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Protein–Disulfide Isomerase Activity of Elongation Factor EF-Tu

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EF-Tu is involved in the binding and transport of the appropriate codon-specified aminoacyl-tRNA to the aminoacyl site of the ribosome. We and others have recently shown that the *Escherichia coli* EF-Tu, in additon to its acknowledged role in translation elongation, displays chaperone-like properties. We report here that EF-Tu, like thioredoxin, protein disulfide isomerase, and DsbA, catalyzes protein disulfide formation (oxidative renaturation of reduced RNase), reduction (reduction of insulin disulfides), and isomerization (refolding of randomly oxidized RNase). In contrast with most protein disulfide isomerases which possess vicinal cysteines and form an intramolecular disulfide upon oxidation, EF-Tu, which does not possess vicinal cysteines, forms intermolecular disulfides upon oxidation, resulting in the appearance of multimeric forms.

Key Words: **elongation factor Tu; protein-disulfide isomerase; oxido-reduction.**

EF-Tu is responsible for binding and transporting the appropriate codon-specified aminoacyl-tRNA to the aminoacyl site of the ribosome (1, 2). It interacts with GTP, aminoacyl-tRNA, ribosomes, and a second protein factor, EF-Ts, which mediates GDP/GTP exchange on EF-Tu. EF-Tu and its eukaryotic counterpart EF-1α, have other functions in addition to that of polypeptide elongation. EF-Tu interacts with the Qβ replicative complex, the transcriptional apparatus and membranes; EF-1α binds to actin filaments and influences the assembly of cytoskeletal polymers (3-8). Both EF-1α and EF-Tu apparently participate in the degradation of N-terminally blocked proteins by the 26S proteasome (9). Recently, we and others have shown that EF-Tu possesses chaperone-like properties in protein folding and renaturation after stress (10, 11). In the present study, we report that EF-Tu displays a protein disulfide isomerase activity.

Protein disulfide isomerases assist protein folding in prokaryotes and eukaryotes (12-14). They catalyze dithiol/disulfide interchange reactions, and promote protein disulfide formation, isomerization or reduction, depending on the imposed redox potential and on the nature of the polypeptide substrate (12-14). In the bacterial cytoplasm, the thioredoxin system (NADPH, thioredoxin reductase and thioredoxin), and the glutaredoxin system (NADPH, glutathione reductase, glutathione and glutaredoxins) have been implicated in the maintenance of proteins in a reduced state (15-17). Protein disulfide isomerases generally contain a Cys-X-X-Cys active site. The first sulfhydryl is exceptionally reactive, acts as the primary nucleophile of protein substrates, and forms a mixed disulfide with these latter (18-20). The second is less reactive, and facilitates disruption of the mixed disulfide by an intramolecular reaction with the first cysteine. The second cysteine of several protein disulfide isomerases (chloroplast thioredoxin, PDI, DsbA) has been mutated with conservation of protein disulfide isomerase activity (21-24); these mutants form a mixed disulfide between their single active-site cysteine and a substrate-protein, and then, glutathione (instead of the missing cysteine) interacts with the mixed disulfide and releases the substrate-protein (24). EF-Tu does not possess a Cys-X-X-Cys sequence. It contains three sulfydryl groups (25). Cysteine 81 (associated with aminoacyltRNA binding) reacts easily with thiol reagents such as p-chloromercuribenzoate, DTNB and N-tosyl-phenylalanine-chloromethane (25-27). Cysteine 137 (associated with guanosine nucleotide binding) can be titrated with DTNB, when EF-Tu is in its nucleotide-free form, in the presence of EDTA (25). The third sulfhydryl group is non-reactive, and can be titrated only after complete denaturation of the protein (25). We have shown recently that EF-Tu displays an impressive reactivity towards Thiol-Sepharose, on which it is retained in conditions in which most proteins are eluted (28), suggesting that it possesses an exceptionally reactive thiol. Accordingly, we show in this

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Abbreviations used: EF-Tu, elongation factor Tu; EF-1α, elongation factor 1α; EF-Ts, elongation factor Ts; PDI, protein disulfide isomerase; DTNB, 5,5′-dithiobis (nitrobenzoic acid).

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study that EF-Tu displays a protein disulfide isomerase activity, catalyzing protein disulfide formation (oxidative renaturation of reduced RNase), reduction (reduction of insulin disulfides), and isomerization (refolding of randomly oxidized RNase).

MATERIALS AND METHODS

Purification of EF-Tu. Crude extracts from the *E. coli* K 12 strain C600 *(leuB6 thi-1 thr-1 supE44)* were prepared by a lysozyme-EDTA method (29). EF-Tu was purified by two successive DEAE-Sephacel chromatographies (column buffer: 20 mM Tris, pH 8.0, 0.2 mM EDTA, 1 mM dithiothreitol, 10% glycerol, elution with a linear 0-0.35 M NaCl gradient in the same buffer), followed by hydroxylapatite chromatography (column buffer: 20 mM Tris pH 7.4, 0.2 mM EDTA, 1 mM dithiothreitol, 10% glycerol, elution with a linear 0-0.1 M gradient of potassium phosphate in the same buffer). The purified protein was dialyzed for 3 hours against 20 mM Tris, pH 8.0, 50 mM KCl, O.1 mM dithiothreitol, 10% glycerol and concentrated by ultrafiltration. EF-Tu was more than 98% pure as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Preparation of reduced and oxidized EF-Tu and determination of SH groups. 40 μl of EF-Tu (12 mg/ml) was incubated for 30 min with 10 mM dithiothreitol, and isolated by gel filtration through a Biogel P-10 column (2 ml bed volume, Bio-Rad Laboratories) equilibrated with oxygen-free 0.1 M potassium phosphate pH 6.8, 1 mM EDTA. SH determination was done immediately by adding 10 μl of each fraction to 100 μl of 1 mM 5,5′ dithiobis(2-nitrobenzoic acid), 5 M guanidine-HCl, 50 mM Tris pH 7.5 (30). The number of SH groups per mol of EF-Tu was calculated from the EF-Tu concentration and from the absorbance at 412 nm (with an adsorption coefficient of the p-nitrothiophenol anion of 13.600 $M^{-}cm^{-1}$ (30)). EF-Tu concentration (MW = 44,000 Da) was calculated from its absorbance at 280 nm, with $A_{1mg/ml}$ = 0.403 calculated from tryptophan and tyrosine content. Oxidized EF-Tu was obtained by incubating reduced EF-Tu for 72 h in air-containing buffer (Tris pH 8, 1 mM EDTA). Titration of EF-Tu with {^{14}C} iodoacetamide (50 mCi/mmol, from Amersham) was done as previously described (19).

Assay of insulin disulfide reduction. The reduction of insulin was assayed by measuring the increase in absorbance at 650 nm (31). The incubation mixture contained the following in a final volume of 100 μl: O.1 M N_2-equilibrated potassium phosphate, pH 6.6, 0.3 mM EDTA, 0.13 mM bovine insulin, 0.3 mM dithiothreitol, in the presence of thioredoxin or EF-Tu as indicated. Insulin stock solutions were prepared as described in Ref. 16.

RNase activity. RNase activity was determined by monitoring the hydrolysis of 2 mM cyclic 2′, 3′ cytidine monophosphate in 0.1 M Tris, pH 7.4 (32): 20 μl of RNase was incubated at 23°C with 100 μl of cyclic CMP. Hydrolysis of cyclic CMP was measured by the increase in absorption at 296 nm. For each experiment, a control without cyclic CMP was made.

Preparation of reduced and denatured RNase. Reduced, denatured RNase A was prepared as described in Ref. 33 by incubating 20 mg of the native enzyme (RNaseA from Sigma) in 1 ml of 0.1 M Tris, pH 8.6 containing O.15 M dithiothreitol and 6 M guanidine hydrochloride. Reduced RNase was separated from dithiothreitol and guanidine hydrochloride using Sephadex G-25 equilibrated with oxygen-free O.O1 M HCl and the fractions were stored under mineral oil.

Preparation of scrambled RNase. For the preparation of scrambled RNase (33), reduced, denatured RNase was made 6 M in guanidine hydrochloride and the pH was adjusted to 8.6 with solid Tris. The sample was then sparged with oxygen and incubated at room temperature in the dark for 3 days. Free thiol was less than 0.1 mol/mol of RNase, and RNase activity was undectable.

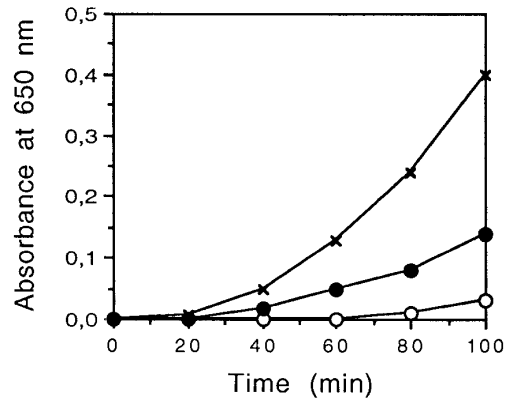

FIG. 1. EF-Tu-catalyzed reduction of insulin by dithiothreitol. The incubation mixtures contained O.1 M N_2-equilibrated potassium phosphate pH 6.6, 0.3 mM EDTA, 0.13 mM bovine insulin, and 0.3 mM dithiothreitol alone (○) or in the presence of 2 μM EF-Tu (●) or 1 μM thioredoxin (×). The reduction of insulin, and its resulting precipitation were monitored by following optical density at 650 nm.

Reactivation of reduced and denatured RNase. Reactivation of reduced RNase by redox buffers was initiated by diluting the reduced enzyme in 50 mM Tris pH 8.2, 1 mM $MgCl_2$, 0.6 mM dithiothreitol, 1 mM oxidized glutathione, at 23°C, in the absence or in the presence of EF-Tu as indicated. Samples were withdrawn at intervals and assayed for RNase activity.

Reactivation of scrambled RNase. Reactivation of scrambled RNase was initiated by diluting it (final concentration 30 μM) in 0.1 M Tris pH 7,4, 1 mM EDTA, 60 μM dithiothreitol at 23°C, in the presence of thioredoxin or EF-Tu as indicated (33). Samples were withdrawn at intervals and assayed for RNase activity.

GDP binding assay. The assay was the standard GDP-exchange nitrocellulose filter assay using 10-100 pmol EF-Tu and 4 μM ^{3}H-GDP, as described in (34).

Materials. Insulin (from bovine pancreas), RNaseA and thioredoxin (from *Spirulina sp.*) were from Sigma. All other products were reagent grade and were also obtained from Sigma. ^{3}H-GDP was obtained from Amersham and was used at 1.5 Ci/mmol. ^{14}C-iodoacetamide (60 mCi/mmol) was also from Amersham. DEAE-Sephacel was from Pharmacia Fine Chemicals, and hydroxylapatite (Bio-Gel HTP) was from BioRad.

RESULTS

Reduction of insulin disulfide bonds. The reduction of insulin by a disulfide oxidoreductase can be assessed by a rapid spectrophotometric assay (31). Insulin contains two polypeptide chains A and B that are linked by two interchain disulfide bonds. When these bonds are broken, the free B chain is insoluble and precipitates, leading to an increase in absorbance at 650 nm. Thioredoxin, PDI and DsbA catalyze the dithiothreitol-dependent reduction of insulin (16, 31, 35). The reduction of insulin (130 μM) by dithiothreitol (0.3 mM) was determined in the presence or absence of EF-Tu (Fig. 1). EF-Tu (2 μM) stimulates insulin precipitation in a dithiothreitol-dependent manner, leading to insulin precipitation after 40 min, instead of 80 min in the absence of EF-Tu. In the same assay, thioredoxin (1

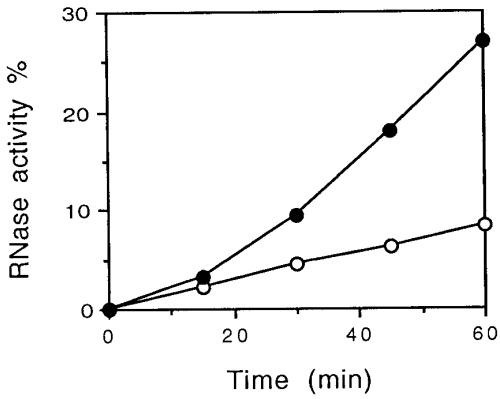

FIG. 2. Oxidative folding of reduced, denatured RNase in the presence of redox buffers. The mixtures (0.6 mM dithiotreitol, 1 mM oxidized glutathione, 1 mM $MgCl_2$, 50 mM Tris, pH 8.2) containing 30 μM reduced denatured RNase alone (○) or in the presence of 30 μM EF-Tu (●) were assayed for RNase activity at the times indicated.

μM) catalyzes insulin reduction, as previously reported (16), and leads to insulin precipitation after 25 min. Like DsbA (31) and protein disulfide isomerase (35), EF-Tu is several times less active than thioredoxin in catalyzing the dithiothreitol-dependent reduction of insulin.

Oxidative folding of reduced RNase in the presence of redox buffers. Reduced, denatured RNase possesses eight SH groups, and its renaturation involves the oxidation of its thiol groups followed by rearrangement of the disulfides to the native conformation (with 4 S-S bridges) (36). Appropriate redox buffers efficiently reactivate reduced RNase, and protein disulfide isomerases can accelerate this reactivation (32). In the presence of 1 mM oxidized glutathione and 0.6 mM dithiothreitol (a redox buffer used for the reactivation of reduced RNase by DsbA (32)), EF-Tu (30 μM) stimulates several fold the reactivation of reduced RNaseA (28% reactivation in 60 min, instead of 8% in the absence of EF-Tu (Fig. 2)). The stimulation we observed is similar to that obtained by others in the presence of 25 μM DsbA (40% reactivation in 60 min under similar conditions (32)).

Refolding of scrambled RNase. Scrambled RnaseA is fully oxidized but inactive as a catalyst of RNA hydrolysis owing to the formation of a complex mixture of species containing an undefined variety of (mainly) intramolecular disulphide bonds. It is obtained by oxidation of reduced RNaseA in the presence of guanidine hydrochloride, and requires interchange of disulfide bonds to acquire the native conformation. Thioredoxin, PDI or DsbA can reactivate scrambled RNase in the presence of dithiothreitol (32-33, 37). 20 μM EF-Tu reactivates scrambled RNase more efficiently than 20 μM thioredoxin, while dithiothreitol alone is unable to reactivate scrambled RNase significantly (Fig. 3).

Copurification of EF-Tu with the protein disulfide isomerase activity. To confirm that the protein disulfide isomerase activity is inherent to EF-Tu, we assayed the fractions obtained during the final step of the purification procedure (hydroxylapatite chromatography) for GDP binding activity (a known property of EF-Tu) ((34), and for a possible capacity to catalyse insulin precipitation. As shown in Fig. 4, both enzymatic activities coincide with the EF-Tu protein peak. Analysis of the EF-Tu preparation by sodium dodecyl sulfate polyacrylamide gel electrophoresis shows a purity of more than 98% without visible material corresponding to thioredoxin (12,000 Da), DsbA (21,000 Da) or DsbC (23,000 Da) (see Fig. 5B, lane 1).

Reduced and oxidized EF-Tu. Most protein disulfide isomerases, such as thioredoxin, PDI and DsbA (12-13, 31), contain a Cys-X-X-Cys sequence in their active site. Upon oxidation, they shift from a dithiol state to a state containing an intramolecular disulfide bond formed by their vicinal cysteines. Less frequently, oxido-reductases, such as the alkyl hydroperoxide reductase AhpC of *Salmonella typhimurium* form intermolecular disulfide bonds upon oxidation (38). EF-Tu does not contain a Cys-X-X-Cys sequence, but contains three thiols. Two of these can be titrated with DTNB in EDTA-containing buffers (25), the third being titratable only upon denaturation of the protein (25). We determined the thiol content and oligomerization state of reduced and oxidized EF-Tu, by titration with DTNB and by SDS-PAGE electrophoresis, respectively.

Reduced EF-Tu was prepared by reduction of EF-Tu with dithiothreitol. EF-Tu was separated from dithiothreitol by filtration through a gel permeation column equilibrated with oxygen-free phosphate/EDTA buffer as described for thioredoxin (30), and its thiol content was determined with DTNB in the presence of guanidine-hydrochloride (30) (Figure 5A). Reduced EF-Tu contains 2.7 SH groups per mol, in accordance

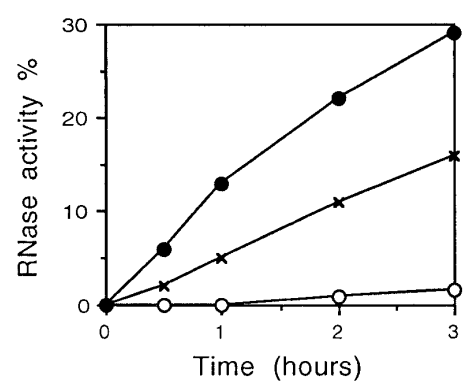

FIG. 3. Refolding of scrambled RNase. The mixtures (0.1 M Tris pH 7,4, 1 mM EDTA at 23°C) containing 60 μM dithiothreitol and 30 μM scrambled RNase alone (○) or in the presence of either 20 μM EF-Tu (●) or 20 μM thioredoxin (×) were assayed for RNase activity at the times indicated.

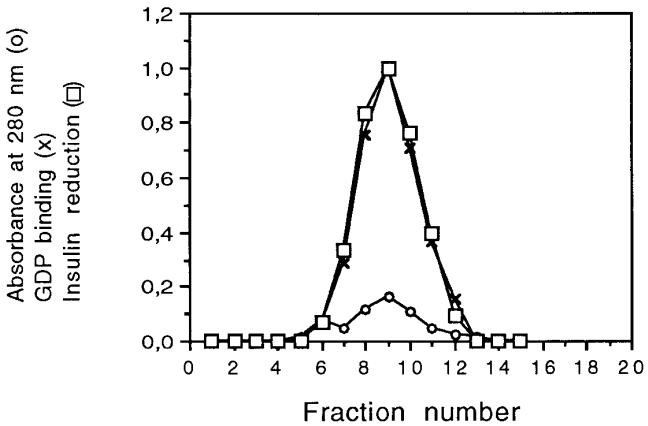

FIG. 4. Copurification of the oxidoreductase activity with EF-Tu. EF-Tu (0.6 mg), after the second DEAE–Sephacel step, was loaded on a hydroxylapatite column (3 ml bed volume) equilibrated in 20 mM Tris, pH 7.4, 100 μM dithiothreitol, 10% glycerol, and eluted with a linear 0–0.1 M gradient of potassium phosphate in the same buffer. The fractions were analyzed for protein content (○), GDP binding (×), and stimulation of insulin precipitation (□). GDP binding, and stimulation of insulin precipitation are normalized with respect to the more active fraction. Stimulation of insulin precipitation is expressed as the ratio between the times required for the onset of insulin precipitation (absorbance 0,05) in the absence, and in the presence of each fraction. GDP binding represents 9 pmol GDP bound per microgram of EF-Tu, similarly to previous results (34). Stimulation of insulin precipitation, and GDP binding were assayed as described under Methods with 20 μl, and 2 μl of each fraction, respectively. Protein determination was performed according to Bradford (48).

with previous results which showed that three thiols can be titrated in EF-Tu in the presence of a denaturant (25) (Fig. 5A). Oxidized EF-Tu (obtained by incubation of reduced EF-Tu in air-containing buffer as described under "Experimental Procedures") contained 1.3 SH group per mol (as determined with DTNB in the presence of guanidine-hydrochloride, not shown). Thus, upon oxidation under our conditions, an average of 1.4 thiol groups per mol of EF-Tu are oxidized.

The change in oligomerization state of EF-Tu during oxidation is shown in Figure 5B. Oxidized EF-Tu, prepared as described under "Experimental Procedures" was run in SDS polyacrylamide gels, either under reducing conditions (in the presence of 2-mercaptoethanol) or under nonreducing conditions (in the absence of 2-mercaptoethanol). EF-Tu migrates with an apparent molecular mass of 44 kDa in SDS polyacrylamide gels run under reducing conditions, as reported by others (39). Under non reducing conditions, however, it migrates as a mixture of 44% monomers and 56% oligomers, mostly trimers (molecular mass of around 130 kDa). Because the migration takes place in the presence of SDS, oligomers result from the formation of intermolecular disulfides. The momomers are not oxidized, as judged from titration with labeled iodoacetamide (not shown), and thus do not contain an intramolecular disulfide bond. They might represent EF-Tu molecules that are unaccessible to oxidation, and which, in the presence of SDS, consequently migrate as monomers (they might be oligomers resulting from the tendency of EF-Tu to aggregate (40)). These results suggest that, in contrast with thioredoxin, which forms an intramolecular disulfide and remains monomeric upon oxidation, EF-Tu forms intermolecular disulfide bonds upon oxidation.

DISCUSSION

EF-Tu displays protein disulfide isomerase activity *in vitro*. Like other protein disulfide oxidoreductases (thioredoxin, protein disulfide isomerase, DsbA), it catalyzes disulfide bond formation, isomerization and reduction of proteins. The protein disulfide isomerase activity of EF-Tu is not significantly lower than that of several *bona fide* protein disulfide isomerases. EF-Tu is three times more active than thioredoxin in reactivating scrambled RNase (this study), and nearly as active as DsbA in catalyzing the oxidative folding of reduced RNase in the presence of redox buffers (32) (PDI is 20-fold more active than thioredoxin in refolding scrambled RNase, however (37)).

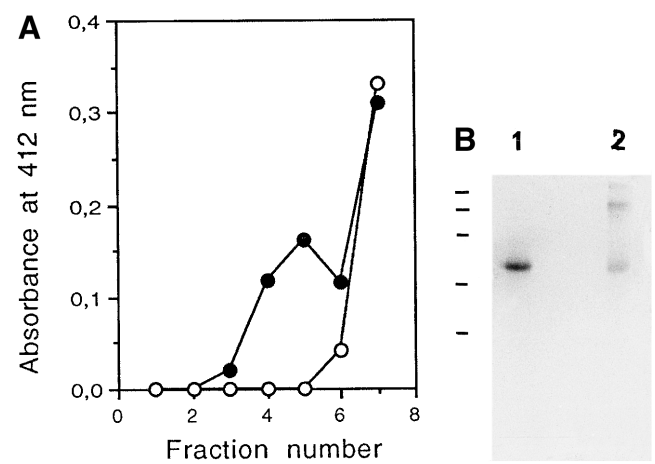

FIG. 5. Analysis of reduced, and oxidized EF-Tu. A) Determination of thiol groups in reduced EF-Tu. 40 μl of reduced EF-Tu (12 mg/ml) (●) or 40 μl of buffer (○) were incubated for 30 min with 10 mM dithiothreitol, and isolated by gel filtration through a Biogel P-10 column as described under "Experimental Procedures". The SH concentration of each fraction was determined with DTNB, by measuring the absorbance at 412 mM as described under "Experimental Procedures". B) Oligomerization state of reduced, and oxidized EF-Tu. Oxidized EF-Tu, prepared as described under "Experimental Procedures" was dissolved in SDS sample buffer (50 mM Tris/HCl, pH 6.8, 1.25%, mass/vol SDS, bromophenol Blue, 20% by vol. glycerol containing 50 mM 2-mercaptoethanol (lane 1) or no 2-mercaptoethanol (lane 2), and run on 12% polyacrylamide gel. The gel was stained with Commassie Blue. Horizontal bars indicate the migration of the molecular weight markers (204,000, 121,000, 78,000, 39,500, 30,700 daltons).

The remarkable efficiency of thioredoxin, PDI and DsbA as protein disulfide oxidoreductases has been explained i) by the specific properties of their first thiol group (which is solvent-exposed, has a high reactivity and a low pKa), which allow the rapid formation of a mixed disulfide with protein-substrates (rearrangements between this mixed disulfide and other thiols of the protein-substrate leads to disulfide isomerization) ii) by their peptide-binding properties (demonstrated for DsbA (41), DsbC (42) and PDI (43)), which also explain their rapid reaction rates with substrate-proteins, iii) by the vicinity of their two active site thiols, which facilitates escape from the mixed disulfide state and release of the substrate protein (by an intramolecular reaction between the second cysteine and the first one engaged in the mixed disulfide). Single cysteine mutants of several protein disulfide isomerases, in which the more reactive thiol has been conserved, are catalytically active (17, 22-24). These mutants form a mixed disulfide with substrate proteins, and then glutathione (instead of the missing cysteine) interacts with the mixed disulfide and releases the substrate-protein (24). Since EF-Tu does not possess vicinal cysteines, and does not appear to form an internal disulfide upon oxidation, we suggest that it might function in the same way as single cysteine mutants of protein disulfide isomerases, by combining the activities of a reactive thiol and of a peptide-binding site associated with its chaperone properties (10, 11). Glutathione (which exists at millimolar concentrations in the bacterial cytoplasm (44)) could possibly fulfill the function of the missing vicinal thiol (24, 43). Interestingly, a single catalytic thiol group was considered during early experiments *in vitro* with PDI, when the presence of an active-site disulfide in PDI had not yet been demonstrated (45).

The biological significance of the protein-disulfide isomerase activity of EF-Tu has not been evaluated. Proteins with chaperone and disulfide isomerase activities like EF-Tu or DnaJ (46) could destabilize disulfide-forming folding intermediates, which might appear despite the reducing environment of the cytoplasm (the tendency to form disulfides in proteins depends not only on the redox potential of the environment, but also on the conformation of the polypeptide (14, 47)). The thioredoxin and glutaredoxin systems seem to play an important role in maintaining cytoplasmic protein reduced (15-17). Nevertheless, the double mutants completely defective for both pathways exhibit some aerobic growth, suggesting that other pathways can substitute for them (15)). Finally, the chaperone (10, 11) and protein-disulfide isomerase activities of EF-Tu suggest that the translational elongation factor is an ancestral protein folding factor, that appeared before dedicated chaperones and protein disulfide isomerases.

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