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Oxidation of translation factor EF-G transiently retards the translational elongation cycle in *Escherichia coli*

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In *Escherichia coli*, elongation factor G (EF-G), a key protein in translational elongation, is particularly susceptible to oxidation. We demonstrated previously that EF-G is inactivated upon formation of an intramolecular disulphide bond. However, the details of the mechanism by which the oxidation of EF-G inhibits the function of EF-G on the ribosome remain to be elucidated. When we oxidized EF-G with hydrogen peroxide, neither the insertion of EF-G into the ribosome nor single-cycle translocation activity *in vitro* was affected. However, the GTPase activity and the dissociation of EF-G from the ribosome were suppressed when EF-G was oxidized. The synthesis of longer peptides was suppressed to a greater extent than that of a shorter peptide when EF-G was oxidized. Thus, the formation of the disulphide bond in EF-G might interfere with the hydrolysis of GTP that is coupled with dissociation of EF-G from the ribosome and might thereby retard the turnover of EF-G within the translational machinery. When we added thioredoxin to the suppressed translation system that included oxidized EF-G, translational activity was almost immediately restored. We propose that oxidation of EF-G might provide a regulatory mechanism for transient and reversible suppression of translation in *E. coli* under oxidative stress.

Keywords: EF-G/GTP hydrolysis/oxidative stress/protein synthesis/redox regulation.

Abbreviations: DHFR, dihydrofolic acid reductase; DTT, dithiothreitol; EF-G, elongation factor G; GDPNP, 5'-guanyl-imidodiphosphate; ROS, reactive oxygen species.

The sensitivity of protein synthesis to oxidative stress has been observed in both prokaryotes and eukaryotes and the mechanism of oxidative damage to the protein-synthetic machinery has been studied extensively (*1*–*3*). Elongation factor G (EF-G), a key protein in translational elongation, is one of the proteins that are strongly oxidized in cells of *Escherichia coli* and *Saccharomyces cerevisiae* that have been exposed to H_2O_2 (*4*, *5*) and in mutant cells of *E. coli* that lack a superoxide dismutase (*6*). Moreover, elongation factor 2, the eukaryotic counterpart of EF-G, is susceptible to oxidation in rabbit (*2*) and rat (*7*) tissues. However, neither the mechanism of the oxidation of the specific translation factors nor the effects of their oxidation on translation have been fully clarified.

Studies with a translation system *in vitro*, derived from the cyanobacterium *Synechocystis* sp. strain PCC 6803, revealed that oxidation of EF-G of *Synechocystis* was critical to the inactivation of protein synthesis (*8*). In this system, the oxidation of EF-G was attributable to the formation of an intramolecular disulphide bond between two specific cysteine residues, Cys105 and Cys242 (*9*). A similar study *in vitro* with a translation system from *E. coli* revealed that EF-G of *E. coli* was also inactivated via the formation of an intramolecular disulphide bond between two specific cysteine residues, Cys114 and Cys266 (*10*). Therefore, we postulated that the specific oxidation of EF-G might provide a general explanation for the oxidative inhibition of protein synthesis in several organisms. However, details of the mechanism by which the oxidation of EF-G inhibits the function of EF-G within the ribosome remained to be elucidated.

A clue to the mechanism was provided by studies of the oxidative damage to EF-G of *E. coli*. Oxidized EF-G was reactivated by the reduction of the disulphide bond by thioredoxin, a ubiquitous protein that mediates dithiol-disulphide exchange (*10*). Such reversibility of oxidative damage suggested that inactivation of protein synthesis via the oxidation of EF-G might be transient rather than permanent. Furthermore, the GTPase activity of EF-G remained at 50% of the control level when translational activity was completely absent under severe oxidizing conditions (*10*), suggesting that the oxidation of EF-G might not completely inhibit the function of EF-G in the ribosome. Taken together, the observations suggest that the oxidation of EF-G might mediate the partial and transient inhibition of the elongation reaction in translation.

During protein synthesis, EF-G binds GTP and is inserted into the A site of the ribosome. Then EF-G drives translocation of peptidyl tRNA from the A site

to the P site. Upon hydrolysis of GTP that is bound to EF-G and subsequent conformational change in EF-G, EF-G dissociates from the ribosome (*11*). In this study, we investigated the effects of the oxidation of EF-G on these various steps. We found that the hydrolysis of GTP and the subsequent dissociation of EF-G from the ribosome were retarded when EF-G was oxidized but the other steps were unaffected. In addition, thioredoxin was able to restore translation to the initial rate via reduction of the oxidized EF-G that had dissociated from the ribosome. Our observations suggest the existence of a previously unrecognized regulatory mechanism that transiently suppresses the translational machinery in a redox-dependent manner.

Materials and Methods

Preparation of recombinant EF-G

Recombinant wild-type and mutated EF-G with a histidine tag at the carboxyl terminus were prepared, in reduced form, as described previously (*10*). The proteins were stored at 4°C in buffer that contained 20 mM HEPES-KOH (pH 7.5), 50 mM NaCl and 20% (w/v) glycerol.

Oxidation and reduction of EF-G

Recombinant EF-G proteins, at a final concentration of 2 μM, were incubated with 0.5 mM H_2O_2 or 1 mM dithiothreitol (DTT) at 4°C for 5 min in buffer that contained 50 mM HEPES-KOH (pH 7.5). These treatments are able to fully oxidize or reduce EF-G (*10*). Residual H_2O_2 and DTT were removed, respectively, by the addition of catalase to the reaction mixture and by passing the proteins through a desalting column (PD Spin Trap G-25; GE Healthcare, Buckinghamshire, UK), as described previously (*10*).

Translation in vitro

Translation *in vitro* was preformed with the PURE system, a reconstituted translation system derived from *E. coli* (*12*). The translation system was generated, in the absence of EF-G and reducing reagents, by mixing 70S ribosomes with the individual components that are required for translation *in vitro*, as described previously (*10*). After the treatment of EF-G with an oxidizing or a reducing reagent, EF-G was added to the translation system that had been prepared without EF-G. Each resultant translation system was incubated at 37°C in the presence of mRNA that encoded dihydrofolic acid reductase (DHFR) as template, ^{35}S-labelled cysteine/methionine and the reagents required for translation, as described previously (*10*).

Measurement of GTPase activity

The GTPase activity of EF-G was measured by monitoring the release of inorganic phosphate from GTP by an end-point assay with BIOMOL GREEN (Enzo Life Sciences, Farmingdale, NY, USA). The reaction mixture contained 30 nM 70S ribosomes from *E. coli*, 200 μM GTP and 30 nM EF-G. Changes in absorbance at 610 nm were measured spectroscopically, as described previously (*10*).

Interaction between EF-G and ribosomes

EF-G at 1 μM, after treatment with 1 mM DTT or 0.5 mM H_2O_2, was incubated with 1 μM 70S ribosomes at 37°C. The mixture was then separated into free EF-G and ribosome-EF-G complexes with a centrifugal filter (pore cut-off size, 100 kDa; Merck Millipore, Darmstadt, Germany) at 18,000 *g* for 3 min at 4°C. EF-G in the supernatant (ribosome-EF-G complexes) and in the filtrate (free EF-G) was quantified by western blotting analysis with EF-G-specific antibodies (*10*). To examine the insertion of EF-G into ribosomes, we incubated EF-G with 2 mM GTP plus 2 mM fusidic acid or with 2 mM GDPNP at 4°C for 5 min after the prior reduction or oxidation of EF-G.

Measurement of translocation activity

Single-cycle translocation was performed as described by Tsuboi *et al.* (*13*). A peptide (MFV)-encoding mRNA (5′-GGGAAAAGA AAAGAAAAGAAA-AUG-UUC-GUU-AAAAGAAAAGAAAA GAAAAUAUUGAAUU-3′), which includes codons encoding Met-Phe-Val, was prepared as described previously (*13*). The pre-translocation (PRE-state) complexes were generated in reaction buffer that contained 2.5 pmol 70S ribosomes, 25 pmol MFV-mRNA and 10 pmol ^{32}P-labelled deacylated fMet-tRNA$_f^{Met}$. The reaction mixture was incubated for 15 min at 37°C with 5 pmol *N*-acetyl-[^{3}H]Phe-tRNA^{Phe} to ensure that this tRNA occupied the A site. Single-cycle translocation was allowed to proceed by incubation of PRE-state complexes at 37°C for 10 min with 2 μM EF-G and 0.1 mM GTP. The resultant post-translocation (POST-state) complexes were then supplemented with 1 mM puromycin. After incubation at 37°C for 15 min, the reaction was stopped by the addition of an equal volume of 0.3 M sodium acetate (pH 5.5). The *N*-acetyl-[^{3}H]Phe-puromycin complexes were extracted with ethyl acetate, and radioactivity in the organic phase was measured by liquid scintillation counting. The amount of [^{32}P]fMet-tRNA$_f^{Met}$ that had been bound to the P site of the PRE-state complexes was also monitored by fixing the complexes on a nitrocellulose filter and measuring radioactivity with liquid scintillation. The amount of the puromycin product was determined as a ratio, relative to the amount of [^{32}P]fMet-tRNA$_f^{Met}$ that had been bound to the P site of the PRE-state complexes. To confirm the accuracy of calculations, the amounts of [^{32}P]fMet-tRNA$_f^{Met}$ and *N*-acetyl-[^{3}H]Phe-tRNA^{Phe} that remained, respectively, at the E and P sites of POST-state complexes were also monitored by fixing the complexes on nitrocellulose filters and measuring radioactivity with liquid scintillation.

Synthesis of short peptides in vitro

We constructed three DNA fragments, namely, ATGTTGTTC, AT GTTCTTCTTGTTC and ATGTTCTTCTTGTTCTTCTTCTTCT TGTTC, which encode the short peptides MLF, MFFLF and MFFLFFFFLF, respectively. We inserted the DNA fragments, separately, into the pET21b vector (Merck Millipore) and individual mRNAs were synthesized by T7 RNA polymerase. The corresponding peptides were synthesized at 37°C with the individual mRNAs as template in the PURE system, as described above. The peptides were extracted with ethyl acetate and the amount of each was measured by liquid scintillation counting.

Results

Effects of oxidation of EF-G on translational activity

We examined the effects of H_2O_2 on the translational activity of EF-G using the PURE system (*12*), a system for translation *in vitro* derived from *E. coli* and generated by mixing the individual components required for translation. Addition of the reduced form of EF-G to the translation system prepared without EF-G resulted in time-dependent synthesis of DHFR (Fig. 1a). However, when EF-G had been treated with 0.5 mM H_2O_2 for 5 min to oxidize EF-G fully, prior to its addition to the translation system, the synthesis of DHFR was completely inhibited (Fig. 1a). In a previous study, we showed that oxidative damage to EF-G involves the oxidation of two specific cysteine residues of EF-G, namely, Cys114 and Cys266, and subsequent formation of an intramolecular disulphide bond, which result in the inactivation of EF-G (*10*). In this study, replacement of Cys114 by serine allowed H_2O_2-treated EF-G to support translation at the same rate as DTT-treated EF-G (Fig. 1b). As shown also in our previous study, when we incubated reduced EF-G with ribosomes in the presence of GTP, GTP was hydrolyzed (Fig. 1c). However, when we oxidized EF-G by treating it with 0.5 mM H_2O_2, the GTPase activity fell by 50%, indicating that the oxidation of EF-G had partially inhibited its GTPase activity. Addition of 2 mM fusidic acid, a specific inhibitor of the GTPase activity of EF-G, to reaction mixtures that contained either the

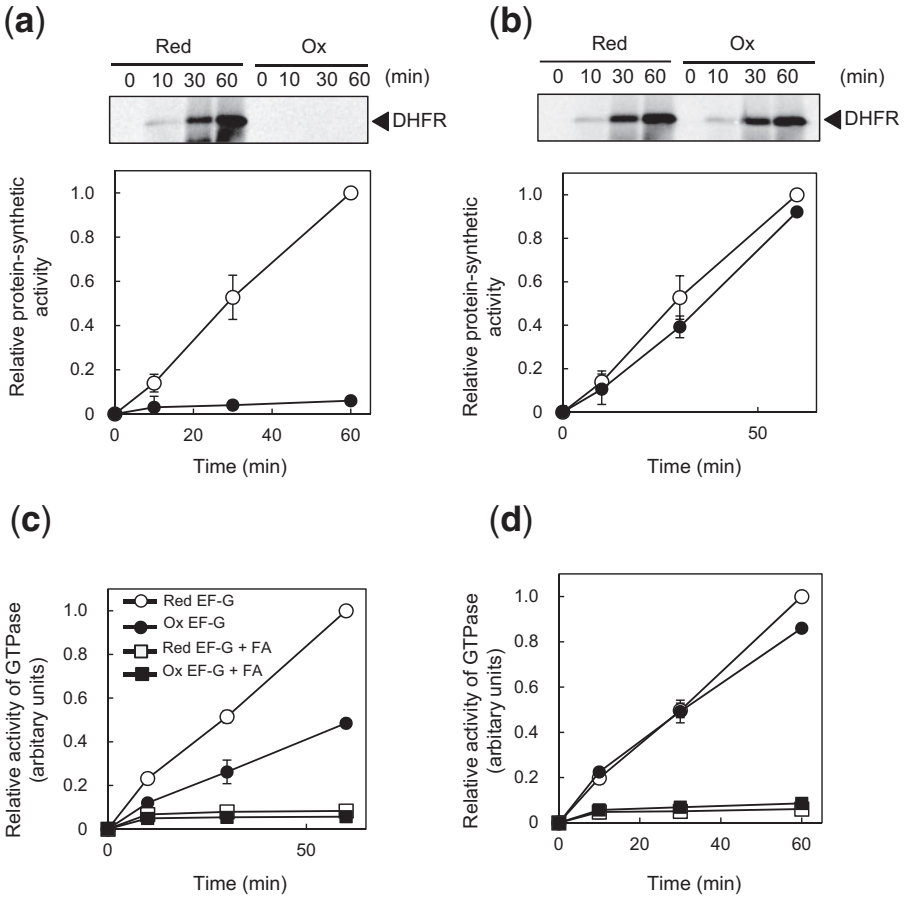

Fig. 1 Effects of H_2O_2 on the translational activity and GTP-hydrolytic activity of wild-type EF-G and its mutant derivative. The translational activity of wild-type EF-G (**a**) and its mutant derivative C114S (**b**) and the GTPase activity of wild-type EF-G (**c**) and the mutant (**d**) are shown. EF-G (2 μM) was treated with 1 mM DTT (Red; open circles) and 0.5 mM H_2O_2 (Ox; closed circles) for 5 min. After residual DTT and H_2O_2 had been eliminated, EF-G was added to the translation system, which had been prepared without EF-G. The reaction mixtures were then incubated at 37°C for the indicated times in the presence of template mRNA that encodes DHFR, and proteins were then fractionated by SDS-PAGE. The ^{35}S-labelled DHFR was visualized by autoradiography. The quantitation of radioactivity from ^{35}S-labelled DHFR is also shown in panels (**a**) and (**b**). GTPase activity was determined by an end-point assay with BIOMOL GREEN. After EF-G had been treated with 5 mM DTT (open circles), 0.5 mM H_2O_2 (closed circles), 5 mM DTT plus 2 mM fusidic acid (FA; open squares) and 0.5 mM H_2O_2 plus 2 mM fusidic acid (closed squares), it was incubated with 70S ribosomes in the presence of 200 μM GTP. Values are mean ± S.D. (bars) of results from three independent experiments.

reduced or the oxidized EF-G almost completely inhibited the GTPase activity, confirming the accuracy of the assay of GTPase activity (Fig. 1c). By contrast, the mutant protein was able to hydrolyze GTP as rapidly as the reduced mutant protein, even when the mutant protein had been treated with 0.5 mM H_2O_2 (Fig. 1d), suggesting that the redox state of Cys114 might be critical for the GTPase activity. Addition of fusidic acid to the mutant protein also inhibited the GTPase activity.

We next examined whether oxidized EF-G might affect the function of reduced EF-G. Figure 2 shows translational activity at various molecular ratios of reduced EF-G to ribosomes. The translational activity was not significantly affected until the ratio of reduced EF-G to ribosomes decreased to 0.5:1 (Fig. 2a and c). However, when oxidized EF-G was added to a translation system that contained reduced EF-G, translational activity was suppressed (Fig. 2b and c). Even when reduced EF-G was present at a molar ratio relative to ribosomes of 0.5:1, the presence of an amount of oxidized EF-G equal to the amount of reduced EF-G decreased the translational activity to 25% of the original rate, suggesting that oxidized EF-G might interfere with the function of reduced EF-G, acting as a competitive inhibitor. In other words, oxidized EF-G might act to impair the activity of functional EF-G that turns over within the ribosome.

Effects of oxidation of EF-G on its interaction with the ribosome

We investigated the interaction between EF-G and the ribosome. After 1 μM EF-G that had been reduced by 1 mM DTT or oxidized by 0.5 mM H_2O_2, it was incubated for designated times with 1 μM ribosomes in the presence of 1 mM GTP. Aliquots of each mixture were then separated into fractions that contained free EF-G and ribosome-EF-G complexes with centrifugal filters with a pore cut-off size of 100 kDa. With increases in incubation time, the amount of reduced EF-G fell dramatically in the supernatant, which contained ribosome-EF-G complexes, but increased in the filtrate,

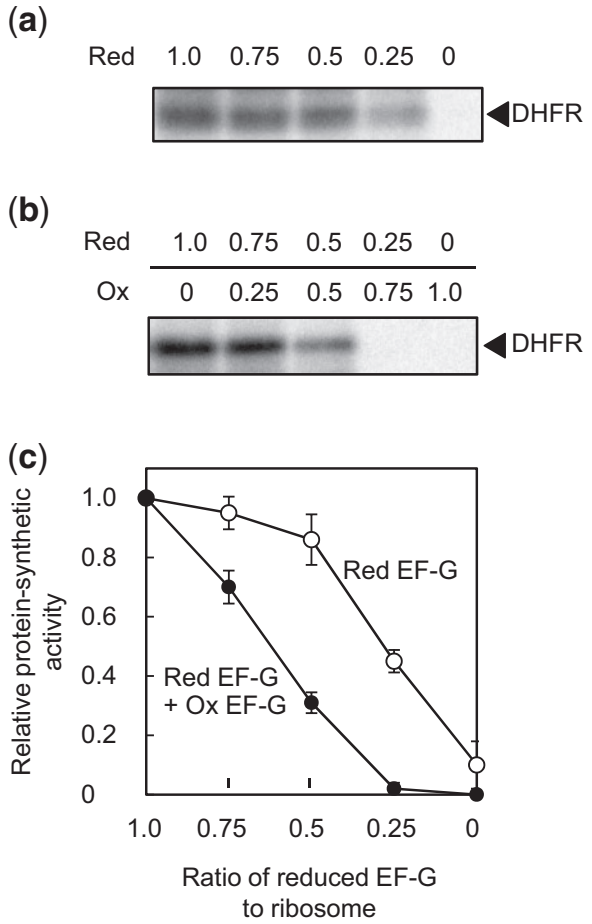

Fig. 2 Effects of oxidized EF-G on the activity of reduced EF-G. (**a**) Wild-type EF-G (1 μM) that had been treated with 1 mM DTT (Red) was mixed with the translation system that lacked EF-G, at the indicated molecular ratios of EF-G to ribosomes, and with mRNA that encodes DHFR as template. After incubation at 37°C for 60 min, proteins were fractionated by SDS-PAGE and DHFR was visualized by autoradiography. (**b**) EF-G that had been treated 0.5 mM H_2O_2 (Ox) was combined with the reduced EF-G at the indicated molecular ratios to ribosomes and then each mixture was added to a translation system under the same conditions as in (**a**). (**c**) The quantitation of translational activity in panels (**a**) and (**b**). Open circles, Translational activity with reduced EF-G; closed circles, translational activity with reduced and oxidized EF-G. Values are mean ± S.D. (bars) of results from three independent experiments.

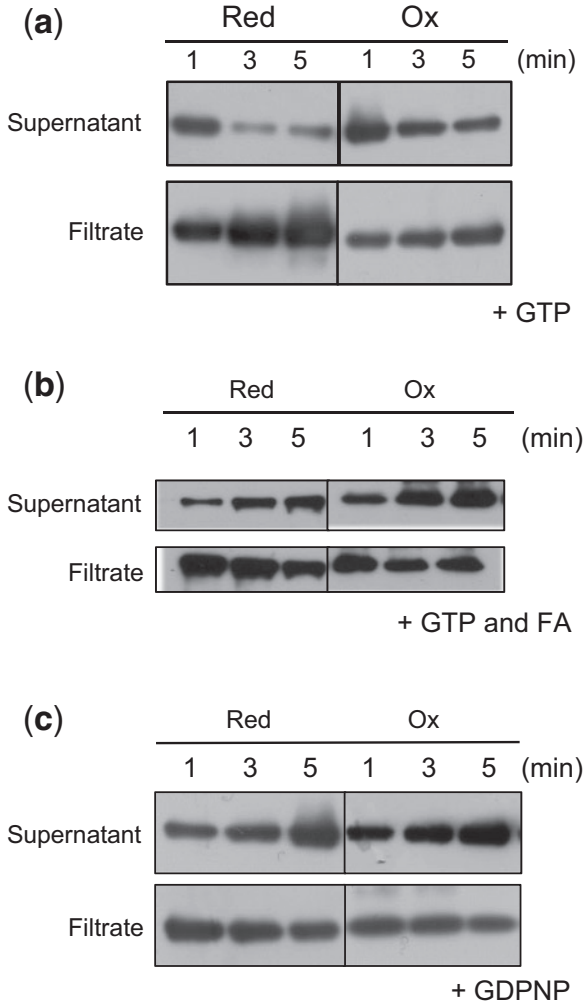

Fig. 3 Effects of H_2O_2 on the interaction between EF-G and ribosomes. (**a**) Wild-type EF-G (1 μM) was treated with 1 mM DTT (Red) or 0.5 mM H_2O_2 (Ox) and then incubated with 70S ribosomes (1 μM) in the presence of 2 mM GTP for the indicated times. Each reaction mixture was separated into supernatant and filtrate with a centrifugal filter. After both fractions had been subjected to SDS-PAGE, EF-G was visualized by western blotting analysis with EF-G-specific antibodies. (**b, c**) Insertion of EF-G into the ribosome. EF-G was incubated with 70S ribosomes in the presence of 2 mM GTP plus 2 mM fusidic acid (**b**) or of 2 mM GDPNP (**c**). Reaction mixtures were fractionated with the centrifugal filters as described above.

which contained free EF-G, suggesting that EF-G might be inserted into and dissociated from ribosomes (Fig. 3a). By contrast, the amounts of oxidized EF-G did not change significantly in either the supernatant or the filtrate, suggesting that the oxidized EF-G might not be similarly inserted into or dissociated from ribosomes.

In order to investigate the insertion of EF-G into the ribosome, we incubated EF-G with ribosomes in the presence of GTP and fusidic acid or of 5′-guanyl-imidodiphospate (GDPNP), a non-hydrolysable analogue of GTP. These treatments enabled us to monitor the insertion step exclusively by fixing EF-G in the GTP-binding form without any accompanying hydrolysis of GTP. In the presence of either fusidic acid or GDPNP, amounts of reduced EF-G increased in the supernatant and decreased in the filtrate with increases in incubation time, indicating that EF-G might be properly inserted into the ribosome but unable to dissociate from it (Fig. 3b and c). The profiles for oxidized EF-G were the same as those for reduced EF-G, suggesting that oxidation of EF-G did not affect the insertion of EF-G into the ribosome. Thus, the results in Fig. 3a indicate that oxidation of EF-G might interfere with the dissociation of EF-G from the ribosome, which is coupled with hydrolysis of GTP.

Effects of oxidation of EF-G on the translocation of tRNA

Prior to the hydrolysis of GTP, EF-G assists in the translocation of peptidyl tRNA from the A site to the P site on the ribosome. Therefore, we examined

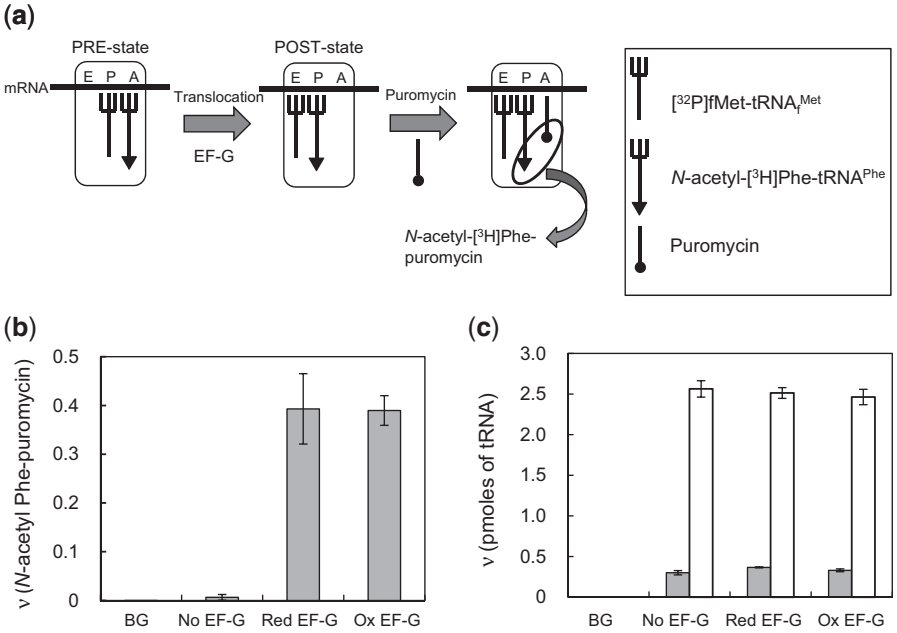

Fig. 4 Effects of the oxidation of EF-G on the translocation of tRNA. (**a**) A schematic representation of the single-cycle translocation assay (for details, see Materials and Methods). (**b**) The amounts of the *N*-acetyl-[^{3}H]Phe-puromycin complex after single-cycle translocation with EF-G that had been treated with 1 mM DTT (Red) or 0.5 mM H_2O_2 (Ox). The values of ν indicate pmoles of *N*-acetyl-[^{3}H]Phe-puromycin formed per pmole of ribosomes. (**c**) The amounts of tRNA bound to POST-state complexes. The values of ν indicate pmoles of *N*-acetyl-[^{3}H]Phe-tRNA^{Phe} (grey bars) and [^{32}P]fMet-tRNA$_f^{Met}$ (white bars) bound to 2.5 pmoles of ribosomes at the P and E sites, respectively. Values are mean ± S.D. (bars) of results from three independent experiments. BG, Background ν values obtained without puromycin in the reactions.

whether oxidation of EF-G might affect the translocation of tRNA. Figure 4a shows a schematic representation of the assay for a single-cycle translocation (*13*). Pre-translocation (PRE-state) complexes were generated with 70S ribosomes that carried [^{32}P]fMet-tRNA$_f^{Met}$ at the P site and *N*-acetyl-[^{3}H]Phe-tRNA^{Phe} at the A site, and EF-G was then added to drive the translocation reaction. The tRNAs at the P and A sites and the mRNA that includes only one codon for Phe-tRNA (UUC) ensure that only a single translocation reaction will occur on each ribosome. We determined the amount of the resulting post-translocation (POST-state) complexes using puromycin, which enters the A site and combines with the aminoacyl residue of the tRNA located at the P site. After reactions with either reduced EF-G or oxidized EF-G, the amounts of *N*-acetyl-[^{3}H]Phe-puromycin complexes were identical (Fig. 4b). In other words, the rates of single-cycle translocation were the same between reduced and oxidized EF-G. There were also no differences in the respective amounts of [^{32}P]fMet-tRNA$_f^{Met}$ and *N*-acetyl-[^{3}H]Phe-tRNA^{Phe} that remained in POST-state complexes regardless of the redox state of EF-G, confirming that all the reactions had occurred appropriately (Fig. 4c). These observations suggested that oxidation of EF-G might not affect the translocation of tRNA.

Effects of oxidation of EF-G on the synthesis of short peptides

We examined whether the oxidation of EF-G might affect the rate of synthesis of short peptides in our translation system. We prepared mRNAs that encoded peptides of three, five and 10 amino residues, respectively. In the presence of oxidized EF-G, the rate of synthesis of the three-amino-acid peptide was ~50% of that in the presence of reduced EF-G (Fig. 5a). However, when the length of the encoded peptide was increased to five and 10 amino acid residues, the synthesis of each peptide was inhibited to a greater extent in the presence of oxidized EF-G (Fig. 5b and c). Thus, partial suppression of each round of elongation appeared to have greater impact on the synthesis of longer peptides. Such suppression might be sufficient to inhibit the synthesis of entire proteins, such as DHFR.

Reactivation of the suppressed translation system by thioredoxin

In our previous study, we showed that oxidized EF-G is reactivated upon the reduction of the intramolecular disulphide bond by thioredoxin, which is a ubiquitous protein that regulates the activity of target proteins by reducing disulphide bonds (*10*). In that study, however, thioredoxin was directly added to oxidized EF-G prior to the assay of translation (*10*) and, therefore, it remained unclear whether thioredoxin might be able to reactivate a translation system that had been suppressed by oxidized EF-G. We prepared translation systems with either reduced or oxidized EF-G, as described above (Fig. 1), and examined the effects of thioredoxin on the synthesis of DHFR by monitoring the incorporation of ^{35}S-labelled cysteine plus methionine. The translation system prepared with oxidized EF-G remained inactive and no synthesis of DHFR was detected (Fig. 6a). However, when thioredoxin

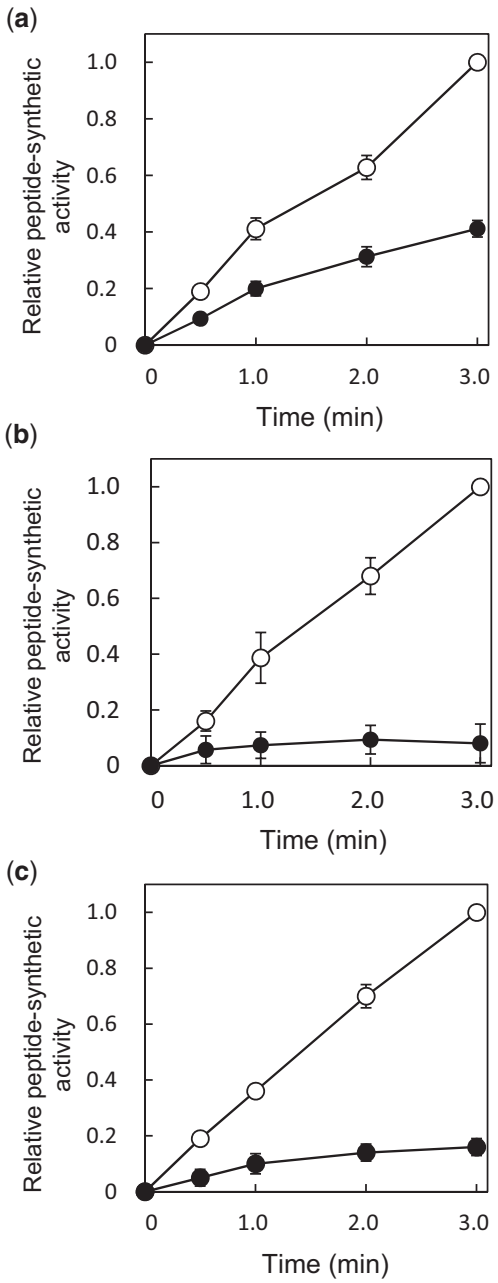

Fig. 5 Effects of the oxidation of EF-G on the synthesis of short peptides. Wild-type EF-G (2 μM) that had been treated with 1 mM DTT (open circles) and 0.5 mM H_2O_2 (closed circles) was used to synthesize three short peptides, namely, MLF (**a**), MFFLF (**b**) and MFFLFFFFLF (**c**), in translation systems that had been prepared without EF-G. Translational activity was quantitated by liquid scintillation counting of ^{35}S-labelled peptides. Values are mean ± S.D. (bars) of results from three independent experiments.

was added to the suppressed translation system, 10 min after the onset of incubation of the translation system, the synthesis of DHFR resumed almost immediately (Fig. 6a). Next, we investigated the recovery of protein synthesis in a very short time and compared it in protein synthesis with the translation system that had been supplemented with reduced EF-G. We found that, initially, e.g. at 0.5 and 1 min, the synthesis of DHFR was slower than that in the presence of reduced EF-G (Fig. 6b). Thus, the recovery of protein synthesis appeared to be delayed, with a brief lag period. These observations suggest that thioredoxin might have reduced the oxidized EF-G that had dissociated from ribosomes. It seems, therefore, that even oxidized EF-G might turn over on the ribosome.

Discussion

Oxidation of EF-G suppresses its GTPase activity and its dissociation from the ribosome

In our previous study, we showed that inactivation of EF-G is attributable to the oxidation of two specific cysteine residues, namely, Cys114 and Cys266, and the subsequent formation of an intramolecular disulphide bond (*10*). In this study, we attempted to determine how the oxidation of EF-G affects the activity of EF-G within the ribosome. Neither the insertion of EF-G into the ribosome nor the translocation of tRNA from the A site to the P site in the ribosome was affected by the oxidation of EF-G. In addition, our previous study showed that the GTP-binding ability of EF-G is also unaffected by the oxidation of EF-G (*10*). However, hydrolysis of GTP by EF-G and subsequent dissociation of EF-G from the ribosome was partially suppressed by oxidation. Taken together, our past and present observations suggest that the oxidation of EF-G might not affect the function of EF-G prior to the hydrolysis of GTP.

Two major hypotheses have been proposed with respect to the order of actions of EF-G within the ribosome; in one, the translocation of tRNA precedes the hydrolysis of GTP (*11*, *14*); and in the other, the hydrolysis of GTP precedes the translocation of tRNA (*15–17*). The former hypothesis suggests that the translocation of tRNA occurs spontaneously in the presence of EF-G, in the absence of energy derived from the hydrolysis of GTP, while the latter suggests that the hydrolysis of GTP is the driving force for the translocation of tRNA. Our observations are in accordance with the former hypothesis since oxidation of EF-G did not affect the translocation of tRNA but did affect the hydrolysis of GTP.

The target cysteine residues Cys114 and Cys266 are located in the GTPase domain of EF-G (*18*) but are far from domain IV, which plays a role in catalyzing the translocation of tRNA via association with the A site on the ribosome (*16*). The location of the target cysteine residues might explain why the oxidation of EF-G affected neither the insertion of EF-G into the ribosome nor the single-cycle translocation but did suppress both the GTPase activity and the dissociation of EF-G from the ribosome.

Mechanism of the inhibition of translation by oxidation of EF-G

Oxidation of EF-G partially suppressed the GTPase activity of EF-G and, also, the dissociation of EF-G from the ribosome during single-cycle translocation. However, oxidation of EF-G completely inhibited the synthesis of DHFR, a protein of 165 amino acid residues. In our analysis of the synthesis of short peptides, the oxidation of EF-G partially inhibited the synthesis of a three-amino-acid peptide but markedly inhibited

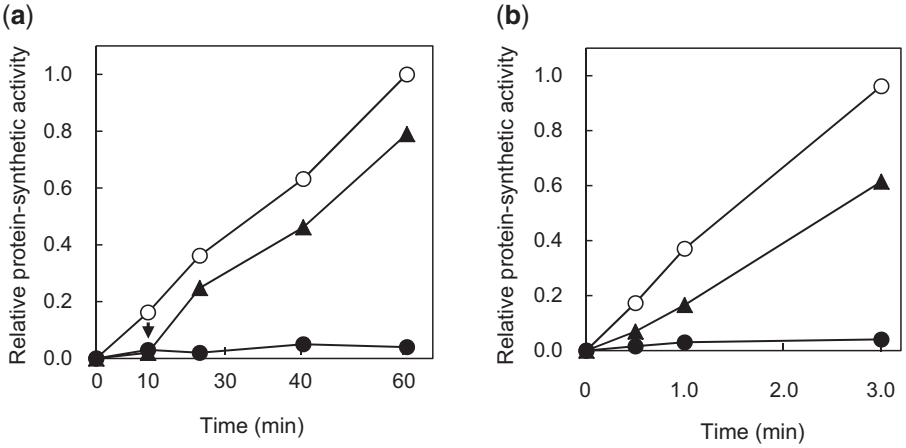

Fig. 6 Effects of thioredoxin on the translation system prepared with oxidized EF-G. (**a**) Restoration of translational activity by thioredoxin. Wild-type EF-G (2 μM) that had been treated with 1 mM DTT (open circles) and 0.5 mM H_2O_2 (closed circles) was added to the translation system prepared without EF-G with DHFR mRNA as template. The translation system that had contained oxidized EF-G was supplemented with 4 μM thioredoxin (Trx) after an initial 10-min incubation (arrow), and translational activity was monitored for indicated times (closed triangles). Translational activity was monitored by quantitating the synthesis of ^{35}S-labelled DHFR by SDS-PAGE and autoradiography, as indicated in the legend to Fig. 1. (**b**) Translational activity over a 3-min period was quantitated by liquid scintillation counting of ^{35}S-labelled DHFR (see text for details). Thioredoxin was added at zero time (at the onset of translation). Values are mean of results from three independent experiments.

the synthesis of five- and 10-amino-acid peptides (Fig. 5). These observations suggest that a single cycle of translational elongation might be partially suppressed by oxidized EF-G, while multiple cycles of elongation might be cumulatively suppressed with increases in the number of cycles. In other words, oxidation of EF-G slows down individual cycles of elongation and, after several cycles, the synthesis of longer peptides and proteins ceases completely.

Thioredoxin was able to reverse the inhibition of protein synthesis almost immediately when it was added to a translation system prepared with oxidized EF-G (Fig. 6a). Cryo-electron microscopy of the ribosome-EF-G complex has revealed that EF-G is deeply buried in the A site of the ribosome (*19*), so it seems unlikely that thioredoxin reduces oxidized EF-G directly within the ribosome. Presumably, oxidized EF-G might turn over slowly within the ribosome, with a lowered rate of dissociation from the ribosome, and thioredoxin might reduce oxidized EF-G that has dissociated from the ribosome. Indeed, the recovery of protein synthesis was delayed with a brief lag period after thioredoxin had been added to the translation system (Fig. 6b). Once oxidized EF-G has been reduced, the elongation cycle can be recovered completely.

A novel mechanism for the regulation of translation

The inhibition of translation via the oxidation of EF-G is a very different phenomenon from inhibition by EF-G-specific antibiotics, such as fusidic acid, which inhibit translation permanently. The transient and reversible inhibition of translation via the oxidation of EF-G might be a mechanism for regulation of protein synthesis under oxidative stress. In the cyanobacterium *Synechocystis* sp. strain PCC 6803, a photosynthetic prokaryote, it has been proposed that inhibition of translation by oxidation of EF-G might be a regulatory mechanism that suppresses protein synthesis under photo-oxidative stress (*20*). When reactive oxygen species (ROS) are abundantly produced by the photosynthetic machinery under strong light, EF-G is specifically oxidized and protein synthesis is suppressed (*8*, *9*). Such suppression of protein synthesis inhibits the repair of photosystem II, preventing further oxidative stress due to photosynthetic reactions, and it can be reversed by the action of thioredoxin under weak light (*20*, *21*). Similarly, in *E. coli*, the oxidation of EF-G might act as an emergency brake for the rapid suppression of protein synthesis in order to limit oxidative stress that might result from an insufficient supply of reducing power and elevated levels of ROS, which originate from respiration, and under stress conditions, such as nutrient starvation. When such unfavourable conditions are eliminated, thioredoxin might immediately restore the cell's capacity for protein synthesis.

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Conflict of Interest

None declared.

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