Psb28 Protein Is Involved in the Biogenesis of the Photosystem II Inner Antenna CP47 (PsbB) in the Cyanobacterium Synechocystis sp. PCC 6803

Marika Dobáková, Roman Sobotka, Martin Tichý, and Josef Komenda*

Institute of Microbiology, Academy of Sciences, 37981 Třeboň, Czech Republic; and Institute of Physical Biology, University of South Bohemia, 37333 Nové Hrady, Czech Republic

The role of the Psb28 protein in the structure and function of the photosystem II (PSII) complex has been studied in the cyanobacterium Synechocystis sp. PCC 6803. The protein was localized in the membrane fraction and, whereas most of the protein was detected as an unassembled protein, a small portion was found in the PSII core complex lacking the CP43 antenna (RC47). The association of Psb28 with RC47 was further confirmed by preferential isolation of RC47 from the strain containing a histidine-tagged derivative of Psb28 using nickel-affinity chromatography. However, the affinity-purified fraction also contained a small amount of the unassembled PSII inner antenna CP47 bound to Psb28-histidine, indicating a structural relationship between Psb28 and CP47. A psb28 deletion mutant exhibited slower autotrophic growth than wild type, although the absence of Psb28 did not affect the functional properties of PSII. The mutant showed accelerated turnover of the D1 protein, faster PSII repair, and a decrease in the cellular content of PSI. Radioactive labeling revealed a limitation in the synthesis of both CP47 and the PSI subunits PsaA/PsaB in the absence of Psb28. The mutant cells contained a high level of magnesium protoporphyrin IX methylester, a decreased level of protochlorophyllide, and released large quantities of protoporphyrin IX into the medium, indicating inhibition of chlorophyll (Chl) biosynthesis at the cyclization step yielding the isocyclic ring E. Overall, our results show the importance of Psb28 for synthesis of Chls and/or apoproteins of Chl-binding proteins CP47 and PsaA/PsaB.
from Arabidopsis (Kashino et al., 2002). This protein was designated as Psb28 (also Psb13 or ycf79). Its amino acid sequence suggests that it is a rather hydrophilic protein without a transmembrane helix and is larger than PsbW (about 13 kD). In the recent crystal structures of the cyanobacterial PSII (Ferreira et al., 2004; Loll et al., 2005), this protein was not identified and it remains an issue of contention whether the protein is a true PSII subunit, a transiently associated assembly factor, or just an impurity of the preparation. The relatively low content of this protein in the isolated preparation suggested that the two latter possibilities are more probable. Very recently, the protein has been detected as a component of PSII complexes in *Synechocystis* depleted of phosphatidylglycerol (Sakurai et al., 2007). It has been proposed that the protein may play a regulatory role during the assembly of PSII. A gene encoding a similar soluble protein has also been found in the genome of Arabidopsis and the protein was designated PsbW-like.

Here, we present a detailed analysis of the role of Psb28 in the structure and function of PSII in *Synechocystis* 6803. The results showed that Psb28 is not a component of the fully assembled dimeric PSII core complex, but it is preferentially bound to PSII assembly intermediates containing the inner antenna CP47. The results support the role of the protein in biogenesis of certain Chl-binding proteins via regulating synthesis of their apoproteins or Chls.

RESULTS

**Psb28 Protein Is Associated with the PSII Assembly Complexes Containing CP47**

Psb28 was previously identified as a minor component of PSII complexes purified using nickel-affinity chromatography from the strain expressing Histagged CP47 (Kashino et al., 2002). To provide more rigorous information about the localization of Psb28, we first screened for its presence in the membrane-associated and soluble protein fractions of a *Synechocystis* whole-cell extract using specific antibodies raised against the last 15 amino acid residues of the protein (Fig. 1A). The vast majority of the protein was found in the membrane fraction. However, the protein was only loosely bound as documented by removal of >90% of the protein by treatment of the membranes with 1 M CaCl₂. Furthermore, the protein was completely washed out from membranes by 0.1 M sodium carbonate or 0.1 M sodium hydroxide. Ninety percent of the protein was removed by treatment with trypsin, which acts only on the cytoplasmic side of this type of membrane preparation, cleaving the D1 protein into specific fragments (Komenda et al., 2002). Thus, Psb28 is the protein peripherally associated with the cytoplasmic side of the membranes. To identify the membrane-binding site of Psb28, we analyzed membrane protein complexes of wild type using two-dimensional (2D) separation of membrane proteins consisting of blue native (BN)-PAGE in one direction and denaturing PAGE in the second direction (2D BN/
SDS-PAGE). The majority (80%–90%) of Psb28 was detected at the end of the BN gel in the low-molecular-weight region, indicating that it is not assembled into any large membrane complex (Fig. 1C, blot). However, a small portion of the protein was found in the position corresponding to the migration of a PSII core subcomplex lacking the inner antenna CP43 (termed RC47), while the dimeric and monomeric core complexes RCC(1) and RCC(2) were seemingly free of this subunit. Because the wild type contains only a small amount of RC47, Psb28 bound to this complex was almost undetectable. Therefore, we also analyzed PSII complexes in the phycobiliprotein-free strain called PAL (Ajlani and Vernotte, 1998), in the site-directed mutant D1-Asn-359His (Kuviková et al., 2005), and in the psbC/slr0228 double-deletion mutant ΔCP43/ΔSlr0228 (Komenda et al., 2006). PAL and D1-Asn-359His are autotrophic mutants with a high RC47:RCC ratio and the nonautotrophic ΔCP43/ΔSlr0228 deletion mutant accumulates a high level of RC47 as the only PSII complex due to the missing genes for CP43 and a FtsH protease involved in regulating the level of PSII assembly intermediates (Komenda et al., 2006). 2D analysis confirmed the presence of Psb28 in RC47 of PAL and the protein was apparent even in the stained gel (Fig. 2A, arrow). Its identity was verified by de novo sequencing of the band using mass spectrometry. In the site-directed mutant D1-Asn-359His, western blotting confirmed the presence of the protein in RC47, but a small amount of Psb28 was also found in the monomeric core complex RCC(1) (Fig. 2B). The Psb28 protein was also clearly detected in RC47 of the ΔCP43/ΔSlr0228 mutant (Fig. 2C). In both latter mutants, the band of unassembled Psb28 exhibited typical smearing approximately starting in the gel region in which the unassembled CP47 is located. This finding raised the question of whether these two proteins might form an unstable complex that is released during native electrophoresis (see below).

Further evidence for the preferential binding of Psb28 to RC47 was obtained using a strain in which the original psb28 gene was replaced by a His-tagged copy. Thylakoids from this Psb28-His/ΔPsb28 strain were solubilized with dodecyl maltoside (DM) and the extract was loaded on the nickel-affinity chromatography column. Bound proteins were analyzed by 2D BN/SDS-PAGE (Fig. 3A) and immunoblotting using antibodies against Psb28, D1, CP47, and CP43 (Fig. 3B). The majority of Psb28-His was present as free protein, but a significant amount was found in RC47. The isolated preparation also contained a small amount of RCC(1). Interestingly, a significant fraction of Psb28-His was associated with the unassembled CP47 confirming the previous indications obtained by analysis of the mutants D1-Asn-359His and ΔCP43/ΔSlr0228.

Investigation of the Psb28 content in several mutants differing in the content of PSII complexes again showed good correlation between Psb28 and CP47. In the mutant ΔCP43, which assembles only RC47 due to the deleted psbC gene, the decreased amount of Psb28 corresponded to about 20% to 30% of the wild-type level of CP47 (Fig. 4A). In the strain lacking CP47, the Psb28 became undetectable. Interestingly, in the absence of a small PSII subunit PsbH, which is bound to CP47, the level of Psb28 was also strongly decreased suggesting the requirement of PsbH for binding of Psb28 to CP47 (Fig. 4A).

The relationship between CP47 and Psb28 was further verified by constructing two double mutants in which the deletion of the psb28 gene was performed in strains lacking the psbC gene and the psbEFLJ operon. In the psbC deletion strain ΔCP43, additional inactiva-
of the psb28 gene led to a 50% decrease in the RC47 level and the unassembled CP47 became almost undetectable (Supplemental Fig. S1). Because the radioactive labeling of D1 was similar in both strains, we assume that the lower level of RC47 is caused by low availability of the newly synthesized CP47 in the absence of Psb28. Accordingly, in the psbEFLJ/psb28 double mutant ΔCYT/ΔPsb28, which accumulates the unassembled CP47, but no other CP47-containing PSI complexes (Komenda et al., 2004), the deletion of the psb28 gene led to a substantial decrease in the steady-state level of the unassembled CP47, whereas the accumulation of the unassembled D1 was increased (Supplemental Fig. S2).

Absence of Psb28 Leads to the Low Cellular Content of PSI But Does Not Affect the Function and Repair of PSII

To elucidate the role of Psb28 in the structure and function of PSII, we characterized the psb28 single deletion mutant (ΔPsb28) in which the psb28 gene was replaced with a zeocin-resistance cassette. The mutant exhibited slower photoautotrophic growth in comparison with wild type and contained a lower cellular level of Chl, especially under low light conditions. However, its light-saturated rate of the overall photosynthesis on a per cell basis was similar to that in wild type (Table I) and the rates of QA reoxidation measured in the presence and absence of the PSII inhibitor.

Figure 3. Analysis of the PSII complexes isolated from the Psb28-His/ΔPsb28 strain. PSII complexes isolated by nickel-affinity chromatography from the Psb28-His/ΔPsb28 strain expressing the His-tagged Psb28 were analyzed by 2D BN/SDS-PAGE and proteins were blotted onto a PVDF membrane, stained (blot stain), and probed with antibodies against CP47, D1, and Psb28. The complex of CP47 and Psb28-His is designated by the arrow.

Figure 4. Accumulation of Psb28 in several PSII mutants differing in accumulation and stability of CP47 (A), level of PSII and PSI in cells of wild-type (WT) and ΔPsb28 analyzed either by western blotting (B) or by 77K Chl fluorescence emission spectra (C). A, Membranes from WT, psbH deletion mutant ΔPsbH, psbC deletion mutant ΔCP43, and psbB deletion mutant ΔCP47 were analyzed by denaturing SDS-PAGE and CP47, D1 and Psb28 were detected using specific antibodies. Correct protein loading was shown by immunodetection with PsaD-specific antibody. 1, 0.5, and 0.25 μg of Chl were loaded onto the gel for each sample. B, Membranes from WT and the psb28 deletion mutant ΔPsb28 grown in the presence of Glc were analyzed by denaturing SDS-PAGE and CP47, CP43, D2, D1, Psb28, and PsaD were detected by specific antibodies. Correct protein loading was shown by protein staining of α- and β-subunits of ATP synthase (AtpAB). 2, 1, and 0.5 μg of Chl were loaded onto the gel for each sample. C, 77K fluorescence spectra of WT cells (solid line) and ΔPsb28 (dashed line) cultivated in the presence of Glc were obtained using an Aminco Bowman Series 2 luminescence spectrometer (Spectronic Unicam). The same number of cells for each strain was excited at 435 nm, spectra were corrected for the sensitivity of the photomultiplier, and normalized to 570 nm (the fluorescence peak of rhodamine used as an internal standard).
Psb28, as well as thermoluminescence characteristics, were identical in wild type and ΔPsb28 (data not shown). These results showed that Psb28 is not important for the function of the fully assembled photochemically active PSII complexes and it corresponded well with the absence of the protein in the dimeric PSII core complex. On the other hand, the rate of PSII-mediated oxygen evolution in the mutant cells largely exceeded that in wild-type cells and this rate was even higher when measured on the per Chl basis, suggesting a decreased level of PSI in the mutant cells (Table I). This was confirmed by a semiquantitative western blot (Fig. 4B), which showed 20% to 40% higher PSII protein levels in the mutant (shown for all four large PSII proteins D1, D2, CP43, and CP47) when the gel was loaded on the basis of Chl content. Because about 80% of Chl in Synechocystis is bound in PSI, the blot did not show a significant decrease in the level of the PSI protein PsAD in the mutant. The results of the protein analyses were supported by the 77K Chl fluorescence emission spectra of cells from both strains (Fig. 4C). The spectra were taken using the same optical density of cells and were normalized using rhodamine as the internal standard (Sobotka et al., 2008). The significantly lower fluorescence maximum of PSI at 725 nm in mutant cells unequivocally confirmed the lower cellular level of PSI.

Because Psb28 was recognized as a component of RC47 in which the selective replacement of D1 most probably takes place (Komenda et al., 2004, 2006), we were interested in whether the absence of Psb28 affects D1 turnover. Pulse-chase experiments (Fig. 5) showed that the turnover of D1 was slightly accelerated in the absence of Psb28. Pulse-chase labeling revealed a half-life of D1 in wild type of about 2.5 h, whereas in the mutant ΔPsb28 it was approximately 2 h. To find out whether the resistance of PSII photochemistry to photodamage was also similar in both strains, we evaluated the time course of light-induced inhibition of oxygen evolution in wild-type and mutant cultures subjected to white light of 500 μmol photons m⁻² s⁻¹, either in the absence or presence of the protein synthesis inhibitor lincomycin. No differences in the decline of oxygen evolution were observed between wild type and ΔPsb28 during both measurements (Fig. 6A). Recovery from photoinhibition under low light conditions was even faster in ΔPsb28 than in wild type as proven by assessment of oxygen evolution in photo-inhibited cells of wild type and ΔPsb28 during subsequent incubation at 50 μmol photons m⁻² s⁻¹ (Fig. 6B). The results showed that the oxygen-evolving PSII complexes of the mutant are equally sensitive to light-induced inactivation as the complexes in wild type, whereas the repair of PSI seems to be slightly more efficient, corresponding with the accelerated turnover of D1 observed in the strain.

We were also interested in whether the absence of Psb28 affects levels of PSII proteins under extreme light conditions (2,000 μmol photons m⁻² s⁻¹). In wild-type cells, these conditions did not lead to significant changes in the content of PSII proteins, whereas in the ΔPsb28 strain a decline of about 50% of the level of D1 was observed (Supplemental Fig. S3A). We also compared the rate of PSII degradation in wild type and ΔPsb28 under conditions where protein synthesis was stopped by lincomycin (Supplemental Fig. S3B). No apparent differences were observed between both strains in the rate of degradation of D1, D2, CP43, CP47, and the α-subunit of cyt b-559. In the wild-type strain, the Psb28 protein was also degraded, whereas the PSI protein PsAD was stable again, confirming the relationship between Psb28 and PSI.

### Table I. Characteristics of the Synechocystis 6803 wild type and the psb28 deletion mutant ΔPsb28 grown at irradiance 10 (LL) or 40 (ML) μmol photons m⁻² s⁻¹

| Strains          | Autotrophic Doubling Timea | Cellular Chl Contemb | Photosynthetic Oxygen Evolution (OD)c | PSI Oxygen Evolution (OD)d | PSI Oxygen Evolution (Chle) |
|------------------|---------------------------|----------------------|--------------------------------------|---------------------------|-----------------------------|
| Wild type LL     | 18.3 ± 0.1                | 2.59 ± 0.05          | 993 ± 151                            | 588 ± 89                  | 216 ± 35                    |
| Wild type ML     | 8.3 ± 0.1                 | 2.54 ± 0.07          | 1,150 ± 191                          | 935 ± 55                  | 374 ± 21                    |
| ΔPsb28 LL        | 22.8 ± 0.2                | 1.60 ± 0.06          | 945 ± 141                            | 1,283 ± 59                | 830 ± 54                    |
| ΔPsb28 ML        | 11.4 ± 0.1                | 1.87 ± 0.04          | 1,020 ± 185                          | 1,353 ± 141               | 719 ± 72                    |

aData in time measured in microtitration plates; means of 10 measurements ± sd; initial OD750 nm of the cultures was 0.005. bChl content in micrograms per OD750, in the cells of both strains cultivated in the presence of Glc; means of three measurements ± sd. cLight-saturated rate of oxygen evolution in nanomoles O₂ per OD750 nm and hours in cells of both strains cultivated in the presence of Glc; means of three measurements ± sd. dLight-saturated rate of oxygen evolution in nanomoles O₂ per OD750 nm and hours in cells of both strains cultivated in the presence of Glc and measured in the presence of 1 mM p-benzoquinone and 5 mM potassium ferricyanide; means of three measurements ± sd. eLight-saturated rate of oxygen evolution in micromoles O₂ per mg Chl and hours in cells of both strains cultivated in the presence of Glc and measured in the presence of 1 mM p-benzoquinone and 5 mM potassium ferricyanide; means of three measurements ± sd.
7). In wild type, at least 10% of D1 was present in the RC47, whereas in ΔPsb28 this complex was almost absent. In wild type, about 5% of overall CP47 was found in the fraction of unassembled proteins (Fig. 7, arrow 1), whereas no free CP47 was found in ΔPsb28. In agreement with the semiquantitative western blot, the overall amount of the stained PSII proteins in the gel loaded on the basis of Chl content was higher in the mutant than in wild type.

Autoradiograms obtained from the same 2D gels showed that, in wild type, the D1 protein was preferentially labeled in both RCC(2) and RCC(1). There was also apparent labeling of D1 in RC47, but not in the RCa complex containing D1, D2, cyt b-559, and PsbI (Fig. 7, autorad; see also Dobáková et al., 2007). In contrast, in membranes of ΔPsb28 about 20% of the labeled D1 was detected in the RCa complex and 30% in the fraction of unassembled proteins (Fig. 7, autorad, arrow 2). Our previous analyses showed that the presence of the RCa complex and unassembled D1 protein in various Synechocystis mutants indicates that the PSII assembly is limited by availability of CP47.
Figure 7. 2D analysis of membrane protein complexes of wild type (WT) and the psb28 deletion strain ΔPsb28. Membranes isolated from radioactively labeled cells of WT and ΔPsb28 grown in the presence of Glc were analyzed by 2D electrophoresis. The obtained gels were stained and exposed to a phosphor imager plate. Complexes are designated as in Figure 1. RCa is a reaction center complex containing D1 and D2, but no CP43 and CP47 inner antennae. pD1 and iD1 indicate the unprocessed and partially processed forms of D1, respectively. Arrows designate important differences between the strains in the accumulation of CP47 (1) and RCa (2); and in the labeling of PSI subunits PsaA/PsaB (3). Each loaded sample contained 6 μg of Chl.

(Komenda et al., 2004, 2005; Dobáková et al., 2007). In agreement with this interpretation, the labeled unassembled CP47 was present in wild type, but absent in ΔPsb28. Surprisingly, the absence of Psb28 also negatively affected the synthesis of a pair of large Chl-binding subunits, the PsaA/PsaB heterodimer of PSI. The radioactive labeling of this heterodimer in wild type exceeded that in ΔPsb28 by several times (Fig. 7, autorad, arrow 3). Overall, the results of radioactive labeling suggested that Psb28 is needed for the efficient synthesis of Chl proteins CP47 and PsaA/PsaB.

To explain the lower Chl level and most probably the related limitation in synthesis of CP47 and PsaA/PsaB observed in the ΔPsb28 mutant, precursor/degredation products of the Chl biosynthetic pathway were quantified in cells of each strain. As shown in Table II, mutant cells contained about 20-fold higher level of protoporphyrin IX than wild type and a substantial amount of this pigment was released by the ΔPsb28 cells into the growth medium (approximately 0.58 mg of protoporphyrin IX in 1 L of cell culture at approximately 0.5 OD750 in comparison with 0.057 found in wild-type culture). Level of magnesium (Mg) protoporphyrin IX monomethyl ester was also highly increased in the mutant cells; in contrast, accumulation of later Chl precursors, protochlorophyllide and chlorophyllide, reached only about 60% to 70% of the wild-type levels. This lower accumulation corresponds well with the total Chl level in the mutant, indicating that the insufficient synthesis of protochlorophyllide limits Chl availability in the ΔPsb28 strain. Consistently, the level of pheophorbide, a product of Chl catabolism, was found to be similar in the mutant and the wild type, suggesting that Chl degradation is not enhanced by deletion of the psb28 gene (Table II). The data suggested that, in the absence of Psb28, synthesis of Chl is significantly less efficient and this inefficiency could be directly related to the inhibition of synthesis of CP47 and PsaA/PsaB. Interestingly, synthesis of the other Chl proteins CP43 and D2 was not affected by the absence of Psb28 and synthesis of D1 was even more pronounced (see Fig. 7; Supplemental Fig. S2).

DISCUSSION
Role of Psb28 in the Structure and Function of PSII

The Psb28 protein has been previously detected as a nonstoichiometric component of the PSII preparation purified using nickel-affinity chromatography from a Synechocystis strain expressing His-tagged CP47 (Kashino et al., 2002). Please note that this purification method pulls down all PSII complexes containing CP47-His, including RCC(2), RCC(1), RC47, and unassembled CP47 and therefore it is not clear to what PSII complex Psb28 actually binds. Very recently,

| Strain                     | Wild Type | ΔPsb28 |
|----------------------------|-----------|--------|
| % of wild type             |           |        |
| Protoporphyrin IX          | 100 ± 18  | 2,340 ± 340 |
| Mg-protoporphyrin IX       | 100 ± 12  | 157 ± 19   |
| Mg-protoporphyrin IX monomethyl ester | 100 ± 21 | 670 ± 82   |
| Protochlorophyllide         | 100 ± 13  | 64 ± 8    |
| Chlorophyllide             | 100 ± 32  | 68 ± 12   |
| Chl                        | 100 ± 9   | 72 ± 6    |
| Pheophorbide               | 100 ± 22  | 119 ± 9   |

Table II. Analysis of components of the Chl biosynthesis/degradation pathway in the wild-type strain and the ΔPsb28 mutant
Sakurai et al. (2007) have also detected the protein in the isolated monomeric PSII complex. However, this complex was obtained by glycerol density gradient centrifugation, which in our hands has insufficient separation ability to resolve RCC(1) and RC47; therefore, the putative monomeric PSII most probably contained both complexes. Here, we show that Psb28 binds preferentially to RC47 and only the very small amount of Psb28 was occasionally found in the monomeric RCC(1) complex. The presence of Psb28 in RCC(1) was typical for strains containing a large amount of RC47, indicating that the protein most probably remains attached for a limited time after binding of CP43 to RC47. However, we have never found the protein in the dimeric complex RCC(2), which is considered to be the native, fully functional competent form of PSII (Barber, 2006). In accordance with this localization, the presence of Psb28 in RCC(1) complexes might be waiting for repair and therefore do not contribute to the overall activity.

The strain expressing His-tagged Psb28 allowed us to preferentially isolate the RC47 complex. However, the eluate from the chromatographic column also contained a complex of Psb28-His with unassembled CP47. Because no RC complexes or unassembled D1 or D2 was detected in the preparation, this complex could not originate from disassembly of RC47 and we assume that it represents a native complex present in vivo. On the other hand, on 2D gels we never observed the complex of CP47 with the nontagged Psb28. However, in the strains with high levels of unassembled CP47 like D1-Asn-359His, the unassembled Psb28 protein is found as a diffuse band smearing from the gel region approximately corresponding to the mobility of the unassembled CP47, suggesting that the CP47-Psb28 complex exists, but is unstable and falls apart during native electrophoresis. We propose that CP47 and Psb28 form a rather unstable precomplex, which is stabilized by the presence of the His-tag attached to Psb28. In this respect, it is interesting that the level of Psb28 is significantly decreased in the mutant lacking the PsbH protein (Fig. 3A), which was previously shown to be essential for the binding of Hli proteins to CP47 (Promnares et al., 2006). We have hypothesized that the Psb28 binding site on CP47 is located in the vicinity of PsbH. Unfortunately, we were not able to verify this proposal using nitrilotriacetic acid-gold labeling of Psb28-His because the protein is present mostly in RC47, which is too small for successful single particle analysis using electron microscopy.

Role of Psb28 Protein in the Synthesis of Chl and Chl-Binding Proteins

The inhibition of the CP47 and PsaA/PsaB synthesis in the psb28 deletion mutant is paralleled by the perturbation in the Chl biosynthesis manifested by the accumulation of intermediates preceding protoporphyrin formation. There are two possible explanations for this observation: (1) Psb28 is required for efficient synthesis of CP47 apoprotein or for Chl binding to it and in its absence the newly synthesized Chl is not consumed and its biosynthesis is stopped at the stage of formation of the isocyclic ring E; (2) Psb28 is needed for the final stage of biosynthesis of Chl that is utilized for synthesis of CP47. The first possibility assuming the direct action of Psb28 on the CP47 apoprotein synthesis seems to be improbable because it assumes the regulatory effect of the unused Chl at one of the latest stages of the biosynthesis pathway. Biosynthesis of heme is known to be regulated at early stages by the end product of the pathway (i.e. heme) in order to prevent accumulation of potentially toxic intermediates and similar mechanism seems to regulate the synthesis of Chl in plants (Meskauskiene et al., 2001). As Mg protoporphyrin monomethyl ester is converted to protoporphyrin by a poorly understood aerobic cyclase enzyme (Minamizaki et al., 2008), the second explanation would presume a direct or indirect role of Psb28 in the cyclase reaction. In this respect, it is interesting that we have detected a substantial amount of the putative aerobic cyclase encoded by the sll1214 gene (Minamizaki et al., 2008), as well as protoporphyrin oxidoreductase in the purified PSII core complex (data not shown). We therefore hypothesize that the Psb28 protein assists the efficient conversion of Mg protoporphyrin monomethyl ester into Chl, which is immediately bound to the CP47 apoprotein.

Although it has been clearly documented that the Psb28 protein is associated with PSI, the absence of Psb28 was also manifested by the limited synthesis of the PsaA/PsaB heterodimer of PSI and by the consequent decrease in the cellular content of PSI. This result suggested the existence of a link between the synthesis of CP47 and PsaA/PsaB. On the other hand, the absence of Psb28 did not affect the synthesis of D2 and CP43 and paradoxically resulted in the acceleration of synthesis and degradation of another PSII Chl-binding protein D1 as documented by the fast PSII repair (Figs. 5 and 6) and by the radioactive protein labeling (Fig. 7). Recent results showing the association of so-called high light-induced proteins with PSI (Promnares et al., 2006; Yao et al., 2007) and their presumable role in the temporary binding of Chl during the PSII repair-related D1 replacement (Vavilin et al., 2007) indicate that Chl molecules released from...
the degraded D1 can be reused. It is possible that the lack of the newly synthesized CP47 allows more Chl precursors to be utilized for the synthesis of Chl consumed during selective D1 turnover. Binding of the newly synthesized Chl would suppress Chl reutilization as an expected rate-limiting step of the repair process. Consequently, the PSI repair becomes faster in the absence of Psb28.

The differences among various Chl-binding proteins in the response to the partial inhibition of Chl synthesis could be explained by their different affinity for Chl. We have recently reported the selective inhibition of Chl synthesis in comparison to other PSI subunits in a Synechocystis Chl-deficient mutant lacking the Gun4 protein required for Mg chelatase activity (Sobotka et al., 2008). However, this mutant contains only about 20% of the wild-type level of Chl, whereas the Psb28-less strain contains at least 60% of the wild-type level, but still the synthesis of CP47 is selectively (at least as concerns PSI1 proteins) inhibited. The different interpretation of these results could be based on the branching and spatial separation of the Chl biosynthesis pathway at the final stages of the process, most probably at the formation of the Chl-specific E ring. In this way, only the branch terminating at CP47 and possibly Psaa/Psab could be affected in the ΔPsb28 strain, whereas the other branch ending at D2 and CP43 remains unaffected or, as in the case of the D1 protein, is even accelerated due to the increased availability of the cyclase substrate. The data from Arabidopsis indicating the localization of the Chl cyclase in both the envelope membrane as well as in thylakoids support this view (Tottey et al., 2003).

**MATERIALS AND METHODS**

**Construction and Cultivation of Cyanobacterial Strains**

The strains used in the study were derived from the Glc-tolerant strain of Synechocystis 6803 (Williams, 1988) referred to as wild type. The following, previously described strains were used in the study: (1) the CP43-less strain ΔCP43 (Vermaas et al., 1988); (2) the CP47 deletion strain ΔCP47 with the pshB gene replaced with the kanamycin resistance cassette; (3) the ΔCTY strain with the pshEF1 operon replaced with a kanamycin resistance cassette (Pakrasi et al., 1998); (4) the pshB deletion mutant ΔpshB with the psh1 gene replaced with a kanamycin resistance cassette (Komenda and Barber, 1995); (5) phycobiliprotein-free strain PAL (Aji and Vermote, 1998); (6) the site-directed mutant D1-Asn-359His (Kaviková et al., 2005); and (7) the pslC sbr0223 double deletion mutant ΔCP43/Δsbr0223 (Komenda et al., 2006).

The Δpsb28 strain was prepared by the replacement of most of the psb28 gene (nucleotides 18-298) with a zeocin resistance cassette using a mega-primer PCR method (Burke and Barik, 2003). We have adapted this method to generate linear deletion constructs containing upstream and downstream regions of the psb28 gene with the zeocin resistance cassette in the middle. In the first step, upstream and downstream regions of psb28 were separately amplified using long fusion primers complementary to the psb28 gene in one direction and the zeocin cassette in the other: (1) 5’-ACATTAATTGGTGGCGCTCTCACGCAAAATGTTACGATCC-3’ and 5’-AACCTAAATCTTCGGGCGGCCAACAAATGTTACGATCC-3’ (the psb28 part is underlined). These fusion primers were used in pairs with psb28 forward and reverse primers: (1) 5’TGGTCCTACGCGATCCGC-3’ and 2) 5’GATTTTGGTCTACGCG-3’. In the second step, the zeocin resistance cassette (Streptovollteichus hindustanus; Invitrogen) was amplified using PCR products from the first step as primers. Finally, the complete deletion construct was amplified using psb28 forward and reverse primers and used for transformation of Synechocystis 6803 cells. Transformants were selected and segregated on zeocin-containing agar plates; their full segregation was confirmed by PCR.

Multiple psb28 deletion strains were obtained by transformation of single mutants lacking pshB and pshEF1 genes using chromosomal DNA from Δpsb28 and their selection was based on additional resistance to zeocin. The Psb28-His strain expressing Psb28 tagged with 6×His at its N terminus under control of the psbA2 promoter (Psb28-His/Δpsb28) was constructed using the pSBa plasmid (Lagarde et al., 2000) in a procedure analogous to that described by Tichy et al. (2003). The resulting strain synthesizing both wild-type and His-tagged forms of Psb28 was transformed with chromosomal DNA from the psb28 deletion mutant and selected by resistance to zeocin. The complete deletion of the psb28 wild-type copies in the Psb28-His strain was confirmed by PCR.

Liquid cultures were grown in 100 to 200 mL of BG11 containing 5 mM Glc using 500-mL Erlenmeyer flasks, aerated using an orbital shaker, irradiated with 30 μmol photons m⁻² s⁻¹ of white light at 29°C, and used when they reached a Chl concentration of about 5 μg mL⁻¹. Solid medium contained in addition 10 mM TES/NaOH, pH 8.2, 1.5% agar, and 0.3% sodium thiosulfate (Pakrasi et al., 1998).

Fluorescence experiments were performed with cells cultivated in double-wall, thermoregulated cultivation cylinders (internal diameter of 35 mm). Here, the culture was maintained at a Chl concentration of 6 to 8 μg mL⁻¹ by regularly diluting with approximately 10 mL of BG11 medium every 150 min. The culture was bubbled with air containing 2% (v/v) CO2 and illuminated with white light at 40 μmol photons m⁻² s⁻¹ at 29°C. For the large-scale cultivation used for isolation of PSI1 complexes, cultures were grown in 10-L flasks (culture volume 6–8 L), stirred by a magnetic stirrer, and bubbled with air.

Measurements of autotrophic growth rates were performed in microtitration plates (culture volume 0.25 mL) under intensive shaking. Optical densities at 750 nm were measured every 6 h using a microplate reader (Tecan Sunrise). Values plotted against time were used for calculation of the doubling time.

**Fluorometric and Polarographic Methods**

The Fv/Fm parameter and kinetics of Chl variable fluorescence decay were measured in dark-adapted cultures (2.5 μg Chl mL⁻¹) using a modulation PAM101 fluorometer (Walz) with an ED-101US cuvette and the dual-modulation kinetic fluorometer (Photon Systems Instruments), respectively (Tichy et al., 2003). The light-saturated steady-state rate of oxygen evolution in cell suspensions was measured polarographically in BG11 medium containing 10 mM HEPES/NaOH, pH 7.0, using 0.5 mM benzobromarone and 1 mM potassium ferricyanide as artificial electron acceptors.

**Preparation of Membranes and Their Protein Analyses**

Cyanobacterial membranes were prepared by breaking the cells using glass beads (Komenda and Barber, 1995) with the following modifications: Cells were washed, broken, and finally resuspended in 25 mM MES/NaOH, pH 6.5, containing 10 μM CaCl₂, 10 μM MgCl₂, and 25% glycerol. The large-scale isolation of PSI complexes for chromatographic purification was performed using a similar procedure in which the cells were resuspended in 20 mL of thylakoid buffer (25 mM MES, pH 6.5, 100 mM NaCl) containing the protease inhibitor cocktail (Roche), the same volume of glass beads was added and the cells were broken eight times for 15 s in the smallest bead-beater container (Biopspec Products) with 5-min interruption for cooling on ice. Glass beads were subsequently removed by filtering and thylakoids were obtained by differential centrifugation.

For separation of membrane and soluble fractions, the broken cells were pelleted (20,000g, 15 min) and the sediment (membrane fraction) was resuspended in the original volume of 25 mM MES/NaOH, pH 6.5, containing 10 μM CaCl₂, 10 μM MgCl₂, and 25% glycerol. The supernatant represented the soluble fraction.

To distinguish membrane-embedded and membrane-associated proteins, the membranes (5 μg Chl) were spun down and resuspended in 25 mM Tris/HCl, pH 7.0, containing 1 mM CaCl₂, 0.1 mM Na₂CO₃ or 0.1 mM NaOH. The suspensions were left on ice with occasional mixing for 4 h, then spun down, resuspended in 25 mM Tris/HCl, pH 7.0, containing 1 mM Suc, and analyzed by SDS-PAGE. The tryptic digestion of thylakoids was performed as described in

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Komenda et al. (2002). After incubation for 4 h, the protease was inhibited with 2 mM Pefabloc SC.

The protein complexes isolated from the thylakoid membranes were solubilized with DM (DM/Chl = 40 [w/w]) and analyzed by BN electrophoresis at 4°C in a 5% to 14% gradient polyacrylamide gel according to Schagger and von Jagow (1991). Samples with the same Chl content (6 μg for gel staining and 1 μg for western blot) were loaded onto the gel.

The protein composition of the complexes was analyzed by electrophoresis in a denaturing 12% to 20% linear gradient polyacrylamide gel containing 7 M urea (Komenda et al., 2002). Complete lanes from the native gel were excised, incubated for 30 min in 25 mM Tris/HCl, pH 7.5, containing 1% SDS (w/v), and placed on top of the denaturing gel; two lanes were analyzed in a single denaturing gel. Proteins separated in the gel were either stained by Coomassie Blue or transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with specific primary antibodies and then with secondary antibody-horseradish peroxidase conjugate (Sigma). The primary antibodies used in the study were raised in rabbits against (1) residues 58 to 86 of the spinach (Spinacia oleracea) D1 polypeptide; (2) the last 12 residues of the D2 polypeptide from Synechocystis 6803; (3) residues 380 to 394 of barley (Hordeum vulgare) CP47; (4) whole isolated CP43 from Synechocystis 6803; and (5) the last 15 C-terminal residues of the Psb28 protein from Synechocystis 6803. For autoradiography, the gel or the membrane with labeled proteins was exposed to x-ray film at laboratory temperature for 2 to 3 d or to a phosphor imager plate (GE Healthcare) overnight.

Samples with the same Chl content were used for the direct comparison and quantification of stained or labeled proteins and were run on a single gel. Quantification of bands was performed using ImageQuant 5.2 software (GE Healthcare).

Radioactive Labeling of Cells

For radioactive labeling, cells containing 75 μg of Chl were resuspended and shaken in 250 μL of BG11 in a microcentrifuge tube for 30 min under illumination with 60 μmol photons m⁻² s⁻¹ and then a mixture of [³⁵S]Met and [³⁵S]Cys (Trans-label; MP Biochemicals) was added (final specific activity 400 μCi mL⁻¹). The suspension was exposed to light at irradiance and temperature indicated and afterward the cells were frozen in liquid nitrogen and used for isolation of thylakoids.

Isolation of Complexes Containing Psb28-His

PSII complexes containing Psb28-His were isolated from thylakoids of the Psb28-His/CYPs28 strain using affinity chromatography on immobilized Ni²⁺-chelated (5) the last 15 C-terminal residues of the Psb28 protein from Synechocystis 6803. For the measurement of Chl concentration, sedimented cells or membranes were resuspended in 0.1% of NH₄OH. Combined supernatants (approximately 1 mL in total) were filtered through a 0.22-μm filter and 100 μL were immediately subjected to HPLC analysis using a Vydac 210TP54 column (250 mm × 4.6 mm, C-18 reversed-phase silica gel). The column was eluted with a linear gradient from 0% of solvent A (methanol and 0.5 M ammonium acetate, 4.6 [v/v]) to 60% of solvent B (methanol and acetone, 64 [v/v]) over 4 min followed by a linear gradient of solvent B. 100% of solvent B was reached after 28 min at a flow rate of 1.1 mL/min at 35°C. HPLC peaks corresponding to protoporphyrin IX, Mg-protoporphyrin IX, Mg-protoporphyrin IX monomethyl ester, protoclorophyllide, and chlorophyllide were identified from their absorption spectra and from comparison with authentic standards (Sigma Aldrich; Frontier Scientific). HPLC fractions containing Chl intermediates were collected and concentrations of the corresponding compounds determined fluorometrically using a Spectronic Unicam series 2 spectrophotometer. Phosphorbidine was quantified spectroscopically.

Analysis of Pigments in Growth Medium

For identification and quantification of tetrpyrroles precipitated in growth medium, a 4-μm cellulose filter from the previous paragraph was frozen in liquid nitrogen, powdered in a 2-mL Eppendorf tube, and dissolved in 1 mL of alkaline acetone. Acetone was then extracted with 400 μL of hexane to remove Chl and presence of tetrpyrroles was analyzed spectrophotoscopically and fluorometrically. Protoporphyrin IX was quantified spectrophotoscopically using an authentic standard (Sigma).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. 2D BN/SDS-PAGE of radioactively labeled membrane proteins from psbc deletion mutant ΔCYPs3 and the double mutant ΔCYPs3/ΔPsb28.

Supplemental Figure S2. 2D BN/SDS-PAGE of radioactively labeled membrane proteins from psbEFL1 deletion mutant ΔCYT1 and the double mutant ΔCYT1/ΔPsb28.

Supplemental Figure S3. Levels of PSII proteins in cells of wild type and ΔPsb28 exposed to high light in the absence and presence of lincomycin.

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