

Responsibility of phosphatidylglycerol for biogenesis of the PSI complex

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Abstract

Phosphatidylglycerol (PG) ubiquitous in thylakoid membranes of photosynthetic organisms was previously shown to contribute to accumulation of chlorophyll through analysis of the *cdsA*[−] mutant of a cyanobacterium *Synechocystis* sp. PCC6803 defective in PG synthesis (SNC1). Here, we characterized effects of manipulation of the PG content in thylakoid membranes of *Synechocystis* sp. PCC6803 on the photosystem complexes to specify roles of PG in biogenesis of thylakoid membranes. SNC1 cells with PG deprivation in vivo, together with the chlorophyll decrease, exhibited a decline not in PSII, but in PSI, at the complex level as well as the subunit levels. On the other hand, the decrease in the PSI complex was accounted for by a remarkable decrease in the PSI trimer with an increase in the monomer. These symptoms of SNC1 cells were complemented in vivo by supplementation of PG. Besides, a reduction in the PG content of thylakoid membranes isolated from the wild type in vitro on treatment with phospholipase A2 (PLA2), similar to the PG-deprivation in SNC1 in vivo, brought about a decrease in the trimer population of PSI with accumulation of the monomer. These results demonstrated that PG contributes to the synthesis and/or stability of the PSI complex for maintenance of the cellular content of chlorophyll, and also to construction of the PSI trimer from the monomer at least through stabilization of the trimerized conformation.

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1. Introduction

Biomembranes are constructed mainly from proteins and lipids that display specific components and/or compositions to the membrane systems. The membrane lipids can be considered not only as structural materials to build lipid bilayers to accommodate membrane proteins and to act as a permeability barrier, but also as factors that interact with the membrane proteins for their proper functioning.

Thylakoid membranes of oxygenic photosynthetic organisms are places for the photosynthetic electron transport and the photophosphorylation systems that participate in conversion of the light energy to chemical one such as ATP and NADPH. A central part of the light-driven energy produc-

tion is taken cooperatively by Photosystem (PS) I and II complexes that are comprised of proteins and cofactors such as chlorophyll (Chl), respectively (e.g., Ref. [1]). Whereas the PSII complex transfers electrons from H₂O to plastoquinone with the use of the light energy, the PSI complex transfers those from plastocyanine (or cytochrome *c*₆) to ferredoxin (or flavodoxin). Four glycerolipids also are included in the thylakoid membranes as the main lipids, i.e., three galactolipids, monogalactosyl diacylglycerol, digalactosyl diacylglycerol, sulfoquinovosyl diacylglycerol (SQDG), and a phospholipid, phosphatidylglycerol (PG) (e.g., Ref. [2]). Among them, PG is the sole lipid that is ubiquitous in the photosynthetic membranes not only of oxygenic photosynthetic organisms, but also of anoxygenic ones [3], which prompted us to investigate the fundamental role of PG in photosynthesis.

Mutants defective in some lipid metabolism, which provide us in vivo systems to down-regulate some specific lipid, have proved valid for evaluation of the roles of lipids

Abbreviations: Chl, chlorophyll; DM, dodecyl β-D-maltoside; PG, phosphatidylglycerol; PS, photosystem; SQDG, sulfoquinovosyldiacylglycerol

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in the membranes through specification of damages to the functioning of the membrane system. Above all, this strategy is essential for elucidation of the mechanism by which lipids contribute to the membrane biogenesis such as complicated biosynthetic process for construction of the protein complexes. Isolated first from the photosynthetic organism as defective in the PG synthesis were disruptants of *Synechocystis* sp. PCC6803 with respect to the *cdsA* gene for cytidine 5'-diphosphate diacylglycerol synthase and the *pgsA* gene for phosphatidylglycerophosphate synthase [4,5]. These two mutants were found to show decreases in the chlorophyll (Chl) content and damages to the conformation and functioning of the PSII complex [4–6], respectively. Mutants defective in chloroplast PG were isolated later from a higher plant, *Arabidopsis thaliana*, as impaired in the *PGS1* gene for phosphatidylglycerophosphate synthase, being reported to exhibit the reduced Chl content, poor development of the thylakoid membranes, and impaired photosynthesis [7–9]. Such injuries in the photosynthetic apparatus indicated involvement of PG in the biogenesis and functioning of thylakoid membranes, although a question remains to be answered of whether the photosynthetic defects in the mutants represent the primary effects of the lesion in PG or the secondary ones.

On the other hand, in vitro system to decline PG content in isolated thylakoid membranes or photosynthetic particles of the wild type through degradation of PG by phospholipase treatment have proposed direct roles of PG for the construction and/or the functioning of the photosynthetic components [10]. However, this strategy leaves problems of some possible artifact that is inevitable for in vitro experiments. It is thus desirable to combine the above in vivo and in vitro systems to manipulate the PG content for identification of direct roles of PG in thylakoid membranes.

Of particular interest to us is the symptom of the reduced Chl content common to the mutants defective in PG synthesis of both the cyanobacterium and the higher plant, suggesting an evolutionarily conserved role of PG in accumulation of the protein complexes containing Chl as a cofactor. Here, we investigated roles of PG in the biogenesis of the photosystem complexes by analyzing photosynthetic apparatus of *cdsA*[−] mutant of *Synechocystis* sp. PCC6803 with manipulation of the PG content in vivo. We also examined whether PG is directly involved in the roles with the use of thylakoid membranes isolated from the wild type, by examining effects of in vitro PG deprivation by treatment with phospholipase A2 (PLA2) on the photosynthetic apparatus.

2. Materials and methods

2.1. Cell culture

Cells of the wild type of *Synechocystis* sp. PCC6803 and its *cdsA*[−] mutant designated as SNC1 were grown photo-

autotrophically in BG-11 medium under constant fluorescent lamp illumination (10 W m^{−2}) at 28 °C in an oblong glass vessel with aeration. The culture medium was supplemented with PG liposomes (6 mM) when indicated, as previously described [4]. The culture of SNC1 cells was serially diluted to below 0.5 in the absorbance at 730 nm during growth in PG-free medium for prevention of cell growth from being retarded.

2.2. Isolation of thylakoid membranes

Thylakoid membranes were isolated according to the method of Hihara et al. [11] with some modification. The cells were harvested by centrifugation at 5000×g (10 min, 4 °C), then washed with HN buffer containing 5 mM HEPES–NaOH, pH 7.5 and 10 mM NaCl, and suspended in the same buffer. The cell suspension where the same volume of zircon beads (100 μm in diameter, Biospec) were added was agitated with a vortex mixer four times for 30 s, each with cooling interval of 1 min, thereafter centrifuged at 3000×g for 15 min at 4 °C to pellet cell debris. The supernatant was centrifuged at 20,000×g for 30 min for collection of thylakoid membranes as pellet, which was then suspended in HN buffer to be stored at −80 °C until use.

2.3. SDS-PAGE and Western analysis

Thylakoid membranes corresponding to 5×10⁶ cells were denatured in a solution containing 5% SDS, 5% 2-mercaptoethanol, and 10% sucrose, then being subjected to SDS-PAGE with a separating gel of 16–22% acrylamide gradient for analysis of the polypeptide pattern, as previously described [12]. On the other hand, for Western analysis, whole cell extracts corresponding to 5×10⁴ cells were denatured in the same way to be subjected to SDS-PAGE with the gel of 10% acrylamide. The proteins of the whole cells separated by SDS-PAGE were then blotted onto PVDF membranes, reacted with antiserum against the D1 protein or PsaA/PsaB, and detected by peroxidase, as we previously described [13]. Specific bands for respective proteins were quantified by NIH Image (1.62).

2.4. Dodecyl β-D-maltoside (DM)-PAGE

Thylakoid membranes corresponding to 1.5×10⁷ cells were incubated on ice for 1 h in a buffer containing 4% DM, 50 mM Tris–HCl pH 7.5, and 10 mM NaCl, thereafter centrifuged at 5500×g (3 min, 4 °C) to pellet insoluble materials. The supernatant was subjected to the disc DM-PAGE, as we previously described [12]. Distribution of Chl on the gel was analyzed with the NIH Image.

2.5. Measurement of the PSI activity

The PSI activity was measured with the reduced form of diaminodurene and methylviologen as the electron

donor and acceptor, respectively. The reaction was performed in a mixture containing thylakoid membranes corresponding to $2.5 \text{ mg Chl ml}^{-1}$, 1 mM ascorbic acid, 5 mM diaminodurene, 2 mM methylviologen, 20 mM DCMU, and 1 mM KCN. O_2 uptake at 30°C was measured with a Clark-type electrode (Rank Brothers, London) with illumination of a tungsten projector lamp, as described previously [12].

2.6. Treatment of wild-type thylakoid membranes with PLA2

Thylakoid membranes of the wild type corresponding to $20 \text{ }\mu\text{g Chl}$ were suspended in a buffer containing 50 mM Tris-HCl pH 7.5 and 10 mM NaCl for adjustment of Chl concentration to $0.5 \text{ }\mu\text{g Chl}\cdot\mu\text{l}^{-1}$. The mixture was then supplied with 3.6-unit of PLA2 (Sigma) to be incubated for 30 min at 30°C , then solubilized with DM for subjection to DM-PAGE or used for lipid analysis as described in Ref. [4].

3. Results

3.1. Identification of the PSI complex as the cause to decrease the Chl content with PG deprivation

SNC1, the mutant of *Synechocystis* sp. PCC6803, was previously produced by disruption of the *cdsA* gene through homologous recombination [4]. This mutant with deficiency in the PG synthesis was not viable in the normal medium, but could grow with supplementation of PG. The shift of SNC1 cells pregrown with PG supplementation to PG-free media thus gives an *in vivo* system to down-regulate the cellular content of PG through dilution of incorporated PG upon cell division. We applied this *in vivo* system to characterize the roles of PG in the construction and functioning of the PSI and PSII complexes.

The shift of the SNC1 cells to the PG-free medium allowed the cells to grow to some extent, but the accumulation of Chl was significantly repressed, producing a decrease in the level of Chl per cell (Fig. 1). In agreement with our previous report, the cellular content of Chl thus diminished in SNC1 up to 36% of the initial level through cell division of 2.8 times (Figs. 1 and 2a). The PG content on a per cell basis was reduced to an undetectable level on the TLC plate 5 days after the shift (data not shown), being estimated to decrease by 48-fold, in view that the cells that divided once showed a reduction by fourfold in the cellular content of PG [4], probably owing to its turnover for the synthesis of other molecules besides simple dilution [14].

We then investigated subunit levels of the PSI and the PSII complexes in SNC1 cells during this PG deprivation to determine the complex(es) responsible for the decrease in the Chl content. A summed level of PsaA and PsaB that form heterodimeric core of PSI was found to decrease to ca. 20% of the initial levels through Western

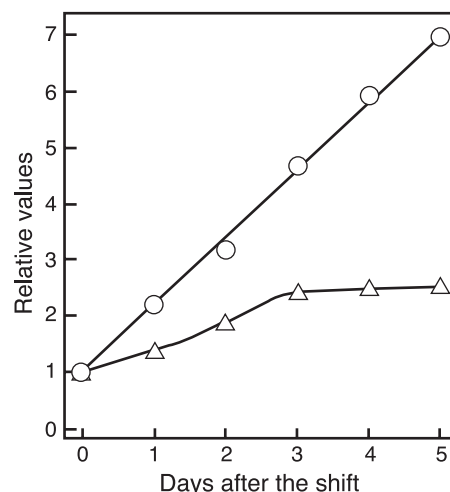


Fig. 1. Growth of SNC1 cells and Chl accumulation of the cell culture. The SNC1 cells pregrown in the presence of PG were transferred to the PG-free medium for further growth for 5 days for monitoring the cell density (circles) and the Chl content of the culture (triangles). The number of cells per milliliter of the suspension was counted with a light microscope, whereas the content of chlorophyll was determined after its extraction from the cells with 100% methanol as described in Ref. [4]. The cell culture before the shift (Day 0) showed $2.4 \times 10^7 \text{ cells ml}^{-1}$ and $0.69 \text{ }\mu\text{g Chl ml}^{-1}$. The values are averages \pm SE of three independent experiments, but bars of SE are within the symbols.

analysis with an antiserum against both PSI subunits, in line with the reduction in the Chl content (Fig. 2a). Similar to PsaA and PsaB, exhibited to be lower in the abundance were other four subunits of small molecular weights of the PSI complex, PsaD, PsaE, PsaF, and PsaL, through SDS-PAGE of thylakoid membrane proteins (Fig. 2c, see below and Fig. 4).

On the other hand, the D1 protein, one of the subunits constituting the PSII reaction center complex, was decreased finally to half the initial level, but with a significant increase transiently, therefore exhibiting an altering pattern quite distinct from that of the Chl (Fig. 2b). These results indicated that the drop of the Chl content with deprivation of PG is accounted for predominantly by the PSI complex, but not by the PSII complex, at least up until day 4.

3.2. Effects of chloramphenicol, an inhibitor of *de novo* synthesis of proteins, on the quantitative decrease in the PSI complex with PG depletion

The steady state level of the PSI complex is determined by a balance between its synthesis and stability of the synthesized complex. To examine whether the decrease in the PSI complex that occurred concomitant with the depletion of PG depends on stability of the preexisting PSI complex we compared the stability of the PSI subunits, PsaA and PsaB, between SNC1 cells before and after PG depletion by Western analysis (Fig. 3). The mutant supplemented with PG was little altered in the levels of

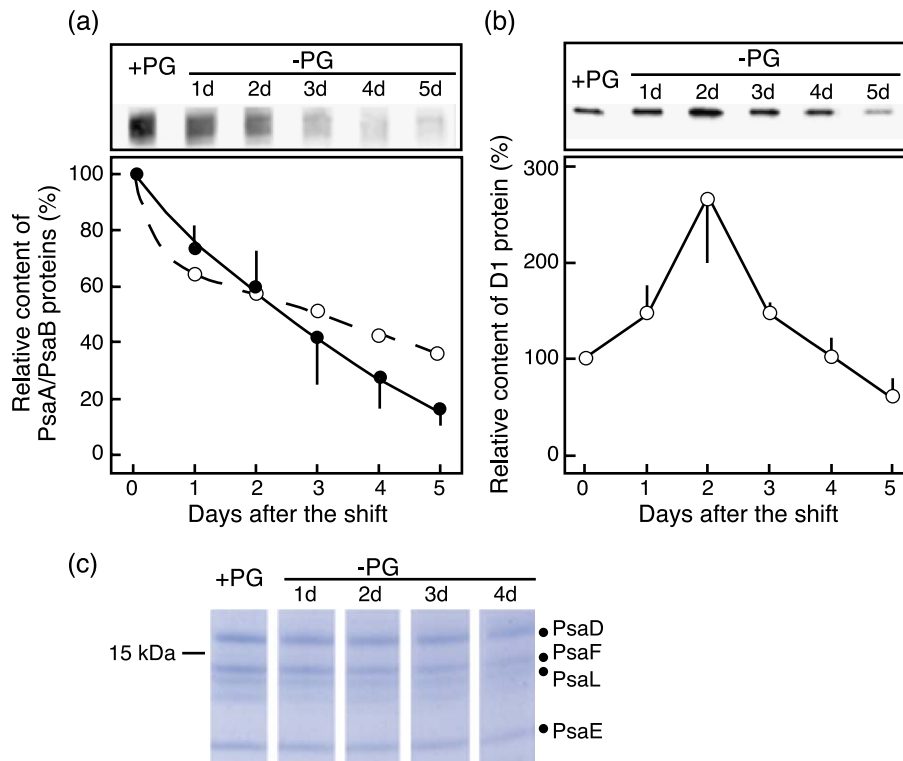


Fig. 2. Changes in levels of the PSI and PSII subunits during deprivation of PG. SNC1 cells pregrown in the presence of PG were transferred to the PG-free medium for further growth for 5 days. As described in Materials and methods, whole cell proteins were prepared for the use for Western analysis with antiserum against (a) PsaA/PsaB or (b) the D1 protein, whereas thylakoid membranes were isolated for SDS-PAGE (c). Samples equivalent to 5×10^4 and 5×10^6 cells were applied to the lanes for Western analysis and SDS-PAGE, respectively. Densitometric measurement was performed with NIH Image (1.62). The Chl content was calculated on the basis of the data in Fig. 1. The values are averages \pm SE of three independent experiments. (a) Closed circles, PsaA/PsaB; open circles, Chl. (b) D1 protein. (c) PSI subunits of small molecular weights. (For colour see online version).

the PSI subunits, PsaA and PsaB, during incubation for 24 h in the presence of chloramphenicol, an inhibitor of protein synthesis, indicating that the preexisting PSI complex was hardly degraded (data not shown). The high stability of PsaA/PsaB was kept even after deprivation of PG (Fig. 3), which showed no evidence to prove the induction of the degradation of the preexisting PSI complex. The decrease in the PSI complex could thus be attributed to the lowered synthesis of the PSI complex that leads to dilution of the complex upon cell division, although there still remains a possibility of involvement of the induction of the degradation system that requires de novo synthesis of proteins for its proper functioning.

3.3. Simultaneous separation of the PSI and PSII complexes through DM-PAGE

Although the PSI complex was deduced to diminish with deprivation of PG from the subunit analysis, there still remains a question of what occurred in the complex in itself. Dodecyl β -D-maltoside (DM)-PAGE of thylakoid membranes was developed previously in a green alga, *Chlamydomonas reinhardtii*, as a method to simultaneously separate thylakoid membrane components into the PSI complex, the PSII core complex, and the light-harvesting complex of PSII on a gel [12]. Thereafter, the

DM-PAGE proved to be valid for evaluation of these complexes not only from quantitative, but also from conformational, aspects [15].

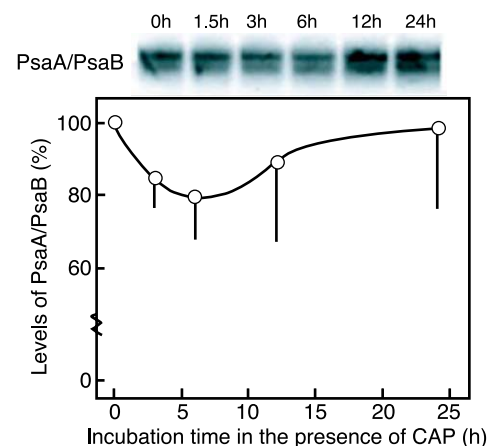


Fig. 3. Effects of PG deprivation on the stability of PSI subunits. SNC1 cells grown in the absence of PG for 5 days were incubated further for 24 h in the presence of an inhibitor of translation, chloramphenicol. Whole cell proteins were prepared for the use for Western analysis with an antiserum against PsaA/PsaB, as described in Materials and methods. Samples equivalent to 5×10^4 cells at 0 h, i.e., before addition of chloramphenicol, were applied to respective lanes. Densitometric measurement was performed with the NIH Image. The values are averages \pm SE of three independent experiments. (For colour see online version).

It was investigated whether the DM-PAGE is applicable to *Synechocystis* sp. PCC6803 for the analysis of the levels and structure of the PSI and the PSII complexes. The thylakoid membranes of the wild type of *Synechocystis* sp. PCC6803 were solubilized with DM, subsequently subjected to the first-dimensional PAGE to yield three green bands (Fig. 4). These bands, designated as bands-1 to -3, were found to include polypeptides through the second-dimensional SDS-PAGE, and thus not to correspond to free Chl, as in the case of *C. reinhardtii* [12].

Bands-1 and -2 exhibited the same protein profiles that were identified to comprise the PSI subunits, PsaA/PsaB, PsaD, PsaF, PsaL, and PsaE in the order of increasing mobility, through comparison with a standard sample of the PSI complex and/or Western analysis (data not shown). As regards band-3, four proteins were included, identified as the PSII subunits such as apoproteins of CP47 and CP43, and D2 and D1 proteins in the order of increasing mobility by their molecular weights and/or Western analysis (data not shown). On the other hand, mobility of the band-1 is close to, but definitely larger than that of, thyroglobuline of 670 kDa, while those of bands-2 and -3 were a little smaller than, and indistinguishable to that of, β -amylase of 200 kDa. On the basis of these results, it was deduced that bands-1 to -3 correspond to the PSI trimer, the PSI monomer, and the PSII monomer, respectively.

DM-PAGE displayed the trimer as a predominant form of the PSI complex in *Synechocystis* sp. PCC6803 (Fig. 4), as was consistent with the previous reports for several cyanobacterial species where the monomer and the trimer of the PSI complex were separated from each other or were identified by other methods such as spectroscopic analysis (e.g. Ref. [16]). In contrast, the PSII dimer that should

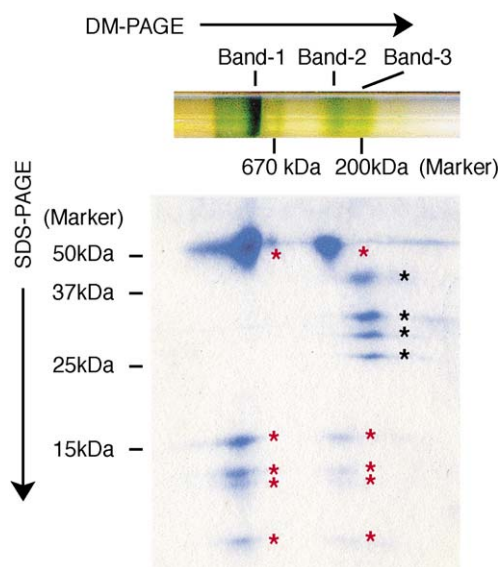


Fig. 4. DM-PAGE of thylakoid membranes of *Synechocystis* sp. PCC6803. Thylakoid membranes equivalent to 1.5×10^7 cells were solubilized with DM, subjected to disc DM-PAGE as the first dimension, then to slab SDS-PAGE as the second dimension, as described in Materials and methods.

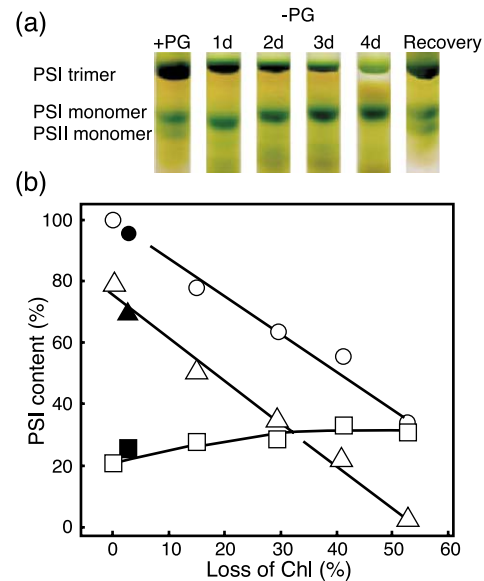


Fig. 5. Changes in behavior of the PSI and PSII complexes during PG deprivation. SNC1 cells grown with PG were transferred to the PG-free medium to grow for further 4 days, thereafter being supplemented with PG again for the recovery of the growth. During this culture, thylakoid membranes were prepared from the SNC1 cells, being subjected to a disc DM-PAGE (a). The distribution of Chl on the gels was densitometrically determined with the NIH Image (b). Triangles, the PSI trimer; squares, the PSI monomer; circles, the monomer plus the trimer. Open symbols indicate alterations in the PSI during PG deprivation, whereas closed ones show values of the cells that recovered its growth with resupplementation of PG. Shown is the representative of two independent experiments with similar results.

prevail in vivo was implied to be dissociated into the monomer in this system, probably owing to severe procedure of the DM-PAGE.

3.4. Quantitatively and conformationally abnormal behavior of the PSI and PSII complexes during PG depletion

We then examined, using DM-PAGE, the distributing pattern of the PSI and PSII complexes in SNC1 during PG deprivation (Fig. 5). The pattern of the PSI complex, initially similar to that in the wild type, exhibited the trimer as a predominant species, drastically decreasing the trimer level with an increase in the monomer one after the onset of deprivation of PG (Fig. 5a). As was compatible with behavior of the PSI subunits (Fig. 2a,c), the PSI complex itself as a whole, i.e., monomer plus trimer, thus proved to decrease in parallel with the Chl content (Fig. 5b). Of another note was that the PSI trimer was the very cause for the decrease in the Chl content (Fig. 5b), eventually with enhancement of a monomer/trimer ratio, which implicated that the PSI complex, with deprivation of PG from SNC1 cells, was being impaired in trimerization of the monomer and/or in conformational stability as the trimer.

To determine if the decreased population of the PSI trimer accompanied deficiency in some PSI subunit, we investigated patterns of the small subunits of the PSI complex in

SNC1 cells before and after the PG removal (Fig. 6). The mutant, during the decrease in the PG content, showed little alteration in subunit compositions of the PSI monomer, indicating that the failure of the mutant to accumulate the PSI trimer is not accounted for by the loss of any PSI subunit as far as we could recognize in our system.

On the other hand, the monomer of the PSII complex in SNC1, although initially present at the level similar to that in the wild type, disappeared as early as 1 day after onset of PG removal (Fig. 5a). Concomitant with diminishing of the PG content, the PSII subunits changed its location to the positions of faster mobility with broader region than usual on a gel of the first-dimensional DM-PAGE (data not shown), which indicated that the structure of the PSII complex became fragile and was broken during DM-PAGE processes.

The decrease in the PG content was thus accompanied by the lesion not only in the quantitative maintenance of the PSI complex (Fig. 2), but also in conformational integrity of both the PSI and PSII complexes as follows: The PSI complex fails to maintain the trimer level, while the PSII complex is disabled to properly keep its subunits assembled at least under some severe condition during DM-PAGE. PG could be responsible for maintenance of the trimer level of the PSI complex through participating first in the synthesis of the complex (Figs. 2 and 3), and second in the formation of the PSI trimer by facilitating trimerization of the monomer and/or stabilizing the trimerized form (Fig. 5). Suggested also as the role of PG was contribution to stabilization of subunit assembly of the PSII complex (Fig. 5).

Despite the striking conformational change in the PSI complex, the PSI activity was little damaged in SNC1 during PG deprivation [$453 \pm 20 \mu\text{mol O}_2 \text{ mg}^{-1} \text{ Chl h}^{-1}$ in SNC1 replete of PG, cf. 478 ± 15 in SNC1 deprived of PG for 4 days (the means \pm SE)]. These results were consistent with previous report as to the other mutant deficient in the PG synthesis of *Synechocystis* sp. PCC6803 owing to disruption of the *pgsA* gene [5]. However, this study,

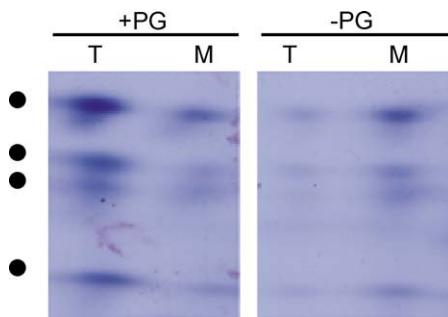


Fig. 6. Effects of PG deprivation on small subunits of the PSI complex. Shown are the portions of gels that cover PSI subunits of small molecular weights after two-dimensional PAGE of the thylakoid membranes. SNC1 cells were grown with PG (+PG), or were transferred to the PG-free medium to grow for 4 days (–PG). Subjected to the first-dimensional DM-PAGE were thylakoid membranes equivalent to 1.5×10^7 cells. T, trimer; M, monomer. (For colour see online version).

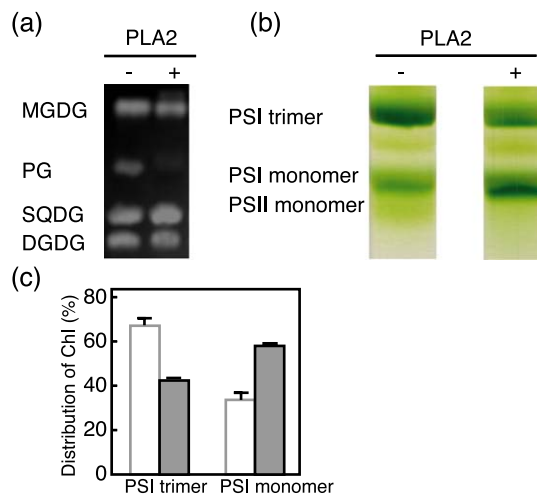


Fig. 7. Effects of PLA2 treatment of wild-type thylakoid membranes on the oligomerized state of the PSI complex. Thylakoid membranes prepared from the wild-type *Synechocystis* sp. PCC6803 cells were treated with PLA2, thereafter solubilized with DM to be subjected to DM-PAGE (a) or used for lipid analysis with TLC (b). Chl distribution between PSI monomer and trimer, which was estimated through densitometric measurements with the NIH Image on the gel after DM-PAGE, is shown as average \pm SE from three independent experiments (c). White and black bars indicate PSI before and after PLA2 treatment, respectively.

distinct from the previous one with no information on the trimerization state of the PSI complex, demonstrated that replacement of the PSI monomer for as much as 87% of the trimer had little detrimental effect on its activity (Fig. 5).

3.5. Chemical complementation in vivo as to the damages to photosystem complexes

There remains a possibility that the lesions in the construction of the photosystem complexes in SNC1 were brought about not directly by the defect in PG, but by its secondary detrimental effects on the fundamental membrane function that is essential for cell viability, e.g., permeability barrier. We therefore cultured the mutant cells that had been depleted of PG in PG-supplemented medium again, thereby examining whether SNC1 cells deprived of PG were damaged so significantly as to fail to grow. The mutant replenished with PG could grow again to exhibit quantitative recovery of the Chl together with the PSI complex as a whole (Fig. 5b), and conformational recovery of the photosystem complexes that includes the trimer formation of the PSI complex and structural tolerance of the PSII complex to DM-PAGE (Fig. 5a,b).

It thus became evident that the detrimental phenotypes of the PSI and PSII complexes were caused not by the secondary effect of PG deprivation that leads to a loss of some essential membrane function. The recovery from the damages to the biogenesis of the photosystem complexes through up-regulation of the PG content in vivo most probably suggested that PG is required for the synthesis of the PSI complex, and also for conformational integrity of the PSI and PSII complexes.

3.6. *In vitro* evidence of involvement of PG in the construction of the PSI complex

To make clear the directness of the roles of PG in sustaining conformational integrity of the PSI and the PSII complexes, isolated thylakoid membranes of the wild type were depleted of PG *in vitro* on treatment with PLA2 for 30 min at 30 °C, for monitoring changes in population of the photosystem complexes by DM-PAGE (Fig. 7). Removal of PG in the isolated thylakoid membranes by more than 80% (Fig. 7a; data not shown), similar to that in SNC cells *in vivo*, resulted in a decreased level of the PSI trimer complementarily with an increased one of the PSI monomer, and also in disappearance of a tight band of the PSII monomer (Fig. 7b,c).

It was noted that this *in vitro* depletion of PG, as in the *in vivo* one, showed no decrease in the PSI activity (data not shown). The results not only strengthened the thought that the trimerized conformation of the PSI complex is little crucial for the functioning of PSI, but also excluded a possibility that the lipase treatment induced some artifact that alters the organization of thylakoid membranes so significantly as to lower the activity. Therefore, we consider that roles of PG at least include direct contribution to stabilization of the trimeric conformation of PSI and the monomeric one of PSII through preventing dissociation into the PSI monomer and decomposition into PSII subunits, respectively.

The level of the trimer dissociation of PSI by PLA2 treatment was much lower than the maximal level observed with SNC1 depleted of PG *in vivo* (Figs. 5 and 7). Lipase treatment for prolonged time up to 90 min, or with threefold more abundant PLA2, stimulated no further breakdown of the trimer in wild-type thylakoid membranes (data not shown), thus indicating the presence of population of PG molecules that are involved in the stabilization of the PSI trimer, but is inaccessible by PLA2, probably owing to the organization of thylakoid membranes.

4. Discussion

4.1. Contribution of PG to the maintenance of the level of the PSI complex

From the previous observation as regards the similar phenotype of the reduced Chl contents for the prokaryotic and eukaryotic mutants defective in thylakoid PG [4,6–9], it could be proposed that PG plays some common role for photosynthetic organisms in the biogenesis of the PSI and/or PSII complexes. With the use of SNC1 as an *in vivo* tool for manipulation of the PG content downward and then upward, it was evidenced that PG is responsible for quantitative maintenance predominantly of the PSI complex, but not of the PSII complex, thereby keeping the Chl content at the regular level (Figs. 1, 2, and 5).

Involvement of anionic phospholipids including PG and its derivative cardiolipin in protein biogenesis has been observed with nonphotosynthetic organisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, through analysis of their mutants impaired in the synthesis of both of these two anionic phospholipids. The roles of PG found included responsibility for translocation of proteins across the inner membranes, integration of proteins into the membranes with proper topology, and translation of membrane proteins [14,17]. Of particular interest was contribution of these phospholipids to translation of protein components of electron transport chain in mitochondria such as subunits of cytochrome *c* oxidase in *S. cerevisiae* [17]. In *Synechocystis* sp. PCC6803, PG might be required for translation of the PSI subunits, especially of PsaA and/or PsaB, deletion mutants of which are known to fail to accumulate the other PSI subunits also in thylakoid membranes [18].

Alternatively, PG might be required by the synthesis of the cofactor(s) of the PSI complex. It is notable that accumulation of *de novo* synthesized PSI complex was remarkably limited with less significant effect on the PSII complex for two cyanobacterial species, *Synechococcus* sp. PCC6714 and *Plectonema boryanum*, and a green alga, *Dunaliella salina*, on repression of Chl synthesis by inhibitors or a genetic defect as to the enzyme for the Chl synthesis [19–21]. It is thus supposed that the PSI synthesis is more closely linked to Chl availability than the PSII synthesis to be repressed sooner by limited availability of Chl. PG might be essential for the normal synthesis of Chl, e.g., for activation of some enzyme concerning the synthesis, therefore being a factor to slow down the Chl synthesis when depleted, with the consequence of the faster decline in the PSI complex than in the PSII complex (Fig. 2).

The role of PG in the quantitative maintenance of the PSI complex will be elucidated in the future through analysis of SNC1 from aspects of Chl synthesis, protein biogenesis of PSI subunits, and their assembly into the PSI complex. The universality of the role in photosynthetic organisms will be evaluated through the similar analysis of *A. thaliana* mutants defective in chloroplast PG [7–9].

4.2. Responsibility of PG for the trimeric form of the PSI complex

Through structural analysis of the photosystem complexes with DM-PAGE not only in SNC1 with the PG content manipulated *in vivo*, but also in the isolated wild-type thylakoid membranes deprived of PG *in vitro*, PG was shown to contribute directly to the formation of the PSI trimer at least through preventing the trimer to dissociate into the monomer (Figs. 5 and 7). Responsibility of PG for regularly oligomerized states of protein complexes through direct association with the complexes was previously demonstrated for the PSII core complex or the light-harvesting chlorophyll *a/b* complex by *in vitro* reconstitu-

tion experiments [22,23]. The dimer of the PSII core complex that should function normally in vivo, e.g., was dissociated into the monomer upon PG removal by treatment with PLA2, while that was stimulated to form from the monomer with PG supplementation [22].

Intriguingly, three molecules of PG were exhibited at the PsaA/PsaB core in the crystal structure of the PSI complex of a cyanobacterium *Thermosynechococcus elongatus* BP-1 [24]. Two of the PG molecules were peripheral lipids, including one that neighbored on the interface of the monomers, while the rest was the innermost lipid close to the PSI core. It is reasonable to speculate that, in this study, PG molecules removed from the isolated thylakoid membranes of wild-type *Synechocystis* sp. PCC6803 in vitro by PLA2 reflected those attached on the periphery of the PSI complex rather than embedded deeply into the complex. A similar case would be applied to by the PG molecules that were removed from SNC1 cells in vivo, in view of the similar decreases in the trimer/monomer ratio concomitantly with little impairment in the PSI activity for this in vivo and in vitro systems of PG deprivation (Figs. 5 and 7). Direct association of PG molecules with the PSI complex particularly in the vicinity of the interface of the monomers in *Synechocystis* sp. PCC6803 would strengthen rigidity of trimer structure through maintaining the regular conformation of the region.

Besides deprivation of PG, the mutational loss of PsaL that is structurally organized to interact with PSI core has been found to dissociate the PSI trimer into the monomer in *Synechocystis* sp. PCC6803 [25–27] and *Synechococcus* sp. PCC7002 [28]. The genes affected were shown to be not only the *psaL* gene itself [25,28], but also the *psaL* or *psaD* gene encoding other PSI subunits. These lines of observation genetically corroborated that PsaL maintains the trimeric form of the PSI complex through the aid of PsaL [26,28] and PsaD [27], in agreement with structural organization of these subunits in the complex [24]. It is thus one way to speculate that PG helps the PSI subunit(s) such as PsaL responsible for the trimer formation be associated with the complex. However, the subunit profile, including PsaL of the PSI monomer, was little changed in SNC1 after deprivation of PG (Fig. 6). Thus, the thought seems inappropriate, although the PSI subunits of smaller molecular weights not recognized in our system remain to be examined, of course, for definitive conclusion. Rather, it could be deduced that PG and the PSI subunits such as PsaL contribute to the trimer formation of PSI in distinct ways, because the defect in the trimer formation was not complemented in PG-deprived SNC1 cells by the presence of PsaL.

On the other hand, we observed no significant damage to the PSI activity, despite abnormal prevalence of the PSI monomer after in vivo depletion of PG in SNC1 cells or in vitro one in the wild-type thylakoid membranes. These results indicated that the trimerized conformation supported by PG is little indispensable for the functioning of the PSI complex. The above disruptants as to the genes of the PSI subunits such as PsaL have similarly been shown to be little

impaired in the PSI activity, proving that not only the subunits themselves, but also the trimeric conformation sustained by the subunits, is not required for the PSI activity. Removal of PG from SNC1 cells in vivo should be incomplete, owing to the strategy to dilute cellular PG dependently on the cell division, while that from the isolated thylakoid membranes of the wild type in vitro left some amount of PG unbroken for probable limited accessibility of PLA2 to PG in the membranes (Fig. 7). Thus, definitive conclusion as to dispensability of PG itself for the PSI activity awaits closer examination.

The PSI complex of higher plants, in contrast to that of cyanobacteria, exists only as the monomeric form [29], which suggests that the PSI complex have adjusted its conformation to keep the structural and functional integrity even as the monomer during the postulated evolution of cyanobacteria to chloroplasts of higher plants. Involvement of PG in construction of the PSI trimer in *Synechocystis* sp. PCC6803 (Figs. 5 and 7), but definitely not in the higher plants, implied that the strategy of the PSI complex to utilize PG have changed also during the evolution. A clue to elucidation of the evolutionary strategy of the PSI complex would be provided through investigation of whether higher plants require PG for the structural and functional integrity of the PSI complex, with the use of the *A. thaliana* mutants defective in chloroplast PG [7–9].

4.3. Responsibility of PG for conformational integrity of the PSII complex

Structural analysis of the photosystem complexes by DM-PAGE of the thylakoid membranes with manipulation of the PG content both in vivo and in vitro also uncovered a direct role of PG in stabilization of subunit assembly of the PSII complex (Figs. 5 and 7). As the PSII complexes prepared from several organisms have been shown to include PG (e.g., Refs. [12,22]), it is plausible that PG is located at the PSII complex also in *Synechocystis* sp. PCC6803, thereby keeping its regular conformation so that the subunits are properly assembled. Such conformation would be related to maintenance of normal structure of the Q_B -binding site of the D1 protein and/or the electron transfer from Q_A to Q_B that were previously shown to be damaged by PG deprivation in SNC1 and the *pgsA*[−] mutant of *Synechocystis* sp. PCC6803 [4–6]. The similar role in the subunit assembly of the PSII complex was previously exhibited for SQDG, the other anionic lipid in the thylakoid membranes, through analysis of a mutant of *C. reinhardtii* deficient in SQDG [15]. PG and SQDG might cooperatively support the normal conformation of the PSII core complex through strengthening forces of subunits to assemble, but in quite distinct ways, in view of the decomposition of the PSII complex induced by the loss of one acidic lipid even in the presence of the other (Figs. 5 and 7, Ref. [15]).

In summary, through analysis of the photosynthetic apparatus of the *cdsA*[−] mutant cells of *Synechocystis* sp.

PCC6803 where the PG content was manipulated in vivo, we identified novel roles of PG in the synthesis and/or stability of the PSI complex, and also for conformational integrity of the PSI and PSII complexes. Besides, the roles of PG in the conformational integrity of the complexes were shown to be direct contribution to stabilize the trimeric conformation of the PSI complex and the subunit assembly of the PSII complex, with the use of the in vitro system to decrease the PG content in the isolated thylakoid membranes of the wild type.

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