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Regulation of Translation by the Redox State of Elongation Factor G in the Cyanobacterium *Synechocystis* sp. PCC 6803*[S]

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Elongation factor G (EF-G), a key protein in translational elongation, was identified as a primary target of inactivation by reactive oxygen species within the translational machinery of the cyanobacterium *Synechocystis* sp. PCC 6803 (Kojima, K., Oshita, M., Nanjo, Y., Kasai, K., Tozawa, Y., Hayashi, H., and Nishiyama, Y. (2007) *Mol. Microbiol.* 65, 936–947). In the present study, we found that inactivation of EF-G (Slr1463) by H_2O_2 was attributable to the oxidation of two specific cysteine residues and formation of a disulfide bond. Substitution of these cysteine residues by serine residues protected EF-G from inactivation by H_2O_2 and allowed the EF-G to mediate translation in a translation system *in vitro* that had been prepared from *Synechocystis*. The disulfide bond in oxidized EF-G was reduced by thioredoxin, and the resultant reduced form of EF-G regained the activity to mediate translation *in vitro*. Western blotting analysis showed that levels of the oxidized form of EF-G increased under strong light in a mutant that lacked NADPH-thioredoxin reductase, indicating that EF-G is reduced by thioredoxin *in vivo*. These observations suggest that the translational machinery is regulated by the redox state of EF-G, which is oxidized by reactive oxygen species and reduced by thioredoxin, a transmitter of reducing signals generated by the photosynthetic transport of electrons.

Reactive oxygen species (ROS)[2] are produced as inevitable by-products of the light-driven reactions of photosynthesis. The superoxide radical, hydrogen peroxide (H_2O_2), and the hydroxyl radical are produced as a result of the photosynthetic transport of electrons, whereas singlet state oxygen (singlet oxygen) is produced by the transfer of excitation energy (1). Exposure of the photosynthetic machinery to strong light promotes the production of ROS and gives rise to oxidative stress (1).

Strong light rapidly inactivates photosystem II (PSII). This phenomenon is referred to as photoinhibition (2–4), and it occurs when the rate of photodamage to PSII exceeds the rate of the repair of photodamaged PSII (5). The actions of ROS in the photoinhibition of PSII have been studied extensively, and several mechanisms for photoinhibition have been proposed (5). Recent studies of the effects of ROS on photodamage and repair have revealed that ROS act primarily by inhibiting the repair of photodamaged PSII and not by damaging PSII directly (5–9). Such studies have also shown that photodamage to PSII is an exclusively light-dependent event; photodamage is initiated by disruption of the manganese cluster of the oxygen-evolving complex upon absorption of light, in particular UV and blue light, with subsequent damage to the reaction center upon absorption of visible light by chlorophylls (10–12).

Inhibition of the repair of PSII has been attributed to the suppression, by ROS, of the synthesis *de novo* of proteins that are required for the repair of PSII, such as the D1 protein, which forms a heterodimer with the D2 protein in the reaction center, in the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*) (6, 7), in *Chlamydomonas* (13), and in plants (14, 15). Analysis of polysomes in *Synechocystis* has demonstrated that ROS inhibit the synthesis *de novo* of proteins primarily at the elongation step of translation, suggesting that some proteins involved in translational elongation might be the targets of inactivation by ROS (6, 7).

A translation system *in vitro* was successfully prepared from *Synechocystis*, and biochemical investigations using this translation system have revealed that elongation factor G (EF-G), a GTP-binding protein that catalyzes the translocation of peptidyl-tRNA (16), is a primary target of inactivation by ROS (17). EF-G is reversibly inactivated by ROS in a redox-dependent manner; it is inactive in the oxidized form and active in the reduced form (17). Moreover, it has been proposed that changes in the activity of EF-G might depend on and be regulated by the redox states of cysteine residues within EF-G (17). However, the specific cysteine residues within EF-G that might be the targets of ROS and might be responsible for redox regulation remain to be determined.

In the present study, we investigated the redox state of Slr1463, the EF-G that is phylogenetically closest to chloroplast EF-G among three homologs of EF-G in *Synechocystis* (17). We determined that two specific cysteine residues in the EF-G of

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S3.
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[2] The abbreviations used are: ROS, reactive oxygen species; DTT, dithiothreitol; EF-G, elongation factor G; PSII, photosystem II; HPLC, high pressure liquid chromatography.

Synechocystis were targets of oxidation by ROS. The resultant disulfide bond between the two cysteine residues was efficiently reduced by thioredoxin. In addition, we observed that EF-G was reduced by thioredoxin *in vivo*. Our findings revealed the mechanism of the ROS-induced inactivation of EF-G and suggested a mechanism for the redox regulation of translation by electrons generated during photosynthesis.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Proteins—EF-G, encoded by *slr1463* in *Synechocystis*, was expressed in *Escherichia coli* BL21 (DE3) as a histidine-tagged recombinant protein and purified, in the reduced form, as described previously (17). The *m*-type of thioredoxin, encoded by *slr0623* in *Synechocystis* (18, 19), was also purified as described previously (20). These proteins were stored in a buffer that contained 20 mM HEPES-KOH (pH 7.5), 50 mM NaCl, and 20% (w/v) glycerol.

Site-directed Mutagenesis of Proteins—Site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) by PCR with a pair of complementary oligonucleotides of 32–44 bases that contained the desired mutations and pET21b/*slr1463* (17) as the template (sequences of primers are available on request). Parental DNA was digested with DpnI to remove methylated parent strands, and the obtained plasmid DNA was used to transform *E. coli* JM109.

Modification of Thiol Groups of Cysteine Residues—The redox state of the cysteine residues in EF-G was monitored by modifying the thiol groups in EF-G with a maleimidyl reagent, methoxypoly(ethylene glycol) maleimide, which has an average molecular mass of 5 kDa (Nihon Yushi, Tokyo, Japan), with subsequent separation of proteins by non-reducing SDS-PAGE on a 7.5% polyacrylamide gel, as described previously (17).

Quantitative Analysis of Thiol Groups—The number of thiol groups per EF-G molecule was determined as described previously (20). From 10 to 50 μM EF-G was incubated with 0.4 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (Sigma) in buffer that contained 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 6 M guanidine HCl. Changes in absorbance at 412 nm were then measured, and the number of reactive thiol groups was determined as described elsewhere (21).

Preparation of Extracts for Translation in Vitro—Extracts for translation *in vitro* were prepared as described previously (17). Cells of *Synechocystis* at the late exponential phase of growth were broken open with glass beads. After centrifugation of the resultant homogenate, the blue-green supernatant that contained thylakoid membranes was used as the cell extract for translation *in vitro*.

Translation in Vitro—Translation *in vitro* was performed by incubation, at 30 °C, of the above mentioned cell extract with *psbA2* mRNA as the template, [^{14}C]leucine, the other 19 amino acids, and various compounds required for translation, as described previously (17). The incorporation of [^{14}C]leucine into proteins was quantified by liquid scintillation counting, and the extent of incorporation of the radiolabeled amino acid was taken as a measure of translational activity. For inhibition of translation by H_2O_2, the cell extract was incubated for 10 min at 30 °C in the presence of 10 mM H_2O_2 together with 40 mM NaN_3, which was necessary for inactivation of catalase and peroxidases that had accumulated at high levels together with other proteins in the extract (17).

Reduction of EF-G by Thioredoxin—EF-G (2 μM) was oxidized by incubation with 500 μM H_2O_2 for 15 min at 25 °C. Then 0.75 μM catalase (Nacalai Tesque, Kyoto, Japan) was added to remove residual H_2O_2, and the oxidized EF-G was incubated for 15 min at 25 °C with dithiothreitol (DTT) at various concentrations in the presence and in the absence of 2 μM thioredoxin. Proteins were then precipitated with 10% trichloroacetic acid and subjected to the thiol modification assay for detection of thiol groups on cysteine residues. To assay the activity of EF-G in translation, EF-G that had been reduced by thioredoxin was separated from thioredoxin, DTT, and catalase on a HisTrap column (GE Healthcare) by the same method as used for the purification of EF-G (17).

Determination of Redox Potential—The redox potential of EF-G was determined as described by Motohashi and Hisabori (22) with a minor modification. EF-G (1 μM) was incubated at 25 °C for 16 h in 50 mM potassium phosphate buffer (pH 7.0) that contained 100 mM oxidized DTT and various concentrations of reduced DTT (0.5 μM to 2 mM) under an atmosphere of nitrogen gas. Proteins were then precipitated with 10% trichloroacetic acid and subjected to the thiol modification assay. Intensities of bands, after staining of proteins with Coomassie Brilliant Blue R-250, were determined with the Scion Image system (available on the World Wide Web). The equilibrium constant and the standard redox potential were calculated, taking a value of −330 mV for the standard redox potential of DTT as a reference, as described in the above cited reference (22).

Redox State of EF-G in Vivo—Wild-type cells and cells of a mutant of *Synechocystis* that lacked NADPH-thioredoxin reductase (19) were grown photoautotrophically at 32 °C in light at 70 μmol of photons m^{-2} s^{-1}, as described previously (6). Cells were disrupted by homogenization with glass beads, and cell extracts were obtained as described previously (17). Proteins in cell extracts (2 μg/assay) were precipitated with 10% trichloroacetic acid and subjected to the thiol modification assay. After separation of proteins by non-reducing SDS-PAGE on a 7.5% polyacrylamide gel and subsequent transfer of proteins to a nitrocellulose membrane, EF-G was detected immunologically with an antiserum that had been raised against EF-G (Slr1463) of *Synechocystis*, as described previously (6).

RESULTS

Oxidation of Two Cysteine Residues by H_2O_2—We monitored the redox state of five cysteine residues of EF-G (Slr1463) by modifying free thiol groups with a maleimidyl reagent (average molecular mass, 5 kDa). Modification of the fully reduced form of EF-G with this reagent resulted in a decrease in the electrophoretic mobility of EF-G on a non-reducing gel (Fig. 1*A*, *lane 2*). Incubation of EF-G with 500 μM H_2O_2 prior to the modification decreased this change in mobility (Fig. 1*A*, *lane 3*), indicating that some of the five cysteine residues were oxidized by H_2O_2.

In order to estimate the number of oxidized cysteine residues in EF-G, we compared the mobility of wild-type EF-G with that of mutated proteins in which specific cysteine residues were

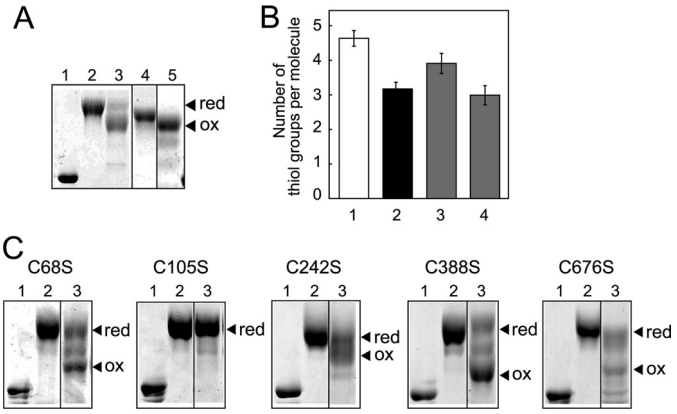

FIGURE 1. **Effects of H_2O_2 on the redox state of cysteine residues in EF-G.** *A*, fractionation on a non-reducing gel of purified EF-G and mutant derivatives that had been treated with a maleimidyl thiol-modifying reagent (*lanes 2–5*) or untreated (*lane 1*). EF-G or a derivative (2 μM) was incubated in the presence of 500 μM H_2O_2 (*lane 3*) or in its absence (*lanes 1*, *2*, *4*, and *5*). Then, after proteins had been treated with the thiol-modifying reagent, they were fractionated by non-reducing SDS-PAGE. *Lanes 1–3*, wild-type EF-G; *lane 4*, C105S; *lane 5*, C68S/C105S. *B*, quantitative analysis of thiol groups. The number of thiol groups per EF-G or its derivatives was determined with 5,5′-dithio-bis(2-nitrobenzoic acid). *Column 1*, reduced form of wild-type EF-G; *column 2*, oxidized form of wild-type EF-G; *column 3*, reduced form of C105S; *column 4*, reduced form of C68S/C105S. Values are means ± S.D. (*bars*) of results from three independent experiments. *C*, fractionation on a non-reducing gel of mutated derivatives of EF-G (C68S, C105S, C242S, C388S, and C676S) that had been treated with the thiol-modifying reagent (*lanes 2* and *3*) or untreated (*lane 1*). Each protein (2 μM) was incubated in the presence of 500 μM H_2O_2 (*lane 3*) or in its absence (*lanes 1* and *2*). Then, after proteins had been treated with the thiol-modifying reagent, they were fractionated by non-reducing SDS-PAGE. *red*, reduced; *ox*, oxidized.

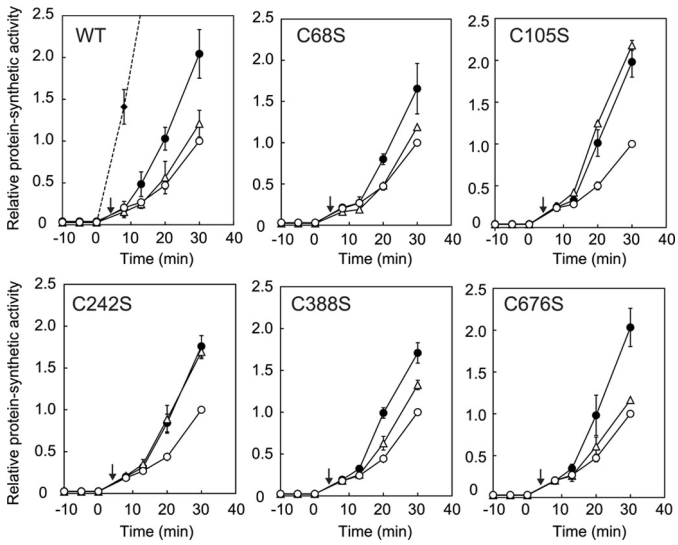

FIGURE 2. **Effects of wild-type and mutated derivatives of EF-G on the recovery of translational activity *in vitro* from H_2O_2-induced inhibition.** Prior to translation reaction *in vitro*, the cell extract was incubated for 10 min in the presence of 10 mM H_2O_2. Components required for translation, including *psbA* mRNA and amino acids, were added at zero time. After incubation for 5 min, 5 μg of wild-type (*WT*) or mutated EF-G were added to the extract, as indicated by *arrows*. ●, reduced form of EF-G or its derivative; △, EF-G or its derivative that had been preincubated with 2 mM H_2O_2; ○, control (buffer used for the preparation of EF-G); ♦, translational activity in the absence of H_2O_2. Radioactivity incorporated into proteins in the cell extract that had been incubated with buffer for 30 min was taken as 1.0. Values are means ± S.D. (*bars*) of results from three independent experiments.

replaced by serine residues. The mobility of the mutated protein C105S fell between the mobilities of the reduced and oxidized forms of wild-type EF-G (Fig. 1*A*, *lane 4*). The mobility of a double-mutated protein, C68S/C105S, was the same as that of the oxidized EF-G (Fig. 1*A*, *lane 5*), indicating that two cysteine residues in EF-G were oxidized by H_2O_2. Since a dimeric form of EF-G was not detected after oxidation of EF-G with H_2O_2 (data not shown), it seems likely that two oxidized cysteine residues formed an intramolecular disulfide bond. Note that Cys^{68} was not a target of oxidation, as discussed below.

We also determined the number of free thiol groups on cysteine residues in EF-G and its mutated derivatives by monitoring changes in absorption after reaction of thiol groups with 5,5′-dithio-bis(2-nitrobenzoic acid). The numbers of free thiol groups per molecule of reduced EF-G, oxidized EF-G, C105S, and C68S/C105S were 4.6, 3.1, 3.9, and 2.9, respectively (Fig. 1*B*), confirming that two cysteine residues were oxidized by H_2O_2.

To identify the cysteine residues that were oxidized by H_2O_2, we replaced individual cysteine residues in EF-G by serine residues and investigated the redox state of cysteine residues by the thiol modification assay. In the presence of 500 μM H_2O_2, the cysteine residues in C68S, C242S, C388S, and C676S were oxidized, whereas the cysteine residues in C105S were not oxidized (Fig. 1*C*). We therefore concluded that Cys^{105} is a target of oxidation by H_2O_2. However, we failed to identify the counterpart of Cys^{105} for formation of a disulfide bond by this method, perhaps because Cys^{105} reacted with another cysteine residue in the mutated protein in which the true counterpart had been replaced by serine.

Effects of Mutation of EF-G on Translation in Vitro—To elucidate the relationship between the redox state of each cysteine residue and translation, we investigated the effects of mutated derivatives of EF-G on translation *in vitro* in a translation system prepared from *Synechocystis*. When we added *psbA2* mRNA, which encodes the D1 protein, to the translation system together with components required for translation, which included [^{14}C]leucine, we observed the incorporation of radioactivity into newly synthesized proteins (Fig. 2, *WT*, *closed diamonds*). In this assay, the major protein synthesized is the D1 protein; traces of other proteins reflect the presence of intrinsic mRNAs in the translation system (17). The addition of 10 mM H_2O_2 to the translation system inhibited translation (Fig. 2, *WT*, *open circles*). This apparent robustness of the translation system against H_2O_2 is due to the presence of catalase and peroxidases in the cell extract (17). The addition of the reduced form of wild-type EF-G to the translation system that had been incubated with H_2O_2 reversed the inhibition of translation to some extent (Fig. 2, *WT*, *closed circles*). By contrast, the oxidized form of wild-type EF-G, generated by incubation of EF-G with 2 mM H_2O_2, was unable to restore translational activity (Fig. 2, *WT*, *open triangles*). These results indicate that EF-G is active in the reduced form and inactive in the oxidized form.

Among mutated derivatives of EF-G, C68S, C388S, and C676S exhibited essentially the same redox response as the wild-type protein (Fig. 2). However, C105S and C242S that had been incubated with H_2O_2 were able to support translation to the same extent as their reduced forms (Fig. 2), indicating that Cys^{105} and Cys^{242} in EF-G are the possible targets of oxidation by H_2O_2 as well as the sites of regulation of the activity of EF-G in a redox-dependent manner.

Identification of Oxidized Cysteine Residues by Peptide Mapping Analysis—To confirm that Cys^{105} and Cys^{242} are the critical targets of oxidation by H_2O_2, we performed peptide mapping analysis of EF-G. We digested the oxidized form of EF-G, which had been obtained by incubation of EF-G with 2 mM H_2O_2, with lysyl endopeptidase and separated the resulting peptide fragments by reversed-phase HPLC. To obtain peptides in their reduced form, we incubated a portion of the peptide fragments with DTT prior to reversed-phase HPLC. We compared the patterns of elution of peptides derived from oxidized EF-G with patterns of elution of the reduced peptides. Three specific peaks, namely LR1, LR2, and LR3, which were present after HPLC of the reduced peptides, were absent from the chromatographic profile of the peptides derived from oxidized EF-G (Fig. S1). Mass spectrometry revealed that LR1, LR2, and LR3 corresponded to peptides that contained Cys^{242}, Cys^{388}, and Cys^{105}, respectively (Table S1). Thus, peptide mapping analysis indicated that these three cysteine residues were potential targets of oxidation. We failed to detect peaks that corresponded to disulfide-linked peptides under oxidizing conditions, probably due to incomplete digestion of EF-G or insufficient separation of peptide fragments. The absence of LR2, which corresponded to the peptide that contained Cys^{388}, in the oxidized EF-G was probably due to further oxidation under strong oxidizing conditions, such as 2 mM H_2O_2.

Regulation of the Redox State of EF-G by Thioredoxin—The redox behavior of EF-G that is mediated by cysteine residues suggested that EF-G might interact with thioredoxin, an oxidoreductase that acts by reducing disulfide bonds in target proteins. We examined whether thioredoxin was able to reduce the disulfide bond in the oxidized form of EF-G. The results of a thiol modification assay showed that the oxidized cysteine residues in 2 μM EF-G that had been incubated with 500 μM H_2O_2 were reduced by subsequent exposure to a high concentration of DTT, such as 5 mM (Fig. 3*A*). By contrast, complete reduction of EF-G was achieved by the addition of 2 μM thioredoxin, even in the presence of only 50 μM DTT (Fig. 3*A*). Thus, thioredoxin can interact with EF-G and reduce an intramolecular disulfide bond.

A triple-mutated derivative of EF-G, in which Cys^{68}, Cys^{388}, and Cys^{676} had been replaced by serine, had a redox response similar to that of wild-type EF-G; the mutated protein was oxidized by H_2O_2 and reduced by thioredoxin (Fig. 3*A*). This result clearly indicated that the remaining (non-mutated) Cys^{105} and Cys^{242} residues formed a disulfide bond under oxidative conditions and that the disulfide bond was reduced by thioredoxin (Fig. 3*B*). By contrast, a different mutated derivative of EF-G, in which Cys^{68}, Cys^{242}, and Cys^{676} had been replaced by serine, was no longer susceptible to oxidation (Fig. 3*A*), an observation that excluded the possibility that a disulfide bond was formed between Cys^{105} and Cys^{388} under oxidative conditions.

Determination of the Redox Potential of EF-G—To evaluate the possibility of a putative cascade of electrons from thioredoxin to EF-G, we determined the redox potential of EF-G with redox buffers that contained various ratios of the reduced form of DTT to the oxidized form. The results of a thiol modification assay showed that EF-G was oxidized in two steps when we decreased the relative level of the reduced form of DTT (Fig. 4).

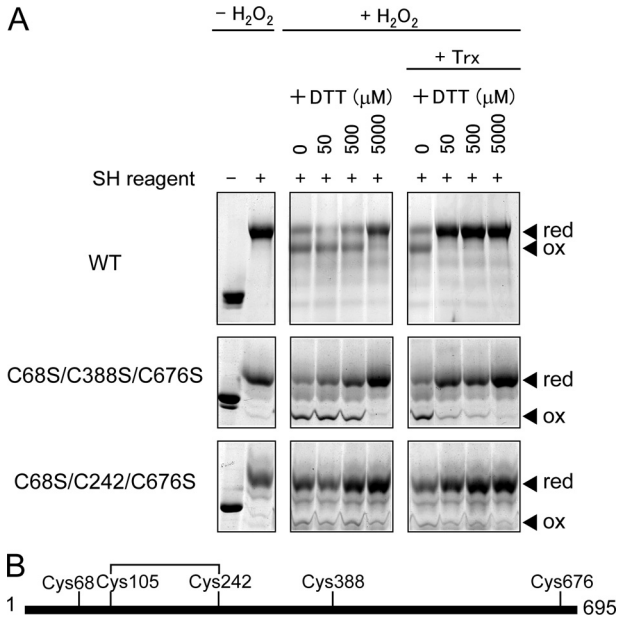

FIGURE 3. **Effects of thioredoxin on the reduction of oxidized EF-G or its mutant derivatives.** *A*, fractionation on a non-reducing gel of wild-type and mutated EF-G that had been treated with the thiol-modifying (SH) reagent (+) or untreated (−). EF-G or a mutant derivative (2 μM) was incubated in the presence of 500 μM H_2O_2 or in its absence and then incubated in the presence of DTT at the indicated concentration plus 2 μM thioredoxin (*Trx*). After proteins had been treated with the thiol-modifying reagent, they were fractionated by non-reducing SDS-PAGE. *red*, reduced; *ox*, oxidized. *B*, schematic representation of the putative disulfide bond in EF-G. The *numbers* indicate the positions of amino acid residues in EF-G.

From its migration on the gel, the first oxidized form of EF-G appeared to be identical to the form generated upon oxidation by H_2O_2; the second form might have been produced by further oxidation of other cysteine residues by the oxidized form of DTT. Since the two-step oxidation complicated the accurate determination of redox potential, we determined the redox potential of the redox-active cysteine residues, namely Cys^{105} and Cys^{242}, using the triple-mutated derivative of EF-G, C68S/C388S/C676S. From the ratios of the reduced form of EF-G to the oxidized form, we determined the redox potential of the target cysteine residues of EF-G to be −221 mV. We also determined the redox potential of thioredoxin-*m* (Slr0623) of *Synechocystis* to be −282 mV, which is the same as that of thioredoxin-*m* (−282 mV) of spinach (22). Thus, EF-G can naturally accept electrons from thioredoxin-*m*.

Functional Regulation of EF-G by Thioredoxin—We investigated whether the reduction of EF-G by thioredoxin affected the activity of EF-G during translation. We incubated oxidized EF-G with 2 μM thioredoxin and 50 μM DTT, namely under conditions used for the thioredoxin-assisted reduction of EF-G (Fig. 3*A*), and then we purified the reduced EF-G. When the reduced protein was added to the translation system *in vitro* that had been incubated with 10 mM H_2O_2 for 10 min, translational activity was restored (Fig. 5*A*). The direct addition of 2 μM thioredoxin plus 10 μM DTT to the translation system that had been incubated with 10 mM H_2O_2 for 10 min also restored translational activity. By contrast, the addition of 10 μM DTT alone did not restore translation (Fig. 5*B*). Thus, the reduction of oxidized EF-G by thioredoxin allowed the EF-G to be active in the translation system.

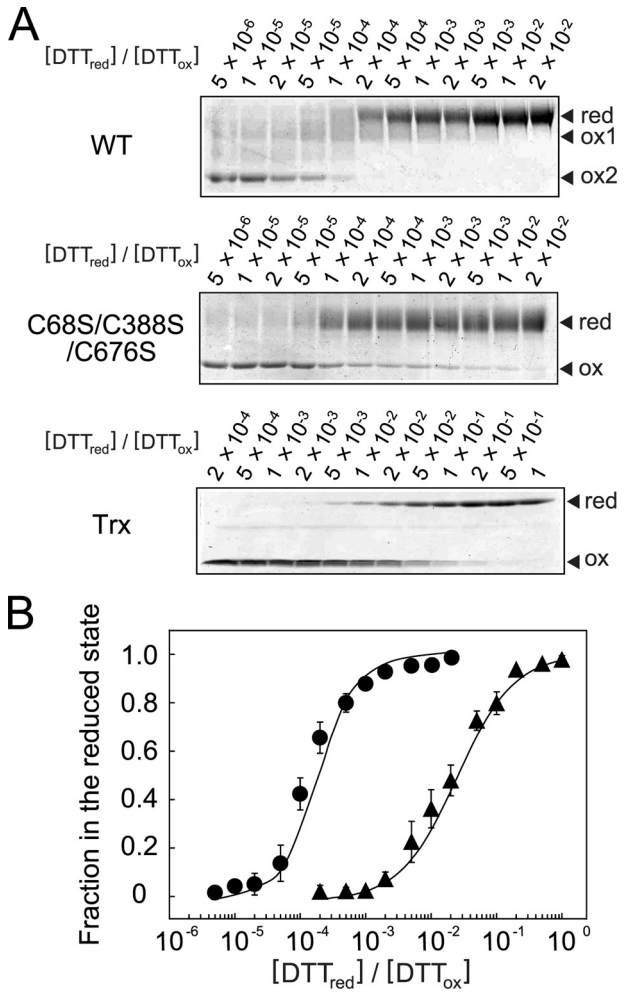

FIGURE 4. **Determination of the redox potentials of EF-G and thioredoxin.** *A*, wild-type (*WT*) and the C68S/C388S/C676S mutant forms of EF-G and thioredoxin (*Trx*) were equilibrated for 16 h with redox buffers that contained various ratios of reduced DTT (DTT_{red}) to oxidized DTT (DTT_{ox}). After treatment with the thiol-modifying (SH) reagent, proteins were fractionated by nonreducing SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250. *red*, reduced; *ox*, oxidized. Two oxidized forms (*ox1* and *ox2*) were detected in wild-type EF-G. *B*, bands of the reduced and oxidized forms of C68S/C388S/C676S (●) and thioredoxin (▲) were quantified, and the fraction of protein in the reduced form was plotted against the value of [DTT_{red}]/[DTT_{ox}]. The data were fitted to a curve, as described elsewhere (22). Values are means ± S.D. (*bars*) of results from three independent experiments.

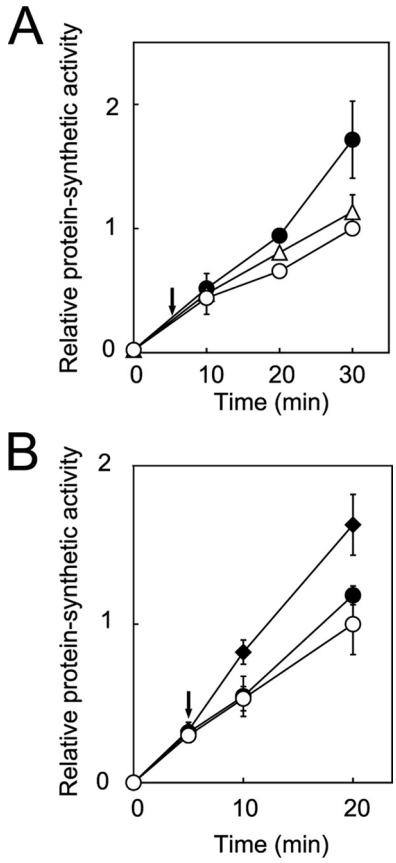

FIGURE 5. **Effects of thioredoxin on the translational activity of oxidized EF-G *in vitro*.** *A*, activation of oxidized EF-G by thioredoxin. Oxidized EF-G was incubated with thioredoxin, and EF-G was then purified. The purified EF-G was added to the translation system *in vitro* that had been incubated with H_2O_2, as indicated by *an arrow*, and translational activity was monitored as indicated in the legend to Fig. 2. ●, EF-G that had been reduced by thioredoxin; △, oxidized EF-G; ○, control (buffer used for the preparation of EF-G). *B*, effects of thioredoxin on the recovery of translational activity from H_2O_2-induced inhibition. Thioredoxin and DTT were added to the translation system *in vitro* after it had been incubated with H_2O_2, as indicated by *arrows*, and translational activity was monitored as indicated in the legend to Fig. 2. ♦, 2 μM thioredoxin plus 10 μM DTT; ●, 10 μM DTT; ○, control (buffer used for the preparation of thioredoxin). Radioactivity incorporated into proteins in the cell extract that had been incubated with buffer for 30 or 20 min was taken as 1.0. Values are means ± S.D. (*bars*) of results from three independent experiments.

Redox State of EF-G in Vivo—We investigated the redox state of EF-G in cells of *Synechocystis* with antibodies specific to EF-G (Slr1463). In cells grown in light at 70 μmol of photons m^{-2} s^{-1}, the optimum light condition for growth, the majority of EF-G was present in the reduced form, whereas the oxidized form was also present to a lesser extent (Fig. 6). From its migration on the gel, the oxidized form was considered to be identical to the form that had been generated upon oxidation by H_2O_2 *in vitro*, namely the form that possessed an intramolecular disulfide bond between the two specific cysteine residues (Fig. 6). When cells were incubated for 1 h in light at 200 μmol of photons m^{-2} s^{-1}, which is considered to be moderately strong light for this organism, levels of the reduced form of EF-G increased to about 90%. This reduction must be due to the acceleration of the photosynthetic transport of electrons. When we further increased the intensity of light to 1,500 μmol of photons m^{-2} s^{-1}, strong light that causes the photoinhibition of PSII (6), some of the EF-G was converted to the oxidized form, most probably as a result of the generation of ROS and the consequent oxidation of EF-G.

In *Synechocystis*, thioredoxins are reduced by two enzymes, namely NADPH-thioredoxin reductase and ferredoxin-thioredoxin reductase (19). Of the two reducing pathways, both of which are derived from the photosynthetic transport of electrons, the pathway via NADPH-thioredoxin reductase is more important for the antioxidant system (19). In a mutant that lacked NADPH-thioredoxin reductase, Δ*ntr*, levels of the reduced form of thioredoxins fell significantly (19). In this same mutant, we found that about 40% of the EF-G was present in the oxidized form, even after incubation in light at 200 μmol of photons m^{-2} s^{-1} (Fig. 6). In strong light at 1,500 μmol of photons m^{-2} s^{-1}, levels of the oxidized form of EF-G in the mutant rose to about 50%, indicating that reduction of EF-G by thiore-

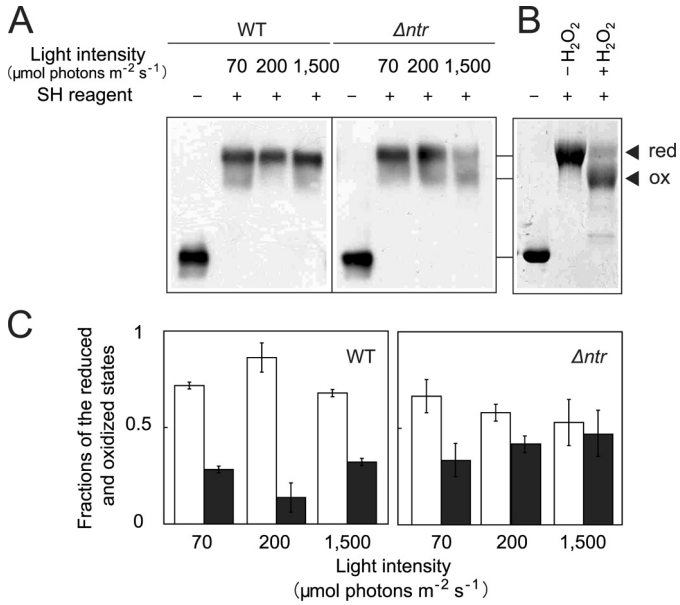

FIGURE 6. **Effects of light intensity on the redox state of EF-G *in vivo*.** *A*, redox states of EF-G *in vivo*. Wild-type cells and cells of Δ*ntr*, which lacks NADPH-thioredoxin reductase, were incubated at 32 °C for 60 min in light at the indicated intensities. Cells were broken with glass beads, and cell extracts were obtained as described under "Experimental Procedures." Proteins in cell extracts were treated with the thiol-modifying (SH) reagent (+) or untreated. After fractionation of proteins by non-reducing SDS-PAGE, EF-G was detected immunologically, as also described under "Experimental Procedures." *B*, redox states of EF-G *in vitro*. The oxidized (*ox*) form of EF-G protein, which had been generated upon oxidation by H_2O_2, and its fully reduced (*red*) form were visualized by staining with Coomassie Brilliant Blue R-250, as indicated in the legend to Fig. 1. *C*, quantification of the bands of the reduced and oxidized forms of EF-G *in vivo* as shown in *A*. Fractions of the reduced and oxidized states are indicated by *white* and *black columns*, respectively. Values are means ± S.D. (*bars*) of results from three independent experiments.

doxin had been interrupted. These observations suggest that EF-G is reduced by thioredoxin-mediated reducing signals that are derived from the photosynthetic transport of electrons.

DISCUSSION

Oxidation of Two Specific Cysteine Residues Inactivates EF-G—Studies of the effects of ROS on the photoinhibition of PSII have revealed that ROS act, at the elongation step of translation, by inhibiting the synthesis of proteins that are required for the repair of PSII (6, 7). Biochemical studies *in vitro* with a translation system derived from *Synechocystis* have demonstrated that EF-G is a primary target, within the translational machinery, of inactivation by ROS (17). EF-G was identified initially as one of the proteins that were most abundantly oxidized in *E. coli*, as assessed by the formation of carbonyl groups, in cells that had been treated with H_2O_2 (23) as well as in cells that lacked a superoxide dismutase (24). However, the chemical nature of the oxidation of EF-G by ROS remained to be determined. In the present study, we demonstrated that inactivation by H_2O_2 of EF-G (Slr1463) in *Synechocystis* was due to the oxidation of two specific cysteine residues and the formation of a disulfide bond.

Replacement of two cysteine residues, namely Cys^{105} and Cys^{242}, by serine rendered EF-G insensitive to inactivation by H_2O_2 and allowed EF-G to function in translation *in vitro* even after exposure to H_2O_2 (Fig. 2). These results support the hypothesis that these two cysteine residues are the targets of oxidation by ROS, and furthermore, they suggest that genetic engineering of these cysteine residues of EF-G might alter the sensitivity of the translation system to oxidative stress.

A model of the three-dimensional structure of EF-G suggests that Cys^{105} and Cys^{242} are located close to each other and to the GTP-binding site (Fig. S2). Oxidation of these cysteine residues with subsequent formation of a disulfide bond might induce a conformational change that decreases the binding affinity of EF-G for GTP or its capacity for hydrolysis of GTP. Alternatively, the putative conformational change might prevent EF-G from entering the P site on the ribosome.

The Target Cysteine Residues Are Conserved in Many Organisms—Alignment of the deduced amino acid sequences of EF-G from various organisms revealed that a pair of cysteine residues that correspond to Cys^{105} and Cys^{242} in EF-G (Slr1463) is strongly conserved in the EF-G proteins of several species of cyanobacteria and other prokaryotes as well as in the chloroplast EF-G proteins of some plants (Fig. S3). Moreover, these cysteine residues are conserved even in anaerobic bacteria, such as *Rhodobacter sphaeroides* ATCC 17025 and *Salmonella typhi* strain CT18. By contrast and unexpectedly, the EF-G proteins of *Thermus thermophilus* and *Bacillus subtilis* lack these cysteine residues. The conserved cysteine residues might play an important role in the regulation of the activity of EF-G, and the susceptibility of these cysteine residues to oxidation might serve as a negative regulator for suppression of translation under oxidative stress *in vivo*.

The Function of EF-G Is Modified by Thioredoxin—Various metabolic processes in chloroplasts and cyanobacteria are controlled by regulatory systems that involve redox reactions (25–27). A fundamental example of such a redox reaction is the reversible reduction of disulfide bonds of cysteine residues by thioredoxin. The redox behavior of two of the cysteine residues in EF-G of *Synechocystis* prompted us to investigate the interaction of EF-G with thioredoxin. Assays involving thiol modification and translation *in vitro* revealed that the disulfide bond in peroxide-oxidized EF-G was reduced by thioredoxin and that the newly reduced form of EF-G was able to function in translation (Figs. 3 and 5).

Thioredoxin affinity chromatography captured a number of putative targets of thioredoxin (25–32). EF-G in spinach chloroplasts (28) and *E. coli* (29) and EF-2 in *Arabidopsis thaliana* (30) and *Chlamydomonas reinhardtii* (31) were identified as candidates for targets of thioredoxin, as was EF-G (Slr1463) in *Synechocystis* (32). In the present study, we demonstrated for the first time, to our knowledge, that EF-G is an actual and functional target of thioredoxin.

Physiological Implications of the Redox Regulation of EF-G by Thioredoxin—In photosynthesis, reducing equivalents that are generated by the photosynthetic transport of electrons are transmitted to various proteins via thioredoxin and regulate their activity. Several enzymes involved in the Calvin cycle, such as glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, and phosphoriburokinase, are activated by thioredoxin (33). ATP synthase in chloroplasts is activated upon reduction of its γ subunit by thioredoxin (34, 35).

The activation of EF-G by the reduced form of thioredoxin suggests that the activity of EF-G might also be regulated by the reducing power that is generated by the photosynthetic transport of electrons and mediated by thioredoxin. We found that the translational activity of EF-G was regulated by its redox state (Fig. 2) (17). Furthermore, Western blotting analysis revealed that the redox state of EF-G is regulated by thioredoxin *in vivo* (Fig. 6). Thus, it seems possible that the translational machinery might be regulated by the photosynthetic transport of electrons in a redox pathway that includes thioredoxin and EF-G. This phenomenon might also explain aspects of the light-dependent regulation of translation. Synthesis of the D1 protein is induced by light at the translational level in plants (36, 37) and algae (38) and at both the transcriptional and the translational level in cyanobacteria (39, 40). In addition, the activation of the synthesis of the D1 protein requires reducing power derived from the photosynthetic transport of electrons (41–43).

Two other homologs of EF-G, namely Sll1098 and Sll0830, have been found in *Synechocystis* (Fig. S3), and they have been shown to function as EF-G (17). Sll1098 has the pair of conserved cysteine residues found in EF-G, but Sll0830 does not. It is possible that Sll1098 might be redox-regulated in the same way as EF-G (Slr1463), whereas Sll0830 might function constitutively. The roles of these two homologs in translation remain to be elucidated.

ROS oxidize a specific pair of cysteine residues in EF-G, and these residues are also targets of thioredoxin. When EF-G is oxidized by ROS, it is no longer able to support translation. The specific cysteine residues in EF-G are strongly conserved in cyanobacteria and chloroplasts (Fig. S3). These observations together suggest a strategy whereby photosynthetic organisms are able to survive under oxidative conditions. Environmental stress, such as strong light, often give rise to oxidative stress, producing ROS via photosynthesis (1). Under severe oxidative conditions, translation might be suppressed to prevent the synthesis of proteins that would otherwise enhance oxidative stress by accelerating the repair of PSII and reactivating or stimulating the photosynthetic transport of electrons. In fact, suppression of translation has been observed both under oxidative stress (6, 7) and when excess reducing power accumulates in the photosynthetic machinery (13–15). Thus, EF-G might act as a safety valve to suppress translation under oxidative stress.

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