of mutations in important genes responsible for

example the electron transport chain or assembly of photosystems can be studied because *Synechocystis* cells can grow with a doubling-rate of 12-15 h heterotrophically with sugar as a carbon source in the medium (Rippka et al. 1979; Grigorieva & Shestakov 1982). All the facts mentioned above point out the role of *Synechocystis* as one of the most important model organisms for studying photosynthetic functions and complex structure. Regarding the close relation to chloroplasts, findings obtained by studying *Synechocystis* help to understand photosynthesis in cyanobacteria and algae and higher plants as well.

Aims of this work

TPR proteins exhibit essential or at least important functions during complex assembly and/or thylakoid membrane biogenesis in photosynthetic organisms. The present study tries to reveal the precise roles of two further TPR proteins encoded in the genome of the photosynthetic cyanobacterium *Synechocystis*.

To investigate the functions of TPR proteins in *Synechocystis* they were systematically inactivated by site-directed mutations. According to the resulting phenotypes, estimated by means of low-temperature chlorophyll fluorescence and oxygen-evolution, mutants were chosen for further characterizations which had defects in the photosynthetic performance.

(1) The first described was the PratA factor, which interacts, as already published with the precursor of the D1 protein and was suggested to support the protease CtpA during maturation of D1. (i) The interaction between PratA and pD1, (ii) the localization of this interaction and (iii) the role of PratA during thylakoid biogenesis was investigated in detail in the present study. The results will hopefully reveal insights into the spatial organization concerning the initial steps of thylakoid membrane biogenesis.

(2) The second part of this work deals with a so far uncharacterized TPR protein encoded by the ORF *slr1644* in *Synechocystis*. The phenotype in the respective mutant showed clear deficits in the photosynthetic performance and furthermore a systematic Yeast Two-Hybrid approach revealed an interaction between 1644p and the light-dependent protochlorophyllide oxidoreductase (POR) (Sato et al. 2007). Analyses in the study focused on (i) verification of the interaction, (ii) characterization of the 1644p protein and its function during chlorophyll synthesis and (iii) its localization and distribution within the membrane portion. Knowledge of this factor will elucidate the processes of photosynthesis complex assembly concerning not only polypeptide subunits, but also pigment incorporation.

RESULTS

This following part is arranged into two parts, which represent two independent studies already published in international peer-reviewed journals. For section I and II, I summarize the results and conclusions and state on the authors' contributions to each publication.

Section I: Schottkowski M., Gkalympoudis S., Tzekova N., Stelljes C., Schuenemann D., Ankele E., Nickelsen J. (2009) Interaction of the periplasmic PratA factor and the PsbA (D1) protein during biogenesis of photosystem II in *Synechocystis* sp. PCC6803. J. Biol. Chem. 284, 1813-1819

This work addresses the characterization of the TPR-protein PratA from *Synechocystis* and its role in the assembly process of PSII. We applied mainly molecular and biochemical approaches to further elucidate the exact function of PratA. Data from Yeast Two-Hybrid analyses clearly show that PratA not only interacts with pD1, but also with the mature protein. Furthermore, the specific binding regions of PratA on the C-terminus of the D1 protein were estimated. The interaction with the membrane protein D1 suggests a D1-dependent membrane association of PratA, which indeed could be shown. 2D-PAGE analysis of either membrane or soluble protein fractions revealed the existence of two different PratA-complexes – one is located in the periplasm with the size of 200 kDa and constitutes to about 80 % of the PratA-pool, the second one was found in the membrane fraction and migrates in the range of 70 kDa. This complex is absent on a 2D-PAGE with D1-deletion strain material, indicating again the D1-dependence. These observations let us propose that the 70 kDa complex consist of PratA and D1. As PratA is usually exclusively located to the periplasm, we tried to localize the PratA/D1-complex with respect to its membrane-type. Therefore we used a sucrose-density centrifugation approach with two consecutive gradients and finally the results clearly substantiate the existence of a specialized membrane fraction, neither plasma nor thylakoid membrane, which is marked by the presence of PratA (PDM = PratA-defined membrane). Additional visualization experiments showed that in the absence of

PratA a CFP-tagged D1 protein accumulates in the periphery of the cell, supporting the idea that PratA is important for processes of early assembly of PSII. Our conclusion is that PratA is located to a novel membrane region, which might represent a specific site where early steps of thylakoid membrane biogenesis occur and which connects plasma- and thylakoid membrane in *Synechocystis*.

My contributions to this work are the membrane isolation and solubilization experiment, the 2D-PAGE- and the sucrose-gradients analyses as well. C. Stelljes generated the mutants and performed the Yeast Two-Hybrid analysis. The peptide-scan and the GST-pull-down assay were done by S. Gkalympoudis, and N. Tzekova together with E. Ankele contributed the microscopic work. The manuscript was written by me and J. Nickelsen, who also revised the final version.

Section II: Schottkowski M., Ratke J., Oster U., Nowaczyk M., Nickelsen J. (2009) Pitt, a novel tetratricopeptide repeat protein involved in light dependent chlorophyll biosynthesis and thylakoid membrane biogenesis in *Synechocystis* sp. PCC 6803. Mol. Plant 2, 1289-1297

In this study initial characterization of the TPR protein encoded by ORF *slr1644*, named Pitt during the investigations, is performed by applying bioinformatical, biophysical, molecular and biochemical approaches. The sequence analysis of Pitt revealed the presence of Pitt-related membrane proteins amongst several photoautotrophic organisms, while none was found in non-photoautotrophs, indicating a conserved function for photosynthesis. After inactivation of the *Pitt* gene, a clear defect in the photosynthetic performance was visible. Further, we were able to verify data obtained in yeast, which suggested an interaction *in vitro* between Pitt and the light-dependent protochlorophyllide oxidoreductase (POR). This finding was supported by the fact that Pitt and POR co-migrate to a substantial amount on native gels. Interestingly, we could show that in the absence of Pitt the POR content is reduced to about 35 % of wild-type level, again suggesting an interaction between

them *in vivo*. This led to the hypothesis, that in a *pitt*-mutant the synthesis or stability of chlorophyll might be affected. And indeed, our data clearly show a retarded light-depending chlorophyll synthesis in the respective mutant. The membrane sublocalization of Pitt seems to depend on the biogenesis factor PratA; and as already shown for the absence of PratA, in a *pitt*-mutant the precursor of the D1 protein accumulates in the PDM subfraction. We conclude that Pitt is involved in initial steps during assembly of protein/pigment complexes in the course of thylakoid membrane biogenesis.

For this work I performed the sequence analysis of Pitt, the generation of the complemented strain, the characterization of Pitt as a membrane protein, the semi-quantitative Western analysis for estimation of the POR accumulation and the 2D-PAGE studies. In addition, I contribute the assay for chlorophyll synthesis and the sucrose-gradient analysis was carried out by me, as well. J. Ratke generated the *pitt*-mutant, produced the antiserum and did the Yeast Two-Hybrid analysis. Measuring of photosynthetic parameters, like O2-evolution and 77k fluorescence, was done by M. Nowaczyk. J. Nickelsen and I wrote the manuscript and it was finally revised by J. Nickelsen.

Section I:

Interaction of the periplasmic PratA factor and the PsbA (D1) protein during biogenesis of photosystem II in *Synechocystis* sp. PCC6803. (2009) by **Schottkowski M**., Gkalympoudis S., Tzekova N., Stelljes C., Schuenemann D., Ankele E., Nickelsen J. J. Biol. Chem. 284, 1813-1819

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Interaction of the Periplasmic PratA Factor and the PsbA (D1) Protein during Biogenesis of Photosystem II in *Synechocystis* sp. PCC 6803*

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The biogenesis of photosynthetic complexes is assisted by a growing number of trans-acting factors in both chloroplasts and cyanobacteria. We have previously shown that the periplasmic PratA factor from *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803) is required for adequate C-terminal processing of the PsbA (D1) subunit of photosystem II (PSII) supporting the idea that the early steps of PSII assembly occur at the plasma membrane. Here we report on the molecular analysis of the interaction between PratA and the D1 protein. Both yeast two-hybrid and glutathione *S*-transferase pulldown assays revealed that PratA binds to the soluble forms of both mature and precursor D1 C-terminal regions. In agreement with that finding, the binding region was mapped to amino acid positions 314–328 of D1 by applying a peptide-scanning approach. Approximately 10–20% of the soluble PratA factor was found to be associated with membranes in a D1-dependent manner. Sucrose density gradient centrifugations allowed the identification of a specific membrane subfraction that contains both PratA and D1 and which might represent a transfer and/or connecting region between plasma and thylakoid membrane. Imaging data obtained with enhanced cyan fluorescent protein-labeled D1 protein in wild-type and *pratA* mutant backgrounds further supported this notion.

Photosystem II (PSII)[2] mediates water oxidation and initiates electron flow during light reactions of photosynthesis in cyanobacteria and in chloroplasts of eukaryotes. Cyanobacterial PSII has been characterized in great detail at the structural level (1–4). However, much less is known about the stepwise biogenesis of this molecular machine, which contains at least 19 protein subunits as well as a complex set of cofactors (5–7). In the prokaryotic model organism *Synechocystis* PCC sp. 6803 (hereafter *Synechocystis* 6803), several proteins have recently been identified that associate transiently with PSII, and inactivation of these leads to severe defects in PSII activity. Such proteins represent good candidates for putative assembly factors.

The protein Slr1471p (OXA1/Alb3/YidC homologous), for instance, has been shown to interact directly with the D1 protein during integration of the latter into the thylakoid membrane (8). The lumenal factor YCF48 (HCF136) is required for both efficient assembly and repair of PSII (9, 10), and the 11-kDa lipoprotein Psb27 has been implicated in facilitating assembly of the manganese cluster of PSII (11, 12). Finally, the 22-kDa Psb29 protein is apparently necessary for accurate biogenesis of the inner antennae of PSII (13).

We have previously shown that the tetratricopeptide repeat protein PratA is involved in the biogenesis of PSII: loss of PratA function affects the maturation of the reaction-center protein D1 (14). In almost all photoautotrophic organisms, D1 is synthesized as a precursor with a C-terminal extension that must be removed to allow assembly of the manganese cluster at the lumenal side of PSII. In *Synechocystis* 6803, this extension consists of 16 amino acids, which are processed in a two-step fashion (15, 16). The precise contribution of the extension to photosynthetic performance is still under debate, but recent analyses of site-directed mutants have revealed that it has a photoprotective function (17, 18). Intriguingly, PratA is a periplasmic protein that was previously shown to interact directly with the soluble C-terminal portion of D1 in yeast two-hybrid studies (19, 14, 20). This finding strongly supports the idea that the early steps in photosystem biogenesis in cyanobacteria take place at the plasma membrane and not in the thylakoids (21, 22). However, recent ultrastructural analyses have failed to unambiguously solve the long-standing question of whether or not a direct connection exists between plasma and thylakoid membranes; such a link would establish a continuum between the periplasm and the thylakoid lumen (23, 24).

Here, we report on the molecular details of the D1-PratA interaction. Based on two-hybrid studies in yeast and *in vitro* assays, we mapped the binding region of PratA on the D1 C terminus. Furthermore, we show that PratA forms part of a complex that also contains the D1 protein. Intriguingly, this complex is apparently not localized to the main thylakoid fraction but is enriched in a specific membrane subcompartment.

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[2] The abbreviations used are: PSII, photosystem II; GST, glutathione *S*-transferase; BN, blue native; CN, colorless native; mD1, mature D1; eCFP, enhanced cyan fluorescent protein.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Analysis—Protein interaction studies in yeast were performed using the MATCHMAKER LexA system (Clontech, see Ref. 14). Cloning of the *PratA* gene (*slr2048*) into the yeast expression vector pJG4-5 and of a DNA fragment encoding the last 68 amino acids of the pD1 protein into pEG202 has been described previously (14). The coding sequence for the C-terminal segment of mD1, which lacks the 16-amino acid extension found in pD1, was amplified by PCR and subsequently inserted into pEG202 via primer-derived EcoRI and XhoI restriction sites. Site-directed mutagenesis of the sequence encoding the C-terminal region of D1 was carried out using the QuikChange kit (Stratagene).

GST Pulldown Assays—GST pulldown assays were performed with recombinant proteins expressed in *Escherichia coli*. After amplification by PCR, the coding region of the *PratA* gene, excluding the transit sequence (14), was cloned into the EcoRI and XhoI sites of the expression vector pGEX-4T-1. GST fusion protein was expressed in *E. coli* BL21 cells. His-tagged PratA protein was produced in *E. coli* M15 cells (Qiagen) after PCR amplification of the same region and subsequent insertion into the SalI and PstI sites of the vector pQE31. Fragments encoding either the C-terminal segment of pD1 (68 amino acids) or mD1 (52 amino acids) were cloned into the EcoRI and XhoI restriction sites in pGEX-4T-1. GST fusion proteins were bound to glutathione-Sepharose 4B (GE Healthcare) for 3 h. The Sepharose beads were then incubated with a mixture of His-tagged PratA protein and GST protein alone for 1 h with gentle rotation, washed five times with washing buffer (20 mM HEPES, pH 7.5, 500 mM NaCl, 1% Tween 20) and finally resuspended in SDS loading buffer. After 10 min at 98 °C samples were subjected to SDS-PAGE and subsequently analyzed by Western blotting.

Peptide Scanning—To map the binding site(s) for PratA in the C-terminal portion of D1, pentadecameric peptides were synthesized (jpt Technologies, Berlin), which overlapped by 12 amino acids and covered the C-terminal 68 amino acids of pD1, and aliquots (5 nmol) of each were immobilized on a filter membrane (PepSpots™ membrane, jpt Technologies). Subsequently, the membrane was incubated at 4 °C overnight with 20 μg of His-tagged-PratA protein. Bound PratA was localized using the αPratA antibody (25).

Membrane Isolation and Solubilization—For preparation of soluble and non-soluble protein fractions, equal amounts of wild-type and *TD41* cells were harvested and resuspended in 500 μl of breaking buffer (50 mM Tris/HCl, pH 7, 20 mM $MgCl_2$, 20 mM KCl). Cells were then mechanically disrupted with glass beads (0.25–0.5 mm diameter) in a mini-beadbeater (Glen Mills), using three 20-s bursts. After centrifugation for 1 min at 15,000 × *g*, the supernatant was loaded onto a 1 M sucrose cushion and centrifuged for 30 min at 94,000 × *g*. The supernatant containing all soluble proteins was collected, and the pellet with membrane-bound proteins was resuspended in breaking buffer containing 5% Triton X-100. Finally the protein fractions were subjected to SDS-PAGE and immunoblotting.

Membranes for solubilization assays (50 μg of protein) were prepared as described (26), sedimented by centrifugation and washed twice in 5 mM HEPES, pH 7.6. The samples were resuspended in 20-μl aliquots of HEPES buffer, to which 20 μl of 5 mM HEPES, pH 7.6 (control), 0.2 M Na_2CO_3, 4 M urea, or 2 M NaCl was added. After a 30-min incubation on ice and centrifugation for 15 min with 18,000 × *g* at 4 °C the supernatants were collected and the pellets were washed twice in HEPES buffer. All samples were then subjected to SDS-PAGE and analyzed by immunoblotting.

Two-dimensional PAGE—For blue native (BN)-PAGE, membranes (500 μg of protein (26)) were sedimented by centrifugation (30 min, 15,000 × *g*, 4 °C) and resuspended in 50 μl of ACA buffer (750 mM ϵ-aminocaproic acid, 50 mM Bis-Tris, pH 7.0, 0.5 mM EDTA). After addition of freshly prepared β-dodecylmaltoside (10% w/v) to a final concentration of 1.5% (w/v), membrane proteins were solubilized for 35 min on ice. After removal of insoluble material by centrifugation, 8 μl of 5% Coomassie solution (750 mM ϵ-aminocaproic acid, 5% Coomassie Brilliant Blue) was added to each supernatant, and the samples were loaded onto a 4.5–12% BN gel. Electrophoresis was performed at 50 V at 4 °C overnight, replacing the blue cathode buffer with fresh, colorless buffer a third of the way through the run, as described previously (27). For the second dimension, a single lane of the BN-PA gel was washed in solubilization buffer (66 mM Na_2CO_3, 2% SDS (w/v), 0.67% β-mercaptoethanol) for 20 min and then placed on top of a 12.5% SDS gel containing 4 M urea. Electrophoresis was carried out at 20 mA for 30 min and then at 4 mA overnight. Subsequently, gels were stained with Coomassie or electroblotted onto nitrocellulose-membranes and probed with various antibodies.

For first-dimension colorless native (CN)-PAGE, Coomassie dye was omitted from samples and cathode buffer. Soluble proteins (500 μg) were mixed with 0.001% Ponceau S and electrophoretically separated as reported before (28).

Separation of Synechocystis Membranes—Equal amounts of cells from each strain in the early exponential growth phase were harvested by centrifugation for 10 min, washed with 5 mM Tris buffer, and resuspended in buffer I (10 mM Tris, 1 mM phenylmethylsulfonyl fluoride, 600 mM sucrose, 5 mM EDTA, 0,2% lysozyme). The suspension was shaken for 2 h at 30 °C and subsequently washed twice in buffer II (20 mM Tris, 1 mM phenylmethylsulfonyl fluoride). Cells were disrupted by passing them twice through a French Press at 1200 p.s.i. DNase I (20 μl) was added, and, after incubation for 15 min at 4 °C, cell debris was sedimented at 4 °C (4500 × *g*, 10 min). The supernatant was adjusted to 50% sucrose by adding 80% sucrose in 10 mM Tris. A 10-ml sample of this solution was overlaid with 8 ml of 39% sucrose, 6 ml of 30% sucrose, and 8 ml of 10% sucrose (all in 10 mM Tris, 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 4 °C for 17 h at 135,000 × *g* the gradient was fractionated into five fractions containing 10% (I), 30% (II), or 50% (V) sucrose. The part containing 39% sucrose was divided into two separate fractions (III and IV) for obtaining a higher resolution in this region of the gradient. The samples were concentrated via ultrafiltration (Millipore) and then separated by SDS-PAGE, blotted onto nitrocellulose membrane, and finally probed with various antibodies.

Fraction number V was diluted with 5 mM Tris buffer to a sucrose concentration of 20% and then centrifuged at 4 °C on a linear (30% to 60%) sucrose gradient for 17 h at 135,000 × *g*. After fractionation, proteins were subjected to immunodetection.

Construction of Strains Expressing eCFP-D1 Fusion Proteins and Confocal Microscopy—For tagging the D1 protein with enhanced cyan fluorescent protein (eCFP), both the promoter and the coding region of the *psbA2* gene (*slr1311*) from *Synechocystis* 6803 were PCR separately amplified from wild-type genomic DNA. The eCFP coding region was amplified from plasmid p2GWC7 (29) and inserted via appropriate primer-derived restriction sites into the N terminus of the *psbA* gene. The fusion gene was then inserted into the SmaI site of the conjugation vector pVZ322 (30) giving rise to plasmid N-eCFP-D1. Wild-type and *pratA* mutant cells were each conjugated with this plasmid by following the protocol of (30). Conjugants were then suspended in 0.3% Gelrite® (w/v) (Serva) and applied to coverslips for fluorescence microscopy. Confocal images were acquired with a TCS-SP5 confocal laser scanning system equipped with an inverted microscope (Leica) and an 63× glycerol immersion objective (numerical aperture, 1.3). For the specific detection of eCFP and chlorophyll, excitation wavelengths were set to 480 nm and 670 nm, respectively.

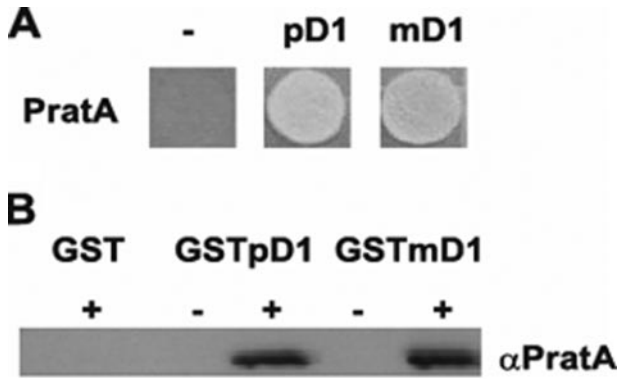

FIGURE 1. **PratA binds the mature D1 protein.** *A*, yeast two-hybrid analysis with strains that express PratA fused to the Gal4 activation domain together with the DNA-binding domain of Gal4, either alone or fused to the C-terminal region of pD1 or mD1. *B*, association of PratA with the C-terminal region of either pD1 or mD1 fused to GST. GST-containing complexes were recovered and co-eluting recombinant PratA protein was detected with an αPratA antiserum. "−/+" indicates the presence of recombinant PratA. As a negative control, GST alone was also tested for the capacity to bind PratA.

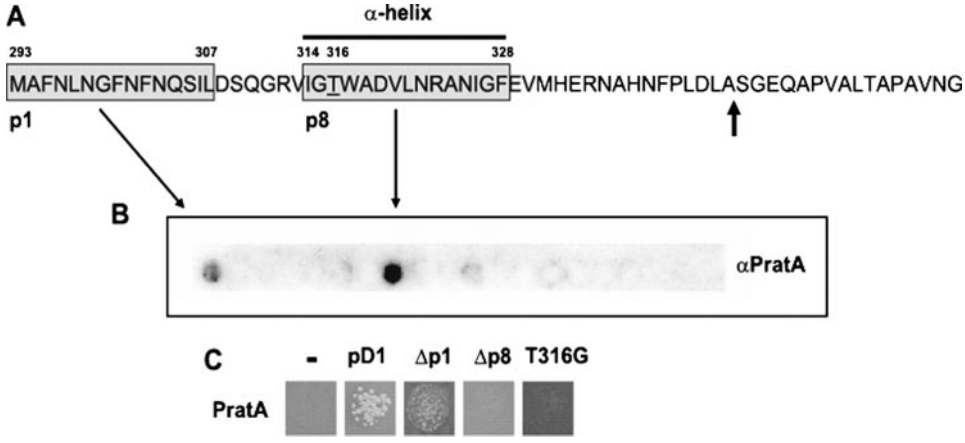

FIGURE 2. **PratA binds to a site within the C-terminal region of D1.** *A*, sequence of the C-terminal 68 residues of D1 (positions 293–360). Nineteen pentadecameric peptides (with 12-amino acid overlaps) representing the entire segment were synthesized, dotted onto a PepSpots™ membrane, and tested for the ability to bind PratA. The PratA-binding peptides 1 and 8 (*B*) are *boxed*, and the single α-helix present in the C-terminal region is indicated by the *overline*. The CtpA processing site is marked with an *arrow*. *B*, peptide blot incubated with recombinant PratA protein and subsequently with αPratA antibody to detect bound PratA. *C*, yeast two-hybrid analysis as in Fig. 1*A*, but with altered versions of the D1 C-terminal segment from which peptide 1 or 8 was deleted or amino acid T316 was mutated as indicated.

RESULTS

PratA Interacts with the Mature D1 Protein—We have previously shown that the soluble, periplasmic tetratricopeptide repeat protein PratA from *Synechocystis* 6803 is involved in the biogenesis of PSII (14). The analysis of pulse-labeled proteins had suggested that the C-terminal processing of the reaction-center protein D1 is affected by a *pratA* mutation. In agreement with this finding, a direct interaction between the soluble C-terminal 68 amino acids of the D1 precursor (pD1) and PratA was documented using a yeast two-hybrid system (14) (Fig. 1).

To test whether PratA binding is specific for the precursor form of the D1 protein, we have now analyzed a version that lacks the C-terminal extension characteristic of the precursor using the same two-hybrid approach in yeast. As shown in Fig. 1*A*, PratA also recognizes the C-terminal segment of the mature D1 (mD1), suggesting that the 16-amino acid extension present in the precursor is not required for binding of PratA. Instead, the 52 amino acids retained in mD1 appear to contain the crucial determinants that mediate the D1-PratA interaction.

Previous attempts to demonstrate this interaction using *in vitro* approaches yielded ambiguous results (14). However, optimization of the conditions used for GST pulldown assays has now allowed us to confirm the specific interaction of the D1 protein and PratA also *in vitro* (Fig. 1*B*). Sepharose-bound GST alone failed to capture recombinant His-tagged PratA protein. In contrast, matrix-bound GST fused to the C-terminal portion of either the precursor or the mature D1 interacted with recombinant PratA, and PratA could subsequently be eluted from the matrix material under denaturing conditions (Fig. 1*B*). As a negative control, *E. coli* proteins from a strain containing no recombinant PratA protein were always analyzed in parallel. No signals were obtained in these cases. Taken together, the data thus strongly suggest that PratA specifically recognizes the C-terminal sequence that is common to pD1 and mD1.

Mapping of the PratA Binding Region in the C-terminal Segment of D1—To determine the one or more regions that mediate this interaction, a peptide scan was performed based on immobilized, pentadecameric peptides with 12-amino acid overlaps and covering the entire C-terminal segment of pD1. Recombinant PratA protein was incubated with the PepSpots™ membrane bearing the whole pep-

tide set, and subsequent decoration with the αPratA antibody revealed that PratA specifically binds to peptide No. 8, corresponding to positions 314–328 of the D1 protein (Fig. 2, *A* and *B*). Peptide No. 1 (positions 293–307) was also recognized by PratA, although the signal was less pronounced. We then analyzed mutant versions of the D1 C terminus that lacked either peptide 1 or 8 in the yeast two-hybrid system. Deletion of amino acids 293–307 had only a limited impact on yeast growth, suggesting that these positions make only a minor contribution, if any, to PratA-D1 complex formation (Fig. 2*C*). In contrast, when residues 314–328 were deleted, no interaction between PratA and the D1 segment was observed (Fig. 2*C*). This indicates that the region represented by peptide 8 indeed contains essential determinants that are strictly required for recognition of PratA. It is intriguing that, based on available structural data of crystallized PSII, this D1 region forms an α-helical structure (4). Furthermore, peptides 7 and 9, each of which overlaps peptide 8 by 12 amino acids, exhibited no detectable PratA binding at all, suggesting that the entire helix and/or its left and right borders are required for recognition by PratA. To substantiate this hypothesis, we replaced the threonine at position 316 with a glycine, because alignments of D1 sequences from various organisms revealed that Thr-316 is the only highly conserved amino acid at the left end (Ile-Gly-Thr) of the helix region. Yeast two-hybrid analysis indeed revealed that the T316G mutation is sufficient to prevent PratA binding completely (Fig. 2*C*).

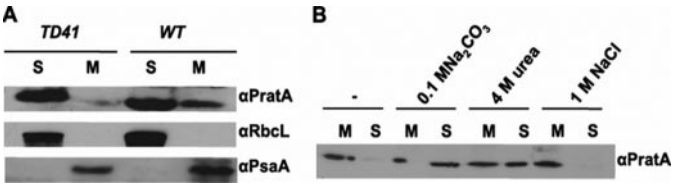

FIGURE 3. **PratA is membrane associated.** *A*, membrane (*M*) and soluble (*S*) protein fractions were prepared from wild-type *Synechocystis* 6803 (*WT*) and the *psbA* deletion strain *TD41*, and separated by ultracentrifugation, subjected to Western blotting, and incubated with the antibodies indicated on the *right margin*. *B*, samples of the membrane fraction from the wild type were treated with the indicated chemicals, and separated into pellet (*M*) and supernatant (*S*) fractions by centrifugation. These fractions were then probed with αPratA antibodies.

PratA Forms Part of Two Different Protein Complexes—Because an association with the D1 protein should result in a membrane localization of the otherwise soluble PratA protein, we measured the amounts of PratA present in soluble and membrane fractions of broken *Synechocystis* 6803 cells (Fig. 3*A*). In the wild-type, ~10–20% of PratA was found to cosediment with the membrane fraction in three independent experiments. In contrast, in the triple *psbA* deletion strain *TD41*, which fails to accumulate any D1 protein (31), only minute amounts of PratA were found to be associated with membranes, supporting the idea of a PratA-D1 interaction (Fig. 3*A*). The nature of the membrane association of PratA was examined further by testing the effects of various potential solubilizing agents (Fig. 3*B*). Treatment of membranes with 1 M NaCl did not release PratA from the membrane, suggesting that non-ionic interactions play a crucial role for its localization. This was confirmed by the finding that 0.1 M Na_2CO_3 or 4 M urea was capable of solubilizing substantial amounts of PratA protein (Fig. 3*B*).

To analyze the membrane-associated and soluble forms of PratA in more detail, two-dimensional gel electrophoresis of protein subfractions was performed. When the membrane fraction was analyzed by two-dimensional BN-SDS-PAGE, PratA was detected together with unassembled D1 protein in the 70-kDa range (Fig. 4*A*). In addition, smaller amounts of PratA material formed a faint smear toward higher molecular mass regions (>100 kDa, Fig. 4*A*). However, various PSII core complexes, such as RCa, RC47, and RCC (32), did not contain any detectable PratA protein. These data are consistent with the idea that a membrane-associated D1-PratA complex forms very early during the biogenesis of PSII. When the *TD41* strain was similarly analyzed, no PratA protein was detected in the membrane fraction, again indicating that the membrane association of PratA is solely mediated via the D1 protein (Fig. 4*A*, compare also Fig. 3*B*). Two-dimensional CN-SDS-PAGE of soluble proteins from the two strains revealed a different picture (Fig. 4*B*). In wild-type cells, the soluble PratA form is found in a complex of ~200 kDa. Subsequent analysis of the *psbA* deletion strain *TD41* revealed

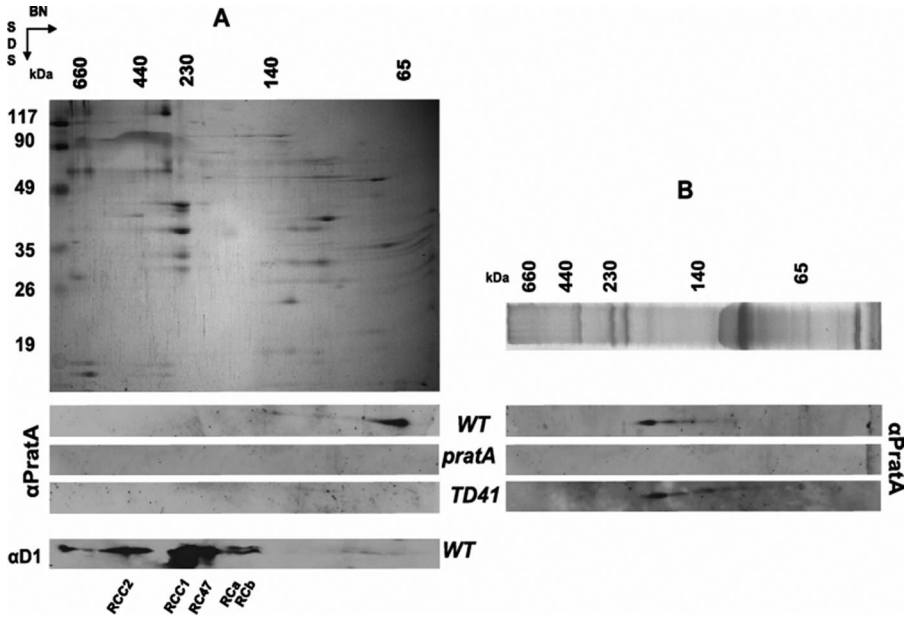

FIGURE 4. **PratA-containing complexes.** *A*, membrane fractions obtained from wild-type, *pratA*, and *TD41* strains were solubilized with 1.2% β-dodecylmaltoside, separated by two-dimensional-BN-SDS-PAGE, and immunoblotted. PratA and D1 were then localized using the respective antibodies. A representative two-dimensional gel loaded with a wild-type extract and stained with Coomassie is shown at the *top*. *B*, similar analysis of soluble proteins, which were separated by two-dimensional CN-SDS-PAGE. The Coomassie-stained gel at the *top* shows the pattern obtained after separation of a wild-type extract by CN-PAGE.

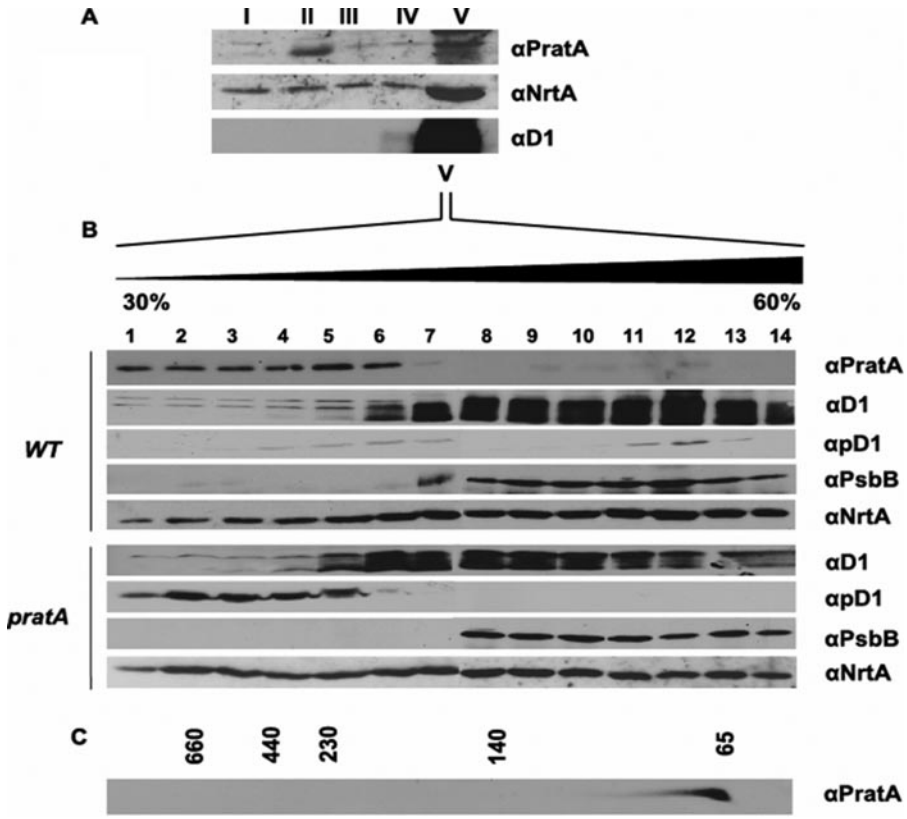

FIGURE 5. **Cellular sublocalization of PratA.** *A*, total cellular proteins were centrifuged through a sucrose step gradient, fractionated into five portions containing 10% (*I*), 30% (*II*), 39% (*III* and *IV*), and 50% (*V*) sucrose and subjected to Western analysis with the indicated antibodies (see "Experimental Procedures"). *B*, the material from *fraction V* in *A* was then centrifuged on a second linear sucrose gradient (30–60%), and fractions were analyzed as in *A* using the indicated antibodies. *C*, the material from *fraction 1* at the *top* of the second gradient was analyzed by two-dimensional BN-SDS-PAGE as in Fig. 4*A*.

that neither the size nor the amount of this complex is altered in the absence of the D1 protein (Fig. 4*B*). This is in striking contrast to the behavior of the smaller, membrane-associated complex. Thus, two PratA complexes that differ in size and localization exist in *Synechocystis* cells. Whether these complexes are functionally related remains to be elucidated.

Repeated attempts to immunoprecipitate the PratA-D1 complex were initiated to confirm its presence *in vivo*. However, presumably due to the low abundance of the complex, evidence for the presence of neither PratA nor D1 in various precipitates remained equivocal (data not shown).

The PratA-D1 Complex Is Localized to a Membrane Subcompartment—All the data presented above strongly suggest that a PratA-D1 complex is formed *in vitro* and *in vivo*. But is this complex associated with the plasma membrane or the thylakoid membrane system in *Synechocystis* 6803? To answer this question, we employed sucrose density gradient centrifugation to separate the two membrane types. Initial centrifugation through a step gradient allowed the partial purification of the plasma membrane, as judged by the immunodetection of the plasma membrane marker NrtA (Fig. 5*A*). However, NrtA was detectable in all fractions, including that containing the thylakoid membranes (Fig. 5*A*, *fraction V*). This indicates that the latter one is still significantly contaminated with plasma membrane material. PratA was mainly found in fraction II and in the thylakoid fraction V, whereas D1 was only detectable in fraction V, even after overexposure of films. We did not further analyze the PratA-containing fraction II, because the main focus of the work was on the characterization of the native D1-PratA complex, which must be localized in the crude thylakoid fraction V of the gradient. To further resolve the membrane mixture present in this fraction, it was subjected to a second centrifugation through a linear 30% to 60% sucrose gradient (Fig. 5*B*). This resulted in a clear separation of PratA (Fig. 5*B*, *fractions 1–6*) from the chlorophyll-containing thylakoid membrane fractions, which contained most of the mature mD1 protein (Fig. 5*B*, *fractions 7–14*). In contrast to mD1, the precursor pD1 protein exhibited a different distribution on the linear sucrose gradient. Roughly similar amounts of pD1 were detected in the PratA-containing *fractions 3–7* and the thylakoid *fractions 11–13*. The inner antenna protein PsbB was identified only in thylakoid *fractions 7–14*. However, due to the lower sensitivity of the antibody used, we cannot exclude the possibility that minor amounts of PsbB are also present in *fractions 1–6*. Hence, the data strongly suggest that PratA is not associated with thylakoid membranes *per se*, but rather with a distinct membrane subfraction, which co-migrates with thylakoids during centrifugation through the step gradient (Fig. 5*A*). NrtA again showed a distribution throughout the whole gradient (Fig. 5*B*). It remains unclear whether this really reflects the sedimentation of the plasma membrane or might represent a special feature of NrtA during sucrose gradient centrifugation. However, no absolute co-fractionation of PratA and neither NrtA nor PsbB was observed in these experiments strongly suggesting that PratA marks a specific membrane subcompartment of *Synechocystis* 6803 cells. To answer the question whether the PratA form in *fractions 1–6* of the linear gradient indeed represents the membrane-associated one two-dimensional-BN-SDS-PAGE was carried out on material from fraction 1 (Fig. 5*B*). The results demonstrated that indeed the smaller membrane-associated PratA complex of 70 kDa was present in this fraction (Fig. 5*C*).

When membranes from the *pratA* mutant were fractionated in the same way, a moderate but significant shift of mD1 material toward the top of the gradient was observed (Fig. 5*B*, compare *lanes 4–7* from *WT* and *pratA*). In contrast, both NrtA and PsbB exhibited the same sedimentation behaviors irrespective of its source. The latter finding indicates that only a PSII subfraction that does not contain appreciable amounts of PsbB is affected by the *pratA* mutation. The strongest impact due to the absence of PratA was detectable for both the amount and the distribution of

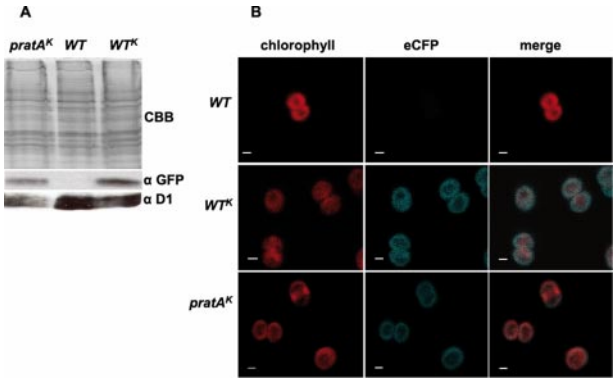

FIGURE 6. **Localization of eCFP-tagged D1 protein in *Synechocystis* 6803.** *A*, Western analysis of eCFP-D1 and D1 accumulation in indicated strains by using respective antibodies. The Coomassie-stained gel is shown as loading control. *B*, wild-type (*WT*) and *pratA* cells conjugated with plasmid N-eCFP-D1 (WT^K and $pratA^K$) and suspended in 0.3% Gelrite were layered on slides, and fluorescence images were taken at excitation wavelengths of 480 nm (*eCFP*) and 670 nm (*chlorophyll*). Chlorophyll autofluorescence is shown in *red* and eCFP fluorescence in *blue*. The *merged images* show the distribution of both signals. *White scale bars* represent 1 μm.

D1 precursor protein. The specific membrane fractions (*1–6*) showed a strong accumulation of pD1, whereas pD1 was missing in the chlorophyll-containing fractions (7–14). Taken together, these data suggest that, in the *pratA* mutant, an early intermediate in PSII assembly is at least partially retained in a membrane subfraction with which the wild-type PratA protein would normally associate. Furthermore, the data underline the formerly described pD1-processing phenotype of the *pratA* mutant (14).

PratA-mediated D1 Localization—The data presented in the previous section suggest that the PratA-D1 complex is localized to a special membrane subcompartment, which forms an intermediate between the plasma membrane and the thylakoid membrane systems. This subcompartment could therefore represent a structural connection between the plasma and thylakoid membranes (23). This would in turn imply that biogenesis of the PSII core starts in the plasma membrane, as has previously been postulated (21). If this is true, one may speculate that PratA is involved in some way in the transfer of the D1 protein from the plasma membrane to the thylakoid membrane. To test this possibility, we used conjugation to construct strains that express an N-terminally eCFP-tagged D1 protein, in addition to the endogenous D1 protein, either in a wild-type or *pratA* genetic background. As depicted in Fig. 6*A* the D1-eCFP fusion protein is expressed abundantly in the conjugated wild-type (WT^K) and at a reduced rate in the *pratA* mutant ($pratA^K$) background. In non-conjugated cells the specific eCFP signal was undetectable. D1 protein accumulation was slightly compromised in conjugated lines suggesting that the tagged D1 version competes to some extent with endogenous D1 (Fig. 6*A*).

The eCFP-tagged D1 protein could be visualized by fluorescence in the wild-type and *pratA* conjugant lines. As expected from the Western analysis a weaker signal was obtained in $pratA^K$. To assess whether the eCFP-D1 fusion protein reaches the thylakoid membrane, we monitored chlorophyll autofluorescence at an emission wavelength of 670 nm. In the wild-type background, the eCFP emission coincided perfectly with the chlorophyll autofluorescence, thus confirming colocalization of the eCFP-D1 fusion protein and the thylakoid membranes (Fig. 6*B*). This indicates that the eCFP tag does not interfere with insertion of the fusion protein into PSII precomplexes. In the *pratA* mutant, in contrast, eCFP emission did not colocalize with thylakoids to the same extent. Instead, it was mainly localized to the periphery of cells and did not show complete coincidence with the chlorophyll fluorescence (Fig. 6*B*). Although the autofluorescence is visible throughout the cell, with the exception of the cytosolic compartment, eCFP in the *pratA* mutant was restricted to a narrow sphere at the periphery of the cells, which may possibly colocalize with the plasma membrane (Fig. 6*B*). This may indicate accumulation of D1 fusion protein in the plasma membrane or the membrane subcompartment identified by our cell fractionation studies. As expected, eCFP emission could not be detected in non-conjugated wild-type cells (negative control, Fig. 6*B*). Taken together, these data provide an additional piece of evidence that the PratA factor might be involved in the transfer of newly synthesized D1 protein from the plasma membrane to the thylakoid membrane system.

DISCUSSION

Interaction of PratA and D1—We previously suggested that the periplasmic tetratricopeptide repeat protein PratA is involved in the C-terminal processing of the D1 protein. However, here we demonstrate, using the yeast two-hybrid system as well as GST pulldown assays, that the C-terminal extension of the D1 protein is dispensable for PratA recognition. This is consistent with our mapping of the PratA binding site to the α-helix between positions 314–328 (peptide 8, see Fig. 2*A*) of the mature D1, and with the fact that alteration of the conserved threonine at position 316 of D1 into a glycine completely prevents binding of PratA. Because membrane association of PratA is mediated by non-ionic interactions, it remains to be seen what the precise determinants for D1 recognition by PratA are. Nevertheless, one can conclude from the peptide scanning data that, in addition to Thr-316, one or more amino acids from the opposite end of the helix (positions 326–328) are also required for efficient PratA binding.

In light of these new findings, the D1-processing phenotype of the *pratA* mutant might be interpretable simply as a secondary effect of a perturbation in the PSII assembly process. For instance, C-terminal D1 maturation has been shown also to be affected in mutants lacking distinct subunits of PSII, *i.e.* PsbH, PsbB, or PsbEFLJ (33). Furthermore, yeast two-hybrid analyses have provided no evidence for a direct interaction of PratA with the C-terminal D1 protease CtpA (data not shown). Thus, the precise molecular function of PratA remains to be dissected.

PratA Forms Part of at Least Two Protein Complexes—Two-dimensional BN-SDS-PAGE revealed that the membrane-associated and soluble forms of PratA are found in complexes that differ significantly in size. The soluble 200-kDa complex is not affected by the absence of the D1 protein. In contrast, formation of the 70-kDa membrane-associated complex is dependent on D1, and its relatively small size suggests that it represents a very early intermediate in PSII assembly that contains at least the D1 protein in addition to PratA. The *Synechocystis* homolog of HCF136/YCF48 has also recently been shown to interact with the D1 precursor protein and the C-terminal processing intermediate iD1, but not with the mature D1 protein, during

the early phase of assembly (10). However, no direct interaction between HCF136 and PratA could be detected in the yeast two-hybrid system (data not shown), suggesting that the two factors may interact only transiently or not at all.

Localization of the PratA-D1 Complex—Based on cell fractionation experiments it had earlier been proposed that the first steps in *de novo* assembly of PSII and PS I take place at the cytoplasmic membrane and not on the thylakoids of *Synechocystis* 6803 (21, 22). For PSII, the D1 and D2 proteins, as well as cytochrome b_{559} subunits, were shown to be present in plasma membrane fractions, whereas the PsbB and PsbC subunits were exclusively detected in thylakoids (21, 34). Furthermore, the PSII subunits in the cytoplasmic membrane accumulated in inside-out rather than right-side-out vesicles, indicating a heterogeneous organization of the plasma membrane (35). This suggests that discrete regions of the plasma membrane harbor sites at which assembly of PSII is initiated (35). Interestingly, in chloroplasts of the green alga *Chlamydomonas reinhardtii*, distinct membrane subfractions around the pyrenoid have been identified as being sites of *de novo* assembly of PSII, whereas D1 repair synthesis was found to occur throughout the entire thylakoid membrane system (36).

Here, using a two-step cell fractionation procedure, we identified a membrane subfraction in which PratA specifically accumulates together with substantial amounts of the precursor pD1 protein. PsbB was not detected in appreciable amounts in these fractions, which is consistent with the abovementioned data suggesting that PsbB is absent from plasma membranes (21, 34). Therefore, it is obviously tempting to speculate that this subfraction represents membrane regions at which initial steps in PSII assembly occur. This idea is further supported by the finding that pD1 accumulation significantly increases in these fractions in the absence of PratA. Extensive additional work will be required to characterize this cellular compartment in greater detail. It will be important to test how its proteomic composition is organized and whether other factors involved in thylakoid membrane biogenesis accumulate there.

Localization of an eCFP-tagged D1 protein at the cell periphery in a *pratA*$^-$ genetic background further supports the idea that PratA is involved in processes related to the preassembly of PSII core complexes at specific sites within the plasma membrane and/or in the transfer of complexes from these sites to the thylakoid membrane. However, the localization effect was not very pronounced, probably due to the fact that the *pratA* mutation leads not to a fully fledged PSII$^-$ phenotype but only to a reduction of PSII levels to 25% of the wild-type value. Hence, *pratA*$^-$ cells can still grow photoautotrophically, and all steps in photosystem biogenesis, including membrane transfer processes, can in principle occur, albeit at reduced rates. Future work will try to establish an inducible imaging system that will allow us to monitor D1-eCFP fluorescence with high temporal resolution.

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REFERENCES

1. Zouni, A., Witt, H. T., Kern, J., Fromme, P., Krauss, N., Saenger, W., and Orth, P. (2001) *Nature* **409,** 739–743
2. Kamiya, N., and Shen, J. R. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 98–103
3. Ferreira, K. N., Iverson, T. M., Maghlaoui, K., Barber, J., and Iwata, S. (2004) *Science* **303,** 1831–1838
4. Loll, B., Kern, J., Saenger, W., Zouni, A., and Biesiadka, J. (2005) *Nature* **438,** 1040–1044
5. Müller, B., and Eichacker, L. A. (1999) *Plant Cell* **11,** 2365–2377
6. Dobakova, M., Tichy, M., and Komenda, J. (2007) *Plant Physiol.* **145,** 1681–1691
7. Nickelsen, J., Nowaczyk, M., and Klinkert, B. (2007) *Prog Bot.* **68,** 57–79
8. Ossenbühl, F., Inaba-Sulpice, M., Meurer, J., Soll, J., and Eichacker, L. A. (2006) *Plant Cell* **18,** 2236–2246
9. Plücken, H., Müller, B., Grohmann, D., Westhoff, P., and Eichacker, L. A. (2002) *FEBS Lett.* **532,** 85–90
10. Komenda, J., Nickelsen, J., Tichy, M., Prasil, O., Eichacker, L. A., and Nixon, P. (2008) *J. Biol. Chem.* **283,** 22390–22399
11. Nowaczyk, M. M., Hebeler, R., Sclodder, E., Meyer, H. E., Warscheid, B., and Rögner, M. (2006) *Plant Cell* **18,** 3121–3131
12. Roose, J. L., and Pakrasi, H. B. (2008) *J. Biol. Chem.* **283,** 4044–4050
13. Keren, N., Ohkawa, H., Welsh, E. A., Liberton, M., and Pakrasi, H. B. (2005) *Plant Cell* **17,** 2768–2781
14. Klinkert, B., Ossenbühl, F., Sikorski, M., Berry, S., Eichacker, L. A., and Nickelsen, J. (2004) *J. Biol. Chem.* **279,** 44639–44694
15. Inagaki, N., Yamamoto, Y., and Satoh, K. (2001) *FEBS Lett.* **509,** 197–201
16. Komenda, J., Kuvikova, S., Granvogel, B., Eichacker, L. A., Diner, B. A., and Nixon, P. J. (2007) *Biochim. Biophys. Acta* **1767,** 829–837
17. Ivleva, N. B., Shestakov, S. V., and Pakrasi, H. B. (2000) *Plant Physiol.* **124,** 1403–1412
18. Kuvikova, S., Tichy, M., and Komenda, J. (2005) *Photochem. Photobiol. Sci.* **4,** 1044–1048
19. Fulda, S., Huang, F., Nilsson, F., Hagemann, M., and Norling, B. (2000) *Eur. J. Biochem.* **267,** 5900–5907
20. Pasch, J. C., Nickelsen, J., and Schünemann, D. (2005) *Appl. Microbiol. Biotechnol.* **69,** 440–447
21. Zak, E., Norling, B., Maitra, R., Huang, F., Andersson, B., and Pakrasi, H. B. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 13443–13448
22. Keren, N., Liberton, M., and Pakrasi, H. B. (2005) *J. Biol. Chem.* **280,** 6548–6553
23. Van de Meene, A. M. L., Hohmann-Marriott, M. F., Vermaas, W. F. J., and Roberson, R. W. (2006) *Arch Microbiol.* **184,** 259–270
24. Liberton, M., Ber, R. H. Heuser, J., Roth, R., and Pakrasi, H. B. (2006) *Protoplasma* **227,** 129–138
25. Reineke, U., Kramer, A., and Schneider-Mergener, J. (1999) *Curr. Top Microbiol. Immunol.* **243,** 23–26
26. Dühring, U., Irrgang, K. D., Lunser, K., Kehr, J., and Wilde, A. (2006) *Biochim. Biophys. Acta* **1757,** 3–11
27. Schägger, H., and von Jagow, G. (1991) *Anal. Biochem.* **199,** 223–231
28. Schägger, H., Cramer, W. A., and von Jagow, G. (1994) *Anal. Biochem.* **217,** 220–230
29. Karimi, M., de Meyer, B., and Hilson, P. (2005) *Trends Plant Sci.* **10,** 103–105
30. Zinchenko, V. V., Piven, I. P., Melnik, V. A., and Shestakov, S. V. (1999) *Russ. J. Genet* **35,** 228–232
31. Nixon, P. J., Trost, J. T., and Diner, B. A. (1992) *Biochemistry* **31,** 10589–10871
32. Komenda, J., Reisinger, V., Müller, B. C., Dobakova, M., Granvogl, B., and Eichacker, L. A. (2004) *J. Biol. Chem.* **279,** 48620–48629
33. Komenda, J., Tichy, M., and Eichacker, L. (2005) *Plant Cell Physiol.* **46,** 1477–1483
34. Bergantino, E., Brunetta, A., Touloupakis, E., Segalla, A., Szabo, I., and Giacometti, G. M. (2003) *J. Biol. Chem.* **278,** 41820–41829
35. Srivastava, R., Battchikova, N., Norling, B., and Aro, E.-M. (2006) *Arch Microbiol.* **185,** 238–243
36. Uniacke, J., and Zerges, B. (2007) *Plant Cell* **19,** 3640–3654

Section II:

Pitt, a novel tetratricopeptide repeat protein involved in light dependent chlorophyll biosynthesis and thylakoid membrane biogenesis in *Synechocystis* sp. PCC 6803. (2009) by **Schottkowski M.**, Ratke J., Oster U., Nowaczyk M., Nickelsen J. (2009) Mol. Plant 2, 1289-1297

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Molecular Plant • Pages 1–9, 2009 RESEARCH ARTICLE

Pitt, a Novel Tetratricopeptide Repeat Protein Involved in Light-Dependent Chlorophyll Biosynthesis and Thylakoid Membrane Biogenesis in *Synechocystis* sp. PCC 6803

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ABSTRACT Biogenesis of photosynthetic pigment/protein complexes is a highly regulated process that requires various assisting factors. Here, we report on the molecular analysis of the *Pitt* gene (*slr1644*) from the cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803) that encodes a membrane-bound tetratricopeptide repeat (TPR) protein of formerly unknown function. Targeted inactivation of *Pitt* affected photosynthetic performance and light-dependent chlorophyll synthesis. Yeast two-hybrid analyses and native PAGE strongly suggest a complex formation between Pitt and the light-dependent protochlorophyllide oxidoreductase (POR). Consistently, POR levels are approximately threefold reduced in the *pitt* insertion mutant. The membrane sublocalization of Pitt was found to be dependent on the presence of the periplasmic photosystem II (PSII) biogenesis factor PratA, supporting the idea that Pitt is involved in the early steps of photosynthetic pigment/protein complex formation.

Key words: *Synechocystis*; TPR protein; POR; thylakoid membrane; chlorophyll.

INTRODUCTION

Light-driven photosynthetic electron flow in cyanobacteria, eukaryotic algae, and plants is mediated by the major complexes of the thylakoid membrane—photosystems I and II (PS I and PS II) and the cytochrome b_6f complex. While the overall structure of this machinery is now well understood at high resolution (Nelson and Ben-Shem, 2004), relatively little is known about the biogenesis of its constituent multisubunit complexes and their co-factors, namely chlorophylls, carotenoids, and iron–sulfur clusters.

Recently, one group of proteins containing so-called TPR (tetratricopeptide repeat) domains has attracted much attention in the context of the biogenesis of the thylakoid membrane system. Typically, a TPR domain consists of multiple copies (3–16) of a degenerate motif that comprises 34 amino acids forming two amphipathic α-helices. The crystal structure of TPR domains shows that these form right-handed superhelices that can serve as a platform to facilitate protein–protein interactions (for review, see D'Andrea and Regan, 2003). Proteins containing TPR domains are found in all kingdoms of life, and have been shown to participate in a variety of different functions, ranging from cell division and RNA metabolism to protein transport and neurogenesis. Several TPR proteins involved in thylakoid membrane biogenesis in chloroplasts have been identified, which act by controlling either posttranscriptional steps of chloroplast gene expression (Boudreau et al., 2000; Vaistij et al., 2000; Sane et al., 2005) or the assembly/stability of photosystems I and II (Peng et al., 2006; Park et al., 2007; Boudreau et al., 1997; Ruf et al., 1997; Stöckel et al., 2006). Also in the cyanobacterium *Synechocystis* 6803, the TPR protein Ycf37 was shown to be required for correct formation of PS I (Wilde et al., 2001; Dühring et al., 2007). Moreover, PSII assembly depends on the so-called PratA TPR-factor, which has been postulated to mark a subcompartment connecting plasma and thylakoid membranes in which biogenesis of photosynthetic complexes takes place (Klinkert et al., 2004; Schottkowski et al., 2009).

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In addition, negative regulation of light-dependent chlorophyll synthesis has been shown to be dependent on the TPR protein Flu in *Arabidopsis thaliana* (Meskauskiene et al., 2001). Via its TPR domains, Flu directly interacts with, and thereby inhibits, glutamyl tRNA reductase, the key enzyme in the early stages of aminolevulinic acid (ALA) synthesis and, thus, the entry point for tetrapyrrole synthesis (Meskauskiene and Apel, 2002). In *flu* mutants, the photosensitizer protochlorophyllide (Pchlde) accumulates in the dark, and rapid bleaching ensues upon transfer of such plants into the light, due to the generation of reactive oxygen (for review, see Kim et al., 2008). These mutants grow normally in continuous light because Pchlde is rapidly converted into chlorophyllide (Chlde) by the light-dependent protochlorophyllide oxidoreductase (POR) and, thus, cannot accumulate.

The membrane-associated POR enzyme belongs to the family of single-subunit short-chain alcohol dehydrogenases that usually form oligomers, and it has been studied extensively with regard to its catalytic activity (for an overview, see Heyes and Hunter, 2005). The enzyme catalyzes the *trans* addition of hydrogen from NADPH across the C17–C18 double bond in the D ring of Pchlde. This reaction requires light, which causes the active site of the enzyme to adopt a more favorable conformation (Sytina et al., 2008). Higher plants contain at least three different POR genes. The genomes of algae and cyanobacteria each harbor only a single POR gene, but encode an additional, structurally unrelated Pchlde reductase (LiPOR) consisting of three subunits, which mediates the light-independent conversion of Pchlde into Chlde (Gomez Maqueo Chew and Bryant, 2007).

In the course of a systematic evaluation of TPR proteins in cyanobacteria, we analyzed the open reading frame (ORF) *slr1644* from *Synechocystis* 6803, the product of which includes five TPR units. Here, we show that this protein, named Pitt, is required for efficient photosynthesis, and affects chlorophyll synthesis both at the level of POR accumulation and thylakoid membrane formation.

RESULTS

Sequence Analysis, Generation, and Characterization of a *pitt* Mutant

ORF *slr1644* from *Synechocystis* 6803 encodes a protein of 290 amino acids that contains five consecutive TPR units. Furthermore, the segment between positions 16 and 38 is predicted to form a transmembrane helix, suggesting that the product of ORF *slr1644* is a membrane protein (Figure 1A). Based on the data presented below, the protein was named Pitt (POR-interacting TPR protein). Protein BLAST searches revealed Pitt-related TPR proteins containing an N-terminal transmembrane domain amongst several photoautotrophs. However, in the genomes of non-photosynthetic organisms like *E.coli*, Yeast, or mice, no Pitt-like factors were identified.

To analyze the function of Pitt, we cloned and disrupted ORF *slr1644* by inserting a kanamycin resistance cassette into the unique HindIII site located 388 bp downstream of the AUG start codon (Figure 1B). After transformation of wild-type cells with this construct, complete segregation of the mutated gene was confirmed by PCR analysis (Figure 1C). The complete absence of the Pitt protein in the mutant was verified by Western analysis using an α-Pitt antibody that had been raised in rabbit (Figure 2A). Fractionation of total cellular proteins from the wild-type into soluble and insoluble phases revealed that Pitt is indeed associated with membranes (Figure 2B). The nature of the membrane association was examined further by testing the effects of various potential solubilizing agents. Treatment of membranes with either 0.1 M Na_2CO_3, 4 M urea, or 1 M NaCl failed to release Pitt from the membrane whereas it was solubilized in the presence of the non-ionic detergent Triton X-100. This suggests that Pitt is indeed an integral membrane protein (Figure 2C).

The *pitt* mutant grew at only about 60% of the wild-type rate under photoautotrophic conditions, but at a similar rate to wild-type when cultured in the presence of 5 mM glucose (Table 1). Thus, the loss of Pitt appeared to significantly affect photosynthetic performance, and, indeed, light-dependent oxygen evolution in the mutant was found to be reduced to one-third of the wild-type level (Table 1). When fluorescence emission spectra were measured at low temperature (77 K), a moderate decrease in chlorophyll emission from PS I at 725 nm was observed, suggesting that the ratio of PSII to PSI is altered in the mutant relative to the wild-type (Figure 3A). However, levels of various PSII and PSI subunits were not significantly affected, as indicated by semiquantitative Western analyses (Figure 3B). Rough estimations after densitometrical scanning of observed signals revealed a reduction of PSI levels standardized to the RbcL signal of only 10% in *pitt* as compared to the wild-type. Therefore, the *pitt* mutation apparently has only a minor effect on the PSI/PSII stoichiometry, suggesting that inactive PSII or PSI complexes accumulate in *pitt*.

Pitt Interacts with POR

Previous systematic two-hybrid studies in yeast had identified Pitt as a putative interaction partner of the light-dependent POR (Sato et al., 2007). To verify this interaction, the *Pitt* and *POR* coding regions were independently analyzed using a yeast two-hybrid approach based on the LexA system (Klinkert et al., 2004). As shown in Figure 4, the interaction between the soluble C-terminal part of Pitt containing the TPR domain and POR in yeast could be confirmed. When the same region of the *Pitt* gene was inserted into both bait and prey vectors, no interaction-dependent growth of cells was detected (Figure 4). This demonstrates that the Pitt TPR domain does not interact with itself, at least in the yeast system. Obviously, more detailed biochemical investigations are required to answer the question of the Pitt oligomerization status *in vivo*.

Further characterization of the Pitt/POR complex was achieved by means of 2-D BN/SDS–PAGE (Figure 5). The two proteins co-migrate, at least partly, under native conditions,

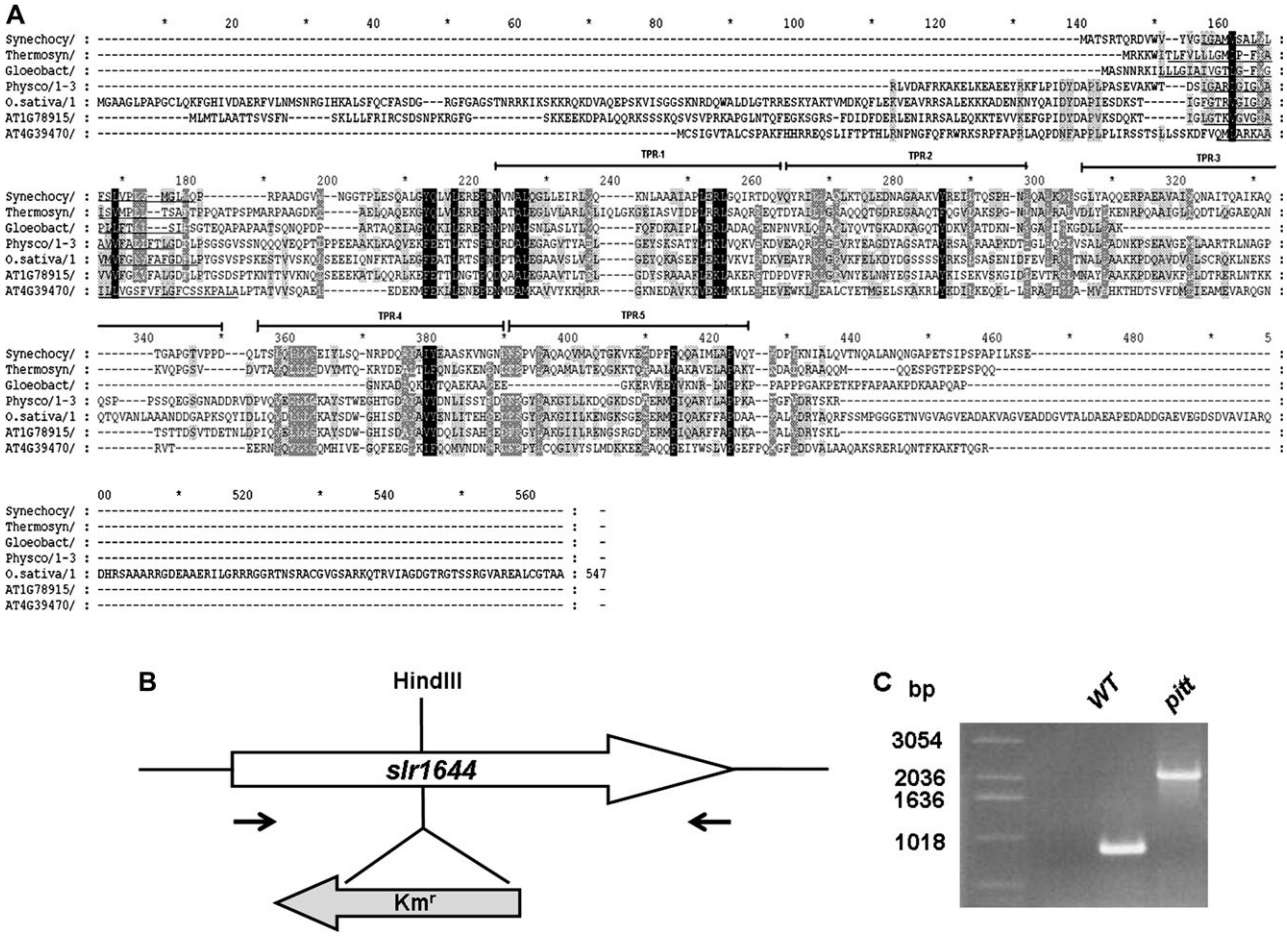

Figure 1. Structure and Inactivation of the *Pitt* Gene.

(A) The amino acid sequence of the *Synechocystis* sp. Pitt (GI:16331053) was aligned with those of related proteins from *Thermosynechococcus elongatus* (GI:22298738), *Gloeobacter violaceus* (GI:37523831), *Physcomitrella patens* (GI: 168029761), *Oryza sativa* (GI: 222636850), and *Arabidopsis thaliana* (AT4G39470 and AT1G78915). Black and gray boxes indicate conserved and related amino acids, respectively. Transmembrane domains are underlined and the five TPR domains in Pitt are each marked by converging arrows.

(B) Strategy for constructing donor plasmids with inactivated *Pitt*. Directions of transcriptions are indicated by open arrows. The solid arrows represent the primers used for PCR-based segregation analysis of *WT* and *pitt* DNA **(C)**.

suggesting that they form one or more complexes in the 60–140-kDa size range. While most of the POR enzyme is associated with the material of higher molecular weights, more than 50% of Pitt appears as free protein in the 35-kDa range (Figure 5). Strikingly, when the *pitt* mutant was similarly analyzed, a reduction in the POR signal was apparent. Furthermore, most of the residual POR enzyme in *pitt* cells was found in smaller complexes in the range of 40–100 kDa (Figure 5). To further estimate the reduction of POR in *pitt*, semiquantitative Western analyses with different amounts of total cellular proteins were performed (Figure 6). Densitometrical evaluation of signal intensities from three independent experiments revealed a reduction in the POR level in *pitt* by 70 ± 2% as compared to the wild-type. Taken together, these data suggest that Pitt is required for the stable accumulation of POR and, in addition, is involved in the organization of high-molecular-weight POR complexes.

Light-Dependent Chlorophyll Synthesis Is Affected in the *pitt* Mutant

The drastic effect on POR accumulation and organization observed in *pitt* cells raised the question of whether chlorophyll synthesis is affected in the mutant. Since *Synechocystis* 6803 harbors a second, light-independent and POR-unrelated system for chlorophyll synthesis, cells were grown under LAHG (light-activated heterotrophic growth) conditions and, upon transfer to light, chlorophyll accumulation was followed by absorption measurements as previously described (He et al., 1998). The data shown in Figure 7 indicate that light-dependent chlorophyll synthesis is indeed significantly reduced in *pitt* cells.

In order to confirm that these effects were directly due to the loss of Pitt, the wild-type *Pitt* gene (including its promoter), on an autonomously replicating plasmid, was reintroduced into the mutant by conjugation (Zinchenko et al., 1999). In the

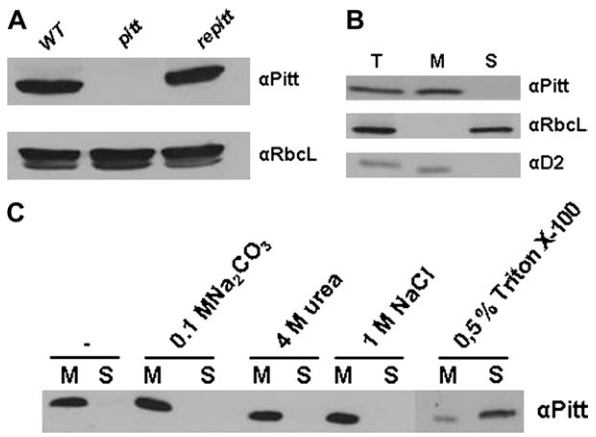

Figure 2. Pitt is a Membrane Protein.

(A) Western analysis of wild-type (WT), mutant (*pitt*) and complemented mutant (*repitt*) cells using an αPitt antiserum. As a loading control, the blot was probed with an αRbcL antibody.

(B) Total (T), membrane (M), and soluble (S) protein fractions (30 μg protein) of wild-type (WT) cells were immunodecorated with the indicated antibodies.

(C) Samples of the membrane fraction from the wild-type (WT) were treated with the indicated compounds, and separated into membrane (M) and soluble (S) fractions by centrifugation.

Table 1. Different Photosynthetical Parameters of WT and *pitt*.

Strain	Rel. growth +[a]	Rel. growth –[b]	PSI/PS II[c]	Chlorophyll[d]	O2 evolution[e]
WT	1	1	12.6 ± 1.2	0.7 ± 0.06	100%
pitt	1.3 ± 0.1	1.8 ± 0.1	9.5 ± 0.8	0.7 ± 0.03	30 ± 9%

a Relative increase in doubling time in the presence of glucose (rel. growth = 1 corresponds to 14.2 ± 1.1 h).
b Relative increase in doubling time in the absence of glucose (rel. growth = 1 corresponds to 26.0 ± 0.4 h).
c Ratio (peak areas) of 77 K chlorophyll fluorescence at 725 nm (PS I) and at 695 nm (PS II); see also Figure 3A.
d Chlorophyll content in mg ml^{-1} culture at OD750 = 1.
e Light-dependent oxygen production (100% corresponds to 170.6 ± 9.0 μmol O_2 per mg chl and h in the presence of glucose).
All measurements include three independent repetitions.

resulting strain (named *repitt*), Pitt accumulation (Figure 2A) and chlorophyll synthesis (Figure 7) were restored. These results indicate that the effects observed in *pitt* cells are entirely attributable to the absence of solely the Pitt protein.

Membrane Sublocalization of Pitt

We have recently shown that a membrane subcompartment exists in *Synechocystis* 6803, which is defined by the PratA factor involved in PSII biogenesis (Klinkert et al., 2004; Schottkowski et al., 2009). This subcompartment has been proposed to represent connection/transfer sites between the plasma and the thylakoid membrane systems at which the initial steps in the biogenesis of photosynthetic complexes—at least partly—take place. To localize Pitt more precisely within the different membrane fractions, *Synechocystis* 6803 membranes were subjected to two consecutive rounds of sucrose gradient centrifugation, exactly as described previously (Schottkowski et al., 2009). Fractionation on the second linear gradient separates PratA from thylakoid fractions, and both Pitt and POR were found mainly in thylakoid fractions 7–14 as defined by the co-fractionation of the thylakoid marker PsbB (Schottkowski et al., 2009). However, some Pitt material was also found in the lighter, PratA-containing fractions 2–6, whereas weaker POR signals were detected in fractions 5 and 6 (Figure 8).

Intriguingly, in the *pratA* mutant line, a dramatic effect on the distribution of POR and, to a lesser extent, Pitt was observed (Figure 8). Here, both proteins accumulate in the lighter fractions that are defined by PratA in the wild-type. This is similar to the situation previously found for pD1 (the precursor of the D1 protein of PSII), suggesting that not only the maturation of D1, but also chlorophyll synthesis/insertion might take place in the membrane system defined by PratA (Figure 8; Schottkowski et al., 2009). When membranes from the *pitt* mutant were analyzed, minor amounts of POR were found to be shifted toward PratA fractions (Figure 8). Unexpectedly, however, the distributions of pD1 on both the *pratA* and the *pitt* gradients were similar (Figure 8). This suggests that, like PratA, Pitt is involved in the migration of PSII precomplexes to thylakoid membranes.

DISCUSSION

Pitt Is an Interaction Partner for POR

Several lines of evidence strongly suggest that the TPR protein Pitt interacts with the light-dependent POR enzyme in *Synechocystis* 6803. (1) Two independent yeast two-hybrid studies have now indicated that the two proteins bind to each other (Sato et al., 2007; Figure 4); (2) electrophoretic fractionation of cyanobacterial membranes on BN-PA gels reveals partial co-migration of Pitt and POR; (3) formation of POR-containing complexes is specifically affected in the *pitt* mutant; (4) the overall level of POR in *pitt* cells is reduced about threefold compared to the wild-type, suggesting that Pitt is involved in the stabilization of POR. Although the two-hybrid data indicate that the two polypeptides interact directly in yeast, the interaction of the native proteins appears to be rather transient, because only a subfraction of Pitt is organized in high-molecular-weight complexes (Figure 5). Moreover, repeated attempts to immunoprecipitate POR using αPitt antiserum yielded inconclusive results, which is consistent with the notion that Pitt/POR complexes are short-lived *in vivo* (data not shown).

Pitt Is Required for Efficient Light-Dependent Chlorophyll Synthesis

The *pitt* mutation clearly affects accumulation of POR and, in agreement with this observation, rates of light-dependent

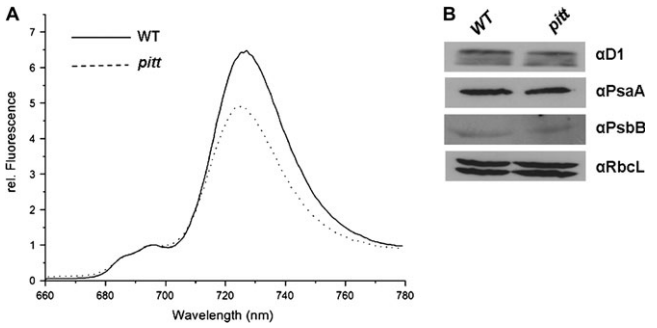

Figure 3. Accumulation of Photosynthetic Complexes in *pitt*.

(A) Low-temperature fluorescence emission spectra of whole cells of indicated strains are shown after excitation of chlorophyll at 435 nm. Spectra were normalized to the emission maxima at 685 nm.

(B) Total proteins of wild-type (WT) and *pitt* were probed with indicated antibodies.

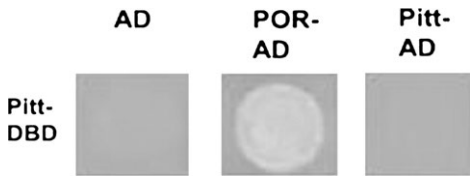

Figure 4. Interaction of Pitt and POR.

Yeast two-hybrid analysis of Pitt fused to the DNA-binding domain of LexA (Pitt–DBD) and either POR or Pitt fused to the activation domain of LexA (POR–AD and Pitt–AD, respectively). Autoactivation of Pitt–DBD by AD alone can be excluded (see first panel).

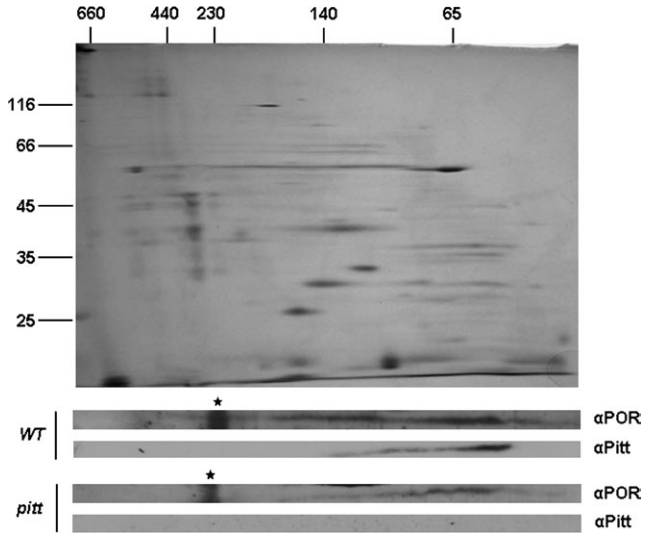

Figure 5. 2-D BN/SDS–PAGE of *Synechocystis* Membranes.

Membrane fractions from wild-type (WT) and *pitt* mutant strains were solubilized with β-dodecylmaltoside, fractionated by 2-D BN/SDS–PAGE and blotted. Pitt and POR were detected with the appropriate antibodies. A representative 2-D gel loaded with wild-type (WT) proteins and stained with Coomassie is shown at the top. Asterisks indicate unspecific cross-reactions of the αPOR antibody.

chlorophyll synthesis decline in *pitt* cells (Figure 7). In *Arabidopsis thaliana*, another TPR protein, named Flu, has previously been shown to regulate the early steps of chlorophyll synthesis by binding to, and thereby inhibiting, glutamyl tRNA reductase (Meskauskiene and Apel, 2002). Therefore, we tested whether Pitt—in addition to its role in determining POR levels—might directly regulate POR activity. We assayed POR activity *in vitro*, using recombinant Pitt and POR proteins expressed in *E. coli* according to Klement et al. (1999). Under the conditions employed, POR activity was independent of the absence or presence of Pitt, suggesting that Pitt does not influence POR activity per se (data not shown). Obviously, this does not rule out the possibility that Pitt is involved in modulating POR enzyme activity, because other necessary factors might be lacking in the *in vitro* system and/or experimental assay conditions might have to be optimized. Indeed, BN–PAGE revealed POR/Pitt complexes of higher molecular weight that might contain additional subunits (Figure 5). More detailed studies will be required to answer this question definitively.

Does Pitt Have Other Functions?

Solubilization experiments indicated that Pitt is an integral membrane protein that is likely to be anchored to the lipid bilayer via its N-terminal transmembrane domain (Figures 1 and 2). In contrast, POR is a peripheral membrane protein that is attached to membranes via its C-terminal domain (Klement et al., 1999; Aronsson et al., 2003; for a recent review, see Belyaeva and Litvin, 2007). Thus, attachment of POR to the membrane system could be a consequence of its interaction with Pitt. However, this scenario seems unlikely, since fractionation experiments showed that membrane association of the residual POR was not altered in the *pitt* mutant (data not shown). These data do not exclude the possibility that Pitt might facilitate localization of POR to distinct membrane subcompartments. In chloroplasts, POR can be localized to stromal thylakoids and/or the inner chloroplast envelope (Masuda and Takamiya, 2004).

Other possible roles for Pitt can also be envisaged, for instance in the spatial organization of chlorophyll synthesis and the biogenesis of chlorophyll-containing protein complexes. The *pitt* mutant exhibits a clear defect in photosynthetic activity at moderate light levels (Table 1). In contrast, deletion of the POR gene (*slr0506*) in *Synechocystis* 6803 has no obvious phenotypical consequences (Cyanobase: http://a.kazusa.or.jp/annotations/2741). This suggests that Pitt has additional, POR-independent functions in photosynthesis, although it should be noted here that in the cyanobacterium *Plectonema boryanum*, a light-dependent decrease in photosynthetic growth rate was observed in a POR deletion strain (Fujita et al., 1998). One such function might be to localize POR for the insertion of pigments into chlorophyll-binding proteins during their assembly into complexes. This idea is supported by the finding that substantial amounts of Pitt are

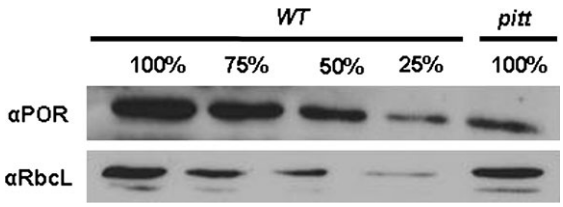

Figure 6. Levels of POR Are Reduced in the *pitt* Mutant.

Western analysis of different amounts of protein extracts (100% correspond to 120 μg protein) from wild-type (WT) and *pitt* cells using the αPOR antiserum. As a loading control the blot was immunodecorated with αRbcL antiserum.

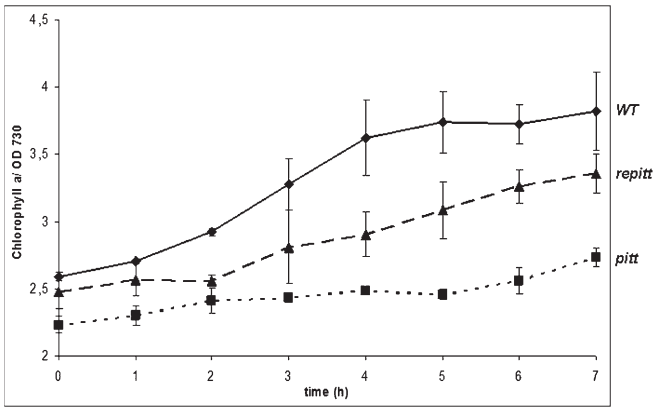

Figure 7. Chlorophyll Synthesis in *pitt* Cells.

Cells were grown under LAHG conditions and, at timepoint 0, cells were shifted to continuous illumination. Chlorophyll amounts of three independent experiments were measured at the given timepoints and the ratio of chlorophyll a (in mg) per cell density was plotted in relation to time. WT, solid line; repitt, dashed line; pitt, dotted line.

present in a lighter membrane subfraction that is defined by the PSII biogenesis factor PratA (Schottkowski et al., 2009). More strikingly, in a *pratA* mutant, both POR and Pitt are shifted towards these lighter membrane fractions, as is pD1, the precursor of the D1 protein (Figure 8). As previously hypothesized, this suggests a perturbation of membrane flow to the thylakoids from biogenesis sites where the initial steps in photosynthetic complex assembly, including pigment insertion, occur (Schottkowski et al., 2009; Keren et al., 2005). The dependence of POR and Pitt localization upon PratA argues that they act together, in a concerted fashion, during PSII precomplex assembly. Moreover, the shift in the distribution of POR material in the *pitt* mutant is less pronounced than that seen in *pratA*, further substantiating a potential role for Pitt in the spatial organization of chlorophyll synthesis and/or pigment insertion into proteins. Surprisingly, membrane localization of pD1 was significantly affected in *pitt* cells, suggesting that Pitt is also involved in the assembly of PSII. Whether this represents a direct or more indirect effect remains to be elucidated.

In conclusion, the available data do not yet allow us to assign a precise molecular function to Pitt, but the effects of its

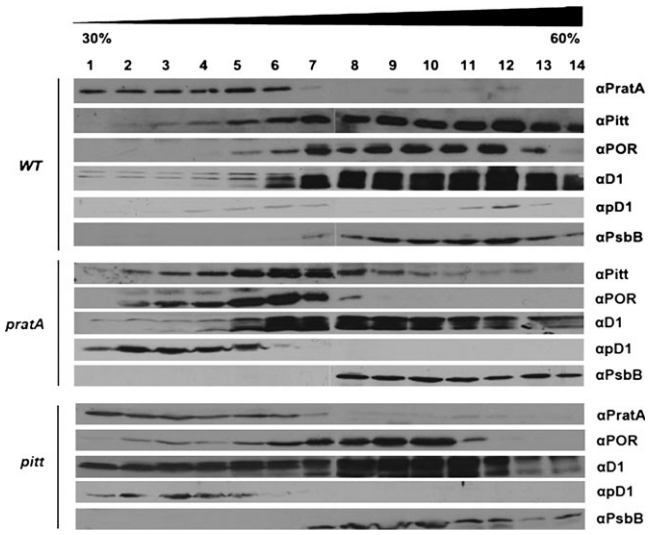

Figure 8. Membrane Sublocalization of Pitt.

Fractionation of cell material from fraction V (Schottkowski et al., 2009) on a linear 30–60% sucrose gradient. After fractionation, proteins were subjected to Western analysis with the indicated antibodies. POR signals in the *pitt* mutant were overexposed to enable visualization of qualitative POR distribution throughout the gradient.

loss, its binding to POR, and its membrane sublocalization permit us to propose that the protein is involved in early steps in the biogenesis of photosynthetic protein/pigment complexes.

METHODS

Sequence Analysis of Pitt

Basic local alignment search was performed with the protein-blast software on the NCBI homepage. Resulting amino acid sequences were aligned using MUSCLE (www.phylogeny.fr; Dereeper et al., 2008). The corresponding TPR-repeats were calculated with the free TPRpred software of the MPI Tübingen (http://toolkit.tuebingen.mpg.de/tprpred) and the prediction of the transmembrane domains was performed with SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/).

Generation and Growth of Strains

Synechocystis 6803 wild-type and mutant strains were grown on solid or in liquid BG 11 medium containing 5 mM glucose (if not otherwise noted) at 30°C at a continuous photon irradiance of 50 μE m^{-2} s^{-1}. The insertion mutant *pitt* was generated by PCR amplification of the wild-type *Pitt* gene using the primer pair *slr1644*/5 (GTGGCAACTTCTAGGACACA) and *slr1644*/3 (TTATTCTGACTTGAGGATGGG), and subsequent cloning of the resulting fragment into the Bluescript pKS vector. A kanamycin resistance cassette was then inserted into the unique *Hind*III restriction site in *Pitt*, and wild-type cells were transformed with the construct as described. For complementation of the resulting mutant *pitt*, the *Pitt* gene, including its

own promoter, was amplified using the primers co*1644*-for (GTCGACTGTTAAACTCCCTGGC) and co*1644*-rev (GTCGACTTATTCTGACTTGAGGATGG), and cloned into the single *Xho*I site of the vector pVZ321, which replicates autonomously in *Synechocystis* 6803 (Zinchenko et al., 1999). Transfer of this construct into *pitt* cells via conjugation was performed as described (Zinchenko et al., 1999).

Antibody Production and Western Analysis

For antibody production, the *Pitt* reading frame without the N-terminal transmembrane region (amino acid positions 38–290) was amplified using the oligonucleotides TH*slr1644*-a (GGATCCGAATTCTTGTGGCAACCCCGTCCCGC) and TH*slr1644*-b (GTCGACTTCTGACTTGAGGATGGGGG). The resulting DNA fragment was inserted into the vector pDrive (Qiagen), sequenced and further subcloned into the *Bam*HI and *Sal*I sites of the expression vector pGex-4T-1. Expression of the GST fusion protein in *Escherichia coli* BL21 and affinity purification on Glutathione Sepharose 4B (GE Healthcare) were performed in accordance with the manufacturer's instructions. A polyclonal antiserum was raised in rabbit (Biogenes). Protein extracts were prepared from *Synechocystis* 6803 and Western analyses were carried out as previously reported (Wilde et al., 2001). The pD1 antibody was raised against the last 16 amino acids of the pD1 protein (Allakhverdiev et al., 2002).

Measurements of Chlorophyll Fluorescence and Oxygen Evolution

Fluorescence emission spectra at 77 K were determined with an SLM-Aminco Series 2 luminescence spectrometer as described previously (Klinkert et al., 2004). Measurements of oxygen evolution during illumination with saturating white light were performed using a Clark-type oxygen electrode (Hansatech) at 30°C.

Yeast Two-Hybrid Analysis

Protein interaction studies in yeast were performed using the MATCHMARKER LexA system (Clontech). A PCR fragment containing the POR coding region was ligated into the pJG4-5 vector. The coding sequence for the mature form of Pitt (lacking 33 amino acids at the N-terminus including the putative transit sequence) was amplified by PCR and subsequently inserted into pEG202 via primer-derived restriction sites. Interaction studies were performed as described earlier (Klinkert et al., 2004).

Cell Fractionation

Total cellular proteins were prepared from 50-ml cultures in the exponential growth phase. Cells were harvested by centrifugation and re-suspended in breaking buffer (50 mM Tris/HCl pH 7, 20 mM $MgCl_2$, 20 mM KCl) supplemented with 0.5% Triton X-100. Cells were broken with glass beads in a mini-beadbeater (Glen Mills), using three 20-s bursts separated by 1-min periods on ice. The suspension was centrifuged for 1 min at 15 000 *g* and the protein concentration in the supernatant was measured. Finally, the samples were fractionated on SDS gels, blotted, and probed with various antibodies.

For separation into membrane and soluble protein fractions, wild-type cells were petleted by centrifugation, re-suspended in breaking buffer w/o detergent, and broken with the help of glass beads as described above. Total cellular proteins were adjusted to 1 M sucrose and centrifuged at 94 000 *g* for 30 min. The pellet was solubilized with breaking buffer containing 0.5% Triton X-100. Equal amounts of soluble and membrane proteins were then subjected to Western analysis.

Membrane sublocalization of Pitt and POR was performed precisely as reported previously (Schottkowski et al., 2009).

Solubilization of Membrane Proteins

Wild-type membranes for solubilization assays (50 μg protein) were sedimented by centrifugation and washed twice in 5 mM HEPES pH 7.6. Pellets were re-suspended in 20 μl of HEPES buffer and 20 μl of 5 mM HEPES pH 7.6 (as control), 0.2 M Na_2CO_3; 4 M urea; 2 M NaCl or 0.5% Triton X-100 was added. After a 30-min incubation on ice and centrifugation for 15 min at 18 000 *g* at 4°C, the supernatants were collected and the pellets were washed twice with HEPES buffer. All samples were fractionated by SDS–PAGE and analyzed by immunoblotting.

2D BN/SDS–PAGE

Preparation of membranes (corresponding to 500 μg mg protein) for blue native (BN)–PAGE in the first dimension was performed according to Dühring et al. (2006). Further steps were carried out as described (Schottkowski et al., 2009).

Chlorophyll Biosynthesis

Strains were grown under LAHG (light-activated heterotrophic growth) conditions (Smart et al., 1991) for 10 d in complete darkness, and exposed to light (5 μE m^{-2} s^{-1}) for 15 min each day. They were then grown in continuous light (20 μE m^{-2} s^{-1}) and chlorophyll a absorption was monitored at different time-points (Moran, 1982). Finally, chlorophyll a content was calculated on a per-cell basis.

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REFERENCES

Allakhverdiev, S.I., Nishiyama, Y., Miyairi, S., Yamamoto, H., Inagaki, N., Kanesaki, Y., and Murata, N. (2002). Salt stress

inhibits the repair of photodamaged photosystem II by suppressing the transcription and translation of psbA genes in Synechocystis. Plant Physiol. **130,** 1443–1453.

Aronsson, H., Sundqvist, C., and Dahlin, C. (2003). POR—import and membrane association of a key element in chloroplast development. Physiol. Plant. **118,** 1–9.

Belyaeva, O.B., and Litvin, F.F. (2007). Photoactive pigment–enzyme complexes of chlorophyll precursor in plant leaves. Biochemistry (Mosc.). **72,** 1485–1477.

Boudreau, E., Nickelsen, J., Lemaire, S.D., Ossenbühl, F., and Rochaix, J.D. (2000). The Nac2 gene of *Chlamydomonas* encodes a chloroplast TPR-like protein involved in *psbD* mRNA stability. EMBO J. **19,** 3366–3376.

Boudreau, E., Takahashi, Y., Lemieux, C., Turmel, M., and Rochaix, J.D. (1997). The chloroplast ycf3 and ycf4 open reading frames of *Chlamydomonas reinhardtii* are required for the accumulation of the photosystem I complex. EMBO J. **16,** 6095–6104.

D'Andrea, L.D., and Regan, L. (2003). TPR proteins: the versatile helix. Trends Biochem. Sci. **28,** 655–662.

Dereeper, A., et al. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic. Acids Res. **36 (Web Server Issue),** W465–W469.

Dühring, U., Irrgang, K.D., Lunser, K., Kehr, J., and Wilde, A. (2006). Analysis of photosynthetic complexes from a cyanobacterial *ycf37* mutant. Biochim. Biophys. Acta. **1757,** 3–11.

Dühring, U., Ossenbühl, F., and Wilde, A. (2007). Late assembly steps and dynamics of the cyanobacterial photosystem I. J. Biol. Chem. **282,** 10915–10921.

Fujita, Y., Takagi, H., and Hase, T. (1998). Cloning of the gene encoding a protochlorophyllide reductase: the physiological significance of the co-existence of light-dependent and -independent protochlorophyllide reduction systems in the cyanobacterium *Plectonema boryanum*. Plant Cell Physiol. **39,** 177–185.

Gomez Maqueo Chew, A., and Bryant, D.A. (2007). Chlorophyll biosynthesis in bacteria: the origin of structural and functional diversity. Annu. Rev. Microbiol. **61,** 113–129.

He, Q., Brune, D., Nieman, R., and Vermass, W. (1998). Chlorophyll *a* synthesis upon interruption and deletion of *por* coding for the light-dependent NADPH:protochlorophyllide oxidoreductase in a photosystem-I-less/*chl*L$^-$ strain of *Synechocystis* sp. PCC 6803. Eur. J. Biochem. **253,** 161–172.

Heyes, D.J., and Hunter, C.N. (2005). Making light work of enzyme catalysis: protochlorophyllide oxidoreductase. Trends Biochem. Sci. **30,** 642–649.

Keren, N., Liberton, M., and Pakrasi, H.B. (2005). Photochemical competence of assembled photosystem II core complex in cyanobacterial plasma membrane. J. Biol. Chem. **280,** 6548–6553.

Kim, C., Meskauskiene, R., Apel, K., and Laloi, C. (2008). No single way to understand singlet oxygen signalling in plants. EMBO Rep. **9,** 435–439.

Klement, H., Helfrich, M., Oster, U., Schoch, S., and Rüdiger, W. (1999). Pigment-free NADPH:protochlorophyllide oxidoreductase from *Avena sativa* L.: purification and substrate specificity. Eur. J. Biochem. **265,** 862–874.

Klinkert, B., Ossenbühl, F., Sikorski, M., Berry, S., Eichacker, L., and Nickelsen, J. (2004). PratA, a periplasmic tetratricopeptide repeat protein involved in biogenesis of photosystem II in *Synechocystis* sp. PCC 6803. J. Biol. Chem. **279,** 44639–44644.

Masuda, T., and Takamiya, K. (2004). Novel insights into the enzymology, regulation and physiological functions of light-dependent protochlorophyllide oxidoreductase in angiosperms. Photosynth. Res. **81,** 1–29.

Meskauskiene, R., and Apel, K. (2002). Interaction of FLU, a negative regulator of tetrapyrrole biosynthesis, with the glutamyl-tRNA reductase requires the tetratricopeptide repeat domain of FLU. FEBS Lett. **532,** 27–30.

Meskauskiene, R., Nater, M., Goslings, D., Kessler, F., op den Camp, R., and Apel, K. (2001). FLU: a negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. Proc. Natl Acad. Sci. U S A. **98,** 12826–12831.

Moran, R. (1982). Formulae for determination of chlorophyllous pigments extracted with *N,N*-dimethylformamide. Plant Physiol. **69,** 1376–1381.

Nelson, N., and Ben-Shem, A. (2004). The complex architecture of oxygenic photosynthesis. Nat. Rev. Mol. Cell Biol. **5,** 971–982.

Park, S., Khamai, P., Garcia-Cerdan, J.G., and Melis, A. (2007). REP27, a tetratricopeptide repeat nuclear-encoded and chloroplast-localized protein, functions in D1/32-kD reaction center protein turnover and photosystem II repair from photodamage. Plant Physiol. **144,** 1233.

Peng, L., Ma, J., Chi, W., Guo, J., Zhu, S., Lu, Q., Lu, C., and Zhang, L. (2006). Low PSII accumulation1 is involved in efficient assembly of photosystemII in *Arabidopsis thaliana*. Plant Cell. **18,** 955–969.

Ruf, S., Kössel, H., and Bock, R. (1997). Targeted inactivation of a tobacco intron-containing open reading frame reveals a novel chloroplast-encoded photosystem I-related gene. J. Cell Biol. **139,** 95–102.

Sane, A.P., Stein, B., and Westhoff, P. (2005). The nuclear gene HCF107 encodes a membrane-associated R-TPR (RNA tetratricopeptide repeat)-containing protein involved in expression of the plastidial psbH gene in *Arabidopsis*. Plant J. **42,** 720–730.

Sato, S., Shimoda, Y., Muraki, A., Kohara, M., Nakamura, Y., and Tabata, S. (2007). A large-scale protein protein interaction analysis in *Synechocystis* sp. PCC 6803. DNA Res. **14,** 207–216.

Schottkowski, M., Gkalympoudis, S., Tzekova, N., Stelljes, C., Schuenemann, D., Ankele, E., and Nickelsen, J. (2009). Interaction of the periplasmic PratA factor and the PsbA (D1) protein during biogenesis of photosystem II in *Synechocystis* sp. PCC 6803. J. Biol. Chem. **284,** 1813–1819.

Smart, B.L., Anderson, S.L., and McIntosh, L. (1991). Targeted genetic inactivation of the photosystem I reaction center in the cyanobacterium *Synechocystis* sp. PCC 6803. EMBO J. **10,** 3289–3296.

Stöckel, J., Bennewitz, S., Hein, P., and Oelmüller, R. (2006). The evolutionary conserved tetratrico peptide repeat protein pale yellow green7 is required for photosystem I accumulation in *Arabidopsis* and copurifies with the complex. Plant Physiol. **141,** 870–878.

Sytina, O.A., Heyes, D.J., Hunter, C.N., Alexandre, M.T., van Stokkum, I.H., van Grondelle, R., and Groot, M.L. (2008). Conformational changes in an ultrafast light-driven enzyme determine catalytic activity. Nature. **456,** 1001–1004.

Vaistij, F.E., Boudreau, E., Lemaire, S.D., Goldschmidt-Clermont, M., and Rochaix, J.D. (2000). Characterization of Mbb1, a nucleus-encoded tetratricopeptide-like repeat protein required for expression of the chloroplast psbB/psbT/psbH gene cluster in *Chlamydomonas reinhardtii*. Proc. Natl Acad. Sci. U S A. **97,** 14813–14818.

Wilde, A., Lünser, K., Ossenbühl, F., Nickelsen, J., and Börner, T. (2001). Characterization of the cyanobacterial ycf37: mutation decreases the photosystem I content. Biochem J. **357 (Pt 1),** 211–216.

Zinchenko, V.V., Piven, I.V., Melnik, V.A., and Shestakov, S.V. (1999). Vectors for the complementation analysis of cyanobacterial mutants. Russian J. Genet. **35,** 228–232.

DISCUSSION

TPR proteins in *Synechocystis*

The genome of *Synechocystis* encodes, depending on search parameter, 22-26 TPR proteins, which have been identified by sequence analysis. Compared to e.g. *A. thaliana*, with 326 encoded TPR proteins, this is a relative small amount. Therefore *Synechocystis* is the perfect model for systematic analysis of their function. The present study elucidated the function of PratA (Section I) and Pitt (Section II). Both factors are important during thylakoid membrane biogenesis. While PratA directly interacts with the D1 protein of PSII (Figure 1 & 2, Section I) and is therefore associated to a membrane subfraction to a substantial amount (Figure 5, Section I), Pitt indirectly influences the chlorophyll synthesis by interacting with the POR (Figure 4 & 6, Section II). Inactivation of Pitt therefore leads to a retarded chlorophyll synthesis (Figure 7, Section II). Pitt-homologues are found in several photoautotrophic organisms hence one may speculate that Pitt and its homologues share similar functions in different organisms (Figure 1, Section II). Besides the already mentioned Ycf3 and Ycf37 biogenesis factors, another TPR protein (slr0151p) might be involved in biogenesis of the thylakoid membrane. The protein is encoded in the so-called Pap-operon (PSII-assembly-protein). During purification of PSII, all encoded proteins were identified as PSII-associated. It was shown that these proteins have binding domains for chlorophyll and billin and are part of a PSII precomplex, suggesting a function for cofactor delivery to PSII during assembly (Wegener et al. 2008).

Inactivation of further, yet uncharacterized TPR proteins led to impaired photosynthetic performance, indicating that more TPR proteins from *Synechocystis* might be important for proper functionality of the photosystems (Shao & Nickelsen, unpublished results).

Thylakoid biogenesis occurs at the cyanobacterial plasma membrane

Where the initial steps of complex assembly and thylakoid membrane biogenesis in cyanobacterial cells occur is still under a strong debate. The present study provides intriguing data that the plasma membrane houses the first steps of thylakoid formation, namely assembly of the first PSII precomplex and the maturation of the D1 protein. Combining already published and novel experimental data, the importance of the plasma membrane to initiate thylakoid biogenesis is supported:

(1) The usually periplasmic TPR protein PratA interacts with the precursor protein of D1 (Klinkert et al. 2004). The present data reveals the presence of two different PratA populations. The majority of the PratA protein forms a soluble periplasm-located 200 kDa complex (Figure 3A & 4B, Section I), whereas a minor amount of PratA is part of a membrane-associated complex (Figure 3A & 4A, Section I). This complex is located in a non-thylakoid membrane fraction which is mediated by the interaction with a D1 subpopulation (Figure 3, Section I). The distribution of thylakoid membranes is indicated by the presence of the CP47 protein, the PSII antenna protein, which is restricted solely to the thylakoids (Figure 5, Section I).

(2) The enzyme CtpA, which is indispensable during maturation of the PSII core protein D1, is exclusively located in pure plasma membrane preparations (Zak et al. 2001).

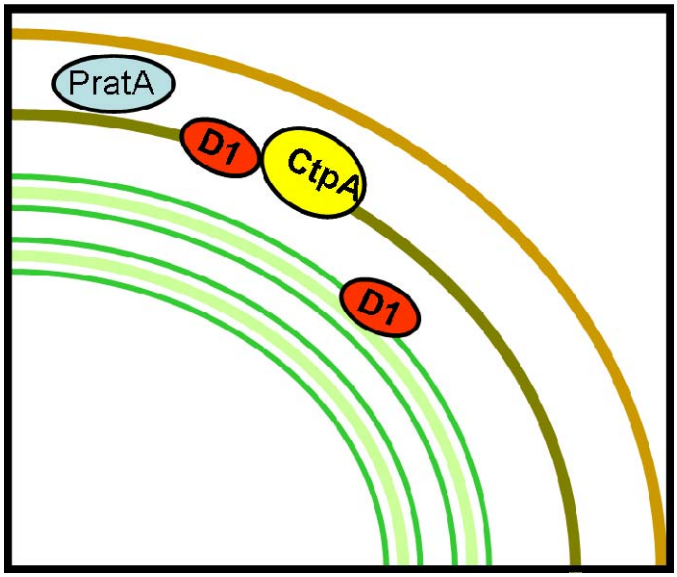

Figure 7: Model of how the periplasmic PratA and the plasma membrane protein CtpA interact with the D1 protein. Shown is the outer membrane (orange), the plasma (brown) and the thylakoid membranes (green).

(3) To a certain amount, core subunits of both, photosystem I and II are found at the plasma membrane, for instance the proteins D1, D2, PsaA and PsaB. Furthermore, several assembly factors (Ycf3 and Ycf4) but not the antenna proteins CP43 and CP47 are located exclusively at the plasma membrane.

(4) A PSII precomplex is formed at the plasma membrane, which consists of the proteins pD1/D1, D2 and cytochrome b559. It was shown that this isolated precomplex has photochemical competence (Keren et al. 2005)

It seems that not the complete plasma membrane harbours a biogenesis apparatus for the photosynthetic complexes, but that these important actions occur at specialized, biogenesis-initiating regions present in this membrane system (Srivastava et al. 2006). During preparation of plasma membrane fractions the existence of a heterogeneous distribution of proteins in the membrane became obvious. Different vesicles were formed– one kind is characterized by proteins like NrtA, a peripheral lipoprotein, and VIPP1. Both proteins accumulated in right-side-out (RSO) vesicles. In contrast the inside-out (ISO) vesicles contained high amounts of the pD1, D1 and PsaD proteins. Indicating that ISO vesicles represent regions of the plasma membrane where biogenesis of thylakoid proteins occurs (Srivastava et al. 2006).

Place of pigment synthesis

Several pieces of data provide satisfying insights in the spatial organization of chlorophyll synthesis in chloroplasts of higher plants. All enzymes necessary for the pigment synthesis pathway till the conversion of protochlorophyllide to chlorophyllide are located in the chloroplast envelope (for review see Joyard et al. 1998). Therefore it is likely that the POR is associated to the envelope, too. But there are other studies suggesting that POR is also located to the thylakoid membrane (Masuda & Takamiya 2004). As POR is not an intrinsic membrane protein, which depends on anchors facilitating its membrane association (Teakle & Griffiths 1993; Rowe & Griffiths 1995), it might be possible that the POR associates to both membranes depending on preparation techniques. The last step of the chlorophyll synthesis, the conversion of chlorophyllide to chlorophyll, appears at the thylakoids, this is due to the association

of the responsible enzyme, the chlorophyll synthase, with those membranes (Joyard et al. 1998).

The understanding of the spatial organization in cyanobacteria is not that advanced compared to higher plants. It was shown that in the cyanobacterium *A. nidulans* precursors of chlorophyll, protochlorophyllide and chlorophyllide as well as the POR are found in preparations of plasma membranes (Peschek et al. 1989). This is in accordance with the situation in higher plants, as PM and envelope share functional and structural relation. Moreover, this study revealed the existence of chlorophyll only in the thylakoids, which would fit to the localization of the chlorophyll synthase at the thylakoids in chloroplasts.

The present work shows that the POR is mainly located to the thylakoid membrane in *Synechocystis* (Figure 8, Section II), which is consistent with a study in higher plants.

Taken together we conclude that not only the biogenesis of polypeptide subunits of the photosynthetic apparatus, but also at least initial steps of the chlorophyll synthesis pathway occur at the plasma membrane of cyanobacteria.

De novo assembly vs. repair cycle

All these data support the role of the plasma membrane as compartment of initial steps of thylakoid formation. Despite there are still concerns about this model. The HCF136 factor from *Synechocystis* which was shown to interact with the pD1 protein and therefore might be important for thylakoid membrane biogenesis is associated to the thylakoid and not the plasma membrane (Dobáková et al. 2009). Furthermore, the authors state that the repair cycle of photosystem II is also affected by the absence of HCF136. This could imply that HCF136 is only involved in the repair cycle and that the *de novo* assembly of photosystems and their repair cycle occur at different regions in cyanobacteria. Data from studies with green algae showed that this is also true for chloroplasts of *C. reinhardtii*. Here specific regions for *de novo* assembly around the pyrenoid are found, while repair seems to occur distributed over the stroma thylakoids throughout the cup-shaped chloroplast (Uniacke & Zerges 2007).

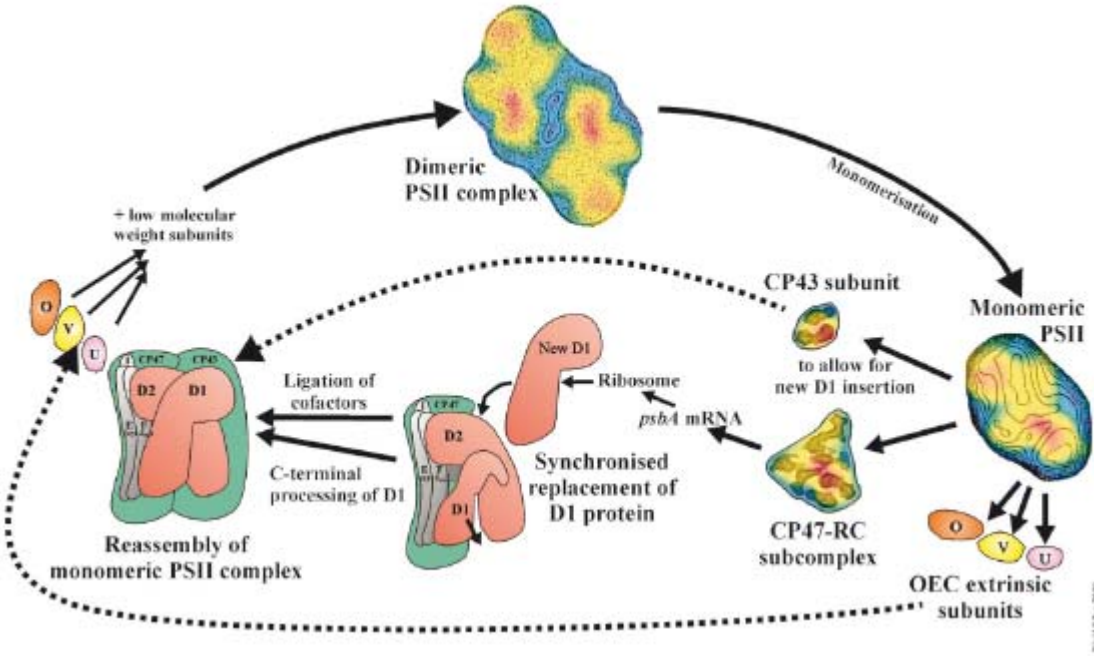

Figure 8: Model of the PSII repair cycle. Dimeric PSII disassembles to the RC47 complex, damaged D1 is replaced by a new synthesized precursor copy, processed and finally the reassembly of cofactors and low-molecular weight subunits occurs (Nixon et al. 2004).

The repair of PSII in *Synechocystis* depends on several proteases, which degrade the damaged D1 protein. The protease FtsH was shown to be located at the thylakoid membrane and furthermore the exchange of the D1 protein occurs in a partially disassembled photosystem II, called RC47 (Komenda et al. 2006; Dobáková et al. 2009). The small complex RC47 still contains the antenna protein CP47 and as CP47 has never been detected in plasma membrane preparations, the repair cycle, in contrast to *de novo* PSII assembly, has to be localized in the thylakoid membranes.

The constantly replacement of photodamaged PSII, especially of the D1 protein, longs for incorporation of a newly synthesized copy of the reaction center protein D1. The maturation of the precursor pD1 depends on the enzyme CtpA, which is supposed to be exclusively located at the plasma membrane. This leads to another challenge: How is maturation of newly synthesized pD1 protein for repair of damaged PSII in the thylakoid membrane achieved?

Analyses of the mobility of protein complexes in thylakoid membranes revealed that complexes are normally completely immobile, but FRAP measurements provide evidence that they become mobile under certain conditions (Sarcina & Mullineaux 2004; Sarcina et al. 2006). One could speculate that during photodamage these conditions exist. In that case, the partially disassembled RC47 complex becomes mobile and migrates to regions in the thylakoids, which are in close connection to the plasma membrane. Secondly the pD1 in RC47 gets in contact to the CtpA by unknown mechanisms and the pD1 is processed to its functional form. Finally RC47 migrates back and active PSII will be formed by reassembly of missing subunits. Indeed, it was shown for *Synechococcus* that under intense red light PSII becomes mobile and the PSII fluorescence accumulates in the thylakoid membrane at specific regions close to the poles of the cell (Sarcina et al. 2006). This scenario reminds of the specific sites for biogenesis in the plasma membrane and leads to the idea that also the thylakoid membrane harbours specific sites for protein synthesis and/or maturation during the repair cycle.

Morphology of the cyanobacterial internal membrane system

To date the transport of protein complexes and pigments from the plasma to the thylakoid membrane in course of *de novo* assembly of photosynthesis complexes is lively discussed.

There are two major alternatives: First, a vesicle transport from the place of initial biogenesis steps to the destination, where this material is needed. Second, connections between both membrane compartments which would allow a migration of complexes along the membrane or from the periplasm to the luminal space.

Vesicles were observed in higher plants chloroplasts and were supposed to represent transport vesicles of lipids and/or proteins from the inner envelope to the thylakoids (Vothknecht & Soll 2005). In cyanobacteria so far only one species is described, in which vesicles are seen by electron tomography. As this cyanobacterium *Microcoleus* sp. is a very ancient species, a conserved presence of vesicles from cyanobacteria to higher plants might be possible (Nevo et al. 2007). The idea of vesicles is supported by the discovery of the vesicle-inducing protein in plastids 1 (Vipp1). This protein is essential for the formation of thylakoids in higher

plants and cyanobacteria. Deletion of Vipp1 in higher plants even prevented the formation of vesicles. In *Synechocystis* the Vipp1 homologue is located at the plasma membrane and despite its location influences the biogenesis of thylakoids (Westphal et al. 2001; Aseeva et al. 2007; Fuhrmann et al. 2009). These findings point out a functional connection between plasma and thylakoid membrane and the necessity of material transport from one to the other area.

Numerous studies were carried out the last years to dismantle the real morphology of the intracellular membrane system in cyanobacteria to support or to deny the possibility that the plasma and thylakoid membrane are connected at specific sites (Kunkel 1982; Spence et al. 2003; Liberton et al. 2006; van de Meene et al. 2006; Nevo et al. 2007). However, the resulting data is not convincing because of its inconsistence. One study, in which ultrarapid freeze-fixation of *Synechocystis* cells facilitates excellent preservation of cellular structures, demonstrates that thylakoid membranes are separate and seem to be a closed compartment within the cell (Liberton et al. 2006). Simultaneously, the researchers state that they found no evidence for vesicle transport in *Synechocystis*. They conclude neither to exclude vesicle transport nor connectivity of both membranes, as membranes in cyanobacteria are a dynamic, depending on development and environment changing system. This was indeed observed for *Synechocystis*: perforations through several sheets of thylakoids are formed on demand to allow traffic of material throughout the whole cell (Nevo et al. 2007).

Several studies provide good evidence for a connectivity of plasma and thylakoid membranes in several cyanobacteria, however there is a remaining lack of evidence for *Synechocystis*. The arrangement of intracellular membranes of *Synechococcus* sp. PCC7002 was analyzed and contacts at numerous points between both membranes are described (Nierzwicki-Bauer et al. 1983), yet this finding might provide evidence for connecting points, but as this cyanobacterium exhibits a very special membrane organization, a validation of membrane connections in cyanobacteria in general is still missing. However, already in 1982 Kunkel found that in four cyanobacteria (*A. cylindrical, D. violaceae, G. alpicola and P. minor*) groups of thylakoids converge at peripheral cytoplasmic points. At these sites cylindric structures, named thylakoid centers (TC), were detected (Kunkel 1982). These thylakoid centers are located within the cytoplasmic membrane and are associated with layers of thylakoids. From the peripheral border of those structures,

four to eight thylakoid membranes radiate to the inner part of the cell. A direct thylakoid-cytoplasmic membrane connection could not be shown, but a significant function of the TCs was suggested due to their location in the plasma membrane, structure and thylakoid attachment (Kunkel 1982).

A recent study provides the first evidence for membrane connectivity in *Synechocystis*. By applying cryopreparation and electron tomography, which provides high-resolution 3-D data, it could be shown that thylakoid membranes converge along the plasma membrane and that at several connections thylakoid centers, associated to both plasma and thylakoid membrane, are present (van den Meene et al. 2006). These connections were proposed to be dynamic, transient fusions, which would allow transfer of materials from the cytoplasmic membrane to the thylakoids.

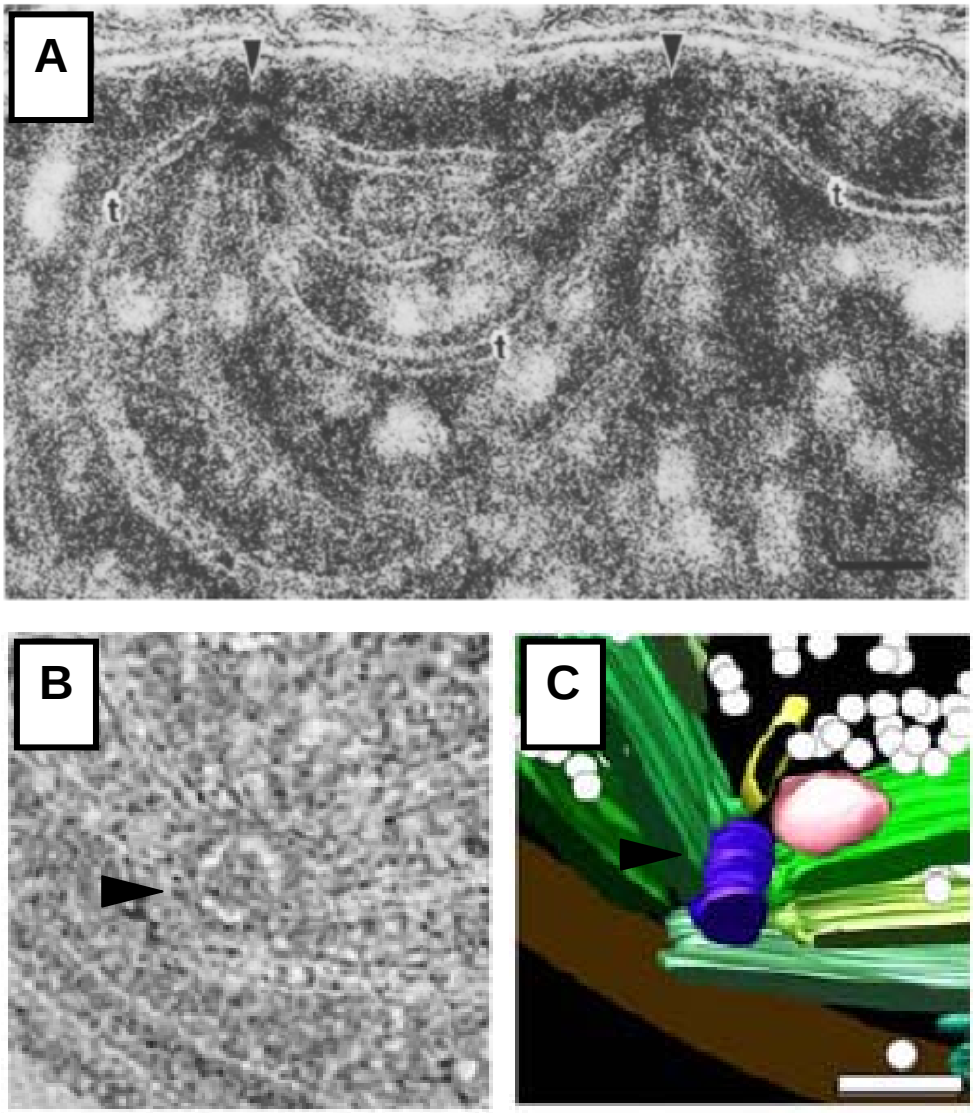

Figure 9: Thylakoid center in different cyanobacterial species. (A) EM-image of two thylakoid centers in *P. minor* with radiating thylakoid membranes (Kunkel 1982). Thylakoid center in *Synechocystis*: (B) EM-image and (C) electron tomography of the corresponding image (van de Meene et al. 2006). Black arrowheads indicate the TCs.

The occurrence of TCs in several cyanobacteria point out their important, yet unclear role in biogenesis of thylakoids. In chloroplasts of spinach TC-like structures are present and it was suggested that they might perform transport from the inner envelope to the thylakoids (Carde et al. 1982). Similar suggestions were made for TCs in *Synechocystis*, where a transfer of PSI and PSII subunits from the cytoplasmic to the thylakoid membrane via this structure was proposed (Hinterstoisser et al. 1993).

The present study provides additional strong evidence that indeed the membrane system in *Synechocystis* is a continuum, which allows transfer of proteins, pigments and lipids from the cytoplasmic to the thylakoid membrane. The present data shows that a special membrane subfraction exists which can be isolated as innate membrane fraction from plasma- and thylakoid membrane and might therefore represent the missing link between both. The PratA factor interacts with the luminal C-terminus of a D1 subpopulation which is to substantial amounts located in this novel PratA-defined membrane (PDM) compartment (Figure 2 & 5, Section I). Furthermore, we were able to detect a weak signal of pD1 in this region in wildtype cells, which accumulates significantly, when PratA is missing (Figure 5, Section I). This observation of accumulated pD1 is in line with earlier findings and might suggest an improper processing or assembly of D1 into new synthesized PSII (Klinkert et al. 2004). In addition, CFP-tagged D1 protein accumulates in the periphery of cells in a *pratA*-mutant (Figure 6, Section I). Taken together the results indicate a function of PratA during transfer of subunits of PSII from the place of biogenesis to the thylakoid membranes, but still its exact function has to be elucidated.

Likewise, Pitt and its interaction partner POR (Figure 4 & 5, Section II) are found to a certain amount in the PDM, while the majority is located at the thylakoid membrane, indicating that both proteins act in a concerted fashion during biogenesis of protein-pigment complexes (Figure 8, Section II). A shift of both factors to lower density sucrose fraction where PratA is found becomes obvious in the absence of PratA. This again is supporting the idea that PratA is involved in transfer of material or in organization of efficient transport (Figure 8, Section II). Interestingly, in the *pitt*-mutant the pD1 protein behaves as in the *pratA*-mutant and seems to stick in the PDM (Figure 8, section II). The accumulation of pD1 might therefore just be seen as a secondary effect of inefficient PSII assembly, which is already described for inactivation of several PSII assisting factors, e.g. PsbH, PsbB or PsbEFLJ (Komenda et al. 2005).

Very recent data revealed that the majority of CtpA is also present in the PDM (Rengstl & Nickelsen, unpublished results). This finding implies that the processing of pD1 does not occur in the cytoplasmic membrane but in the PratA-defined one, thus this membrane subfraction might be specialized in certain steps of thylakoid membrane biogenesis. This hypothesis is supported by the finding that in PratA-defined gradient fractions also a chlorophyll precursor is present (Rengstl &

Nickelsen, unpublished results). This confirms earlier studies in which proto- and chlorophyllide were found to accumulate in the thylakoid centers in *A. nidulans* and *Synechocystis*, respectively (Peschek et al. 1989; Hinterstoisser et al. 1993).

We were able to isolate the special membrane as a distinct fraction in sucrose gradients, also shown for TC (Hinterstoisser et al. 1993), which is neither plasma nor thylakoid membrane. If this novel PDM subfraction shares structural and/or functional similarity or even equality with the already described thylakoid center remains to be elucidated.

Working model for the spatial organization of thylakoid biogenesis

Based on the present data combined with earlier published facts, a working model of processes occurring at the internal membrane system in *Synechocystis* during thylakoid membrane biogenesis and complex assembly can now be drawn.

We propose a model for the membrane system of *Synechocystis* with transient or permanent connecting regions between plasma and thylakoid membrane at certain points at the periphery of the cells. These connections are represented by a membrane fraction distinct from cytoplasmic membrane and the thylakoids, which can be isolated in a sucrose gradient. This special membrane subfraction is characterized by the presence of a minor part of the PratA factor (Figure 3A & 5, Section I), which seems to facilitate efficient transport/transfer of photosynthetic complex subunits to the thylakoids and/or to organize the connecting region. The majority of PratA is part of a periplasm-located 200 kDa complex, the function of which is still under investigation (Figure 4B, Section I).

As initial steps of thylakoid membrane biogenesis occur in the novel PDM subfraction, we refer to this region as “biogenesis center” (BC). It is tempting to speculate that the ISO vesicles, which seem to represent regions of early thylakoid formation, share similarities with the PDMs.

PratA interacts with unassembled pD1 at the PDM; therefore this interaction must be present before the assembly of the first PSII precomplex, consisting of the proteins D1, D2 and cytochrome b559. These core subunits are inserted into the membrane in a co-translational manner, but if they are inserted into the plasma membrane or into the PDM remains to be elucidated. Our data suggests that this

early complex is assembled in the biogenesis center and transported from there to the thylakoids where subsequently all missing subunits are assembled and finally the fully active photosystem II reaction center is connected to the OEC. The presence of the CtpA protein at the PDM indicates that the C-terminal extension of pD1 is cleaved immediately before or during transport from the BC to the thylakoids. Furthermore, the localization of CtpA raises new ideas regarding the mode of the repair cycle in *Synechocystis*, as discussed later. We believe that the discrepancy between the localization of CtpA at the plasma membrane in former studies or in the PDM fraction in the present work is due to different preparation procedures. It is possible that the PDM subfraction became associated to the cytoplasmic membrane during preparation, but through improved procedures we are now able to isolate it as innate membrane fraction.

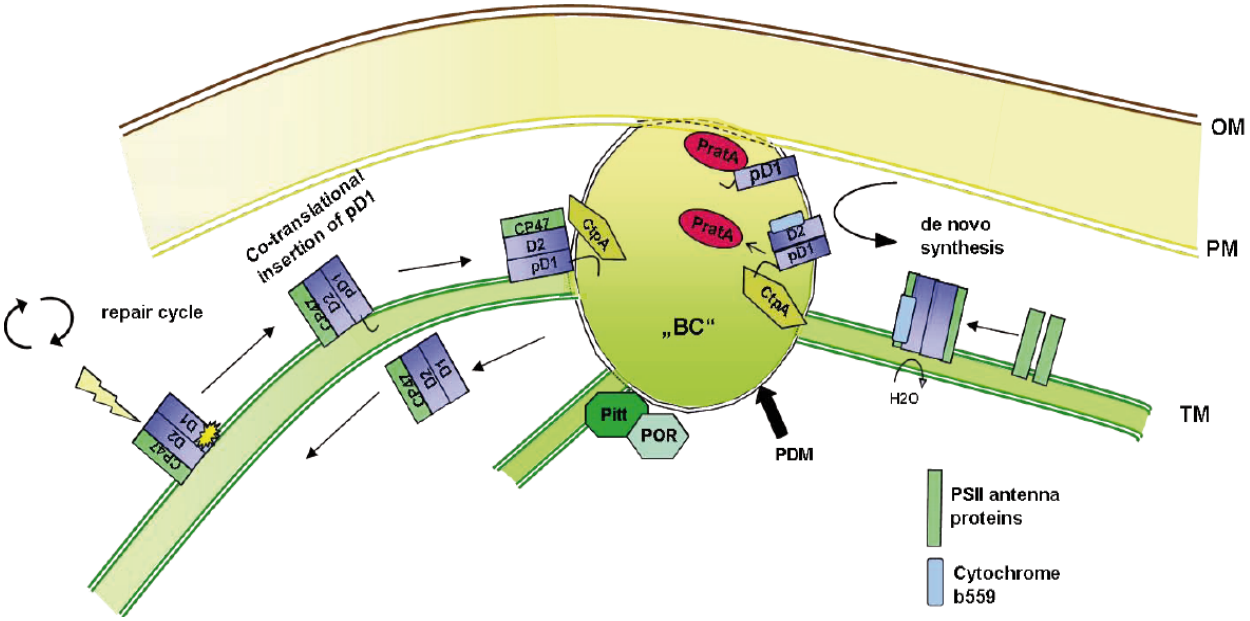

Figure 10: Working model of the spatial organization of thylakoid membrane biogenesis in *Synechocystis*. *De novo* assembly and the PSII repair cycle are differentially localized. Outer membrane (OM), plasma membrane (PM), thylakoid membranes (TM) and the PratA-defined membrane (PDM) are depicted. PDMs enclose the biogenesis center (BC), where early steps of thylakoid membrane biogenesis occur. For more details refer to the text.

The spatial organization of chlorophyll synthesis in *Synechocystis* remains to be elucidated. The present data provides strong evidence for the localization of the light-dependent POR, which drives one of the last steps of the chlorophyll synthesis

pathway. The Pitt protein together with the POR is mainly located in the thylakoid membrane, while a small amount is present in the PDM fraction (Figure 8, Section II). This together with yet unpublished data about accumulation of chlorophyll precursor in the PDM, indicate that the synthesis of chlorophyll might occur in or adjacent to the BC. Because already in the first precomplex of PSII chlorophyll is incorporated (Keren et al. 2005), one could suggest that either chlorophyll synthesis is not restricted to the area of the BC or a transport of chlorophyll throughout the membrane system is present.

It seems likely that the repair cycle of PSII, which is constantly exposed to photodamage, appears separated from the *de novo* assembly of the photosynthetic complexes in *Synechocystis*. In our model the damaged PSII disassembles to a certain amount, then proteases degrade and remove the core protein D1 and a newly synthesized precursor copy of D1 is integrated into the RC47 complex, still located in the thylakoids. This idea is in accordance with an earlier study and also is supported by weak signals of pD1 in the thylakoid fractions (Jansén et al. 2002; Figure 5, Section I). During or after insertion of pD1 RC47 migrates through the thylakoid membrane towards the biogenesis center, where the CtpA protease is located. At the border of thylakoids to PDM the C-terminal extension of pD1 is exposed into the lumen and CtpA gets in contact to the extension, which is in turn cleaved. Thereafter the RC47 complex moves back to the thylakoids and the reassembly takes place, to gain back full functionality.

The hypothesis of membrane continuity in *Synechocystis* raises more questions: if the membrane system is a continuum, are consequently the periplasm and the thylakoid lumen a contiguous compartment, too? One study provides evidence that this is not the case. GFP was engineered with a Tat-signal peptide and transported across the plasma membrane to the periplasm. The resulting GFP-fluorescence was solely present in the periplasm and a diffusion of GFP to the lumen was not obvious (Spence et al. 2003). This suggests that in the periplasm and thylakoid lumen, despite the connectivity of both respective membranes, a free and spontaneous diffusion of materials from one to the other compartment is not possible. One could

think about the presence of special “checkpoints” organizing the correct transport from periplasm to thylakoid lumen and vice versa.

The present work helps to elucidate the long-time contrary discussed question concerning spatial organization of thylakoid membrane biogenesis by providing strong evidence for special biogenesis sites in *Synechocystis*. The possible existence of a biogenesis-initiating region (biogenesis center) is substantiated by the identification of specific biogenesis sites in the chloroplast of *C. reinhardtii* (Uniacke & Zerges 2007). These sites, called T-zone, are punctuate regions around the pyrenoid, to which membranes in different stages of thylakoid biogenesis are located. Those membranes are similar to chloroplast envelope, but associated with the thylakoids, light-activated RNA-binding proteins as well as translation factors.

As a next step it would be highly fascinating to visualize connecting structures in *Synechocystis*. Therefore we would recommend Immuno-gold labelling of PratA and ultrastructure analysis by high-resolution microscopy.

REFERENCES

Anbudurai PR., Mor TS., Ohad I., Shestakov SV., Pakrasi HB. (1994) The *ctpA* gene encodes the C-terminal processing protease for the D1 protein of the photosystem II reaction center complex. Proc. Natl. Acad. Sci USA **91**, 8082-8086

Anderson LK. and Toole CM. (1998) A model for early events in the assembly pathway of cyanobacterial phycobilisomes. Mol. Microbiol. **30**, 467-474

Armstrong GA. (1998) Greening in the dark: light-independent chlorophyll biosynthesis from anoxygenic photosynthetic bacteria to gymnosperms. J. Photochem. Photobiol. **43**, 87-100

Aseeva E., Ossenbuehl F, Sippel C., Cho WK., Stein B., Eichacker LA., Meurer J., Wanner G., Westhoff P., Soll J., Vothknecht UC. (2007) Vipp1 is required for basic thylakoid membrane formation but not for the assembly of thylakoid protein complexes. Plant Physiol. Biochem. **45**, 119-128

Atanassova A., Sugita M., Sugiura M., Pajpanova T., Ivanov I. (2003) Molecular cloning, expression and characterization of three distinctive genes encoding methionine aminopeptidases in cyanobacterium *Synechocystis* sp. strain PCC6803. Arch. Microbiol. **180**, 185-193

Baker ME. (1994) Protochlorophyllide reductase is homologous to human carbonyl reductase and pig 20-beta-hydroxysteroid dehydrogenase. Biochem. J. **300**, 605-607

Barber J. and Andersson B. (1992) Too much of a good thing: light can be bad for photosynthesis. Trends Biochem. Sci. **17**, 61-66

Barber J. (2008) Photosynthetic generation of oxygen. Phil. Trans. R. Soc. B **363**, 2665-2674

Barber J. and Murray JW. (2008) The structure of the Mn_4Ca^{2+} cluster of photosystem II and its protein environment as revealed by X-ray crystallography. Phil. Trans. R. Soc. B **363**, 1129–1138

Beale SI. (1999) Enzymes of chlorophyll biosynthesis. Photosynth. Res. **60**, 43-73

Blatch GL. and Laessle M. (1999) The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. Bioessays **21**, 932-939

Boudreau E., Takahashi Y., Lemieux C., Turmel M., Rochaix JD. (1997) The chloroplast ycf3 and ycf4 open reading frames of *Chlamydomonas reinhardtii* are required for the accumulation of the photosystem I complex. EMBO J. **16**, 6095-6106

Boudreau E., Nickelsen J., Lemaire SD., Ossenbuehl F., Rochaix JD. (2000) The *Nac2* gene of *Chlamydomonas* encodes a chloroplast TPR-like protein involved in *psbD* mRNA stability. EMBO J. **19**, 3366-3376

Bowyer JR., Packer JC., McCormack BA., Whitelegge JP., Robinson C., Taylor MA. (1992) Carboxyl-terminal processing of the D1 protein and photoactivation of water-splitting in photosystem II. Partial purification and characterization of the processing enzyme from *Scenedesmus obliquus* and *Pisum sativum*. J. Biol. Chem. **267**, 5424-5433

Carde JP., Joyard J., Douce R. (1982) Electron microscopic studies of envelope membranes from spinach plastids. Biol. Cell **44**, 315-324

Choen RO., Nixon PJ., Diner BA. (2007) Participation of the C-terminal region of the D1-polypeptide in the first steps in the assembly of the Mn4Ca cluster of photosystem II. J. Biol. Chem. **282**, 7209-7218

Cormann KU., Bangert JA., Ikeuchi M., Roegner M., Stoll R., Nowaczyk MM. (2009) Structure of Psb27 in solution: implications for transient binding to photosystem II during biogenesis and repair. Biochemistry **48**, 8768-8770

D'Andrea LD. and Regan L. (2003) TPR proteins: the versatile helix. Trends Biochem. Sci. **28**, 655-662

Dobáková M., Sobotka R., Tichý M., Komenda J. (2009) Psb28 is involved in the biogenesis of the photosystem II inner antenna CP47 (PsbB) in the cyanobacterium *Synechocystis* sp. PCC6803. Plant Physiol. **149**, 1076-1086

Drath M., Baier K., Forchhammer K. (2009) An alternative methionine aminopeptidase, MAP-A, is required for nitrogen starvation and high-light acclimation in the cyanobacterium *Synechocystis* sp. PCC 6803. Microbiology **155**, 1427-1439

Duehring U., Irrgang KD., Lunser K., Kehr J., Wilde A. (2006) Analysis of photosynthetic complexes from a cyanobacterial ycf37 mutant. Biochim. Biophys. Acta **1757**, 3-11

Duehring U., Ossenbuehl F., Wilde A. (2007) Late assembly steps and dynamics of the cyanobacterial photosystem I. J. Biol. Chem. **282**, 10915-10921

Felder S., Meierhoff K., Sane AP., Meurer J., Driemel C., Pluecken H., Klaff P., Stein B., Westhoff P. (2001) The nucleus-encoded *HCF107* gene of *Arabidopsis* provides a link between inter-cistronic RNA processing and the accumulation of translation-competent *psbH* transcripts in chloroplasts. Plant Cell **13**, 2127-2141

Ferreira KN., Iverson TM., Maghlaoui K., Barber J., Iwata S. (2004) Architecture of the photosynthetic oxygen-evolving center. Science **303**, 1831-1838

Fromme P., Kern J., Loll B., Biesiadka J., Saenger W., Witt HT., Krauss N., Zouni A. (2002) Functional implications on the mechanism of the function of photosystem II including water oxidation based on the structure of photosystem II. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **357**, 1337-1344

Fuhrmann E., Gathmann S., Rupprecht E., Golecki J., Schneider D. (2009) Thylakoid membrane reduction affects the photosystem stoichiometry in the cyanobacterium *Synechocystis* sp. PCC 6803. Plant Physiol. **149**, 735-744

Fulda S., Huang F., Nilsson F., Hagemann M., Norling B. (2000) Proteomics of *Synechocystis* sp. strain PCC6803. Identification of periplasmic proteins in cell growth at low and high salt concentrations. Eur. J. Biochem. **267**, 5900-5907

Fujita S., Inagaki N., Yamamoto Y., Taguchi F., Matsumoto A., Satoh K. (1995) Identification of the carboxyl terminal processing protease for the D1 precursor protein of the photosystem II reaction center of spinach. Plant Cell Physiol. **36**, 1169-1177

Goebl M. and Yanagida M. (1991) The TPR snap helix: a novel protein repeat motif from mitosis to transcription. Trends. Biochem. Sci. **16**, 173-177

Golbeck JH. (2003) The binding of cofactors to photosystem I analyzed by spectroscopic and mutagenic methods. Annu. Rev. Biophys. Biomol. Struct. **32**, 237-256

Gomez Maqueo Chew A. and Bryant DA. (2007) Chlorophyll biosynthesis in bacteria: the origin of structural and functional diversity. Annu. Rev. Microbiol. **61**, 113-129

Giglione C., Vallon O., Meinnel T. (2003) Control of protein life-span by N-terminal methionine excision. EMBO J. **22**, 13-23

Grigorieva GA. and Shestakov SV. (1982) Transformation in the cyanobacterium *Synechocystis* PCC6803. FEMS Microbiol. Lett. **13**, 367-370

Hankamer B., Morris E., Nield J., Carne A., Barber J. (2001) Subunit positioning and transmembrane helix organisation in the core dimer of photosystem II. FEBS Lett. **504**, 142-151

He Q. and Vermaas W. (1998) Chlorophyll a availability affects *psbA* translation and D1 precursor processing in vivo in *Synechocystis* sp. PCC 6803. Proc. Natl. Acad. Sci. U S A **95**, 5830-5835

Hinterstoisser B., Cichna M., Kuntner O., Peschek GA. (1983) Cooperation of plasma and thylakoid membranes for the biosynthesis of chlorophyll in cyanobacteria: the role of thylakoid centers. J. Plant Physiol. **142**, 407-413

Hippler M., Rimbault B., Takahashi Y. (2002) Photosynthetic complex assembly in *Chlamydomonas reinhardtii*. Protist **153**, 197-220

Hirano T., Kinoshita N., Morikawa K., Yanagida M. (1990) Snap helix with knob and hole: essential repeats in *S. pombe* nuclear protein nuc2+. Cell **60**, 319-328

Hoffman-Falk H., Mattoo AK., Marder JB., Edelman M., Ellis RJ. (1982) General occurrence and structural similarity of the rapidly synthesized, 32,000-dalton protein of the chloroplast membrane. J. Biol. Chem. **257**, 4583-4587

Huesgen PF., Schuhmann H., Adamska I. (2009) Deg/HtrA proteases as components of a network for photosystem II quality control in chloroplasts and cyanobacteria. Res. Microbiol. (epub ahead of print)

Ivleva NB., Shestakov SV., Pakrasi HB. (2000) The carboxyl-terminal extension of the precursor D1 protein of photosystem II is required for optimal photosynthetic performance of the cyanobacterium *Synechocystis* sp. PCC 6803. Plant Physiol. **124**, 1403-1411

Jansén T., Kanervo E., Aro EM., Mäenpää P. (2002) Localisation and processing of the precursor form of photosystem II protein D1 in *Synechocystis* 6803. J. Plant Physiol. **159**, 1205-1211

Joyard J., Teyssier E., Miege C., Berny-Seigneurin D., Marechal E., Block MA., Dorne AJ., Rolland N., Ajlani G., Douce R. (1998) The biochemical machinery of plastid envelope membranes. Plant Physiol. **118**, 715-723

Kaneko T., Sato S., Kotani H., Tanaka A., Asamizu E., Nakamura Y., Miyajima N., Hirosawa M., Sugiura M., Sasamoto S., Kimura T., Hosouchi T., Matsuno A., Muraki A., Nakazaki N., Naruo K., Okumura S., Shimpo S., Takeuchi C., Wada T., Watanabe A., Yamada M., Yasuda M., Tabata S. (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res. **3**, 109-136

Kaneko T. and Tabata S. (1997) Complete genome structure of the unicellular cyanobacterium *Synechocystis* sp. PCC6803. Plant Cell Physiol. **38**, 1171-1176

Kashino Y., Lauber WM., Carroll JA., Wang Q., Whitmarsh J., Satoh K., Pakrasi HB. (2002) Proteomic analysis of a high active photosystem II preparation from the cyanobacterium *Synechocystis* sp. PCC6803 reveals the presence of novel polypeptides. Biochemistry **41**, 8004-8012

Keren N., Liberton M., Pakrasi HB. (2005) Photochemical competence of assembled photosystem II core complex in cyanobacterial plasma membrane. J. Biol. Chem. **280**, 6548-6553

Keren N., Ohkawa H., Welsh EA., Liberton M., Pakrasi HB. (2005) Psb29, a conserved 22-kD protein, functions in the biogenesis of photosystem II complexes in *Synechocystis* and *Arabidopsis*. Plant Cell **17**, 2768-2781

Klinkert B., Ossenbuehl F., Sikorski M., Berry S., Eichacker LA., Nickelsen J. (2004) PratA, a periplasmic tetratricopeptide repeat protein involved in biogenesis of photosystem II in *Synechocystis* sp. PCC6803. J. Biol. Chem. **279**, 48620-48629

Komenda J., Reisinger V., Mueller BC., Dobáková M., Eichacker LA. (2004) Accumulation of the D2 protein is a key regulatory step for assembly of photosystem II reaction center complex in *Synechocystis* PCC6803. J. Biol. Chem. **279**, 48620-48629

Komanda J., Tichý M., Eichacker LA. (2005) The PsbH protein is associated with the inner antenna CP47 and facilitates D1 processing and incorporation into PSII in the cyanobacterium *Synechocystis* PCC 6803. Plant Cell Physiol. **46**, 1477-1483

Komenda J., Barker M., Kuviková S., de Vries R., Mullineaux CW., Tichý M., Nixon PJ. (2006) The FtsH protease slr0228 is important for quality control of photosystem II in the thylakoid membrane of *Synechocystis* sp. PCC 6803. J. Biol. Chem. **281**, 1145-1151

Komenda J., Nickelsen J., Tichý M., Prášil O., Eichacker LA., Nixon PJ. (2008) The cyanobacterial homologue of the HCF136/YCF48 is a component of an early assembly complex and is important for both the efficient assembly and repair of photosystem II in *Synechocystis* sp. PCC6803. J. Biol. Chem. **283**, 22390-22399

Kunkel DD. (1982) Thylakoid centers: structures associated with the cyanobacterial photosynthetic membrane system. Arch. Microbiol. **133**, 97-99

Kurisu G., Zhang H., Smith JL., Cramer WA. (2003) Structure of the cytochrome b6f complex of oxygenic photosynthesis: tuning the cavity. Science **302**, 1009-1014

Kuvikova S., Tichý M., Komenda J. (2005) A role of the C-terminal extension of the photosystem II D1 protein in sensitivity of the cyanobacterium *Synechocystis* PCC6803 to photoinhibition. Photochem. Photobiol. Sci. **4**, 1044-1048

Liberton M., Berg RH., Heuder J., Roth R., Pakrasi HB. (2006) Ultrastructure of the membrane systems in the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. Protoplasma **227**, 129-138

Loll B., Kern J., Saenger W., Zouni A., Biesiadka J. (2005) Towards complete cofactor arrangement in the 3.0 A resolution structure of photosystem II. Nature **438**, 1040-1044

Masuda T. and Takamiya, K. (2004) Novel insights into the enzymology, regulation and physiological functions of light-dependent protochlorophyllide oxidoreductase in angiosperms. Photosynth. Res. **81**, 1-29

Mattoo AK., Pick V., Hoffman-Falk H., Edelman M. (1981) The rapidly metabolized 32,000-dalton polypeptide of the chloroplast is the “proteinaceous shield” regulating photosystem II electron transport and mediating diuron herbicide sensitivity. Proc. Natl. Acad. Sci. USA **78**, 1572-1576

Menke W. (1962) Structure and chemistry of plastids. Annu. Rev. Plant Physiol. **13**, 27-44

Meskauskiene R., Nater M., Goslings D., Kessler F., op den Camp R., Apel K. (2001) FLU: A negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. PNAS **98**, 12826-12831

Meurer J., Pluecken H., Kowallik KV., Westhoff P. (1998) A nuclear-encoded protein of procaryotic origin is essential fort he stability of the photosystem II in *Arabidopsis thaliana*. EMBO J. **17**, 5286-5297

Naver H., Boudreau E., Rochaix JD. (2001) Functional studies of Ycf3: its role in assembly of photosystem I and interactions with some of its subunits. Plant Cell **13**, 2731-2745

Nelson N. and Ben-Shem A. (2004) The complex architecture of oxygenic photosynthesis. Nat. Rev. Mol. Cell Biol. **5**, 971-982

Nelson N. and Yocum CF. (2006) Structures and functions of photosystems I and II. Annu. Rev. Plant Biol. **57**, 521-565

Nevo R., Charuvi D., Shimoni E., Schwarz R., Kaplan A., Ohad I., Reich Z. (2007) Thylakoid membrane perforations and connectivity enable intracellular traffic in cyanobacteria. EMBO J. **26**, 1467-1473

Nickelsen J., Nowaczyk MM., Klinkert B. (2007) Function of genetic material: assembly factors of the photosynthetic machinery in cyanobacteria. Prog. Botany **68**, 57-79

Nierzwicki-Bauer SA., Balkwill DL., Stevens jr. SE. (1983) Three-dimensional ultrastructure of a unicellular cyanobacterium. J. Cell Biol. **97**, 713-722

Nixon PJ., Trost JT., Diner BA. (1992) Role of the carboxy terminus of polypeptide D1 in the assembly of a functional water-oxidizing manganese cluster in photosystem II of the cyanobacterium *Synechocystis* sp. PCC 6803: assembly requires a free carboxyl group at C-terminal position 344. Biochemistry **31**, 10859-10871

Nixon PJ., Barker M., Boehm M., de Vries R., Komenda J. (2004) FtsH-mediated repair of the photosystem II complex in response to light stress. J. Exp. Bot. **56**, 357-363

Nowaczyk MM., Hebeler R., Schlodder E., Meyer HE., Warscheid B., Roegner M. (2006) Psb27, a cyanobacterial lipoprotein, is involved in the repair cycle of photosystem II. Plant Cell **18**, 3121-3131

Ohad I., Kyle DJ., Hirschberg J. (1985) Light-dependent degradation of the Qb-protein in isolated pea thylakoids. EMBO J. **4**, 1655-1659

Ossenbuehl F., Inaba-Sulpice M., Meurer J., Soll J., Eichacker LA. (2006) The *Synechocystis* sp. PCC6803 Oxa1 homolog is essential for membrane integration of reaction center precursor protein D1. Plant Cell **18**, 2236-2246

Ozawa S., Nield J., Terao A., Stauber EJ., Hippler M., Koike H., Rochaix JD., Takahashi Y. (2009) Biochemical and structural studies of the large Ycf4-photosystem I assembly complex of the green alga *Chlamydomonas reinhardtii*. Plant Cell **21**, 2424-2442

Park S., Khamai P., Garcia-Cerdan JG., Melis A. (2007) REP27, a tetratricopeptide repeat nuclear-encoded and chloroplast-localized protein, functions in D1/32-kD reaction center protein turnover and photosystem II repair from photodamage. Plant Physiol. **143**, 1547-1560

Peng L., Ma J., Chi W., Guo J., Zhu S., Lu Q., Lu C., Zhang L. (2006) LOW PSII ACCUMULATION 1 is involved in efficient assembly of photosystem II in *Arabidopsis thaliana*. Plant Cell **18**, 955-969

Peschek GA., Hinterstoisser B., Wastyn M., Kuntner O., Pineau B., Missbichler A., Lang J. (1989) Chlorophyll precursors in the plasma membrane of a cyanobacterium, *Anacystis nidulans*. J. Biol. Chem. **264**, 11827-11832

Pluecken H., Mueller B., Grohmann D., Westhoff P., Eichacker LA. (2002) The HCF136 protein is essential for assembly of the photosystem II reaction center in *Arabidopsis thaliana*. FEBS Lett. **532**, 85-90

Porra RJ. (1997) Recent progress in porphyrin and chlorophyll biosynthesis. Photochem. Photobiol. **65**, 492-516

Rippka R., Deruelles J., Waterbury JB., Herdman M., Stanier RY. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111**, 1-61

Roose JL. and Pakrasi HB. (2004) Evidence that D1 processing is required for manganese binding and extrinsic protein assembly into photosystem II. J. Biol. Chem. **279**, 45417-45422

Roose JL. and Pakrasi HB. (2007) The Psb27 protein facilitates manganese cluster assembly in photosystem II. J. Biol. Chem. **283**, 4044-4050

Rowe JD. And Griffiths WT. (1995) Protochlorophyllide reductase in photosynthetic prokaryotes and its role in chlorophyll synthesis. Biochem. J. **311**, 417-424

Ruediger W., Hedden P., Koest HP., Chapman DJ. (1977) Esterification of chlorophyllide by geranylgeranyl pyrophosphate in a cell-free system from maize shoots. Biochem. Biophys. Res. Commun. **74**, 1268-1272

Ruf S., Koessel H., Bock R. (1997) Targeted inactivation of a tobacco intron-containing open reading frame reveals a novel chloroplast-encoded photosystem I-related gene. J. Cell Biol. **139**, 95-102

Saenger W., Jordan P., Krauss N. (2002) The assembly of protein subunits and cofactors in photosystem I. Curr. Opin. Struct. Biol. **12**, 244-254

Sarcina M. and Mullineaux CW. (2004) Mobility of the IsiA chlorophyll-binding protein in cyanobacterial thylakoid membranes. J. Biol. Chem. **27**, 36514-36518

Sarcina M., Bouzovitis N., Mullineaux CW. (2006) Mobilization of photosystem II induced by intense red light in the cyanobacterium *Synechococcus* sp. PCC7942. Plant Cell **18**, 457-464

Sato S., Shimoda Y., Muraki A., Kohara M., Nakamura Y., Tabata S. (2007) A large-scale protein protein interaction analysis in *Synechocystis* sp. PCC6803. DNA Res. **14**, 207-216

Satoh K. and Yamamoto Y. (2007) The carboxyl-terminal processing of precursor D1 protein of the photosystem II reaction center. Photosynth. Res. **94**, 203-215

Shestakov SV., Anbudurai PR., Stanbekova GE., Gadzhiev A., Lind LK., Pakrasi HB. (2004) Molecular cloning and characterization of the ctpA gene encoding a carboxyl-terminal processing protease. Analysis of a spontaneous photosystem II-deficient mutant strain of the cyanobacterium *Synechocystis* sp. PCC 6803. J. Biol. Chem. **269**, 19354-19359

Sikorski RS., Boguski MS., Goebl M., Hieter P. (1990) A repeating amino acid motif in CDC23 defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis. Cell **60**, 307-317

Sobotka R., Duehring U., Komenda J., Peter E., Gardian Z., Tichý M., Grimm B., Wilde A. (2008) Importance of the cyanobacterial Gun4 protein for chlorophyll metabolism and assembly of photosynthetic complexes. J. Biol. Chem. **283**, 25794-25802

Spence E., Sarcina M., Ray N., Moller SG., Mullineaux CW., Robinson C. (2003) Membrane-specific targeting of green fluorescent protein by the Tat pathway in the cyanobacterium *Synechocystis* PCC6803. Mol. Microbiol. **48**, 1481-1489

Spence E., Bailey S., Nenninger A., Moller SG., Robinson C. (2004) A homologe of Albino3/OxaI is essential for thylakoid biogenesis in the cyanobacterium Synechocystis sp. PCC6803. J. Biol. Chem. **279**, 55792-55800

Srivastava R., Battchikova N., Norling B., Aro EM. (2006) Plasma membrane of *Synechocystis* PCC6803: a heterogeneous distribution of membrane proteins. Arch. Microbiol. **185**, 238-243

Stroebel D., Choquet Y., Popot JL., Picot D. (2003) An atypical haem in the cytochrome b(6)f complex. Nature **426**, 413-418

Susek RE., Ausubel FM., Chory J. (1993) Signal transduction mutants of Arabidopsis uncouple nuclear CAB and RBCS gene expression from chloroplast development. Cell **74**, 787-799

Suzuki YJ. and Bauer CE. (1995) A prokaryotic origin for light-dependent chlorophyll biosynthesis in plants. Proc. Natl. Acad. Sci. USA **92**, 3749-3753

Teakle GR. and Griffiths WT. (1993) Cloning, characterization and import studies on protochlorophyllide reductase from wheat (*Triticum aestivum*). Biochem. J. **296**, 225-230

Uniacke J. and Zerges W. (2007) Photosystem II assembly and repair are differentially localized in *Chlamydomonas*. Plant Cell **19**, 3640-3654

Vaistij FE., Boudreau E., Lemaire SD., Goldschmidt-Clermont M., Rochaix JD. (2000) Characterization of Mbb1, a nucleus-encoded tetratricopeptide-like repeat protein required for expression of the chloroplast *psbB*/*psbT*/*psbH* gene cluster in *Chlamydomonas reinhardtii*. PNAS **97**, 14813-14818

van de Meene AML., Hohmann-Marriott MF., Vermaas WFJ., Roberson RW. (2006) The three-dimensional structure of the cyanobacterium *Synechocystis* sp. PCC6803. Arch. Microbiol. **184**, 259-270

van Wijk KJ., Roobol-Boza M., Kettunen R., Andersson B., Aro EM. (1997) Synthesis and assembly of the D1 protein into photosystem II: processing of the C-terminus and identification of the initial assembly partners and complexes during photosystem II repair. Biochemistry **36**, 6178-6186

Vavilin DV. and Vermaas WFJ. (2002) Regulation of the tetrapyrolle biosynthetic pathway leading to heme and chlorophyll in plants and cyanobacteria. Physiol. Plant. **115**, 9-24

Vothknecht U. and Soll J. (2005) Chloroplast membrane transport: Interplay of prokaryotic and eukaryotic traits. Gene **354**, 99-109

Wegener KM., Welsh EA., Thornton LE., Keren N., Jacobs JM., Hixson KK., Monroe ME., Camp II DG., Smith RD., Pakrasi HB. (2008) High sensitivity proteomics assisted discovery of a novel operon involved in the assembly of photosystem II, a membrane protein complex. J. Biol. Chem. **283**, 27829-27837

Westphal S., Heins L., Soll J., Vothknecht UC. (2001) *Vipp1* deletion mutant of *Synechocystis*: a connection between bacterial phage shock and thylakoid biogenesis? PNAS **98**, 4243-4248

Wettern M., Ohad I. (1984) Light-induced turnover of thylakoid polypeptides in *Chlamydomonas reinhardtii*. Isr. J. Bot. **33**, 253-263

Wilde A., Haertel H., Huebschmann T., Hoffmann P., Shestakov SV., Börner T. (1995) Inactivation of a *Synechocystis* sp. strain PCC 6803 gene with homology to conserved chloroplast open reading frame 184 increases the photosystem II-to-photosystem I ratio. Plant Cell **7**, 649-658

Wilde A., Lunser K., Ossenbuehl F., Nickelsen J., Boerner T. (2001) Characterization of the cyanobacterial ycf37: mutation decreases photosystem I content. Biochem. J. **357**, 211-216

Wilde A., Mikolajczyk S., Alawady A., Lokstein H., Grimm B. (2004) The *gun4* gene is essential for cyanobacterial porphyrin metabolism. FEBS Lett. **571**, 119-123

Xiong J. and Bauer CE. (2002) Complex evolution of photosynthesis. Annu. Rev. Plant Biol. **53**, 503-521

Zak E., Norling B., Maitra R., Huang F., Andersson B., Pakrasi HB. (2001) Initial Stepps of biogenesis of cyanobacterial photosystems occur in plasma membranes. Proc. Natl. Acad. Sci. USA **98**, 13443-13448

Zouni A., Lueneberg C., Fromme P., Schubert WD., Saenger W., Witt HT. (1998) Characterization of crystals of photosystem II from the thermophilic cyanobacterium Synechococcus elongates. In Photosynthesis: Mechanisms and Effects, g. Garab, ed. (Dordrecht, Kluwer), pp. 925-928

Zouni A., Jordan R., Schlodder E., Fromme P., Witt HT. (2000) First photosystem II crystals capable of warer oxidation. Biochim. Biophys. Acta **1457**, 103-105

List of abbrevations

2D-PAGE	two dimensional polyacrylamid gelelectrophorese
A	Ångström
ALA	5-aminolevulinic acid
ATP	adenosine triphosphate
BC	Biogenesis center
Ca	calcium
CFP	cyan fluorescent protein
CP47	PSII antenna protein CP47
CP43	PSII antenna protein CP43
C-termiuns	carboxyl terminus
CtpA	carboxyl-terminal processing protease
Cyt-b559	subunit cytochrome b559 of the cytochrome b_6f-complex
D1	PSII reaction center protein D1
D2	PSII reaction center protein D2
DPOR	light-independent protochlorophyllide oxidoreductase
FRAP	fluorescence recovery after photobleaching
GFP	green fluorescent protein
GUN	genome uncoupled
HCF	high clorophyll fluorescence
kDa	Kilodalton
Mg	magnesium
Mn	manganese
NADP	nicotinamide adenine dinucleotide phosphate
N-terminus	amino terminus
OEC	oxygen-evolving complex
ORF	open reading frame
PDM	PratA-defined membrane
Pitt	POR-interacting TPR protein
PM	plasma membrane
POR	light-dependent protochlorophyllide oxidoreductase
PratA	processing-associated TPR protein A
PSI	photosystem I

PSII	photosystem II
PCC	Pasteur culture collection
pD1	precursor protein of D1
Q_A	primary electron carrier in PSII
Q_B	secondary electron carrier in PSII
RC	reaction center
RC47	reaction center lacking CP43
TC	Thylakoid center
TM	thylakoid membrane
TPR	tertratricopeptide-repeat
T-zones	translation zones
VIPP	vesicle-inducing protein in plastids 1
Ycf	hypothetical chloroplast ORF

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Schottkowski M., Gkalympoudis S., Tzekova N., Stelljes C., Schuenemann D., Ankele E., Nickelsen J. (2009) Interaction of the periplasmic PratA factor and the PsbA (D1) protein during biogenesis of photosystem II in *Synechocystis* sp. PCC6803. J. Biol. Chem 284, 1813-1819

Ehrenwörtliche Versicherung

Ich versichere hiermit ehrenwörtlich, dass die vorgelegte Dissertation von mir selbstständig und ohne erlaubte Beihilfe angefertigt wurde.

München, den

Marco Schottkowski

Erklärung

Hiermit erkläre ich

- dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.

- dass ich mich **nicht** anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

München, den

Marco Schottkowski

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