

Characterization of the interaction between the nucleotide exchange factor EF-Ts from nematode mitochondria and elongation factor Tu

Takashi Ohtsuki, Masayuki Sakurai, Aya Sato and Kimitsuna Watanabe*

Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan

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ABSTRACT

Caenorhabditis elegans mitochondria have two elongation factor (EF)-Tu species, denoted EF-Tu1 and EF-Tu2. Recombinant nematode EF-Ts purified from *Escherichia coli* bound both of these molecules and also stimulated the translational activity of EF-Tu, indicating that the nematode EF-Ts homolog is a functional EF-Ts protein of mitochondria. Complexes formed by the interaction of nematode EF-Ts with EF-Tu1 and EF-Tu2 could be detected by native gel electrophoresis and purified by gel filtration. Although the nematode mitochondrial (mt) EF-Tu molecules are extremely unstable and easily form aggregates, native gel electrophoresis and gel filtration analysis revealed that EF-Tu-EF-Ts complexes are significantly more soluble. This indicates that nematode EF-Ts can be used to stabilize homologous EF-Tu molecules for experimental purposes. The EF-Ts bound to two eubacterial EF-Tu species (*E.coli* and *Thermus thermophilus*). Although the EF-Ts did not bind to bovine mt EF-Tu, it could bind to a chimeric nematode–bovine EF-Tu molecule containing domains 1 and 2 from bovine mt EF-Tu. Thus, the nematode EF-Ts appears to have a broad specificity for EF-Tu molecules from different species.

INTRODUCTION

Mitochondria from the nematode are known to have an unusual translation system that employs two types of extremely truncated tRNAs (1–4), namely the T arm-lacking (5) and the D arm-lacking tRNAs (6), and their corresponding elongation factor (EF)-Tu species that have been denoted EF-Tu1 (7) and EF-Tu2 (8), respectively. These EF-Tu molecules have been characterized and it is known that EF-Tu1 has a C-terminal extension of about 57 amino acids (7) not found in any other known EF-Tu. In addition, EF-Tu2 has a unique specificity for the aminoacyl moiety of seryl-tRNA (8). In contrast, little is known about nematode mitochondrial (mt) EF-Ts, a factor that facilitates the catalytic use of EF-Tu by

promoting the exchange of GDP for GTP on EF-Tu (9). At present, only the cDNA sequence of the EF-Ts homolog in *Caenorhabditis elegans* has been reported (10). This cDNA sequence reveals that the nematode mt EF-Ts protein bears a putative mitochondria-specific transit peptide sequence at its N-terminus (10). On the basis of amino acid sequence homology, the nematode mt EF-Ts protein appears to fall into the long EF-Ts category, which includes EF-Ts molecules from mammalian mitochondria and eubacteria (excluding *Thermus* and cyanobacteria), rather than into the short EF-Ts category, which includes EF-Ts proteins from *Thermus*, cyanobacteria and plastids (10). These observations indicate that we can expect the nematode EF-Ts homolog to act like the well-characterized bovine mt EF-Ts (11–16) or *Escherichia coli* EF-Ts (17,18) molecule. This is particularly the case with respect to the former, since the nematode mt EF-Ts amino acid sequence shares more homology with bovine mt EF-Ts than with the *E.coli* EF-Ts.

The interaction between EF-Tu and EF-Ts was analyzed in detail by X-ray analysis of the crystal structure of EF-Tu-EF-Ts complexes of *E.coli* (17) and *Thermus thermophilus* (19). Nematode EF-Ts is more homologous to *E.coli* EF-Ts than to *T.thermophilus* EF-Ts. However, *C.elegans* EF-Ts has only 24% amino acid identity with that of *E.coli* (10). As for the amino acid residues of *E.coli* EF-Ts that have been shown to interact with *E.coli* EF-Tu (17), only a few positions, such as Arg12, Asp80, Phe81, Gly126 and His149 (*E.coli* numbering) are conserved in nematode EF-Ts (10). Most of these residues occur in the N-terminal half of EF-Ts. Residues in the C-terminal half of *E.coli* EF-Ts that interact with EF-Tu domain 3 (17) are poorly conserved in *C.elegans* EF-Ts. Thus, as in the interaction between EF-Ts and EF-Tu of *E.coli*, the N-terminal half of *C.elegans* EF-Ts may interact with domain 1 of EF-Tu, whereas the interaction involving domain 3 may be quite different in *C.elegans* EF-Ts.

The activity and binding specificity for EF-Tu of bovine mt EF-Ts has been well characterized (12,13,16,20). Bovine mt EF-Ts forms an extremely tight complex with bovine mt EF-Tu (16,20). Furthermore, when bovine mt EF-Ts was expressed in *E.coli*, it was found to form a stable complex with *E.coli* EF-Tu (12,13). In contrast, *E.coli* EF-Ts does not seem to be able to bind bovine mt EF-Tu since a recombinant bovine mt EF-Tu expressed in *E.coli* could be purified as a free protein separate from *E.coli* EF-Ts (21). Work with

*To whom correspondence should be addressed. Tel/Fax: +81 471 36 3606; Email: kw@kwl.t.u-tokyo.ac.jp

E. coli-bovine EF-Ts chimeras also revealed that the N-terminal half of bovine mt EF-Ts, which includes the N-terminal domain and the subdomain N in the core domain, is important for its tight binding to EF-Tu (14).

The activity and EF-Tu binding specificity of the *C. elegans* EF-Ts homolog has not been previously investigated. In this work, we confirmed that this molecule is a proper EF-Ts as it is able to stimulate the guanine nucleotide exchange and the translational activity of EF-Tu and bind to both of the *C. elegans* mt EF-Tu proteins. We were able to purify complexes formed between *C. elegans* mt EF-Tu and EF-Ts, and found that these complexes are much more soluble than that of *C. elegans* EF-Tu alone. This suggests that *C. elegans* mt EF-Ts could be used as a tool to stabilize EF-Tu. Studies of the binding of *C. elegans* mt EF-Ts to various EF-Tu molecules revealed the broad specificity range of the EF-Ts.

MATERIALS AND METHODS

Buffers

Buffer A contained 50 mM HEPES-KOH (pH 7.5), 150 mM KCl, 10 mM MgCl₂, 1% glycerol, 5 mM β-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride. Buffer C contained 50 mM HEPES-KOH (pH 7.5), 1 M NH₄Cl, 10 mM imidazole, 1% glycerol and 5 mM β-mercaptoethanol. HiQ-A buffer consisted of 20 mM Tris-HCl (pH 7.7), 5 mM MgCl₂, 50 mM KCl, 1% glycerol and 1 mM dithiothreitol (DTT). HiQ-B buffer is similar to the HiQ-A buffer except that the concentration of KCl is 500 mM. Tu-Ts buffer contained 50 mM Tris-HCl (pH 7.5), 150 mM KCl, 5 mM EDTA, 3% glycerol and 1 mM DTT. PD buffer contained 50 mM HEPES-KOH (pH 7.6), 150 mM KCl, 10% glycerol and 5 mM β-mercaptoethanol.

Construction of a plasmid for the expression of the *C. elegans* EF-Ts homolog bearing a C-terminal His tag

The plasmid pET-yk141g2 contains the cDNA sequence of the predicted mature *C. elegans* EF-Ts homolog, i.e. it encodes a protein comprised of amino acids Ala21-Glu316 from its precursor sequence (10). It was constructed by placing the cDNA between the *Nco*I and *Xho*I sites of pET-15b (Novagen) and was kindly provided by Dr Y. Watanabe, who prepared it from the cDNA clone yg141g2 originally provided by Dr Y. Kohara (National Institute of Genetics, Japan). pET-yk141g2 was used to construct an expression vector encoding the *C. elegans* EF-Ts homolog bearing a C-terminal His tag. To add the six residue histidine tag to the EF-Ts C-terminus, the 17 bp sequence at the end of the EF-Ts coding sequence in pET-yk141g2 (5'-TAATTAGATAAAAGTGG-3' in the coding strand, which is followed by the stop codon TAG) was replaced by the 18 bp sequence 5'-CACCATCATCAT-CATCAT-3' (in the coding strand) using the QuickChange site-directed mutagenesis kit (Stratagene) according to the supplier's manual.

Expression and purification of the *C. elegans* EF-Ts homolog

The *E. coli* strain BL21(DE3)pLysS (22) was transformed by the expression vector encoding the *C. elegans* EF-Ts homolog and grown and harvested as described (7). All purification

procedures as described below were performed at 4°C. Cellular pellets (~30 g) were resuspended with 30 ml of buffer A and lysed by sonication. The paste was then centrifuged for 1 h at 45 000 r.p.m. with a 70Ti rotor (Beckman) and the supernatant was collected and applied to a HiTrap chelating column (5 ml) (Amersham Biosciences) previously charged with Ni²⁺ ions and equilibrated with buffer A. The column was washed with 30 ml of buffer C and then with 30 ml of buffer A containing 25 mM imidazole. Elution was performed using an imidazole gradient ranging from 25 to 150 mM in buffer A applied at a flow rate of 0.5 ml/min. The fraction that included the EF-Ts was dialyzed against 500 ml of HiQ-A buffer for 4 h with two buffer changes. The dialyzed fraction (15 ml) was loaded onto an Econo-Pac High Q Cartridge (1 ml) (Bio-Rad) at a flow rate of 0.5 ml/min and the column was then rinsed with 5 ml of HiQ-A buffer at an equivalent flow rate. The flow-through, which now contained pure EF-Ts, was collected. The protein concentration was estimated by the dye-binding assay employed by the Protein Assay Kit (Bio-Rad). Bovine serum albumin (BSA) was used as the standard.

Preparation of EF-Tu variants

Recombinant proteins of *C. elegans* mt EF-Tu1 and EF-Tu2, bovine mt EF-Tu, *T. thermophilus* EF-Tu and the two chimeric nematode-bovine EF-Tu variants BmCe3' and BmCe3 were expressed in *E. coli* and purified on a Ni²⁺-NTA agarose column (Qiagen) as described (7,8). *Caenorhabditis elegans* mt EF-Tu1 bearing a C-terminal His tag was prepared using an expression vector containing N-terminal His-tagged cDNA encoding the predicted mature sequence of EF-Tu1 (a protein comprised of the Gly39-Pro496 sequence of its precursor protein) (7). This vector was amplified by PCR and inserted between the *Sph*I and *Bgl*II sites of pQE-70 (Qiagen), which was then used to transform *E. coli* strain BL21. The recombinant protein bearing the additional amino acid sequence RSHHHHH at its C-terminus was then expressed and purified as described (7). *Escherichia coli* EF-Tu was purified from its native source as described (23). The protein concentration of each EF-Tu was estimated using the Protein Assay Kit (Bio-Rad) with BSA as the standard.

Measurement of guanine nucleotide exchange with EF-Tu

The assay was performed basically according to Schwartzbach and Spemulli (20). Briefly, 80 pmol EF-Tu and various amounts of EF-Ts were incubated for 10 min at 37°C in a final volume of 50 μl reaction mixture containing 25 mM Tris-HCl (pH 7.6), 50 mM NH₄Cl, 10 mM MgCl₂ and 50 μM [³H]GDP, then the [³H]GDP bound to EF-Tu in each sample was counted as described (20).

In vitro translation

Bovine mt translation factors, namely ribosomes (prepared as described; 24), EF-G [partially purified according to Chung and Spemulli (25)] and tRNA^{Phe} were kindly provided by Dr C. Takemoto. Poly(U)-dependent poly(Phe) synthesis was performed *in vitro* using these translation factors as described (25) except that 9 pmol [¹⁴C]Phe-tRNA of bovine mitochondria was used in each 20 μl reaction mixture and the concentrations of EF-Tu and EF-Ts were 0.8 and 0.5 μM,

respectively. The radioactivity retained on the filter in the absence of EF-Tu and EF-Ts (blank) was subtracted from the radioactivity obtained in the presence of EF-Tu (and EF-Ts) in each assay.

Native gel electrophoresis

Native gel electrophoresis of EF-Tu and EF-Ts was performed at 4°C (450 V, 2 h) on a 1 mm × 10 cm × 10 cm 9% polyacrylamide gel (acrylamide:bisacrylamide 29:1) containing 8 mM Tricine–NaOH (pH 8.2), 1 mM EDTA and 5% glycerol. The buffer used contained 8 mM Tricine–NaOH (pH 8.2) and 1 mM EDTA. To analyze the interaction between EF-Tu and EF-Ts, recombinant EF-Tu and EF-Ts were mixed on ice in a reaction mixture of 6.5 μl that included 50 mM Tris–HCl (pH 7.5), 70 mM KCl, 1.5 mM EDTA, 10% glycerol, 1 mM DTT, 0.004% bromophenol blue, 38 pmol EF-Ts and either 12.5 or 25 pmol EF-Tu. Each reaction was then loaded on the gel, electrophoresed, and stained with Coomassie brilliant blue R-250 (NACALAI Tesque Inc.).

EF-Ts pull-down assay

His-tagged *C.elegans* mt EF-Ts (2 μg/tube) in the absence or presence of *E.coli*, *T.thermophilus* or bovine mt EF-Tu (4 μg/tube) were incubated at 4°C for 10 min in 50 μl of PD buffer. The mixture was centrifuged at 17 000 g for 7 min. The supernatant was mixed with Ni²⁺-NTA magnetic agarose beads (Qiagen) (20 μl of 5% bead slurry suspended in PD buffer) and incubated at 4°C for 15 min with weak agitation. The beads were collected using a magnet and washed twice with 400 μl of PD buffer containing 10 mM imidazole, then eluted twice with 30 μl of buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM KCl, 5 mM EDTA, 10% glycerol, 400 mM imidazole and 5 mM β-mercaptoethanol. The eluents were desalted on a Centri-Sep spin column (Applied Biosystems) and concentrated to 5 μl. The samples were analyzed by SDS–PAGE.

Analysis and purification of the *C.elegans* mt EF-Tu·EF-Ts complexes by gel filtration

Caenorhabditis elegans mt EF-Tu purified on a Ni²⁺-NTA column (7,8) and the EF-Ts purified as described above were mixed at a molar ratio of 1:1.5. The mixture was dialyzed against Tu·Ts buffer for 15 h with two buffer changes. The sample was concentrated to less than 1 ml using an Ultrafree-15 centrifugal filter device (Millipore) and the insoluble fraction was removed by centrifugation. The sample was loaded on a HiPrep 16/60 Sephacryl S-200 column (Amersham Biosciences) at a flow rate of 0.5 ml/min and eluted with Tu·Ts buffer. Each of the gel filtrated fractions was analyzed by SDS–PAGE.

RESULTS

Expression and purification of the *C.elegans* EF-Ts homolog

Although the recombinant *C.elegans* EF-Ts homolog expressed in *E.coli* cells was almost separated from *E.coli* proteins by use of the Ni²⁺-HiTrap chelating column (Fig. 1, lanes 4 and 7) and repeated column washes (Fig. 1, lanes 2 and 3), the preparation still contained some impurities. One protein,

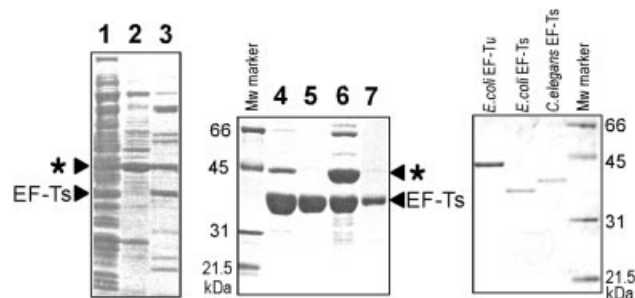


Figure 1. SDS–PAGE analysis of the recombinant *C.elegans* EF-Ts preparations at various purification steps using Ni²⁺ column and HighQ column chromatography. Lane 1, the flow-through after loading the sample onto a Ni²⁺ column; lane 2, the first wash with buffer C; lane 3, the second wash with buffer A that included 25 mM imidazole; lanes 4 and 7, the Ni²⁺ column fraction that included the EF-Ts homolog [To detect possible impurities, lane 4 was overloaded with the sample. To show that there is no second protein co-migrating with *C.elegans* EF-Ts, such as *E.coli* EF-Ts, a smaller volume of sample (one-eighth of the amount loaded onto lane 4) was loaded in lane 7]; lane 5, the flow-through after loading the sample from the Ni²⁺ column onto the HighQ column; lane 6, the wash with HiQ-B buffer. The SDS–PAGE standard (Bio-Rad), purified *E.coli* EF-Tu and recombinant *E.coli* EF-Ts were used as protein size markers.

shown by an asterisk in Figure 1, which apparently associates with the EF-Ts homolog, was particularly difficult to remove. The size of this protein suggested that it could be *E.coli* EF-Tu (~43.2 kDa). This EF-Tu-like protein could eventually be removed by passing the 15 ml Ni²⁺-HiTrap preparation containing 72 mg protein (Fig. 1, lane 4) through a HighQ column. After running through 5 ml of HiQ-A buffer, most of the loaded EF-Ts was recovered (~60 mg) (Fig. 1, lane 5) in pure form. After that, the column was washed with 5 ml of HiQ-B buffer; the fraction eluted with the buffer contained the impurities as well as the EF-Ts (~11 mg) (Fig. 1, lane 6). Pure materials (lane 5) were used in all assays (Figs 2–6).

Nematode mt EF-Ts can stimulate guanine nucleotide exchange and *in vitro* translation

To verify that the *C.elegans* EF-Ts homolog is indeed an EF-Ts, we first examined its ability to stimulate poly(U)-dependent poly(Phe) synthesis mediated by EF-Tu molecules. A bovine mt *in vitro* translation system was used because a homologous nematode mt translation system is as yet unavailable. The EF-Ts homolog stimulated poly(U)-dependent poly(Phe) synthesis activity of *T.thermophilus* EF-Tu but not bovine mt EF-Tu (Fig. 2A). The poly(Phe) synthesis activity of bovine mt EF-Tu can be activated by bovine mt EF-Ts (13). Poly(Phe) synthesis using mt EF-Tu does not appear to be maximal in this assay because the addition of increased amounts of bovine mt EF-Tu increased poly(Phe) synthesis (data not shown). Thus, the observation that the EF-Ts homolog did not change the activity of bovine mt EF-Tu cannot be explained by the presence of an already saturating amount of mt EF-Tu activity in this assay. As will be seen, this differential activity of the *C.elegans* EF-Ts homolog is consistent with its EF-Tu binding specificity, as it complexes with *T.thermophilus* EF-Tu but cannot bind bovine mt EF-Tu (see below). The observation that the *C.elegans* EF-Ts homolog stimulates the translational activity of *T.thermophilus* EF-Tu agrees with the results of filter binding

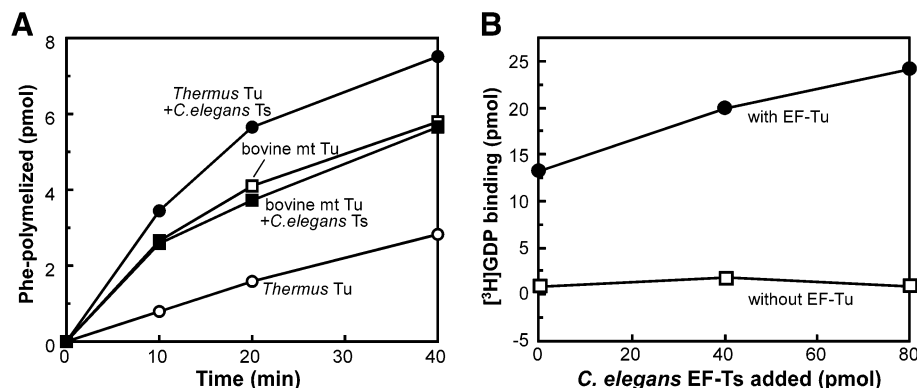


Figure 2. Stimulation of the activity of EF-Tu by the *C.elegans* EF-Ts homolog. (A) *In vitro* poly(U)-dependent poly(Phe) synthesis (25) was performed using bovine mt ribosome (2.0 A₂₆₀ units/ml, 125 U/ml, 63.4 nM), EF-G [2500 U/ml (saturated amount); unit definition as in Chung and Spremulli (25)] and bovine mt Phe-tRNA^{Phe} in the presence of *T.thermophilus* EF-Tu alone (open circle), *T.thermophilus* EF-Tu plus *C.elegans* EF-Ts homolog (filled circle), bovine mt EF-Tu alone (open square) or bovine mt EF-Tu plus *C.elegans* EF-Ts homolog (filled square). Aliquots of the reaction mixture (18 μl) were withdrawn at appropriate times and the radioactivity of the hot TCA-insoluble material was measured. (B) Stimulation of GDP exchange activity of EF-Tu by *C.elegans* EF-Ts. The EF-Ts stimulates the GDP exchange of *T.thermophilus* EF-Tu (filled circle). [³H]GDP binding to EF-Ts was not detected in the absence of EF-Tu (open square).

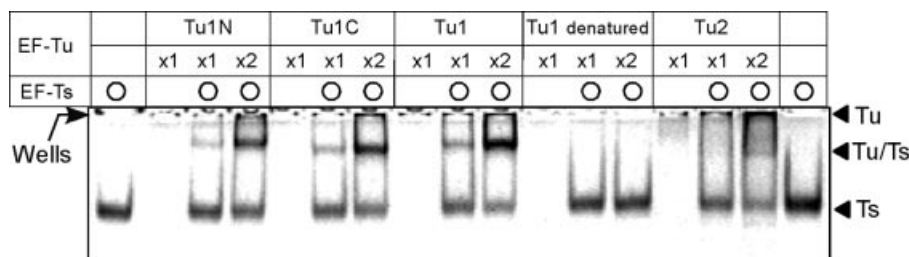


Figure 3. *Caenorhabditis elegans* mt EF-Ts can bind to nematode EF-Tu molecules. The ability of *C.elegans* mt EF-Ts to bind to *C.elegans* mt EF-Tu1 and EF-Tu2 was analyzed by loading a mixture of 38 pmol EF-Ts and/or 12.5 (×1) or 25 pmol (×2) of EF-Tu onto a native gel. Several different forms of EF-Tu1 were assessed, namely EF-Tu1 with an N-terminal His tag (Tu1N), EF-Tu1 with a C-terminal His tag (Tu1C), EF-Tu1 lacking a His tag (Tu1) and heat-denatured EF-Tu1 (Tu1 denatured). Heat denaturation of EF-Tu1 was performed at 90°C for 5 min. The positions of the wells are indicated by an arrow on the left side of the gel.

assays of EF-Tu-GDP (Fig. 2B). The assays demonstrated that the *C.elegans* EF-Ts homolog stimulates the GDP exchange of *T.thermophilus* EF-Tu (Fig. 2B). Unfortunately, GDP exchange with bovine and nematode mt EF-Tu was hardly detectable, even with nematode mt EF-Ts (data not shown). This is reminiscent of previous failure to detect GDP exchange with bovine mt EF-Tu even with bovine mt EF-Ts by the same assay (20). All these observations strongly suggest that the *C.elegans* EF-Ts homolog is indeed a functional EF-Ts.

Binding of nematode mt EF-Ts to nematode mt EF-Tu proteins

To assess whether *C.elegans* EF-Ts can bind to either or both *C.elegans* mt EF-Tu molecules (EF-Tu1 and EF-Tu2), the binding of recombinant *C.elegans* EF-Ts to recombinant Tu proteins was analyzed by native PAGE. In the absence of *C.elegans* EF-Ts, EF-Tu1 and EF-Tu2 stayed in the wells of the gel (Fig. 3). In contrast, when *C.elegans* EF-Ts was present, complexes were formed and these migrated into the gel (Fig. 3). While the band of the EF-Tu2-EF-Ts complex in the native PAGE gel is rather diffuse, later experiments with gel filtration chromatography clearly revealed the presence of the complex (Fig. 4C), thus confirming the association. With

regard to EF-Tu1, we assessed the ability of *C.elegans* EF-Ts to bind to EF-Tu1 bearing an N-terminal His tag (Tu1N), a C-terminal His tag (Tu1C) or no tag (Fig. 3). Heat-denatured EF-Tu1 was also tested. *Caenorhabditis elegans* EF-Ts bound to the EF-Tu1 molecules bearing N- or C-terminal His tags as well as to untagged EF-Tu1, which suggests that the N- and C-termini of EF-Tu1 are not important for its binding to *C.elegans* EF-Ts. Denatured EF-Tu1 did not bind to *C.elegans* EF-Ts, indicating that the native conformation of EF-Tu1 is necessary for its binding to *C.elegans* EF-Ts. Thus, *C.elegans* EF-Ts binds to both EF-Tu1 and EF-Tu2 of *C.elegans* mitochondria, demonstrating that nematode EF-Ts could be a functional EF-Ts in *C.elegans* mitochondria.

Reconstitution and isolation of *C.elegans* mt EF-Tu-EF-Ts complexes

The complexes formed between recombinant *C.elegans* mt EF-Ts and EF-Tu1 or EF-Tu2 could be efficiently separated from EF-Tu and EF-Ts proteins by gel filtration using a HiPrep Sephacryl S-200 column (Fig. 4A and C). The three peaks on the gel filtration chromatograms for EF-Tu1 (Fig. 4A) and EF-Tu2 (Fig. 4C) were analyzed by SDS-PAGE and the middle peak (denoted fractions 2 and 4) was found to comprise

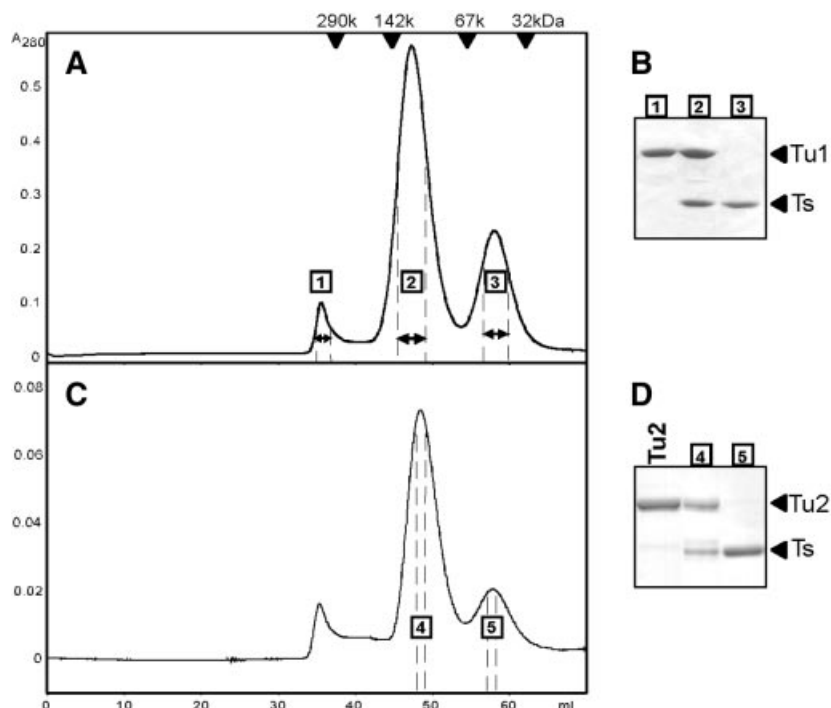


Figure 4. *Caenorhabditis elegans* mt EF-Tu-EF-Ts complexes can be purified by HiPrep Sephacryl S-200 column chromatography. (A) Gel filtration of *C. elegans* mt EF-Tu1 and EF-Ts. Peak positions of molecular weight marker proteins (Oriental Yeast Co.) are indicated. (B) Each peak in (A) was analyzed by SDS-PAGE. Before loading, 100 μ l of fraction 1 was concentrated to 5 μ l and 3 μ l aliquots of fractions 2 and 3 were loaded on the gel without concentrating the proteins. (C) Gel filtration of *C. elegans* mt EF-Tu2 and EF-Ts. (D) Each peak in (C) was analyzed by SDS-PAGE. Before loading, 10 μ l of fraction 4 and 50 μ l of fraction 5 were concentrated to 5 μ l solutions and loaded on the gel. The lane indicated by Tu2 contains *C. elegans* mt EF-Tu2 without a His tag (2 μ g).

the EF-Tu-EF-Ts complex (Fig. 4B and D). Native PAGE analysis of fraction 2 in the gel filtration chromatogram for EF-Tu1 confirmed that it contained EF-Tu1-EF-Ts complexes (data not shown). EF-Tu1 and EF-Tu2 were eluted faster than the EF-Tu-EF-Ts complexes, which suggests that these molecules aggregate in the Tu-Ts buffer in the absence of EF-Ts (Fig. 4A and C). Similar patterns were observed for EF-Tu1 and EF-Tu2 when gel filtration was performed using a Superdex 75 HR 10/30 (Amersham Biosciences) gel filtration column with the same buffer, although in this system the separation of EF-Tu-EF-Ts complexes from EF-Ts was poor (data not shown).

Ability of nematode mt EF-Ts to bind to different EF-Tu molecules

To characterize the binding specificity of *C. elegans* mt EF-Ts, its binding to heterologous EF-Tu proteins was analyzed. The addition of *C. elegans* EF-Ts to *E. coli* EF-Tu and *T. thermophilus* EF-Tu caused the EF-Tu bands to disappear and a new band containing the EF-Tu-EF-Ts complex to appear slightly below the EF-Ts band (Fig. 5A). This suggests that *C. elegans* mt EF-Ts can bind to both of these EF-Tu molecules. The complexes between bacterial EF-Tu molecules and *C. elegans* mt EF-Ts were further confirmed by pull-down assay (Fig. 5B). In contrast, *E. coli* EF-Ts did not appear to complex with either EF-Tu1 or EF-Tu2 from *C. elegans* (Fig. 5C). Thus, the binding specificity of *C. elegans* mt EF-Ts differs from that of *E. coli* EF-Ts (Fig. 5D).

Although *C. elegans* mt EF-Ts could bind to bacterial EF-Tu, it could not bind to bovine mt EF-Tu (Fig. 6), which was confirmed by pull-down assay (Fig. 5B) and gel filtration analysis (data not shown). The ability of *C. elegans* EF-Ts to bind to two chimeric EF-Tu constructs composed of bovine mt and nematode mt EF-Tu domains was also analyzed (Fig. 6). BmCe3 contains domains 1 and 2 of bovine mt EF-Tu while BmCe3' contains domain 1-3 of bovine mt EF-Tu. Both of these variants can bind to Met-tRNA^{Met} from *E. coli* or *C. elegans* mitochondria (7). Although the BmCe3-EF-Ts band was not separated from the BmCe3 band on the native PAGE gel, the intensity of the *C. elegans* EF-Ts band was reduced when BmCe3 was added, indicating that *C. elegans* mt EF-Ts binds to BmCe3 (Fig. 6). In contrast, *C. elegans* mt EF-Ts did not bind to the BmCe3' chimera (Fig. 6). These observations indicate that domains 1 and 2 of bovine mt EF-Tu do not prevent *C. elegans* mt EF-Ts binding, unlike domain 3 of bovine mt EF-Tu.

DISCUSSION

Structural features of *C. elegans* mt EF-Ts

EF-Ts molecules from *E. coli* and *T. thermophilus* have been well characterized. Crystals of these two molecules complexed with EF-Tu revealed that they interact with EF-Tu differently, as *E. coli* EF-Tu and EF-Ts form a heterodimer (Tu-Ts) (17,26) while the *T. thermophilus* factors form a dyad symmetric heterotetramer [Tu-(Ts)₂-Tu] (19,26,27). The

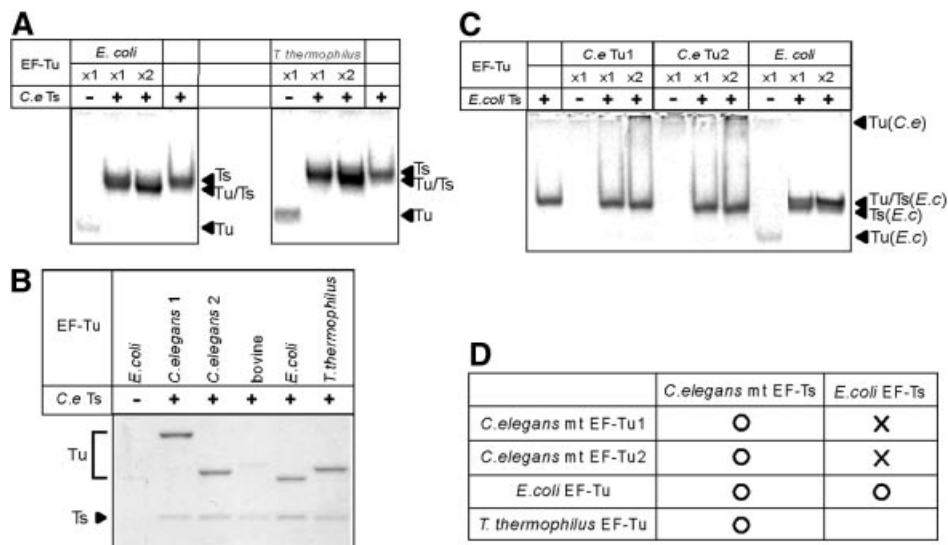


Figure 5. Analysis of binding specificity of *C.elegans* mt EF-Ts to various EF-Tu molecules. (A) Native gel analysis of *C.elegans* mt EF-Ts mixed with EF-Tu from *E.coli* or *T.thermophilus*. (B) *Caenorhabditis elegans* mt EF-Ts was pulled down by using Ni-NTA magnetic agarose beads in the presence of *C.elegans* mt, bovine mt or bacterial EF-Tu. The fractions pulled down were analyzed by 10% SDS-PAGE. (C) Native gel analysis of *E.coli* EF-Ts mixed with *C.elegans* mt EF-Tu1 or EF-Tu2 or *E.coli* EF-Tu. (D) Summary of the binding specificities of nematode mt and bacterial EF-Tu molecules to nematode mt and bacterial EF-Ts proteins.

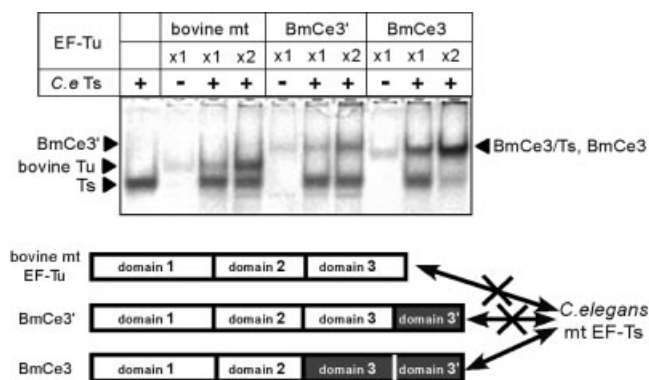


Figure 6. Native PAGE analysis of the ability of *C.elegans* mt EF-Ts to bind to bovine mt EF-Tu (bovine mt) and chimeric nematode-bovine EF-Tu variants (BmCe3' and BmCe3). The lower part of the figure indicates a summary of the binding specificity of *C.elegans* mt EF-Ts to the EF-Tu variants. White boxes with black letters show the bovine mt domains and gray filled boxes with white letters show the nematode mt domains. Domain 3' represents the C-terminal 57 amino acid extension that is specific for *C.elegans* mt EF-Tu1.

amino acid sequence of *C.elegans* mt EF-Ts resembles more closely that of *E.coli* EF-Ts than that of *T.thermophilus* EF-Ts. The peak retention volumes of *C.elegans* mt factors in the gel filtration chromatogram are also suggestive of the formation of a EF-Ts·EF-Tu heterodimer (83.5 kDa for EF-Tu1·EF-Ts and 77.8 kDa for EF-Tu2·EF-Ts) (Fig. 4A and C). The peak retention volume of *C.elegans* mt EF-Ts suggests the EF-Ts is a 32.8 kDa monomer.

Purification of the *C.elegans* mt EF-Tu·Ts complex

Caenorhabditis elegans mt EF-Tu1 and EF-Tu2 are particularly unstable, which makes handling them very difficult. For

example, they are easily precipitated during dialysis against low salt solutions as well as by concentrations of EF-Tu1 and EF-Tu2 that exceed ~2 and ~1 mg/ml, respectively. Furthermore, they aggregate under conditions of native gel analysis (Fig. 3) and gel filtration (Fig. 4). Such instability and low solubility complicates their use in experiments that require high protein concentrations or various solution conditions (e.g. crystallization for X-ray analysis). In contrast, the *C.elegans* mt EF-Tu·EF-Ts complexes are highly soluble (>10 mg/ml) and appear to be more stable than the EF-Tu alone under a wide variety of conditions. Recently it was reported that *E.coli* EF-Ts could act as a structural chaperone to improve the solubility of unstable EF-Tu mutants (28). *Caenorhabditis elegans* mt EF-Ts could be similarly used to increase the solubility and stability of nematode EF-Tu molecules. Thus, the procedure we developed to purify nematode mt EF-Tu·EF-Ts complexes may be a useful tool for the analysis of nematode mt EF-Tu molecules.

Specificity of *C.elegans* mt EF-Ts for different EF-Tu molecules

Bovine mt EF-Ts expressed in *E.coli* is known to form an extremely tight complex with *E.coli* EF-Tu *in vivo* (13). The complex cannot be dissociated completely even in the presence of 8 M urea or 8 M guanidine hydrochloride (13). In this work, a similar phenomenon was observed for *C.elegans* mt EF-Ts when it was expressed in *E.coli*. When we tried to purify the recombinant *C.elegans* mt EF-Ts protein on a Ni²⁺ column, we found it was difficult to remove a protein contaminant whose molecular weight agreed with that of *E.coli* EF-Tu. The binding between the two proteins was loose enough, however, to allow the EF-Tu-like protein to be largely eliminated by repeated column washes (Fig. 1, lanes 1–4). Native gel analysis confirmed that *C.elegans* mt EF-Ts and *E.coli* EF-Tu can form a heterologous complex (Fig. 5A).

Caenorhabditis elegans mt EF-Ts also bound to *T.thermophilus* EF-Tu and to the two mt EF-Tu molecules from *C.elegans* itself, suggesting that it has broad specificity. Such broad specificity is probably required for it to be able to recognize the two distinct EF-Tu species in the *C.elegans* mitochondria. Notwithstanding this broad specificity, the EF-Ts was unexpectedly not able to bind to bovine mt EF-Tu (Fig. 6). The lack of binding of *C.elegans* mt EF-Ts to bovine mt EF-Tu was examined further by using chimeric nematode–bovine EF-Tu molecules. *Caenorhabditis elegans* mt EF-Ts was able to bind to a chimeric *C.elegans* mt EF-Tu that contained domains 1 and 2 of bovine mt EF-Tu but not to a chimera that contained domain 3 of bovine mt EF-Tu, indicating that domain 3 of bovine mt EF-Tu prevents the binding of EF-Ts but domains 1 and 2 do not.

A previous study has shown that strong binding of bovine mt EF-Ts to EF-Tu is due to its N-terminal half and that the N-terminal half of bovine mt EF-Ts enables it to bind heterologous EF-Tu (14). The crystal structure of the *E.coli* EF-Tu-EF-Ts complex shows that the N-terminal half of EF-Ts binds to domain 1 of EF-Tu (17). The amino acid identity between the N-terminal halves of *C.elegans* mt EF-Ts and bovine mt EF-Ts (40.0%) is much higher than that between *C.elegans* mt EF-Ts and bacterial EF-Ts. For example, the homology with *E.coli* EF-Ts was only 27.7%. Thus, it is likely that the N-terminal half of *C.elegans* mt EF-Ts also enables it to bind heterologous EF-Tu proteins like bacterial EF-Tu and the chimera bearing domains 1 and 2 from bovine mt EF-Tu. The fact that *C.elegans* mt EF-Ts cannot bind the EF-Tu chimera bearing bovine domain 3 can be explained as follows. The crystal structure of the *E.coli* EF-Tu-EF-Ts complex reveals that the C-terminal half of EF-Ts interacts with domain 3 of EF-Tu (17). The amino acid identity between the C-terminal halves of the *C.elegans* and bovine mt EF-Ts molecules is not very high (22.9%) and thus it is likely that the C-terminal half of *C.elegans* mt EF-Ts cannot support binding to domain 3 of bovine mt EF-Tu.

In conclusion, *C.elegans* mt EF-Ts recognizes a common structure shared by *C.elegans* mt EF-Tu1 and EF-Tu2, *E.coli* EF-Tu and *T.thermophilus* EF-Tu, as well as domains 1 and 2 from bovine mt EF-Tu. Further analysis using EF-Tu mutants or crystallographic analysis will elucidate the molecular mechanism by which EF-Tu is recognized by the EF-Ts.

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