Chaperone Properties of Mammalian Mitochondrial Translation Elongation Factor Tu<sup>*</sup><sup>[1]</sup>

Hiroaki Suzuki, Takuya Ueda, Hideki Taguchi, and Nono Takeuchi<sup>1</sup>

From the Department of Medical Genome Sciences, Graduate School of Frontier Sciences, University of Tokyo, Building FSB-401, 5-1-5, Kashiwanoha, Kashiwa, Chiba Prefecture 277-8562, Japan

The main function of the prokaryotic translation elongation factor Tu (EF-Tu) and its eukaryotic counterpart eEF1A is to deliver aminoacyl-tRNA to the A-site on the ribosome. In addition to this primary function, it has been reported that EF-Tu from various sources has chaperone activity. At present, little information is available about the chaperone activity of mitochondrial EF-Tu. In the present study, we have examined the chaperone function of mammalian mitochondrial EF-Tu (EF-Tumt). We demonstrate that recombinant EF-Tumt prevents thermal aggregation of proteins and enhances protein refolding <i>in vitro</i> and that this EF-Tumt chaperone activity proceeds in a GTP-independent manner. We also demonstrate that, under heat stress, the newly synthesized peptides from the mitochondrial ribosome specifically co-immunoprecipitate with EF-Tumt and are destabilized in EF-Tumt-overexpressing cells. We show that most of the EF-Tumt localizes on the mitochondrial inner membrane where most mitochondrial ribosomes are found. We discuss the possible role of EF-Tumt chaperone activity in protein quality control in mitochondria, with regard to the recently reported <i>in vivo</i> chaperone function of eEF1A.

The main function of the translation elongation factor Tu (EF-Tu)<sup>2</sup> and its eukaryotic counterpart eEF1A is to deliver aminoacyl-tRNA to the A-site on the ribosome. In addition to this, multiple secondary functions have been reported for EF-Tu. EF-Tu is an essential host-donated subunit of the replicative complex of Qβ phage, and it may interact with the transcriptional apparatus as a positive regulator of RNA synthesis (1). Higher eukaryotes, such as Arabidopsis, sense microbes via bacterial EF-Tu. EF-Tu activates a signaling event and defense responses in plants (2). The association of eEF1A with the actin cytoskeleton has been reported in Dictyostelium amoebae, yeast, and mammals (3–5). This interaction is essential for the regulation of the actin cytoskeleton and cell morphology (6).

The ribonucleoprotein complex containing eEF1A and a non-coding RNA called HSR1 regulates the activation of heat-shock transcription factor 1 in response to heat shock in mammalian cells (7). eEF1A-2, an eEF1A isoform that is specifically expressed in neurons, cardiomyocytes, and myotubes, interacts with peroxiredoxin I to protect cells against apoptotic death induced by oxidative stress (8).

Recently, it has been reported that EF-Tu displays properties of a chaperone. EF-Tu from <i>Escherichia coli</i> and maize chloroplasts is able to suppress the thermal aggregation of proteins <i>in vitro</i> (9, 10). EF-Tu from <i>E. coli</i> and Thermus thermophilus, as well as rabbit eEF1A, are able to promote protein refolding <i>in vitro</i> (9, 11, 12). <i>E. coli</i> EF-Tu displays a protein disulfide isomerase activity <i>in vitro</i> (13). Rabbit reticulocyte eEF1A binds to the ribosome nascent-chain complex during translation. Moreover, eEF1A can bind to unfolded polypeptides that are no longer associated with the ribosome (12). Remarkably, the chaperone activity of eEF1A has been linked to <i>in vivo</i> functions, as demonstrated by yeast genetic screening of the suppressor of the rad23Δrpn10Δ strain (14). Rad23 and Rpn10 play synergistic roles in the recognition of ubiquitinated proteins, and the loss of both proteins causes growth and proteolytic defects. eEF1A interacts with the ubiquitinated nascent peptide on the ribosome, as well as with the proteasome subunit Rpt1. Therefore, eEF1A suppresses the rad23Δrpn10Δ mutation by mediating the proteasome degradation of co-translationally damaged proteins in place of Rad23 and Rpn10 (14). eEF1A is also involved in the quality surveillance of newly synthesized proteins in yeast.

Accumulating evidence points to an important role for mitochondrial (mt) protein quality control in the pathogenesis of human diseases, such as neurological disorders, aging, cancer, and various neuromuscular syndromes (15–17). Indeed, disease-causing mutations in components of the mt protein control system have been identified, including a membrane-bound ATP-dependent AAA protease and the mt chaperonin heat-shock protein (HSP) 60 (18, 19). Functional impairment of these proteins causes hereditary spastic paraplegia, a genetic disorder that is characterized by axonal degeneration in humans. It is noteworthy that the ATP-dependent m-AAA protease regulates mt ribosome assembly and translation by controlling the proteolytic maturation of L32, an essential mt ribosomal protein (20). This suggests that the pathogenesis of neurodegenerative disorders, such as hereditary spastic paraplegia, is related to a defect in mt protein synthesis, as well as to the accumulation of misfolded proteins in the mitochondria.

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1 To whom correspondence should be addressed: Tel./Fax: 81-47-136-3648; E-mail: nono@k.u-tokyo.ac.jp.

2 The abbreviations used are: EF-Tu, translation elongation factor Tu; COII and COIV, cytochrome oxidase subunits II and IV; CS, citrate synthase; EF-Tumt, mitochondrial translation elongation factor; F1-ATPase-β, F1-ATP synthetase β subunit; GFP, green fluorescent protein; HSP, heat shock protein; IM, inner membrane; mt, mitochondrial; BSA, bovine serum albumin.
In the present work, we have studied the chaperone function of mammalian mt EF-Tu (EF-Tumt). We demonstrate that mammalian EF-Tumt displays chaperone activity \textit{in vitro}, in a GTP-independent manner. We have investigated the interaction between EF-Tumt and the newly synthesized peptides from mt ribosomes under stress conditions. Localization of EF-Tumt in mitochondria has also been analyzed. Based on these observations, we discuss the possible role of EF-Tumt chaperone activity in the quality control of misfolded newly synthesized polypeptides in mitochondria.

**MATERIALS AND METHODS**

**Cells and Culture Conditions**—293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Intergen) in the presence of penicillin (100 units/ml) and streptomycin (100 units/ml). Cells were routinely passaged twice a week and grown at 37 °C in 5% CO2. 293T derivatives that stably overexpressed human EF-Tumt (hEF-Tumt) were maintained as above, but in the presence of Zeocin (0.3 mg/ml). 293T/mock, 293T/hEF-Tumt[WT], and 293T/hEF-Tumt[D156N] were established by transfection of 293T cells with the plasmids pcDNA3.1/Zeol(+), hEF-Tumt[WT]-pcDNA3.1/Zeol(+), and hEF-Tumt[D156N]-pcDNA3.1/Zeol(+), respectively.

**Plasmids**—BMtu/pET24c, the \textit{E. coli} expression vector for C-terminal histidine-tagged bovine mt EF-Tu (bEF-Tumt), was a gift from Prof. Linda Spremulli, University of North Carolina. hEF-Tumt[WT]-pcDNA3.1/Zeol(+), the mammalian expression vector for C-terminal 3× FLAG-tagged hEF-Tumt, was constructed as follows. A DNA fragment carrying the coding sequence for hEF-Tumt (GenBank™ accession number BC001633) was obtained by reverse transcription-PCR, using poly(A) RNA from HeLa cells and primers containing the 3× FLAG sequence and cloned into pcDNA3.1/Zeol(+) (Invitrogen). hEF-Tumt[D156N]-pcDNA3.1/Zeol(+) was constructed with a QuikChange mutagenesis kit (Stratagene), using the primers 5′-GTATGTGAACAAGGCTAACGCTGTCCAGGACTC-3′ and 5′-GAGTCTCCTGGACACCGCTTAGCCTTTCACATAC-3′.

**In Vitro Chaperone Assay**—Recombinant bEF-Tumt for the \textit{in vitro} chaperone assay was expressed using BMtu/pET24c in \textit{E. coli} BL21(DE3) and purified as described (21), in the presence of GDP to yield the GDP form. Recombinant \textit{E. coli} EF-Tu was expressed and purified as described previously (22). Citrate synthase (CS) and L-malate dehydrogenase were purchased from Roche Applied Science. Kirromycin was obtained from Sigma.

The thermal aggregation of CS was tested as described (25). In separate trials, CS (0.2 μM) was mixed with various amounts of purified recombinant bEF-Tumt in 20 mM Tris-HCl buffer (7 mM MgCl2, 100 mM KCl, 15 μM GDP, and 10% (v/v) glycerol, pH 7.5) in a total volume of 1.5 ml in covered quartz cuvettes. The three controls were CS alone, CS mixed with BSA, and CS mixed with ovalbumin. Samples were incubated at 43 °C for the indicated times, and CS stability was estimated by monitoring light scattering during incubation at 320 nm by spectrophotometry (JASCO Corp., FP-6500).

The refolding of GFP was performed as described (26). GFP (100 μM) was denatured with an equal volume of 0.2 N HCl for 2 min at 25 °C. Spontaneous and EF-Tu-assisted refolding was initiated by diluting 3 μl of the denatured protein to a final volume of 1.5 ml in a solution containing 20 mM Tris-HCl (pH 7.5), 100 mM KCl, and 10% (v/v) glycerol. GTP or GDP was added where indicated. The final concentration of GFP in the refolding reaction was 100 nM. The refolding reaction was carried out for 15 min at 18 °C. Refolding of GFP was monitored by GFP fluorescence spectrophotometry with excitation at 400 nm and emission at 510 nm. The relative fluorescence of GFP was plotted.

For the rhodanese refolding assay, recombinant rhodanese (final concentration, 33 μM) was denatured in 200 mM sodium phosphate buffer (pH 7.4) containing 1 mM β-mercaptoethanol and 8 mM urea for 45 min at 25 °C. Spontaneous and EF-Tu-assisted refolding was initiated by diluting the indicated amount of denatured enzyme into a final volume of 160 μl in a solution containing 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 50 mM sodium thiosulfate, 10 mM MgCl2, 100 mM KCl, and 10% (v/v) glycerol. In addition, 1 mM GTP or GDP was added where indicated. The final concentration of rhodanese in the refolding reaction was 100–300 nM. The refolding reaction was carried out for 30 min at 20 °C. Enzyme activity was used as a measure of the successful refolding of rhodanese into its native conformation. Enzyme activity was measured by colorimetry of the complex formed between ferric ions and one of the reaction products, thiocyanate at 460 nm, as described (27).

To analyze EF-Tu-GDP- and EF-Tu-GTP-dependent stimulation of rhodanese refolding, EF-Tu-GDP and EF-Tu-GTP were prepared as follows. EF-Tu-GTP was formed by incubating EF-Tu (final concentration, 10 μM) in the presence of 20 μM GTP, 4 mM phosphoenolpyruvate, and 10 μg of pyruvate kinase in 50 μl of PK buffer (50 mM Tris-HCl (pH 7.5), 10 mM KCl, 1.5 mM MgCl2, 10% (v/v) glycerol) at 30 °C for 15 min. EF-Tu-GDP was similarly prepared but in the absence of GTP and pyruvate kinase. After incubation, aliquots of samples were directly used for rhodanese refolding. Samples without EF-Tu were used in the refolding assay to assess EF-Tu-dependent refolding of rhodanese. GDP, GTP, and phosphoenolpyruvate had no effect on substrate-protein renaturation.

**Isolation of Mitochondria from Human Cells**—Mitochondria were isolated according to a previously published procedure (28), with slight modifications. Briefly, cells were washed with phosphate-buffered saline and collected in MSED buffer (20 mM HEPES-KOH, pH 7.4, 225 mM mannitol, 75 mM sucrose, 0.2 mM Mg(OAc)2, 1 mM EDTA, 1 mM dithiothreitol), before homogenization by nitrogen cavitation (200 p.s.i. for 15 min) in a cell disruption bomb (Parr Instrument Co., Moline, IL). The cell lysate was centrifuged at 750 × g for 20 min, and the supernatant was centrifuged again at 3,000 × g for 30 min. The resulting intact mt-enriched pellet was washed twice with MSED buffer.

Mitoplasts were obtained using a phosphate swelling-shrinking method as described previously (29) with minor modifications. Purified mitochondria were swollen by the addition of...
swelling buffer (10 mm KH$_2$PO$_4$, pH 7.4) at a concentration of 500 µg of mt protein/ml and incubated for 20 min at 4 °C with gentle mixing. An equal volume of shrinking buffer (10 mm KH$_2$PO$_4$, pH 7.4, 32% (w/v) sucrose, 30% (v/v) glycerol, 10 mm MgCl$_2$) was added, and the samples were incubated for an additional 20 min at 4 °C. The suspension was centrifuged at 10,000 × g for 30 min, yielding a pellet composed of mitoplasts. Mitoplasts were washed three times in MSED buffer and resuspended in MSED buffer at a protein concentration of ~30 µg/µl. The mitoplast suspension was frozen in liquid nitrogen and stored at ~80 °C until use.

**In Organello Translation, Co-immunoprecipitation, and the Stability of Mitochondrial Gene Products—**In vitro labeling of mitochondrial translation products was performed in isolated mitoplasts, as previously described (30). Briefly, mitoplasts were resuspended to a protein concentration of 1 mg/ml in translation buffer (0.6 M sorbitol, 150 mM KCl, 15 mM K$_2$HPO$_4$, 20 mM HEPES-KOH, 12.7 mM MgSO$_4$, 0.3% (w/v) BSA, 4 mM ATP, 0.5 mM GTP, 1.13 mg/ml α-ketoglutarate, 2.33 mg/ml phosphoenolpyruvate, 10 µg/ml of each amino acid except methionine, 26.6 mg/ml pyruvate kinase, pH 7.5). After 5 min of incubation at 25 °C, [35S]methionine (2 GBq/ml, 50 mCi/mmol, Institute of Isotopes Co., Ltd., Hungary) was added (final concentration, 1.8 mCi/mmol), and the incubation was continued for an additional 60 min. Translation was stopped by the addition of unlabeled methionine (100 mM) and pyruvokin (50 µg/ml). Mitoplasts were washed three times with washing buffer (0.6 M sorbitol, 1 mM EDTA, 5 mM methionine). The mitoplasts were suspended in LiDS sample buffer (40 mM Tris-HCl, 2% (w/v) LiDS, 10% (v/v) glycerol, 10% (w/v) bromphenol blue, 350 mM β-mercaptoethanol, pH 6.8), subjected to 15% (w/v) SDS-PAGE (0.1% (w/v) bisacrylamide), and visualized on a BAS5000 bio-imaging analyzer using an Imaging Plate (Fujifilm).

To analyze the interaction of *in organello* translated proteins with FLAG-tagged EF-Tumt, co-immunoprecipitation analysis was performed as follows. After *in organello* translation, the washed mitoplasts were lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 1.0% (v/v) Triton X-100) and centrifuged at 10,000 × g for 15 min. The supernatant was subjected to immunoprecipitation with FLAG-specific antibodies. Antibodies against F1-ATP synthetase β subunit (F1-ATPase-β) were used to test for the nonspecific binding of *in organello* translated proteins to non-EF-Tumt protein.

To analyze the stability of newly synthesized mitochondrial translation products within the mitochondria, pulse-chase analysis was performed as described (31). Briefly, after *in organello* translation, incorporation of radioactivity into newly translated polypeptides was stopped by the addition of cold methionine. Mitochondria were isolated and washed three times with ice-cold washing buffer. To assess the stability of the newly synthesized polypeptides, mitochondrial pellets were resuspended in translation buffer and samples were further incubated at the indicated temperature. Aliquots were withdrawn at various time points, dissolved into LiDS sample buffer, subjected to SDS-PAGE, and analyzed by BAS5000 using an Imaging Plate (Fujifilm).

**Subfractionation of Mitochondria—**For subfractionation of mitochondria (29), mitoplasts were sonicated in MSED buffer at 10 watts for 20 s in total. KCl, urea, and RNase A were included in the MSED buffer where indicated. Samples were centrifuged at 150,000 × g for 1 h. The supernatants were saved as the matrix fractions. The inner-membrane (IM) pellets were washed once in MSED buffer and centrifuged at 150,000 × g for 30 min. The IM pellets were solubilized in radioimmune precipitation assay buffer (25 mM Tris-HCl, pH 8.2, 50 mM NaCl, 0.5% (v/v) Nonidet P-40, 0.5% (v/v) deoxycholate, 0.1% (w/v) SDS, and protease inhibitors (complete mini, EDTA-free, Roche Applied Science)). All procedures were performed at 4 °C. Protein concentrations were determined by the bicinechonic acid method (Pierce). The presence of mitochondrial ribosomes was determined as described previously (32). Northern blotting for mitochondrial 16S ribosomal RNA was carried out with the oligomer 5’-GGAATGCTGGAGGTATGTGT-3’.

**Antibodies—**Monoclonal antibodies to human HSP60 (clone LK1) and FLAG M2 antibodies were from Sigma-Aldrich. Polyclonal antibodies to the bovine mitochondrial EF-Tu-T complex were the kind gift of Prof. Linda Spremulli, University of North Carolina. Polyclonal antibodies to the recombinant bEF-Tumt were raised in our laboratory. Monoclonal antibodies against human cytochrome oxidase subunit II (COII), cytochrome oxidase subunit IV (COIV), and F1-ATPase-β were purchased from Molecular Probes.

**RESULTS AND DISCUSSION**

**Recombinant Bovine EF-Tumt Exhibits Chaperone Properties in Vitro—**We first examined whether bEF-Tumt protects proteins from thermal aggregation. The substrate protein CS was incubated at 43 °C, and its thermal aggregation was monitored by light scattering. In the absence of EF-Tumt, aggregate formation was observed (Fig. 1A, green line). Thermal aggregation of CS was suppressed in the presence of EF-Tumt in a dose-dependent manner. Near-complete inhibition of aggregate formation was observed at ~3× molar excess of EF-Tumt over CS. The light-scattering trace did not reach horizontal in the presence of adequate EF-Tumt, due to the aggregation of a small fraction of the recombinant EF-Tumt (data not shown). The chaperone-like activity was not observed with the control proteins BSA or ovalbumin (Fig. 1B).

We next examined whether bEF-Tumt promotes the refolding of denatured proteins *in vitro*. Acid-denatured recombinant GFP was diluted in neutral reaction buffer, and its refolding was monitored by GFP fluorescence. In the absence of EF-Tumt, only ~40% of the GFP refolded spontaneously, whereas ~80% refolded in the presence of EF-Tumt (Fig. 2, blue lines). This effect was not observed with BSA (Fig. 2, green line). This indicated that the spontaneous refolding of GFP was promoted in the presence of EF-Tumt, because EF-Tumt prevented the aggregation of GFP during its refolding.

The chaperone activity of EF-Tumt was not restricted to a specific substrate. Thermal aggregation of l-malate dehydrogenase was also suppressed (data not shown), and the refolding of urea-denatured rhodanese was promoted in the presence of bEF-Tumt (see Figs. 3 and 4). Thus, recombinant bEF-Tumt displays chaperone properties *in vitro*, as reported for EF-Tu from other organisms.
Chaperone Activity of EF-Tumt Is GTP-independent—The molecular mechanism of EF-Tu chaperone activity has been unclear. A large conformational difference is observed between EF-Tu complexed with GDP and EF-Tu complexed with GTP. EF-Tu-GDP has a relatively closed conformation, whereas the EF-Tu-GDP complex, formed upon GTP hydrolysis or after binding of GDP, has an open conformation (33). It remains controversial as to which form interacts with unfolded proteins and whether the conformational change accompanying GTP hydrolysis is required for chaperone activity.

Kudlicki et al. (11) proposed that the flexing of EF-Tu between the EF-Tu-GDP (open) form and the EF-Tu-GTP (closed) form is a major factor in its chaperone-like refolding activity. They observed that the *T. thermophilus* EF-Tu is less active in rhodanese refolding in the presence of 1 mM GDP than in the presence of 1 mM GTP (11). They interpreted this to mean that the conformational change of EF-Tu accompanying GTP hydrolysis is important for its chaperone activity. Consistently, they observed that the renaturation activity of the *T. thermophilus* EF-Tu is reduced in the presence of kirromycin and pulvomycin, antibiotics that specifically bind to EF-Tu and stabilize it in its closed and open conformations, respectively (11).

In contrast, Caldas et al. (9) proposed that EF-TuGTP is less active than EF-Tu-GDP. In their experiments, *E. coli* EF-Tu-GTP was prepared by treating EF-Tu-GDP with pyruvate kinase, as described in (34), and the EF-Tu-GDP form was found to be significantly more active than the EF-Tu-GTP form for CS and α-glucosidase refolding (9).

To gain insight into the mechanism of EF-Tu chaperone activity, we carried out the same experiments as Kudlicki et al. and Caldas et al. using EF-Tumt, as well as *E. coli* EF-Tu. First, the guanine nucleotide-dependent EF-Tu activity in *in vitro* refolding of denatured rhodanese was analyzed, according to the method of Kudlicki et al. Denatured rhodanese was incubated with EF-Tumt at 20 °C in the absence or presence of 1 mM GDP or 1 mM GTP. The activity of the refolded enzyme was measured at the indicated time points. Given the *K*ₐ of EF-Tu for guanine nucleotides, 1 mM GDP or GTP is sufficient to form EF-Tu-GDP or EF-Tu-GTP, respectively (35, 36). Guanine nucleotide itself has little effect on rhodanese refolding. In contrast to the observation of Kudlicki et al. that *T. thermophilus* EF-Tu is less active in the presence of 1 mM GDP than in the presence of the 1 mM GTP (11), refolding of rhodanese was promoted by EF-Tumt irrespective of the guanine nucleotide present in the reaction (Fig. 3A). A similar result was obtained with *E. coli* EF-Tu (Fig. 3B). Furthermore, we observed that the refolding of GFP by EF-Tumt (Fig. 2) is independent of the presence of guanine nucleotides (data not shown). This indicated that both EF-Tu-GDP and EF-Tu-GTP interact with unfolded proteins and that the conformational change of EF-Tu accompanying GTP hydrolysis may not be important for its chaperone activity. The apparent discrepancy between our observations and those of Kudlicki et al. might be due to the source of the EF-Tu. *T. thermophilus* EF-Tu may interact with unfolded proteins more strongly than EF-Tumt or *E. coli* EF-Tu. The conformational change of *T. thermophilus* EF-Tu may be required for its chaperone activity to release the substrate.

Next, the influence of EF-Tu-GDP and EF-TuGTP on the refolding of rhodanese was analyzed according to the method of Caldas et al. Denatured rhodanese was incubated at 20 °C in the presence of EF-Tu-GDP or EF-Tu-GTP. The latter were prepared by treating EF-Tu-GDP with pyruvate kinase, as described previously (34). The resulting reaction mixture, containing EF-Tu-GTP as well as pyruvate kinase, was used directly in the refolding assay. The activity of the refolded enzyme was measured at the indicated time points, and the results were expressed as EF-Tu-dependent stimulation of rhodanese refolding. We obtained essentially the same results as Caldas et al., using CS and α-glucosidase as substrates (9).
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EF-Tu-specific antibiotics such as kirromycin and pulvomycin inhibit rhodanese refolding in the presence of EF-Tu, which is one of the bases for the proposal by Kudlicki et al. that the flexing of the EF-Tu conformation between the EF-Tu-GDP open configuration and the EF-Tu-GTP closed configuration is important for its chaperone activity (11). We asked whether kirromycin might inhibit rhodanese through interactions with denatured rhodanese rather than through interactions with EF-Tu, because kirromycin inhibited EF-Tumt-assisted refolding of rhodanese (data not shown) without targeting EF-Tumt (37). We found that kirromycin inhibited rhodanese refolding in the presence of pyruvate kinase (Fig. 4C, PK + kirromycin). Me2SO alone had no inhibitory effect (Fig. 4C, PK + DMSO). This indicates that kirromycin inhibits rhodanese refolding through interactions with denatured rhodanese rather through interactions with EF-Tu, which would lock EF-Tu in its closed conformation. Thus, there is no evidence that flexing is important for EF-Tu chaperone activity, at least in the case of EF-Tumt and E. coli EF-Tu.

Taken together, we suggest that (i) both EF-Tu-GDP (open form) and EF-Tu-GTP (closed form) are able to interact with unfolded proteins (Fig. 3, A and B); (ii) EF-Tu-GTP (closed form) may interact with unfolded proteins to a somewhat lesser extent than EF-Tu-GDP (Fig. 4, A and B) (9); and (iii) flexing of EF-Tu between EF-Tu-GDP (open form) and EF-Tu-GTP (closed form) is not required for its chaperone activity, at least when unfolded protein binding to EF-Tu-GDP is relatively weak (Figs. 3A, 3B, and 4C) (11).

EF-Tumt Specifically Interacts with Misfolded, Newly Synthesized Polypeptides from Mitochondrial Ribosomes—After demonstrating that EF-Tumt interacts with unfolded proteins in vitro, we examined whether EF-Tumt interacts with unfolded proteins in mitochondria. There are several possible unfolded proteins that could interact with EF-Tumt in mitochondria. These include proteins that are denatured following stress, proteins undergoing importation into mitochondria from the cytosol, and nascent polypeptides on the mt ribosome. Rabbit reticulocyte eEF1A has been identified as a protein that inter-

![FIGURE 3. Chaperone activity of EF-Tu is independent of guanine nucleotide.](image)

Denatured rhodanese (final concentration, 0.2 μM) was incubated at 20°C without EF-Tu (open circle) or in the presence of 1 μM EF-Tu without guanine nucleotide (black triangle, Tu[GNP-]) or with 1 mM GDP (black circle, Tu[GDP+]) or 1 mM GTP (black square, Tu[GTP+]). The activity of the refolded enzyme was measured at the indicated time points and expressed as a percentage of the activity of the same amount of native enzyme incubated at 20°C under the same conditions. A, EF-Tumt; B, E. coli EF-Tu.

![FIGURE 4. Influence of EF-Tu-GDP and EF-Tu-GTP on the refolding of rhodanese.](image)

Denatured rhodanese (final concentration, 0.3 μM) was incubated at 20°C in the presence of 1 μM EF-Tu-GDP (black circle, EF-Tu GDP) or 1 μM EF-Tu-GTP (open circle, EF-Tu GTP). The activity of the refolded enzyme was measured at the indicated time points and the results are expressed as the EF-Tu-dependent stimulation of rhodanese renaturation. EF-Tu-GDP and EF-Tu-GTP were prepared as described under “Materials and Methods.” A, EF-Tumt; B, E. coli EF-Tu; C, the effect of pyruvate kinase on rhodanese refolding. Denatured rhodanese (final concentration, 0.3 μM) was incubated at 20°C in the presence of pyruvate kinase alone (open circle, PK), pyruvate kinase plus kirromycin (black circle, PK + kirromycin), or pyruvate kinase plus Me2SO (cross, PK + DMSO). The activity of the refolded enzyme was measured at the indicated time points, and the results are expressed as the pyruvate kinase-dependent stimulation of rhodanese renaturation. The amount of pyruvate kinase used was the same as in the assay mixture with EF-Tu-GTP in A and B.

EF-Tumt-GDP was significantly active in substrate refolding, whereas the EF-Tumt-GTP form had little activity (Fig. 4A). A similar result was obtained with E. coli EF-Tu (Fig. 4B). It is important to note here that pyruvate kinase was present in the refolding reaction with EF-Tu-GTP and that pyruvate kinase itself had rhodanese refolding activity (Fig. 4C, PK). As a result, the EF-Tu-GTP-dependent stimulation of rhodanese refolding is underestimated due to the background refolding activity of pyruvate kinase. It is difficult to assess precisely how much less active EF-Tu-GTP is compared with EF-Tu-GDP in this assay system. Considering the results in Fig. 3, it remains possible that the chaperone activity of EF-Tu-GTP is approximately equal to that of EF-Tu-GDP.
FIGURE 5. EF-Tumt interacts with misfolded, newly synthesized polypeptides. A, mitochondrial gene products were labeled by in organello translation, using mitoplasts from hEF-Tumt WT-overexpressing cells (WT), hEF-Tumt[D156N]-overexpressing cells (D156N), and 293T cells stably transfected with pcDNA3.1 (mock), at different temperatures (25 °C and 42 °C). Both hEF-Tumt WT and hEF-Tumt[D156N] were expressed as C-terminal 3×FLAG-tagged proteins. After in organello translation, mitochondrial extracts were immunoprecipitated with FLAG-M2 antibody for hEF-Tumt (Tu) and with a non-related antibody (anti-F1-ATPase-β) for the control (ctl). Total (total) and immunoprecipitated (IP) samples were subjected to SDS-PAGE and visualized by BAS5000 using an Imaging Plate (Fujifilm). β, the total and immunoprecipitated samples in A were subjected to Western blotting with F1-ATPase-β antibody (upper panel) and EF-Tumt antibody (lower panel). IB, immunoblotting. White and black arrows indicate FLAG-tagged EF-Tumt and endogenous EF-Tumt, respectively.

B, EF-Tumt-overexpressing cells were established as described under “Experimental Procedures.” Usually, FLAG-tagged EF-Tumt is overexpressed 1- to 2-fold over endogenous EF-Tumt, as assessed by immunoblotting of mitochondrial extracts from each cell line with anti-EF-Tu antibody (Fig. 3B, total). Isolated mitoplasts from FLAG-tagged EF-Tumt-overexpressing cells were incubated in the translation buffer in the presence of [35S]methionine to label newly synthesized polypeptides. After in organello translation, mitoplasts were lysed and FLAG-tagged EF-Tumt was immunoprecipitated.

Theoretically, 13 polypeptides that are encoded by mitochondrial DNA should have been labeled during in organello translation. However, extra polypeptide bands with molecular weights that did not correspond to mitochondrial DNA-encoded proteins were observed (Fig. 5A). These might be either proteolytic products or SDS-resistant aggregates of newly synthesized polypeptides, as observed with in organello translation with yeast mitochondria (38). The specific labeling of newly synthesized polypeptides was confirmed by two methods: (i) chloramphenicol specifically inhibited the labeling of newly synthesized polypeptides during in organello translation, whereas cyclohexamide did not (supplemental data); and (ii) after in organello translation, the mitoplasts were lysed, and two mt oxidative phosphorylation-related proteins, COII and F1-ATPase-β, were immunoprecipitated. Analysis of the immunoprecipitation adduct revealed that the mitochondrial DNA-encoded COII was labeled, whereas the nuclear DNA-encoded F1-ATPase-β was not (supplemental data).

When in organello translation was carried out at 25 °C, the labeled, newly synthesized polypeptides did not co-immunoprecipitate with EF-Tumt (Fig. 5A, left, 25 °C, IP). In contrast, when translation was carried out at 42 °C, significant amounts of labeled polypeptides were co-immunoprecipitated with EF-Tumt (Fig. 5A, right, 42 °C, IP, lanes Tu). It is unlikely that these labeled polypeptides co-aggregated with FLAG-tagged EF-Tumt, because EF-Tumt is stable (Fig. 1) and active at 42 °C (Fig. 5A, total), and immunoprecipitation was carried out after the mitochondrial lysate had been centrifuged to remove aggregates. The co-immunoprecipitation of the labeled polypeptides was specific for EF-Tumt, because little co-immunoprecipitation by an unrelated antibody, anti-F1-ATPase-β, was observed at 42 °C (Fig. 5A, right, 42 °C, IP, lanes ctl).

To assess the results of immunoprecipitation of FLAG-tagged EF-Tumt and the control protein F1-ATPase-β, each immunoprecipitation adduct was analyzed by immunoblotting using anti-EF-Tumt antibody and anti-F1-ATPase-β, respectively (Fig. 5B, IP: FLAG-tagged EF-Tumt, lower panel; F1-ATPase-β, upper panel). It was confirmed that the differences in the amounts of labeled polypeptides co-immunoprecipitated with FLAG-tagged EF-Tumt were not due to differences in the efficiency of immunoprecipitation.

It has been reported that the yeast eEF1A mutant, eEF1A[D156N], is more effective at eliminating damaged proteins due to its enhanced interaction with misfolded peptides (14). The corresponding aspartic acid (Asp) in EF-Tumt, which is generally a conserved residue in EF-Tu, was mutated to asparagine (Asn), and in organello translation was carried out using mitoplasts from the EF-Tumt[D156N]-overexpressing cell line. Consistent with the above report, we observed enhanced interaction between the mutant EF-Tumt[D156N] and newly synthesized polypeptides at 42 °C (Fig. 5A, right, 42 °C, IP, D156N).

These observations indicate that EF-Tumt specifically interacts with newly synthesized polypeptides, presumably when they cannot fold correctly under stress conditions, such as heat stress (see also Fig. 8). At present, it is not clear whether the
interaction of EF-Tumt with misfolded, newly synthesized polypeptides is co-translational or post-translational. The interaction was slightly enhanced when co-immunoprecipitation was carried out after puromycin treatment (data not shown). This may imply that EF-Tumt interacts with misfolded newly synthesized polypeptides post-translationally. It is consistent with the chaperone-like activity of EF-Tumt observed in vitro (Figs. 1 and 2).

**Newly Synthesized Polypeptides Are Destabilized at High Temperature in the EF-Tumt-overexpressing Cell—Yeast eEF1A interacts with the nascent peptide on the ribosome and mediates the degradation of co-translationally damaged protein by the proteasome (14). We demonstrated that EF-Tumt interacts with unfolded proteins, especially with misfolded, newly synthesized polypeptides in mitochondria (Fig. 5). Next, we investigated whether EF-Tumt is involved in the degradation of misfolded, newly synthesized polypeptides in mitochondria, where it would play a role similar to that of eEF1A.

The stability of newly synthesized polypeptides in mitochondria was investigated by pulse-chase analysis. In organello translation was carried out for 1 h at 25 °C. The mitoplasts were further incubated in the translation buffer at different temperatures (25 °C, left panel; 42 °C, right panel) for the indicated time periods, subjected to SDS-PAGE, and visualized by BAS5000 (Fujifilm).

**FIGURE 6.** Newly synthesized polypeptides are destabilized at high temperature in EF-Tumt-overexpressing cells, especially in EF-Tumt(D156N)-overexpressing cells. The stability of newly synthesized polypeptides in mitochondria was investigated by pulse-chase analysis. Mitochondrial gene products were labeled by in organello translation for 1 h at 25 °C, using equal amounts of mitoplasts from HEF-Tumt(WT)-overexpressing cells (WT), HEF-Tumt(D156N)-overexpressing cells (D156N), and 293T cells stably transfected with pcDNA3.1 (mock). After the labeling was stopped by extensive washing, mitoplasts were further incubated in the translation buffer at different temperatures (25 °C, left panel; 42 °C, right panel) for the indicated time periods, subjected to SDS-PAGE, and visualized by BAS5000 (Fujifilm).

**FIGURE 7.** EF-Tumt associates with the mitochondrial inner membrane, independent of mitochondrial ribosomes. A, the nature of the interaction between EF-Tumt and the mitochondrial inner membrane was analyzed by sub-fractionation of mitochondria. Mitoplasts were sonicated in MSED buffer in the presence of KCl (0, 250, or 500 mM) and urea (6 M), and the matrix and inner membrane fractions were separated by centrifugation. Each fraction was analyzed by Western blotting for HSP60 (an inner membrane-associated protein), COI (an inner membrane-embedded protein), EF-Tumt, and EF-Tsmt. B, sub-fractionation of mitoplasts was carried out in the presence of RNase A. Each matrix and inner-membrane fraction was analyzed by Western blotting for HSP60, COI, EF-Tumt, and Ef-Tsmt. C, the complete digestion of mitochondrial ribosomes by RNase A in B was confirmed by Northern blotting for mitochondrial 16 S ribosomal RNA.
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EF-Tumt Associates with the Inner Mitochondrial Membrane, Independently of the mt Ribosome—Most mt ribosomes are associated with the mt IM (32). To obtain more insight into the in vivo function of EF-Tumt chaperone activity, we analyzed the interaction of EF-Tumt with the mt IM and with mt ribosomes by submicrohondrial fractionation.

Purified mitoplasts were subfractionated into the IM and matrix. The separation of each compartment was verified by immunoblotting the subfractions for mt compartment-specific proteins, using antibodies against COIV, an IM-embedded protein, and HSP60, an IM-associated protein. Subfractionation was carried out in the presence of KCl (0–500 mM) and in the presence of 6 M urea, to determine the nature of the interactions of the proteins with the IM. COIV was found in the IM fraction, regardless of the salt concentration, and was partially released into the matrix in the presence of urea (Fig. 7A, COIV). HSP60 was found partially in the IM fraction, regardless of the salt concentration, and was completely released into matrix in the presence of urea (Fig. 7A, HSP60). The association of these proteins with the IM was governed by hydrophobic interactions, because salt had little effect. EF-Tumt was found predominantly in the IM fraction in the absence of salt and was released into the matrix at higher salt concentrations. Almost all of EF-Tumt was found in the matrix in the presence of urea. These results indicate that, like HSP60, EF-Tumt associates with the IM under physiological conditions (~150 mM KCl) but by a combination of ionic and hydrophobic interactions. It is noteworthy that EF-Tsmt, the guanine nucleotide exchange factor of EF-Tumt, preferentially resides in the matrix under physiological conditions (Fig. 7A, EF-Tsmt).

Because, like most mt ribosomes (32), EF-Tumt associated with the IM, we examined whether the interaction of EF-Tumt with the IM was mediated by mt ribosomes. After subfractionation in the presence or absence of RNase A, mt ribosomes were detected by Northern blotting for 16 S ribosomal RNA (32). As shown in Fig. 7C, RNase A completely digested the mt ribosomes in the IM. However, a significant amount of EF-Tumt was still associated with the IM regardless of RNase A treatment (Fig. 7B). These results indicate that EF-Tumt associates with the mt IM under physiological conditions and that this association is independent of mt ribosomes.

Biological Role of EF-Tumt Chaperone Activity in Mitochondria—In this study, we show that mammalian EF-Tumt displays chaperone properties. EF-Tumt prevents thermal aggregation of proteins (Fig. 1) and enhances protein refolding (Fig. 2) in vitro. The chaperone activity of EF-Tumt is GTP-independent (Figs. 3 and 4). EF-Tumt interacts with misfolded newly synthesized peptides from the mt ribosome (Fig. 5), and the newly synthesized polypeptides are destabilized in EF-Tumt-overexpressing cells when mitochondria are exposed to heat stress (Fig. 6). Most EF-Tumt localizes to the mt IM, where most mt ribosomes are found (Fig. 7).

Based on these observations, as well as on the in vivo chaperone function of eEF1A (14), we hypothesize that EF-Tumt exercises chaperone activity in mitochondria in the following manner (Fig. 8). Under stress conditions, EF-Tumt may recruit misfolded, newly synthesized peptides to an mt protease complex near the ribosome. Because EF-Tumt is able to interact with unfolded proteins post-translationally, it may also recruit damaged proteins to the mt protease complex.

eEF1A binds to actin filaments and microtubules both in vitro and in vivo (39), in a manner that is reminiscent of the ability of chaperones to control the aggregation state of multimeric proteins (40, 41). The binding of eEF1A to actin and aminoacyl-tRNA is mutually exclusive (42). The possible competition between aminoacyl-tRNAs and unfolded protein for EF-Tumt awaits future analysis. However, it would be reasonable to assume that EF-Tumt acts as a translation factor in an EF-Tumt-GTP-aminoacyl-tRNA complex under normal conditions, whereas EF-Tu-GDP, whose level increases under stress conditions, would act as a chaperone.

eEF1A is involved in protein quality control through its interaction with proteasomes (14). Prokaryotes and mitochondria lack proteasomes. Thus, EF-Tumt might be involved in an mt protein quality-control system that is different from that of eEF1A in eukaryotes. EF-Tu, together with GroEL, ClpA, etc.,
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has been purified as an Hsp31-interacting protein from E. coli (43). It has been suggested that EF-Tu, in cooperation with Hsp31, the GroEL/ES chaperone system, and the ClpA/ClpP protease system, is involved in protein/peptide degradation. The human mt homologues of GroEL, ClpP, and Hsp31 are HSP60, hClpP, and DJ-1, respectively. Therefore, it might be possible that EF-Tumt interacts with an mt protease complex as depicted in Fig. 8. Pim 1 is the main protease responsible for the degradation of soluble matrix proteins in mitochondria (44). The ATP-dependent m-AAA protease is localized in the mt inner membrane, where its proteolytic site is exposed to the matrix. It is implicated in the biogenesis of inner membrane protein complexes and in the degradation of non-assembled subunits (45). Pim 1 and m-AAA protease are candidate interaction partners of EF-Tumt. To extend our hypothesis concerning EF-Tumt chaperone activity in mitochondria, we are currently investigating EF-Tumt-interacting proteins.

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