Sulfite-stress induced functional and structural changes in the complexes of photosystems I and II in a cyanobacterium, *Synechococcus elongatus* PCC 7942

Satomi Kobayashi, Mikio Tsuzuki, and Norihiro Sato

1School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Horinouchi 1432-1, Hachioji, Tokyo 192-0392, Japan
2JST, CREST, Chiyoda-ku, Tokyo, 102-0075, Japan

*Corresponding author: E-mail, nsato@ls.toyaku.ac.jp; Tel, 042-676-6716; Fax, +042-676-6721.

(Received February 17, 2015; Accepted May 17, 2015)

Excess sulfite is well known to have toxic effects on photosynthetic activities and growth in plants, however, so far, the behavior of the photosynthetic apparatus during sulfite-stress has not been characterized as to the responsible proteins or genes. Here, the effects of sulfite on photosystem complexes were investigated in a cyanobacterium, *Synechococcus elongatus* PCC 7942, a possible model organism of chloroplasts. Culturing of the cells for 24 h in the presence of 10 mM sulfite retarded cell growth of the wild type, concomitantly with synthesis of Chl and phycobilisome repression. The excess sulfite simultaneously repressed photosynthesis by more than 90%, owing largely to structural destabilization and resultant inactivation of the PSII complex, which seemed to consequently retard the cell growth. Notably, the PsbO protein, one of the subunits that construct the water-splitting system of PSII, was retained at a considerable level, and disruption of the *psbO* gene led to higher sensitivity of photosynthesis and growth to sulfite. Meanwhile, the PSI complex showed monomerization of its trimeric configuration with little effect on the activity. The structural alterations of these PS complexes depended on light. Our data provide evidence for quantitative decreases in the photosystem complex(es) including their antenna(e), structural alterations of the PSI and PSII complexes that would modulate their functions, and a crucial role of *psbO* in PSII protection, in *Synechococcus* cells during sulfite-stress. We suggest that the reconstruction of the photosystem complexes is beneficial to cell survival.

**Keywords:** photosynthesis • photosystem I • photosystem II • *psbO* • sulfite stress • *Synechococcus.*

**Abbreviations:** 2D-PAGE, 2-dimensinal PAGE; DGDG, digalactosyl diacylglycerol; DM, dodecyl-ß-D-maltoside; GLC, gas-liquid chromatography; MALDI TOF/TOF-MS, matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry; MGDG, monogalactosyl diacylglycerol; p-BQ, p-benzoquinone; PG, phosphatidylglycerol; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species; SQDG, sulfoquinovosyl diacylglycerol; TLC, thin-layer chromatography.

**Introduction**

Sulfur dioxide (SO_2) is one of the major air pollutants that generates acid rain, being emitted industrially through the combustion of fossil fuels including oil and coal and also naturally through volcanic eruptions. Acid rain could cause bleaching of the leaves of crops and forest trees and their consequent death. Air pollution by SO_2 today prevails in the industrial areas of developing countries like China (Lu et al. 2010) and was highlighted by the eruption of Icelandic volcano Eyjafjallajökull in 2010 (Heue et al. 2011).

SO_2 is taken up by plants mainly through the stomata, and then dissolved in the apoplastic space to enter the mesophyll cells (Furihara et al. 1990, Rennenberg and Herschbach 1996). When dissolved in the aqueous phase, SO_2 can be converted to a mixture of H_2SO_3, HSO_3^−, and SO_3^2− (sulfite), their proportions depending on the pH and temperature of the solution (Perrin 1982). Sulfite is an intermediate metabolite in the S-assimilating pathway: sulfate, a major ambient S-source, incorporated into plant cells is activated to become adenosine 5′-phosphosulfate, which is then reduced to sulfite for utilization in the synthesis of S-containing organic compounds such as Cys and Met (Saito 2004). On the other hand, sulfite is a nucleophilic compound that attacks biological matter such as DNA, proteins, and lipids, e.g. cleaving the disulfide bonds of proteins such as thioredoxin, which is a reaction designated as sulfitolysis (Würfel et al. 1990). Moreover, sulfite can be harmful for plants in particular, since it enhances the formation of reactive oxygen species (ROS) through photo-oxidation in chloroplasts (Asada and Kiso 1973). In plants, application of SO_2 at below toxic levels can thus be utilized effectively in the S-assimilating pathway, whereas above a certain threshold it can cause damage to physiological processes (Rennenberg 1984). So far, the ability of tolerance to sulfite could be enhanced in seed plants by over-expressing the gene for sulfite oxidase or reductase, which partially repressed an increase of sulfite concentrations in the plants fumigated with sulfite (e.g. Lang et al. 2007, Yarmolinsky et al. 2013).

Plants exposed to excess SO_2 show depression of photosynthesis as one of the major physiological impairments, before
The photosynthetic apparatus of cyanobacteria include essentially the same functional units as those of seed plants, such as photosystem I (PSI) and photosystem II (PSII) complexes, although some different aspects have also been revealed in light-harvesting complexes (Liu et al. 2005; phycobilisomes, cf. Chla/b-binding light-harvesting complexes I and II in seed plants), subunit composition of the water splitting complex at PSII (Enami et al. 2008; PsbO, PsbU, and PsbV, cf., PsbO, PsbP, and PsbQ in seed plants), and higher-order structure of the PSI complex (Grotjohann and Fromme 2005; trimeric conformation, cf. monomeric one in seed plants). Likewise, components and compositions of thylakoid membranes lipids in cyanobacteria resemble those in seed plants. It has thus been regarded that cyanobacteria are the ancestor of chloroplasts of seed plants. Some cyanobacterial species, especially those in which genomic DNA sequences are determined and genetic manipulation protocols are established, has been used as model organisms of chloroplasts. However, little attention to phytotoxicity of sulfite has been paid to the photosynthetic apparatus or gene expression involved in sulfate assimilation in aquatic photosynthetic organisms including cyanobacteria, although such information would lead to a comprehensive understanding of the process of acclimation of plants to sulfite-stress, and further to its application to development of ability to detoxify sulfite in plants.

In this study, we investigated the sulfite toxicity toward photosynthesis in a cyanobacterium, *Synechococcus elongatus* PCC 7942 (hereafter referred to as *Synechococcus*), with a focus on thylakoid membranes, thereby revealing sulfite-induced functional and/or structural alterations in the complexes of photosystems I and II. On the basis of the results, a process of acclimation to sulfite-stress in the cyanobacterial cells will be discussed.

### Effects of sulfite on the cell growth of *Synechococcus*

The purpose of this study was to clarify how the photosynthetic apparatus behaves in response to sulfite-stress in *Synechococcus* cells and thereby to understand the mechanism by which the cells become acclimated to the stress. We first investigated the effects of application of sulfite to a culture of *Synechococcus* cells on their growth and determined the contents of Chl and phycobilisomes (Fig. 1). Standing culture of *Synechococcus* cells in a 48-well plate revealed that their growth evaluated with OD730 values was little affected at 2.5 mM sulfite (data not shown; Fig. 1A, upper panel). However, dose-dependent retardation of cell growth became obvious with a further increase in the sulfite concentration to show 52.1 ± 3.1% and 15.8 ± 2.0% growth at 7.5 mM and 15.0 mM sulfite, respectively, relative to the control level (a 2.5-fold increase in OD730 from 0.23). A similar trend as to cell growth was observed with the cells cultured with aeration in a larger scale for quantification of Chl and phycobilisome (Fig. 1A, lower panel). The growth of the cells was normal in the presence of 2 mM sulfite, however, it was retarded with an increase in the sulfite concentration to 10 or 50 mM. In parallel, similar patterns were observed for the Chl and phycobilisome contents (Fig. 1B, C). Moreover, it became evident that, upon exposure to the severest stress of sulfite at 50 mM, the contents of Chl and phycobilisomes decreased gradually on the basis of the OD730 value, reaching 69% and 66% at 24 h, respectively, relative to the controls (Fig. 1D, E). These results indicated that contents of the PSI and/or PSII complexes per OD730 decreased during imposition of sulfite-stress. However, little deleterious effect on re-growth in the normal medium was observed with the cells that had been stressed by sulfite (Fig. 1F, upper panel). To preclude light-induced damage to the sulfite-stressed cells during the recovery process, we cultured the cells under the very low-light conditions (0.3 W m⁻²). Standing culture for 5 and 10 days increased OD730 values by 4.6 ± 0.4 and 11.2 ± 1.2 folds relative to the initial level (OD730 = 0.03), respectively, in the control (non-stressed) cells whereas that increased the values by 4.5 ± 0.7 and 10.9 ± 1.8 folds in the cells that had been imposed on 15 mM sulfite for 6 d as in Fig. 1A (upper panel). Therefore, the sulfite-stress did not seem to significantly increase the death of the cells. Consistently, in culture with aeration for 24 h, irrespective whether sulfite treatment was performed or not, the proportion of the cell population that was apparently disturbed as to integrity of the cell membranes was less than 2%, and thus was very small (Supplementary Fig. S1, data not shown). Despite the integrity of the cell membranes, the cells that had been stressed with 10 mM sulfite for 24 h in culture with aeration showed lower viability than the normally grown cells, on subsequent growth on an agar plate containing the regular BG11 medium (Fig. 1F, lower panel, SO₄²⁻, cf. Control). The decreased cell survival-level might reflect some physiological damage to the cells in the liquid culture, which could become serious, only when the cells are placed on the agar plate and thus are subjected to a desiccation stress. Since 10 mM sulfite allowed the cells to grow only slowly, without lowering the viability in the liquid medium, we hereafter utilized this concentration for our study of the process of acclimation to sulfite-stress.

There are two possibilities as to how sulfite delayed the cell growth. One is that it was the consequence of the chemical
toxicity of sulfite inducing oxidative stress and/or sulfitolysis. Alternatively, in view of the inclusion of only 0.3 mM sulfate as a sole S-source in the regular BG11 medium, 10 mM sulfite apparently was too abundant as an external S-source, leading possibly to an over-flow of S into the S-assimilation pathway that disturbed S-metabolism. To examine these two possibilities, we here investigated the viability of cells that had been cultured with aeration for 24 h in the presence of 10 mM sulfate.
Liquid-culturing in advance with 10 mM sulfate, distinct from that with 10 mM sulfite, had no detrimental effects on cell viability on an agar plate containing the regular medium (SO₄²⁻, cf. Control). It would thus be the chemical toxicity of sulfite that perturbed physiological aspects of sulfite-stressed *Synechococcus* cells.

**Effects of sulfite on photosynthesis**

We here examined the effects of sulfite on photosynthesis to determine the cause of the repressed cell growth (Fig. 2). CO₂-dependent photosynthetic activity showed little alteration 1 h after the addition of sulfite and thereafter gradually decreased to reach 47% of the initial level by 4 h (Fig. 2A), and finally to as low as 9.5% at 24 h (Fig. 2B). It thus seemed that the lesion in photosynthesis was the cause of the seriously retarded cell growth (Fig. 1A). Since quantitative decreases were detected in the photosynthetic systems (Fig. 1D, E), our research hereafter was focused on the effects of sulfite on the behavior of PSI and PSII. The PSII activity was lowered to 84% of the initial level in 4 h, and thus seemed more tolerant to sulfite than CO₂-dependent photosynthesis at this time point (Fig. 2C). Later, however, similar to CO₂-dependent photosynthesis, PSII activity exhibited a drastic decrease to 37% of the initial level at 8 h and finally to 0.2% at 24 h (Fig. 2C). Notably, the absolute values of the PSII activity became almost indistinguishable from those of CO₂-dependent photosynthesis at 8 or 24 h (Fig. 2D). It should be mentioned that PSII activity became almost zero at 24 h after sulfite application. However, the functional integrity of the PSII complex should be kept in vivo, at least to an extent that can account for the low residual O₂ evolution through photosynthesis detected with CO₂ as an electron acceptor (Fig. 2D). In contrast, the PSI activity was maintained at a normal level even at 24 h (Fig. 2E). These results clearly demonstrated that photosynthesis at 8 or 24 h was limited by the impaired PSI activity at least, but definitely not by the PSII activity.

We then investigated whether or not the damage to photosynthesis depends on light. Culturing of the cells with sulfite in the dark, similar to that in the light, caused CO₂-dependent photosynthesis to decrease to 58% of the initial level in 4 h, and to almost zero in 24 h (Fig. 2B). The changing patterns of the PSI activity were also similar for sulfite-stressed cells in the light and in the dark (Fig. 2C). It thus seemed that photosynthesis is impaired by sulfite, irrespective whether light or dark conditions were imposed. However, the cells stressed by sulfite in the light were inferior to those stressed in the dark as to the viability on an agar plate containing the regular medium, suggesting that the light caused additional injury to some physiological process (Fig. 2F).

**Effects of sulfite on the structures of the PSI and PSII complexes**

To investigate the effects of sulfite on the structures of the PSI and PSII complexes, we applied the two-dimensional (2D) PAGE system for the membrane proteins in *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*, Sato et al. 2004) to *Synechococcus*. For the first dimension, native PAGE was performed with the use of a non-ionic detergent, dodecyl-β-D-maltoside (DM), for solubilization of the membranes, whereas in the second one, SDS-PAGE was used. The DM-PAGE gel of the membrane proteins from normally grown cells of *Synechococcus* exhibited three Chl-containing bands 1 to 3 (Fig. 3A). Bands 1 and 2 migrated to the same positions of PSI trimer (larger than 670 kDa) and PSII monomer (a little larger than 200 kDa) of *Synechocystis*, respectively, whereas the position of band 3 was the same as that of PSII monomer of *Synechocystis* (ca. 200 kDa). Then, the gel was subjected to SDS-PAGE, which revealed that the constituent proteins were similar for bands 1 and 2 (Fig. 3A), comprising at least a protein of 50 kDa and four proteins of low molecular weight (equal to or less than 15 kDa). The molecular weights of these constituent proteins were thus very similar to those of PSII monomer or trimer of *Synechocystis*, which we previously separated by the 2D-PAGE. Above all, one of these low-molecular weight proteins were identified as PsAD by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI TOF/TOF-MS analysis; score, 238; match, 6; coverage, 55%). From these lines of evidence concerning positions of the bands 1 and 2, and their protein composition, it could be deduced that the bands 1 and 2 were PSII trimer and its monomer, respectively, and that their subunits are composed of a mixture of proteins corresponding to PsAA and PsAB, and other PSI subunits of low molecular weight including PsAD, as we previously observed with *Synechocystis*.

Meanwhile, band 3, similar to PSII monomer we previously isolated from *Synechocystis*, included at least four proteins of molecular weights from 24 to 39 kDa, which was judged from a migration pattern of molecular-weight marker proteins. Moreover, the protein of the highest molecular weight was identified as PsBB by MALDI TOF-MS analysis (score, 108; matches, 16; coverage, 31%). The results, together with the same mobility of the band 3 as the *Synechocystis* PSI monomer (Fig. 3A; Sato et al. 2004), would indicate that this band is the PSI monomer. The PSI dimer, the higher-order structure of which should predominate as cyanobacterial PSI, seemed to be dissociated into the monomer on this DM-PAGE, as in the case of *Synechocystis* (Sato et al. 2004).

We then isolated thylakoid membranes from cells that had been exposed to sulfite for 4, 8 and 24 h, respectively, to examine structural alterations in the PSI and PSII complexes (Fig 3B, C). Proteins of thylakoid membranes corresponding to 10 μg Chl were subjected to DM-PAGE. As to the PSI complex, the DM-PAGE gel revealed a time-dependent decrease in the content of the PSI trimer with an increase in that of the PSI monomer (Fig. 3B). Accordantly, the PSI complex at 24 h consisted almost exclusively of the monomer, as if at the expense of the trimer. In line with these observations, the following SDS-PAGE revealed that the respective PSI subunits were decreased in quantity in the trimer whereas they were increased in the monomer (Fig. 3C). These results showed that the alteration in the PSI complex during the sulfite-stress was due definitely to a quantitative change in the protein complex per se and not to one in Chl a molecules bound to the complex. The addition of sulfite should thus lead to dissociation of the PSI trimer into the PSI monomer.
monomer and/or preferential synthesis of the PSI monomer relative to the trimer. The former case is highly probable in view of the limited synthesis of Chl during imposition of sulfite-stress (Fig. 1B). Meanwhile, the PSII complex seemed stable, at least during the sulfite-stress for the first 4 h when evaluated on the DM-PAGE gel (Fig. 3C), which was consistent with no remarkable decrease in the PSII activity at this early phase of sulfite-stress. However, in the next 20 h, the PSII complex exhibited a time-dependent and gradual decrease to an undetectable level (Fig. 3B). This decrease in the PSII complex was
accompanied by ones in the PSII subunits (Fig. 3C), which demonstrated that the PSII monomer per se disappeared.

Apart from those of the authentic complexes of PSI and PSII, a novel pigmented band exhibiting higher mobility than that of the PSII monomer appeared abruptly at 8 h, and, together with the PSI monomer, became major at 24 h (Fig. 3B). This novel band could be eluted electrically from the gel, and, on spectroscopic analysis, was found to give two peaks at 490 and 670 nm in the absorption spectrum, which seemed to correspond to those of carotenoid and Chl $\alpha$, respectively (Fig. 3D). Among several proteins that co-migrated with the novel band in an SDS-PAGE gel at 24 h, only two proteins exhibited quantitative behavior similar to that of this novel band during the first 8 h, coinciding well with two upper subunits of PSII monomer, PsbB and probable PsbC, respectively, in molecular mass (Fig. 3C; see proteins denoted by open circles). Also note that the PsbO protein (red circle), which was identified by MALDI-TOF, was retained at considerable levels throughout the experiment. (D) Absorption spectra of pigmented proteins isolated from the disc gel containing the novel band.

The mechanism by which the conformations of the photosystem complexes are altered in the cells subjected to sulfite-stress

We here investigated the mechanism for the conformational change of the PSI and PSII complexes in *Synechococcus* cells during imposition of sulfite (Fig. 3B, in vivo). The dark conditions considerably repressed the monomerization of the PSI complex and degradation of the PSII complex, in parallel with no appearance of the novel band. It thus seemed that the individual protein components for assembly into the PSII complex were retained at considerable levels without severe degradation in the membranes of the sulfite-stressed cells.
stimulated, but, contrarily, repressed by application of sulfite (\(\pm SO_3^{2-}\), cf. \(-SO_3^{2-}\)). Meanwhile, the PSII complex from the illuminated thylakoid membranes appeared as a smear band on a gel, which implied some structural instability of the PSII complex (\(-SO_3^{2-}\), cf. Cont), however, the instability was not further enhanced by sulfite (\(\pm SO_3^{2-}\), cf. \(-SO_3^{2-}\)). Thus, we could not obtain any evidence that supports a thought that sulfite directly stimulates structural changes of the PSI and PSII complexes.

The PSI trimer is prevented from being dissociated into the monomer by PG, one of the thylakoid membranes lipids, which the X-ray structure of the complex included (Jordan et al. 2001). Lipids in the cells, which predominantly comprise thylakoid membranes, were then analyzed, it being revealed that the relative content of PG was similar for both cells cultured in the presence and absence of sulfite (Fig. 4, 16.8 ± 2.3% and 16.6 ± 1.3%, respectively, relative to total lipids). No remarkable effect of sulfite was observed also on the compositions of the other lipids, including SQDG, the synthesis of which needs sulfite as a substrate (Fig. 4). It thus seemed improbable that the increasing PSI monomer to trimer ratio in cells exposed to sulfite depends on quantitative changes in the membrane lipids including PG.

**Effects of disruption of the psbO gene on physiological aspects of the cells**

Some membrane proteins, distinct from the PSI and PSII subunits, were distributed at the same positions with little effect on the abundance on the 2D-PAGE gel (Fig. 3C), irrespective of whether before or after imposition of the sulfite-stress. Such proteins included a 33 kDa one (Fig. 3C, see a protein denoted by a red circle). The MALDI TOF-MS analysis dismantled that this protein was PsbO (Score, 142; matches, 12; coverage, 62%), one of the subunits that construct the water-splitting system of PSII. It is known that the PsbO protein is dispensable in cyanobacteria for growth under normal conditions (Bockholt et al. 1991). To investigate whether or not the PsbO protein is important for the functional stability of the photosynthetic apparatus under sulfite-stress conditions, we generated a disruptant as to the psbO gene (\(\Delta psbO\)) in *Synechococcus*. As shown in Fig. 5A and B, we inserted a cassette of the chloramphenicol resistant gene into the psbO gene in the genome of *Synechococcus* cells, and confirmed the disruption of all genomic copies by PCR. As a result, the mRNA or protein product of psbO was completely absent from the \(\Delta psbO\) cells (Fig. 5B).

The growth of the \(\Delta psbO\) cells, as compared with that of the WT ones, was not so deleteriously affected under the normal conditions (Fig. 5D), as was previously reported (Bockholt et al. 1991). Accordingly, similar results were obtained for the content of Chl or phycobilisome (Fig. 5E, F). However, growth of the \(\Delta psbO\) cells was markedly repressed at as low as 2 mM sulfite (Fig. 5D), which hardly injured the wild-type (WT) cells (Fig. 1A). Concomitantly observed in the culture at 2 mM sulfite were stimulated decreases in the contents of Chl and phycobilisome per OD_{730} in the \(\Delta psbO\) cells (Fig. 5E, F). These features of the \(\Delta psbO\) cells were similar to those of the WT cells that were cultured at 10 mM sulfite. Accordingly, culturing of the cells for 24 h in the presence of 2 mM sulfite remarkably reduced the viability of the \(\Delta psbO\) disruptant on an agar plate, but not that of the WT (Fig. 5C). Furthermore, CO_{2}-dependent photosynthesis in the \(\Delta psbO\) cells was lowered to 48% of the initial level after culturing for 4 hr even at as low as 1 mM sulfite, in contrast to that in the WT, which was retained at more than 80%, demonstrating the much higher sensitivity of photosynthesis in \(\Delta psbO\) cells (Fig. 5G).

**Discussion**

**Sulfite-induced inactivation of photosynthesis**

The cell growth of *Synechococcus* was repressed on application of 10 mM sulfite, but scarcely at 2 mM sulfite (Fig. 1A). The
Fig. 5 Effects of ΔpsbO on sulfite-induced changes in growth, cellular contents of Chl and phycobilisome, and photosynthesis. Plasmid construct for generation of a disruptant as to the psbO gene in Synechococcus (A), and confirmation of the disruption of psbO by PCR (left panel, B). Absence of the psbO mRNA (middle panel, B) or that of the PsbO protein (right panel, B) in the disruptant was confirmed through RT-PCR and two-dimensional PAGE of the thylakoid proteins, respectively. Expression of rnpB mRNA was shown as an internal control. The position of the PsbO protein is indicated by a red arrow. (C) WT or ΔpsbO cells, which had been cultured for 24 h in the presence of 2 mM sulfite or its absence, (continued)
defect in the growth at 10 mM sulfite could be correlated to both a decrease in ability to construct photosystems and cessation of photosynthesis (Fig. 1D, E, Fig. 2A, B). The sulfite dose that effectively impairs the photosynthetic apparatus and causes chlorosis is 8–10 mM in seed plants such as Nicotiana tabacum and Arabidopsis thaliana (Xia et al. 2012, Yarmolinsky et al. 2013). It thus seemed that the toxic concentrations of sulfite are similar for seed plants and Synechococcus. Meanwhile, fumigation of A. thaliana leaves with 8 mM sulfite, e.g. caused a 4.2-fold increase in sulfite concentration to 0.5 μmole g⁻¹ fresh (Yarmolinsky et al. 2013). The concentration of sulfite in Synechococcus cells should be investigated in the future. As to the functions of photosystems I and II, quite different responses to sulfite, i.e. little decrease in the PSI activity despite an almost complete loss of the PSII activity, became apparent in Synechococcus (Fig. 2C–E). A specific decrease in the PSII activity was also observed in the photosynthetic electron transport system of chloroplasts, which were isolated from Spinacia oleracea plants after fumigation with SO₂ (Shimazaki and Sugahara 1979). It is thus likely that the response of photosynthetic electron transport to sulfite-stress is fundamentally similar for chloroplasts and their postulated ancestor, cyanobacteria. The overall similarity of Synechococcus to chloroplasts of seed plants would indicate the usefulness of Synechococcus as a model organism of chloroplasts for investigation of how the photosynthetic apparatus of seed plants behaves in response to sulfite-stress. It is likely that decreases in contents of the PSI and/or PSII complexes per OD₇₃₀ in response to sulfite-stress (Fig. 1D, E) would reflect repression in the synthesis of the PS complexes including their antennae. At present, it is unclear whether some regulatory mechanism participates in such decreases or not. In either case, the decreased levels of the complexes would consequently help prevent the sulfite-stressed cells from absorbing excess light energy and therefore from generating ROS (Fig. 6).

Notably, it seemed that the primary effect of sulfite that accounts for the initial phase of the decrease in photosynthesis (0 to 4 h in Fig. 2D) is due to some other photosynthetic function than PSII, although PSII eventually limits photosynthesis at a later phase of sulfite-stress. In leaves of S. oleracea, it was
proposed that SO₂-fumigation leads to generation of H₂O₂ through a chain reaction depending on photosynthetic electron transport and to resultant oxidation of SH-groups of the enzymes that constitute the Calvin-Benson cycle, such as gly-ceraldehyde 3-phosphate dehydrogenase, which thereby depresses their light activation process, as the primary injury in photosynthesis (Asada and Kiso 1973, Tanaka et al. 1982a, Tanaka et al. 1982b). Moreover, oxidative stress caused by H₂O₂ itself or that induced by interruption of the Calvin-Benson cycle inhibited synthesis of the D1 protein during the repair of PSII after photodamage in a cyanobacterium, Synechocystis sp. PCC 6803 or a green alga, Chlamydomonas reinhardtii (Nishiyama et al. 2001, Takahashi and Murata 2006). On the other hand, there remains the possibility that, through sulfitolysis, sulfite, directly attacks the SH-groups of proteins, e.g. the enzymes of the Calvin-Benson cycle (Würfel et al. 1990). Future work is needed to elucidate the sequential events that proceed in the photosynthetic apparatus in Synechococcus cells after application of sulfite, including the mechanism by which sulfite directly or indirectly exerts its toxicity. The functional maintenance of PSI specifically, of the two photosystems, has often been observed with photosynthetic microorganisms including cyanobacteria, green algae, and diatoms when stressed by nutritional depletion (Berges et al. 1996, Wykoff et al. 1998) and seemed to represent one of the common strategies for acclimation to environmental stresses that limit the functioning of PSI in such organisms.

**Structural fragility of PSII induced by sulfite application in Synechococcus**

When we focused on the PSI complex the sulfite-stressed cells were found to retain Cytb₅₅₃, PsbO and probable CP47 and CP43 at appreciable levels, although a large part of the PSII complex disappeared on the DM-PAGE gel (Fig. 3B, C). The new PSII-related band definitely lacked the subunit of the lowest molecular weight in PSII monomer, but there remained the possibility that the new band contained the third largest subunit (Fig. 3C). Anyway, it seemed that the PSII complex became structurally fragile upon exposure to cells of sulfite-stress. This structural fragility could be explained in two distinct ways: one is that the PSII complex was disassembled into the respective protein components in vivo in the thylakoid membranes. The other is that the PSII complex was barely stable as to structure in vivo, but was broken down in vitro during the process of preparation of the membranes or on DM-PAGE of the membrane proteins. It is widely accepted that the integral-membrane core subunits of PSI including Cytb₅₅₃ tend to be rapidly degraded when unassembled (e.g. Nilsson et al. 1990). Therefore, the latter explanation seems more probable. The destabilization and concomitant inactivation of PSII in the light might be regarded as an injury that jeopardizes the cell viability (Fig. 2F). It was probable that the light conditions caused the impaired photosynthetic apparatus to generate ROS to an intolerable level for the cell growth. At the same time, the inactivation of PSII would prevent over-reduction of the electron transport chain and explosive generation of ROS in consequence, through repression of the linear electron transport during photosynthesis. The structural and functional damage to PSII actually seemed to benefit cells for survival in a liquid culture (Figs. 1F & 6). Moreover, consistent with above idea concerning ROS, dark conditions, which repress photosynthesis, could help prevent the damage by sulfite for cell survival (Fig. 2F). Interestingly, cells stressed by sulfite in the dark showed no obvious damage to PSII structure despite a marked decrease in PSI activity (Figs. 2C & 3B). Damage processes of PSI might differ between cells stressed in the light and in the dark.

The mobility of the PsbO protein on the gel was distinct from those of integral-membrane subunits of the PSII complex when the membrane proteins prepared from normally grown cells were subjected to DM-PAGE (Fig. 3C). It therefore seemed that, in this study, PsbO was released from the intrinsic PSII complex in vitro during the preparation of the membranes, thereafter being trapped in the luminal space of the membranes. Quantitative maintenance of the PsbO protein at a considerable level during the imposition of the sulfite-stress might reflect stable assembly of PsbO into the intrinsic structure of the PSII complex in vivo, as we suggested above. Our study demonstrated that PsbO is responsible for the functional stability of the photosynthetic apparatus, and ultimately for cell survival when cells are stressed with sulfite (Fig. 5C-G). PsbO might prevent the PSII complex from rapid inactivation by sulfite through protection of Mn-cluster and stabilize the PSI activity that is still retained at very low level in the cells exposed to the stress for 24 h (Fig. 6, Bricker et al. 2012). It was previously reported that PsbO is responsible for protection of the PSI activity against high-temperature stress in Synechocystis (Kimura et al. 2002).

**Monomerization of PSI in response to sulfite application in Synechococcus**

Sulfite-stress induced monomerization of the PSI complex with little deleterious effect on its activity (Fig. 2E, 3B). We previously reported that PSI activity was little impaired after monomerization of the PSI trimer, which was induced by PG-depletion in vivo with the use of a mutant defective in PG synthesis or in vitro by treatment of isolated thylakoid membranes with phospholipase A₂ (Sato et al. 2004). Likewise, monomerization of the PSI trimer by disruption of the psaL gene had little impact on the PSI activity (Chitnis and Chitnis 1993). It could thus be supposed that the PSI complex can fully exert its activity per se, irrespective of it being in the monomeric or trimeric conformation, and that the conformational change in PSI that is induced by sulfite-stress might contribute to other functional aspects of PSI than regulation of its own activity.

Kubota et al. (2010) reported that the monomeric form of the PSI complex is associated specifically with subunits of the NADH dehydrogenase 1 complex in Synechocystis, suggesting an important role of the PSI monomer in the cyclic electron transport through formation of a super-complex with NDH-1, as is the case of the plant counterpart. In this context, it is of
note that SO$_2$-fumigation of *S. oleracea* plants lowered the rate of ATP synthesis depending on the linear electron transport during photosynthesis, but never deleteriously affected the rate depending on cyclic electron transport, suggesting the contribution of the active PSI to cyclic photophosphorylation (Shimazaki and Sugahara 1979). Since a plant PSI, distinct from a cyanobacterial one, only adopts a monomeric conformation, the active participation of PSI in the cyclic electron transport in plants exposed to sulfite should never accompany alteration of the higher-order conformation of PSI, as observed with *Synechococcus*. However, it is possible that, in sulfite-stressed cells of *Synechococcus*, the PSI monomer interacts with NADH dehydrogenase 1 for facilitation of the cyclic electron transport for the synthesis of ATP (Fig. 6). ATP is necessary for the synthesis and/or functioning of proteins involved in the maintenance of vital cellular processes or protection of the cells from extreme environmental stress conditions (Murata et al. 2012). These proteins would include a series of P-type ATPases that, respectively, transport specific ions for the ionic homeostasis in the cells (Chan et al. 2010).

Future work will be elucidation of the molecular mechanism underlying monomerization of the PSI complex and structural/functional destabilization of the PSII complex in *Synechococcus* under sulfite-stressed conditions, including possible development of a cyclic electron transport system and regulation of gene expression. A study along these lines would provide a basic architecture for a comprehensive understanding of how the photosynthetic apparatus acclimates to sulfite-stress in cyanobacteria and would further contribute to elucidation of how plants modulate their photosynthetic apparatus under sulfite-stress conditions, not only from biochemical and physiological aspects, but also from an evolutionary one.

**Materials and Methods**

**Cyanobacterial strains and growth conditions**

The cyanobacterial strains used were *Synechococcus* and a mutant of it, the *psbO* gene of which was disrupted (see below). The cells were cultured at 30°C in a glass tube containing BG11 medium, with illumination (10 W m$^{-2}$) and aeration, as we previously described (Sato et al. 2000). The cells were pre-cultured until it was confirmed that the OD$_{730}$ value became ca. 0.3 to 0.4 (Murata et al. 2012). These proteins would include a series of P-type ATPases that, respectively, transport specific ions for the ionic homeostasis in the cells (Chan et al. 2010).

Future work will be elucidation of the molecular mechanism underlying monomerization of the PSI complex and structural/functional destabilization of the PSII complex in *Synechococcus* under sulfite-stressed conditions, including possible development of a cyclic electron transport system and regulation of gene expression. A study along these lines would provide a basic architecture for a comprehensive understanding of how the photosynthetic apparatus acclimates to sulfite-stress in cyanobacteria and would further contribute to elucidation of how plants modulate their photosynthetic apparatus under sulfite-stress conditions, not only from biochemical and physiological aspects, but also from an evolutionary one.

**Determination of Pigment Contents and Photosynthesis**

Measurements of the contents of Chl *a* and phycobilisome and of photosynthetic activities were performed as we previously described (Sato et al. 2000, Sato et al. 2004). A Clark-type electrode (Rank Brothers, Cambridge, UK) was used for measurement of following photosynthetic activities. Photosynthetic O$_2$ evolution was measured in the BG-11 medium containing cells and 10 mM NaHCO$_3$. PSI activity was measured with the reduced form of diaminodurene (5 mM) and methylviologen (2 mM) as the electron donor and acceptor, respectively. PSII activity was measured with p-benzoquinone (2 mM) as an electron acceptor (Sato et al. 2004). The electrode chamber was filled with 5 ml of reaction mixture, kept at 30°C, and illuminated at saturating light with a tungsten projector lamp.

**Lipid Analysis**

The total lipids were extracted from the cells and then separated into individual lipid classes by thin-layer chromatography (TLC). The spots of lipids were visualized by spraying with primulin. Fatty acid methyl esters were then prepared from the total lipids and each lipid class with 5% anhydrous methanolic HCl for analysis by capillary gas-liquid chromatography (GLC), as described previously (Aoki et al. 2004). The lipid contents were estimated with arachidonic acid as an internal standard.

**Disruption of the *psbO* gene in *Synechococcus***

For amplification of the coding region of the *psbO* gene in *Synechococcus*, PCR was performed with following two primers, 5'-AATACCATTCGCCCTCTAGCTG-3' and 5'-AATCCTGGCCGTAGAATGAC-3', as we previously described (Sato et al. 2000). A product of 1.0 kbp was ligated to the pGem T-EASY vector (Promega), cut with Xhol at the center and blunt-ended. The kanamycin-resistant gene obtained from pKM7093 by HinclI digestion was then inserted. The resultant plasmid containing the disrupted *psbO* gene was used to transform wild-type (WT) cells of *Synechococcus* by homologous recombination, as we described previously (Sato et al. 2000). The disruption of the putative gene on the genome was confirmed by PCR with the primers described above. Furthermore, impaired expression of *psbO* was examined at the transcript level in the *psbO* disruptant by semi-quantitative reverse transcription (RT)-PCR, as described by Tabei et al. (2007). The primers used for amplification of a part of *psbO* cDNA were 5'-ACTGGCTTGGCCAACAAATGG-3' and 5'-AAGGAA ATTCCTCTTGGACC-3'. The nrb8 gene for a subunit of ribonucleosine P was used as an internal control (Lee et al. 1998). The primers for the semi-quantitative RT-PCR as to nrb8 were 5'-AAGTCCGGGCTCCAAAGA-3' and 5'-TAACCGG GGTCTGTTCTCT-3'.
use of ferricyanide, and hydroquinone and ascorbate, as oxidizing and reducing reagents, respectively (Aoki et al. 2004).

References


