Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery

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Absorption of excess light energy by the photosynthetic machinery results in the generation of reactive oxygen species (ROS), such as H₂O₂. We investigated the effects *in vivo* of ROS to clarify the nature of the damage caused by such excess light energy to the photosynthetic machinery in the cyanobacterium *Synechocystis* sp. PCC 6803. Treatments of cyanobacterial cells that supposedly increased intracellular concentrations of ROS apparently stimulated the photodamage to photosystem II by inhibiting the repair of the damage to photosystem II and not by accelerating the photodamage directly. This conclusion was confirmed by the effects of the mutation of genes for H₂O₂-scavenging enzymes on the recovery of photosystem II. Pulse labeling experiments revealed that ROS inhibited the synthesis of proteins *de novo*. In particular, ROS inhibited synthesis of the D1 protein, a component of the reaction center of photosystem II. Northern and western blot analyses suggested that ROS might influence the outcome of photodamage primarily via inhibition of translation of the *psbA* gene, which encodes the precursor to D1 protein.

Keywords: cyanobacterium/D1 protein/H₂O₂-scavenging enzyme/photosystem II/reactive oxygen species

Introduction

Oxygen is essential for most living organisms but is also a precursor to reactive oxygen species (ROS), which can damage cellular components such as proteins, lipids and nucleic acids (Halliwell and Gutteridge, 1990). In oxygenic photosynthesis, various ROS, such as the superoxide radical (O₂⁻), hydrogen peroxide (H₂O₂) and the hydroxyl radical (·OH), are generated as a result of the photosynthetic transport of electrons (Asada, 1996, 1999). When the absorption of light energy by chlorophylls exceeds the capacity for utilization of the energy in photosynthesis, the generation of ROS is greatly accelerated.

High-intensity light is an environmental factor that can damage the photosynthetic machinery of plants and it is often the cause of reductions in the growth and productivity of crop plants (Long *et al.*, 1994). The main target of photodamage is photosystem II (PSII), which is a complex of proteins and pigments that is the site of the photochemical reaction and the subsequent transport of electrons from water to plastoquinone. Photodamage to PSII is mainly due to damage to the D1 protein, which forms a heterodimer with the D2 protein in the reaction center of PSII, and the subsequent rapid degradation of the D1 protein (Barber and Andersson, 1992; Prášil *et al.*, 1992; Aro *et al.*, 1993; Andersson and Barber, 1996).

A number of reports have suggested that ROS are involved in the photodamage to PSII (Prášil *et al.*, 1992; Aro *et al.*, 1993; Andersson and Barber, 1996). ROS such as O₂⁻ (Chen *et al.*, 1992), H₂O₂ (Ananyev *et al.*, 1992; Miyao *et al.*, 1995) and ·OH (Miyao *et al.*, 1995) were shown to induce the cleavage of the D1 protein directly *in vitro*. Besides such ROS, ¹O₂, which is generated by transfer of excitation energy in the reaction center of PSII, is often considered to trigger degradation of the D1 protein (Vass *et al.*, 1992; Hideg *et al.*, 1994; Telfer *et al.*, 1994; Okada *et al.*, 1996). Since the cited studies were conducted *in vitro* using thylakoid membranes or PSII complexes, the action of ROS in living cells in which the ROS-scavenging system is fully active is still in question.

In the present study, we examined whether ROS, such as H₂O₂, act primarily by accelerating the damage to PSII directly or by inhibiting the repair of the photodamage to PSII *in vivo*, using the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*). We found that increased intracellular concentrations of ROS inhibited repair of the photodamage to PSII. Labeling of proteins *in vivo* and northern and western blot analyses demonstrated that ROS primarily inhibited the synthesis of the D1 protein *de novo* at the translational level at both the initiation and elongation steps.

Results

Light and ROS act synergistically in the photodamage to PSII

Incubation of *Synechocystis* cells in strong light at 1.5 mmol photons/m²/s had no significant effect on the activity of PSII over a 2-h period (Figure 1A; open circles). However, in the presence of chloramphenicol, an inhibitor of protein synthesis, similar incubation resulted in the inactivation of PSII (Figure 1B; open circles). These observations suggested that, in light at 1.5 mmol photons/m²/s, the rate of repair of photodamage that required the synthesis of proteins *de novo* was higher than the rate of photodamage to PSII.

Fig. 1. Light-induced inactivation of PSII in the presence of reagents that accelerate the production of ROS in wild-type *Synechocystis*. Cells were exposed to light at 1.5 mmol photons/m²/s with standard aeration. (A) In the presence of methyl viologen at 2 μ M (open triangles) and at 5 μ M (open squares) and in its absence (open circles); filled circles: in the presence of 5 μ M methyl viologen in darkness. (B) The same as (A) but in the presence of 200 μ g/ml chloramphenicol. (C) In the presence of H₂O₂ at 0.5 mM (open triangles) and at 2 mM (open squares) and in its absence (open circles); filled circles: in the presence of 2 mM H₂O₂ in darkness. (D) The same as (C) but in the presence of 200 μ g/ml chloramphenicol. PSII activity was monitored in terms of the photosynthetic evolution of oxygen in the presence of 1 mM 1,4-benzoquinone as the electron acceptor. The activity taken as 100% was 527 ± 46 μ mol O₂/mg chlorophyll/h. Values are means $\pm$ SD (bars) of results from four independent experiments.

We next examined the actions of ROS by modulating their intracellular concentrations in cells using reagents that induce the production of ROS. Methyl viologen is an acceptor of electrons that accelerates the production of O₂⁻ and H₂O₂ in light (Asada, 1996). The presence of methyl viologen during the incubation of cells in light at 1.5 mmol photons/m²/s enhanced the apparent photodamage to PSII (Figure 1A; open triangles and squares). However, when repair of PSII was blocked by the presence of chloramphenicol, the effect of methyl viologen was abolished (Figure 1B; open triangles and squares). In darkness, methyl viologen had no effect on the activity of PSII (Figure 1A; closed circles).

Exogenously supplied H₂O₂ also accelerated the photodamage to PSII (Figure 1C), suggesting that the agent that accelerated photodamage during incubation in the presence of methyl viologen might have been H₂O₂. The presence of chloramphenicol during the incubation further accelerated the photodamage and abolished the effect of H₂O₂ (Figure 1D). Incubation in darkness in the presence of H₂O₂ had no effect on the activity of PSII (Figure 1C; closed circles). These observations suggested that the enhanced photodamage to PSII in living cells might have been due to inhibition of the repair of PSII and not to direct damage to PSII.

A mutant defective in H₂O₂-scavenging enzymes with enhanced sensitivity to photodamage to PSII

Enzymes that scavenge H₂O₂, such as catalase peroxidase (Tichy and Vermaas, 1999) and thioredoxin peroxidase

Fig. 2. Light-induced inactivation of PSII in wild-type *Synechocystis* and in *katG*⁻/*tpx*⁻ mutant cells. Cells were exposed to light at 1.5 mmol photons/m²/s in the presence of 200 μ g/ml chloramphenicol and in its absence. The other experimental conditions were the same as described in the legend to Figure 1. (A) Wild-type cells. (B) *katG*⁻/*tpx*⁻ cells. Open triangles and circles: in the presence and absence of chloramphenicol, respectively. PSII activity was monitored in terms of the photosynthetic evolution of oxygen. The oxygen-evolving activity of *katG*⁻/*tpx*⁻ cells that was taken as 100% was 560 ± 44 μ mol O₂/mg chlorophyll/h. Values are means $\pm$ SD (bars) of results from four independent experiments.

(Yamamoto *et al.*, 1999), which are encoded by the *katG* and *tpx* genes, respectively, appear to maintain intracellular concentrations of H₂O₂ at low and non-toxic levels. Targeted mutagenesis of both the *katG* and *tpx* genes in *Synechocystis* cells enhanced photodamage to PSII but had no significant effect on such photodamage in the presence of chloramphenicol (Figure 2). These observations supported the hypothesis that ROS might inhibit the repair of photodamage to PSII.

Inhibition by exogenous H₂O₂ of the repair of photodamage to PSII

We examined inhibition of repair by H₂O₂ directly by monitoring the recovery of PSII activity after photodamage (Figure 3). When wild-type cells were incubated in strong light at 3.0 mmol photons/m²/s, PSII activity fell to 20% of the original level. During subsequent exposure of cells to weak light, the activity of PSII returned to the original level (Figure 3A). The presence of H₂O₂ during the incubation in weak light suppressed the repair of the photodamage to PSII. Targeted mutagenesis of the *katG* and *tpx* genes delayed the repair of photodamage (Figure 3B). Furthermore, repair in *katG*⁻/*tpx*⁻ mutant cells was completely eliminated in the presence of 0.5 mM H₂O₂. Removal of H₂O₂ from the mutant cells by exogenously

Fig. 3. Repair of photodamage to PSII and the effects of H_2O_2 after light-induced inactivation in wild-type *Synechocystis* and *katG-/tpx-* cells. Cells were exposed to light at 3 mmol photons/ m^2/s for 60 min (wild type) or 40 min (*katG-/tpx-*) without aeration to induce ~80% inactivation of PSII. Cells were then incubated in light at 70 μmol photons/ m^2/s with standard aeration in the presence of 0.5 mM H_2O_2 (open triangles), 2 mM H_2O_2 (open squares) or 200 $\mu\text{g}/\text{ml}$ chloramphenicol (filled triangles), and in the absence of these reagents (open circles). (A) Wild-type cells. (B) *katG-/tpx-* cells. Diamond symbols indicate repair after 0.1 μM catalase had been added, at the time indicated by the vertical arrow, to a suspension of cells that had been incubated in the presence of 0.5 mM H_2O_2 . Values are means $\pm$ SD (bars) of results from three independent experiments.

supplied catalase allowed repair to proceed (Figure 3B; open diamonds).

Inhibition by ROS of the synthesis of the D1 protein

We used western blot analysis to examine changes in the level of the D1 protein when intracellular concentrations of ROS were increased by the presence of methyl viologen during incubation in light at 1.5 mmol photons/ m^2/s (Figure 4). The presence of methyl viologen decreased the apparent level of D1 protein. However, the decrease in the level of pre-existing D1 protein, assessed in the presence of chloramphenicol, was unaffected by methyl viologen. Therefore, we postulated that ROS, produced by the photosynthetic transport of electrons that was mediated by methyl viologen, inhibited the synthesis of the D1 protein *de novo* but did not affect the degradation of this protein.

We examined the above-mentioned hypothesis by monitoring the incorporation of [^{35}S]methionine into

Fig. 4. Changes in the level of the D1 protein during the light-induced inactivation of PSII in wild-type cells. (A) Results of western blot analysis. (B) Quantitation of the results shown in (A). Cells were exposed to light at 1.5 mmol photons/ m^2/s with standard aeration in the absence (lane 1; open circles) and in the presence (lane 2; open triangles) of 5 μM methyl viologen. Cells were also exposed to light in the presence of 200 $\mu\text{g}/\text{ml}$ chloramphenicol (lane 3; filled circles) and in the presence of 200 $\mu\text{g}/\text{ml}$ chloramphenicol plus 5 μM methyl viologen (lane 4; filled triangles). Thylakoid membranes were isolated from cells at the indicated times. Values are means $\pm$ SD (bars) of results from three independent experiments.

proteins and, in particular, into the D1 protein (Figure 5). The synthesis of the D1 protein *de novo* was markedly suppressed in the presence of methyl viologen and H_2O_2 . The rate of synthesis of the D1 protein in *katG-/tpx-* cells was lower than that in wild-type cells, suggesting that inhibition by ROS of the synthesis of the D1 protein in *katG-/tpx-* cells might have been more severe than that in wild-type cells. Furthermore, not only the synthesis of the D1 protein *de novo* but also the synthesis of almost all other proteins was suppressed in the presence of ROS (Figure 5A).

Effects of ROS on the expression of *psbA* genes

To determine whether ROS might inhibit the synthesis of the D1 protein at the transcriptional level, we examined the expression of *psbA* genes for the D1 protein in the presence of increased intracellular concentrations of ROS. In *Synechocystis*, the D1 protein is encoded by a small multigene family that consists of the *psbA1*, *psbA2* and *psbA3* genes (Jansson *et al.*, 1987). The *psbA1* gene is non-functional, while the *psbA2* and *psbA3* genes are expressed in response to light (Tyystjärvi *et al.*, 1998) and both encode the identical D1 protein.

We monitored changes in the level of a mixture of *psbA2* and *psbA3* transcripts by northern blot analysis (Figure 6). The presence of methyl viologen did not affect

Fig. 5. The synthesis of the D1 protein *de novo* in the presence of ROS in wild-type *Synechocystis* and in *katG*⁻/*tpx*⁻ cells, as monitored in terms of the incorporation of radioactive [³⁵S]methionine into proteins of thylakoid membranes. (A) Lanes 1–3, wild-type cells were labeled for 20 min in the absence of added reagents (lane 1), in the presence of 5 μM methyl viologen (lane 2) and in the presence of 2 mM H₂O₂ (lane 3). Lane 4, *katG*⁻/*tpx*⁻ cells were labeled in the absence of any reagent. The arrow indicates the D1 protein. (B) Changes in the level of labeled D1 protein that had been incorporated into thylakoid membranes in wild-type cells in the absence of any added reagents (open circles), in the presence of 5 μM methyl viologen (open triangles), and in the presence of 0.5 mM H₂O₂ (open squares); filled circles indicate labeled D1 protein in *katG*⁻/*tpx*⁻ cells in the absence of any added reagents. Values are means ± SD (bars) of results from three independent experiments.

the light-induced expression of the *psbA* genes. At 0.5 mM, H₂O₂ did not significantly affect the induction of the expression of these genes at the early stage (zero to 10 min) of incubation. However, at 20 min it repressed the level of *psbA* transcripts to 70% of the control level. Targeted mutagenesis of the *katG* and *tpx* genes had no effect on induction of the expression of the *psbA* genes. Inhibition by chloramphenicol of the translation of the *psbA* transcripts also had no effect on the induction of expression during a 20-min incubation, and the level of *psbA* transcripts was maintained subsequently. These observations suggest that the inhibition by ROS during the synthesis of the D1 protein *de novo* might not occur primarily at transcription.

We also examined the action of ROS on the synthesis of the precursor to D1 protein (pre-D1) at the translational level. Figure 7 shows that the level of pre-D1 increased during exposure of wild-type cells to strong light and reached a maximum at 40 min. The presence of 0.5 mM H₂O₂ significantly depressed induction of the synthesis of pre-D1. These observations suggest that the main target of

Fig. 6. Effects of ROS on the light-induced expression of *psbA* genes in wild-type *Synechocystis* and *katG*⁻/*tpx*⁻ cells. (A) Results of northern blot analysis. (B) Quantitation of the results in (A). Prior to exposure to light, cells were incubated at 30°C in darkness for 60 min. Wild-type cells were exposed to light at 1.5 mmol photons/m²/s in the absence of any added reagents (control; open circles) and in the presence of 5 μM methyl viologen (MV; open triangles), 0.5 mM H₂O₂ (open squares) and 200 μg/ml chloramphenicol (Cm; filled triangles); filled circles indicate induction of the expression of *psbA* genes in *katG*⁻/*tpx*⁻ cells in the absence of any added reagents. Total RNA (5 μg) was loaded in each lane of the gel. Values are means ± SD (bars) of results from three independent experiments.

inhibition by ROS during the synthesis of the D1 protein might be translation.

Effects of ROS on the translation of *psbA* mRNAs

To identify the step in the translation of *psbA* mRNAs that was inhibited by ROS, we examined the effects of H₂O₂ on the distribution of *psbA* mRNAs in membrane-bound and cytosolic polysomes as well as in a polysome-free form in wild-type cells (Figure 8). In *Synechocystis*, *psbA* mRNAs are first associated with polysomes in the cytosol (translation initiation) and, after translational elongation has proceeded to a certain extent, *psbA* mRNA-ribosome complexes are targeted to thylakoid membranes leading to the subsequent elongation (Tyystjärvi *et al.*, 2001; see Discussion).

Figure 8 shows that, during incubation in light at 1.5 mmol photons/m²/s, almost all the *psbA* mRNA was in the form of polysomes, which were either associated with or dissociated from thylakoid membranes. The level of *psbA* mRNA that was not associated with polysomes was

Fig. 7. Changes in the level of the pre-D1 protein during the light-induced inactivation of PSII in wild-type cells. Cells were exposed to light at 1.5 mmol photons/m²/s in the absence of H₂O₂ (open circles), in the presence of 0.5 mM H₂O₂ (open squares), and in the presence of 200 µg/ml chloramphenicol (open triangles). Thylakoid membranes were isolated from cells at the indicated times. Values are means $\pm$ SD (bars) of results from three independent experiments.

very low. The presence of 0.5 mM H₂O₂ significantly decreased the relative level of *psbA* mRNAs in membrane-associated polysomes. These findings suggest that the elongation step in the translation of *psbA* mRNAs might be strongly inhibited by H₂O₂. In addition, the presence of 0.5 mM H₂O₂ increased the level of *psbA* mRNA that was not associated with polysomes, suggesting that the binding of *psbA* mRNAs to ribosomes, namely the initiation of translation, might be also inhibited by H₂O₂.

Discussion

In the present study we investigated the actions of ROS in the photodamage to PSII and its repair in *Synechocystis* cells. Conditions that produced ROS stimulated the apparent photodamage to PSII (Figure 1). Thus, it appeared that ROS might act primarily by inhibiting repair of the photodamage to PSII *in vivo* (Figure 3A). Inactivation of H₂O₂-scavenging enzymes also inhibited the repair of the photodamage to PSII (Figure 3B).

Which ROS affect the repair of photodamage?

When the light energy absorbed by the photosynthetic machinery exceeds the energy that is required for photosynthesis, O₂⁻ is generated on the acceptor side of photosystem I (Asada, 1996). In *Synechocystis*, O₂⁻ is disproportionated by an Fe-containing superoxide dismutase to H₂O₂, which is then scavenged by catalase peroxidase and thioredoxin peroxidase (Tichy and Vermaas, 1999; Yamamoto *et al.*, 1999). However, strongly oxidative conditions, for example, the combination of light and the presence of methyl viologen, allow the photosynthetic machinery to produce excessive amounts of O₂⁻ and H₂O₂. In such cases, a small fraction of the H₂O₂ is converted to the hydroxyl radical ·OH by the

Fig. 8. Effects of H₂O₂ on the distribution of *psbA* mRNAs that are free or are associated with polysomes in wild-type cells. Cells were incubated at 30°C in darkness for 60 min and then exposed to light at 1.5 mmol photons/m²/s for 20 min in the presence of 0.5 mM H₂O₂ and in its absence. Cells were disrupted and membrane-bound polysomes (M), cytosolic polysomes (C) and polysome-free RNA (F) were prepared as described in the text. RNA was isolated from each fraction and subjected to northern blotting with a labeled fragment of the *psbA2* gene as the probe. The amount of RNA in each lane corresponded to that from each individual fraction, and each fraction was derived from cells equivalent to 5 µg chlorophyll. (A) Gel-electrophoretic pattern. The results shown are representative of the results of four independent experiments, each of which gave similar results. (B) Quantified results. Values are means $\pm$ SD (bars) of results from four independent experiments. T represents the total of the three fractions of *psbA* mRNA.

action of transition metal cations, such as Fe²⁺ and Cu²⁺ (Asada, 1996).

Among the various ROS, O₂⁻ is highly reactive and ·OH is extremely reactive, whereas H₂O₂, which is the most abundant ROS, is the least reactive. We can ask which of the three ROS is active in the inhibition of repair. It is unlikely that O₂⁻ is the major contributor to the inhibition because exogenously supplied H₂O₂, which cannot be converted to O₂⁻, was as effective as the presence of methyl viologen.

Inhibition by ROS of the repair of photodamage to PSII was reversible (Figure 3B), an observation that suggests that the repair system might be inhibited directly by H₂O₂ and/or ·OH and not by the secondary products of ROS, such as lipid peroxides (Wise, 1995).

Effects of ROS on the de novo synthesis of proteins

Western blot analysis indicated that ROS did not enhance the degradation of the pre-existing D1 protein when protein synthesis was blocked by the presence of chloramphenicol. However, degradation of the pre-existing D1 protein occurred more slowly than inactivation of PSII. This difference might have been caused by the presence of inactivated D1 protein, which was detected by western blot analysis. Since degradation of inactivated D1 protein is coordinated with the synthesis *de novo* of active D1 protein (Komenda and Barber, 1995), it is likely that inhibition of its synthesis *de novo* by either ROS or

chloramphenicol led to the accumulation of inactivated D1 protein.

Labeling of proteins *in vivo* provided direct evidence for the inhibition by ROS of the synthesis of the D1 protein *de novo* (Figure 5). Thus, the enhanced sensitivity of the *katG⁻tpx⁻* cells to strong light might be explained by the inhibition by ROS of the synthesis of the D1 protein *de novo*. It is noteworthy, in this context, that not only the synthesis of the D1 protein *de novo* but also the synthesis of almost all other proteins was suppressed in the presence of ROS (Figure 5). Thus, it seems likely that ROS might inhibit the synthesis of most proteins in *Synechocystis*.

Translational initiation and elongation as the primary target of ROS

Northern blot analysis indicated that ROS had only a slight effect on the accumulation of *psbA* transcripts (Figure 6). Thus, since ROS inhibited the synthesis of pre-D1 *de novo* (Figures 5 and 7), it seemed likely that ROS might act primarily by inhibiting translation.

The distribution of *psbA* mRNAs (Figure 8) revealed that ROS markedly decreased the level of *psbA* mRNA–polysome complexes that were associated with thylakoid membranes. Recently, Tyystjärvi *et al.* (2001) demonstrated that, in *Synechocystis*, synthesis of the D1 protein *de novo* is regulated at the level of transcription of *psbA* genes and at the elongation step in the translation of *psbA* mRNA. The transcriptional machinery in *Synechocystis* is different from that in chloroplasts. In the latter, the synthesis of D1 protein is redox-regulated at the initiation of the translation of *psbA* mRNA via binding of the nuclear-encoded protein complex to the 5′-untranslated region of *psbA* mRNA (Hirose and Sugiura, 1996; Yohn *et al.*, 1996; Trebitsh *et al.*, 2000).

In *Synechocystis*, *psbA* mRNAs associate first with ribosomes in the cytosol (translation initiation) and then elongation starts. After a 17-kDa nascent chain of the D1 protein has been synthesized, the *psbA* mRNA–ribosome complexes are targeted to thylakoid membranes where further elongation of the D1 protein occurs (Tyystjärvi *et al.*, 2001). Our observations together with these observations suggest that the major sites of inhibition by ROS in our system are both initiation and elongation steps of translation. However, it remains possible that ROS might also inhibit the targeting of *psbA* mRNA–polysome complexes to thylakoid membranes.

Ayala *et al.* (1996) demonstrated that oxidative stress due to cumene hydroperoxide inhibits protein synthesis in rat liver by inactivating elongation factor 2, an important participant in the elongation step of translation. In *Escherichia coli*, H₂O₂ inactivates elongation via carbonylation of elongation factor G (Tamarit *et al.*, 1998). Our results are consistent with these earlier findings.

Materials and methods

Cells and culture conditions

Wild-type *Synechocystis* sp. PCC 6803 and *katG⁻tpx⁻* mutant cells were grown photoautotrophically (Gombos *et al.*, 1994) at 34°C in BG-11 medium under light at 70 µmol photons/m²/s, with aeration by sterile air that contained 1% CO₂. Cells at a density of 5 ± 0.5 µg/ml chlorophyll were used directly for studies of photodamage and repair.

Conditions for photodamage and repair, and measurements of photosynthetic activity

Cells were incubated in light at 1.5 mmol photons/m²/s (unless otherwise noted) to induce photodamage to PSII or in light at 70 µmol photons/m²/s to induce the repair of photodamaged PSII. These experiments were performed at 30°C at a concentration of cells that corresponded to 5 ± 0.5 µg/ml chlorophyll.

The activity of PSII was measured at 30°C by monitoring the photosynthetic evolution of oxygen, which was determined from the concentration of oxygen in a suspension of cells, measured with a Clark-type oxygen electrode in the presence of 1.0 mM 1,4-benzoquinone, as described previously (Gombos *et al.*, 1994). Concentrations of chlorophyll were determined as described by Arnon *et al.* (1974).

Targeted mutagenesis

The nucleotide sequences of the *katG* and *tpx* genes of *Synechocystis* were obtained from the CyanoBase website [CyanoBase Website (Online) <http://www.kazusa.or.jp/cyano/>] Chromosomal DNA was extracted from *Synechocystis* cells, purified with ISOPLANT (Nippon Gene, Osaka, Japan), and used as template for amplification by PCR. A 2.26-kb fragment of DNA that contained the *katG* gene was amplified by PCR with primers 5′-TCATATGGGACCCCAACCCGCCAGG-3′ and 5′-AGATATCTAGCCCTAGGGAGATCAAA-3′. The amplified fragment of DNA was ligated into the TA cloning vector pT7Blue-T (Novagen, Madison, WI). A plasmid with a disrupted *katG* gene was constructed by inserting a kanamycin-resistance gene cassette (Km^r; 1.2 kb) into the *Eco*O109I site of the *katG* gene in pT7Blue-T. The resultant plasmid was designated pKatG-Km^r. Wild-type *Synechocystis* cells were transformed with this plasmid to generate *katG⁻* cells as described elsewhere (Williams, 1988). Transformed cells were selected on agar-solidified BG-11 medium supplemented with 25 µg/ml kanamycin. To produce *katG⁻tpx⁻* cells, *katG⁻* cells were transformed with pTPX-Sp^r/Sm^r, a plasmid with a disrupted *tpx* gene (Yamamoto *et al.*, 1999), and transformed cells were selected on agar-solidified BG-11 medium supplemented with 30 µg/ml spectinomycin and 25 µg/ml kanamycin. Complete segregation of the *katG* and *tpx* genes in *katG⁻tpx⁻* cells was confirmed by PCR with appropriate primers, as described elsewhere (Yamamoto *et al.*, 1999).

Western blot analysis

Thylakoid membranes were isolated from wild-type cells that had been incubated in light at 1.5 mmol photons/m²/s for designated times, as described previously (Gombos *et al.*, 1994). Proteins in isolated thylakoid membranes, equivalent to 0.63 µg of chlorophyll, were separated by SDS–PAGE on a 12.5% polyacrylamide gel that contained 6 M urea. Separated proteins were blotted onto a nitrocellulose membrane and allowed to react with a D1-specific antiserum that had been raised against a synthetic oligopeptide that corresponded to the AB loop of the D1 protein (amino acids 55–78, counted from the N-terminus) of *Synechocystis* or with a pre-D1-specific antiserum that had been raised against a synthetic oligopeptide that corresponded to the C-terminal extension of pre-D1, the precursor to the D1 protein of *Synechocystis*. Each band of immunologically reactive protein was detected with peroxidase-linked secondary antibodies and enhanced chemiluminescence western blot detection reagents (Amersham Pharmacia Biotech, UK). Levels of the D1 and pre-D1 proteins were determined densitometrically.

Labeling of proteins in vivo

Cell cultures were supplemented with 10 nM [³⁵S]methionine (>1000 Ci/mmol; Amersham Pharmacia Biotech) and then incubated at 30°C for designated times in light at 1.5 mmol photons/m²/s in the presence of the indicated reagents or in their absence (Sippola *et al.*, 1998). Labeling was terminated by the addition of non-radioactive methionine to a final concentration of 1 mM and immediate cooling of samples on ice, and thylakoid membranes were isolated. After separation of proteins equivalent to 0.67 µg of chlorophyll by SDS–PAGE, as described above, labeled proteins on the gel were visualized by exposure of the dried and fixed gel to X-ray film. Levels of labeled D1 protein were determined densitometrically.

Northern blot analysis

Northern blot analysis was performed as described previously (Los *et al.*, 1997). A 1.0-kb fragment of DNA that contained the coding region of the *psbA2* gene was amplified by PCR with primers 5′-AACGACTCT-CCAACAGCGCGAAA-3′ and 5′-CGTTCGTGCATTACTTCAAA-CCG-3′. The amplified fragment of DNA was ligated into the TA

cloning vector pT7Blue-T. The plasmid was digested at the *HincII* and *NcoI* sites within the insert. The resultant 700-bp fragment of DNA was conjugated with alkaline phosphatase (Alkphos Direct kit; Amersham Pharmacia Biotech) and the conjugate was used as the probe for northern blot analysis. After hybridization, the blot was soaked in CDP-star solution (Amersham Pharmacia Biotech) and signals from hybridized mRNA were detected with a luminescence image analyzer (LAS-1000; Fuji-Photo Film, Tokyo, Japan).

Isolation of polysomes

Polysomes were isolated from wild-type *Synechocystis* cells as described by Tyystjärvi *et al.* (2001) with minor modifications. Subsequent procedures were performed at 0–4°C. Cells equivalent to 125 µg of chlorophyll were first washed with a buffer that contained 50 mM Tris-HCl pH 8.5, 400 mM sucrose, 10 mM MgCl₂, 30 mM EDTA and 500 µg/ml chloramphenicol, and then they were washed with medium A, which contained 50 mM Tris-HCl pH 8.5, 400 mM sucrose, 10 mM MgCl₂ and 500 µg/ml chloramphenicol. Cells were suspended in 200 µl of medium A that had been supplemented with 200 units of ribonuclease inhibitor (SUPERase-In; Ambion, Austin, TX) and a cocktail of protease inhibitors (Complete, Mini, EDTA-free; Roche, Mannheim, Germany). The suspension was homogenized with an equal volume of zirconia silica beads (diameter, 0.1 mm; BioSpec Products, Inc., Bartlesville, USA) on a vortex mixer which was operated at maximum speed for 3 min with five interruptions of 30 s each on ice. After addition of 700 µl of medium B, which was the same as medium A except that it was prepared without sucrose, the homogenate was centrifuged at 650 g for 5 min to remove unbroken cells and cell debris. The resultant supernatant was centrifuged at 18 000 g for 20 min to yield a membrane fraction (the pellet) and a cytosolic fraction (the supernatant).

The pelleted membranes were resuspended in 700 µl of medium B and then Tween-20 was added to a final concentration of 2% (v/v). The cytosolic fraction (900 µl) was also supplemented with 2% Tween-20. After incubation for 10 min on ice, insoluble materials were removed from these fractions by centrifugation at 18 000 g for 5 min. The resultant supernatants were further centrifuged at 270 000 g for 1 h, on a cushion of 300 µl of a solution that contained 1 M sucrose, 50 mM Tris-HCl pH 8.5, 10 mM MgCl₂ and 500 µg/ml chloramphenicol, for collection of polysomes as the pellet. The supernatant from the cytosolic fraction was used for northern blot analysis of free RNAs. RNAs were isolated from the polysome and free-RNA fractions as described previously (Los *et al.*, 1997) and dissolved in 50 µl of H₂O. Two-microliter aliquots of the solutions were subjected to northern blot analysis as described above.

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