Thylakoid Membrane Reduction Affects the Photosystem Stoichiometry in the Cyanobacterium *Synechocystis* sp. PCC 6803[1][C]

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Biogenesis of thylakoid membranes in both chloroplasts and cyanobacteria is largely not understood today. The vesicle-inducing protein in plastids 1 (Vipp1) has been suggested to be essential for thylakoid membrane formation in Arabidopsis (*Arabidopsis thaliana*), as well as in the cyanobacterium *Synechocystis* sp. PCC 6803, although its exact physiological function remains elusive so far. Here, we report that, upon depletion of Vipp1 in *Synechocystis* cells, the number of thylakoid layers in individual *Synechocystis* cells decreased, and that, in particular, the content of photosystem I (PSI) complexes was highly diminished in thylakoids. Furthermore, separation of native photosynthetic complexes indicated that PSI trimers are destabilized and the monomeric species is enriched. Therefore, depletion of thylakoid membranes specifically affects biogenesis and/or stabilization of PSI in cyanobacteria.

In chloroplasts and cyanobacteria the energy transfer between PSI and PSII is regulated in a light-dependent manner (for a recent review, see Kramer et al., 2004). The two photosystems are connected by the cytochrome b₆f complex, and electron transfer from PSII via the cytochrome b_6f complex to PSI is believed to be regulated by the redox state of the plastoquinol pool potentially also involving the cytochrome b_6f complex (Fujita et al., 1987; Murakami and Fujita, 1993; Schneider et al., 2001, 2004; Pfannschmidt, 2003; Volkmer et al., 2007). Transfer of light energy to the two photosystems is mediated by light-harvesting complexes, and in cyanobacteria light is harvested by the soluble extramembranous phycobilisomes. The efficient energy transfer to PSI and PSII has to be balanced to synchronize the function of the two photosystems. In response to changing light intensities and qualities, energy coupling between the phycobilisomes and the photosystems changes, which allows a rapid adjustment of light absorbance by the individual photosystems. Furthermore, besides this short-term adaptation mechanism, it has been shown in many studies that on a longer term in cyanobacteria the ratio of the two photosystems changes depending on the light conditions (Manodori and Melis, 1986; Murakami and Fujita, 1993; Murakami et al., 1997). Upon shifting cyanobacterial cells from low-light to high-light growth conditions, the PSI-to-PSII ratio decreases due to selective suppression of the amount of functional PSI. In recent years, some genes have already been identified that are involved in this regulation of the photosystem stoichiometry (Hihara et al., 1998; Sonoike et al., 2001; Fujimori et al., 2005; Ozaki et al., 2007).

Whereas in chloroplasts of higher plants and green algae the amounts of the two photosystems change in response to changing light conditions (Melis, 1984; Chow et al., 1990; Smith et al., 1990; Kim et al., 1993), it has already been noted a long time ago that the chloroplast ultrastructure also adapts to high-light and low-light conditions (Melis, 1984). Chloroplasts of plants grown under low light or far-red light have more thylakoid membranes than chloroplasts of plants grown under high light or blue light (Anderson et al., 1973; Lichtenthaler et al., 1981; Melis and Harvey, 1981). There appears to be a direct correlation between the chlorophyll content and the amount of thylakoids per chloroplast because light harvesting is increased by enhanced chlorophyll and thylakoid membrane content per chloroplast. Thus, chloroplasts adapt to high light both by a reduction of thylakoid membranes and by a decrease in the PSI-to-PSII ratio.

Thylakoid membranes are exclusive features of both cyanobacteria and chloroplasts, and it still remains mysterious how formation of thylakoid membranes is organized. Many cellular processes, like lipid biosynthesis, membrane formation, protein synthesis in the cytoplasm and/or at a membrane, protein transport,

protein translocation, and protein folding have to be organized and aligned for formation of internal thylakoid membranes. The recent observation that deletion of the *vipp1* gene in Arabidopsis (*Arabidopsis thaliana*) results in complete loss of thylakoid membranes has indicated that Vipp1 is involved in biogenesis of thylakoid membranes. Further analysis has suggested that Vipp1 could be involved in vesicle trafficking between the inner envelope and the thylakoid membrane of chloroplasts (Kroll et al., 2001). Because of this, the protein was named Vipp1, for vesicle-inducing protein in plastids 1. Depletion of Vipp1 strongly affected the ability of cyanobacterial cells to form proper thylakoid membranes (Westphal et al., 2001) and, consequently, also in cyanobacteria Vipp1 appears to be involved in formation of thylakoid membranes. A Vipp1 depletion strain of Arabidopsis is deficient in photosynthesis, although the defect could not be assigned to a deficiency of a single photosynthetic complex, but appeared to be caused by dysfunction of the entire photosynthetic electron transfer chain (Kroll et al., 2001). Therefore, depletion of Vipp1 in Arabidopsis seems to affect thylakoid membrane formation rather than the assembly of thylakoid membrane protein complexes (Aseeva et al., 2007). However, for cyanobacteria, it is not clear yet how diminishing the amount of thylakoid membrane layers would affect the amount and stoichiometry of the two photosystems.

Here, we present the generation and characterization of a Vipp1 depletion strain of the cyanobacterium *Synechocystis* sp. PCC 6803. Upon depletion of Vipp1, a decrease in thylakoid membrane pairs in the generated mutant strain and, furthermore, a significant decrease in active PSI centers was observed. Moreover, trimerization of PSI also appeared to be impaired in the mutant strain. These results suggest that thylakoid membrane perturbations caused by the Vipp1 depletion directly affects PSI assembly and stability in cyanobacterial thylakoid membranes.

RESULTS AND DISCUSSION

Whereas it has been suggested that depletion of Vipp1 in Arabidopsis affects thylakoid membrane formation per se, but not the assembly of individual thylakoid membrane protein complexes, it is unclear how depletion of thylakoid membranes would affect the photosystem content and/or stoichiometry in cyanobacteria. Therefore, we have generated and characterized a Vipp1 depletion strain to analyze a potential connection between the amount of thylakoid membranes and the photosystem stoichiometry in *Synechocystis* PCC 6803 more thoroughly.

Disruption of the *vipp1* Gene in *Synechocystis*

Because the physiological function of Vipp1 is still mysterious, we aimed to characterize the physiologi-

cal effects of a Vipp1 depletion in *Synechocystis* in more detail. To this end, the *vipp1* (*sll0617*) gene (Kaneko et al., 1996) was disrupted in *Synechocystis* PCC 6803 by insertion of a kanamycin resistance cassette into a single *Pst*I site of the gene (Fig. 1A). After transformation of *Synechocystis* wild-type cells with the plasmid containing the interrupted gene, complete segregation of the mutant should be obtained by growing the cells for various generations on medium containing increasing amounts of kanamycin. *Synechocystis* contains several identical genome copies and, in each of these, the gene has to be inactivated to obtain a completely
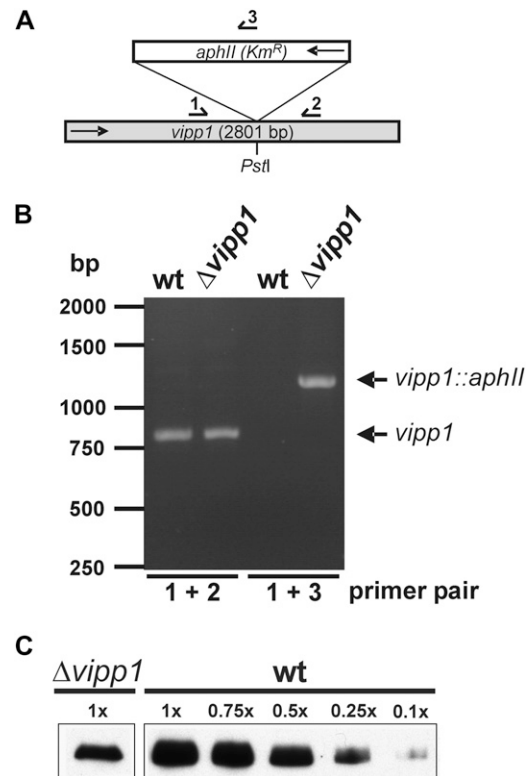

Figure 1. Disruption of *vipp1* in *Synechocystis* sp. PCC 6803. A, Strategy for disruption of *vipp1* in the genome of *Synechocystis* sp. PCC 6803. The *aphII* gene confers resistance to kanamycin (*Km^R*). Arrows indicate the direction of the *aphII* and the *vipp1* gene. Numbered arrows indicate primers, which were used for segregation control (compare B). B, The *vipp1* gene cannot be deleted completely in *Synechocystis*. Amplification of the *vipp1* gene from wild-type (wt) and Δ*vipp1 Synechocystis* genomic DNA resulted only in an amplification of the wild-type fragment (800 bp) and no fragment, which corresponds in size to the interrupted *vipp1* gene (approximately 2,000 bp) was observed. When a primer pair was used, one primer of which anneals to the 5′-end of the *vipp1* gene and the other anneals to the *aphII* gene (compare A), a fragment of about 1,200 bp was amplified, which demonstrates that the *aphII* gene is interrupted in some genomic *vipp1* copies. C, Immunoblot analysis of total cellular extracts from *Synechocystis* wild type and the Vipp1 depletion strain grown at 120 μmol s^{-1} m^{-2}. Five micrograms protein have been separated by SDS-PAGE and subsequently analyzed by immunoblot analysis using an α-Vipp1 antibody. For comparison, for the wild-type cell extract, 3.75, 2.5, 1.25, and 0.5 μg protein have also been analyzed.
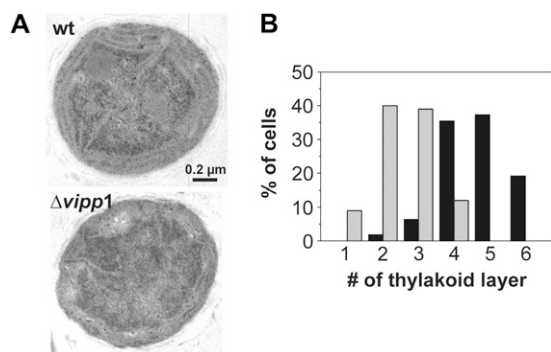
Figure 2. Depletion of Vipp1 results in a *Synechocystis* strain with less thylakoid membranes. A, Representative electron micrographs of *Synechocystis* wild-type (wt) and Vipp1 depleted cells are shown. B, Under the presented growth conditions, the wild-type cells (black bars) had on average four to six layers of thylakoid membranes, whereas the mutant strain (gray bars) had only two to three thylakoid layers. *Synechocystis* cells were grown at 20 μmol/(m^2 s) as described in "Materials and Methods."

segregated disruption strain. To check the segregation state of the *Synechocystis* $\Delta vipp1$ strain, we amplified the *vipp1* gene from genomic *Synechocystis* DNA by PCR. The wild-type *vipp1* gene is approximately 800 bp and the size of the PCR fragment should increase to about 2,000 bp when the *aphII* gene (kanamycin resistance cassette) is introduced into the gene. As can be seen in Figure 1B, besides the wild-type fragment, no fragment corresponding in size to the interrupted *vipp1* gene was amplified by PCR when genomic DNA from the *vipp1* merodiploid strain was used as a template. However, when primers were used, which anneal at the 5'-end of the *vipp1* gene and within the *aphII* gene (see Fig. 1A), a fragment with the expected size was amplified. These observations verify that some of the *vipp1* gene copies are interrupted by the *aphII* gene in the *vipp1* merodiploid strain. However, most of the genomic *vipp1* copies remained noninterrupted and, even after growing the cells for 4 years on selective medium, no completely segregated strain has been obtained. This highly supports the assumption that the *vipp1* gene is essential for cell viability.

Because at least in some genomic copies the *vipp1* gene was interrupted, we tested whether the Vipp1 content in the depletion strain was decreased when compared to wild-type cells. After growing the cells in liquid cultures enriched with 2% CO_2, a significant depletion of Vipp1 in total cellular extracts was observed by immunoblot analysis (Fig. 1C). Based on this analysis, we conclude that the total Vipp1 content in the mutant strain was significantly decreased when compared to wild-type cells.

Because it has been shown that Vipp1 is involved in formation of thylakoid membranes in chloroplasts, we subsequently characterized the ultrastructure of *Synechocystis* wild-type and Vipp1 depleted cells by electron microscopy (EM), and we determined and compared the amount of thylakoid membrane pairs

in the two strains. As can be seen in Figure 2A, after depletion of Vipp1, *Synechocystis* cells contain less thylakoid membrane pairs than the wild-type strain. To subsequently quantify the observed difference in more detail, we determined the number of thylakoid membrane pairs in wild-type and Vipp1 depleted cells. As can be seen in Figure 2B, under the presented growth conditions, the mutant cells contain on average stacks of two to three pairs of thylakoid layers, whereas the wild-type cells contain on average four to five layers of thylakoid membrane pairs. Therefore, depletion of Vipp1 results in a reduction of thylakoid membrane pairs. Nevertheless, it has to be noted that, although we have tested numerous growth conditions, we were not able to generate a *Synechocystis* strain with no organized thylakoids and no photosynthetic activity, in contrast to the results reported in Westphal et al. (2001). We always observed a significant amount of internal thylakoid membranes coupled with photosynthetic activity (compare below). Besides the observed reduction in thylakoid membrane pairs in the *vipp1* merodiploid strain, the EM data further suggest that the overall thylakoid membrane morphology is disturbed in the mutant strain and the thylakoid membranes are less well arranged than in the wild-type strain. This observation further suggests that Vipp1 is involved in thylakoid membrane biogenesis in cyanobacteria and depletion of Vipp1 might result in formation of less well-organized thylakoid membrane pairs (as further discussed below).

Chlorophyll and Photosystem Content in Vipp1 Depleted Cells

For cyanobacterial wild-type cells, it is well established that shifting cyanobacterial cells from low light to higher light intensities results in a general decrease in the amount of the two photosystems in thylakoid membranes, as well as in a specific decrease of the PSI-
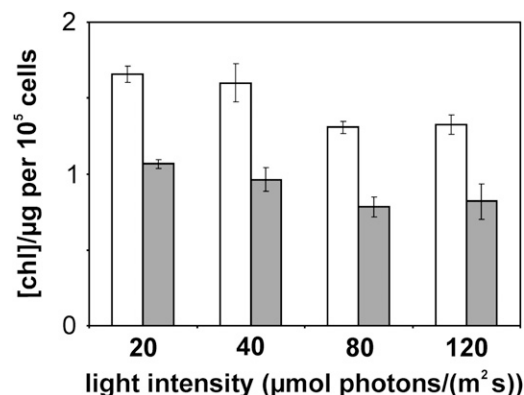

Figure 3. Chlorophyll content in *Synechocystis* wild-type and Vipp1 depleted cells. Chlorophyll content of *Synechocystis* wild-type (light bars) and Vipp1 depleted cells (gray bars) was determined as described in "Materials and Methods" after growing the cells at different light intensities [20, 40, 80, 120 μmol/(m^2 s)]. Chl is shown for 10^5 cells.
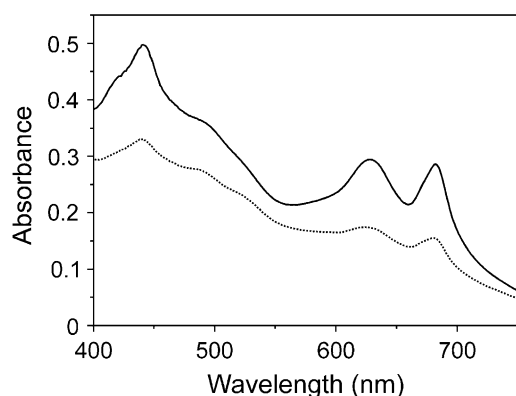
Figure 4. Absorbance spectra of *Synechocystis* wild-type and *vipp1* merodiploid cells. Cell suspensions were diluted to 3×10^5 cells/mL (for details see the text), and spectra were recorded in the range between 350 to 750 nm. Solid line, Wild-type cells; dashed line, *vipp1* merodiploid cells.

to-PSII ratio (see introduction). Therefore, we have determined and compared the chlorophyll content of wild-type and Vipp1 depleted cells at different growth light intensities. As can be seen in Figure 3, the chlorophyll content per cyanobacterial cell significantly decreased in both wild-type and *vipp1* merodiploid cells when the cyanobacterial cells were grown under increasing light intensities. Furthermore, the chlorophyll content per cell was at any light intensity lower in *vipp1* merodiploid cells when compared to the wild-type strain. For a meaningful comparison of the cellular chlorophyll content, we have quantified the amount of *Synechocystis* cells per OD_{750}, and *Synechocystis* cells were counted in a Thoma counting chamber (see "Materials and Methods"). Whereas the overall cell shape and size of the mutant strain appeared not to have changed due to the *vipp1* gene interruption, the amount of cells corresponding to an $OD_{750} = 1$ was significantly altered. An $OD_{750} = 1$ corresponded to 1.98×10^5 cells mL^{-1} for the wild type and to 2.88×10^5 cells mL^{-1} for the mutant strain at identical growth conditions. Thus, at the same OD_{750}, the two cultures contain significantly different amounts of cells. Therefore, a simple normalization of cells based on OD_{750} measurements would have resulted in rather inaccurate data comparison. This observed difference in light scattering was taken into account, and for all further measurements the wild-type and mutant strains were compared on a per-cell basis. The results presented in Figure 3 clearly suggest that the amount of chlorophyll-containing membrane protein complexes (photosystems) decreases with increased light intensities and that in the *vipp1* merodiploid strain the total amount of chlorophyll-containing membrane protein complexes is lower at any tested light intensity when compared to wild-type cells.

To further characterize the effect of the thylakoid membrane perturbation and reduction on pigment-binding membrane protein complexes, absorbance

spectra of whole *Synechocystis* wild-type and Vipp1 depleted cells were recorded using identical amounts of cells (Fig. 4). These spectra further support the observation that in *vipp1* merodiploid cells the cellular concentration of chlorophyll-binding proteins is significantly lower when compared to wild-type cells. Based on the absorbance spectra, the ratio of phycobilisomes to chlorophyll-binding proteins can be estimated (Bennett and Bogorad, 1973). Whereas the pigment content per cell has changed in the mutant strain, the ratio of phycocyanin/chlorophyll, which corresponds to the phycobilisome-to-photosystems ratio, remains largely unchanged (0.51 ± 0.0015 in wild-type cells and 0.53 ± 0.002 in the Vipp1 depletion strain).

The observed decrease in the photosystem content per cell could be caused by a general reduction of the content of both PSI and PSII. However, it is also possible that the cellular concentration of one photosystem is more affected. Because in *Synechocystis* about 90% of all chlorophylls are bound to PSI (Shen et al., 1993), a reduction of PSI, which is often observed when, for example, the photosynthetic electron transfer chain is affected due to mutations (Schneider et al., 2001, 2004; Berry et al., 2002; Volkmer et al., 2007), would result in a more dramatic reduction of the cellular chlorophyll content than reduction of solely PSII. To address the question of whether the cellular content of PSI and PSII is equally affected by the Vipp1 depletion, we have determined the relative amounts and activities of the two photosystems.

The relative amounts of the two photosystems can be estimated by fluorescence emission spectroscopy at 77K. After excitation of chlorophylls at 435 nm, PSI has a maximal fluorescence emission at 725 nm and PSII at 685 and 695 nm. In Figure 5, fluorescence emission spectra of *Synechocystis* wild-type and *vipp1* merodiploid cells measured after growth at different light
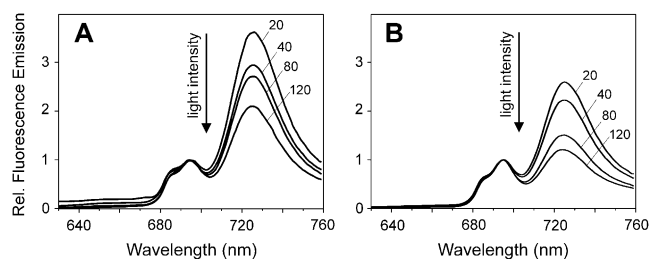

Figure 5. Light-dependent regulation of the PSI-to-PSII ratio in wild-type and *vipp1* merodiploid cells. A and B, 77K fluorescence emission spectra of *Synechocystis* wild-type (A) and *vipp1* merodiploid (B) cells after excitation of chlorophylls at 435 nm. Increase of the growth light intensity [20, 40, 80, 120 μmol/(m^2 s)] results in a decreased PSI-to-PSII ratio in both the wild-type and Vipp1 depletion strain. At each line, the light intensity, at which the cells were grown, is indicated. Under all tested light intensities, the PSI-to-PSII ratio was decreased when compared to wild-type cells grown under identical growth conditions. For better comparability, the spectra were normalized at 695 nm and the fluorescence emission at 695 nm was set at 1.
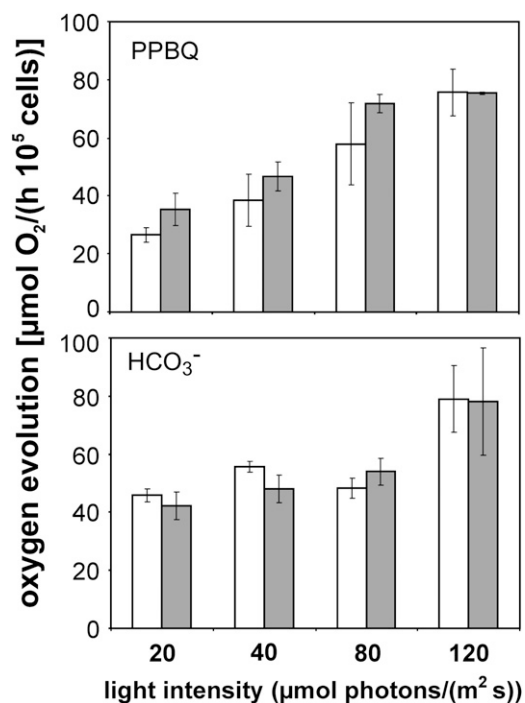
Figure 6. Oxygen evolution of wild-type (light bars) and *vipp1* merodiploid (gray bars) *Synechocystis* cells. Cells were grown at different light intensities and oxygen evolution was measured using either PPBQ (top) or HCO_3^- (bottom) as electron acceptor. The statistical significance of the observed difference in oxygen evolution was calculated by a one-tailed *t* test. For all the observed oxygen evolution rates, the differences between the means were due to chance ($P > 0.05$) and thus statistically not relevant.

intensities are shown. The spectra clearly support the results obtained by UV/VIS spectroscopy and strengthen the assumption that the ratio of PSI to PSII has changed in the mutant when compared to the wild-type strain. The PSI-to-PSII ratio is regulated in a highly dynamic fashion in cyanobacteria and it has been observed that in cyanobacteria the ratio of PSI to PSII depends on the light conditions used for cell growth (Murakami and Fujita, 1993). With increasing light intensities, the amount of PSI is down-regulated, whereas the PSII concentration within membranes remains largely constant. Because of this observation, it appeared possible that reduction of thylakoids resulted in disturbed regulation of the PSI-to-PSII ratio and that the Vipp1 depletion has somehow frozen the cyanobacterial cells in a high light state with less PSI centers in the thylakoid membranes. In wild-type *Synechocystis* cells, the PSI-to-PSII ratio changed as expected, and with increasing light intensities the amount of PSI centers decreased. However, as for the wild type, we also observed similar changes in the PSI-to-PSII ratio in *vipp1* merodiploid cells and also in these cells the ratio of the two photosystems was regulated in a light-dependent manner. When compared to wild-type cells, the relative amount of PSII was higher under all tested growth conditions. Com-

pared to the wild type, either dramatically more PSII centers are present per mutant cell or less PSI. Because the chlorophyll content per cell was significantly decreased in the mutant strain, it appeared unlikely that the amount of PSII was increased, which would have resulted in an increase of chlorophylls per cell. Therefore, we conclude that depletion of thylakoid membranes directly affects accumulation of PSI in *Synechocystis* cells. Nevertheless, the different cellular concentrations of internal thylakoid membranes and chlorophylls could in general result in different reabsorption of fluorescence emission and, therefore, the 77K fluorescence measurements strongly indicated, but not firmly established, a changed PSI-to-PSII ratio. Because of this, we have subsequently determined the activities of PSI and PSII in *Synechocystis* wild-type and Vipp1 depleted cells to further support the spectroscopic observations.

Amount and Activity of PSI and PSII

To determine the activity of PSII, the photosynthetic activity of whole *Synechocystis* cells was measured as described in "Materials and Methods." Wild-type and the *vipp1* merodiploid *Synechocystis* strain were grown under various light intensities and the rate of oxygen evolution was determined using phenyl-*p*-benzoquinone (PPBQ) as an electron acceptor at PSII. When normalized to the amount of cells (see above), these measurements suggest that the PSII activity in the *vipp1* merodiploid strain has not significantly changed at any light intensity when compared to wild-type cells (Fig. 6). Nonetheless, it appears possible that the PSII
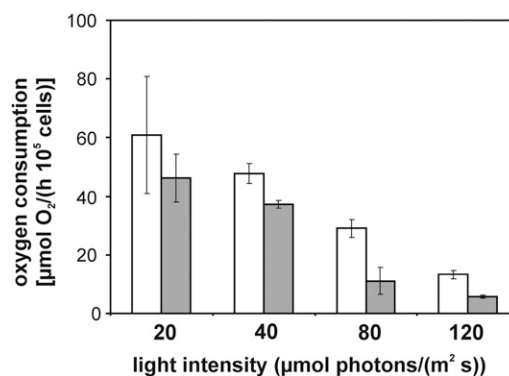

Figure 7. Determination of the PSI activity. PSI activity of *Synechocystis* cells grown at different light intensities was measured as oxygen consumption due to the Mehler reaction (for details, see "Materials and Methods"). Oxygen consumption of wild-type (white bars) and *vipp1* merodiploid (gray bars) cells was normalized to 10^5 cells. The statistical significance of the observed difference in oxygen consumption was calculated by a one-tailed *t* test. For the observed oxygen consumption rates at 40, 80, and 120 μmol photons/(m^2 s), the differences were statistically relevant at a 1% level ($P < 0.01$). Due to the relatively high scattering of the measured rates at 20 μmol photons/(m^2 s), the observed difference between the means were statistically not relevant ($P > 0.05$).
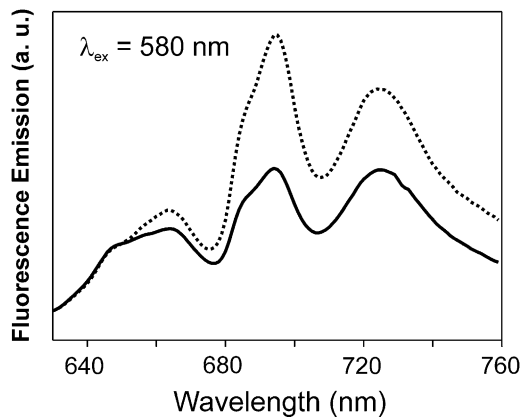
Figure 8. 77K fluorescence emission spectra of *Synechocystis* wild-type (solid line) and Δ*vipp1* cells (dotted line) after excitation of phycobilisomes at 580 nm. Cells were grown at 120 μmol/(m^2 s). The spectra were normalized at 650 nm. a. u., Arbitrary units.

activity is slightly increased per cell in the *vipp1* merodiploid strain at light intensities up to 80 μmol/(m^2 s).

When oxygen evolution at PSII is measured with HCO_3^- as an electron acceptor, the activity of the entire photosynthetic electron transfer chain can be determined. Interestingly, when normalized to the amount of cells, with this acceptor, the oxygen evolution was about the same for the wild type and the mutant strain within the range of the error (Fig. 6). These data further support the assumption that, upon thylakoid membrane reduction, the amount of active PSII per cell is not significantly altered.

To subsequently determine the PSI activity in *Synechocystis* wild-type and mutant cells, oxygen consumption at PSI due to the Mehler reaction was

measured. As can be seen in Figure 7, the activity of PSI was determined to be decreased relative to the wild-type strain at any growth condition in line with the observations described above. Taken together, these measurements indicate reduced PSI content per cell in the *vipp1* merodiploid strain relative to wild-type cells and an unchanged or just marginally increased PSII content. However, even with a reduced amount of PSI, the mutant cells still appear to be able to operate the electron transfer chain at wild-type rates, as shown in Figure 6. Thus, the PSI reduction does not significantly affect the electron transfer chain from water (PSII) via PSI to HCO_3^-.

As discussed above, whereas the overall content of the photosystems has been changed in the mutant strain and this strain contains less PSI, the ratio of phycobilisomes to chlorophyll-containing protein complexes has not changed in the *vipp1* merodiploid strain. To subsequently analyze the energy coupling between phycobilisomes and the two photosystems, 77K fluorescence emission spectra were recorded after excitation of phycobilisomes at 580 nm. After excitation of the phycobilisomes, the fluorescence emission at 685 and 695 nm was increased in the mutant strain relative to the fluorescence emission of phycobilisomes at 650 and 665 nm (Fig. 8), which suggested an increased energy transfer from phycobilisomes to PSII. Because the ratio of phycobilisomes/chlorophyll is largely unchanged in the mutant strain and because only the content of PSI is reduced in the *vipp1* merodiploid cells, the ratio of phycobilisomes to PSII has in fact increased, which is in line with the observed increase in phycobilisomes coupled to the PSII reaction centers (emission at 685 and 695 nm). However, whereas the cellular content of PSI is decreased in the mutant strain, energy transfer from phycobilisomes to
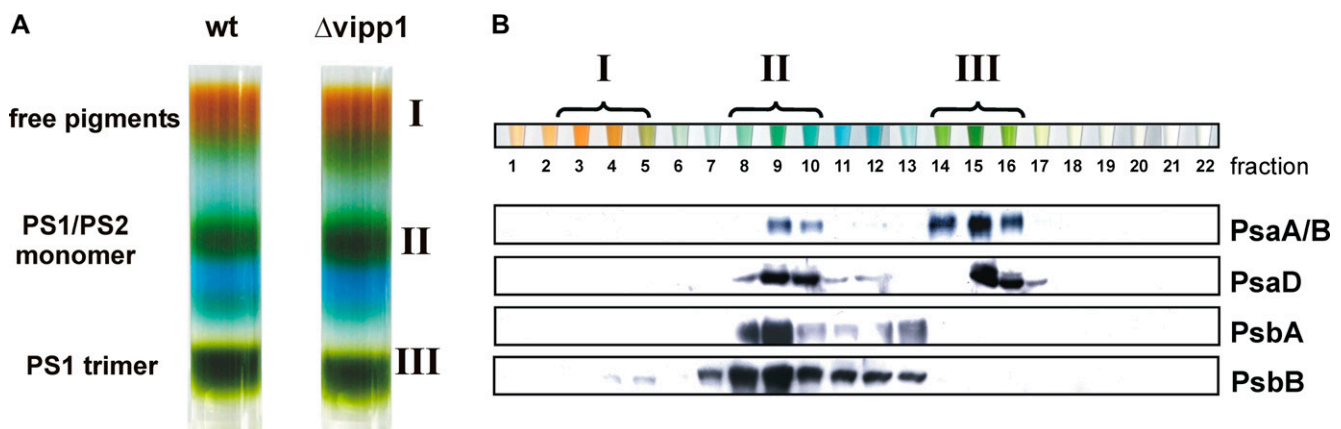

Figure 9. Separation of membrane protein complexes from wild-type and Vipp1 depleted *Synechocystis* cells. A, Membrane proteins from *Synechocystis* wild-type (wt) and Vipp1 depleted cells were solubilized by β-DM and membrane protein complexes were separated on a 10% to 30% Suc density gradient. Three colored bands (I–III) were clearly resolved. In wild-type membranes, band II of the gradient contained about 3.5 times more chlorophyll than band III, whereas in the depletion strain, this ratio was lowered to about 1.8. B, Individual fractions of the gradient from wild-type membranes were analyzed by immunoblot analysis. The individual membrane protein complexes were identified using antibodies directed against PSI (PsaA, B, D) and PSII (PsbA, B) subunits. Fraction 1 corresponds to the top of the Suc gradient.

PSI was also increased when compared to wild-type cells (emission at 720 nm). These observations suggest that, although the ratio of the two photosystems has changed, the *vipp1* merodiploid cells maintain efficient energy coupling between phycobilisomes and PSI. Thus, by an increased energy transfer from phycobilisomes to PSI, the cells may adjust to the decrease in the PSI content and, thereby, retain efficient electron transfer rates, as observed in the whole-cell electron transfer measurements (Fig. 6).

To further characterize the relative amounts of PSI and PSII in the thylakoid membranes of *Synechocystis* wild-type and mutant cells, we have used Suc gradient centrifugation and blue native (BN)-PAGE analyses. These techniques can be used to separate the native photosystems from each other, as well as to analyze the oligomeric state of the photosystems. Whereas PSI forms trimers in cyanobacteria (Kruip et al., 1994), PSII forms dimers (Boekema et al., 1995). However, whereas both photosystems form distinct oligomers in membranes, the monomeric species are also active and so far the physiological role of the oligomerization is largely enigmatic.

As can be seen in Figure 9, when thylakoid membranes of *Synechocystis* wild type and the *vipp1* merodiploid strain were extracted and separated on a 10% to 30% Suc gradient, differences in the photosystem composition can be observed: To compare the relative amounts of monomeric and trimeric PSI, we have determined the chlorophyll concentration of bands II and III of the Suc gradient. The ratio of chlorophylls enclosed in bands II and III of the gradient was about 3.5 for the wild type and lowered to approximately 1.8 for the mutant strain. Thus, the amount of PSI trimers is significantly reduced in membranes isolated from mutant cells when compared to the wild-type situation. In line with a general PSI reduction, the relative amount of monomeric PSII is increased. In contrast to the trimeric PSI species, in the Suc gradient dimeric PSII is almost not resolved. However, the significantly increased intensity of the PSI/PSII monomer band could not only be caused by the apparent increase of PSII relative to PSI, but also by an increase of the monomeric PSI species. Furthermore, in the mutant cells, we observed more free pigments after centrifugation, which might indicate a general destabilization of PSI and/or PSII in this strain. These observations might indicate that not only the amount of PSI is reduced in thylakoid membranes of the Vipp1 depletion strain, but also the ratio of monomeric PSI to trimeric PSI has changed. To further strengthen the assumption that Vipp1 has an impact on PSI trimerization in membranes, we have additionally analyzed the composition of thylakoid membrane proteins by BN-PAGE. As can be seen in Figure 10, when followed by BN-PAGE, the amount of PSI trimers is also reduced in the mutant cells accompanied by an increase in monomeric PSI. For the wild type, the PSI trimer band contained about 2.5 times more PSI than the monomer band, whereas in the depletion strain the

ratio was shifted to about 0.5. Thus, in the mutant strain, the trimeric PSI species is highly destabilized when compared to wild-type membranes. Because the Suc gradients and BN-PAGE gels were loaded on a chlorophyll basis and because in the mutant strain less PSI is present, the relative amount of PSII appears to be increased in the mutant strain when compared to the wild type, in line with the presented spectroscopic observations. Nevertheless, it has to be emphasized that, because, due to technical problems, the gels cannot be loaded on a per-cell basis, this observed relative increase does not reflect the concentrations within a living cell. BN-PAGE, as well as Suc density analysis, only allows comparison of the relative amounts of PSI and PSII in thylakoid membranes. However, besides the observed reduction of PSI trimers, which was also observed after the Suc density gradient centrifugation, the BN-PAGE analysis (Fig. 10) also indicates that the stability of PSII dimers is slightly decreased in the mutant strain because the relative amount of PSII monomers to PSII dimers has changed in the mutant membranes. Thus, the Vipp1 depletion and the resulting thylakoid membrane perturbation appear to affect not only the PSI-to-PSII ratio,
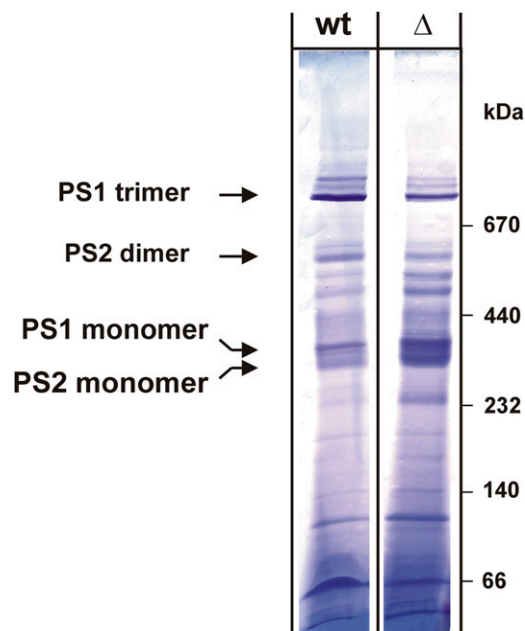

Figure 10. Coomassie-stained BN-PAGE gel of membrane protein complexes isolated from wild-type (wt) and Vipp1 depleted (Δ) *Synechocystis* cells. For identification of the photosystems, the individual membrane protein complexes were identified with antibodies directed against PSI and PSII subunits (see Fig. 9 legend) after separation in a second dimension by SDS-PAGE (data not shown). Densities of the PSI monomer and trimer bands in BN-PAGE gels were determined using the program Scion Image (Scion Corp.). Based on these measurements, the wild type contained at least 2.5 times more trimeric over monomeric PSI, whereas in the depletion strain, this ratio was shifted to approximately 0.5. [See online article for color version of this figure.]

but also oligomerization of both photosystems, although PSI seems to be more affected than PSII.

On the Function of Vipp1

Biogenesis of thylakoid membranes is neither understood in chloroplasts nor in cyanobacteria. The discovery that Vipp1 could be involved in thylakoid membrane formation in chloroplasts was a major breakthrough and indicated a direct link between a single protein and thylakoid membrane organization via a vesicular trafficking system (Kroll et al., 2001). It has subsequently been shown that Vipp1 can form large oligomeric ring structures (Aseeva et al., 2004), although the exact physiological function of Vipp1 still remains mysterious.

The results presented in this study suggest an in vivo function of Vipp1 not only in thylakoid membrane organization, but also in biogenesis of cyanobacterial membrane-bound protein complexes. A Vipp1 depletion strain of Arabidopsis is deficient in photosynthesis, although the defect could not be assigned to a deficiency of a single photosynthetic complex, but appeared to be caused by dysfunction of the entire photosynthetic electron transfer chain (Kroll et al., 2001). Therefore, it has been suggested that depletion of Vipp1 in Arabidopsis affects thylakoid membrane formation rather than the assembly of thylakoid membrane protein complexes (Aseeva et al., 2007). In contrast, depletion of the *vipp1* gene product resulted in a rather specific decrease of functional (trimeric) PSI in *Synechocystis*, whereas the amount of active PSII was not significantly altered. Therefore, the observed effect seems to be more specific for PSI and Vipp1 appears to be involved in biogenesis and/or stabilization of PSI in thylakoid membranes. However, based on the presented results, we cannot clearly distinguish between a direct effect of Vipp1 on PSI biogenesis and/or stability and a more indirect effect of Vipp1 on, for example, membrane formation, which could subsequently affect PSI stability or biogenesis in the membrane. In several recent studies, it has been observed that defects in thylakoid membrane formation in chloroplasts and cyanobacteria are caused by depletion of important lipids or of pigments (for a recent overview, see Vothknecht and Westhoff, 2001). After deletion of genes involved in myxoxanthophyll biosynthesis, thylakoid membranes in the resulting *Synechocystis* cells were highly disorganized and myxoxanthophyll biogenesis is therefore critical for thylakoid membrane organization (Mohamed et al., 2005). In chloroplasts, defects in thylakoid membrane formation have been linked to deficiencies in chlorophyll biosynthesis (Falbel and Staehelin, 1994; Runge et al., 1995). Furthermore, synthesis of galactolipids is essential for thylakoid membrane formation (Kobayashi et al., 2007) and also negatively charged phospholipids appear to be indispensable for thylakoid membrane formation and function (as summarized in Frentzen, 2004). Taken together, many factors are involved in formation, stabilization, and organization of thylakoid membranes in chloroplasts and cyanobacteria, and Vipp1 could therefore be involved in processes other than a vesicular transfer, as originally suggested.

CONCLUSION

Taken together, we show here that in the cyanobacterium *Synechocystis* sp. PCC 6803 the synthesis of active PSI depends on the amount of thylakoid membranes present per cell. Down-regulation of the amount of active PSI appears to be a general adaptation mechanism in cyanobacteria. Vipp1 could be directly involved in such mechanisms, resulting in changes of the PSI-to-PSII ratio in response to, for example, increasing light intensities. In contrast, depletion of the *vipp1* gene product resulted in a specific decrease of functional (trimeric) PSI in *Synechocystis*, whereas the amount of functional PSII was not significantly altered and, thus, the observed effect seems to be more specific for PSI.

MATERIALS AND METHODS

Growth Conditions

Synechocystis wild type and the *vipp1* merodiploid strain were grown at 30°C in BG11 medium supplemented with 10 mM Glc (Rippka et al., 1979). Kanamycin (30 μg/mL) was added to the growth medium for growth of the *vipp1* merodiploid strain. The growth medium was aerated with air enriched with 2% CO_2 and cells were grown under constant illumination of 20 to 120 μmol/(m^2 s).

Population sizes for the different *Synechocystis* strains in a cell suspension were determined by direct microscopic counts using a Thoma counting chamber.

Escherichia coli DH5α, which was used for plasmid propagation, was grown in Luria-Bertani medium at 37°C according to standard procedures (Sambrook and Russel, 2001). Luria-Bertani broth and agar were supplemented with 100 μg/mL ampicillin when needed.

Heterologous Expression of Vipp1 from *Synechocystis*

For raising antibodies against the cyanobacterial Vipp1 protein in rabbits, the *vipp1* gene from *Synechocystis* was amplified by PCR using the two primers vipp5′ 5′-tatgccatatgggattatttgaccgtttagg-3′ and vipp3′ 5′-atacgggatccttaca-gattatttaaccgacgacg-3′, and genomic *Synechocystis* DNA as a template. The PCR product was restriction digested with NdeI and BamHI and ligated to the equally restriction-digested plasmid pET19b (Merck). The resulting plasmid pET-SynVipp1 was used to express the Vipp1 protein with an N-terminal His-tag in *E. coli* BL21 (DE3) cells. For protein expression, cells were grown in 1-L Luria-Bertani medium to an OD_{600} of approximately 0.6 and protein expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. After 3 h, cells were harvested and resuspended in 50 mL 20 mM HEPES, pH 7.5, 5 mM EDTA buffer. Cells were subsequently broken by ultrasonic treatment and afterward inclusion bodies were sedimented by centrifugation at 10,000g for 10 min. Proteins were solubilized in buffer A (50 mM sodium phosphate, pH 7.6, 50 mM NaCl) supplemented with 150 mM SDS. After 10-min incubation at room temperature, the samples were centrifuged at 10,000g, and the supernatant was loaded onto a nickel-nitrilotriacetic acid agarose column (Qiagen). The column was washed two times with buffer A + 150 mM SDS, two times with the same buffer at pH 7.2, and proteins were finally eluted in buffer with 10 mM SDS at pH 6.5.

Preparation of Cyanobacterial Membranes

Synechocystis cells were harvested in the mid-log growth phase and pelleted by centrifugation (5,000g, 10 min, 4°C). Cells were resuspended in

buffer (50 mM HEPES, pH 7.0, 5 mM $MgCl_2$, 25 mM $CaCl_2$, 10% [v/v] glycerol) and disrupted in a bead beater homogenizer (BioSpec) using 0.5-mm glass beads. Glass beads, unbroken cells, and cell debris were removed by centrifugation for 5 min at 4°C and 5,000g. After centrifugation at 100,000g and 4°C for 40 min, the membrane pellet was resuspended in a buffer at a chlorophyll concentration of about 1 mg chl/mL.

SDS-PAGE and Immunoblot Analysis

For immunoblot analysis, membranes were extracted with SDS sample buffer and proteins were loaded on a 12% polyacrylamide gel. Subsequently, SDS-PAGE proteins were transferred to a polyvinylidene difluoride membrane using a semidry blotter from Bio-Rad. The rabbit primary antibodies and the goat anti-rabbit secondary antibody (Sigma) were used at dilutions of 1:2,000 and 1:10,000, respectively. To visualize the cross-reacting protein bands, membranes were incubated with the ECL kit from Pierce.

Suc Density Gradient Centrifugation and BN-PAGE Analyses

For analyses of membrane protein complexes in *Synechocystis* cells, the membranes were always prepared freshly as described in Dühring et al. (2006).

Thylakoid membranes were extracted with 1% *n*-dodecyl-β-D-maltoside and separation of protein complexes in a 10% to 30% Suc density gradient was done as described in Rögner et al. (1990). Individual fractions of the gradient were analyzed by immunoblot analyses to determine the fractions containing PSI or PSII. Antibodies were directed against the PSI subunits PsaA, PsaB, and PsaD and against the PSII subunits PsbA and PsbB. PsaA/B, PsbA, and PsbB antibodies were a kind gift of M. Rögner (Ruhr-University Bochum, Germany). The antibody directed against PsaD was from Agrisera.

For BN-PAGE analyses, *Synechocystis* membranes were extracted with 1% *n*-dodecyl-β-D-maltoside, and membrane protein complexes were separated exactly as described in detail in Dühring et al. (2006, 2007). Individual lanes were separated in a second dimension on a 12% SDS gel (Laemmli, 1970) and PSI and PSII were identified by immunoblot analyses using the antibodies described above.

Inactivation of the *vipp1* Gene

Inactivation of the *Synechocystis vipp1* gene (*sll0617*) was essentially carried out as described (Westphal et al., 2001). A kanamycin resistance cassette, derived from pBSL14 (Alexeyev, 1995), was introduced into a single *Pst*I site within the *vipp1* gene of pET-SynVipp1. Five micrograms of the plasmid were used to transform *Synechocystis* wild-type cells following the procedure described in Williams (1988). For selection of kanamycin-resistant mutants, solid BG11 medium was supplemented with increasing kanamycin concentrations (5–50 μg/mL).

Absorbance and 77K Fluorescence Emission Spectroscopy

Absorbance spectra of whole cells in the visible range were recorded with a Perkin-Elmer Lambda 25 spectrophotometer. Low-temperature fluorescence emission spectra were recorded at 77K with an Aminco Bowman Series 2 fluorimeter. Monochromators were set to a slit width of 4 nm. Cells were adjusted to a chlorophyll concentration of about 3 μM in BG11 and frozen in liquid nitrogen. Chlorophylls were excited at 435 nm and phycobilisomes at 580 nm.

Chlorophyll concentration was determined according to Porra et al. (1989) after extraction of whole cells with 100% methanol. Phycocyanin/chlorophyll content was determined from whole-cell absorbance spectra as described (Bennett and Bogorad, 1973).

Oxygen Evolution and Consumption Measurements

Oxygen evolution was measured in 1-mL cell suspension (OD_{750} of approximately 1, about 5 μg/mL chlorophyll) using a fiber-optic oxygen meter (PreSens) under actinic light (600 μmol photons m^{-2} s^{-1}). *Synechocystis* wild-type and Vipp1 depleted cells were harvested in the mid-log phase and

diluted to an OD_{750} = 1 in BG11 medium. Three independent cultures of each analyzed strain were measured three times each. Photosynthetic rates were determined in the presence of 10 mM $NaHCO_3$ and PSII activity in the presence of 0.5 mM PPBQ.

PSI activity was measured as oxygen consumption in the presence of 20 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 2 mM NaN_3, 200 μM 2,3,5,6-tetramethyl-1,4-phenylenediamine, 5 mM sodium ascorbate, and 200 μM methyl viologen. A baseline was recorded in dark and light. Oxygen consumption due to the Mehler reaction was determined as corrected oxygen consumption in the light by oxygen consumption in the dark.

EM

The morphology of *Synechocystis* cells was studied by examining ultrathin sections with a PHILIPS CM10 electron microscope (Fei Company) equipped with a Bioscan camera (model 792; Gatan). The electron micrographs taken at an acceleration voltage of 80 kV were processed by a digital image system (digital micrograph; Gatan). The specimens were prepared routinely (Golecki, 1988) for thin sectioning by collecting the cells by centrifugation and fixing in 2% glutardialdehyde in cacodylate buffer (pH 7.0) and 1% osmium tetroxide in veronal acetate buffer (pH 7.0). After embedding in epoxy resin ultrathin sections were produced. The sections were stained with uranyl acetate followed by lead citrate.

To determine the number of thylakoid layers per cell, more than 100 cells of each *Synechocystis* wild type and Vipp1 depletion strain were evaluated on ultrathin sections.

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