Sulfoquinovosyl-diacylglycerol (SQDG) is one of the four lipids present in the thylakoid membranes. Depletion of SQDG causes different degrees of effects on photosynthetic growth and activities in different organisms. Four SQDG molecules bind to each monomer of photosystem II (PSII), but their role in PSII function has not been characterized in detail, and no PSII structure without SQDG has been reported. We analyzed the activities of PSII from an SQDG-deficient mutant of the cyanobacterium *Thermosynechococcus elongatus* by various spectroscopic methods, which showed that depletion of SQDG partially impaired the PSII activity by impairing secondary quinone (Q$_b$) exchange at the acceptor site. We further solved the crystal structure of the PSII dimer from the SQDG deletion mutant at 2.1 Å resolution and found that all of the four SQDG-binding sites were occupied by other lipids, most likely PG molecules. Replacement of SQDG at a site near the head of Q$_b$ provides a possible explanation for the Q$_b$ impairment. The replacement of two SQDGs located at the monomer–monomer interface by other lipids decreased the stability of the PSII dimer, resulting in an increase in the amount of PSII monomer in the mutant. The present results thus suggest that although SQDG binding in all of the PSII-binding sites is necessary to fully maintain the activity and stability of PSII, replacement of SQDG by other lipids can partially compensate for their functions.

Thylakoid membranes of oxygenic photosynthetic organisms contain four types of lipids, monogalactosyl-diacylglycerol (MGDG), digalactosyl-diacylglycerol (DGDG), sulfoquinovosyl-diacylglycerol (SQDG), and phosphatidylglycerol (PG). The relative content of these lipids is largely conserved among different organisms, with MGDG, DGDG, and SQDG comprising the majority of the lipids and PG as a minor class. The roles of lipids can be classified in two ways: one is to form the lipid bilayer, and the other one is to associate with some specific sites of membrane protein(s) to function as an essential part of that protein(s). MGDG and DGDG are non-ionic lipids and are considered to function mainly as structural lipids for the formation of the lipid bilayer, whereas SQDG and PG are acidic lipids and are probably required to maintain the balance of negative charges in the thylakoid membranes. In addition, all of these lipids are found to bind directly to photosynthetic membrane–protein complexes, such as photosystem II (PSII), photosystem I (PSI), and cytochrome $b_{6}f$ (5–8), and therefore may be required to maintain the integral functions of these protein complexes. In fact, deletion of PG by inactivating the genes for PG synthesis has been shown to inhibit the growth of cyanobacteria, which is caused mainly by the decrease in the activity and stability of PSII, indicating that PG plays an important role in the PSI activity (9, 10).

The roles of SQDG have been studied in various organisms by mutagenesis to inactivate the genes responsible for the SQDG synthesis. It was found that the extent of the effects caused by deletion of the genes for SQDG synthesis is different among different organisms. In *Arabidopsis thaliana* and two species of cyanobacteria (*Synechococcus elongatus* PCC 7942 and a marine cyanobacterium *Synechococcus* sp. PCC 7002), inactivation of the genes for SQDG synthesis resulted in very little effects on their growth, oxygen-evolving activity, and the sensitivity to herbicides such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) under normal growth conditions (11–13). The effects of inactivation of the gene for SQDG synthesis,
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However, became much more severe under PG-limiting conditions caused by phosphate (P) deficiency or by genetic disruption of the PG biosynthesis pathway (3, 11, 14). In Chlamydomonas reinhardtii, deletion of the gene for SQDG synthesis resulted in a decrease of oxygen-evolving activity by 30–40% and an increase in the sensitivity to DCMU (15–17). In addition, the mutant cells showed a lower resistance to heat or hydroxylamine (NH2OH) treatment (17, 18), suggesting an impairment in the oxygen-evolving complex of PSII. In contrast, deletion of the gene for SQDG synthesis completely inhibited the growth of Synechocystis sp. PCC 6803 cells concomitant with a decrease in the PSI activity (19). The inhibition of growth caused by deletion of the gene for SQDG synthesis, however, was found to be caused by the inhibition in DNA synthesis instead of the inhibition of the photosynthetic activities (20), although it is not clear why deletion of the SQDG gene inhibits DNA synthesis in Synechocystis sp. PCC 6803 but not in other cyanobacteria, and how this inhibition occurs. Recently, the gene sqdB responsible for SQDG synthesis was disrupted in a thermophilic cyanobacterium Thermosynechococcus elongatus BP-1, and the resulting mutant (∆sqdB) was characterized in terms of growth and photosynthetic activities (3). It was found that the ∆sqdB mutant had a slightly decreased growth rate and O2-evolving activity than the WT under normal growth conditions, and both PSII dimer and PSI trimer were monomerized, with the PSII dimer almost disappearing based on the blue native-PAGE analysis. The relative content of PG was remarkably increased in the sqdB-deletion mutant under phosphate-sufficient conditions, and the effects became much more severe under phosphate-limiting conditions. These results suggested that SQDG may play important roles in photosynthesis and PSII, but its role is partly compensated for by PG, resulting in a phenotype similar to that of WT under phosphate-sufficient conditions. However, it is unclear whether the role of SQDG is general as a structural lipid in maintaining the integrity of the lipid bilayer or specific in maintaining the structures and functions of the photosynthetic systems, and whether the SQDG-binding sites in the mutant have been occupied indeed by PG or other lipids.

High-resolution structural analysis of the PSII dimer from the thermophilic cyanobacterium Thermosynechococcus vulcanus has revealed binding of four SQDG molecules in a PSII monomer (Fig. 1A) (5, 21, 22). Among them, two are located in the monomer–monomer interface (Fig. 1, A, B, and D), SQDG-1, SQDG-2, corresponding to SQDG418/A and SQDG103/L, respectively, in the PDB code 3WU2). The third one is located in the boundary between the CP47 and D2 proteins and near the quinone electron acceptor Qb (SQDG-3, corresponding to SQDG412/A of PDB code 3WU2), and the final one is located in an area between PsbX and PsbF (cytochrome b559 B-subunit) (SQDG-4, SQDG407/D in the PDB code 3WU2) (Fig. 1, A, C, and E). In the structure of the PSI dimer from T. elongatus analyzed at 3.6 Å resolution (23), one of the two SQDGs (SQDG-1) located in the monomer–monomer interface of the dimer was not found, suggesting that SQDG may be required for the dimerization of the PSI monomer in the assembly process. However, the exact roles of SQDG in PSI are still unclear, because no structure of SQDG-deleted PSII has been solved, so it is unclear whether the binding sites of SQDG are replaced by other lipids/molecules or are completely empty upon depletion of SQDG, and whether there are any structural changes in the SQDG-binding regions caused by the deletion of SQDG.

To examine the roles of SQDG in PSII in more detail, we characterized the functions of PSII from the sqdB-deletion mutant ∆sqdB of T. elongatus by FTIR spectroscopy, thermoluminescence (TL), and delayed fluorescence (DL) measurements in either thylakoid membranes or purified PSII dimers. We further crystallized and analyzed the crystal structure of PSII dimers purified from the SQDG-deletion mutant. Based on the results obtained, we discuss the functions of SQDG in PSII from a structural point of view.

Results

Analyses on the content of PSII dimer and oxygen evolution

Previously it was shown by blue native-PAGE analysis that inactivation of the sqdB gene in the thermophilic cyanobacterium Thermosynechococcus vulcanus remarkably affected the stability of the PSII dimer, resulting in a large increase in the content of the PSII monomer and an almost disappearance of the PSII dimer in the mutant (3). In the procedure we used to purify the PSII dimer, the elution pattern from the ion-exchange column chromatography constantly showed that the fraction containing the PSII monomer was much larger than that of the dimer (Fig. 2). This contrasts with the situation observed in WT where the fraction of the PSII dimer is much larger than that of the PSII monomer, indicating that inhibition of SQDG synthesis destabilized the PSII dimer, in agreement with the previous results (3). The purification procedure we used here, however, still yielded a considerable fraction of PSII dimer, allowing us to perform the subsequent crystallization and structural analysis of the PSII dimer. The difference in the amount of PSII dimers observed in the previous study and this study may be due to the different purification procedures and/or analysis methods employed. In both cases, however, it is clear that inhibition of SQDG synthesis destabilized the PSII dimer, resulting in an increase in the amount of PSII monomer. All analyses described below are performed with the PSII dimers from the mutant and compared with the PSII dimers of WT.

The ∆sqdB mutant cells have been shown to have slightly decreased oxygen-evolving activity in the presence of PSII electron acceptors (3). We further compared the oxygen-evolving activities of isolated thylakoid membranes and PSII dimers between WT and ∆sqdB with 0.4 mM phenyl p-benzoquinone and 1 mM potassium ferricyanide as electron acceptors. The results showed that the oxygen-evolving activity of the mutant samples decreased by about 19–32% in the thylakoid membranes and purified PSII dimers. This decrease is comparable with those observed with the mutant cells of T. elongatus reported previously (3) and also with the previous report in the C. reinhardtii mutant with the SQDG synthesis gene inactivated (15–17). To examine the causes for the decrease of PSII activity in detail, we performed spectroscopic analyses with the WT and ∆sqdB samples.
Spectroscopic analyses (TL, FTIR, and DL measurements) of the PSII activities

The S-state transitions of the WT and ΔsqdB thylakoid membranes were examined by TL in either the presence or absence of DCMU. As shown in Fig. 3, the TL glow curves in both the presence and absence of DCMU were very similar between the WT and the ΔsqdB mutant, suggesting that the redox potential of the S2/S1 couple at the electron donor side and those of QA/QA and QB/QB at the acceptor side were not significantly changed by the inhibition of SQDG synthesis.

The properties of S-state transition were further examined by flash-induced FTIR spectroscopy with purified PSII dimers from WT and the ΔsqdB mutant. As shown in Fig. 4A, the 1st and 2nd flash-induced FTIR difference spectra exhibited negative bands in the symmetric COO⁻ stretching region (1450–1300 cm⁻¹), whereas the 3rd and 4th flash-induced difference spectra exhibited positive bands in this region, in the WT-PSII.

Figure 1. Location of SQDG molecules in a PSII dimer of WT (PDB code 3WU2). A, overall structure of WT—PSII dimer with a top view from the cytoplasmic side. B and C, surface model view of the enlarged boxed region in shown in A. D and E, view with a rotation of 90° relative to B and C, respectively, in a cartoon model. All four SQDG molecules in each PSII monomer are located within the membrane close to the stromal side, with their negatively charged headgroups facing the stromal surface. In the monomer–monomer interface, two SQDGs (SQDG-1 and -2) bind to each monomer in a fashion resembling a clamp. SQDG-3 is close to the Qb cavity and binds to a position about 10 Å away from Qb across the D1-loop. SQDG-4 is located between PsbX and PsbF with its tail close to the isoprenoid tail of Qb. Color codes: SQDG, yellow stick; Qb, cyan stick; PG, orange stick.

Figure 2. Anion-exchange column chromatography of crude PSII samples after solubilization with 1.0% β-DDM from WT (blue lines) and the ΔsqdB mutant (orange lines). Q-Sepharose high performance was used for the column, and the samples were eluted with a linear gradient of NaCl from 170 to 300 mM, and the eluent was monitored by absorption at 280 nm.

Spectroscopic analyses (TL, FTIR, and DL measurements) of the PSII activities

The S-state transitions of the WT and ΔsqdB thylakoid membranes were examined by TL in either the presence or absence of DCMU. As shown in Fig. 3, the TL glow curves in both the presence and absence of DCMU were very similar between the WT and the ΔsqdB mutant, suggesting that the redox potential of the S2/S1 couple at the electron donor side and those of QA/QA⁺ and Qb/Qb⁻ at the acceptor side were not significantly changed by the inhibition of SQDG synthesis.

The properties of S-state transition were further examined by flash-induced FTIR spectroscopy with purified PSII dimers from WT and the ΔsqdB mutant. As shown in Fig. 4A, the 1st and 2nd flash-induced FTIR difference spectra exhibited negative bands in the symmetric COO⁻ stretching region (1450–1300 cm⁻¹), whereas the 3rd and 4th flash-induced difference spectra exhibited positive bands in this region, in the WT-PSII.
These features are identical to those of active PSII reported previously (24, 25), and they represent the structural changes in the \( S_1 \rightarrow S_2, S_2 \rightarrow S_3, S_3 \rightarrow S_4 \), and \( S_4 \rightarrow S_0 \) transitions, respectively. The spectral features of 5th/9th, 6th/10th, 7th/11th, and 8th/12th flashes were similar to those of 1st, 2nd, 3rd, and 4th flashes, respectively, although their intensities were gradually decreased by increasing the flash numbers (Fig. 4A). The oscillation of the FTIR difference spectra is attributed to the misses in the \( S \)-state transitions, which increase the mixture of other \( S \)-state transitions at increased flash numbers (25). All of these spectral features were found to be similar in the \( \Delta sqdB \) mutant PSII (Fig. 4A), suggesting that absence of SQDG did not affect the structures and reactions of the water-oxidizing center significantly.

Oscillation of the \( S \)-state transition was examined by plotting the intensity difference between the FTIR peaks at 1437 and 1401 cm\(^{-1}\) against the flash number, with normalization against the same intensity difference observed at the 1st flash spectrum. Typical period-four oscillation patterns were observed in both WT and the mutant PSII samples (closed circles in Fig. 4, B and C). The oscillation pattern was simulated with a conventional method based on the equation employed previously, assuming an average efficiency for the different \( S \)-state transitions (26). Good fittings were obtained for both WT and \( \Delta sqdB \) mutant PSIIIs (open triangles in Fig. 4, B and C), and their average efficiencies were estimated to be 88 ± 2 and 86 ± 2%, respectively. These average efficiencies were almost
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the same between the WT and ΔsqdB mutant PSII dimers. Because the present FTIR measurements of S-state transition were performed in the presence of ferricyanide as an exogenous electron acceptor, the results obtained suggest that inhibition of SQDG synthesis did not affect the PSII donor side significantly.

The electron transfer properties of PSII from the ΔsqdB mutant were further examined by DL measurements, which originates from the charge recombination and back reaction from the PSII acceptor side. As shown in Fig. 5, the DL intensity from the WT thylakoid membranes exhibited a typical period-four oscillation during 12 flashes of illumination (black line). The oscillation pattern of the DL intensities from the ΔsqdB mutant sample (Fig. 5, red line) was remarkably changed compared with the WT, with almost no further oscillations after the 7th flash. Because the donor side and the electron transfer from Qₐ to Qₖ were not much changed in the mutant based on the FTIR and TL results shown above, the DL results indicate that inhibition of SQDG synthesis affected the exchange of plastoquinone with free plastoquinone after reduction of Qₖ at the acceptor side.

Crystal structure analysis of the ΔsqdB–PSII dimer

The structure of the PSII dimer from the ΔsqdB mutant was analyzed at a resolution of 2.1 Å with X-ray diffraction data collected at a wavelength of 1.0 Å (Table 1). The overall structure of the ΔsqdB–PSII dimer obtained is very similar to that of the WT PSII, with a root-mean-square deviation of 0.32 Å between the Ca atoms of WT and ΔsqdB–PSII. To examine the SQDG-binding sites in detail, we calculated the SQDG-omitted |Fₒ|−|Fₑ| difference map for the mutant PSII. The results showed that all of the four SQDG-binding sites have positive electron densities, indicating that the four SQDG-binding sites are not empty but have been occupied by some other molecules in the mutant PSII (Figs. 6 and 7). Among them, the 2|Fₒ|−|Fₑ| map for the binding sites of SQDG-1, SQDG-2, and SQDG-3 clearly showed fatty acid tails of diacylglycerol (Figs. 6, A–C, and 7), suggesting that these sites are replaced by other types of lipids. However, the electron densities for their headgroups are distorted and not clearly defined, which prevented us to assign the lipid classes into these sites unambiguously. Among the other classes of lipids, DGDG has a much larger headgroup and cannot fit with the electron density, whereas the headgroups of MGDG and PG are rather similar and therefore cannot be distinguished based on the 2|Fₒ|−|Fₑ| and |Fₒ|−|Fₑ| maps. We also collected datasets at 1.9 Å wavelength and analyzed the structure to 2.4 Å resolution to locate the possible phosphorus atoms by its anomalous signal; however, no anomalous signals were observed in these three sites (Fig. 7). Thus, we modeled the molecules in these SQDG-binding sites as unknown lipids. The headgroup for the SQDG-4–binding site can be modeled to a PG molecule, and the anomalous scattering data collected at 1.9 Å wavelength also showed apparent anomalous signals (Fig. 7); thus, this binding site is occupied by a PG molecule.

To examine the effects of sqdB deletion on the composition of lipid classes in the purified PSII dimer, we analyzed the lipid

Table 1

| Data collection | Wavelength (Å) | 1.000 | 1.900 |
|----------------|---------------|-------|-------|
|                | Space group   | P2₁,2₁,2₁ | P2₁,2₁ |
|                | Unit cell (Å) | a = 122.3, b = 228.4, c = 287.1 Å | a = 121.5, b = 228.4, c = 286.7 Å |
|                | Resolution (Å) | 20–2.10 (2.22–2.10) | 20–2.40 (2.46–2.40) |
|                | Unique reflections | 459,862 | 601,068 |
|                | Redundancy     | 6.8 (6.7) | 21.74 (20.00) |
|                | Completeness   | 99.5 (98.5) | 100 (99.8) |
|                | Rmerge         | 0.087 (0.086) | 0.128 (1.090) |
|                | I/σ(I)         | 13.7 (2.1) | 18.2 (3.2) |
|                | CC(1/2)        | 0.998 (0.870) | 0.999 (0.916) |

| Refinement statistics | Rmerge | 0.157 | 0.155 |
|-----------------------|--------|-------|-------|
|                       | Rfree  | 0.198 | 0.205 |
|                       | RMSD (bond) (Å) | 0.021 | 0.018 |
|                       | RMSD (angle) (degrees) | 2.564 | 2.420 |
|                       | Ramachandran plota |        |       |
|                       | Favored (%) | 97.4 | 97.1 |
|                       | Allowed (%) | 2.4 | 2.7 |
|                       | Outliers (%) | 0.2 | 0.2 |

a Values in the parentheses indicate those for the highest resolution shell.

b Ramachandran plot was calculated with the MolProbity.
contents of the PSII dimers before and after crystallization and compared them with that of the WT PSII. The results showed that although the numbers of MGDG and DGDG are not much different between the WT and mutant PSII (data not shown) and are also consistent with those found in the crystal structure, the number of PG is 5–6 per monomer in WT but increased to around 9 in the ΔsqdB mutant. This suggests that most of the SQDG molecules have been replaced by PG in the mutant PSII.

**Structural changes caused by inactivation of the sqdB gene**

Among the two SQDGs bound in the interface between the two monomers within a dimer, SQDG-1 connects D1 from one monomer (A-monomer) with CP47 from the other monomer (B-monomer). The headgroup of SQDG-1 is hydrogen-bonded with D1–Trp-20 and D1–Asn-26 of the A-monomer and with CP47–Trp-113 and CP47–Tyr-117 of the B-monomer (Fig. 8A). Although depletion of SQDG did not affect the structures of the residues surrounding this binding site significantly, replacement by an unknown diacyl chain results in the breakage of most of the hydrogen bonds, leading to a disconnection between D1 of the A-monomer and CP47 of the B-monomer (Fig. 8A). Even if it is replaced by a PG molecule based on the result of lipid analysis, it can form a hydrogen bond with CP47–Trp-113 only (Fig. 9, A and B). This apparently weakens the interactions between the two monomers, resulting in the destabilization of the dimer in the mutant.

SQDG-2 is also located in the monomer–monomer interface. In WT, SQDG-2 is surrounded by PsbL, PsbM, and PsbT subunits from the A-monomer and CP47 from the B-monomer, and it forms hydrogen bonds with PsbL–Arg-14 of the A-monomer and CP47–Arg-18 and CP47–Trp-115 of the B-monomer (Fig. 8B). In the ΔsqdB mutant, this SQDG is replaced again by an unknown lipid. One of the two fatty acid tails of this unknown lipid was shifted from the original position of the SQDG-2 tail and away from the A-monomer, leaving a space close to the PsbT and PsbM subunits. This space was filled by a new β-dodecyl maltoside (β-DDM) molecule in the mutant PSII. However, the unknown lipid and β-DDM mole-
cule formed almost no hydrogen bonds with the neighboring subunits. Modeling of a PG molecule into this site does not change the situation (Fig. 9, C and D). Thus, the connection between the A- and B-monomers via SQDG-2 was also broken in the mutant PSII. This, together with the breakage of the bridge via SQDG-1 described above, will apparently destabilize the dimer, leading to the significant monomerization of PSII in the mutant as observed in the present and previous studies.

The headgroup of SQDG-3 is surrounded by D1 and CP43, and one of its fatty acid tails is close to PsbJ, PsbK. The head of SQDG-3 is hydrogen-bonded with D1–Asn-267, D1–Ser-270, CP43–Gln-28, and CP43–Trp-36 directly and with D2–Phe-232 through a water molecule (Fig. 8C). Because of the replacement of SQDG-3 by an unknown diacyl chain, these hydrogen bonds were also broken in the ΔsqdB mutant. Modeling of a PG molecule in this site does not help to maintain most of the hydrogen bonds with the surrounding residues (Fig. 9, E and F). Although the structure of the surrounding residues was not changed significantly again by this replacement, two residues hydrogen-bonded to SQDG-3, namely D1–Asn-267 and D1–Ser-270, are located in a loop close to the headgroup of Qb, where D1–Ser-264 is hydrogen-bonded to the head of Qb (Fig. 8C). The breakage of the hydrogen bonds between D1–Asn-267, D1–Ser-270, and SQDG-3 may affect the interaction of this loop with Qb, thereby affecting the exchange process of this bound quinone acceptor with free plastoquinones.

SQDG-4 is surround by D2, PsbF, and PsbX, and its fatty acid tails are close to the isoprenoid tail of QB. In the WT, the headgroup of SQDG-4 is hydrogen-bonded with D2–Arg-24, D2–Arg-26, PsbF–Val-118 directly, and with PsbE–Glu-7 through a water molecule (Fig. 8D, left panel). In the ΔsqdB mutant, this SQDG is clearly replaced by a PG molecule, which maintains the hydrogen bonds with D2–Arg-24, D2–Arg-26, and PsbF–Val-18 (Fig. 8D, right panel). The phosphorus atom is fitted in the same position as that of the sulfur atom in SQDG of the WT structure. Only the water molecule connecting SQDG-4 and PsbE–Glu-7 was lost in the mutant, resulting in the loss of the hydrogen bond between the PG molecule and PsbE–Glu-7. The tail of the PG molecule is also located in a position very similar to that of SQDG-4 and close to the tail of

Figure 7. Comparison of 2|Fo| − |Fc| and |Fo| − |Fc| electron densities and the anomalous difference densities in the four SQDG-binding sites between WT and ΔsqdB. A–D, violet, green, and red mesh indicate 2|Fo| − |Fc|, maps contoured at 0.8 σ, SQDG-omitted |Fo| − |Fc| maps (positive) contoured at 3.5 σ, and anomalous difference maps countered at 4.0 σ, respectively. In WT, the density maps and anomalous difference maps were calculated from the datasets refined to 2.1 and 2.5 Å resolution collected at 0.9 and 1.9 Å wavelengths, respectively. In the WT structure, SQDG molecules depicted in yellow are fitted based on the omit 2|Fo| − |Fc| and |Fo| − |Fc| densities.
Thus, the function of SQDG-4 may well be maintained by the PG molecule in this binding site.

Discussion

In this study, we examined the effects of inactivation of the sqdB gene that results in a mutant unable to synthesize SQDG, on the stability and activity of PSII dimer from the thermophilic cyanobacterium *T. elongatus*. FTIR and TL measurements showed that the absence of the SQDG synthesis has little effect on both the electron donor and acceptor sides, which is in agreement with the results of structural analysis showing that the overall structure of the ΔsqdB–PSII is very similar to that of the WT–PSII. A slight decrease in the oxygen-evolving activity was observed upon inhibition of SQDG synthesis, which is presumably caused by the effects on the QB exchange process at the acceptor side as shown by the DL measurements. An important effect of the inhibition of SQDG synthesis is the destabilization of PSII dimer, leading to a significant increase in the PSII monomer. This is in agreement with the previous results (3). However, our purification procedures used still gave rise to a certain fraction of PSII dimers, which is different from the previous analysis showing that almost all of the PSII dimer was lost in the mutant (3). This may be due to the differences in the solubilization conditions and/or analysis methods used between the present and previous studies.

The slight effects of SQDG absence on PSII activity observed in the thermophilic cyanobacterium in the present and previous studies (3) are similar to those observed with other organisms (except *Synechocystis* 6803) and may be taken as evidence to indicate that SQDG is dispensable for the photosynthetic growth and PSII activity (13). However, our structural analysis of the ΔsqdB mutant PSII clearly showed that all of the four SQDG-binding sites have been occupied by other lipid molecules. Among them, the lipid molecules replacing SQDG-1 to SQDG-3 could not be identified unambiguously from the electron density map. In the structure of WT–PSII analyzed at the same resolution, the $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ maps showed clear densities for these SQDG-binding sites (Fig. 7). This suggests that the alternative lipid molecules do not fit well with these sites and therefore cannot bind to the nearby residues specifically, resulting in the distortions in the electron densities. In contrast, the electron densities for the fatty acid tails in these three sites are more or less observed, allowing us to assign the

**Figure 8. Comparisons of the structures of the four SQDG-binding sites between WT and the ΔsqdB mutant.** A and B, comparison of the structure of the SQDG-1– and SQDG-2– binding sites between WT and the ΔsqdB mutant. The structures of the WT and the mutant are superimposed, and the residues of WT are colored, and those of the mutant are depicted in gray. SQDGs are depicted in yellow, and the unknown lipid molecules in the mutant are depicted in cyan. A bound detergent molecule, β-DDM, was depicted in green in B. C, comparison of the structure of the SQDG-3– binding site between WT and the ΔsqdB mutant. For the sake of clarity, only residues of WT are shown except for the unknown lipid of the mutant, which is shown in cyan. D, comparison of the structure of the SQDG-4– binding site between WT (left side) and the ΔsqdB mutant (right side). In all panels, except D, the hydrogen-bond distances are shown in Å. D, hydrogen-bond distances were omitted for the sake of clarity.
positions of the tails based on the electron density map. How-
ever, the electron densities corresponding to the headgroup of
the unknown lipids were very weak or not observed, suggesting
that binding of the new, unknown lipids to these three sites are
nonspecific and weak, which in turn indicates that these sites
have evolved to accommodate SQDG best. Because MGDG and
DGDG have relatively larger headgroups than that of PG, we
expect that it would be easier to visualize them if these sites
were replaced by MGDG and/or DGDG. The absence of elec-
tron densities for the headgroups may therefore suggest that
these sites have mostly been replaced by PG. This is in agree-
ment with the results of lipid analysis showing that the PG con-
tent is increased significantly in the mutant PSII, whereas those
of MGDG and DGDG remained unchanged compared with the
WT. The replacement of SQDG by PG in the mutant also agrees
with a number of studies reported previously that phosphate is
required to maintain the photosynthetic growth of mutants
defective in SQDG synthesis (3, 12, 19, 27), although the weak

Figure 9. Modeling of PG molecules into the three SQDG-binding sites and their possible hydrogen bonds with nearby residues in the mutant. The three SQDG-binding sites (SQDG-1, SQDG-2, and SQDG-3) were tentatively assigned to PG molecules (B, D, and F) and superimposed with their 2|Fo| – |Fc| maps contoured at 0.8σ (violet) and SQDG-omitted |Fo| – |Fc| maps (green, positive) contoured at 3.5σ, respectively, in the mutant PSII dimers, and compared with the structures of SQDGs in the WT PSII (A, C, and E). Dotted lines indicate the hydrogen bonds between SQDGs and nearby residues in the WT–PSII and possible hydrogen bonds between PG and nearby residues (including water molecules) in the ΔsqdB–PSII. PG, SQDG, and β-DDM molecules are depicted as sticks in pink, yellow, and green, respectively.
electrode densities of the headgroup in these binding sites in the mutant may suggest that part of the binding sites were occupied by a mixture of different lipids. However, the binding site for SQDG-4 was shown to be replaced by a PG molecule based on its electron density as well as the anomalous signal of the phosphorus atom.

The effects of SQDG absence on the stability of the PSII dimer and the exchange of bound Q_{B} can be explained based on the results of the structural analysis. Among the four SQDG molecules in PSII, SQDG-1 and SQDG-2 are located in the interface between the two PSII monomers and contribute to the formation of the dimer by connecting different subunits from the two monomers by a number of hydrogen bonds formed between the headgroup of SQDG and nearby amino acid residues. Replacement of these two lipids by unknown lipids resulted in the breakage of the hydrogen bonds. This may have caused destabilization of the PSII dimer, resulting in the increase of PSII monomer observed in the present and previous studies (3). Even if these two sites are replaced by PG in the mutant, due to its different structure of the headgroup as well as the weak binding to the nearby residues, most of the hydrogen bonds may have been weakened or lost, resulting in the destabilization of the PSII dimer. This indicates that only SQDG can fulfill the full function to stabilize the PSII dimer in these two binding sites, and replacement by other lipids was not enough to fully compensate for the function of SQDGs.

SQDG-3 is located at a position close to Q_{B}, and its headgroup forms hydrogen bonds with residues of a D1 loop (including D1–Ser-270) that also forms hydrogen bonds with the headgroup of Q_{B} (D1–Ser-264). Replacement of this SQDG by an unknown lipid in the ΔsqdB mutant broke the hydrogen bonds with the D1 loop, which may in turn affect the hydrogen bonds between this D1 loop and the head of Q_{B}. This may be the cause for the changes observed in the Q_{B} exchange property in the mutant. Indeed, alteration of D1–Ser-270 to alanine has been shown to affect the binding of bromoxynil to the Q_{B}-binding site, resulting in a phenotype that is more susceptible to bromoxynil (28). Similar high sensitivity to herbicides was also reported previously in several species depleted of SQDG (14, 16). This suggests that the interactions of D1–Ser-270 and other nearby residues with SQDG may provide a cap to protect the Q_{B}-binding site, and breakage of these interactions results in the destabilization of the Q_{B}-binding site, leading to the easy exchange by and higher sensitivity toward herbicides.

Finally, SQDG-4 was replaced by a PG molecule, and hydrogen bonds of SQDG-4 with nearby amino acid residues were largely maintained by the PG molecule. Thus, this replacement may not affect the PSII activity significantly.

In conclusion, we succeeded in the crystallization and its structural analysis at a high resolution with PSII dimers derived from a single lipid deletion mutant. The apparent effects of failure to synthesize SQDG on the growth and PSII activity of the thermophilic cyanobacterium are very small. However, structural analysis revealed that all of the four SQDG-binding sites were replaced by other lipids, mostly PG, which apparently have contributed to maintain the photosynthetic growth and activity of the mutant. This in turn suggests that the four SQDG-binding sites have to be occupied by some lipid molecules, with SQDG being the optimum choice, and its functions can be compensated in part by other lipids, mostly PG. However, the replacement cannot completely reproduce the hydrogen bonds mediated by SQDG between the A- and B-subunits and in the vicinity of Q_{B}, leading to the decrease in the PSII dimer stability and alterations of the Q_{B} exchange property. Thus, these sites have evolved to best accommodate SQDG in the thermophilic cyanobacterium. Our results thus demonstrated that the effects of lipid depletion and their functions in membrane proteins and their complexes must be examined on the basis of structural analysis.

**Experimental procedures**

**Cell culture and purification**

The ΔsqdB strain was constructed by deleting the sqdB gene from *T. elongatus* as described previously (3), which was confirmed by PCR analysis. Liquid culture of cells was grown at 45 °C with bubbling of air containing 3% (v/v) CO_{2} under constant red LED light at an intensity of 20 μmol photons m^{-2} s^{-1} in a plant growth chamber (BIOTRON LH-410PF-SP), in the presence of 15 μg/ml chloramphenicol. For purification of the PSII dimer sample, 40 liters of mutant cells were grown for 7–9 days in the absence of chloramphenicol, and the light intensity was increased gradually from 20 to 100 μmol photons m^{-2} s^{-1}. The cultured cells were harvested and disrupted by a lysozyme treatment and a freeze-thawing method (29, 30). The ΔsqdB-PSII dimers were purified as described previously (29, 30) and finally stored in a buffer containing 5% (w/v) glycerol, 20 mM Mes-NaOH (pH 6.0), 20 mM NaCl, 3 mM CaCl_{2}. All procedures for the preparation were performed under dim green light.

**Oxygen evolution and TL and DL measurements**

Oxygen evolution of thylakoid membranes and PSII dimers was measured with a Clark-type oxygen electrode (Hansatech Instruments) under continuous, saturating illumination at 30 °C in the storage buffer, with 0.4 mM phenyl-p-benzoquinone and 1 mM potassium ferricyanide as electron acceptors at a chlorophyll (Chl) concentration of 10 μg of Chl/ml. TL measurements were performed using a laboratory-built apparatus as described previously (31). For measurement of the Q band, thylakoids (250 μg of Chl ml^{-1}) in the presence of 50 μM DCMU were illuminated with continuous white light (~55 milliwatts cm^{-2} at the sample point) from a halogen lamp (MEJIRO PRECISION PHL-150) for 10 s at ~20 °C. For measurement of the B band, thylakoids (250 μg of Chl ml^{-1}) were illuminated with a single xenon flash at 5 °C. The sample was quickly cooled down and then warmed at a rate of 40 °C min^{-1} to record the TL glow curves.

For DL measurements, thylakoids (250 μg of Chl ml^{-1}) were illuminated with a series of xenon flashes (0.5 Hz) at 25 °C. The DL intensity at 1.8 s after each flash was normalized based on the maximum DL intensity of each sample during the measurement with the train of flashes and was plotted against the flash number.

**FTIR measurements**

PSII core complexes were washed in a buffer containing 1 mM Mes-NaOH (pH 6.0), 5 mM NaCl, 5 mM CaCl_{2}, and 0.03%
Structure and function of PSII from a SQDG-deficient mutant

β-DDM and concentrated to ~4.5 mg of Chl ml⁻¹ by ultrafiltration (Vivaspin 500, molecular mass cutoff 100 kDa). An aliquot of 10 μl of the concentrated PSII was mixed with 1 μl of 100 mM potassium ferricyanide on a CaF₂ plate (25 mm in diameter) and dried by a stream of nitrogen gas on ice in the dark. The resultant sample was resuspended with 1 μl of 200 mM Mes-NaOH (pH 6.0) and sandwiched with another CaF₂ plate as described previously (26). The sample temperature was kept at 10 °C by circulating cold water through a copper holder.

Light-induced FTIR difference spectra were recorded using a Bruker IFS-66/S spectrophotometer equipped with an MCT detector (InfraRed D313-L) at 4 cm⁻¹ resolution. The samples were stabilized at 10 °C in the dark for 4 h before the spectra were recorded. For the measurements of S-state transitions, flash illumination was performed by a Q-switched Nd:YAG laser (Quanta-Ray GCR-130; 532 nm, ~7 ns full width at half-maximum) with a power of ~7 mJ pulse⁻¹ cm⁻² at the sample position. The samples were illuminated by two preflashes (1 Hz) followed by dark adaptation for 30 min to synchronize all centers to the S₀-state. Twelve flashes were applied to the sample at intervals of 10 s, and a single-beam spectrum (20 scans) was measured twice before the 1st flash and once after each flash. The samples were then dark-adapted for 30 min again. This whole measurement scheme was repeated six times, and the spectra were averaged (120 scans in total). Difference spectra upon individual flashes were calculated to provide structural changes during S-state transitions, whereas a difference spectrum between the two spectra before the 1st flash represented the noise level. Spectral fitting and simulation of the oscillation pattern were performed using Igor Pro (WaveMetrics Inc.) according to the equation described previously (26).

Lipid analysis

Lipids in the WT and ΔsqdB PSII samples were extracted by the method described previously (32) and separated by TLC. Each lipid class was quantified by GC as described previously (33, 34).

Crystallization and structural analysis

The purified ΔsqdB–PSII dimers were crystallized using the oil batch method under conditions similar to those previously described (5, 30, 35) with slight modifications as follows. In the first step of crystallization, many multiclustered and needle-clustered crystals were obtained; they were collected individually, washed by a crystallization buffer containing 10%

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