Oxidation of a Cysteine Residue in Elongation Factor EF-Tu Reversibly Inhibits Translation in the Cyanobacterium Synechocystis sp. PCC 6803*

Received for publication, November 23, 2015, and in revised form, January 17, 2016. Published, JBC Papers in Press, January 19, 2016, DOI 10.1074/jbc.M115.706424

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Translational elongation is susceptible to inactivation by reactive oxygen species (ROS) in the cyanobacterium *Synechocystis* sp. PCC 6803, and elongation factor G has been identified as a target of oxidation by ROS. In the present study we examined the sensitivity to oxidation by ROS of another elongation factor, EF-Tu. The structure of EF-Tu changes dramatically depending on the bound nucleotide. Therefore, we investigated the sensitivity to oxidation in vitro of GTP- and GDP-bound EF-Tu as well as that of nucleotide-free EF-Tu. Assays of translational activity with a reconstructed translation system from *Escherichia coli* revealed that GTP-bound and nucleotide-free EF-Tu were sensitive to oxidation by H2O2, whereas GDP-bound EF-Tu was resistant to H2O2. The inactivation of EF-Tu was the result of oxidation of Cys-82, a single cysteine residue, and subsequent formation of both an intermolecular disulfide bond and sulfenic acid. Replacement of Cys-82 with serine rendered EF-Tu resistant to inactivation by H2O2, confirming that Cys-82 was a target of oxidation. Furthermore, oxidized EF-Tu was reduced and reactivated by thioredoxin. Gel-filtration chromatography revealed that some of the oxidized nucleotide-free EF-Tu formed large complexes of >30 molecules. Atomic force microscopy revealed that such large complexes dissociated into several smaller aggregates upon the addition of dithiothreitol. Immunological analysis of the redox state of EF-Tu in vivo showed that levels of oxidized EF-Tu increased under strong light. Thus, resembling elongation factor G, EF-Tu appears to be sensitive to ROS via oxidation of a cysteine residue, and its inactivation might be reversed in a redox-dependent manner.

In photosynthetic organisms, reactive oxygen species (ROS)2 are inevitably produced as byproducts of photosynthetic reactions. Photosynthetic transport of electrons produces the superoxide radical and hydrogen peroxide (H2O2), whereas photosynthetic transfer of excitation energy produces singlet oxygen (1). Production of these ROS is stimulated under strong light, resulting in oxidative stress (1). Studies of the effects of ROS on photosystem II (PSII) revealed that ROS act primarily by inhibiting the repair of photodamaged PSII, thereby stimulating the photoinhibition of PSII (2–5). Inhibition of the repair of PSII has been attributed to the suppression by ROS of the synthesis of the D1 protein, a protein in the reaction center of PSII (2, 3, 6, 7). However, ROS not only induces the suppression of the synthesis of the D1 protein but also that of almost all of the thylakoid proteins, indicating that the protein-synthetic machinery itself is sensitive to ROS (2, 3).

Analysis of polysomes in the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*) demonstrated that the elongation step of translation is particularly sensitive to ROS (2, 3). Biochemical analysis of the *Synechocystis* translation system revealed that EF-G, a protein essential for translational elongation, is a target of inactivation by ROS (8, 9). Moreover, EF-G of *Synechocystis* was inactivated via the oxidation of two specific cysteine residues, namely, Cys-105 and Cys-242, and subsequent formation of an intramolecular disulfide bond between them (9). Expression in *Synechocystis* of mutated EF-G, in which Cys-105 was replaced by serine, enhanced both protein synthesis and the repair of PSII under strong light (10). However, the extent of the protective effects of the mutation was as limited as 20%, and this modest effect suggested that the target of ROS might not be only EF-G but also some other factor(s) involved in translational elongation (10).

Elongation factor Tu (EF-Tu), another protein that is essential for translational elongation, binds aminoacyl-tRNA in its GTP-bound form and delivers it to the A site of the ribosome. Upon hydrolysis of the bound GTP, EF-Tu dissociates from the ribosome (11). Several studies have suggested that EF-Tu might be oxidized under oxidizing conditions (12–14). For example, elongation factor G; EF-Tu, elongation factor Tu; FTR, ferredoxin-thioredoxin reductase; NBD-CI, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole; PSII, photosystem II.
EF-Tu was identified as one of the proteins that was abundantly carboxylated in *Bacillus subtilis* cells that had been treated with H$_2$O$_2$ (12). Treatment of *Escherichia coli* cells with nitric acid resulted in the modification of thiol groups of various proteins, and EF-Tu was found to be one of these modified proteins (13). Furthermore, EF-Tu was also identified as an S-nitrosylated protein in *Mycobacterium tuberculosis* cells that had been treated with acidified nitrite (14). However, the mechanism of oxidation of EF-Tu and the effects of oxidation of EF-Tu on translation remain to be clarified.

A clue to the understanding of the mechanism of oxidation of EF-Tu is provided by the presence of a specific cysteine residue that is strongly conserved in various species of bacteria (15, 16). EF-Tu of *Synechocystis* includes a single cysteine residue, namely, Cys-82, and this residue corresponds to the residue that is strongly conserved. In EF-G of *Synechocystis* and *E. coli*, a specific pair of cysteine residues that is also conserved in various organisms is the target of oxidation by ROS (8, 9, 17, 18). These observations together led us to postulate that Cys-82 in EF-Tu might be a potential target of oxidation by ROS.

In the present study we investigated the sensitivity to H$_2$O$_2$, of Cys-82 in EF-Tu from *Synechocystis* and the effects of the oxidation of this residue on translational activity in a reconstituted translation system derived from *E. coli*. We found that Cys-82 of EF-Tu was oxidized by H$_2$O$_2$ with formation of an intermolecular disulfide bond and sulfenic acid in both its GTP-bound and nucleotide-free forms. Oxidized EF-Tu was unable to function in the translation system. By contrast, the GDP-bound form was insensitive to oxidation and inactivation by ROS. Furthermore, thioredoxin was able to reduce and reactivate the oxidized forms of EF-Tu.

**Experimental Procedures**

Preparation of Reconstituent Proteins—The *sll1099* and *slr0623* genes of *Synechocystis*, which encode EF-Tu and m-type thioredoxin, respectively, were cloned into the pET21b vector (Novagen, Darmstadt, Germany), and the resultant plasmids were used to transform *E. coli* BL21 (DE3). Proteins were expressed with a histidine tag at the carboxyl terminus and were purified, in reduced forms, by nickel affinity chromatography with a HiTrap chelating column (GE Healthcare) in buffer that contained 20 mM HEPES-KOH (pH 7.5), 450 mM NaCl, and 7 mM β-mercaptoethanol as described previously (9). For purification of GDP-bound EF-Tu (EF-Tu-GDP), 10 mM MgCl$_2$ and 20 µM GDP were added to the buffer, whereas for purification of nucleotide-free EF-Tu neither MgCl$_2$ nor GDP was added. EF-Tu-GDP was stored in storage buffer that contained 20 mM HEPES-KOH (pH 7.5), 50 mM NaCl, 20% (w/v) glycerol, 10 mM diithiothreitol (DTT), and 10 mM MgCl$_2$; nucleotide-free EF-Tu was stored in the same storage buffer prepared without MgCl$_2$. To prepare GTP-bound EF-Tu (EF-Tu-GTP), EF-Tu-GDP was mixed with 1 mM GTP, 10 mM MgCl$_2$, 2 mM phosphoenolpyruvate, and 0.08 unit/μl pyruvate kinase. The mixture was incubated for 30 min at 37 °C and stored in storage buffer. Before the assays, DTT and nucleotides were removed by passing proteins through a desalting column (PD spinTrap™ G-25; GE Healthcare). Thioredoxin was stored in the storage buffer.

**Detection of the Redox State of Cys-82**—The redox state of the cysteine residue in EF-Tu was monitored by modifying the thiol group with a maleimydyl reagent, methoxypolyethylene glycol maleimide, which has an average molecular mass of 5 kDa (Nihon Yushi, Tokyo, Japan), and subsequent separation of proteins by non-reducing SDS-PAGE on a 12.5% polyacrylamide gel, as described previously (9).

**Translation in Vitro**—Translation in vitro was performed with the PURE system, an artificial reconstituted translation system derived from *E. coli* (19). The translation system was generated in the absence of EF-Tu and reducing reagents by mixing 70S ribosomes with the individual components that are required for translation, such as translation factors, amino acids, and GTP. After treatment of EF-Tu from *Synechocystis* with H$_2$O$_2$ or DTT, the treated EF-Tu was added to the translation system that had been prepared without EF-Tu (17). The resultant translation system was incubated at 37 °C for 1 h in the presence of mRNA that encoded dihydrofolic acid reductase (DHFR) as template, $^{35}$S-labeled cysteine/methionine for detection of synthesis of proteins de novo, and the reagents required for translation as described previously (17).

**Detection of Disulfide Bonds**—EF-Tu that had been treated with 0.5 mM H$_2$O$_2$ or 1 mM DTT was subjected to non-reducing SDS-PAGE and stained with Coomassie Brilliant Blue. After separation, the stained bands of oxidized and reduced EF-Tu were excised from the gel and were subjected to analysis by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry on an Autoflex system (UltraFlexXtreme-TK2; Bruker Daltonics, Bremen, Germany).

**Detection of Sulfenic Acid**—Sulfenic acid was detected with 4-(3-azidopropyl)cyclohexane-1,3-dione (DAz-2), a reagent that reacts specifically with sulfenic acid, as described previ-
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ously with minor modification (20, 21). Oxidized and reduced EF-Tu (20 µg) were incubated in 50 mM HEPES-KOH (pH 7.5) with DAAz-2 at 37 °C for 1 h, and then DAAz-2-labeled EF-Tu was conjugated with biotin by further incubation at 37 °C for 2 h. The reaction was terminated by the addition of cold acetone, and proteins were pelleted by centrifugation at 15,000 rpm for 15 min at 4 °C. The pelleted proteins were resuspended in 10% SDS, separated by SDS-PAGE, and transferred to a PVDF membrane. The membrane was incubated with streptavidin that had been conjugated with horseradish peroxidase, and protein products were visualized with the ECL-Plus chemiluminescence system (GE Healthcare). Sulfenic acid in oxidized EF-Tu was also detected with 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl). NBD-Cl reacts with both sulfenic acid and free thiol groups to generate absorption spectra with a peak at 347 nm and a peak at 420 nm, respectively (20). Nucleotide-free EF-Tu was incubated with 5 mM DTT or 1 mM H2O2, and after reaction with NBD-Cl the respective absorption spectra were recorded.

Gel-filtration Chromatography—Proteins were applied to a gel-filtration column (10 mm × 300 mm; Superdex 200; GE Healthcare) that had been equilibrated with buffer that contained 50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, and 10 mM MgCl2. Proteins were eluted with the same buffer at a flow rate of 0.5 ml min⁻¹ at 25 °C, and absorption of eluted proteins was monitored at 280 nm.

Atomic Force Microscopy—Nucleotide-free EF-Tu that had been treated with 1 mM H2O2 for 5 min was diluted to 0.1–10 nm with buffer that contained 20 mM HEPES-KOH (pH 7.5) and 50 mM NaCl. Aliquots of 5 µl were placed on a mica surface that had been coated with 3-aminopropyl triethoxysilane and were incubated at room temperature for 10 min before analysis as described previously (22). Dynamic changes in the structure of EF-Tu were observed by high speed atomic force microscopy, which was developed by Ando et al. (22, 23). Changes in the structure of EF-Tu were monitored continuously at a rate of 600 ms per frame at room temperature, and recorded images were analyzed with the custom software Kodec (22, 23).

Redox State of EF-Tu in Vivo—Wild-type cells and cells of ftr-v (ssr0330)-disrupted mutant, which is derived from a glucose-tolerant wild-type strain of Synechocystis (24), were grown photoautotrophically at 32 °C under light at 70 µmol of photons m⁻² s⁻¹, as described previously (8). Cells in the culture at an optical density at 730 nm of 1.0 were exposed to light at 2000 µmol of photons m⁻² s⁻¹ for the designated times. Redox reactions were terminated by the addition of 20 mM N-ethylmaleimide and 20 mM NaN3 10 s before turning off the light. Cells were harvested and broken by homogenization with glass beads, and cell extracts were obtained as described previously (8). Proteins in cell extracts were precipitated with 10% trichloroacetic acid. After separation of proteins by non-reducing SDS-PAGE on a 10% polyacrylamide gel and subsequent transfer of proteins to a nitrocellulose membrane, EF-Tu was detected immunologically, as described previously (8), with an antisera that had been raised in a rabbit against EF-Tu of Synechocystis.

Results

Quantification of Nucleotides Bound to EF-Tu—The structure of EF-Tu depends on the nature of the nucleotide bound to it (25–27). Therefore, we postulated that the sensitivity of EF-Tu to oxidation might differ among the three different forms of the protein, namely, the GTP-bound form (EF-Tu-GTP), the GDP-bound form (EF-Tu-GDP), and the nucleotide-free form. We prepared the three forms individually from the recombinant protein and quantitatively the bound nucleotides in each form. In preparations of EF-Tu that had been purified in the presence of GDP and Mg²⁺, >80% of the EF-Tu was associated with GDP (Fig. 1), and we designated this form EF-Tu-GDP. When EF-Tu-GDP was incubated with GTP, phosphoropyruvate, and pyruvate kinase, >80% of the EF-Tu was associated with GTP (Fig. 1), and we designated this form EF-Tu-GTP. By contrast, when EF-Tu was purified in the absence of GDP and Mg²⁺, no GDP or GTP was associated with EF-Tu (Fig. 1), and we designated this form nucleotide-free EF-Tu.

Sensitivity of Cys-82 to Oxidation—We monitored the redox state of Cys-82, the single cysteine residue of EF-Tu, by modifying the free thiol group with a maleimidyl reagent (average molecular mass, 5 kDa). When EF-Tu-GTP that had been reduced with DTT was treated with the maleimidyl reagent, its apparent molecular mass increased during SDS-PAGE on a non-reducing gel, indicating that the thiol group of Cys-82 had bound this reagent (Fig. 2A). Incubation of EF-Tu-GTP with H2O2 at various concentrations before incubation with the maleimidyl reagent resulted in changes in electrophoretic mobility that indicated that Cys-82 had been oxidized and had failed to bind the maleimidyl reagent. Increases in the concentration of H2O2 gradually decreased the levels of the reduced form and increased the levels of two oxidized forms, whose molecular mass suggested that they might be an oligomer and a monomer, respectively (Fig. 2, A, upper image, and B). However, Cys-82 of EF-Tu-GDP was minimally oxidized by H2O2, suggesting that the binding of GDP might render EF-Tu insensitive to oxidation (Fig. 2, A, middle image, and B). By contrast, nucleotide-free EF-Tu was more susceptible to oxidation by H2O2 than was EF-Tu-GTP, yielding two oxidized forms, which
suggested that the absence of a bound nucleotide might render EF-Tu hypersensitive to oxidation (Fig. 2, A, lowest image, and B). When Cys-82 was replaced by serine, no oligomers of EF-Tu-GTP, EF-Tu-GDP, or nucleotide-free EF-Tu were formed in the presence of H$_2$O$_2$, suggesting that the oxidation of Cys-82 might be responsible for oligomerization (Fig. 2 C).

**Translational Activity of EF-Tu in Vitro**—We examined the translational activity of EF-Tu in the PURE system, a system for translation in vitro that is derived from *E. coli* and is generated by mixing the individual components required for translation (19). The addition of the reduced form of EF-Tu-GTP to the translation system that had been prepared without EF-Tu resulted in the synthesis of DHFR (Fig. 3 A). However, when EF-Tu-GTP had been treated with H$_2$O$_2$ before its addition to the translation system, the rate of synthesis of DHFR fell as the concentration of H$_2$O$_2$ was increased (Fig. 3 A). By contrast, in the case of mutant EF-Tu-GTP in which Cys-82 had been replaced by serine, the synthesis of DHFR was barely affected by the presence of H$_2$O$_2$, suggesting that the oxidation of Cys-82 might be responsible for the inactivation of EF-Tu-GTP (Fig. 3 B). However, the translational activity of EF-Tu-GDP as well as that of its mutant derivative EF-Tu-GDP C82S was unaffected by H$_2$O$_2$, suggesting that the binding of GDP might render EF-Tu insensitive to inactivation by oxidation (Fig. 3 C). The addition of only 0.1 mM H$_2$O$_2$ depressed the translational activity to $\approx$20% that of the original level (Fig. 3 A and C). The nucleotide-free mutant derivative EF-Tu C82S was resistant to inactivation by H$_2$O$_2$ (Fig. 3B). These results suggested that the absence of a bound nucleotide might enhance the susceptibility of EF-Tu to inactivation via oxidation of Cys-82.

**Formation of Intermolecular Disulfide Bonds and Sulfenic Acid in Preparations of Oxidized EF-Tu**—To determine the fate of Cys-82 in the oxidized oligomeric form of EF-Tu, we performed peptide-mapping analysis of nucleotide-free EF-Tu. Reduced and oxidized forms of nucleotide-free EF-Tu, which had been obtained by incubation with 1 mM DTT and 0.5 mM H$_2$O$_2$, respectively, were subjected to non-reducing SDS-PAGE. The monomeric proteins in the reduced form and the
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oligomeric proteins in the oxidized form were digested with trypsin, and the resultant peptide fragments were analyzed by MALDI-TOF mass spectrometry. In the analysis of the reduced form, we detected a peptide fragment with a molecular mass of 1768.88 that corresponded to a peptide derived from the combination of two peptides that included Cys-82 via formation of a disulfide bond. Theoretical and observed monoisotopic mass values for peptide fragments that included Cys-82

| Condition | Expected Mass | Mass Difference | Peptide including Cys-82–Cys-82 |
|-----------|---------------|----------------|-------------------------------|
| Reducing  | 1768.88       | +0.64          | ND                            |
| Oxidizing | ND            | −0.61          | 3420.91                       |

* Difference between given and expected values.

### FIGURE 3

Effects of H$_2$O$_2$ at various concentrations on the translational activity of wild-type (WT) EF-Tu and its C82S mutant derivative. A, each form of wild-type EF-Tu was treated with H$_2$O$_2$ at the indicated concentrations for 5 min. After catalase had been added to eliminate residual H$_2$O$_2$, oxidized EF-Tu was mixed with the aliquots of the PURE system that had been generated without EF-Tu. Reaction mixtures were incubated at 37 °C for 60 min in the presence of template mRNA that encoded DHFR and $^{35}$S-labeled cysteine/methionine. Proteins were then fractionated by SDS-PAGE, and translational activity was analyzed in terms of the synthesis of $^{35}$S-labeled DHFR. Quantitation of the intensity of bands due to $^{35}$S-labeled DHFR is shown in the graphs. EF-Tu-GTP ($\bullet$), EF-Tu-GDP ($\square$), and nucleotide-free EF-Tu ($\triangle$) are shown. B, translational activity of each form of C82S EF-Tu in the presence of H$_2$O$_2$ at various concentrations. C, translational activity of nucleotide-free EF-Tu in the presence of H$_2$O$_2$ at low concentrations. The arrows indicate DHFR. Values are the means ± S.D. (bars) of results from three independent experiments.

TABLE 1

| Peptide including Cys-82 | Peptide including Cys-82–Cys-82 |
|--------------------------|--------------------------------|
| Expected                  | 1768.24                       |
| Reducing                  | 1768.88                       |
| Oxidizing                 | ND                            |

in the oxidized monomers might form sulfenic acid. To examine the possible formation of sulfenic acid in the oxidized monomer, we treated nucleotide-free EF-Tu with DAz-2, a reagent that reacts specifically with sulfenic acid, and then we examined the formation of EF-Tu-DAz-2 complexes by Western blotting analysis with streptavidin-conjugated horseradish peroxidase. Sulfenic acid-containing EF-Tu was detected at the position of the EF-Tu monomer after incubation with low concentrations of H$_2$O$_2$, but this reaction product was absent in the analysis of the reduced form of nucleotide-free EF-Tu that had been prepared by incubation with DTT (Fig. 4B). Formation of sulfenic acid in CrtJ, a protein that is known to form sulfenic acid under oxidizing conditions (20), was also detected (Fig. 4C), supporting our conclusion that sulfenic acid was formed at Cys-82 in the oxidized monomer of nucleotide-free EF-Tu (Fig. 4B). Formation of sulfenic acid was also confirmed by treatment of EF-Tu with NBD-Cl, another reagent that binds specifically to sulfenic acid. Oxidized EF-Tu that had been incubated with NBD-Cl exhibited enhanced absorbance at 347 nm, indicating the presence of sulfenic acid, whereas treatment of reduced EF-Tu with NBD-Cl resulted in a decrease in absorbance at 347 nm and an increase at 420 nm, indicating the presence of free thiol groups (Fig. 4D).

Reduction and Reactivation of Oxidized EF-Tu by Thioredoxin—The redox behavior of EF-Tu, as mediated by its single cysteine residue, suggested that EF-Tu might interact with thioredoxin, a small redox protein that regulates the activity of target proteins by reducing disulfide bonds (29, 30). We investigated whether the disulfide bond in oxidized EF-Tu might be reduced by thioredoxin. The thiol-modification assay demonstrated that the oxidized cysteine residues in preparations of nucleotide-free EF-Tu that had been incubated with H$_2$O$_2$ were reduced by $m$-type thioredoxin at various concentrations in the presence of 50 μM DTT. Thus, thioredoxin was able to reduce both oxidized monomers and dimers of EF-Tu (Fig. 5A). Reduction of oxidized monomers by thioredoxin also confirmed that the oxidized monomers had formed sulfenic acid at Cys-82. Thioredoxin is able to reduce sulfenic acid but unable to reduce sulfenic acid or sulfonic acid (31–33).

We next examined whether the reduction of EF-Tu by thioredoxin affected the activity of EF-Tu in translation. We incubated oxidized nucleotide-free EF-Tu with thioredoxin at various concentrations in the presence of 50 μM DTT, namely, under the conditions used for the thioredoxin-assisted reduc-
 tion of EF-Tu. When the treated EF-Tu was included in the translation system prepared without EF-Tu, translational activity was restored (Fig. 5B). By contrast, the addition of 50 μM DTT alone did not restore translation. These results confirmed the hypothesis that oxidized EF-Tu might be reactivated by thioredoxin.

**FIGURE 4. Detection of sulfenic acid in oxidized EF-Tu.** A, effects of dithiothreitol on the redox state of Cys-82 in oxidized EF-Tu. Nucleotide-free EF-Tu was incubated in the presence of 0.5 mM H₂O₂ for 5 min. After removal of residual H₂O₂ by catalase, EF-Tu was treated with DTT at the indicated concentrations and then modified by reaction with a maleimidy reagent (SH reagent). Proteins were fractionated by non-reducing SDS-PAGE. B, nucleotide-free EF-Tu was treated with 5 mM DTT or with H₂O₂ at the indicated concentrations and was then subjected to labeling with DAz-2, a reagent that specifically reacts with sulfenic acid. DAz-2-labeled proteins were visualized by immunoblotting analysis with streptavidin-conjugated horseradish peroxidase. C, detection of sulfenic acid in CrtJ as a positive control. CrtJ was treated with 5 mM DTT or 0.5 mM H₂O₂ and was then labeled with DAz-2. D, detection of sulfenic acid in oxidized EF-Tu with NBD-Cl. NBD-Cl reacts with both sulfenic acid and free thiol groups to generate absorption spectra with a peak at 347 nm and a peak at 420 nm, respectively. Nucleotide-free EF-Tu was incubated with 5 mM DTT (dashed line) and 1 mM H₂O₂ (solid line). After reaction with NBD-Cl, the respective absorption spectra were recorded.

**FIGURE 5. Effects of thioredoxin on the redox state of residue Cys-82 and the translational activity of oxidized EF-Tu.** A, changes in the redox state of residue Cys-82 in oxidized nucleotide-free EF-Tu. Nucleotide-free EF-Tu was incubated in the presence of 0.5 mM H₂O₂ or in its absence. After removal of residual H₂O₂ by catalase, EF-Tu was incubated with 50 μM DTT plus thioredoxin (Trx) at various concentrations. After proteins had been treated with the thiol-modifying reagent (SH reagent), they were fractionated by non-reducing SDS-PAGE. B, recovery of translational activity of oxidized nucleotide-free EF-Tu in the presence of thioredoxin. After oxidized nucleotide-free EF-Tu had been incubated with 50 μM DTT plus H₂O₂ at various concentrations, each sample was added to the translation system *in vitro*. The resultant synthesis of radio-labeled DHFR was monitored as described under “Experimental Procedures.”

Determination of the Molecular Mass of EF-Tu under Native Conditions—After the three types of EF-Tu had been treated with 1 mM H₂O₂ or with 5 mM DTT, they were subjected to gel-filtration chromatography on a Superdex 200 column. Most of the oxidized EF-Tu-GTP was eluted at positions that corresponded to molecular masses of ~90 and 44 kDa, respectively (Fig. 6). This elution profile confirmed that under oxidizing conditions, EF-Tu-GTP existed as both dimers and monomers (44 kDa), as observed on the non-reducing gel shown in Fig. 2A. However, oxidized EF-Tu-GDP was eluted at the position that corresponded to monomers. EF-Tu-GDP was eluted at the position that corresponded to monomers after it had been treated with either H₂O₂ or DTT. By contrast, the elution profile of oxidized nucleotide-free EF-Tu included a major peak corresponding to a molecular mass of ~1400 kDa and a minor peak corresponding to a molecular mass of ~90 kDa, indicating that oxidized nucleotide-free EF-Tu might form large complexes that consist of >30 molecules in addition to dimers. However, most of the reduced nucleotide-free EF-Tu eluted at the position of dimers, suggesting that the reduced form of nucleotide-free EF-Tu might exist as dimers without the formation of an intermolecular disulfide bond.
Redox-dependent Structural Changes in Nucleotide-free EF-Tu—We analyzed the structure of the large complexes of oxidized nucleotide-free EF-Tu by high speed atomic force microscopy. Some of the oxidized nucleotide-free EF-Tu was present as large complexes with a diameter of ~20 nm (Fig. 7A, left image). However, when 100 mM DTT was added directly to the preparation of oxidized nucleotide-free EF-Tu, the large complexes dissociated into many small aggregates (Fig. 7A, right image). By contrast, the addition of DTT did not affect the structures of the nucleotide-free C82S mutant that had been treated with H₂O₂ (Fig. 7B). When we focused on a single large complex of oxidized nucleotide-free EF-Tu, we found that it dissociated into many small aggregates in 30 to 40 s (Fig. 7C). The dissociation of the large complexes by DTT was probably initiated by reduction of intermolecular disulfide bonds and/or the sulfenic acid formed at Cys-82.

Redox State of EF-Tu in Vivo—We monitored changes in the redox state of EF-Tu in cells of Synechocystis under strong light that causes oxidative stress. After cells were exposed to strong light at 2000 μmol of photons m⁻² s⁻¹, cell extracts were subjected to non-reducing SDS-PAGE, and EF-Tu was detected by Western blotting analysis with antibodies specific to EF-Tu. To fix the redox state of EF-Tu in vivo by inhibiting the reducing activity of thioredoxins, we added N-ethylmaleimide, a cell-permeable thiol-modifying reagent, to cell suspensions just before turning off the light. Before illumination, most of EF-Tu was found at the position that corresponded to monomers (Fig. 8A). When cells were exposed to strong light, a portion of EF-Tu was found at the position that corresponded to the oxidized oligomers that had been generated upon oxidation by H₂O₂ in vitro, and levels of the oxidized oligomers increased during prolonged illumination (Fig. 8A). It appeared that EF-Tu might exist as reduced monomers under weak light, whereas under strong light some portion of EF-Tu might form oxidized oligomers and also, most likely, oxidized monomers with sulfenic acid at Cys-82, as observed in vitro. However, the low levels of oxidized oligomers suggested that exogenous addition of N-ethylmaleimide to cell suspensions might not be able to inhibit thioredoxins completely in vivo.

In Synechocystis, ferredoxin-thioredoxin reductase (FTR) mediates the transfer of photosynthesis-derived electrons to thioredoxins, and in ftr-v mutant that lacks the variable subunit of the FTR heterodimer, levels of reduced thioredoxins are significantly decreased (24). Our immunological analysis showed that in ftr-v mutant cells, levels of oxidized oligomers of EF-Tu increased significantly under strong light and reached 70% that of the total levels of EF-Tu in 180 min (Fig. 8). These results indicated that lower levels of reduced thioredoxins accelerated the oxidation of EF-Tu under strong light. Thus, it seems that reducing power that is generated from the photosynthetic transport of electrons might be transmitted to EF-Tu via thioredoxins and that excess ROS might interfere with the reduction of EF-Tu, yielding oxidized EF-Tu.

Discussion

Inactivation of EF-Tu Is Due to the Oxidation of a Cysteine Residue—The particular sensitivity of EF-Tu to oxidation in several species of bacteria has been reported (12–14), but the mechanism of its oxidation and the effects of its oxidation on translation remain to be clarified. In the present study we demonstrated that EF-Tu of Synechocystis is inactivated by H₂O₂ via
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EF-Tu was in its nucleotide-free form, translational activity disappeared much more rapidly than the oxidation of Cys-82 in the presence of \( \text{H}_2\text{O}_2 \); the addition of only 0.1 mm \( \text{H}_2\text{O}_2 \) depressed the translational activity to 20% that of the original level but, under these conditions, 70% of Cys-82 remained in its reduced form (Figs. 2 and 3), implying that disruption of the translational activity of EF-Tu preceded the oxidation of Cys-82. This apparent discrepancy might be related to the unusual structure of the oxidized nucleotide-free form, which aggregates as large complexes of >30 molecules of EF-Tu (Figs. 6 and 7). It seems plausible that the large complexes might include not only oxidized EF-Tu but also the more abundant reduced EF-Tu as discussed below.

Replacement of Cys-82 with serine rendered EF-Tu resistant to \( \text{H}_2\text{O}_2 \) by preventing oligomerization and by protecting the translational activity from disruption by \( \text{H}_2\text{O}_2 \) (Figs. 2 and 3). Genetic engineering of a specific cysteine residue in EF-Tu might allow alteration of the sensitivity of the translation system to oxidative stress. Elevated concentrations of \( \text{H}_2\text{O}_2 \), such as 2 mm, slightly decreased the translational activity of the C82S mutant form of EF-Tu. Thus, strongly oxidative conditions might oxidize additional amino acid residues, as suggested previously (34).

Role of Cys-82 in EF-Tu—Alignment of the deduced amino acid sequences of EF-Tu from various organisms revealed that Cys-82 is strongly conserved in the EF-Tu of several species of cyanobacteria, prokaryotes, and plants (16, 35). In analyses of bacteria, this particular cysteine residue was found in 111 (89%) of 125 aligned sequences, and substitution by alanine and methionine was found in only 13 (10%) and 1 (1%) sequence, respectively (16).

Crystallographic analysis of EF-Tu-GDP from \( E. \text{coli} \) suggested that the carbonyl oxygen of the backbone of Cys-81 (corresponding to Cys-82 in EF-Tu of \( \text{Synechocystis} \)) might form a hydrogen bond with a water molecule that coordinates with a \( \text{Mg}^2+ \) ion, which is in turn essential for the ability of EF-Tu to bind GTP and GDP (25). Furthermore, mutation of Cys-81 in EF-Tu of \( E. \text{coli} \) revealed that replacement of Cys-81 with glycine impaired the ability of EF-Tu to bind both aminoacyl-tRNA and nucleotides (36). Another study of mutations in EF-Tu of \( E. \text{coli} \) demonstrated that replacement of Cys-81 with either serine or methionine did not affect the affinity of nucleotides for EF-Tu but decreased the affinity of aminoacyl-tRNA for binding to EF-Tu (16). By contrast, replacement with alanine had no obvious effects on the binding affinity of nucleotides and aminoacyl-tRNA for EF-Tu (16). In view of the limited variability at the site of the cysteine residue, De Laurentiis et al. (16) proposed that the side chain of Cys-81 might not interact directly with either nucleotides or aminoacyl-tRNA but might play a role in stabilizing the ternary complex that consists of EF-Tu, GTP, and aminoacyl-tRNA. In our study, the C82S mutant EF-Tu of \( \text{Synechocystis} \) was able to function in translation similarly to wild-type EF-Tu. The compatibility of cysteine with serine, an isosteric amino acid (thiol versus hydroxyl group), suggests that the thiol group of Cys-82 might not be essential for the function of EF-Tu in translation. However, the susceptibility of Cys-82 to oxidation in \( \text{Synechocystis} \) suggests that this specific cysteine residue in

oxidation of Cys-82, with subsequent formation of an intermolecular disulfide bond between two EF-Tu molecules and sulfenic acid in individual molecules. It seems that the oxidized EF-Tu with sulfenic acid might not be a transient but a stable oxidized form. It is reported that 1-Cys methionine sulfoxide reductase B (MSRB1) of \( \text{Arabidopsis} \) (32) and CrtJ of \( R. \text{capsulatus} \) (20) form a stable sulfenic acid at redox-sensitive cysteine residues.

The sensitivity of EF-Tu to oxidation is strongly affected by the nucleotide that is bound to it. The GTP-bound form was sensitive to oxidation by \( \text{H}_2\text{O}_2 \), whereas the GDP-bound form was much more resistant to oxidation. In the absence of a bound nucleotide, EF-Tu was hypersensitive to oxidation by \( \text{H}_2\text{O}_2 \). The redox state of Cys-82 in the GTP- and GDP-bound forms of EF-Tu was closely correlated with the factor’s translational activity. For example, treatment of EF-Tu-GTP with 1 mm \( \text{H}_2\text{O}_2 \) oxidized 50% of Cys-82 residues and decreased the translational activity by 50% (Figs. 2 and 3). However, when
EF-Tu might regulate the function of EF-Tu in translation in a redox-dependent manner.

Effects of the Bound Nucleotide on the Sensitivity of EF-Tu to Oxidation—EF-Tu undergoes a drastic conformational change when GDP is replaced by GTP and vice versa. The transition from the GDP-bound form to the GTP-bound form rotates domain I by ~90° relative to domains II and III, resulting in a change from an open to a closed conformation (25). Although the structure of nucleotide-free EF-Tu is unavailable, it is likely that its structure might be very different from those of the GDP- and GTP-bound forms. However, we do not yet understand why the GTP-bound form, with a closed conformation, is so much more sensitive to oxidation than the GDP-bound form, which has an open conformation.

The crystal structures of EF-Tu-GDP of E. coli and of EF-Tu-GTP of Thermus aquaticus suggest that helix B in the switch II region might play a key role in the structural transition from the GDP- to the GTP-bound form. Moreover, the distance between Cys-81 and Gly-94, an invariant residue downstream of helix B, changes dramatically during this transition (25). In the GTP-bound form, side chains around Cys-81 are tightly packed, and the distance between Cys-81 and Gly-94 is as short as 4.1 Å. By contrast, in the GDP-bound form the distance increases to 10.2 Å, and Tyr-87 moves to the position that was occupied by Cys-81 in the GTP-bound form (16, 37). In addition, the orientation of helix B in the GTP-bound form is shifted in the GDP-bound form by 42 Å (37, 38). Thus, the environment around Cys-81 changes drastically during the transition. Taking the crystallographic data into account, we postulate that during the transition from the GDP- to the GTP-bound form, a change in the electrostatic interactions of side chains around Cys-82 might occur, increasing the reactivity of Cys-82 such that it can easily be oxidized by H₂O₂ in EF-Tu of Synechocystis rather than a change that facilitates the spatial accessibility of H₂O₂ to Cys-82.

Formation of Large Complexes of Oxidized Nucleotide-free EF-Tu—In our analysis of nucleotide-free EF-Tu, we found that the reduced form was present only as a dimer under native conditions (Fig. 6). It is possible that nucleotide-free EF-Tu forms a dimer easily via non-covalent bonding, such as hydrophobic interactions and not via formation of a disulfide bond. We found that some oxidized nucleotide-free EF-Tu existed as large complexes of >30 molecules. Given that in preparations of oxidized nucleotide-free EF-Tu, Cys-82 in its reduced state was more abundant than Cys-82 in its oxidized state in the presence of 1 mM H₂O₂ (Fig. 2), we postulate that the mechanism for formation of the large complexes is as follows. In the absence of nucleotides, EF-Tu exists as dimers in which two Cys-82 are located in close proximity to one another. The addition of H₂O₂ promotes the formation of an intermolecular disulfide bond via Cys-82 between the two molecules. The resulting conformational change endows oxidized dimers with adhesive properties such that a core develops to trigger the aggregation of many reduced molecules, presumably due to exposure of hydrophobic surfaces. Earlier studies demonstrated that nucleotide-free EF-Tu of E. coli and Thermus thermophilus are extremely unstable: both are rapidly inactivated at moderately high temperatures such as 30 °C (39, 40). The particular instability of nucleotide-free EF-Tu might be explained in part by its strong sensitivity to oxidation and the subsequent formation of the large complexes that we observed. Our high speed atomic force microscopy observations revealed the globular structure of the large complexes and confirmed that each large complex of oxidized nucleotide-free EF-Tu consists of many small aggregates that adhere to one another (Fig. 7). The addition of DTT to a preparation of oxidized nucleotide-free EF-Tu resulted in the dissociation of large complexes into many smaller ones, suggesting that when the oxidized core is reduced, interactions between reduced forms of EF-Tu might disappear immediately. However, it remains to be determined whether large aggregates of nucleotide-free EF-Tu exist in vivo.

Physiological Implications of the Redox Sensitivity of EF-Tu—Photosynthetic organisms utilize captured light not only as a source of chemical energy that drives metabolism but also as a source of reducing agents that regulates the expression and activity of various proteins in response to changes in light conditions (29, 41). Thioredoxins, small multifunctional redox proteins, play important roles in the thiol-based redox regulation of target proteins through the reduction of specific disulfide bonds, thereby modulating the activity of these proteins (41, 42). In chloroplasts and cyanobacteria, thioredoxins receive reducing equivalents from the photosynthetic transport of electrons via ferredoxin-thioredoxin reductase (24, 29, 41). Thioredoxin-affinity chromatography captured a number of putative targets of thioredoxin (42–45), and the EF-Tu proteins in Synechocystis (43), spinach chloroplasts (44), and Chlamydomonas reinhardtii (45) were identified as candidate targets of thioredoxin. In the present study we demonstrated for the first time to our knowledge that EF-Tu is an actual and functional target of thioredoxin in Synechocystis.

The activation of EF-Tu by the reduced form of thioredoxin-µ suggests that the translational activity of EF-Tu might be regulated by the reducing power that is generated by the photosynthetic transport of electrons and mediated by thioredoxin. Our analysis in vivo supported the hypothesis that the reducing power is transmitted to EF-Tu via thioredoxin (Fig. 8). The resulting reduction of EF-Tu might activate the synthesis de novo of proteins, such as the D1 protein, that are essential for the repair of PSII. However, under strong light, accelerated production of ROS might result in the oxidation of EF-Tu in addition to the oxidation of EF-G in Synechocystis, suppressing protein synthesis (4, 9, 10). Under such 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, as a consequence, the photosynthetic transfer of electrons, producing excess ROS. Thus, the particular sensitivity of Cys-82 of EF-Tu to oxidation might act as a transient regulator to suppress both protein synthesis and photosynthesis. Future studies should be directed toward clarification of the molecular mechanism by which the oxidation of EF-Tu inhibits the function of this elongation factor on the ribosome as well as the physiological significance of Cys-82 in EF-Tu in the response of photosynthesis to environmental stress.
Author Contributions—R. Y. and Y. N. designed the study and wrote the paper. T. N. and K. Y. performed mass spectrometry. H. J. performed HPLC analysis of nucleotides. Y. H. and T. Hisabori contributed to redox analysis and discussion. T. K. and T. U. prepared the PURE system. T. Haruyama and H. K. performed AFM analysis. All authors analyzed the results and approved the final version of the manuscript.

Acknowledgments—We thank Saki Inaizumi, Hirokazu Nagao, Tomohisa Niimi, and Yoichi Tsumuraya (Saitama University) for skilled technical assistance and Takayuki Shimizu and Shinji Masuda (Tokyo Institute of Technology) for providing CrtJ.

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